Vincenza Faraco *Editor*

Lignocellulose Conversion

Enzymatic and Microbial Tools for Bioethanol Production

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Preface

Bioethanol represents one of the most promising biofuels, exhibiting several advantages, such as high octane number, low cetane number high heat of vaporization and, most importantly, reduction of greenhouse gas emissions. A variety of biomass feedstock have been explored for ethanol production including sucrose-rich crops such as sugarcane and sugar beet, starch-rich crops such as maize and grain sorghum, and lignocellulosic materials such as woody biomass, herbaceous perennials, and various wastes.

In the United States, the Department of Energy has set a goal of 60 billion gallons of renewable fuels per year to be produced by 2030. In the European Union there is a mandatory target to substitute 10 % of transportation fuels with renewable fuels by 2020. Production of ethanol from corn starch in United States has almost reached its full capacity. Moreover, ethanol production from this edible feedstock poses concerns about competition with food and feed supplies. The only sustainable alternative substrate for ethanol production is lignocellulosic biomass.

Lignocellulosic biomasses are the most abundant renewable resources on Earth. The use of lignocellulosic materials for second-generation ethanol production would minimize the conflict between land use for food (and feed) and energy production. Moreover, these raw materials are less expensive and they present a more even geographical distribution than the conventional agricultural feedstock. A large fraction of lignocelluloses is represented by residual biomass such as agro-industrial wastes, agricultural and forest crop residues, and the organic and paper fractions of municipal solid waste that would represent the key response to the need of increasing renewable energy production. It is worth noting that only small amounts of cellulose, hemicellulose, and lignin composing agricultural residues are currently exploited, the majority being considered wastes. Moreover, second-generation ethanol production and use show lower greenhouse gas emissions than the first-generation fuels, reducing environmental impact, particularly in terms of climate change.

Lignocellulose consists of three types of polymers—cellulose, hemicellulose, and lignin-bonded by both non-covalent and covalent cross linkages. Cellulose is a highly crystalline linear polymer that is composed of D-glucose units linked by β-1,4 glycosidic bonds. Hemicellulose is also a polysaccharide, accounting for

around 25–35 % of dry wood. It is a very heterogeneous and ramified polymer, consisting of a mixture of various monosaccharides, such as xylose and arabinose (both 5-carbon sugars) and glucose, mannose and galactose (all 6-carbon sugars), and glucuronic acid. Lignin is present in the cellular wall to give structural support, mechanical resistance, impermeability, and defense against microbial attack and oxidative stress. It is an amorphous heteropolymer formed from phenylpropane units joined together by non-hydrolyzable linkages.

Lignocellulose conversion into ethanol commonly involves i) a pretreatment to remove the barrier of lignin and expose plant cell wall polysaccharides, ii) enzymatic saccharification of sugars with a (hemi)cellulolytic enzyme cocktail, and iii) fermentation of the sugars with ethanologenic microorganisms. Pretreatment involves the use of acids, alkalis, and/or organic solvents. Numerous pretreatment strategies have been developed such as physical treatment, chemical treatment (alkaline or acid), biological treatment, physicochemical treatment, i.e., steam explosion, liquid hot water, ammonia fiber expansion, supercritical fluid treatment, and thermochemical treatment. Biological pretreatments are also investigated to reduce use of toxic reagents. After pretreatment, the released cellulose and hemicelluloses are hydrolyzed to monomeric sugars (hexoses and pentoses) using acid or enzymatic methods. Enzymatic hydrolysis by (hemi)cellulases is the preferred method because of the higher conversion yields and less corrosive and toxic conditions compared to the acid hydrolysis. Fermentation of all free sugars into ethanol is carried out by yeasts or bacteria.

The cost of enzymes used in the process is considered as one of the key bottlenecks for producing fuels and chemicals from lignocellulosic biomass. Several efforts are underway to reduce the cost and maximize enzyme production. Some of the strategies include improving the performance of the enzymes by increasing the specific activity (through direct evolution and site directed mutagenesis) and thereby minimizing enzyme dosage or reduce the cost of enzyme production by improving cellulase titers during fermentation (through process engineering approaches by using cheap substrates including biomass, producing enzymes near biorefinery, or expression of enzyme in plants).

The enzymatic hydrolysis may take place in a separate step followed by fermentation called separate hydrolysis and fermentation process, or it may take place together with the fermentation in a simultaneous saccharification and fermentation of hexoses process or simultaneous saccharification and co-fermentation of both hexoses and pentoses. The ultimate objective is a one-step consolidated bioprocessing of lignocellulose to bioethanol, in which all the steps occur in a single reactor where a single microorganism or microbial consortium converts pretreated biomass into ethanol without added enzymes.

In this book, the main tools, the current technological developments, and future prospects in cellulosic ethanol production and research are described.

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Chapter 1
Introduction: Potential of Cellulosic Ethanol

Takashi Watanabe

Abstract Conversion of lignocellulosic biomass is emerging as one of the most important technologies for sustainable production of renewable fuels and chemicals due to its widespread availability, large quantity, non-competitiveness with food supply, potential as platform for green chemicals, and high mitigation effects on GHG emissions. The process for cellulosic ethanol production by enzymatic saccharification and fermentation consists of pretreatments exposing plant cell wall polysaccharides, production of reducing sugars with a (hemi) cellulolytic enzyme cocktail, and fermentation of the sugars with ethanologenic microorganisms. Simultaneous saccharification and co-fermentation (SSCF) and consolidate bioprocess (CBP) have been studied as cost-effective integrated processes for bioethanol production. For this purpose, ethanologenic microorganisms have been engineered to co-utilize hexoses and pentoses at a similar rate and secrete or display hydrolases on the cell surfaces. In this chapter, the role of bioethanol in sustainable society, its potential as new platform chemicals, and the current technological developments and future prospects in bioethanol research are overviewed.

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1.1 Development of Sustainable Society Through Cellulosic Fuel Ethanol

Excessive use of fossil resources causes global warming and depletes accessible crude oil. Humans have acquired the technology to consume and convert crude oil and gain a broad range of benefits from it, but this has also led to massive emissions of carbon dioxide into the environment. Unless our society shifts away from the consumption of crude oil and fossil fuels to the re-cyclical use of renewable resources such as biomass, it is difficult to secure sustainability of human life. The conversion of biomass into biofuels, chemicals, energy, and new materials is becoming vital to solving these problems. Production of bioethanol plays a central role in the conversion system due to its high productivity and applicability as liquid fuel and chemical resource. Table 1.1 lists the main ethanol producing plants all over the world. Bioethanol has several known advantages such as high octane number, low cetane number, and high heat of vaporization. Due to these properties bioethanol has better antiknock characteristics (Balat et al. 2007). However, fuel ethanol is water-miscible and its calorific value is lower than that of gasoline. The low vapor pressure of ethanol, together with its single boiling point, is disadvantageous for engine start at low ambient temperatures. Therefore, mixed use with high colorific fuels is essential for the application of bioethanol to transportation fuels.

Cellulosic biomass from plant cell walls is the largest renewable organic resource on Earth and its use does not compete directly with food supply. Therefore, along with its conversion to fuels, chemicals, energy, and new materials, maintaining a balance between its production and consumption becomes one of the decisive factors for solving the problems caused by the rapid consumption of fossil resources. Biorefineries contribute to the security of energy and chemical resources in countries which heavily depend on import of fossil resources.

The primary sources of lignocellulosic biomass include woody feedstock (softwoods or hardwoods), agricultural wastes (such as corn stover, sorghum, sugarcane bagasse, rice straw, wheat straw, empty fruit bunch from oil palm and date palm, agave bagasse from tequila industry), perennial grasses (switchgrass, miscanthus, canary grass, erianthus, napier grass, giant reed, and alfalfa), and municipal solid waste (MSW). Among renewable resources, woody biomass is the largest organic resource on Earth. Use of woody biomass potentially provides incentive in forest plantation, and it provides great potential to renovate local communities, economy, and environment associated with the forests. The use of wood harvested from forests as a source of chemical components leads to the decrease in the consumption of fossil resources, and at the same time the

Table 1.1 Main ethanol producing plants

Company Web-site	Location	Raw materials
Iogen Corporation http://www.iogen.ca	Canada	Wheat, oat and barley straw
Abengoa Bioenergy http://www.abengoabioenergy.com	Europe (Spain) USA	Grain, DDG Wheat/barley straw
Broin jointly with US DoE, DuPont and Novozymes http://www.poetenergy.com/news/showRelease.asp?id=13	USA Iowa	Corn fiber and stover
Verbio Vereinigte BioEnergie AG http://www.verbio.de/en/desktopdefault.aspx	Germany	Grain Rapeseed oil
British Sugar http://www.britishsugar.co.uk	United Kingdom	Sugar beet
Agrana http://www.agrana.com	Austria Hungaria	Cereals
Tereos http://www.tereos.com/en	France, Czech Republic, Brazil.	Sugar beet, wheat and sugar cane
Inbicon http://www.inbicon.com/pages/index.aspx	Denmark	Wheat straw
Sekab http://www.sekab.com	Sweden	Forestry products
Saint-Louis Sucre www.saintlouis-sucre.com	France	Beet or molasses
Cristal Union www.cristal-union.fr	France	Beet
Tereos www.tereos.com	France	Wheat and sugar beet
SÜdzucker www.suedzucker.de	Germany	Wheat
Sauter www.sauter-logistik.de	Germany	Rye
Kwst www.kwst.com	Germany	Sugar beet or molasses
Agroethanol AB www.agroetanol.se	Sweden	Wheat
Mossi & Ghisolfi http://www.gruppomg.com/index.php	Italy	Arundo donax
DINS Sakai http://dins-sakai.jp/	Japan	Waste construction wood
Lignol Innovations Inc http://www.lignol.ca/	USA	Woody biomass
Pacific Ethanol Inc. http://www.pacificethanol.net/	USA	Wheat straw, corn cob, woody biomass
American Process Inc. http://www.americanprocess.com/	USA	Woody biomass

(continued)

Table 1.1 (continued)

Company Web-site	Location	Raw materials
Archer Daniels Midland http://www.adm.com/en-US/Pages/default.aspx	USA	Corn stem
ICM Inc. http://www.icminc.com/	USA	Corn stover, switchgrass
Mascoma Corporation http://www.mascoma.com	USA	Waste wood
Logos Technologies, Inc. http://www.logos-technologies.com/	USA	Corn stem, switchgrass, woody biomass
ZeaChem, Inc. http://www.zeachem.com/	USA	Hybrid poplar and other cellulosic feedstocks
BlueFire Renewables http://bfreinc.com/	USA	Waste wood
POET, LLC http://poet.com/	USA	Corn stover, corn stem

conversion process generates economic benefits that can be returned to maintaining forest growth. This requires a balance between nurturing forests and their utilization, with careful evaluation of artificially cultivated forests and their influence on the environment, society, and economy. However, forests are unevenly distributed. The use of agricultural wastes and by-products as feedstock for ethanol production gives significant environmental advantages, since it increases the efficiency of the utilization of the solar energy converted by crop plants without exploitation of additional natural resources such as land and water. The choice of feedstock, either plant species or waste material, depends on local conditions and economy.

One of the main reasons for using bioethanol is to reduce greenhouse gas (GHG) emissions. GHGs are gases that impair the Earth's ability to radiate thermal energy to space. An appropriate method to examine the environmental impacts of GHG is well-to-wheel analysis (WTW) or more precisely for bioethanol, field-to-wheels analyses with the Greenhouse gases, Regulated Emissions, and Energy use in Transportation (GREET) model (Argonne National Laboratory 2012; Han et al. 2011; Dunn et al. 2011; Wang et al. 2012a, b). Bioethanol produced from lignocellulosics by saccharification and fermentation processes have been reported to have much lower life cycle fossil energy use and GHG emissions than conventional petroleum-derived gasoline and diesel (Sheehan et al. 2003; Wang 2005; Larsen et al. 2009). Wang (2005) reported that GHGs emission reductions for different gasoline–ethanol blends made by corn, on first-generation technologies, are 18–26 and 21–29 % for E10 and E85 gasoline, respectively. For cellulosic ethanol, it is estimated that GHG emissions will be reduced by about 85 % for E10 and E85. With regard to corn ethanol, some authors concluded that it offers reductions in life cycle GHG emissions when compared with gasoline (Liska et al. 2009; Wang et al. 2011). On the other hand, most analyses of cellulosic ethanol

Table 1.2 Well-to-Wheel GHG emissions associated with the lignocellulosic ethanol and alternative fuel pathways. Adapted from GREET 1 (2009), Zhang et al. (2010)

Greenhouse gas (GHG) emissions ($g\ CO_2$ eq./km driven)	WTT	TTW	WTW
Conventional gasoline	58	241	300
Ethanol from farmed tree	−248	220	−28
Ethanol from harbecious lignocellulosics	−173	220	47
Stover ethanol	−185	220	35
Forest rediue ethanol	−142	220	78
Corn ethanol	−17	220	203
Brazil sugarcane ethanol	−158	220	62

Notes WTT well-to-tank, *TTW* tank-to-wheel, *WTW* well-to-wheel

reported significant reductions in life cycle GHG emissions when compared with those from baseline gasoline. Reductions of 63–118 % have been reported (Borrion et al. 2012; MacLean and Spatari 2009; Monti et al. 2012; Mu et al. 2010; Wang et al. 2011; Whitaker et al. 2010). In another study of 2012, Wang (Wang et al. 2012a) reported that ethanol from corn, sugarcane, corn stover, switchgrass, and miscanthus can reduce life cycle GHG emissions by 19–48, 40–62, 90–103, 77–97, and 101–115 %, respectively.

Argonne National Laboratory compared well-to-wheel (WTW) GHG emissions associated with the lignocellulosic ethanol and alternative fuel pathways (Table 1.2) (GREET 1 2009; Zhang et al. 2010). They reported that, while farmed tree-derived ethanol succeeded in reducing WTW greenhouse gas emissions by −28 g CO_2 eq./km driven, WTW GHG emission from corn ethanol exceeded 200 g CO_2 eq./km driven, although the emission is lower that from gasoline. Hence, bioethanol can decrease CO_2 emissions when lignocellulosic biomass is used as a raw material rather than grain starch. Emissions of GHG from lignocellulosic biomass are influenced by various factors, such as land-use change (LUC), transportation, processing industry, cultivation, and harvest systems including supply of fertilizers, irrigation, and land management. An important variable in GHG emissions by LCA studies is the contribution to GHG emissions of N_2O, which evolves from nitrogen fertilizer application and organic matter decomposition in soil (Stehfest and Bouwman 2006; Cherubini and Jungmeier 2010). Emissions from fields vary depending on soil type, climate, crop, tillage method, and fertilizer application rates (Larson 2006). The uncertainties in actual emissions are magnified by the high global warming potential of N_2O, 298 times greater than CO_2.

1.2 Biethanol as Feedstock for Chemical Industry

The creation of cellulosic biorefineries is of great significance for the new industry. In the long-term perspectives, new platforms converting lignocellulosics to chemicals should be established, and research on the production of new platform

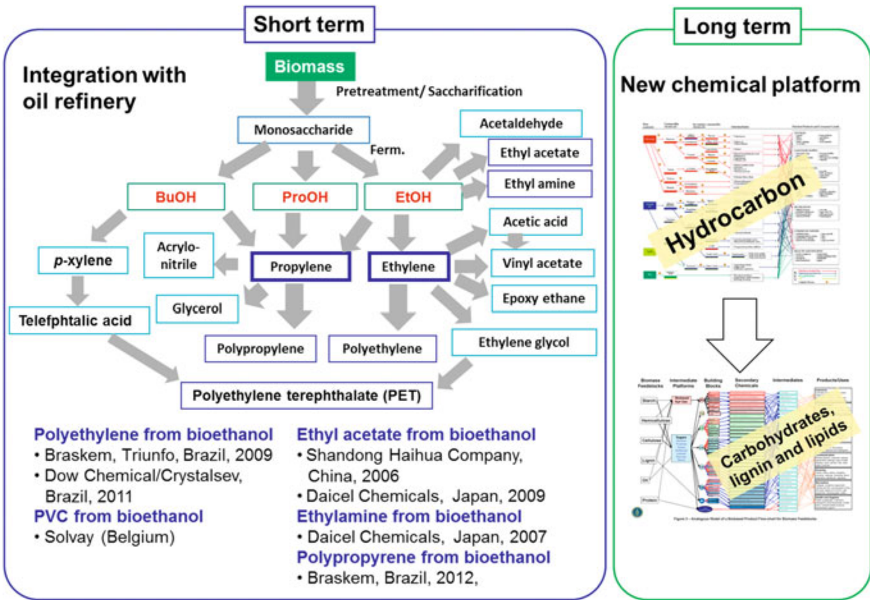

Fig. 1.1 Short-term and long-term perspectives in production of chemicals from biomass in biorefineries. *Note* Ref. for long term platform is adapted from Werpy et al. 2004

compounds has been a active concern. The United States Department of Energy selected 12 types of platform compounds in biorefineries and presented chemicals derived from them at an early stage (Werpyet al. 2004).

In the short-term perspective, integration of biofuel production system into oil refinery is more feasible. As shown in Fig. 1.1, ethylene and propylene can be produced from bioethanol, biopropanol, and biobutanol. Production of polyethylene and polypropylene from this route is commercialized in Braskem and a group of Dow Chemical and Crystalsev in Brazil. Production of triethylamine from bioethanol is industrialized by Daicel Co. Ltd, in Japan in 2007. Triethylamine is a raw feedstock for the production of quaternary ammonium compounds for textile auxiliaries and quaternary ammonium salts of dyes. Triethylamine can be used as a catalyst and acid neutralizer for condensation reactions and also as an intermediate for manufacturing medicines, pesticides, and other chemicals. Production of ethyl acetate from bioethanol is industrialized by Shandong Haihua Company in China and Daicel Co. Ltd. Bioethanol can be converted to acetaldehyde, acetic acid, epoxyethane, and ethylene glycol. Production of polyvinylchloride is planned by Solvay in Belgium. Polyethylene terephthalate (PET) can be synthesized from bioethanol and biobutanol via ethylene glycol and terephthalic acid. Thus, in recent years, an increasing number of research and development projects have been launched worldwide for production of chemicals from bioethanol.

The process producing second-generation bioethanol includes pretreatments and enzymatic saccharification of plant biomass. This process also serves as a core technology in microbial production of chemicals other than ethanol from lignocellulosics. Intensive research has been conducted to convert lignocellulosic biomass into a wide variety of fermentation products such as succinic acid, fumaric acid, malic acid, 3-hydroxypropionic acid, aspartic acid, glutamic acid, and itaconic acid. These compounds, together with those produced by thermochemical process, are the key platform compounds for biorefinery proposed by DOE (Table 1.3) (Werpy 2004).

1.3 Technological Advances and Tasks for Cellulosic Ethanol Production

Bioethanol production from lignocellulosic materials relies on technologies that hydrolyze cellulosic biomass to fermentable sugars. The first step in the overall process of lignocellulosic fermentation breaks the lignin barrier and disrupts the crystalline structure of cellulose (pretreatment). This is the most rate limiting step in the overall process. The pretreatment processes involve physical, chemical, thermochemical, and biological treatments (Table 1.4). Post pretreatment, the recalcitrant lignocellulosic biomass becomes susceptible to enzymatic hydrolysis (Stephanopoulos 2007). The pretreated biomass is hydrolyzed with enzymes and fermented by yeasts or bacteria to ethanol (Joshi et al. 2011).

Four biologically mediated events occur during conversion of pretreated lignocellulose to ethanol: (i) production of depolymerizing enzymes (cellulases and hemicellulases), (ii) hydrolysis of the polysaccharide constituents of pre-treated biomass, (iii) fermentation of the hexose sugars present, and (iv) fermentation of pentose sugars present (Lynd et al. 2002). The enzymatic hydrolysis may take place in a separate step followed by fermentation called separate hydrolysis and fermentation (SHF) process, or it may take place together with the fermentation in a simultaneous saccharification and fermentation of hexoses (SSF) process or simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses. The ultimate objective would be a one-step CBP of lignocellulose to bioethanol, in which all four of these steps occur in a single reactor where a single microorganism or microbial consortium converts pretreated biomass into ethanol without added enzymes.

The process SHF involves separate hydrolysis and fermentation by running the reactions in two reactors. Pretreated lignocellulosic material is in a first reactor degraded to monomeric sugars by enzymatic hydrolysis and thereafter fermented to ethanol in a second, separate reactor (Petrova and Ivanova 2010). After that, the mixture is distilled to remove the ethanol. In a second reactor, the xylose is fermented to ethanol, and the ethanol is again distilled. The main advantage of this method is that the two processes (hydrolysis and fermentation) can be performed at

Table 1.3 Building blocks proposed by DOE (Werpy et al. 2004)

Building block	Chemical structure	Derivatives
1,4-Diacids (Succinic, Fumaric and Malic acids)	succinic acid; fumaric acid; malic acid	(Products from succinic acid):1,4-Butanediol, Succindiamide, 1,4-Diaminobutane, Succinonitrile, Dimethyl succinate, *N*-Methyl-2-pyrrolidone (NMP), 2-Pyrrolidone, Tetrahydrofuran, γ-Butyrolactone, Polybutylene succinate, Polyethylene succinate, Polybutylene succinate/adipate, 4,4-Bionolle, Succinimides, Butyrate, Succinicanhydrides, Maleic anhydride
2,5-Furandicarboxylic acid		Succinic acid, 2,5-Furandicarbaldehyde, 2,5-Dihydroxymethylfuran, 2,5-Dihydroxymethyltetrahydrofuran, 2,5-bis(Aminometyl)-tetrahydrofuran
3-Hydroxypropionic acid		Acrylic acid, Methyl acrylate, Acrylamide, Acrylonitrile, Propiolactone, Ethyl 3-hydroxypropionate, Malonic acid, 1,3-Propanediol
Aspartic acid		2-Amino-1,4-butanediol, Amino-2-pyrrolidone, Aspartic anhydride, Amino-γ-butyrolactone, 3-Aminotetrahydrofuran, Substituted amino-diacids, Pharma and sweetener intermediates
Glucaric acid		α-Ketoglucarates, Polyhydroxypolyamides, Glucarodiactone, Glucaro-δ-lactone, Glucaro-γ-lactone, Glucaric acid esters and salts
Glutamic acid		Glutaric acid, 1,5-Pentandiol, 5-Amino-1-butanol, Pyroglutamic acid, Prolinol, Proline, Pyroglutaminol, Norvoline, Glutaminol, Polyglutamic acid
Itaconic acid		2-Methyl-1,4-butanediamine, Itaconic diamide, 3-Methylpyrrolidine, 3- & 4-MethylNMP, 2-Methyl-1,4-BDO, 3-Metyl THF, 3- & 4-Methyl-GBL
Levulinic acid		2-Methyl-THF, Acrylic acid, δ-Aminolevulinate, Diphenolic acid, β-Acetylacrylic acid, Levulinate esters, 1,4-Pentanediol, Angelilactones, γ-Valerolactone
3-Hydroxybutyrolactone		γ-Butenyl-lacotone, Epoxy-lactone, Acrylate lactone, 2-Amino-3-hydroxytetrahydrofuran, 3-Aminotetrahydrofuran, 3-Hydroxytetrahydrofuran
Glycerol		1,3-Propanediol, Propylene glycol, Branched polyesters and nylons, Mono-, di-, or triglycerate, Diglyceraldehyde, Glycerol carbonate, Glyceric acid, Glycidol

(continued)

Table 1.3 (continued)

Building block	Chemical structure	Derivatives
Sorbitol		Propyrene glycol, Ethylene glycol, Glycerol, Lactic acid, 2,5-Anhydrosugars, 1,4-Sorbitan, Isosorbide
Xylitol/arabinitol	xylitol L-arabinitol	Propylene glycol, Ethylene glycol, Glycerol, Lactic acid, Mixutures of hydroxyfurans, Xylaric acid

Table 1.4 Pretreatments for enzymatic saccahrification of lignocellulosic biomass

Physical treatment	Milling	Ball mill, roll mill, hammer mill, rod mill, grinder, refiner, cryomill, wet mill, etc.
Thermochemical treatment	Hot water treatment	Hydrothermolysis, Compressed hot water, Steam treatment Supercritical water[a], Subcritical water[a]
	Steam explosion	Steam explosion, Ammmonia fiber explosion (expansion) (AFEX)
	Irradiation	Microwave[b], Electron beam, γ-Irradiation Sonication
Chemical treatment	Acid	Conc. $H_2SO_4^a$, dil. H_2SO_4, HCl[a], H_3PO_4 Lewis acid, Organic acids
	Alkali	NaOH, Ammonia, Na_2SO_3, $Ca(OH)_2$
	Oxidant	Ozone, H_2O_2, Peracetic acid, Organic peroxiside
	Organic solvent	Organosolvolysis (EtOH, HCOOH, AcOH, Alcohols with high boiling point, etc.)
Biological treatment	Wood rot fungi	White rot fungi Brown rot fungi

[a] These processes can be applied to hydrolysis without enzymes
[b] Microwave irradiation can be used for hot water treatment and organosovolysis with a wide variety of catalysts

their own individually optimal conditions. This technique exhibits issues of high capital cost, long processing time, high contamination risk and the major drawback is that end products, i.e., glucose and cellobiose released in cellulose hydrolysis, strongly inhibit the cellulase efficiency.

The simultaneous saccharification and fermentation (SSF) process combines the hydrolysis and fermentation into one single step, so reducing hexoses sugars produced in cellulose hydrolysis are simultaneously fermented to ethanol by fermentative microorganisms. The pentoses are fermented before the hydrolysis of cellulose in a separate fermenter. The benefits of this technique is that it reduces the number of steps in the process, reduces sugar inhibition of enzymes, improves cellulose conversion rates, increases ethanol yield, lowers enzyme loadings, and eliminates the need for separate reactors for saccharification and fermentation (Lynd et al. 2005). The disadvantages of using SSF in comparison to the separate hydrolysis and fermentation (SHF) process are (Hamelinck et al. 2005; Lynd et al. 2005; Olofsson et al. 2008): (i) the operating temperature for enzymatic hydrolysis is typically (37–38 °C) higher than that of fermentation; (ii) much of the sugar released by cellulose hydrolysis is used for the growth of yeast necessary to ensure good ethanol production. Nevertheless, the overall ethanol yield in SSF has been reported to be higher than if the enzymatic hydrolysis and fermentation are carried out separately (SHF).

The simultaneous saccharification and co-fermentation (SSCF) represents hydrolysis of the cellulose and co-fermentation of pentose and hexose sugars by xylose- and glucose-fermenting microorganisms in one reactor. This technology is superior to SSF technology in terms of cost-effectiveness, higher yield, and shorter processing time (Lynd et al. 2005; Chandel et al. 2007). Besides reduced capital cost (Wingren et al. 2003), The SSCF process offers several advantages which

include continuous removal of end products of enzymatic hydrolysis that inhibit cellulases or β-glucosidases (Olofsson et al. 2008) and higher ethanol productivity and yield than separate hydrolysis and fermentation (Tomás-Pejó et al. 2008; Alfani et al. 2000). To operate a SSCF process at high content of water-insoluble-solids (WIS) it is required to achieve high concentrations of ethanol.

Consolidated bioprocessing (CBP) is an alternative processing strategy in which enzyme production, substrate hydrolysis, saccharification, and fermentation are accomplished in a single step and in one reactor (Lynd et al. 2005; Demain et al. 2005; Van Zyl et al. 2007; Cardona and Sánchez 2007). It is important to note that, in CBP, only one microbial consortium is employed for both the production of cellulase and fermentation. This process has the potential of lowering production costs of bioethanol, reducing energy inputs, and enhancing conversion efficiencies in comparison to SSF or SSCF based processes.

The following paragraphs discuss the technological and scientific advances relevant to cellulosic ethanol production.

1.3.1 Strategies to Enhance Enzymatic Hydrolysis of Lignocellulosic Biomass

Technologies to hydrolyze polysaccharides in plant cell walls for bioethanol production from lignocelluloses include methods using supercritical water, subcritical water, strong acids, and hydrolases. Hydrolysis of polysaccharides with supercritical water, subcritical water, and strong acids usually requires no pretreatment to rupture the plant cell walls because the water and acids can penetrate into the cell wall regions to contact the polysaccharides. On the other hand, for enzymatic hydrolysis, pretreatment is necessary because enzymes cannot access to the polysaccharides due to their large molecular size and coverage of the polysaccharides with lignin. Nevertheless, the enzymatic hydrolysis process is the current centerpiece because it has higher potentials to decrease use of harsh and polluting conditions and production cost of bioethanol than the other methods.

Many physico-chemical, structural, and compositional factors hinder access and hydrolyzability. In enzymatic hydrolysis, the reaction starts from direct physical contact between the enzymes and exposed polysaccharides (Kawakubo et al. 2010). However, nonspecific adsorption of enzymes on lignin decreases the reactivity, even after the rupture of the lignin network (Palonen et al. 2004; Nakagame et al. 2010; Börjesson et al. 2007; Tu et al. 2009, Rahikainen et al. 2011). The crystalline structure, degree of polymerization, surface area, porosity, and particle size of polysaccharides affect the hydrolysis rate, depending on the enzyme load and composition of the enzyme mixtures. Concerning the possible change in the degree of crystallinity or the dimensions of the crystallites during hydrolysis, diverse results for different substrates have been obtained. Some studies have shown that the crystallinity is slightly increased by enzymatic hydrolysis of cellulose (Cao and Tan 2005; Wang et al. 2006), while others have

reported that this index has remained unchanged during the hydrolysis (Penttilä et al. 2010; Gama and Mota 1997). A single factor such as crystallinity index is not enough to understand the enzyme–substrate interaction even in the hydrolysis of pure cellulose. In hydrolysis of microcrystalline cellulose (Avicel) digested with the *Trichoderma reesei* cellulase system, it was suggested that the enzymes act on the surface of cellulose bundles and are unable to penetrate into the nanopores of wet cellulose (Penttilä et al. 2010).

In hydrolysis of pretreated biomass, structures of the surrounding lignin and hemicelluloses significantly affect the accessibility and hydrolysis rate. In enzymatic hydrolysis of woody biomass pretreated by organosolvolysis and kraft pulping processes, linear dependency of the exposure of crystalline and non-crystalline cellulose surfaces for enzymatic saccharification yield were found, but this correlation was not observed for the substrates pretreated by hydrothermolysis and ball-milling (Kawakubo et al. 2010). Thermal and thermochemical pretreatments expose cell wall polysaccharides but a part of core structures of pentoses and hexoses are decomposed by the process, decreasing the sugar yield by enzymatic hydrolysis, with concomitant production of fermentation inhibitors such as furfural and 5-hydroxymethyl furfural.

The high costs of the biomass fractionation step due to cellulases and hemicellulases production costs are still major bottlenecks in commercialization of lignocellulose bioconversion to ethanol. Strenuous efforts have been made to minimize the enzyme loading for saccharification and the cost for enzyme production. To decrease the enzyme loading, research is directed to understanding of the role of each monocomponent enzyme in hydrolysis of pretreated biomass. These studies include analysis of non-productive adsorption of enzymes on lignin, cellulose, and pretreated biomass, screening of new enzymes by metagenomic approaches, and modification of monocomponent enzymes by protein engineering with rational design and direct evolution. Minimum set of enzymes depends on pretreatment process and the source of lignocellulosics.

Filamentous fungus *Trichoderma reesei* is known to be hyper producer of cellulolytic enzymes and is widely used for commercial scale production of cellulases and hemicellulases. A series of *T. reesei* strains knocking out one of the cellulase genes have been used for the functional analysis of monocomponent enzyme. The mutant series is a powerful tool to analyze the key enzyme for biomass degradation but the interaction of each enzyme with different types of pretreated biomass is still not fully understood. In the *T. reesei* genome, two cellobiohydrolases (CBH), eleven endoglucanases (EG), and seven β-glucosidases (BGL) are encoded. The *T. reesei* genome also contains sixteen hemicellulases. To improve the saccharification of lignocellulosic biomass by *T. reesei*, fungal secretomes have been applied to supplement enzyme cocktails from *T. reesei*. Recently, roles of GH61 including *T. reesei* EGIV in cellulose degradation attract considerable attention. *T. terrestris* GH61 was characterized originally as endoglucanase but this enzyme was found to be oxidoreductase (Langston et al. 2011). Endoglucanase activity of this enzyme is weak but it accelerates cellulase degradation when it was added to cellulase mixtures. The accelerating effect of GH61

was dependent on divalent metal ions, and recently it was proved that GH61s are copper-dependent lytic polysaccharide monooxygenases (Quinlan et al. 2011). GH61 acts in synergy with cellobiose dehydrogenase (CDH) (Bey et al. 2013; Horn et al. 2012). The redox system of GH61 in cellulose degradation is becoming important for construction of minimum set of enzyme cocktail for biomass degradation. Lignin degradation products from pretreated biomass may serve as the electron donor necessary for the oxidative degradation of cellulose with GH61 and molecular oxygen. CBM33 has similar functions with GH61. In addition to hydrolases and oxidoreductases, proteins loosening crystalline cellulose such as swollenin and expansin have received significant attention. The research is expanding to screen and characterize the non-hydrolyzing proteins from various microorganism origins including bacterial expansin (Georgelis et al. 2012).

Carbohydrate-binding module (CBM) of cellulase is a protein component adsorbing the enzyme on non-soluble substrate, cellulose, and hemicelluloses. However, CBM also preferentially binds to lignin, mainly via hydrophobic interaction, and the adsorbed enzyme rapidly loses its activity. Under high substrate concentration, CBM was found to play no crucial role for adsorption on cellulose. Therefore, use of CBM-deleted cellulase for enzymatic hydrolysis of pretreated biomass was proposed to avoid the unfavorable binding of enzymes on lignin in hydrolysis of pretreated biomass (Viikari et al 2012). To decrease the non-productive binding of enzymes, use of surfactants, polyethylene glycol, and masking proteins (e.g., BSA and skim milk) has been examined (Börjesson et al. 2007; Yang and Wyman 2006; Eckard et al. 2012). However, use of these additives is still not practical due to their high cost. For reduction of the enzyme cost, suppression of the non-productive binding and/or recycling of enzymes after hydrolysis or fermentation process are essential.

Hyperproduction of cellulase is another factor to decrease the enzyme costs. The research includes overexpression of enzymes by introduction and modification of translational regulators and/or by alteration of constitutive and inducible promoters, suppression of catabolite repression (e.g., deletion or modification of *cre1* for *T. reesei*), development of natural inducers and their response system, and optimization of fermentation process (Nakari-Setala et al. 2009; Ilmen et al. 1996).

On-site production of enzymes has also a big potential to reduce the cost for enzyme production due to no shipping cost, no need for enzyme concentration, and availability of carbon source from pretreated biomass.

1.3.2 Genetic Engineering of Ethanologenic Microorganisms to Improve Fuel Ethanol Production

To decrease the enzyme loading, genetic engineering of ethanologenic microorganisms expressing cellulolytic and hemicellulolytic enzymes have been studied. Cell surface display or secretion of enzymes is selected for this purpose (Matano

et al. 2012; Fan et al. 2012; Kojima et al. 2012; Linger et al. 2010). Because cellulose is a crystalline insoluble polysaccharide, solid–solid interaction by the cell surface display system limits accessibility of the enzymes. Assembly of enzymes on cell surface at high density may break the limitation. As found in cellulosome from anaerobic bacteria, optimized alignment of catalytic modules and CBMs significantly increases the reactivity (Fan et al. 2012).

In ethanol fermentation, fermentation inhibitors such as furfural, 5-hydroxy methyl furfural, vanillin, vanillic acid, syringaldehyde and syringic acid significantly affect the production efficiency. Lignin-degrading enzymes like laccase were assembled on the cell surface of yeast to decompose fermentation inhibitors (Nakanishi et al. 2012). Because laccase uses molecular oxygen as an electron acceptor, the process requires at least two stage reactions with different levels of oxygen concentration. Lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and other peroxidases are also the candidate for detoxification, but feeding of H_2O_2 should be controlled by pump or *in situ* oxidase reaction. In addition to the degradation and separation of inhibitors, breeding of inhibitor-resistant ethanologenic microorganisms by metabolic engineering has been studied. Integrative omics analysis is a powerful tool to make a strategy for the transformation. Process engineering such as adsorption of inhibitors on ion exchange resin and activated charcoal, dithionite and sulfite treatment, overliming, neutralization, membrane filtration, extraction with organic solvents, and evaporation are possible approaches for detoxification (Chandel et al. 2011), but the additional process increases the production cost of bioethanol to weaken competitiveness in the market. Biotechnological approaches and innovation of pretreatments and separation technique are the key issues to solve the problem of fermentation inhibition.

For cost-effective bioethanol production, utilization of monosaccharides other than glucose is necessary. Converting xylose and other sugars (e.g., arabinose, mannose, and galactose) into ethanol increases the total yield of bioethanol. Two different strategies can be applied for this purpose. The first is transformation of microorganisms able to naturally ferment a wide variety of sugars, including glucose and xylose, to introduce or enhance fermentation ability of ethanol production. *Escherichia coli* is one of those microorganisms able to ferment a wide range of sugars, including xylose, arabinose, glucose galactose, mannose, and glucuronic acids, and has been investigated to introduce an ethanologenic process from pyruvic acid, and suppress unfavorable side pathways. Another strategy is to transform ethanologenic microorganisms to utilize a wide variety of sugars. This process has been applied to *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Zymobacter palmae*, and other ethanologenic microorganisms. Bioethanol was produced from dilute-acid pretreated yellow poplar by SSCF with the ethanologenic bacterium *Zymomonas mobilis,* which was transformed to utilize xylose and glucose (McMillan et al. 1999). Yanase et al. (2012) produced ethanol from the hydrolysate of wood biomass containing glucose, mannose, and xylose as major sugar components. This transformation was accomplished by introducing genes encoding mannose and xylose catabolic enzymes from *Escherichia coli*.

Expression of *E. coli manA*, *xylA*, *xylB*, *tal*, and *tktA* in *Z. mobilis* broadened the range of fermentable sugar substrates for *Z. mobilis*. Much work has been directed toward obtaining *S. cerevisiae* strains able to ferment pentose sugars. Several laboratory and industrial yeast strains have been engineered to co-ferment D-xylose and L-arabinose (Becker and Boles 2003; Karhumaa et al. 2007) and to co-ferment xylose and cellobiose (Cho et al. 1999). Zhou et al. (2012) described the metabolic engineering of an *S. cerevisiae* strain, including overexpression of the *Piromyces* xylose isomerase gene (XYLA), *Pichia stipitis* xylulose kinase (XYL3), and genes of the non-oxidative pentose phosphate pathway (PPP). *P. stipitis* is able to utilize xylose and other important hexoses (Jeffries et al. 1996). However, different studies have been directed to increase its capability to degrade polysaccharides. For instance, Den Haan and Van Zyl (2003) enhanced the xylanolytic ability of *P. stipitis* by co-expressing both xylanase of *T. reesei* and *Aspergillus kawachii* and xylosidase of *Aspergillus niger* encoding genes.

In utilization of sugars other than glucose, difficulties in simultaneous utilization of glucose and other sugars have been recognized. The problem arises due to the difference in sugar uptake and metabolic flow rate. Integrated omics analysis is useful to make a strategy to solve the problems. As for the first problem, research is directed to increasing the uptake efficiency of sugars other than glucose, or suppressing the glucose uptake rate by modifying sugar transportation systems such as phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) and glucose transporter. More simple approaches are mixed cultivation of two different microorganisms and two-step cultivations although efficiency for cost reduction is limited.

1.3.3 Consolidated Bioprocessing for Cellulosic Bioethanol Production

As an alternative to using several steps to convert pretreated lignocellulosics into ethanol, consolidated bioprocessing (CBP) has been studied (Olson et al. 2012). CBP requires a highly engineered microorganism able to produce effective hydrolases, for high ethanol titer and productivities, using both hexoses and pentoses from a high solid pretreated material. In addition, the microorganism for CBP should have a high resistance to ethanol and fermentation inhibitors. Pretreatments for CBP should be optimized to achieve high enzymatic hydrolysis rate in the initial phase of fermentation.

For high productivity from sugars to target chemicals, development of genome-scale engineering including minimum genome cell factory is attractive (Esvelt and Wang 2013). Microorganisms are transformed to eliminate unnecessary genes to work as a cell factory with minimum genome. Metabolic flow is simulated to attain a targeted fermentation product from sugars through the introduction of foreign genes, the elimination of competitive pathways, and disruption of byproducts formation. Redox balance should be optimized through the metabolic flow.

Development of cell factory and customization for various chemical products is a trend to establish integrated biorefinery. It is necessary to develop cell factories with state-of-the-art science in biorefineries, and their success largely depends on big projects led with national government support. This technology is suitable for production of various chemicals but applicable to bioethanol production.

1.3.4 Toward Industrialization of Cellulosic Ethanol Biorefinery

In commercial production of bioethanol, overall production process from plant cultivation, biomass collection, construction and operation of mill, and transportation of end products including bioethanol and waste materials should be taken into account. Especially, cost of total mill equipment significantly affects the selling price of bioethanol. Therefore, pretreatment, saccharification, fermentation, purification, and wastewater treatment processes should be designed to simplify the equipment necessary for the conversion. This means that technologies requiring expensive equipment do not always lead to decrease the production cost even if conversion efficiency of the process is high.

For industrialization of second-generation bioethanol, conversion technology of residual lignin is also important. In most cases, residual lignin is planned to use as the source for energy recovery due to its higher calorific value than carbohydrates. This route is advantageous for energy balance but its contribution to the cost balance is small. If value-added chemicals are produced from the residual lignin, the process makes the cost balance better. A major ongoing research conducted worldwide is gasification of lignin into syngas (CO/H_2) and subsequent conversion into liquid fuels and chemicals. By this route ethanol can be produced from the residue in addition to other chemicals. Conversion of the residual lignin into functional polymers or other value-added products such as carbon fiber, engineering plastics, and separator for battery has been studied, aiming at total utilization of lignocellulosic biomass. Systematic design of pretreatments is necessary to cope with both bioethanol production and value-added products from lignin.

Research into second-generation bioethanol is a wide-ranging interdisciplinary research field. The book “Lignocellulose conversion: Enzymatic and microbial tools for bioethanol production” covers the most relevant aspects concerning cellulosic ethanol production from lignocellulosic raw materials, pretreatments, enzymes for saccharification, fermentation microorganisms, and CBP.

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Chapter 2
Sources for Lignocellulosic Raw Materials for the Production of Ethanol

Yitzhak Hadar

Abstract Production of ethanol from non-crop plant cell walls represents a sustainable solution for biofuel production due to the abundance of these renewable resources on our planet. The resources for cellulosic raw material can be either dedicated bioenergy feedstocks such as fast growing trees and energy grasses, or those based on by-products and waste materials such as crop residues and municipal solid waste. The processing of lignocellulosic into biofuel still requires expensive and harsh pretreatments, some of which are not acceptable from environmental point of view. This is due to the high level crystallinity of the cellulose and cross linking of the carbohydrates with the lignin that form a barrier preventing efficient and economic biomass enzymatic digestion. The advances in plant genetic engineering enable genetic modifications of the plant cell wall structure and function and may provide solutions that will help to overcome the difficulty in utilizing energy crops and trees. Despite the current technological difficulties related to processing of the complex cell wall polymers into fermentable sugars, the demand for renewable liquid fuel motivates the search for practical solutions and development of innovative technologies.

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2.1 Introduction

Most ethanol produced to date as biofuel is generated from edible crops by fermentation of sugars from sugarcane (*Saccharum officinarum*) or conversion of starch from corn (*Zea mays*). However, this "first generation" approach led to the "*food versus fuel*" conflict and dilemma leading to search for alternative biomass sources for the "next generation biofuels" mostly based on cellulose (Kullander 2010; Valentine et al. 2012). Plant cell walls are the most abundant renewable resource on our planet with 150–170 × 10^9 tons produced annually (Pauly and Keegstra 2008). Thus, the production of ethanol from the cell walls of non-crop plants or non-edible parts of plants is considered a sustainable solution for biofuel production. This is despite the current difficulties related to the costs, high energy inputs, and harsh conditions required to process the complex cell wall polymers into fermentable sugars. The complex composition of lignocellulosic materials is a key factor affecting the efficiency of bioethanol production during the conversion processes (Jordan et al. 2012; Himmel et al. 2007; Dixon 2013) The major components of plant cell walls are cellulose, hemicellulose, and lignin that comprise around 90 % of its dry biomass (Gibson 2012; Harris and Stone 2008; Pauly and Keegstra 2008). The principal cell wall polysaccharide is cellulose, composed of hydrogen bonded chains of β-1,4-linked glucose. Cellulose is coated with hemicellulose. The most abundant type of hemicellulose is xylan, a polymer of β-1,4-linked xylose which may have branches containing other sugars such as arabinose or glucuronic acid, depending on the plant species. The saccharification of cellulose and hemicellulose releases glucose and xylose that can in turn be fermented to ethanol. Lignin, a complex polymer of hydroxylated and methoxylated phenylpropanoids, cross-links plant secondary cell walls to provide mechanical strength and hydrophobicity and it contributes to defense against pathogens. The percentage of lignin content in cell wall varies between plants and is a crucial parameter affecting the decomposition efficiency of the polysaccharides.

Among the various issues hampering competitive sustainable utilization of lignocellulosic materials for commercial fuel production, the choice of biomass feedstock is of major importance, and it is discussed in this chapter. Preferentially, biomass feedstock needs to be of high cellulose content and a non-crop species, such as fast growing trees or grasses that can grow on marginal soils. Such species would not compete for land use with food crops and could be grown at relatively dry zones. To reduce the environmental and economical impacts of their utilization, efforts should be made to use low quality water such as treated waste water, if

irrigation is required. These energy crops cell wall components can be modified via genetic engineering in order to facilitate hydrolyzing enzymes accessibility and reduce cost of pretreatments. Other sources of biomass are by-products, such as agricultural wastes or crop residues, agro-industrial by-products, and municipal solid waste (Limayem and Ricke 2012; Sanchez and Cardona 2008).

In addition to plant species and cell wall chemical composition, other requests for the choice of feedstock include: minimal energy input required for processing the biomass to ethanol; eco-friendly pretreatment of the lignocellulosic raw material; minimum production of yeasts inhibitors such as furfural and phenolic compounds by pretreatment; low price of production and processing of lignocellulose to fermentable sugars; stable supply of feedstock to ensure a continuous operation of the factory all-year round; optimized supply chain management from harvest to collection, storage, and transport on a local base. In addition, geography may dictate specific strategy for specific regions, i.e., different plants may be economically grown in a tropical area in comparison to temperate ones.

Faraco and Hadar (2011) focused on the potential of bioethanol fuel production from lignocellulosic residues in the Mediterranean Basin. Residues from cereal crops, olive trees, and tomato and grape processing are abundant lignocellulosic wastes in France, Italy, Spain, Turkey, and Egypt, where their use as raw materials for ethanol production could give rise to a potential production capacity of 13 Mtoe of ethanol. Due to the lack of sufficient amounts of agricultural residues in all the other Mediterranean countries, use of the cellulosic content of municipal solid waste as feedstock is also an option. A maximum potential production capacity of 30 Mtoe of ethanol could be achieved from 50 % of the 180 million tons of waste currently produced annually in the Mediterranean Basin (Faraco and Hadar 2011).

Another investigation showing the possibility to identify unique biomass sources on a local basis was conducted in North–East India (Sasmal et al. 2012). This study focuses on characterization of three plant species: nut husk (*Areca catheu*), moj (*Albizia lucida*), and bonbogori (*Ziziphus rugosa*), available in the analyzed region. Physical and chemical analysis of these lignocellulosic biomass samples showed that they can serve as potential sources for biofuel production.

In another study, Puri et al. (2012) analyzed the prospects, challenges, and feedstock for biofuel production in Australia, where the largest renewable resources for biofuel production revealed to be forest plantations, based on Eucalyptus trees, agricultural residues, and organic by-products, mostly bagasse.

Another important point of local nature should be the alternative use of the feedstock, either for energy production by other technologies or for different purposes such as animal feed or soil amendment. For example, wheat straw is considered a nuisance and inexpensive bioethanol feedstock in Europe and North America but it can be difficult to obtain during droughts in the Middle East.

Cellulosic raw materials including dedicated bioenergy feedstocks (trees and energy grasses) and those based on by-products and waste material (plant residues and municipal solid waste) are described in this chapter.

2.2 Annual and Perennial Dry Energy Grasses

Forages and particularly cultivated grasses can be considered prominent candidates for cellulosic biomass production and among the various forms of biomass available for ethanol production, they seem to be very promising as future biomass feedstock. Their cultivation and processing are the focus of widespread research. The major herbaceous energy crops that have been selected for bioethanol production are switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus spp.* Anderss.), canary grass (*Phalaris arundinacea*), giant reed (*Arundo donax* L.), alfalfa (*Medicago sativa* L.), and Napier grass (*Pennisetum purpureum*). Besides avoiding "*food versus fuel*" conflict, they are considered to have energetic, economic, and environmental advantages over food crops for ethanol production (Hill et al. 2006; Chandel and Singh 2011). After a perennial grass is established, the major expenses are for nitrogen fertilizers and harvest. These plants can grow in marginal and erosive soils and respond to nitrogen fertilization with remarkable increase in biomass yield (Muir et al. 2001).

Switchgrass is native to North America, tall grass prairies are known for their rapid growth during the warm months to heights of two meters. Commercial switchgrass cultivation was studied mostly in the US and Canada. This plant can be grown in many environments, including swamplands, plains, and streams, and along the shores and interstate highways. It is self-seeding and resistant to many diseases and pests, and can produce high yields with low applications of fertilizer and other chemicals. It is also tolerant of poor soils, flooding, and drought; furthermore, it improves soil quality and prevents erosion due to its type of root system (Parrish and Fike 2009).

Miscanthus is another viable feedstock for cellulosic ethanol production. This species of grass is native of Asia and can grow up to 3.5 meters tall with little water or fertilizer inputs. It is similar to switch grass with respect to cold and drought tolerance and water use efficiency (Ng et al. 2010). *Miscanthus* is commercially grown in the European Union as a combustible energy source (Brosse et al. 2012). The cellulose and lignin levels vary between the different crops of *Miscanthus* with the highest cellulose content in range of 40 % (Chandel and Singh 2011), suggesting its highest potential for ethanol yield.

Napier grass is tropical grass native to the African grasslands. It requires very little supplement of nutrients for growth. It can be harvested 3–4 months after planting and then at intervals of 6–8 weeks for up to 5 years with a annual dry biomass yield per hectare of 40 tons (Woodard and Prine 1993). Its fibers can be used for obtainment of polymer derivatives and composites in addition to bioethanol (Reddy et al. 2012). It was suggested to use Napier grass, in addition to other grasses, in several tropical countries including countries like Thailand, Philippines, Kenya, and Brazil (Wongwatanapaiboon et al. 2012). In addition to the high biomass produced, it has been shown that hybrid giant Napier grass is an effective salt tolerant plant that can grow well in saline soil and reduce the saline soil pH (Ma et al. 2012).

The bioethanol industry needs a continuous and reliable supply of biomass that can be produced at a low cost and with minimal use of water, fertilizer, and arable land. Byrt et al. (2011) reviewed several studies focusing on a number of key existing and potential energy crops which are C_4 plants, and compared the photosynthesis rate, the composition of the plant cell wall as well as use of water, fertilizer, sugar yields, biomass yields, and the calculated ethanol production yield. They showed, based on calculations, that ethanol yields from C_4 sugar crops, such as sugarcane, sugar beet, and sweet sorghum may be ranging from 4000 to 8000 L/Ha, and exceed the ethanol yields from starch from grain crops. However, data directly comparing the performance of potential biofuel feedstock crops under different climate and field conditions is lacking. As pointed out by Byrt et al. (2011), these data should be collected regionally, to be able to assess which crop may give the highest bioethanol yield under different environments. They proposed that plant scientists have not yet even started to explore the rich genetic resources available for improving C_4 grasses and breeding of biofuel crops such as miscanthus and switchgrass, sorghum and sugarcane. C_4 biofuel feedstock improvement is only in its infancy considering the efforts invested in agricultural selection and breeding of corn over thousands of years (Byrt et al. 2011).

2.3 Forest Woody Feedstock

Fast growing short rotation forest trees can play an important role as feedstock for bioenergy production (Seguim 2011). However, forests are unevenly distributed. Forests play important environmental role in preservation of marginal land and reducing CO_2 levels in the atmosphere. Forest woody feedstock account for approximately 370 million tons per year of lignocellulosic biomass in the US (Perlack et al. 2005), other countries rich in forests are for example, Canada, the Russian Federation, Brazil, and China. Together, these countries account for more than half of the total forest area worldwide. Sources of woody materials include residues left in natural forest, forestry wastes, such as sawdust from sawmills, wood chips and branches from dead trees, and cultivated short rotation energy forest plantations utilizing several fast growing tree species.

There are two types of woody materials, softwoods, or hardwoods. Softwoods originate from conifers and gymnosperm trees (Sanchez and Cardona 2008). Unlike hardwoods, softwoods possess lower densities and grow faster. These trees comprise of evergreen species such as pine, cedar, spruce, cypress, fir, hemlock, and redwood. Hardwoods are mainly found in the Northern hemisphere and include trees such as poplar, willow, oak, cottonwood, and aspen. In the US, hardwood species account for over 40 % of the trees (Perlack et al. 2005). The genus *Populus* (cottonwood) which includes 35 species is the most abundant fast growing species suitable for bioethanol production. An advantage of woody biomass over agricultural plants is the flexibility in harvesting times as they do not depend on seasonality. Trees also contain less ash compared to crops and are of

higher density, due to the thick secondary wall, which makes their transportation more economical.

Nieminen et al. (2012) reviewed the different options for optimizing wood development in bioenergy trees. They concluded that tree breeding has been, thus far, very challenging due to the trees' long generation time. However, new breeding possibilities are emerging through the development of high-throughput technologies in molecular genetics. They describe traits, including stem morphology and xylem cell dimensions that could be modified to enhance wood production as well as hormonal and molecular regulation of wood development (Nieminen et al. 2012).

2.4 Municipal Solid Waste

In the European Union countries, over 250×10^6 tons of Municipal Solid Waste (MSW) are produced each year, with an annual growth of 3 %. In 1990, each individual in the world produced an average of 250 kg of MSW generating in total 1.3×10^9 tons of MSW (Beede and Bloom 1995). Ten years later, this amount almost doubled leveling at 2.3×10^9 tons (Al-Salem et al. 2009). MSW treatment and recycling in general have both economical and environmental implications that should be considered also for ethanol production (Chester and Martin 2009; Kalogo et al. 2007; Stichnothe and Azapagic 2009). In many countries, the MSW is separated into several fractions, thus the biodegradable organic components of MSW consisting of paper and cardboard, kitchen waste and garden waste, could be converted into biofuel (Li et al. 2007), although these are not an ideal feedstock, due to the diversity in the MSW components and less than perfect source separation and existence of contaminations. However, it may be useful in regions were more suitable raw materials are lacking or scarce. For example, with the advances in cellulosic ethanol technologies, the Mediterranean could use the cellulosic content of MSW as a transportation fuel feedstock and simultaneously reduce externalities associated with land filling. It was calculated by Faraco and Hadar (2011) that if assuming between 60 and 90 % practical yields for ethanol production, the Mediterranean could produce between 17 and 25 billion liters per year of ethanol from 50 % of the 180 million tons of waste currently produced annually. The organic fraction of MSW contains lignocellulose in the form of waste paper products and food residues which could be an adequate raw material for ethanol production (Schmitt et al. 2012). For example, Li et al. (2012) achieved 53 % conversion of the cellulose and hemi-cellulose by enzymatic hydrolysis in buffer solution containing 6 wt % lignocellulosic MSW concentrate incubated at 40 °C for 12 h. An important parameter for successful hydrolysis was the particle size ranging 150–300 μm. Li et al. (2012) suggested that 1 ton of the fiber originated from MSW can produce 154 L of bioethanol.

In addition to bioethanol, energy can be produced from MSW via incineration or anaerobic digestion and production of CH_4-enriched biogas. Another useful product produced from the organic fraction on MSW is compost applied as soil conditioner.

2.5 Agricultural Residues

Agricultural crop residues include field residues and processing residues. Agricultural wastes are renewable and abundant resources. Field residues represent materials left in an agricultural field after harvesting the crop, and they include straw and stalks, leaves, and seed pods. Processing residues, such as husks, seeds, bagasse and roots, are those materials left after the processing of the crop into a usable resource. Harvesting of cereals, vegetables, and fruits generates huge amounts of crop residues.

Rice straw, wheat straw, corn stover, and sugarcane bagasse are the major agricultural wastes in terms of quantity of biomass availability (Kim and Dale 2004). Kim and Dale (2004) estimated that there are 73.9×10^6 ton of dry wasted crops in the world that could potentially produce 49.1 GL year^{-1} of bioethanol. About 1.5×10^9 ton year^{-1} of dry lignocellulosic biomass from seven crops is also available for conversion to bioethanol. The total potential bioethanol production from crop residues and wasted crops is 491 GL year^{-1} (Kim and Dale 2004).

Sugarcane is among the principal agricultural crops cultivated in tropical countries. Bagasse is the residue obtained from the sugarcane after it is crushed to obtain the juice used for sugar and ethanol production, thus it has an advantage over other crop residues as it is a by-product, already collected to the ethanol refinery and does not require special and costly collection and transportation. The annual world production of sugarcane is 1.6 billion tons, and it generates 279 million tons of biomass residues (bagasse and leaves). For the past three decades, bagasse and leaves have been explored for use in lignocellulosic bioconversion (Chandel et al. 2012). Theoretically, one ton of bagasse could yield up to 300 L of ethanol. In addition to ethanol, sugarcane bagasse has been successfully converted into many value-added products such as xylitol, organic acids, and industrial enzymes (Chandel et al. 2012).

In addition to the crops cultivated globally, some crops have local importance. Nevertheless, they can be very significant for the region economy and a substantial source of biomass for biofuel. An example is represented by olive groves (Faraco and Hadar 2011). Cultivation of olives in the Mediterranean results in huge amounts of lignocellulosic residues (olive tree pruning residues and olive mill solid waste).

Olive oil production represents one of the most important economic agro-food sectors in the Mediterranean Basin. Southern Europe (Spain, Italy, and Greece) is the world's largest producer of olive oil, accounting for 79 % of world olive oil

production in 2005, when they produced around 2 million tons of olive oil (UNCTAD 2007). The olive oil industry generates two downstream by-products, olive mill solid waste, and olive mill wastewater which pose environment hazard and pollution. Crude olive mill solid waste, the leftover solids following the pressing of olives, contains a mixture of skin, pulp, and seeds. It comprises approximately 35 % of the olives starting weight. The newer two-stage extraction technology significantly reduces the amount of wastewater produced, but generates a new type of waste, a solid residue called olive pulp (OP) resulting in approximately 8 million tons of OP generated per year in Southern Europe (UNCTAD 2007). At present, OP is either discarded to the environment or combusted with low economic value.

The use of all of the residues generated from both harvesting and processing of olives in the Mediterranean as raw materials for ethanol production could be of importance for the countries involved. Several authors analyzed the technological feasibility and potential of converting wastes from the olive industry to ethanol. Based on recent literature, Faraco and Hadar (2011) analyzed the feasibility and potential of olive trees pruning as well as olive mill waste as a source for fermentable sugars.

A major concern in the utilization of crop residues is the residue collection methodology and efficiency. According to Perlack et al. (2005), most operations are needed to pick up the residues left on the ground after the crops have been harvested. Collection of residues from these crops involves multiple passes of equipment over fields and results in removal of only 40 % of the biomass. Perlack et al. (2005) envisaged future residue collection technology with the potential of collecting up to 75 % of the residues. The equipment to be developed is expected to be single-pass system that would reduce costs by collecting the grain and residue together and reduce soil compaction. As with other raw materials, when discussing the use of crop residues as raw material for biofuel, their alternative uses should be considered. Of special importance is their use as soil conditioner and for increasing the levels of soil organic matter, with important effects on soil structure, preventing erosion, the supply of nutrients, acidification, and water-holding capacity of soils, all affecting soil fertility and health (Lal 2005; Recosky and Forcella 1998; Tarkalson et al. 2006; Wilhelm et al. 2004). These effects can be maintained harvesting only fraction of the residues or by returning the left-over from the processed biomass to the field. These residues are rich in lignin and also contain unreacted cellulose and hemicellulose (Mosier et al. 2005).

Besides ethanol, biodiesel, produced from plants, such as *Jatropha curcas*, or algae rich in lipids is considered an important future biofuel. Although biodiesel is not the focus of this paper, it should be mentioned that once the lipids have been extracted, the leftover solids are composed of mostly carbohydrates. These carbohydrates are potential substrates that could be fermented to produce ethanol. In such a case, several biofuels are produced from one biomass source (Jones and Mayfieldt 2012).

In conclusion, the differences and variation between the many crop residues, some of which available on local basis only and during different seasons, require the development of flexible conversion processes.

2.6 Cell Wall Engineering

Although the availability of renewable cellulosic feedstock is almost unlimited around the globe, the processing of lignocellulose into fermentable sugars and biofuel still requires expensive and harsh pretreatments, due to the high level crystallinity of the cellulose and cross linking of the carbohydrates with the lignin that form a barrier preventing efficient and economic biomass enzymatic digestion. Some pretreatments are not acceptable from environmental point of view. The advances in plant genetic engineering enable genetic modification of plants and this is widely used in commodity crops such as corn, soybean, and cotton. Safe and environmentally accepted genetic engineering technology can be used to modify cell wall structure and function and may provide a solution that will help to overcome the difficulty in utilization energy crops and trees. Limited number of environmental studies conducted so far, failed to indicate any strong effects of lignin-modified transgenic trees on the ecosystem (Pilate et al. 2012). Nevertheless, Nonic et al. (2012) reviewing the possible use of genetically modified trees in EU countries concluded that it is important to develop recommendations for the use of genetically engineered trees for forestry and plantations, taking into account socio-economic analyses as well as acceptance by the public. Indeed, as the cell wall structure and composition is providing the plant strength, defense mechanism against pathogens, and protection against other biotic and abiotic stress, the genetic modification of plant cell walls could unexpectedly lead to alteration of plant growth and development and result in harmful effects such as poor plant fitness (Jung et al. 2012b). Nevertheless, large efforts and investments are made in recent years toward this direction. The various possibilities for improvement of plant performance as feedstock for ethanol production were reviewed in recent years (Abramson et al. 2010; Xie and Peng 2011; Wang and Brummer 2012; Mizrachi et al. 2012; Jung et al. 2012b; Cook and Devoto 2011). In addition to modifying genes directly related to cell wall synthesis, another approach to increase the suitability of a crop as a feedstock is to increase biomass yield or increase plant productivity in general by affecting photosynthesis rate by over-expressing genes like phosphoenolpyruvate carboxylase, fructose-1, 6-bisphosphatase and sedoheptulose-1, 7-bisphosphatase (Lefebvre et al. 2005). Enhancement of trees growth and performance via genetic engineering for biomass production was reviewed by Harfouche et al. (2011).

The genes that can be modified include structural genes as well as transcription factors that function as positive or negative regulators of lignin or cellulose synthesis (Wang and Dixon 2012).

2.7 Targeting Lignin Biosynthesis

A prerequisite for lignin structure and function modification is deep understanding of its biosynthetic pathway and the ability to predict the outcome of alternation of any of the many genes involved in the process. Vanholme et al. (2012a) reviewed the current knowledge on lignin and its precursors' biosynthesis and discussed the characteristics of alternative lignin monomers and criteria to meet for the purpose of increased susceptibility of the biomass to the depolymerization. They described in detail several types of compounds that may be used as alternative monomers for lignin biosynthesis: monomers that directly produce a readily cleavable functionality in the polymer; hydrophilic monomers; monomer conjugates linked via a readily cleavable functionality; monomers that minimize lignin–polysaccharide cross linking and monomers that give rise to shorter lignin polymers (Vanholme et al. 2012a).

Eudes et al. (2012) described a strategy developed in Arabidopsis for the overproduction of rare lignin monomers to reduce lignin polymerization degree via incorporation of side-chain-truncated lignin monomers. In this work, the expression of the bacterial hydroxycinnamoyl-CoA hydratase-lyase (HCHL) in Arabidopsis was restricted to the lignifying tissues using a secondary cell wall-specific promoter, thus avoiding adverse phenotypes observed in previous studies. HCHL cleaves the propanoid side-chain of hydroxycinnamoyl-CoA lignin precursors to produce the corresponding hydroxybenzaldehydes so that plant stems expressing HCHL accumulate in their cell wall higher amounts of hydroxybenzaldehyde and hydroxybenzoate derivatives. Engineered plants did not show reduction in total lignin, sugar content, or biomass yield compared with wild-type plants. However, cell wall analyses revealed an increased amount of unusual C_6C_1 lignin monomers and lignin with a reduced molecular weight. These plants showed an increase in saccharification of pretreated stem cell walls (Eudes et al. 2012).

Jung et al. (2012a) achieved a reduction of the recalcitrance of sugarcane biomass by reducing lignin content via RNA interference suppression of lignin biosynthesis. Downregulation of the sugarcane caffeic acid *O*-methyltransferase (*COMT*) gene by 67–97 % reduced the lignin content by 3.9–13.7 %, respectively. The syringyl/guaiacyl ratio in the lignin was reduced from 1.47 in the wild type to values ranging between 1.27 and 0.79. The yields of fermentable glucose were 96 and 135 mg glucose g^{-1} in the wild type and the best transgenic plant, respectively, without pretreatment. After dilute acid pretreatment, the fermentable glucose yield was increased to 190 and 288 mg glucose g^{-1} in the wild type and the best transgenic plant, respectively. These observations demonstrate that a moderate reduction in lignin can reduce the recalcitrance of sugarcane biomass without compromising plant performance (Jung et al. 2012a).

The lignin content of a feedstock has been proposed as one of the key agronomic traits impacting biofuel production from lignocellulosic biomass. 4-Coumarate:coenzyme A ligase (4CL) is one of the key enzymes involved in the monolignol biosynthetic pathway. Xu et al. (2011) showed that silencing of 4CL in

switchgrass leads to reduced lignin content and improved fermentable sugar yields. RNA interference of *Pv4CL*1 reduced extractable 4CL activity by 80 %, leading to a reduction in lignin content with decreased guaiacyl unit composition. Altered lignification patterns in the transgenic plants did not compromise biomass yields. Dilute acid pretreatment of the low lignin transgenic biomass resulted in significant increase of 57 % in cellulose hydrolysis efficiency (Xu et al. 2011).

As was shown in the aforementioned examples, engineering of a specific gene in the lignin biosynthetic pathway can lead to positive results. However, lignin engineering may also results in profound metabolic consequences in the plant. Vanholme et al. (2010, 2012b) used a systems biology approach to study the response of the plant to lignin disruption. They studied 20 *Arabidopsis thaliana* mutants, each mutated in a single gene of the lignin biosynthetic pathway and analyzed them using transcriptomic and metabolomic approaches. By combining metabolomic and transcriptomic data in a correlation network, system-wide consequences of the perturbations were revealed and genes with a putative role in phenolic metabolism were identified. Together, these data provide insight into biosynthesis of lignin and the metabolic network it is embedded in, and provide a system view of the plant's response to pathway perturbations (Vanholme et al. 2012b). This system biology study was performed using the model plant *Arabidopsis*, rather than on a specific energy crop. However, it is reasonable to assume that it is feasible to translate cell wall research from *Arabidopsis* to commercial crops. In addition, the existing *Arabidopsis* mutant collections and natural accessions are the best available genetic bases to reveal, through systems biology, how mutations in cell wall recalcitrance genes affect biosynthesis in other metabolic and developmental processes—information that will be crucial for the rational design of bio-energy crops (Vanholme et al. 2010). Other examples are discussed by Pilate et al. (2012) with emphasis on the evaluation of performance of transgenic trees in field trial for assessing the effects of lignin modification on wood properties and trees physiology and performance. They suggest a threshold of about 20 % reduction in lignin content to avoid negative effects such as winter mortality of the transgenic trees. Evaluation under field conditions is of great importance for the identification of new directions to improve wood properties for applications such as pulp and paper manufacturing and ethanol production (Pilate et al. 2012).

2.8 Targeting Cellulose Structure and Function

Cellulose is naturally resistant to enzymatic hydrolysis. The chains of covalently linked glucose molecules form microfibrils which have a firmly condensed structure. Cellulose microfibrils are insoluble in water; therefore cellulolytic enzymes have a small surface area to act upon. Reducing the recalcitrance of cellulose to enzymatic hydrolysis into a fermentable form of sugar via decreasing

the crystallinity and increasing the accessibility into the glucan chain is the aim of many studies.

Overexpression of glycoside hydrolases was suggested as a mean to modulate the cell wall. The *Arabidopsis* endo-(1-4)-β-glucanase protein (Cel1) accumulates in young, expanding tissues, playing a key role in disruption of cell wall during cell elongation of rapidly growing tissues (Shani et al. 1997; Shani et al. 2004) Heterologous overexpression of *cel1* in poplar trees or of poplar endoglucanase (*PaPopCel1*) in *Arabidopsis* resulted in longer internodes, increased cell elongation, and subsequent biomass accumulation (Park et al. 2003; Shani et al. 2004). Mechanical analysis, studying leaf blade extension at constant load and breakage at changing load, was conducted. An elongation *versus* load curve demonstrated higher elongation rates in transgenic *Arabidopsis* leaf blades when compared to wild type, it was speculated that the cell wall of these transgenic plants contained less cross-linked polymers (Park et al. 2003). Similar results were obtained upon expression of *Aspergillus niger* xyloglucanase in poplar trees. Both stem length and cellulose content increased (Park et al. 2004).

In conclusion, overexpression of endoglucanases can enhance plant growth, but may also result in undesirable effects; therefore attempts should be made to use tissue-specific promoters for targeting the overexpressed gene.

Cellulose binding modules (CBM) expression is another mean to enhance cell wall biosynthesis. Plant growth and biomass can be increased by bacterial CBMs transgenically expressed in the cell wall (Shoseyov et al. 2006). Shoseyov et al. (2006) suggested that this effect is the result of separation of the cellulose-biosynthesis polymerization and crystallization steps. Accelerated cell and plant growth have also been observed in transgenic tobacco, poplar, and potato plants expressing a cell wall-targeted *Clostridium cellulovorans* CBM (Levy et al. 2002; Safra-Dassa et al. 2006). The role of CBM expression in increased plant growth rates could have been potential in yield enhancement and can be applied to many biofuel feedstocks. This genetic modification can change the carbon partitioning between source and sink tissues by creation of stronger sinks in cellulose synthesizing cells, leading to enhanced growth, biomass, and yield (Abramson et al. 2010).

Harris et al. (2009) genetically modified the cellulose synthase of *Arabidopsis* and reduced the crystallinity of cellulose and improved its biochemical conversion to fermentable sugars. In the mutant studied, a 34 % lower biomass crystallization index and 151 % improvement in the efficiency of conversion from raw biomass to fermentable sugars was measured, relative to that of the wild type. They later showed that the cellulose microfibril crystallinity was reduced by mutating the C-terminal transmembrane region residues of cellulose synthase (Harris et al. 2012).

Manipulation of cellulose synthase can result in negative effects. Joshi et al. (2011) studied the possibility of overexpression of an aspen secondary wall-associated cellulose synthase gene in transgenic aspen (*Populus tremuloides L.*) but they unexpectedly observed silencing of the transgene as well as its endogenous counterparts. The main axis of the transgenic aspen tree stopped growing, and weak branches showed a weeping growth pattern. The transgenic stems containing

reduced amounts of crystalline cellulose produced typical collapsed or irregular xylem vessels that had altered secondary wall morphology. These results demonstrate the fundamental role of secondary wall cellulose within the secondary xylem in maintaining the strength and structural integrity required to establish the vertical growth of trees. In another study, Hoenicka et al. (2012) studied the effect of overexpression of the flowering promoting factor 1 gene (*FPF*1) from *Arabidopsis* on wood formation in hybrid poplar. They found a strong effect on wood formation but no effect on flowering time. Wood density was lower in the transgenic plants, despite the significantly reduced vessel frequency which was compensated by thinner fiber cell walls. Chemical screening of the wood by pyrolysis GC/MS showed that *FPF*1 transgenic plants have higher fractions of cellulose and glucomannan products as well as lower lignin content.

As demonstrated above, cellulose and lignin biosynthetic pathways have been extensively studied. However, modifications of other wall matrix components during secondary growth have been the focus of only few studies, despite their importance as the third component of the secondary cell wall which is cross-linked to both lignin and cellulose (Cook and Devoto 2011; Park et al. 2004; Lee et al. 2009). For example, Bindschedler et al. (2007) studied the downregulation of UDP-glucuronate decarboxylase (the enzyme responsible for UDP-xylose synthesis) in transgenic tobacco. Several of the down-regulated antisense plants showed high glucose to xylose ratios in xylem walls due to less xylose-containing polymers. However, unexpectedly, this result did not lead to improvements in cellulose extractability. Goulao et al. (2011) observed differential expression of several genes involved in hemicellulose and pectin in xylem or phloem of stem regions undergoing secondary growth in *Eucalyptus globulus*. These results suggest that hemicellulose and pectin biochemistry in wood formation and architecture could be a target for gene manipulations. It seems that more attention should be given to the research in this direction.

2.9 Concluding Remarks

The growing demand for liquid fuel accelerates the research and applications in the areas of development and exploitation of cellulosic raw materials. It is estimated that as much as 1.3 billion dry tons of biomass could be produced annually in the US only, by 2030 (Perlack et al. 2005). If 90 % of the sugars derived from the biomass will be utilized for bioethanol production, it will be sufficient for approximately 130 billion gallons of cellulosic ethanol, equivalent on an energy basis to approximately 87 billion gallons of gasoline (Carroll and Somerville 2009). Beringer et al. (2011) calculated the global bioenergy potentials of biomass sources under environmental and agricultural constraints and concluded that they may provide between 130 and 270 EJ $year^{-1}$ in 2050, equivalent to 15–25 % of the World's future energy demand. The sources for cellulosic feedstocks are diverse and include dedicated bioenergy crops (tress and energy

grasses) and by-products and waste material (agricultural wastes, plant residues, and municipal solid waste). The use of agricultural wastes and by-products is of special importance, from environmental point of view, since it increases the efficiency of the utilization of the solar energy converted by crop plants without exploitation of additional natural resources such as land and water. Although the principles of utilization of plant biomass are ubiquitous, the choice of feedstock either plant species or waste material depends on local conditions and economy. These new potential markets for biofuels can provide new economic possibilities to rural and remote regions. However, a whole life-cycle assessment (LCA) is required to ensure that social and potential environmental problems in addition to financial performance are taken into account. Inconsistencies in the assumptions applied to biofuel LCA and lack of standardization may lead to variable and even conflicting estimates (Davis et al. 2009). Misleading estimates may impair the sustainability of cellulosic ethanol.

Despite the availability of the lignocellulosic biomass, the processing into fermentable sugars and to biofuel still requires expensive and harsh pretreatments, some of which are not acceptable from environmental point of view. This is the result of the high level crystallinity of the cellulose and cross linking of the carbohydrates with the lignin that form a barrier that prevents efficient enzymatic digestion. Development of improved analytical techniques and enquiring additional basic knowledge on the interrelationship among the major cell wall components, hemicellulose, lignin, pectin, and cellulose could be an important step toward improvement of energy crops (Jordan et al. 2012). The advances in plant genetic engineering enable genetic modification of plant cell wall structure and function and may provide a solution that will help to overcome the difficulty in utilization energy crops and trees. The major advances in crop plant genetic molecular biology may accelerate this trend, however, cell wall composition, structure, and function have not been the focus in the breeding and molecular studies of most food crops, where the focus is on yield quantity and quality and resistance to pests, thus much basic knowledge is missing in that area and can be a fertile topic for research (Carroll and Somerville 2009). However, this should be done with caution as designing plants for improved degradability may result in adverse effects on plant development and survival. The ultimate goal should be “matching optimized feedstock traits (phenotypes) with low-input processing technology” (Ellis 2012). Using genetic engineering technologies will require caution to avoid nondesirable traits and should also address ecological concerns.

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Chapter 3
The Pretreatment Step in Lignocellulosic Biomass Conversion: Current Systems and New Biological Systems

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Abstract The demand for liquid fuels is constantly increasing, and ethanol from lignocellulosic biomass might be one of the most important solutions for this problem. Although biomass may be cheap, the costs of processing it can be high. Many technologies for converting biomass into bioethanol have been developed, which include the pretreatment of biomass, enzymatic saccharification of the pretreated biomass, and fermentation of the hexose and pentose sugars released by hydrolysis, and saccharification. In this chapter, the most frequently used and new biological pretreatment methods of lignocellulosic biomass are discussed. The common initial pretreatment steps of a lignocellulosic material such as drying, grinding, and granulometric classification are presented, and then the thermo-chemical and biological treatments including biopulping and enzymatic pretreatment are also discussed.

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3.1 Introduction

A major problem in the processing of lignocellulosic materials is the natural resistance of these materials to the conversion process required to generate fermentable sugars due to the presence of lignin and the degree of crystallinity of cellulose.

A suitable pretreatment of biomass is essential to the success of subsequent steps of saccharification and fermentation; the goal is to break the lignin seal and disrupt the crystalline structure of cellulose (Mosier et al. 2005a). Pretreatment is responsible to separate the components of the lignocellulosic biomass, reducing the crystallinity of the material, making the cellulose accessible, and removing the lignin (Sun and Cheng 2002).

Van Dyk and Pletschke (2012) reported the most important factors limiting hydrolysis of the biomass by cellulolytic and hemicellulolytic enzymes, providing evidence that lignin has an effect on hydrolysis and a correlation between the percentage of lignin and release of sugars from lignocellulose substrates can be established. Several reasons have been proposed to explain how the presence of lignin reduces hydrolysis: the lignin provides a physical barrier which limits accessibility of cellulases or hemicellulases to their substrate; the cellulases become non-specifically adsorbed to the lignin which reduces the productive hydrolysis of the substrate; lignin may also directly inhibit the hydrolytic enzymes; not only just the presence of lignin, but its type and distribution have an impact on enzymatic hydrolysis.

The definition of the best pretreatment and the conditions under which it occurs depend on the type of lignocellulosic material. The main types of pretreatment include the thermo-chemical methods, such as steam explosion, followed by chemical methods with alkalis or acids, and biological method, with enzymes or whole cells.

Physical treatments such as drying, grinding, and granulometric classification are initial steps common to most processes that involve the use of lignocellulosic biomass. These are essential steps for the standardization of the material, ensuring that the particle size, flow, and reaction properties are the same for the same kind of raw material.

Thermo-chemical methods cause the disruption of the material's structure, degradation of hemicellulose and cellulose and lignin transformation, thus facilitating the subsequent hydrolysis of cellulose. Steam explosion, alkali washing, and dilute acid hydrolysis are some thermo-chemical methods for pretreating and hydrolyzing lignocellulosic material. Steam explosion causes the material explosion due to the high temperature and pressure. Acid hydrolysis is the most commonly used pretreatment method, and sulfuric acid is the most frequently used acid, but other acids, such as hydrochloric, phosphoric, and nitric, are also reported. Organic or aqueous-organic solvents as well as catalysts, such as oxalic, salicylic, and acetylsalicylic acids, can be used in the organosolv pretreatment of lignocellulosic materials at temperatures of 150–200 °C. A variety of organic solvents, such as alcohols, esters, ketones, glycols, organic acids, phenols, and ethers, have been used.

Microbial treatment is also capable of lignin removal, but it requires longer retention times than thermo-chemical methods. Enzymatic treatments, which take only a few hours and are very selective, are therefore more suitable than fungal treatments.

This chapter discusses traditional methods for pretreatment of lignocellulosic materials, and new, in-development methods which aim to improve lignin removal from lignocellulosic biomasses.

3.2 Physical Treatments

All materials used as substrate for biotechnological processes need a pretreatment in order to adapt the material to the use and to give a homogenous and linear process response. Among the physical pretreatment steps the most important are drying, grinding, and size classification operations discussed as follow.

3.2.1 Drying and Concentration

Drying is the removal of a volatile substance, usually water, from a solid material to a gaseous unsaturated phase through thermal evaporation. The moisture content

of a material (its humidity) is associated to its water activity (a_w). Drying reduces a_w, thus preventing deterioration, microbial growth, chemical redox processes, and reducing enzymatic activity. Drying also reduces the volume and weight of the material, thus reducing transportation, packing, and storage costs (Coulson and Richardson 1991; Pessoa and Kilikian 2005).

For agro industrial residues, drying is frequently adopted as a pretreatment: residues with high water activity, such as brewer′s spent grain, grape skins, or citrus peels must have their humidity reduced for posterior treatment, or processing to recover directly fermentable sugar or any other component of interest; if the material is to be grinded, drying is still necessary so that it attains an adequate hardness (dry fibers are less flexible and more brittle than dry materials).

Part of the liquid content of a residue may be removed by mechanical processes such as centrifugation or pressing. In order to remove the remainder humidity, thermal drying methods are essential. In these operations, the vaporization happens below the boiling point of water; it is a complex process that involves simultaneous heat and mass transfer, resulting in significant changes in the physical, chemical, and structural properties. When heating the water, loss can cause cellular structural stress, microstructure alteration, producing or increasing porosity, and material shrinkage (Laopoolkit and Suwannaporn 2011).

Moisture of biomass differs largely with the crop, part of the plant, harvest season, and harvest stage as well as the processing raw material technology. For example, Shinners and Binversie (2007), studying corn biomass residues, found that when the harvested corn grain moisture was less than 30 %, the total stalk moisture ranged from 66 to 47 %, depending on the part of the stalk. The whole stalk moisture ranged from 69 to 56 %, while the moisture was from 63 to 45 % for the top-three-quarters, 52 to 32 % for the top-half, and 36 to 27 % for the top-quarter of the stalk. This is economically important: in order to transport 1 tonne of dry solids with 69 % humidity, 2.23 extra tonnes of water is transported, while the biomass with 56 % humidity carries 1.27 tonnes of water.

If the weather is adequate, a good option for crop residues is to partially dry it on the field before baling and transporting, although this procedure can in some cases increase microbiological charge and/or cause partial residue degradation. For residues produced in the industry, such as sugarcane bagasse or palm empty fruit bunches, the drying may be done using flue gases or steam from the plant (Hasibuan and Daud 2004; Sosa-Arnao et al. 2004). For these operations, the driers are usually either drum or pneumatic driers.

3.2.2 Grinding Process

Grinding is a unit operation of size particle reduction, where the size of the solid material is reduced by impact, compression or shear. The advantages of the particle size reduction include: (1) increasing the ratio surface/volume; (2) standardizing the

particle size, improving the homogeneity of the substrate (McCabe et al. 1993; Gauto and Rosa 2011). Large pieces of biomass are first triturated with crushers, producing relatively large particles which are then grinded or milled, producing smaller particles and fine dust (Gauto and Rosa 2011; Coulson and Richardson 1991).

Particle uniformity in terms of size will provide a uniform process, as far as fermentative process and hydrolysis processes are concerned; even chemical and biochemical reactions that can be considered surface phenomena, depend on the surface area available to the microorganism activity (Izumi et al. 2010; Hendriks and Zeeman 2009).

Substrate morphology and size affect surface area and so the chemical/enzymatic accessibility to the material (Santos et al. 2005). In general, the smaller the particles, the better are the reactions yields. However, there are serious restrictions to this rule and a limit to decrease material particle size. Solid medium must have a proper granulometry because very small particles can cause clumping and clogging of the fermentative medium, affecting negatively gaseous changes (respiration/aeration), heat and gases dissipation, microbial growth, and the process yield.

Kumar et al. (2003) tested three granulometries of sugarcane bagasse for citric acid production; they found the lowest production with particles between 0.64–1.2 mm; the best production was reached with particles between 1.2–1.6 mm and with particle of 1.6–2.0 mm the production was lower. Yuan et al. (2011) working with grinded wheat stem in granulometries around 1.0, 5.0, and 10 mm at the production of hydrogen, acetate, and butyrate, found the best results with the smaller particles.

3.2.3 Granulometric Separation

After drying and reduction in size, the material classification in different granulometries is required to achieve a better material homogenization and to ensure less influence of this parameter in the future process. The simplest and most common method is the mechanical separation that consists of passing the material through a series of sieves with meshes progressively smaller, under vibration. Fractions classified according to the meshes of the screen are obtained; particles larger than the desired size may be recycled to the mill, while very small particles may be separated for other processes such as burning. The medium size of particles with regular shape (i.e.: sphere, cylinder, and others) is determined according to their geometric parameters (i.e.: length, height, diameter, depending on the case). From these data it is possible to build a granulometric distribution curve of each milling process (Gomide 1983) and adapt the equipment for better efficiency. In lab scale, a set of sieves separates efficiently the fractions of a milled material. In industrial scale, however, a continuous sieving process must be used.

Table 3.1 Hemicellulose (HCel), Cellulose (Cel) and Lignin (Lig) fates in steam explosion pretreatment of biomasses

Biomass	P (atm)	T (°C)	Water content	Catalyst (% g/g of water)	Time min	Yield (%)			Reference
						HCel	Cel	Lig	
Sugarcane bagasse	13	190	50 %	No	15	82.7	11.8	7.9	Rocha et al. (2012)
Wheat straw	10	180	nd	Acid (0.9 % H_2SO_4)	10	85	25	nd	Ballesteros et al. (2010)
Pinus patula	25	225	nd	3 % SO_2	5	98.5	NS	MNS	Chacha et al. (2011)
Eucalyptus	20	210	10.6	No	4	81.4	NS	MNS	Supantamart et al. (2009)
Switchgrass	10	180	10	5 % SO_2		75	6	22	Garlock et al. (2011)

NS not solubilized; *MNS* modified, not solubilized; *nd* not determined; SO_2

3.3 Thermo-Chemical Treatments

Many thermo-chemical methods have been used for pretreating and hydrolyzing agroindustrial lignocellulosic wastes. The most important thermo-chemical methods are steam explosion, alkali or acid treatment, and organosolv. The aim of the pretreatment is to expose the carbohydrates (cellulose and hemicellulose) to be hydrolyzed through the mechanical expansion of the fiber or through digestion of the binding material such as lignin.

3.3.1 Steam Explosion

Steam explosion is one of the most studied methods for the pretreatment of lignocellulosic biomass, using vapor and, in some cases, a catalyst (alkali or acid) (Soccol et al. 2011). The grinded biomass is submitted to high-pressure saturated steam, at temperatures varying from 160 to 260 °C and in pressures from 0.69 to 4.83 MPa; after a short period of time, usually ranging from 2 to 30 min, the reactor is suddenly decompressed, which makes the material undergo an explosion. The process causes the disruption of the material's structure and the degradation of hemicellulose, cellulose, and lignin due to the high temperature and the material expansion, thus facilitating the subsequent hydrolysis of cellulose (Öhgren et al. 2007). Table 3.1 shows some examples of steam explosion pretreatment of biomasses:

Although harsher conditions guarantee a higher depolymerization of the biomass, these can cause the formation of possibly toxic or fermentation-inhibiting molecules such as furfural and hydroxymethyl furfural; both incubation times and temperatures are directly proportional to the amount of these by-products. One way of comparing different treatments is to use a severity index or factor, which

combines temperature and time in a single factor $R_0 = \exp(T\text{-}100/14.75)*t$, where T is the temperature (in degrees Celsius) and t is the time (in seconds) (Heitz et al. 1987)

3.3.2 Alkaline Pretreatment

Alkaline pretreatment has received a lot of attention lately because it can remove lignin from biomass, thus improving the reactivity of the remaining polysaccharides and removing acetyl groups and various uronic acid substitutions on hemicellulose (Chen et al. 2011). It is mainly used to recover cellulose partially degraded. The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin, and hemicelluloses. Dilute NaOH treatment of lignocellulosic material causes swelling, leading to an increase of material internal surface area, a decrease in the degree of polymerization, a decrease in the cellulose crystallinity degree, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Soccol et al. 2011; Fan et al. 1987).

3.3.3 Acid Hydrolysis

Dilute acid hydrolysis can be an effective thermo-chemical pretreatment process to improve lignin separation and it is considered an efficient method to produce reducing sugars from hemicelluloses and cellulose. Sulfuric acid is the most commonly used acid in pretreatment and hydrolysis (Lavarack and Griffin 2002) but other reagents, such as hydrochloric, nitric, and phosphoric acids can also be used (Gámez et al. 2006; Rodríguez-Chong et al. 2004). The use of acetic acid and hydrogen peroxide has been reported by Tan et al. (2010) as a method for removing lignin prior to bagasse enzymatic hydrolysis.

According to Chen et al. (2011), pretreatment using dilute sulfuric acid has been considered as one of the most cost-effective methods. The mixture of biomass and dilute acid solution is usually controlled at a moderate temperature by means of conventional heating or microwave-assisted heating, which is another effective route to pretreat biomass. During hot acid pretreatment, some of the polysaccharides are hydrolyzed, mostly hemicelluloses. The resulting free sugars can be degraded to furfural (from pentoses) and to 5-hydroxy-methyl-furfural (HMF) (from hexoses) mainly at very high temperatures. These compounds are inhibitory for microorganisms, and their production means loss of fermentable sugars. Organic acids such as maleic and fumaric have been suggested as alternatives to avoid HMF formation (Kootstra et al. 2009).

Although in acid hydrolysis the use of low concentration is the rule, unusual conditions may prove efficient. For example, Sun et al. (2011) used very high

concentrations of sulfuric acid in a continuous, three-step hydrolysis of bamboo biomass, recovering up to 81.6 of the sugars and 90.5 % of the acid used. Another unusual strategy is to use very low acid concentration (0.07 % sulfuric acid, for example) and high temperature (225 °C) in percolation reactors. Using this technique, Torget et al. (2000) hydrolyzed up to 90 and 99 % of the cellulose from prehydrolyzed poplar wood in 10 and 20 min, respectively; the prehydrolysis consisted in removing hemicellulose and amorphous cellulose through a previous acid hydrolysis, with the same acid concentration but at 175 °C, leaving crystalline cellulose and lignin

3.3.4 Organosolv

This method consists in the use of an organic liquid and water, with or without the addition of a catalyst (acid or alkali). Organosolv pretreatments efficiently remove lignin from lignocellulosic materials through the partial hydrolysis of lignin bonds, resulting in a pulp enriched in cellulose. The addition of a catalyst can enhance the selectivity of the solvent with respect to lignin. Most of the hemicellulose sugars are also solubilized by this process (Mesa et al. 2011; Sun and Cheng 2002). This technique presents advantages when compared with aqueous-based processes. In particular, the recovery of lignins and polyoses from the liquor is easily performed by distillation with the simultaneous recycling of solvents (Novo et al. 2011). Optimization may lead to efficient lignin removal: Astner (2012) used a Taguchi design to treated switchgrass and poplar mixtures (9:1 m/m) with water:ethanol:methyl isobutyl ketone (50:34:16) acidified with sulfuric acid (up to 0.1 M), obtaining concentrated cellulose, hemicellulose sugars, and lignin fractions at 120–140 °C; the lignin content in the organic solvent fraction was of 92 %, and the recovery near 100 %. The posterior conversion of cellulose through enzymatic or acid hydrolysis is facilitated: from biomass of *Pinus radiata*, Araque et al. (2008) obtained up to 99.5 % of the theoretical ethanol fermentation yield, after an organosolv pretreatment using 50 % acetone in water at 195 °C and pH 2 for 10 min.

3.3.5 Ammonia Fiber Expansion

Ammonia fiber expansion (AFEX) is a process in which liquid ammonia is added to the biomass under moderate pressure (0.7 to 27 MPa) and temperature (70–200 °C) before rapidly releasing the pressure (Bals et al. 2010). This process decrystallizes the cellulose, hydrolyses hemicellulose, removes and depolymerises lignin, and increases the size and number of micropores in the cell wall, thereby significantly increasing the rate of enzymatic hydrolysis (Mosier et al. 2005a).

As reported by Krishnan et al. (2010), the AFEX pretreatment improved the accessibility of cellulose and hemicelluloses in bagasse during enzymatic

hydrolysis by breaking down the ester linkages and other lignin carbohydrate complex bonds. The maximum glucan conversion of the AFEX pretreated bagasse and cane leaf residue by cellulases was approximately 85 %, and the supplementation with hemicellulases during enzymatic hydrolysis improved the xylan conversion to 95–98 %. Similarly, Dale et al. (1996) showed that AFEX at 90 °C, 1:1NH_3, and 15–30 % water improves enzymatic hydrolysis of corn fiber, ryegrass straw, and switchgrass 4–5 times, ensuring the achievement of high sugar conversions with the same enzyme load, or the reduction of enzyme use; the initial hydrolysis is very rapid, with 80 % of the sugar yield being achieved in 5–10 h.

3.3.6 Liquid Hot Water

Liquid hot water process (LHW) is a biomass thermal treatment where only water without any chemical is used. Even without a chemical catalyst addition, the pH of the solution may turn into acid or basic due to the different chemical composition of the biomass used. Compared to the other thermal-chemical pretreatments, hydrothermal presents the advantage of not using chemicals, enhancing the hemicellulosic sugar recovery with low costs. Lignin solubilization in hot water, at temperatures above 200 °C, is reported, but because of its high reactivity degree, recondensation reactions occur causing its rapid precipitation. Anyway, an efficient structure modification takes place, facilitating further lignin removal and enzymatic cellulose hydrolysis. Comparing different pretreatment technologies and different solvents to the retreatment of rye straw, Wörmeyer et al. (2011) and Ingram et al. (2011) found that LHW (water at 260 °C, 10:1 volume:biomass) extracted lignin of better quality than aquasolve (LHW at 200 °C followed by enzymatic digestion of the carbohydrates linked to lignin) and organosolv. As for the biomass left after extraction of the lignin, the treatment ensures and easier access for hydrolysis and less toxicity of the hydrolysate: Mosier et al. (2005b), treated corn stover with LHW at 190 °C for 15 min, obtaining a biomass suitable for slow enzyme hydrolysis and posterior fermentation, with a conversion of 90 % of the cellulose into glucose.

3.4 Biological Treatments

Effluents from chemical pretreatment of lignocellulosic biomass conversion contain substances reported to be toxic and mutagenic (Herpoël et al. 2002). These environmental considerations gave rise to intensive investigations on biological treatments of lignocellulosic biomass in order to reduce substantially the need for chemical substances.

3.4.1 Ligninolytic Abilities of Fungi

Lignin decomposition in nature is primarily attributed to the metabolism of white-rot basidiomycetes, since they degrade lignin more rapidly and extensively than other microorganisms (Falcón et al. 1995). These fungi produce several ligninolytic enzymes (laccases, manganese peroxidases, and lignin peroxidases) that catalyze the oxidation of an array of aromatic substrates, producing aromatic radicals, and altering the microstructure of the vegetable cell walls (Giardina et al. 2000, 2010; Maijala et al. 2008). Some white-rot fungi that preferentially attack lignin more readily than hemicellulose and cellulose are *Ceriporiopsis subvermispora*, *Phellinus pini*, *Phlebia* spp., *Pleurotus* spp,. and *Phanerochaete chrysosporium*. The latter has been shown to successfully biopulp wood chips without the need of autoclaving or nutritional enrichment (Wong 2009; Pandey et al. 2000). Many white-rot fungi, however, exhibit a pattern of simultaneous decay characterized by degradation of all cell wall components. Examples of this group include *Trametes versicolor*, *Heterobasidion annosum,* and *Irpex lacteus* (Wong 2009). Lignin degradation is mainly attributed to the secondary metabolism or to restricted availability of nitrogen, carbon or sulphur, and it is normally not degraded as sole carbon and energy sources (Silva et al. 2010).

There are four major groups of ligninolytic enzymes produced by the white-rot fungi: lignin peroxidase (LiP; 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductase; EC 1.11.1.14), manganese dependent peroxidase (MnP; Mn(II):hydrogen-peroxide oxidoreductase or manganese peroxidase; EC 1.11.1.13), versatile peroxidase (VP; EC 1.11.1.16), and laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2). However, the process of lignin biodegradation can be further enhanced by the action of other enzymes such as glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (veratryl alcohol oxidase; EC 1.1.3.7), pyranose 2-oxidase (glucose 1-oxidase; EC 1.1.3.4), cellobiose/quinone oxidoreductase (EC 1.1.5.1), and cellobiose dehydrogenase (EC 1.1.99.18) (Wong 2009).

3.4.1.1 Biopulping

Pulping is one of the steps of the refining process of lignocellulosic biomass, whose aim is to recover cellulosic fibers from the integral biomass. The pulping process is usually performed by mechanical and/or chemical treatments that selectively separate lignin and hemicelluloses and preserve the cellulose molecules (Llano et al. 2012; Simão et al. 2011). Pulp can be obtained not only from woody biomass but also from agro industrial residues.

Biopulping is the process of treating lignocellulosic materials with microorganisms, usually prior to the mechanical/chemical pulping. According to Maijala et al. (2008), this pretreatment reduces energy demands and improves the quality of the pulp. Ferraz et al. (2008) pointed out that the extent of lignin removal during fungal pretreatment is not related to the energy savings in biomechanical pulping

or to the increase in delignification rates observed in organosolv and kraft pulping. These benefits would be more related to the depolymerization of lignin during the initial stages of biotreatment and also to the esterification of oxalate produced by the fungi from the fibers. For industrial applications, biopulping is still considered a slow process since it can demand many days or weeks. Nevertheless, beneficial effects of biopulping are mainly obtained at the early stages when weight losses are below 5 %, usually during the first week, when the biomass becomes softer and easier to disrupt (Ferraz et al. 2008).

Biopulping has been also defined as the solid state fermentation of wood chips (Pandey et al. 2000). Solid state fermentation is an interesting process to perform biological delignification because it mimics the natural environment of lignin-degrading fungi. The guiding principles to design solid-state fermenters for biological delignification should be, first, to provide optimum conditions for the activity of the fungus through effective mixing, heat removal, oxygen, and water supply and, second, to keep the equipment as simple and inexpensive as possible (Reid 1989; Ali and Zulkali 2011). High moisture content (around 55–60 %) should be maintained during the biotreatment step to ensure an optimal colonization of the biomass by the fungal hyphae, and the degree of asepsis should be consistent with the resistance of the fungus against contamination (Ferraz et al. 2008).

Yaghoubi et al. (2008) developed a process for the biochemical pulping of rice, wheat, and barley straws by *Ceriporiopsis subvermispora*. The raw materials were chopped to 0.5–2.0 cm pieces, sterilized, humidified with a nutrient solution and corn steep liquor and incubated in the presence of *C. subvermispora*. After the biotreatment the straws were cooked in the presence of Kraft liquor and the resulting pulp was used as raw material for paper making. For the biopulping process, nine variables (fungus/substrate—5:1000/5:100 g per kg; liquid volume—120/200 mL per 40 g; pH—3.5/6.0; incubation time—12/18 days; incubation temperature—25/30 °C; nutrients solution's volume—0.2/1.5 mL per 40 g; corn steep liquor—0.4/4 g per 40 g; cooking time—20/30 min and chemicals/substrate percentage—5/8) were screened by a Plackett–Burman design on the basis of their effects on the final kappa number, which represents the residual lignin content (mL of 0.1 N potassium permanganate solution per gram of moisture-free pulp). Significant variables were found to be liquid volume, incubation temperature, cooking time, and chemicals/substrate ratio, and their optimized values were 100 mL per 30 g, 35 °C, 19 min and 2 % (w/w), respectively. Other variables were fixed at: fungus/substrate 6:1000 g per kg; pH 5.0; incubation time 18 days; nutrients solution 1.0 mL per 30 g and CSL 3.5 g per 30 g. The biological treatment of rice, wheat, and barley straws decreased the kappa number by 34, 21, and 19 %, respectively, compared to the control samples. Moreover, the tensile index (tensile strength in N m^{-1} per basis weight in g m^{-2}) and burst factor (bursting strength in g cm^{-2} per grammage in g m^{-2}) of hand sheets produced from the treated materials were improved by 37–62 %, and by 33–45 %, respectively, when compared to the control straws.

Giles et al. (2011) studied the two-stage fungal biopulping of wood prior to enzymatic hydrolysis to produce soluble glucose. *Liriodendron tulipifera* wood chips were exposed to liquid culture suspensions of *C. subvermispora* and *Postia placenta* (a brown rot fungus) and incubated at 28 °C, for 30 days when cultivated separately at the same time or for 60 days when cultivated successively in the same substrate. Treatments consisting of *C. subvermispora* followed by *P. placenta* exhibited 6 ± 0.5 % mass loss and increased the yield of enzymatic hydrolysis by 67–119 %. Similar results were obtained by the same research group when the biopulping process was conducted in aerobic polypropylene spawn bags as experimental bioreactors. However, the biotreated wood did not exhibit significant increases in soluble sugars after enzymatic hydrolysis of the ground material (40 mesh) using a 1:1.75 mixture of cellulase and β-glucosidase (22 FPU/g substrate), with a 3 % biomass loading volume, incubated at 50 °C for 72 h. Authors suggested, as a hypothesis, that the inoculation using nitrogen rich medium may have inhibited lignin-specific fungal hydrolysis of the wood (Giles et al. 2012).

Ferraz et al. (2008) reported the results of a large-scale biopulping process of *Eucalyptus grandis* by *C. subvermispora* in a 50-tonne chip pile. After 60 days of biodegradation, the wood chips were refined on a mill-scale by using a two-stage thermo-mechanical process. The average energy consumption for producing thermo-mechanical pulps with 450–470 Canadian Standard Freeness (CSF) was 913 and 745 kWh/tonne for control and biotreated wood chips, respectively. In the case of thermo-chemical mechanical pulps with similar CSF, energy consumption was 1,038 and 756 kWh/tonne for control and biotreated wood chips, respectively.

3.4.1.2 Fungal Delignification

Kerem et al. (1992) compared the fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium* in lignocellulose degradation during solid-state fermentation on cotton stalks. They reported that growth of *P. chrysosporium* on cotton stalks resulted in the disappearance of 55 % of the initial organic matter within 15 days of fermentation, whilst the lignin loss amounted to 35 % of the original amount. Growth of *P. ostreatus* resulted in the disappearance of only 20 % of original organic matter, whilst the lignin loss amounted to 45 % of the original amount.

Li et al. (2001) analyzed compositional changes of cottonseed hull substrate during *P. ostreatus* growth. Lignin was primarily degraded in the spawn run and primordial formation period, and little was degraded after this time period. After 45 days of incubation, lignin content decreased from an initial 17 % (as % of dry matter) to a final of 11 %.

Fackler et al. (2006) evaluated the delignification kinetics of *Dichomitus squalens*, *Phlebia brevispora, Phlebia radiate, Phlebia tremellosa*, three strains of *Ceriporiopsis subvermispora*, and also of the white rot ascomycete *Hypoxylon fragiforme* and the basidiomycete *Oxyporus latemarginatus* for a period of 2 weeks. The amount of delignification achieved by the selected white rot fungi

ranged from 7.2 % for *C. subvermispora* to 2.5 % for *P. radiata*. Reid (1989) estimated a cost of C\$ 720 per batch for a hypothetical process to delignify 10 tons of aspen wood. The duration of the batch was considered as 8 weeks and the highest cost was attributed to the wood (C\$56 per ton).

Li et al. (2008) analyzed delignification of wheat straw by *Fusarium concolor* and observed removal of 13.07 % of the lignin and loss of 7.62 % of the holocellulose by fungal degradation after 5 days of incubation. The degradation of lignin continued slowly with a loss of 65 % after 14 days of incubation.

Gupta et al. (2011) reported fungal delignification of lignocellulosic biomass (*Prosopis juliflora/Lantana camara*) by *Pycnoporus cinnabarinus*, demonstrating that it improves the saccharification of cellulosics. The fungus degraded a higher amount of lignin in *P. juliflora* (13.13 %) than in *L. camara* (8.87 %). The fungal delignification in both the substrates was higher during the first 15 days, and thereafter no significant improvement in lignin degradation was observed. Moreover, an increment of 21.1–25.1 % sugar release was obtained when fungal treated substrates were enzymatically hydrolysed as compared to the hydrolysis of unfermented substrates.

The biotreatment of sugarcane bagasse developed by Karp et al. (2012) using *P. ostreatus* cultivated in solid state fermentation reduced the lignin content from 31.89 to 26.36 % after 5 days and to 20.79 % after 15 days.

3.4.2 Enzymatic Pretreatment

Although several basidiomycetes strains were shown to be able to reduce the lignin content of lignocellulosic biomass, as reported above, the rate of fungal delignification was too low for industrial use (Reid and Paice 1994; Kondo 1995). Enzymatic treatments, which take only a few hours and are very selective, are therefore more suitable than fungal treatments. Several studies have demonstrated the effectiveness of ligninolytic fungal enzymes to delignify cellulosic biomass, as described below.

3.4.2.1 Laccases

Laccases are blue multicopper oxidases able to oxidize a variety of phenolic compounds including polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, with concomitant reduction of molecular oxygen to water (Dwivedi et al. 2011). They oxidize phenols and phenolic lignin substructures by one-electron abstraction with formation of radicals that can re-polymerize or lead to depolymerization (Higuchi 1989). These enzymes have been found to oxidize also non-phenolic compounds in the presence of a mediator (e.g., 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate or ABTS) (Wong 2009). Because of these characteristics, laccases can be employed for delignification and removal

of phenolic compounds in several areas, as biofuels, food products, pulp and paper, textiles, nanobiotechnology, soil bioremediation, synthetic chemistry, and cosmetics (Couto and Herrera 2006).

The overall reaction catalyzed by these phenoloxidases is 4 benzenediol + O_2 $\leftrightarrow$ 4 benzosemiquinone + $2H_2O$ (Wong 2009). The laccase molecule is a monomeric, dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites named T1, T2, and T3. In the resting enzymes, all four coppers are in the 2^+ oxidation state (Couto and Herrera 2006; Wong 2009). Both intracellular and extracellular isoenzymes may be produced from a single organism. The monomeric proteins have a molecular mass ranging from 50 to 110 kDa (Thurston 1994).

The highest amounts of laccases are produced by white-rot fungi. Fungal laccases are secreted into the medium by the mycelium of filamentous fungi (Couto and Toca-Herrera 2007). Examples of microorganisms that produce laccase with high activity are *Trametes pubescens* (740,000 U/L) (Galhaup et al. 2002), *Coriolus hirsutus* (83,830 U/L) (Koroleva et al. 2002), *Trametes hirsuta* (19,400 U/L) (Rodríguez-Couto et al. 2006), *Trametes versicolor* (16,000 U/L) (Font et al. 2003), *Pycnoporus cinnabarinus* (10,000 U/L) (Meza et al. 2006), *Neurospora crassa* (10,000 U/L) (Luke and Burton 2001) and *Pleurotus ostreatus* (80,000 U/L) (Lettera et al. 2011).

3.4.2.2 Lignin Peroxidases, Manganese Dependent Peroxidases and Versatile Peroxidases

Besides laccases, other important enzymes that can be used for delignification are lignin peroxidases, manganese dependent peroxidases, and versatile peroxidases. Both LiP and MnP belong to the class of peroxidases that oxidize their substrates by two consecutive one-electron oxidation steps with intermediate cation radical formation. Due to its high redox potential, the preferred substrates for LiP are nonphenolic methoxyl-substituted lignin subunits and the oxidation occurs in the presence of H_2O_2 (Tuor et al. 1995; Wong et al. 2009) whereas MnP acts exclusively as a phenol oxidase on phenolic substrates using Mn^{2+}/Mn^{3+} as an intermediate redox couple (Tuor et al. 1995). Versatile peroxidases are a group of enzymes, primarily recognized as manganese peroxidases, which exhibit activities on aromatic substrates similar to that of LiP. These enzymes are not only specific for Mn(II), but also oxidize phenolic and non-phenolic substrates that are typical for LiP, including veratryl alcohol, methoxybenzenes, and lignin model compounds in the absence of manganese (Wong 2009).

Significant amounts of these enzymes have been produced by submerged fermentation and solid state fermentation. Some examples are lignin peroxidase (75,376 U/L) and manganese peroxidase (4,484 U/L) produced by the fungus *Mucor racemosus* in submerged culture (Bonugli-Santos et al. 2010); lignin peroxidase (2,600 U/L) and manganese peroxidase (1,375 U/L) produced by *Phanerochaete chrysosporium* in solid state fermentation of steam exploded wheat

straw (Fujian et al. 2001); and versatile peroxidase (7,300 U/L) produced by genetically modified *Pleurotus ostreatus* in submerged fermentation (Tsukihara et al. 2006).

3.4.2.3 Enzymatic Delignification

Herpöel et al. (2002) investigated wheat straw pulp after combined treatment with commercial xylanases and laccases from *Pycnoporus cinnabarinus* followed by an alkaline extraction carried out at 10 % consistency at a temperature of 70 °C for 90 min. The rate of sodium hydroxide applied was 2.5 % on a dry pulp basis. After each stage, pulp was filtered and washed with distilled water. Pulp treated as described above without enzymes was used for control. Xylanases are already used for enzyme-aided bleaching in several mills, allowing improvements in the subsequent chemical delignification (Viikari et al. 1994). New sequential treatments combining xylanase and laccase mediator system in two steps were developed followed by alkaline treatment that were effective to remove 60 % of lignin in wheat straw pulp (Herpoël et al. 2002).

Gutiérrez et al. (2012) evaluated the delignification of wood (*Eucalyptus globulus*) and non-wood (*Pennisetum purpureum*) feedstocks using laccase from *Trametes villosa*, with 1-hydroxybenzotriazole (HBT) as mediator and alkaline extraction. 48 and 32 % of the lignin from *E. globulus* and *P. purpureum* were removed, respectively, when using 50 U/g laccase and 2.5 % HBT. The enzymatic pretreatment (25 U/g) increased the glucose and ethanol yields by 61 and 12 % in 72 h and by 4 and 2 g/L in 17 h for both *E. globulus* and *P. purpureum*, respectively.

Although nowadays there is a great expectation of industries for implementing lignolytic enzymes for delignification and for bleaching systems, there is no real concept on these discoveries capable of fulfilling the efficiency of its application to date. Very promising results have been obtained using lignolytic enzymes for delignification, but it still shows high cost of application, or even restriction in performance or technical feasibility, which depends on the enzymatic system. So Call and Call (2005) reported new generation of enzymatic systems for delignification and bleaching such as the mediated oxidoreductase systems (like the laccase mediator system) as already mentioned, and others have different but significant drawbacks.

Possible alternatives to these existing enzymatic concepts could be: (1) the hydrolase mediated oxidation system containing as main components hydrolases like lipases, special ketone compounds, fatty acid or fat compounds, and peroxide (e.g., H_2O_2) as cooxidant (Call 1998, 2000; Kazlauskas and Bornscheuer 1998; Rüsch et al. 2000); and other new enzymatic approaches, including methods that generate reactive oxygen species or reactive nitrogen species (Call 2001, 2002). These new systems can delignify with the aid of the active components: peroxynitrous acid (PNA) or dicyclopentadienyl transition metal complexes (mainly ferrocene) activating the peroxide provided; and special generated

organosulphonic peracids or enzymatically activated sulphite that can generate in combination with ketones dioxirane (Call and Call 2005).

3.5 "Combined" Treatments

The pretreatment of biomasses may benefit from the use of more than one processing technology, either simultaneous or sequential. Several studies have investigated the effects of combination of different processing technologies for the pretreatment of lignocellulosic biomasses.

Rughani and McGinnis (1989) combined the rapid steam hydrolysis (RASH) with the organosolv methods for the pretreatment of mixed hardwoods. The enzymatic rate studies indicated that the RASH process helps in increasing the accessibility of cellulose to enzymatic hydrolysis and increased the amount of soluble lignin while the organosolv process only removed solubilized lignin. Three alcohols (methanol, ethanol, and butanol) were studied using a combined RASH organosolv process. At lower temperatures there were small differences between the alcohols; however, at higher temperatures all alcohols were equally effective. At longer RASH times, the percentage of glucose in the final product and the amount of solubilized lignin increased.

Jurcoane et al. (2009) investigated the efficiency of combined pretreatment (different concentrations H_2SO_4 + 30 min at 121 °C) followed by enzymatic hydrolysis. Testing different concentration of H_2SO_4, good results were obtained for Gavott maize whole crop when was used a combined pretreatment (3 % H_2SO_4 + 30 min at 121 °C) followed to enzymatic hydrolysis (36,88 % of reducing sugars) and for Gavott Maize Straw when was used a combined pretreatment (2 % H_2SO_4 + 30 min at 121 °C) followed to enzymatic hydrolysis (42,22 % of reducing sugars), resulting in an increment of 3.9-fold higher and 3.6-fold higher, respectively, comparing with untreated samples.

Bjerre et al. (1996) performed the wet oxidation combined with the alkaline hydrolysis treatment on the wheat straw to increase the percentage of polysaccharides for the enzymatic hydrolysis. By using a specially constructed autoclave system, the wet oxidation process was optimized with respect to both reaction time and temperature. The best conditions (20 g/L straw, 170 °C, 5–10 min) gave about 85 % w/w yield of converting cellulose to glucose.

Chen et al. (2010) treated the straw using the 1-Butyl-3-methylimidazolium chloride [(BMIM)C1] (ionic liquid pretreatment) after steam explosion. The first step allows degrading the hemicellulose into soluble reducing sugar. Then, lignin with high molecular weight in steam exploded straw was separated from cellulose due to its insolubility in (BMIM)C1. The enzymatic hydrolysis rates and fermentability of straw treated with (BMIM)Cl were improved. The maximum hydrolysis rate was 100 % and cellulase activity was 118.64 FPU/(g dry substrates).

Chen et al. (2008) studied the pretreatment method coupling steam explosion with alkaline peroxide for wheat straw to increase the cellulose content of substrate and ethanol yield in simultaneous saccharification and fermentation (SSF). After the complex pretreatment, the cellulose content in wheat straw increased from 31.5 to 67.2 %. In the hydrolysate of wheat straw pretreated with the complex method and steam explosion, the glucose concentration was 110.9 and 67.8 g/L, respectively. The optimal conditions for SSF were 40 °C, 120 h, cellulase loading 40 FPU/(g wheat straw), yeast inocula 10 % (v/v) and substrate concentration 16.7 % (w/v). Under the optimal conditions, the total ethanol concentration in SSF of wheat straw pretreated with steam explosion and alkaline peroxide reached 51.5 g/L, and an overall yield of 81.1 % was obtained.

Mesa et al. (2011) demonstrated that the combination of a dilute-acid pretreatment followed by the organosolv pretreatment with NaOH under optimized conditions (60 min, 195 °C, 30 % (v/v) ethanol) was efficient for the fractionation of sugarcane bagasse for subsequent enzymatic hydrolysis, yielding a residual solid material containing 67.3 % (w/w) glucose, which was easily recovered by enzymatic hydrolysis. Novo et al. (2011) developed a process using glycerol-water mixtures and obtained a pulp with a residual lignin amount lower than 8 %; extent of delignification close to 80 %; and residual cellulose content higher than 80 %.

Regarding the delignification process developed by Rocha et al. (2012), the steam explosion pretreated bagasse was reacted with a NaOH solution 1.0 % (w/v), using a solid–liquid ratio of 1:10 (w/v). The operation was carried out at 100 °C for 1 h, and there was an excellent removal of lignin from the biomass (92.7 ± 3.9 %). The process hydrolyzed 31.1 ± 3.5 % of the cellulose and the percentage of hemicellulose hydrolysis was 94.7 ± 0.9 %.

The electromagnetic field used in microwaves may create non-thermal effects that also accelerate the destruction of crystal structures. The process developed by Binod et al. (2012) using microwave-alkali (1 % NaOH) followed by acid pretreatment (1 % H_2SO_4), and enzymatic hydrolysis gave an overall reducing sugar yield of 0.83 g/g dry sugarcane bagasse.

Kadimaliev et al. (2003) compared the effect on the birch and pine sawdusts of the combination of the biological pretreatment by the fungus *Panus* (*Lentinus*) *tigrinus* with three different physical/chemical treatments: ammonia (5 % solution of NH_4OH at 165 °C for 10 min) or sulfuric acid (2.5 % solution of H_2SO_4) or ultrasound (in a UM-4 device at a frequency of 22 kHz for 10 min). The results showed that the decrease in lignin contents during solid-phase cultivation of *P. tigrinus* on birch and pine sawdusts is 45 and 32 %, respectively. The alkaline and acid modifications of the substrate decreased the lignin consumption by the fungus in both birch (15 %) and pine (12 %) sawdusts, while the ultrasound increased lignin consumption (about 5 %) and may be recommended for accelerating biodegradation of lignocellulosic substrates.

Two novel two-step pretreatments for enzymatic hydrolysis of rice hull were proposed by Yu et al. (2009). They consisted of an ultrasonic pretreatment (25 °C for 10, 20, 30, 40, 50, and 60 min, using the ultrasonic system 250 W, 40 kHz) or H_2O_2 pretreatment (1, 2, 3, 4, and 5 % (W/V) for 48 h), and a subsequent

biological treatment with *Pleurotus ostreatus*). For 6–60 days at 28 °C. The *P. ostreatus* pretreatment of untreated rice hull for 18 days led to 18 % degradations of lignin. Degradation of lignin was greatly enhanced by the combined pretreatment of H_2O_2 and fungus, and the delignification rate was about two times higher than that of the sole fungal pretreatment.

The combination of biological pretreatment by a white rot fungus *Echinodontium taxodii* or a brown rot fungus *Antrodia sp*. 5,898 with mild acid pretreatment (0.25 % sulfuric acid at varied temperature) were evaluated by Ma et al. (2010), under different pretreatment conditions for enzymatic hydrolysis and ethanol production from water hyacinth (*E. crassipes*). The reducing sugar yield from enzymatic hydrolysis of co-treated water hyacinth increased 1.13-2.11-fold than that of acid-treated water hyacinth at the same conditions. The following study on separate hydrolysis and fermentation with *Saccharomyces cerevisiae* indicated that the ethanol yield from co-treated water hyacinth achieved 0.192 g/g of dry matter, which increased 1.34-fold than that from acid-treated water hyacinth (0.146 g/g of dry matter).

Exhaustive hot water extraction (HWE) and liquid hot water (LHW) pretreatment were evaluated for their effects on degradation of biomass feedstocks (i.e., corn stover, wheat straw, and soybean straw) by *Ceriporiopsis subvermispora* (Wan et al. 2011). HWE (85 °C for 10 min) partially removed water soluble extractives, and subsequently improved fungal degradation on wheat straw while it had little or no effect on the fungal degradation of corn stover and soybean straw. In contrast, LHW (170 °C for 3 min) pretreatment improved the fungal degradation of soybean straw; thus, lignin removal of 36.70 % and glucose yield of 64.25 % were obtained from the combined LHW and fungal pretreatment.

Yu et al. (2010a) used the fungal treatment with *Irpex lacteus* to enhance the delignification and xylan loss during mild alkaline pretreatment and subsequent enzymatic conversion. The biotreatment with *I. lacteus* enhanced the alkaline delignification of cornstalks significantly. The lignin loss varied from 23.28 % (15 min, 30 °C) to 75.67 % (120 min, 75 °C) for the raw cornstalks and from 27.81 % (15 min, 30 °C) to 80.00 % (120 min, 75 °C) for the biotreated cornstalks. While about 60 % of lignin loss required 90-min alkaline pretreatment at 60 °C for the raw cornstalk, 45-min pretreatment for the biotreated cornstalk. The combination pretreatment of the biotreatment and alkaline pretreatment could further improve the enzymatic digestibility of glucan. The glucan digestibility obtained after the combination pretreatment varied from 58.50 % (15 min, 30 °C) to 93.86 % (120 min, 60 °C), and the maximum digestibility of glucan increased 14 % in comparison with that obtained after the sole pretreatment.

The effects of biological treatment prior to alkaline/oxidative pretreatment O/A (alkaline liquor with 0.0016 % NaOH and 3 % (vol/vol) H_2O_2 at room temperature for 16 h) using three white-rot fungi (*Ganoderma lucidum*, *Trametes versicolor,* and *Echinodontium taxodii*) were evaluated for the enzymatic hydrolysis of corn straw by Yu et al. (2010b). *Trametes versicolor*, *Ganoderma lucidum,* and *Echinodontium taxodii* caused 54.6, 32.7, and 42.2 % lignin loss, respectively, after a 30-day pretreatment at 25 °C. The lignin content of the corn straw

decreased more significantly after biological and A/O pretreatment than after A/O pretreatment alone. Biological pretreatment and A/O pretreatment decreased the lignin content of corn straw (from 12.4 to 7.8 % and 7 %, respectively); however, the lignin content decreased more significantly (6.2 %) when the two pretreatment methods were combined.

Fissore et al. (2010) treated wood chips of *Pinus radiata* softwood with the brown rot fungus *Gloeophyllum trabeum* for periods from 4 to 12 weeks at 27 °C. Lignin was not severely attacked by brown rot fungi and no losses were observed until 2 months of biodegradation. Undecayed and 4-week decayed wood chips were delignified by alkaline (NaOH solution) or organosolv (ethanol/water) processes to produce cellulosic pulps. The chemical composition of alkaline pulps indicated that carbohydrates were solubilized in higher amounts than lignin, and glucan amount is lower in pulps from decayed wood (77 % glucan) than in control pulps (83 % glucan). The higher solubilization of carbohydrates in decayed wood resulted also in pulps with higher amount of residual lignin (17 %). Organosolv cooking of *P. radiata* wood chips with ethanol/water was more selective for delignification than the alkaline process. Low residual lignin amount and higher glucan content were obtained for pulps of 4-week decayed wood as compared with control pulps. Chemical pulps and milled wood from undecayed and 4-week decayed wood chips were pre-saccharified with cellulases for 24 h at 50 °C followed by simultaneous saccharification and fermentation (SSF) with the yeast *Saccharomyces cerevisiae* IR2-9a at 40 °C for 96 h for bioethanol production. The combination of brown rot fungus and organosolv processes resulted in a calculated production of 210 mL ethanol/kg of wood.

Baba et al. (2011) treated the softwood chip of Japanese cedar (*Cryptomeria japonica*) with selective white rot fungi prior to the ethanolysis to increase the sugar yield. The treatment with a biopulping fungus, *Ceriporiopsis subvermispora* FP-90031, and a new fungal isolate *Phellinus sp*. SKM2102 for 8 weeks increased the sugar yield to 35.7 and 40.8 %, respectively. The best pretreatment conditions in terms of overall sugar yield including a soluble fraction were obtained by ethanolysis after the fugal treatment with *Phellinus sp*. SKM2102, resulting in production of 42.2 g of total reducing sugars per 100 g of the fungus-pretreated biomass. After the combined pretreatment, simultaneous saccharification, and fermentation of the water-insoluble pulp fraction were carried out using *Saccharomyces cerevisiae*. Ethanol production from undecayed Japanese cedar wood was negligible but pretreatments with the two fungi significantly increased the ethanol production, in combination with ethanolysis: after 72 h, a trace amount (0.42 g/l) of ethanol from the untreated wood was determined, while 9.82 g/l and 8.94 g/l of ethanol were produced from the wood treated with the 2 fungi for 8 weeks.

Saad et al. (2008) performed fungal pretreatment on sugarcane straw before organosolv pulping. Sugarcane straw measuring about 2.5 × 1.5 cm was washed with water and sterilized, and presented a moisture content of 60 % before inoculation. The material was then inoculated with fungal mycelium and incubated at 27 °C. The best conditions of fermentation time and fungal mycelium load were 15 days and 250 mg/kg. The highest lignin and hemicelluloses loss was 27.7 and

16.9 %, respectively, after 30 days of fermentation. However, there was a significant lignin removal from the cell walls of straw of 24.9 % in 15 days. Lignin degradation exceeded cellulose degradation by 2.4-fold. The biological pretreatment had a positive effect on the acetic acid reduction (21.5 %) during the acetosolv pulping process. The pulping kinetics, carried out at 120 °C, showed that for biopulps the final lignin content was around 7.5 % with a reduction of 40 % in the pulping time to reach 12.5 % pulp lignin.

Salvachúa et al. (2011) investigated the potential of fungal pretreatment combined with a mild alkali treatment (0.1 % sodium hydroxide 5 % w/v, at 50 °C and 165 rpm for 1 h) in the process of ethanol production from wheat straw. The fungal treatment was performed by solid state fermentation during a period of 21 days. Glucose yield at the end of the pretreatment with *Poria subvermispora* and *Irpex lacteus* reached 69 and 66 % of the cellulose available in the wheat straw, respectively, with an ethanol yield of 62 % in both cases. Around 90 % of glucose was converted to ethanol, indicating that no inhibitors were generated during the process.

3.6 Conclusions

The abundance of lignocellulosic biomass in the nature and also its large scale generation as a byproduct in several agroindustrial processes has been motivating researches to pretreat these materials and generate a broth with simpler fermentable sugars (like hexoses and pentoses). Aspects that are aimed are not only economical ones, but also environmental, like the use of enzymatic pretreatment and biopulping, conjugated with classical strategies, as steam explosion and thermal treatment.

The production of fuels, as ethanol, from these treated materials (second generation fuels) is one of the possible alternatives, and the challenge is to make it as economical as possible with the new technologies that have been developed recently.

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Chapter 4
The Saccharification Step: *Trichoderma Reesei* Cellulase Hyper Producer Strains

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Abstract One of the major applications of cellulases is to produce fermentable sugars from lignocellulosic biomass for biofuels production. The filamentous fungus *Trichoderma reesei* is known to be hyper producer of cellulases and hemicellulases and it is widely used for commercial scale production of these enzymes using novel fermentation techniques. Some of the *T. reesei* industrial strains produce over 100 g/l of cellulases. However, there are still technical and economic constraints to the development of cheap commercial cellulase production process. Here, we bring together and discuss the results on *T. reesei* as cellulase producer, the different kinds of enzymes it expresses, recent genomic, genetic, and metabolic engineering approaches that have helped to improve the biomass degrading enzyme mixture and the strategies adopted to reduce the cost of enzymes during fermentation process. Current efforts and some future perspectives for reducing the cost of enzymes by using cheaper substrates, recycling enzyme during the hydrolysis and fermentation process, and on-site enzyme production in the biorefinery facility are also discussed.

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4.1 Introduction

Import of crude oil in United States (US) and European Union (EU) alone constituted 60 and 80 %, respectively, in 2009. Other developing countries like India and China are completely dependent on foreign oil to meet their liquid transportation fuel demand. In recent years, an increase in the cost of crude oil has initiated extensive research and development to produce large-scale alternate liquid transportation fuels from renewable resources. In the US, the Department of Energy (DOE) has set a goal of producing 60 billion gallons of renewable fuels per year by 2030. In the EU there is a mandate to produce 25 % of its transportation fuel using renewable resources by 2030 (Himmel et al. 2007). Production of ethanol from sugarcane juice (Brazil) and corn starch (US) has almost reached its full capacity and both methods are often criticized for using feedstock for food to make biofuels. The only sustainable alternative substrate for making ethanol is lignocellulosic biomass. The primary sources of lignocellulosic biomass include agricultural wastes (corn stover, sorghum, sugarcane bagasse, rice straw, wheat straw, empty fruit bunch from oil palm and date palm, Agave bagasse from tequila industry), Perennial grasses (switchgrass, miscanthus), woody biomass, and municipal solid waste (Gomez et al. 2008).

A biorefinery is a facility that produces fuels and chemicals from lignocellulosic biomass using a combination of process technologies (Fig. 4.1). The sugar polymers (cellulose and hemicellulose) present in biomass are depolymerized by efficiently degrading glycosidic bonds using microbial enzymes followed by microbial fermentation of sugars to fuels and chemicals (Menon and Rao 2012). This concept resembles a petroleum refinery, which produces different fuels and chemicals from crude oil. Compared to a petroleum refinery, producing fuels and chemicals in a biorefinery has several advantages: energy security, environmental benefits, and sustainability (Huber and Dale 2009; Fitzpatrick et al. 2010). Technologies for several different aspects of the biorefinery process are currently being developed. They include: (i) biomass production (breeding, cultivation, harvesting); (ii) transportation of biomass; (iii) biomass storage and preprocessing;

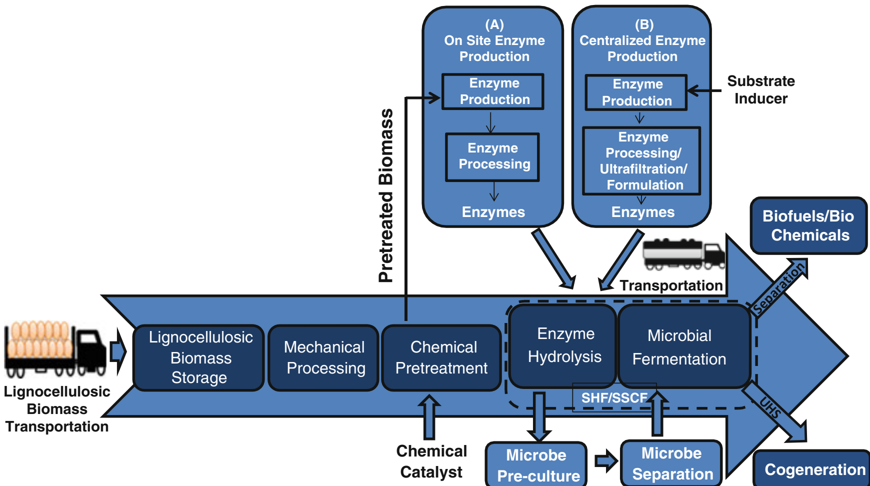

Fig. 4.1 Biorefinery process flow diagram for producing biofuels and biochemical. Here, two different scenarios of enzyme production are shown (**a**) On-site enzyme production and (**b**) Centralized enzyme production. SHF: separate hydrolysis and fermentation; SSCF: simultaneous saccharification and co-fermentation; UHS: unhydrolyzed solids

(iv) pretreatment; (v) enzyme hydrolysis; (vi) microbial fermentation, and (vii) product separation (Balan et al. 2012). Though ethanol is projected to be widely produced in the biorefinery, other fuels and chemicals are also pursued using biological processing route. Some of the chemicals produced using biological route could be transformed to higher value products using a hybrid combination of biochemical and catalytic routes (Ohara 2003). Depending on geographical location and availability of feedstock, the biorefinery can be operated as a large centralized facility or as smaller decentralized facility (Sanders et al. 2007; Lyko et al. 2009).

Several microbes could naturally grow on a wide variety of biomass substrates by efficiently degrading cellulose and hemicellulose and are a good source for new lignocellulosic biomass degrading enzymes. Enzymes that are needed to hydrolyze sugar polymers are cellulases and hemicellulases (Bouws et al. 2008; Kumar et al. 2008). Scientists have been looking for several hypercellulase/hemicellulase producing strains for quite some time. One of the best hyper producers of extracellular cellulolytic enzymes is the mesophilic, filamentous fungus, *Trichoderma reesei (Trichoderma viride)*, first recognized during World War II, when it destroyed cotton fabric US Army tents (Cherry and Fidantsef 2003a, b). The genome sequence of *T. reesei* QM6a was published in 2008 (Martinez et al. 2008a, b). For commercial production of enzymes, several *T. reesei* strains (Rut-30, RL-P37, and MCG-80) have been developed and are currently being used in the industry (Merino and Cherry 2007). Some of these industrial strains produce more than 100 g/l of cellulase/hemicellulase enzymes (Cherry and Fidantsef 2003a, b).

Typical enzyme concentrations used to hydrolyze cellulose are 15–30 filter paper units of cellulase per g of cellulose. On the other hand, for starch hydrolysis 0.2 k Novo unit of amylase per g and 3.26 amyloglucosidase units of glucoamylase per g are needed. This shows that enzyme concentration needed for cellulose degradation is 40-fold to 100-fold higher than that of starch hydrolysis. As a result, the cost of enzymes for cellulose degradation is much higher and it is considered as one of the key bottlenecks for producing fuels and chemicals from lignocellulosic biomass. Several efforts are underway to reduce the cost and maximize enzyme production yield (Carroll and Somerville 2009; Wilson 2009). Some of the strategies include (i) improving the performance of the enzymes by increasing the specific activity (through direct evolution and site directed mutagenesis) and thereby minimizing enzyme dosage (ii) reducing the cost of enzyme production improving cellulase titers during fermentation (through process engineering approaches using cheap substrates including biomass, producing enzymes near biorefinery or expressing enzymes in plants). An overview of *T. reesei* as a cellulase producer, different kinds of enzymes they express, recent genomic, genetic, and metabolic engineering approaches that have helped to improve the biomass degrading enzyme mixture, and strategies pursued to reduce the cost of enzymes are presented in this chapter.

4.2 Cellulase and Hemicellulase Genes in *Trichoderma reesei*

4.2.1 T. reesei Genome: General Characteristics

T. reesei has a genome with a sequence length of around 34 Mbp (Martinez et al. 2008a, b). Gene modeling using a combination of homology and ab initio method predicted 9,129 genes in the genome with an average gene length of 1,793 (bp), and 3.1 exons per gene. Among the 9,129 genes, only 200 genes encode glycoside hydrolases (GHs). The GH genes' number was surprisingly low considering the plant polysaccharide degradation efficiency of *T. reesei.* It was also indicated that the set of plant degrading enzymes of *T. reesei* is smaller than any other sequenced plant cell wall degrading fungus. *T. reesei* also has less carbohydrate-binding module (CBM)-containing proteins among the Sordariomycetes. Totally, ten cellulases are encoded in the *T. reesei* genome including two cellobiohydrolases (CBHI/CEL7A and CBHII/CEL6) and eight endoglucanases (EGII/CEL5A, EG-VIII/CEL5B, EGI/CEL7B, CEL12A/CEL12A, EGV/CEL45A, EGIV/CEL61A, EGVII/CEL61B) (Table 4.1). The *T. reesei* genome also contains 16 hemicellulases and among the pectin degrading enzymes, only members of the GH28 family were found. Only seven cellulases (CBHI, CBHII, EGI, EGII, EGIII, EGIV, and EGV) and two β-glucosidases (BGLI and BGLII) have been characterized (Amore and Faraco 2012; Aro et al. 2005). Two major endo-β-1,4-xylanases XYNI and

Table 4.1 Cellulase and hemicellulase enzymes characterized from *Trichoderma reesei*

Enzyme	Former name	CAZY nomenclature	GH family	Amino acids	Mol Wt (kDa)	P.I	Structure (PDB ID)	Ref
β-glucosidase	BGLII	CEL1A	1	466	52.2	5.3	3AHY	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	BGV	CEL1B	1	484	55.1	5.5	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	BGLI	CEL3A	3	744	78.4	6.4	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	CEL3B	CEL3B	3	874	93.9	5.7	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	BGLIV	CEL3C	3	834	91	5.5	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	CEL3D	CEL3D	3	700	77.1	6	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	CEL3E	CEL3E	3	765	83	6	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGII	CEL5A	5	418	44.15	5	3QR3	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGVIII	CEL5B	5	438	46.9	4.3	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Cellobiohydrolase	CBHI	CEL6A	6	471	49.7	5.1	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Cellobiohydrolase	CBHII	CEL7A	7	514	54.1	4.6	2V3I, 1EGN	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGI	CEL7B	7	459	48.2	4.7	1EG1	Ouyang et al. (2006), Seiboth et al. (2011)

(continued)

Table 4.1 (continued)

Enzyme	Former name	CAZY nomenclature	GH family	Amino acids	Mol Wt (kDa)	P.I	Structure (PDB ID)	Ref
Endoglucanase	CEL12A	CEL12A	12	234	25.16	6.9	1OLQ, 1H8V	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGV	CEL45A	45	242	24.4	4.2	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGIV	CEL61A	61	344	35.5	5.3	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGVII	CEL61B	61	249	26.8	7.8	2VTC	Ouyang et al. (2006), Seiboth et al. (2011)
Xyloglucanase	EGVI	CEL74A	74	818			NA	Ouyang et al. (2006), Seiboth et al. (2011)
Xylanase	XYNI	XYN11A	11	223	24.1	8.1	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Xylanase	XYNII	XYN11B	11	229	24.6	5	3LGR, 2DFB	Ouyang et al. (2006), Seiboth et al. (2011)
Xylanase	XYNIII	XYN10A	10	347	38.1	7.1	1FH7, 1CLX, 1XYZ…	Ouyang et al. (2006), Seiboth et al. (2011)
Acetyl Xylan Esterase	AXE1	AXE1	CE5	302	47	7	NA	Margolles-Clark et al. (1996a)
α-L-arabino-furanosidase	ABFI	ABFI	54	500	51	7.5	NA	Poutanen (1988)
α-Galactosidase	AGLI	AGLI	27	444	50	5.2	1SZN	Margolles-Clark et al. (1996b)

NA not available

XYNII (EC 3.2.1.8) (Törrönen et al. 1992; 1994); and one β-xylosidase, BXLI (EC 3.2.1.37) (Herrmann et al. 1997a, b) have been characterized. The secretory mechanism of extracellular enzymes is not yet fully understood, but an analysis of the membrane trafficking system suggests that *T. reesei* has a very diverse system (Martinez et al. 2008a, b).

4.2.2 T. reesei Genome Encoded Cellulases and Hemicellulases

Cellobiohydrolases (CBHs): CBHs comprise 80–85 % of the total secreted cellulase protein and the major cellobiohydrolase component, CBHI, accounts for 50–60 % (Gritzali and Brown Ross 1979). CBHI and CBHII have a molecular weight of 57 and 56 kDa, respectively, and act on cellulose chains from non-reducing and reducing ends (Sun et al. 2008a, b; Teeri 1997). They both have a cellulose-binding domain (CBD) and a catalytic domain (CD), which is also observed in many other cellulases. CBHII contains two essential Gly residues located at positions 212 and 217 which take part in the initial binding sites with predominantly hydrogen bonds (Divne et al. 1998). There are two steps for glycosidic hydrolysis by CBHI producing cellobiose as the main product. A nucleophilic substitution at the anomeric C1 atom is involved in both steps. In the first step, a negatively charged carboxyl group in Glu 217 acts as a nucleophile with general acid-catalytic assistance from the carboxyl residue forming a glycosyl-enzyme intermediate. In the second step, water attacks this intermediate with general base-catalytic assistance from the deprotonated carboxyl residue, displacing the nucleophile (Divne et al. 1998). In the tunneled active site of CBHI, there are many H-bonds and indole-glycosyl interactions create a large steric confinement which may be the main factor explaining why substituted celluloses, like carboxymethyl cellulose (CMC), are poor substrates for CBHI (Divne et al. 1998).

Endoglucanases (EGs): EGs comprise around 10–15 % of the total secreted protein by *T. reesei* (Gritzali and Brown Ross 1979). EGI has a sequence that is 45 % identical to CBHI and the respective proteins have similar folding pattern suggesting a common ancestor (Penttilä et al. 1986). The EGI protein contains 437 aminoacidic residues and has a molecular weight of around 46–58 kDa (Messner et al. 1988; Penttilä et al. 1986). The secretion of this protein is directed by a signal peptide of 22 aa which is cleaved in the mature protein at the N-terminal. It is heavily glycosylated near the C-terminal (Penttilä et al. 1986). EGIII is believed to have evolved by divergent evolution from a common ancestor from *Schizophyllum commune* (Saloheimo et al. 1988). It contains relatively long introns and encodes a protein (EG3) whose main product is cellodextrins with a degree of polymerization of 2–5 with a turnover rate of 10–200 per minute (Saloheimo et al. 1988). Like CBHI and EGI, EGIV contains a CBD near the C-terminal and has a MW of ~56 kDa. Unlike most EGs, EGIV's main byproduct is cellobiose, but it was

deemed as an EG because of its ability to hydrolyze CMC, which cannot be hydrolyzed by CBHs (Karlsson et al. 2001; Saloheimo et al. 1997). EGIV has a molecular mass of 57 kDa and has an endoglucanase activity that is very small compared to EGI (Karlsson et al. 2001). EGV encodes a relatively small protein of 242 amino acids (aa) (MW of 22.8 kDa) whose catalytic core is also small compared to other cellulases (Saloheimo et al. 1994). Its shape is believed to be rod-like, which may enable the enzyme to penetrate the cellulose fibers better than other cellulases (Saloheimo et al. 1994).

β-glucosidases (BGLs): BGLs do not technically belong to the cellulases because they do not directly act on cellulose but are commonly included in the cellulase complex because of their synergetic effect with CBHs and EGs. The role of BGLs is hydrolysis of cellobiose and short cello-oligomers into glucose. It appears that β-glucosidase gene expression is regulated separately from CBHs and EGs (Jackson and Talburt, 1988). *T. reesei* produces intracellular (Inglin et al. 1980), extracellular (Chen et al. 1992) and mycelium associated β-glucosidase (Jackson and Talburt 1988). The *bgl1* gene encodes an extracellular β-glucosidase while the *bgl2* gene product is an intracellular β-glucosidase (Mach et al. 1995, 2006; Saloheimo et al. 2002). It has been shown that the *bgl1* gene product is required for rapid induction of the cellulase complex (Fowler and Brown 1992). When sophorose is used as an inducer, the β-glucosidase from the *bgl1* gene is secreted. However, when methyl- β-o-glucoside or gentiobiose is used as an inducer, β-glucosidase is typically mycelium-associated and the protein produced was not associated with *bgl1* gene (Mach et al. 1995). The levels of β-glucosidase are also correlated to fungal morphology. In vegetative hyphae there are very low levels of β-glucosidase while in conidiogenous cells or germinating conidia high levels of β-glucosidase are found. Based on this, it was postulated that conidia formation and germination may be coupled with β-glucosidase formation (Jackson and Talburt 1988).

Xylanases (XYNs): The two endo-β-1,4-xylanases, XYNI and XYNII, contribute around 90 % of the xylanase activity in the *T.reesei* enzyme complex (Rauscher et al. 2006). XYNI and XYNII have a MW of 19 and 21 kDa, and an optimum pH range of 2.5–4.0 and 4.5–5.5, respectively (Törrönen et al. 1992). The *xyn2* gene codes for a protein of 223 aa having two N-glycosylation sites and contains one intron of 108 nucleotides (Saarelainen et al. 1993). *Xyn1* and *xyn2* have very similar gene sequences except for the first 100 N-terminal aa's, and the secondary structure of both xylanases consists of primarily β-sheets (Törrönen et al. 1992). Throughout most of these types of xylanases, the aa's are typically conserved at the positions of the β-turns, suggesting a common ancestor (Törrönen et al. 1992). β-xylosidase production by *T. reesei* was also reported (Herrmann et al. 1997a, b).

4.3 Random Mutagenesis of *T. reesei*—Cellulase Hyper-Producer Strains

Random mutagenesis by treating a microorganism with mutagens (e.g. N-nitroguanidine or UV light) and then screening for the mutants with desired features is a widely used method and an efficient way to obtain a desired microbial strain. This method has been successfully applied to *T. reesei* strains development. Because of the worldwide interests in alternative fuels production from cellulosic biomass, cellulase production and *T. reesei* strain development was of great interest in 1970 s and 1980 s (Peterson and Nevalainen 2012). Random mutagenesis was widely used at that time for improving cellulase production/activity, reducing catabolite repression and alleviating end-product inhibition.

The success of random mutation highly depends on the screening/selection method used. Montenecourt and Eveleigh (1979) summarized the screening methodologies for cellulases production strains. Different substrates together with colony inhibitors oxgall and Phosfon D were used for screening the mutants with desired cellulase activities. For instance, to screen a mutant with enhanced production of all the cellulases, acid swollen cellulose was used as the substrate, while CMC was used as the substrate for selection of a mutant with high endoglucanase activities. Several efficient plate screening techniques were developed for selecting β-glucosidase producer mutants. For instance, one method was developed using esculin and ferric ammonium citrate in the agar medium. β-glucosidase splits esculin into glucose and esculetin which reacts with ferric ammonium citrate and forms a black precipitate. Another method applied cellobiose and 2-deoxyglucose. The mutants that could not produce β-glucosidase must use 2-deoxyglucose which is toxic and causes the death of the mutants, while the mutants producing β-glucosidase are able to utilize cellobiose. For screening a mutant with the catabolite de-repression feature, high concentrations of a catabolite repressor (e.g. glucose or glycerol) were applied in the selection medium. The use of 2-deoxyglucose is particularly useful because it can be used as a catabolite repressor as well as an antimetabolite (Montenecourt and Eveleigh 1979).

A successful case of random mutagenesis on *T. reesei* was conducted by Mandels et. al. (1971), who irradiated the conidia of QM6a with high energy electrons generated by a linear accelerator and then screened the mutants in a cellulose medium. A mutant strain QM9123 was isolated, which secretes twice as much cellulase as the wild type, QM6a (Mandels et al. 1971). Later, another mutant QM 9414 was obtained, which has an even higher capacity of cellulase production (Mandels 1975). Another random mutagenesis effort generated the renowned hypercellulolytic strain RUT-C30 (Peterson and Nevalainen 2012). The native strain QM6a was first treated by UV light and then a catabolite repression resistant mutant M7 was isolated. Further mutagenesis by N-nitroguanidine and screening on an acid swollen cellulose plate with oxgall, Phosfon D and 5 % glycerol led to the isolation of NG14, which produced around 20 times the filter paper activity compared to QM6a (Montenecourt and Eveleigh 1977, 1979;

Peterson and Nevalainen 2012). Subsequent mutagenesis on NG14 using UV light and selection on cellobiose and a 2-deoxyglucose medium obtained RUT-C30, which is a catabolite de-repression strain that produces 15–20 times higher cellulase activity than QM6a (Montenecourt and Eveleigh 1979).

4.4 Metabolic Engineering and Protein Engineering

Several transformation systems (e.g., agrobacterium-mediated transformation) have been developed for efficient manipulation of *T. reesei* (Guangtao et al. 2010; Steiger et al. 2011; Yao et al. 2007). Metabolic engineering of *T. reesei* for cellulase and hemicellulase production have been focusing on study and manipulation of transcriptional regulators (Kubicek et al. 2009). Protein engineering research for improving individual cellulase/hemicellulase includes rational design and direct evolution (Zhang et al. 2006; Wen et al. 2009). Several detailed reviews have been published in the past on protein engineering (Chandel et al. 2012; Peterson and Nevalainen 2012; Elkins et al. 2010). In some cases, point mutations were done to improve the thermo tolerance or specific activity of the enzymes. In many other instances, cellulose and hemicellulose degrading enzymes with multiple activities could be produced using gene-fusion techniques. Since cellulase comprise of catalytic, linker, and cellulose-binding domains, there can be n-number of possibilities of mixing and matching different domains to create novel enzymes with superior activities. Though *T. reesei* have 12 β-glucosidases in their genome, most of them were found to be intracellular and secreted enzyme had lesser cellobiase activities. Companies like Novozyme (http://www.novozymes.com) and Genencor International Inc. (http://www.genencor.com) have already developed a genetically modified *T. reesei* strain that shows higher β-glucosidase activity. Though several synergistically acting enzymes from other organisms are expressed in *T. reesei* for improving their enzyme activity, the details are yet to be revealed (Gusakov 2011).

4.4.1 Transcriptional Regulators

Production of cellulases and hemicellulases by *T.reesei* is tightly regulated by transcriptional regulators and requires an inducer (e.g., cellulose and lactose) for cellulase/hemicellulase gene expression to occur (Kubicek et al. 2009). Currently, three positive transcriptional activators (Xyr1, Ace2, and the Hap2/3/5 complex) and two negative regulators (Ace1and Cre1) have been identified. Xyr1 is a central regulatory protein which belongs to the class of zinc binuclear cluster proteins. All inducible cellulase promoters were found containing *consensus sequences* for Xyr1 (Kubicek et al. 2009). Xyr1 not only activates the most important hydrolase genes involved in the degradation of xylan and cellulose, including *cbh1, cbh2, egl1, bgl1, xyn1, xyn2* and *bxl1* (Mach-Aigner et al. 2008; Pucher et al. 2011), but

it is also involved in the regulation of xylose and lactose metabolism (Seiboth et al. 2007; Stricker et al. 2006). It was found by Mach-Aigner et al. (2008) that the *xyr1* gene was not induced by any cellulases inducers. However, Portnoy et al. (2011) indicated that it was induced by lactose and D-galactose. *Xyr1* transcription was repressed by Ace1 as well as by glucose through carbon catabolite repression mediated by Cre1 (Mach-Aigner et al. 2008). Deletion of the *xyr1* gene results in elimination of all cellulase and some hemicellulase production (Akel et al. 2009). Although it is well known that Xyr1 is the main regulator for cellulase and hemicellulase production, how to use this transcriptional regulator for producing cellulase and hemicellulase at a higher yield is still under investigation (Kubicek et al. 2009).

Cre1 has two zinc fingers of the C2H2 type involved in base recognition (Ilmen et al. 1996; Strauss et al. 1995) and it confers negative regulation of transcription. Unlike Xyr1, Cre1 only has direct control of some major hydrolases, most notably *cbh1* and *xyn1*, whereas other hydrolytic genes such as *cbh2, xyn2,* and *bgl1* are not Cre1 regulation dependent (Ilmen et al. 1996; Mach et al. 1995; Margolles-Clark et al. 1997). It has been illustrated that in the presence of glucose, Cre1 binds to specific sites in the *cbh1* promoter and hence it represses cellulase production (Ilmen et al. 1996). Deletion or modification of *cre1* is a way to resolve the catabolite repression issue (Nakari-Setala et al. 2009). The hypercellulolytic strain *T. reesei* Rut-C30 was found having a truncated *cre1* gene which is the reason for its catabolite derepression property (Ilmen et al. 1996; Nakari-Setala et al. 2009; Peterson and Nevalainen 2012). Zou et al. (2012) substituted the binding sites of Ace2 and Hap2/3/5 for the Cre1 binding sites within the *cbh1* promoter and thus improved the efficiency of the promoter.

Transcription factors Ace1, Ace2, and Hap2/3/5 complex are also involved in the regulation of cellulase formation in *T. reesei.* Ace1 has been described as a cellulase repressor (Aro et al. 2003) while Ace2 is described to promote cellulase production (Aro et al. 2001). Ace1, containing three Cys2His2 –type zinc fingers, is a repressor of cellulase and xylanase genes (Aro et al. 2003). It binds to eight sites in the *cbh1* promoter containing the core sequence 5'AGGCA (Saloheimo et al. 2000). An increase of all the main cellulase and xylanase expression was observed for the Δ*ace1* strain when cultured on sophorose and cellulose (Aro et al. 2003), which may be due to the fact that Ace1 also acts as a repressor of *xyr1*, as mentioned before (Mach-Aigner et al. 2008). Ace1 also competes with Xyr1for binding sites in the *xyn1* promoter and thus it represses the transcription of *xyn1* (Rauscher et al. 2006).

Ace2 is a zinc binuclear cluster protein like Xyr1. In contrast to Ace1, expression of Ace2 helps to increase cellulase expression (Aro et al. 2001). Deletion of *ace2* leads to lower transcript levels of major cellulases (CBHI, CBHII, EGI, and EGII) and xylanase (XYNII) when cellulose was used as an inducer, but it was unaffected when sophorose was used as an inducer (Aro et al. 2001). Ace2 binds to the strong *cbh* promoter at the 5'-GGCTAATAA site (Aro et al. 2001), and it has been suggested that phosphorylation and dimerization are needed for the binding of Ace2 to the target promoter (Stricker et al. 2008).

Hap2/3/5 complex binds to a CCAAT box of *cbh2*-activating element (CAE) in the *cbh2* promoter and acts as a transcriptional enhancer (Zeilinger et al. 2001).

4.4.2 Rational Design and Direct Evolution of Individual Cellulase/Hemicellulase

The idea of rational design is to modify the aminoacidic sequence of a protein in order to achieve dramatic impacts on the protein performance (Sheehan and Himmel 1999). Rational design for improving cellulases requires detailed information of the protein structure, protein structure/function relationships and how the protein interacts with the substrate to make the catalysis reaction to occur (Zhang et al. 2006). Site-directed mutagenesis, secondary structure element exchange, and whole domain exchange of fusion proteins are examples of how protein modifications can be achieved. The success of enzyme enhancement (e.g., increased activity) is usually limited to well-understood proteins, and is commonly applied to the amino acidic sites near the active site or the binding pocket in the 3-dimensional structure (Zhang et al. 2006). Based on the limited understanding of insoluble cellulose substrates, reaction complexity of the cellulase enzymes and the arsenal of enzymes needed to work in synergy to degrade lignocellulosic biomass, the cellulase complex needs much more investigation before effective methods can be developed. Cellulases working on the insoluble substrates have a complex mechanism to degrade the cellulose. For instance, six steps were proposed for CBHI of *T. reesei* to work on cellulose: binding to substrate via CBM (carbohydrate-binding module), recognizing a reducing end of a cellulose chain, threading the cellulose chain, forming a catalytically active complex, hydrolyzing the cellulose, and expulsing the product (Chundawat et al. 2011). Because of the complexity of their action, site-directed mutagenesis has met some difficulties toward improving the properties of the cellulases (Zhang et al. 2006). However, there are few successful cases concerning cellulases from fungi. For example, Wohlfahrt et al. improved the pH stability of the CBHII from *T. reesei* by mutagenesis of the non-active site residues (Wohlfahrt et al. 2003). Voutilainen et al. enhanced the thermo stability and activity of a CBHI from another fungus, *Talaromyces emersonii,* by introducing an additional disulfide bridge to the catalytic module (Voutilainen et al. 2010). Chen et al. increased the thermo stability of a xylanase from *Aspergillus niger* F19 by introducing five arginine substitutions and a disulfide bond to the enzyme (Chen et al. 2010).

Direct evolution mimics the natural random mutation and selection through recombinant DNA technology. It does not require the knowledge of enzyme structure and enzyme-substrate interactions, but relies on the screening method for evaluating the mutants (Zhang et al. 2006). CMC plus Congo red staining is a widely applied screening method for endoglucanase mutants (Lin et al. 2011). The Endoglucanase activity can be determined by the “halos” on the solid agar plates

in which higher hydrolysis correlates with the size of the halos. DNA techniques that have been applied for directed evolution include family shuffling, DNA shuffling, error-prone Polymerase Chain Reaction (PCR), and SCHEMA (Lin et al. 2011). Improved enzyme activity, thermostability, and pH adaptability are the desired characteristics (Goedegebuur et al. 2005; Han et al. 2009; Lin et al. 2011; Trivedi et al. 2011; Wang et al. 2005; Xia and Wang 2009). For instance, Nakazawa et al. (2009) carried out directed evolution of *T. reesei* EGIII using error-prone PCR and selected a mutant exhibiting broader pH stability, better thermo stability as well as higher activity when compared to the wild type EGIII. Moreover, Hokanson et al. (2011) enhanced the thermo stability of the GH11 xylanase II from *T. reesei* through a directed evolution method.

4.5 Fermentation Technologies for Enzyme Production

4.5.1 Nutrients for T. reesei Growth and Enzyme Production

T. reesei is a mesophilic fungus having the metabolic pathways to utilize all the lignocellulose carbohydrates (Amore and Faraco 2012) and little nutrient requirements for growth (Mandels and Weber 1969). Hence, it can grow fairly easily on most carbon sources. Its growth rate is rapid on glucose, fructose, and glycerol and relatively slow on cellulose and lactose (Messner and Kubicek 1991). Glucose was shown to give higher cell biomass yield when compared to lactose, arabinose, or their mixtures (Xiong et al. 2004a). Nevertheless, glucose is a repressor of cellulase production via the action of Cre1. However, cellulase expression was still seen in a glucose medium after glucose was depleted with no inducer present (Ilmen et al. 1997). Oligosaccharides released from the cell walls of the starving fungus or sophorose generated from the glucose by the action of β-glucosidase were possibly acting as inducers in this case (Ilmen et al. 1997). When grown on carbohydrates, the fungus produces acidic compounds and the growth continues until pH drops below 2.5. In contrast, when grown on peptone the pH increases and growth continues until the pH reaches 7.5 (Mandels and Weber 1969). *T. reesei* can produce cellulases in a defined medium with simple nutrient salts and cellulose, but the addition of peptone has been shown to improve protein production. In another study, peptone was shown to have little effect on improvement of cellulase activity but increased β-glucosidase activity (Esterbauer et al. 1991). Tween 80, a commonly used surfactant, has been shown to increase cellulase production. It was theorized that this increase is due to loosening of the *T. reesei* cell wall and thus facilitating the entrance and exit of compounds from the cell (Reese and Maguire 1969).

4.5.2 Inducers for Cellulase and Hemicellulase Production

Cellulose and xylan were thought to be the natural inducers for cellulase and hemicellulase production. When considering the insoluble nature of xylan and cellulose, which are unable to enter the fungal cell, it has been suggested that the natural inducer was low molecular weight hydrolysis products (such as oligosaccharides and their derivatives), which can penetrate the cell and affect the fungal metabolism (Haltrich et al. 1996). Commonly used inducers including sophorose, lactose, and sorbose are discussed here.

The disaccharide sophorose is a very powerful soluble inducer of *T. reesei* cellulases and it was suggested to be the natural inducer (Sternberg and Mandels 1979; Sternberg and Mandels 1982). It has been demonstrated that the formation of sophorose from cellobiose was mediated by the trans-glycosylation activity of β-glucosidase (Vaheri et al. 1979). In addition, when β-glucosidase was inhibited, a substantial decrease in the synthesis of EGs was observed with cello-oligodextrins but not with sophorose as inducers (Kubicek 1987). These results further indicate the importance of β-glucosidase and sophorose in the induction of hydrolytic enzymes.

Lactose (D-galactosyl- β-1,4-D-glucoside) is another widely used cellulase inducer. A clear advantage of lactose compared to cellulose as an inducer is its solubility, yet lactose consumption has been shown to be slower and cellulase yields were lower compared to cellulose (Warzywoda et al. 1983). Because natural lactose only occurs in the milk of mammals, it is unlikely that it is the natural inducer for cellulose production (Kubicek et al. 2009). Extracellular hydrolysis of lactose into D-galactose and D-glucose is the initial step of lactose catabolism in *T. reesei.* The extracellular hydrolysis of lactose raised the question of the potential of the monomers ability to induce cellulase expression (Kubicek et al. 2009). Seiboth et al. (2003) found that neither D-glucose nor D-galactose or any mixture of these two resulted in cellulase induction even when the carbon catabolite repressor Cre1 was absent. Based on this, it is believed that the stereospecificity of the D-galactopyranose which is released from the cleavage of lactose by β-galactosidase plays a key role in the induction of cellulase by lactose (Kubicek et al. 2009).

Monosaccharides generally inhibit cellulase expression through carbon catabolite repression via the action of Cre1 or through end-product feedback inhibition. The only monosaccharide found to have a cellulase-inducing effect is L-sorbose (Kawamori et al. 1986; Nogawa et al. 2001). Sorbose affects cellulase formation at a transcriptional level and has been proposed to inhibit β-1,3-glucan synthetase, which changes the composition of the fungus cell wall and reduces the degradation of inducers (Nogawa et al. 2001).

XYNI and XYNII are not co-regulated, but are both formed in the presence of xylan or xylobiose, but only one is formed in the presence of sophorose (Hrmová et al. 1986; Senior et al. 1989). Cellulose, sophorose, xylan, xylobiose, and L-arabitol can induce expression of most of the tested hemicellulase genes including

two β-xylanases, β-mannase, acetyl xylan esterase, β-xylosidase, and many others (Margolles-Clark et al. 1997). In the presence of glucose, most of the hemicellulase genes are repressed but de-repressed expression was observed once glucose was depleted (Margolles-Clark et al. 1997). *Xyn2* transcription occurs at a low basal level when the fungus is grown on glucose as the sole carbon source and is elevated in the presence of xylan, xylobiose, or sophorose (Zeilinger et al. 1996).

4.5.3 Fermentation Conditions

The culture medium pH is a critical factor affecting many aspects of *T. reesei* fermentation including germinating time, growth rate, morphology, and enzyme production. Germination time is the shortest in the pH range of 3–5 and elongated when the pH becomes too acidic or alkaline (Lejeune et al. 1995). Maximal growth rate is also in the pH range of 3–5 with decreased growth rate in more acidic or alkali medium (Brown and Zainudeen 1977; Lejeune et al. 1995). On cellulose and xylan-based media, it was observed that cellulase production is favored at a pH around 4.0 while xylanase production favored at a pH around 7.0 (Bailey et al. 1993). In another study on lactose medium, the highest xylanase activity was observed at pH 6.0 and optimum cellulase production at a pH range of 4.0–5.0 (Xiong et al. 2004b). Different xylanases also favor different pH. Xiong et al. (2004b) found that XYNI favors pH 4.0, XYNIII favors pH 6.0, and XYNII favors both. Relatively high production of β-glucosidase was also found at high pH values (Juhász et al. 2004).

Agitation rate also plays a crucial role in enzyme production. Enzyme production is an energy intense reaction and requires a lot of ATPs. High agitation rate typically results in high dissolved oxygen level and hence facilitates aspiration and ATP production. However, high agitation rate also causes high shear stress and affects hyphae growth (Ahamed and Vermette 2010). When a 2.6 L fermenter is used, the optimal agitation speed for cellulase production was determined to be 300 rpm (Mukataka et al. 1988). In contrast to the total cellulase activity, the optimal agitation speed for endoglucanase and β-glucosidase production was 200 and 400 rpm, respectively (Mukataka et al. 1988). Effect of culture media composition is also known to have significant impact on cellulase production by *T. reesei* (Ahamed and Vermette 2008, 2010).

4.5.4 Morphology

Filamentous fungi are able to develop three major morphologies during submerged fermentation: pellets, mycelial aggregates (clumps), and filamentous mycelia (Cox et al. 1998). In bioreactors, the filamentous or clump mycelia are undesirable because they increase the viscosity of the medium and also wrap around the

impellers. The pellet form is a desirable morphology, especially for industrial production, not only because of the reduced viscosity but also due to the improved culture rheology (enhanced mass and oxygen transfer) and the reduced energy cost for agitation and aeration (Suijdam et al. 1980). Numerous factors in the fermentation affect the fungus morphology, including agitation speed (see above), medium, pH, polymer additives, surface active agents, and inoculum size (Metz and Kossen 1977; Papagianni 2004; Ferreira Susana et al. 2009). It has been postulated that increasing the number of tips of hyphae increases protein production (Juge et al. 1998; Pluschkell et al. 1996) because it is believed that in filamentous fungus, protein secretion occurs at the tip of growing hyphae (Peberdy 1994; Punt et al. 1994). Since the tips are more porous, they allow proteins to exit more easily through the cell wall (Punt et al. 1994; Wosten et al. 1991). Correlation between fungal physiology and cellulase production is yet to be clearly understood. Several articles have shown that cellulase production is directly influenced by the fungal morphology during fungal fermentation (Grimm et al. 2005 and Ferreira Susana et al. 2009).

4.6 Current Efforts and Future Perspective of Reducing the Cost of Enzymes

Costs for producing enzymes used to hydrolyze pretreated biomass represent about one-third of the total hydrolysis processing cost (Walker and Wilson 1991; Lynd et al. 2005). It has been projected that the cost of enzymes is 2665 dollars per mega gram. Over the past 30 years, the cost of enzymes has been considerably decreased, but it is still considered to be high. About 10–20 Filter Paper Unit (FPU) of enzymes is required per gram of cellulose to achieve 90 % sugar conversion in 72 h (Chandel et al. 2012; Peterson and Nevalainen 2012). Though some of the reported *T. reesei* QM 6a strains produce an enzyme concentration of about 20 FPU ml^{-1} at a rate of 150 FPU $L^{-1}h^{-1}$, the cost of pure cellulose substrate used in the process is very high. Several efforts have been taken to lower the cost of enzymes as described below.

4.6.1 Enzyme Production Using Lignocellulosic Biomass

One of the main sources for the high cost of cellulase production is the substrate cost. Currently, pure cellulose power and inorganic salts are used as media with artificial inducers (Qu et al. 1991). Several artificial inducers (like lactose, cellobiose) are used in the industry to induce cellulase and hemicellulase production in *T. reesei* (Table 4.2). In order to reduce the cost of enzyme production, these artificial inducers could be replaced by natural inducers. The oligosaccharides

Table 4.2 Protein production using *T. Reesei* Rut-30 strain on different substrates

Substrate	Cellulase (FPU/ml)	Xylanase IU/ml	Reference
Cellulose+ yeast extract	5.02		Ahamed and Vermette 2008
Cellulose-Yeast nitrogen base-CMC	1.4		Ahamed and Vermette 2008
Plasma-assisted pretreated wheat straw-unwashed-sterilized by autoclave	0.1	84.0	Rodriguez-Gomez et al. 2012
Plasma-assisted pretreated wheat straw-washed-sterilized by autoclave	0.4	106.0	Rodriguez-Gomez et al. 2012
Wet oxidized wheat straw	<0.37		Thygesen et al. 2003
Rice straw	0.38		Colina et al. 2009
Sticks of rice straw	0.6		Sun et. al. 2008
Sticks of rice straw (alkaline pretreatment)	1.07		Sun et. al. 2008
Sticks of rice straw (acid pretreatment)	0.3		Sun et. al. 2008
Steam-pretreated spruce	0.45	13.2	Juhasz et al. 2005
Steam-pretreated spruce	0.8		Szengyel et al. 2000
Steam-pretreated willow	0.56	57.0	Juhasz et al. 2005
Steam-pretreated willow	1.6		Szengyel et al. 1997; Zacchi 1996
Steam-pretreated willow	0.64		Palmqvist 1997
Steam-pretreated willow + hydrolisate	0.6		Chahal 1982
Steam-pretreated corn stove	1.2	64.4	Juhasz et al. 2005
Steam-exploded poplar nonwashed	1		Szakacs and Tengerdy, 1997
Steam-exploded poplar washed	3.7		Szakacs and Tengerdy, 1997
Pretreated poplar wood	1.4		Shin et al. 2000
Steam-exploded wood	4.3		Xiong et al. 2005
Oat husk hydrolysate (acid)	0.5	276.0	Xiong et al. 2005
Spruce fiber hydrolysate (acid)		30.0	Xiong et al. 2005
Homogenized dairy manure (optimized)	1.72		Wen et al. 2005
Bagasse pretreated with hot water	0.6		Bigelow and Wyman 2002
Pulverized newspaper sludge	1.7		Shin et al. 2000
Old corrugated cardboard	2.27		Szijarto et al. 2004

produced during the hydrolysis of cellulose play important roles in the natural cellulase induction (Ladisch et al. 1981). Pure cellulosic materials (avicel, cotton, filter paper) have often been used both as substrates and as the source of inducers during fermentation processes for cellulase production (Lee and Fan 1983; Ahamed and Vermette 2008). Several limitations do exist while using solid substrates, such as an increase in viscosity and problematic agitation and reduced oxygen transfer efficiency of the bioreactors. To overcome these problems, lower solid concentrations are often used to get higher cellulase yields (Szengyel et al. 1997). If a cheap source of oligosaccharides is available, they could be directly used for

induction. In many cases both native and pretreated biomass are used for induction (Dashtban et al. 2011). For pretreated biomass, a longer lag phase was observed when compared to controls with media containing glucose (Lo et al. 2005; Juhasz et al. 2005; Lau et al. 2012). These lag phases were attributed to the presence of inhibitory products produced during pretreatment (Palmqvist et al. 1997; Chundawat et al. 2010). When Ammonia Fiber Expansion (AFEX^{TM}) pretreated wash stream was used as inducing medium, more hemicellulases were produced when compared to cellulases. Also, continuous culture produces more enzymes than batch culture, since oligosaccharides are known to be hydrolyzed before the cell concentration is high enough to produce a high concentration of cellulases (Lo et al. 2010).

Many researchers are looking for cheap sources of substrate like clarifier sludge and digester fine from paper mill, pretreated sugarcane bagasse, sorghum straw, corn stover (Yu and Koo 1999; Szakacs and Tengerdy 1997) for producing enzymes. In a larger lignocellulosic biorefinery, pretreated biomass could be used to produce enzymes that will substantially reduce the cellulase production costs.

4.6.2 Improvement of the Fermentation Process

4.6.2.1 Batch *Versus* Fed Batch Substrate Loading

Concentration of substrate and how the substrates are loaded during fermentation can influence the cellulase enzyme productivity. It has been reported that as the substrate concentration in the fermentation tank increases, the cellulase productivity raises: 2–3FPU/ml at 2 % substrate loading and 1–15 FPU/ml at 8 % substrate loadings (Esterbauer et al. 1991). Also, higher cellulase concentrations and volumetric rates were reported for fed batch fermentation when compared to batch fermentation (Hendy et al. 1984).

4.6.2.2 Solid State Fermentation (SSF) *Versus* Submerged Fermentation (SmF)

SSF process is done by growing the microbes on moist solid materials in the absence of free water. Here the substrate used in the process is used slowly and steadily. Although this method is best suited for fungus, the residence time for the process is too long and product separation is tedious. On the other hand, SmF process is done using free flowing liquid substrate or in substrate slurry. This is best studied for bacteria and the separation and purification of product is much easier (Bailey and Tähtiharju 2003; Subramaniyam and Vimala 2012). Most of the cellulase production in the industry is done using SmF. However, most of the aerobic microorganisms produce cellulases at high titers during SSF which is similar to natural environment. About 10-fold reduction in production cost has

been reported when cellulases were produced in SSF when compared to SmF (Singhania et al. 2010). This is due to several advantages SSF have which include high volumetric productivity, higher titer of enzymes, lesser waste generation, low catabolic repression. Given its advantages, SSF could be promising technology for the future.

4.6.3 Fast Downstream Processing for Maximum Product Recovery

Downstream processing of enzymes could account for 50 % of the total cost of enzyme production. Traditional technologies used in the industry are centrifugation (solid liquid separation), filtration, and ultrafiltration (to remove waste and concentrate the enzymes) (Beilen and Li 2002). Efficient enzyme bioseparation process will bring down the cost of enzyme production. Some of the advanced technologies include two-phase extraction, reverse micelle extraction, cloud-point extraction, and field-assisted (electric, magnetic, and acoustic) separation methods (Keller et al. 2001; Karumanchi et al. 2002).

4.6.4 Recycling Enzymes During Bioconversion

One of the strategies to reduce the cost of enzyme is recycling the enzyme during the enzyme hydrolysis step (Tu et al. 2007). The most economical way to recover the enzyme after hydrolysis is done by re-adsorption of free cellulases onto fresh lignocellulosic substrates. About 80–85 % of enzymes activities could be removed using this approach (Lee et al. 1995). In few cases, ultrafiltration methods were also used to retain almost all the enzyme which could be used for subsequent cycle of enzyme hydrolysis. In another study, about 30–50 % of enzymes have been demonstrated to be recycled after each cycle of hydrolysis process using fast Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Co-Fermentation (SSCF) process which takes just two days to complete both hydrolysis and fermentation process when compared to traditional process which takes close to 10 days (Galbe and Zacchi 1993; Lynd et al. 2005; Jin et al. 2012).

4.6.5 Other Approaches to Reduce the Cost of Enzyme Production

There are two models widely followed for supplying enzymes to the biorefinery (Fig. 4.1). The first includes production of enzymes in a centralized large-scale processing facility which concentrates and formulates the enzymes and ships them to the different biorefinery locations. There are several drawbacks to this approach.

Pure substrates, like cellulose and inducers, add additional costs to the process, ultrafiltration techniques used to concentrate the enzymes are energy intensive and shipping enzyme solution are very inefficient. The second approach includes production of enzymes at the site of the biorefinery. In this approach, there are several advantages, such as concentration of the enzymes is unnecessary, the pretreated substrate available in the biorefinery can be used as substrate for enzyme production and there are no shipping costs. Many enzyme companies are trying to adopt the second approach in order to reduce the cost of enzymes for making biofuels.

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Chapter 5
The Saccharification Step: The Main Enzymatic Components

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Abstract In the lignocellulosic biorefinery, enzymatic hydrolysis (i.e., saccharification) is one of the major bottlenecks due to the recalcitrance of plant cell wall. Recent data from genome sequencing have yielded abundant information to select genes of interest putatively involved in lignocellulose degradation. This chapter describes current knowledge on the strategies developed by micro-organisms for lignocellulose degradation with an overview of the various classes of microbial lignocellulose-acting enzymes involved in the saccharification step. In the last part of the chapter, some of the data related to the contribution of fungal secretomes and fungal enzymes to the improvement of lignocellulose degradation are reported.

Abbreviation

CAZy	Carbohydrate active enzyme
CBM	Carbohydrate binding module
CDH	Cellobiose dehydrogenase
CE	Carbohydrate esterase
DP	Degree of polymerization
GH	Glycoside hydrolase

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5.1 Introduction

Lignocellulosic biomass conversion to simple sugars is widely studied for subsequent fermentation to bioethanol or industrial chemicals but processes needed to achieve it are both complex and costly. The three types of polymers, cellulose, hemicellulose, and lignin are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages and their proportions are variable depending on plant species. In the lignocellulose biorefining, enzymatic hydrolysis (i.e., saccharification) is one of the major bottlenecks due to the recalcitrance of plant cell wall. Current costs for enzymatic saccharification still remain the largest contributor to the overall cost of lignocellulosic ethanol production. A major challenge is the development of a suitable efficient and economically viable hydrolysis process step. Currently, *Trichoderma reesei* is the most extensively used fungus in industry due to its capacity to secrete high level of cellulases. However, conversion is still not optimal due to the heterogeneous composition of plant biomass. Possible strategies to circumvent *T. reesei* weaknesses in lignocellulosic biomass hydrolysis include the search for enzymatic activities able to supplement those of *T. reesei* industrial enzyme preparations. The identification of novel enzymes that are either distantly related to or absent from the *T. reesei* genome is a promising option to generate more competitive enzyme cocktails.

5.2 Microbial Strategies for Lignocellulose Degradation

5.2.1 Bacteria: Multi-Enzyme Systems

Most of the bacterial genomes that encode cellulases are soil and marine saprophytes, many of which encode a range of enzymes for cellulose hydrolysis and also for the breakdown of the other constituents of plant cell walls (hemicelluloses and pectins) (Mba Medie et al. 2012). To efficiently hydrolyze cellulose, bacteria have developed different multi-enzyme systems. They produce numerous individual, extra-cellular enzymes with a wide array of binding modules (Maki et al. 2012). Bacterial glycoside hydrolases are often multimodular (www.CAZy.org) providing increased function and synergy. Anaerobic bacteria have developed the cellulosome as a strategy to efficiently hydrolyse cellulose. Cellulosomes are multienzymatic complexes that contain predominantly high molecular weight cellulases, cellulose binding modules, and structural proteins (Lamed et al. 1987; Bayer et al. 1998). The cellulosomes have been described for the first time in the genus *Clostridium* (Bayer et al. 1983) and have been identified later in other genera such as *Ruminococcus* (Ding et al. 2001). The biochemical and genetic studies have shown that the different elements of cellulosome are strongly related to each other through a structural protein called scaffoldin. Scaffoldin exhibits cohesin domains interacting with the dockerin domains of catalytic components. Therefore, the overall architecture of cellulosome is determined by the specificity and the strength of the cohesin-dockerin interaction. Cellulose binding modules are also part of the cellulosome architecture by allowing its specific binding to the substrate. The energy consumption that is necessary to the production of such complex formation is compensated by (i) the binding of substrate at a short distance of cell through cellulosome CBMs limit losses of hydrolized products for the bacteria and (ii) the combination in a restrained area of the complementary activities necessary for degradation of the substrate allows the maximum efficiency of each enzyme.

5.2.2 Fungi: Different Decay Strategies

Filamentous fungi belonging to the phyla Basidiomycota and Ascomycota play a key role in recycling nutrients in forest ecosystems. They are known to produce a high number and broad variety of extracellular enzymes with different, complementary catalytic activities to degrade lignocellulose-rich materials (Sigoillot et al. 2012). Fungal lignocellulolytic enzymes have therefore been extensively studied for the hydrolysis of renewable biomass resources.

5.2.2.1 Classification of Filamentous Fungi

Plant cell wall-degrading filamentous fungi are classified depending on the appearance of wood after decomposition. Three groups have been defined: soft-rot, brown-rot, and white-rot fungi that display different effects onto the lignocellulose, suggesting different degradation mechanisms (Sigoillot et al. 2012).

Soft-rot fungi include mainly ascomycete fungi such as species belonging to the genera *Aspergilli* or *Neurospora* (Martinez et al. 2005). These fungi mainly degrade polysaccharides in the surface layers of plants. Degradation leads to darkening and softening of the wood. Lignin is generally not attacked, although it has been shown that fungi responsible for soft-rotting of wood produce some enzymes of lignin modification, such as laccases and peroxidases (Liers et al. 2006).

Brown-rot fungi mainly metabolize cellulose and hemicellulose. Lignin undergoes only partial alterations. At an advanced stage of degradation, wood residue exhibits cube-shape and has a brownish color due to the predominant presence of oxidized lignin. The brown-rot fungi are basidiomycetes, including *Gloephyllum trabeum*, *Coniophora puteana,* and *Postia placenta,* which are among the most studied (Daniel et al. 2007; Martinez et al. 2009; Irbe et al. 2011).

White-rot fungi are able to effectively degrade all wood components. Degradation of lignin is more effective than in the case of brown-rot and soft-rot fungi, as it can lead to its complete mineralization to carbon dioxide (Cullen and Kersten 2004). Delignified wood takes a whitish appearance. The white-rot fungi capable of causing selective delignification of wood only belong to basidiomycetes (Martinez et al. 2005), such as *Phanerochaete chrysosporium* (Van den Wymelenberg et al. 2006), *Phanerochaete carnosa* (MacDonald et al. 2012), *Pleurotus ostreatus* (Piscitelli et al. 2011), and *Pycnoporus cinnabarinus* (Lomascolo et al. 2011) that are considered as models for the study of white-rot fungi enzymatic mechanisms.

5.2.2.2 Fungal Mechanisms of Lignocellulose Degradation

In ascomycetes, including *Aspergilli* such as *Aspergillus niger* (Pel et al. 2007) and *Aspergillus nidulans* (Galagan et al. 2005), genome sequencing revealed a high number of putative glycoside hydrolases targeting cellulose and hemicellulose of plant cell walls. In the case of *Podospora anserina*, a coprophilic ascomycete that grows on undigested biomass in herbivore dung, many putative GHs have been identified, including cellulases among which 33 GH61 genes. A large number of xylanases have been identified together with one of the highest content in CBMs of all fungal genomes sequenced to date (Espagne et al. 2008; www.cazy.org). The cereal parasite *Fusarium graminearum* genome was also sequenced and its analysis led to the prediction of more than 100 secreted enzymes involved in the degradation of various components of plant cell walls, including 30 enzymes targeting cellulose and nearly 50 acting on hemicelluloses (Brown et al. 2012). By multiplying the enzymes attacking different areas of plant cell wall with

complementary activities, all these fungi ensure an effective degradation of the plant cell wall, which leads to the release of sugars useful for their growth.

In *Postia placenta*, a brown-rot basidiomycete fungus, the strategy for biomass degradation is very different. The publication of its genome (Martinez et al. 2009) and other studies (Van den Wymelenberg et al. 2010) suggest specific degradation mechanisms. The number of glycoside hydrolases (GH) identified in the genome is relatively small, and among them a very limited number is represented by cellulose degrading enzymes. These predictions were corroborated by proteomic studies of its secretome (Ryu et al. 2011) that revealed the presence of only four enzymes potentially active on cellulose. This limited set of enzymes cannot explain the ability of the fungus to hydrolyze plant cell wall polysaccharides. In fact, it is likely that a cooperative action between GH and oxidative enzymes including the depolymerization of the polysaccharide chains by Fenton reactions is responsible for this degradation. The same type of mechanism has been identified in *Gloeophyllum trabeum*, another brown-rot fungus (Cohen et al. 2002; Daniel et al. 2007).

Basidiomycete white-rot fungi are characterized by the presence of many sugar-degrading enzymes but also oxidative enzymes that can initiate lignin depolymerization reactions. The genome of *Phanerochaete chrysosporium* was the first basidiomycete genome sequenced (Martinez et al. 2004). Its analysis showed the presence of more than 160 putative GHs, including endoglucanases, cellobiohydrolases, and β-glucosidases required for cellulose degradation, and many activities targeting hemicelluloses. Lignin peroxidases (LiP) and manganese peroxidases (MnP) have also been found redundantly, since 10 LiP genes and 5 MnP genes were identified, as well as various glyoxal oxidases with which LiP and MnP work in synergy. The secretion of these enzymes in cultures of *P. chrysosporium* was confirmed by proteomic analyses (Ravalason et al. 2008; Van den Wymenlenberg et al. 2010).

5.3 Microbial Enzymes Involved in the Saccharification Step

5.3.1 The CAZy Classification

Carbohydrate-active enzymes (CAZy) were initially classified according to their activity and substrate specificity in the classification of the International Union of Biochemistry and Molecular Biology (IUBMB) with attribution of corresponding Enzyme Commission (EC) numbers. More recently, they have been grouped in the CAZy classification (CAZy database—www.cazy.org) based on comparison of their amino acid sequence, structure and catalytic mechanism (Henrissat 1991; Cantarel et al. 2009). This classification gathers the enzymes involved in the modification of carbohydrates into several groups, among which the GH that cleave glycosidic bonds and the carbohydrate esterases (CE) that allow

de-acylation of sugars. Carbohydrates-Binding Modules (CBM), which are non-catalytic modules involved in substrate binding, are also referenced. Enzymes belonging to the same CAZy family may have different substrate specificities and reciprocally several enzymes acting on the same substrate can be classified in different families. In January 2013, the CAZy database included 131 GH families, 16 CE, and 66 CBM families. More information on GH families can be found in CAZypedia, available at http://www.cazypedia.org/.

5.3.2 Cellulose-Acting Enzymes

Different characteristics of the cellulose influence the efficiency of enzymatic hydrolysis such as the degree of polymerization (DP), crystallinity, particle size, and surface area. Regarding the crystallinity for example, it was shown that it has a strong impact on the rate of hydrolysis. In general, it is considered that the enzymatic attack preferentially occurs in amorphous regions, which should lead to an increase of the degree of crystallinity in remaining cellulose, but several studies have shown that this index actually varied only little during hydrolysis (Penttilä et al. 2010).

Enzymatic hydrolysis of cellulose requires the combined action of at least three types of GH capable to hydrolyse the β-1,4 covalent bonds that connect glucose units in the cellulose fiber. Endoglucanases (endo-1,4 β D-glucanases, EC 3.2.1.4) randomly cleave β-1,4 bonds in cellulose chains thereby generating new reducing-ends and non-reducing ends. They belong to families GH5, GH6, GH7, GH9, GH12, GH45, and GH74 (Table 5.1) and display various structures such as $(\beta/\alpha)_8$ barrels, jelly rolls, or sevenfold β-propellers. Exoglucanases (cellulose 1,4-β-cellobiosidases, EC 3.2.1.91), also known as cellobiohydrolases (CBH), work in a processive manner and release cellobiose groups from the chains extremities. They belong to families GH6 and GH7 (Table 5.1). Some CBH are able to work only on reducing ends while others cleave cellobiose units only at the non-reducing ends. β-glucosidases (1,4-β-glucosidase, EC 3.2.1.21) cleave cellobiose and gluco-oligosaccharides released upon action of the two other types of cellulases and release glucose residues. β-glucosidases belong to families GH1 and GH3 (Table 5.1).

Table 5.1 Fungal enzymes involved in cellulose degradation

Activity	EC number	CAZy families
Endoglucanase	EC 3.2.1.4	GH5, GH6, GH7, GH9, GH12, GH45, GH74
Cellobiohydrolase	EC 3.2.1.91	GH6, GH7
Polysaccharide Mono-Oxygenase (PMO)	EC -	GH61
Cellobiose dehydrogenase (CDH)	EC 1.1.99.18	–
β-glucosidase	EC 3.2.1.21	GH1, GH3

The synergistic action of the different types of cellulases is an essential phenomenon in the hydrolysis of cellulose. Two types of synergies have been identified, one endo–exo between endoglucanases and cellobiohydrolases (Jalak et al. 2012) and one exo–exo between two cellobiohydrolases, one acting on reducing end and the other one acting on non-reducing end (Teeri 1997).

In addition to the classical cellulose-acting GH described above, it is now recognized that other enzymes may have a role in the degradation of cellulose. GH61 family enzymes are known to significantly improve the hydrolysis of lignocellulose by acting in synergy with other cellulolytic enzymes. Originally, they were classified as GH because they had low endoglucanase activity (Karlsson et al. 2001; Koseki et al. 2008), but after further purification, no effect was measured toward crystalline cellulose or hemicellulosic substrates. Recently, the three-dimensional structure of a *Trichoderma terrestris* GH61 was solved (Harris et al. 2010) and it showed a 3D structure very different from that of GHs. Since then, several studies (Qinlan et al. 2011; Westereng et al. 2011; Langston et al. 2011) have demonstrated that GH61 are oxidative enzymes (PMO, polysaccharide monooxygenases) acting in synergy with cellobiose dehydrogenase (CDH) (Bey et al. 2013; Horn et al. 2012; Dimarogona et al. 2012), which brings a new model of cellulose degradation (Fig. 5.1).

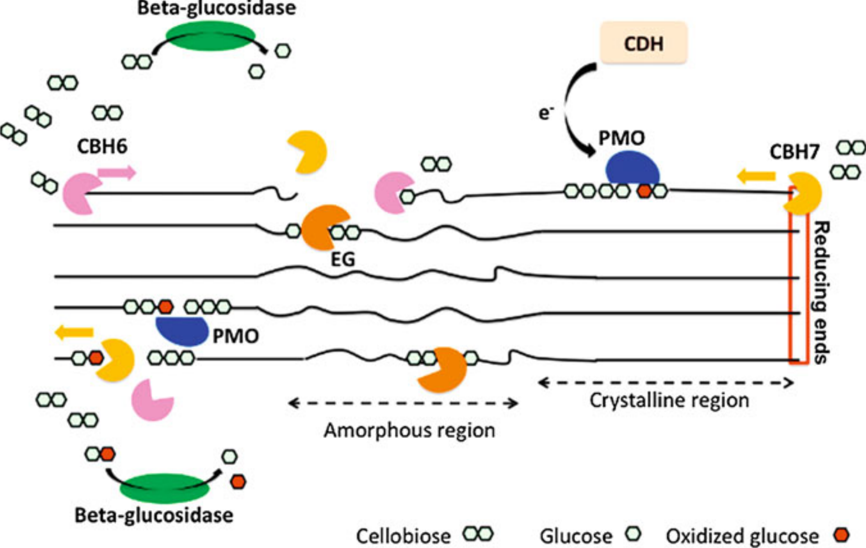

Fig. 5.1 A simplified scheme of the current view on the enzymatic degradation of cellulose involving cellobiohydrolases (CBH), endoglucanases (EG), polysaccharide monooxygenases (PMOs). Cellobiose dehydrogenase (CDH) is a potential electron donor for PMOs. EGs and PMOs cleave internally cellulose chains releasing chain ends that are targeted by CBHs. CBHs generate cellobiose or oxidized cellobiose that are subsequently hydrolyzed by β-glucosidase. From Dimarogona et al. (2012)

5.3.3 Hemicellulose-Acting Enzymes

As in the case of cellulose, hemicellulose hydrolysis requires the intervention of several types of enzymes with complementary activities working at different levels of the hemicelluloytic network, either on the main chain of sugars or to disconnect substitutions. The complexity of hemicelluloses and their close association with the other components of plant cell wall results in the need for a large variety of enzymes with complementary activities for their deconstruction. Two types of enzymes are predominantly involved, GH and CE that cleave ester bonds between the acetyl or feruloyl groups and hemicellulose chains or at the junction between hemicellulose and lignin (Shallom and Shoham 2003).

5.3.3.1 Hemicellulases Involved in Main Chain Degradation

The most abundant component of hemicellulose in the cell walls of monocots (i.e., cereals) is β-1,4-xylan, which consists of β-1,4-linked D-xylose residues substituted with L-arabinosyl, 4-O-methyl-glucuronosyl, and acetyl side chains (Waldron and Faulds 2007). Arabinoxylan degradation involves xylanases (endo-1,4-β-D-xylanase, EC 3.2.1.8), which hydrolyse β-1,4 xylan chains and release xylo-oligosaccharides. Most of xylanases belong to families GH10 and GH11 and some to families GH5, GH7, GH8, and GH43 (Table 5.2) (Collins et al. 2005; Polizeli et al. 2005). Xylanases can display various structures, such as "β-jelly roll" for GH11 (Törrönen and Rouvinen 1995), $(\beta/\alpha)_8$ barrel for GH10 (Paës et al. 2012) or $(\alpha/\alpha)_6$ barrel for GH8 (Berrin and Juge 2008). Their action on the main chain of xylans is followed by β-D-xylosidases (EC 3.2.1.37), which cleave xylobiose and xylo-oligosaccharides and release xylose monomers. Xylosidases are classified in families GH3, GH39, GH43, GH52, and GH54 (Table 5.2).

Table 5.2 Fungal enzymes involved in hemicellulose degradation

Activity	EC number	CAZy families
Xylanase	EC 3.2.1.8	GH5, GH7, GH8, GH10, GH11, GH43
β-Xylosidase	EC 3.2.1.37	GH3, GH39, GH43, GH52, GH54
Mannanase	EC 3.2.1.78	GH5, GH26, GH113
Mannosidase	EC 3.2.1.25	GH1, GH2, GH5
β-Glucosidase	EC 3.2.1.21	GH1, GH3
α-Arabinofuranosidase	EC 3.2.1.55	GH43, GH51, GH62
α-Glucuronidase	EC 3.2.1.139	GH67
α-Galactosidase	EC 3.2.1.22	GH4, GH27, GH36, GH57, GH97, GH110
Acetyl xylan esterase	EC 3.1.1.72	CE1, CE2, CE3, CE4, CE5, CE6, CE7, CE12, CE16
Acetyl esterase	EC 3.1.1.6	CE16
Feruloyl esterase	EC 3.1.1.73	CE1
Glucuronoyl methyl esterase	EC 3.1.1-	CE15

Mannans are the major component of hemicellulose in softwood. The main backbone can be constitued of mannose residues only or of mannose residues and glucose residues (glucomannan). β-mannanases (endo-β-1,4-D-mannanase, EC 3.2.1.78) that belong to families GH5, GH26, and GH113 (Table 5.2) are endo-hydrolases that cleave β-1,4 bonds of mannan chains and produce new reducing and non-reducing ends. Most β-mannanases are capable of degrading oligosaccharides from a DP (DP, number of monomeric units) of 4 but some are also active on mannotriose (DP of 3). This parameter depends on the active site organization. In addition to their ability to hydrolyse mannan fibers, which is their main function, some β-mannanases are able to perform transglycosylation of manno-oligosaccharides. The hydrolytic action of β-mannanases on mannan is supplemented by β-mannosidases (EC 3.2.1.25, families GH1, GH2 and GH5 (Table 5.2)). The latter release mannose and manno-oligosaccharides from the non-reducing ends produced upon mannanase action. Both β-mannanases and mannosidases display $(\beta/\alpha)_8$ structures (www.cazy.org). In the case of glucomannan, β-glucosidases can also act to cleave the bond between one mannose residue and one glucose residue (Table 5.2). The action of these enzymes on galactoglucomannan strongly depends on the number and pattern formed by the substituted galactoses and other substitutions, and therefore depends on the action of auxiliary enzymes (Moreira and Filho 2008).

5.3.3.2 Debranching Hemicellulases

In the case of wheat straw arabinoxylan, xylanases and xylosidases require the coordinate action of accessory enzymes that will remove residues substituted at C2 and/or C3 of xylose residues. α-L-arabinofuranosidases (EC 3.2.1.55) are included in families GH43, GH51 (Table 5.2), and GH62. They hydrolyze covalent bonds between L-arabinose and D-xylose residues and are required for the complete degradation of arabinoxylans. They act only at the non-reducing ends and can have special specificities for α-1,2 or α-1,3 bonds. Synergy between xylanases and arabinofuranosidases was demonstrated by de Vries et al. (2000).

Glucuronic acids substituted on the main chain of xylans are cleaved by α-glucuronidases from family GH67 (Table 5.2) (Ruile et al. 1997). Esterases play a role to disconnect ester-bound groups from hemicellulose main chains. The acetyl groups at C2 or C3 of xylose residues are cleaved by acetyl xylan esterases (EC 3.1.1.72) from families CE1, CE2, CE3, CE4, CE5, CE6, CE7, CE12, and CE16 (Table 5.2) (Biely 2012); ferulic acids are disconnected by feruloyl esterases (EC 3.1.1.73) from CE1 family, and the methyl groups that can be carried by glucuronic acid substitutions are cleaved by glucuronoyl methyl esterases (EC 3.1.1.-) family CE15 (Li et al. 2008).

Galactose residues that are connected to mannan and glucomannan main chains via α-1,6 glycosidic bonds are cleaved by α-galactosidases (EC 3.2.1.22) from families GH4, GH27, GH36, GH57, GH97, GH110 (Table 5.2). Acetyl esterases

(EC 3.1.1.6, CE16) remove acetyl groups thus allowing access to the backbone for main-chain degrading enzymes.

5.4 Optimization of Enzymatic Cocktails for the Saccharification of Lignocellulosic Feedstocks

Currently, *Trichoderma reesei* is the most extensively used fungus in industry due to its capacity to secrete high level of cellulases. The release of the *T. reesei* genome has shown that its CAZy machinery is globally comparable to other saprophytic fungi (Martinez et al. 2008; Cantarel et al. 2009; Kubicek et al. 2011). However, compared to other filamentous fungi, the *T. reesei* genome is poor in terms of number and diversity of enzymes that are likely to be involved in biomass degradation (Martinez et al. 2008). The lack of key lignocellulosic enzymes in *T. reesei* opens opportunities to generate more competitive enzyme cocktails. Results related to the contribution of fungal secretomes and fungal enzymes to the improvement of lignocellulose degradation are reported here. Some of these studies have been developed in a high-throughput mode taking advantage of robotic automated methods to assess enzymatic conversion of the biomass (e.g. Berlin et al. 2007; Navarro et al. 2010).

5.4.1 Fungal Secretomes

To improve the saccharification of lignocellulosic biomass by *T. reesei*, several studies assessed the supplementation of *T. reesei* enzymatic cocktail with fungal secretomes (mixture of secreted enzymes). For instance, Shrestha et al. (2011) have isolated novel fungal species (mainly ascomycetes) from decaying bioenergy grasses, among which some were able to convert *Miscanthus* biomass. However, the dry weight loss was only 8–13 % over 4 weeks. There has also been growing interest in the potential of plant-pathogenic fungi to optimize hydrolysis of lignocellulosic biomass (Gibson et al. 2011). A large-scale screening using 156 ascomycetes revealed that the plant pathogens were more active than the non-pathogens on several lignocellulosic substrates (King et al. 2011). Couturier et al. (2012) analyzed 20 filamentous fungi for which genomic data are available for their biomass-hydrolysis potential. Most of the fungal secretomes tested individually supplemented the industrial *T. reesei* enzymatic cocktail and the most striking effect was obtained with the phytopathogen *Ustilago maydis* that improved the release of total sugars by 57 % and of glucose by 22 %. Proteomic analyses (LC-MS/MS) of its secretome indicated a specific enzymatic mechanism. As *T. reesei* is devoid of oxido-reductases, some of the putative *U. maydis* oxidases identified in *U. maydis* secretome are likely responsible for the observed

increase in saccharification. Recent data from literature on the synergy between GH61 and oxido-reductases (Langston et al. 2011) might also explain the efficiency of *U. maydis* secretome in combination with *T. reesei* that contains a GH61 enzyme (Couturier et al. 2012). Interestingly, a similar approach led to the identification of the phytopathogenic fungus *Fusarium verticillioides* that revealed a high number of hemicellulases and pectinases of interest in its secretome (Ravalason et al. 2012). Natural fungal diversity in wood-decaying species (CIRM collection of filamentous fungi: http://cirm.esil.univ-mrs.fr/crbmarseille) was explored for biomass deconstruction (Berrin et al. 2012). Among 74 fungal isolates collected in temperate and tropical forests, 19 isolates led to an improvement in biomass conversion of at least 23 %. Among the isolates, the *Trametes gibbosa* BRFM 952 secretome performed best, with 60 % improved conversion, a feature that was not a universal feature to the *Trametes* and related genera. Enzymatic characterization of the *T. gibbosa* secretome revealed a high activity on crystalline cellulose, higher than that of the *T. reesei* cellulase cocktail. Therefore white-rot basidiomycetes could be an interesting source of lignocellulose-active enzymes (Berrin et al. 2012; Falkoski et al. 2012) to supplement cocktails originated from ascomycetes (e.g. *Aspergillus niger, T. reesei*).

Obviously, exploration of fungal biodiversity through their secretomes is currently one of the most relevant methods to find new enzymes of interest to improve the saccharification of biomass. Further transcriptomic and proteomic studies together with controlled synergy experiments using fungal lignocellulolytic enzymes are now required. However, a full exploitation of the data requires access to microbial genomic information, which is a major challenge to gain a better knowledge of this biodiversity.

5.4.2 Fungal Enzymes

Another approach to improve the saccharification of lignocellulosic biomass by *T. reesei* is the supplementation of its enzymatic cocktail with fungal enzymes. It is well known that cellulase enzymatic cocktails secreted by *T. reesei* are very poor in β-glucosidase activities and that optimal hydrolysis of pretreated biomass often requires supplementation with a commercial β-glucosidase preparation (e.g. Novozymes SP188). Characterization and comparative analyses of fungal β-glucosidases are therefore essential to enhance conversion and avoid inhibition of saccharification by cellobiose (Ramani et al. 2012; Chauve et al. 2010).

"Accessory" enzymes, such as hemicellulases and oxido-reductases, are also believed to stimulate cellulose hydrolysis by removing non-cellulosic polysaccharides that coat cellulose fibers (Berlin et al. 2007). For example, several genes encoding putative polysaccharide-degrading enzymes were selected from the coprophilic fungus *Podospora anserina* using comparative genomics (Couturier et al. 2011). Among ascomycetes, the genome of *P. anserina* revealed the potential of this coprophilic fungus to hydrolyse recalcitrant lignocellulosic residues. It

contains a significantly higher number of putative cellulases and xylanases compared to other fungi. Three hemicellulases among which two mannanases, *Pa*Man5A and *Pa*Man26A, and one xylanase, *Pa*Xyn11A, efficiently individually complemented the secretome of the industrial *T. reesei* CL847 strain and significantly improved the release of reducing sugars and glucose upon hydrolysis of micronized wheat straw or spruce (Couturier et al. 2011). Helper enzymes can loosen up the lignocellulosic structure via degradation of hemicellulosic substrates thus increasing the surface area of cellulose attacked by *T. reesei* cellulases. The fact that the boosting effect is more striking with the addition of mannanase (Pham et al. 2010, Couturier et al. 2011) might be due to the close association of softwood glucomannan with cellulose microfibrils whereas xylan is interconnected to lignin. Moreover, the fusion of mannanases to a CBM1 module can potentiate the action of the enzyme toward mannan-containing lignocellulosic substrates (Pham et al. 2010). With addition of relevant xylan- or mannan-acting enzymes to commercial cocktail, one could consider to lower the dosage of *T. reesei* cocktail with the same glucose yield (Gao et al. 2009, Meyer et al. 2009) since cellulose would be more accessible to cellulases. The solubilisation of hemicellulose could have also been enhanced by acetyl esterases (Biely 2012). A recent study has shown that the supplementation of xylanase with an acetyl esterase from *T. reesei* enhances the solubilization of xylan and cellulose from wheat straw and giant reed, thus indicating a layered structure of xylan and cellulose chains in the cell wall substrates (Zhang et al. 2011).

Lignin removal is also an important technical issue for the conversion of lignocellulosic feedstock into ethanol. Most biological pretreatments for delignifying lignocellulosic materials employ lignin-degrading fungi, mainly belonging to the group of white-rot basidiomycetes (Salvachúa et al. 2011) but such pretreatments require long application periods and consume a fraction of the plant polysaccharides. Gutiérrez et al. (2012) have investigated the ability of the high redox-potential laccase from the basidiomycete *Trametes villosa* to remove lignin and make cellulose accessible to hydrolysis, when applied on the whole lignocellulosic biomass in combination with a redox mediator. The enzymatic treatment of the laccase enzyme on Eucalypt and Elephant grass feedstocks increased the glucose (by 61 and 12 % in 72 h) and ethanol (by 4 and 2 $g.L^{-1}$ in 17 h) yields from both lignocellulosic materials, respectively, as compared to those without enzyme treatment (Gutiérrez et al. 2012). Bey et al. (2011) also investigated the contribution of CDH in the saccharification process using a commercial *T. reesei* enzymatic cocktail that was supplemented with the CDH from *Pycnoporus cinnabarinus*. A significant enhancement of the degradation of wheat straw was observed with (i) the production of a large amount of gluconic acid, (ii) the increase of hemicellulose degradation, and (iii) the increase of the overall degradation of the lignocellulosic material. Similar results were also obtained by Turbe-Doan et al. (2012) with CDHs from *Coprinopsis cinerea* and *P. anserina*.

5.5 Conclusions and Perspectives

An overview of the various classes of microbial lignocellulose-acting enzymes involved in the saccharification step has been presented in this chapter. Several approaches have been developed by scientists to optimize microbial enzymatic cocktails. Novel enzymes identified through genome mining and/or exploration of microbial diversity have already permitted to improve significantly the saccharification step. Several hundred novel microbial genomes are at various stages of sequencing worldwide and will become available in the next few years; it will open new avenues for enzyme discovery. Due to structural differences between plant cell walls depending on their origin, it is now essential to adapt enzymatic cocktails for efficient conversion of diverse lignocellulosic biomasses into fermentable sugars. New prospects are also considering the improvement of lignocellulose-acting enzymes using molecular evolution to adapt enzymes to industrial processes. These approaches will contribute to unlock the bottlenecks of biomass deconstruction.

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Chapter 6
Extremophilic (Hemi)cellulolytic Microorganisms and Enzymes

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Abstract The *second generation* bioethanol represents a main challenge in global efforts to utilize renewable resources rather than fossil fuels. However, the close association of cellulose and hemicelluloses to lignin in the plant cell wall makes it difficult to degrade lignocellulose into fermentable sugars. Consequently, pre-treatments are necessary to make the polysaccharides more accessible to the enzymes, but the high temperature and extreme pH conditions required give rise to problems when using conventional enzymes in the saccharification step (Galbe and Zacchi 2002). Microorganisms thriving in habitats characterized by harsh conditions, and the enzymes derived therein, represent a helpful tool in the development of bioethanol production processes. In fact, they allow bioconversions at non-conventional conditions under which common biocatalysts are denatured. The use of high operational temperatures allows energy savings by reducing the cooling cost after high temperature pretreatments, and, in ethanol production, thermophilic conditions permit ethanol evaporation allowing harvest during fermentation.

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6.1 Extremophilic Microorganisms

The study of extremophiles and extremozymes started in the late 1960s. In the following sections we will summarize the state-of-the-art on (hyper)thermophiles, halophiles and alkaliphiles involved in (hemi)cellulose degradation.

6.1.1 (Hyper)thermophiles

Depending on their optimal growth temperature, thermophilic microorganisms are grouped in thermophiles (45–80 °C) and hyperthermophiles (80–122 °C). The latter are dominated by the Archaea domain, but some Bacteria, such as *Thermotoga* and *Aquifex,* tolerate temperatures around 100 °C. Degradation of cellulosic and hemicellulosic substrates among thermophiles is mostly due to Bacteria species with few Archaea species.

6.1.1.1 (Hyper)thermophilic Bacteria

The major rate-limiting step in the conversion of lignocellulose is represented by the hydrolysis of cellulose, since in plant biomass it has a high order of crystallinity and is scarcely accessible to microbial or enzymatic attack. Not many microorganisms are able to degrade pure crystalline cellulose, and two main concepts are considered for its microbial degradation: free cellulases and large multi-enzyme complexes (cellulosomes). Crystalline cellulose degradation via cellulosomes was firstly described in *C. thermocellum* (T 60 °C). This microorganism solubilized to an extent of 95 % the cellulose microcrystals from the alga *Valonia ventricosa*, whose crystallinity is close to 100 % (Boisset et al. 1999).

The order *Thermoanaerobacteriales* includes several species that utilize (hemi)cellulose as growth substrates. The most thermophilic species belong to the genus *Caldicellulosiruptor*, but, while all species hydrolyze hemicellulose, not all degrade crystalline cellulose. *C. kristjanssonii* (T 78 °C) and *C. bescii* (T 80 °C), which is the most thermophilic cellulolytic bacterium characterized to date, can grow on crystalline cellulose and unprocessed plant biomasses (Bredholt et al. 1999; Yang et al. 2009, 2010). Recently, a novel cellulolytic bacterium has been isolated from Obsidian Pool in Yellowstone National Park. The microorganism,

designated *C. obsidiansis* sp. nov. (T 78 °C), exhibits fermentative growth on arabinogalactan, xylan, Avicel, filter paper, dilute acid-pretreated switchgrass, and poplar, whereas is unable to grow on lignin (Hamilton-Brehm et al. 2010).

Decomposition of lignocellulose by complex microbial communities represents a promising alternative for biomass conversion. In particular, thermophilic consortia are a potential source of enzymes adapted to harsh reaction conditions. Wongwilaiwalin et al. (2010) obtained from a high-temperature bagasse compost a stable thermophilic lignocellulolytic microbial consortium highly active on cellulosic biomass. A microbial consortium including anaerobic bacteria of genera *Clostridium* and *Thermoanaerobacterium,* efficiently degraded rice straw, corn stover, and industrial eucalyptus pulp sludge. In a recent study, compost-derived microbial consortia were adapted on switchgrass at 60 °C: high abundance of thermophilic bacteria as *Rhodothermus marinus* and *Thermus thermophilus* were observed (Gladden et al. 2011).

In addition to traditional bioethanol production processes, consolidated bioprocessing (CBP), which combines in one step saccharification with fermentation using a whole cells-based approach, represents an alternative method with outstanding potential for low-cost processing of lignocellulosic biomass (Lynd et al. 2005). No ideal CBP microbe able to degrade efficiently lignocellulose and, at the same time, to utilize the released sugars to produce ethanol is currently available. A newly discovered thermophilic microorganism, *Geobacillus* sp. R7 (T 60 °C), is a facultative anaerobic bacterium isolated from soil samples of the Homestake gold mine, South Dakota. It produces a thermostable cellulase when grown on extrusion-pretreated agricultural residues such corn stover and prairie cord grass, and ferments lignocellulosic substrates to ethanol in a single step (Zambare et al. 2011).

Xylan represents the second most abundant polysaccharide in lignocellulose; however, the number of characterized thermophilic microorganisms that utilize xylan exceeds the number of cellulose-degrading ones. Xylan-utilizing microorganisms are widely distributed within the order *Thermoanaerobacteriales*. *T. zeae* (T 68 °C), isolated from industrial environments, utilizes cracked corn and xylan, but not cellulose, and produces ethanol as the main product after glucose fermentation (Cann et al. 2001). Xylan-degrading bacteria have also been found in the genera *Thermotoga*. *T. hypogea* sp. nov. (T 70 °C) produces trace amounts of ethanol during xylan fermentation (Fardeau et al. 1997).

In comparison with the anaerobic thermophilic bacteria few aerobic have been described to produce cellulases and xylanases. The aerobic thermophiles *R. marinus* produces a highly thermostable cellulase (Cel12A), and three glycoside hydrolases belonging to family GH10 of the Carbohydrate Active enZyme (CAZy) database (http://www.cazy.org) (Alfredsson et al. 1988; Cantarel et al. 2009). *Aquifex aeolicus*, isolated in the Aeolic Islands in Sicily (Italy), represents one of the most thermophilic bacteria since its growth temperature can reach 95 °C (Deckert et al. 1998). From this source, a single cellulase, able to hydrolyze CMC but not Avicel, has been reported to date. Table 6.1 summarizes other bacteria not mentioned in the text.

Table 6.1 Thermophilic (hemi)cellulolytic bacteria

Microorganism	Growth T (°C)	Growth conditions	References
Acidothermus cellulolyticus	55	Aerobic	Mohagheghi et al. (1986)
Alicyclobacillus acidocaldarius	60	Aerobic	Wisotzkey et al. (1992)
Clostridium stercorarium	65	Anaerobic	Madden (1983)
Dictyoglomus thermophilum	73	Anaerobic	Saiki et al. (1985)
Moorella strain F21	60	Anaerobic	Karita et al. (2003)
Spirochaeta thermophila	70	Anaerobic	Aksenova et al. (1992)
Thermoanaerobacter cellulolyticus	75	Anaerobic	Taya et al. (1988)
Thermotoga neapolitana	80	Anaerobic	Jannasch et al. (1988)
Thermotoga petrophila	80	Anaerobic	Takahata et al. (2001)

6.1.1.2 Hyperthermophilic Archaea

Among Archaea, the genus *Pyrococcus* and *Sulfolobus* have been found to produce cellulases and xylanases (Maurelli et al. 2008). Microorganisms belonging to the genus *Pyrococcus* have been isolated from hydrothermally heated sea vents with T 90–100 °C. The genomes of *P. furiosus* (Fiala and Stetter 1986), *P. horikoshii* (Gonzalez et al. 1998), *P. abyssi* (Cohen et al. 2003) and *Thermococcus kodakaraensis* KOD1 (Fukui et al. 2005), encode a variety of cellulases, however, none of these microorganisms grows on crystalline cellulose.

Within the order *Sulfolobales*, *Sulfolobus* species are commonly isolated from acidic thermal pools (T up to 90 °C). A specific strain *S. solfataricus* (Oα) can grow on xylan as sole carbon source (Cannio et al. 2004), but crystalline cellulose is not a growth substrate for any species of *Sulfolobus* so far reported. Recently, Perevalova et al. (2005) demonstrated that *Desulfurococcus fermentans,* an obligately anaerobic archaeon isolated from a freshwater hot spring of the Uzon caldera (Kamchatka Peninsula, Russia) growing optimally at 82 °C, is capable of growing on crystalline cellulose.

As reported above, few archaea species are known to be able of degrading lignocellulose. To identify new species able to decompose biomasses at high temperatures, analyses of 16S rRNA genes in DNA samples from terrestrial hot springs and deep-sea vents may reveal hyperthermophilic microbes recalcitrant to culture and with potential unknown hydrolyzing properties. Kublanov and co-workers (2009) identified many hot springs (T 68–87 °C) in Kamchatka Peninsula for in situ enrichment on microcrystalline cellulose of thermophilic species. Denaturing gradient gel electrophoresis analysis of 16S rRNA gene fragments obtained after PCR with Archaea-specific primers, revealed the presence of uncultivated micrcounganisms. They were closely related to uncultured organisms from Iceland and Kamchatka hot springs belonging to the *Crenarchaeota* phylum “unknown *Desulfurococcales*”. Recently, a sediment collected from a 94 °C geothermal pool in northern Nevada, USA, was used as inoculum for enrichment trials in laboratory. After two consecutively enrichments with Avicel and filter

paper, an anaerobic consortium consisting of three archaeal species able of growth on cellulose sources was identified (Graham et al. 2011). These data represent a new benchmark in searching for new microbial species capable of degradation of lignocellulose at high temperatures.

6.1.2 Halophiles

Saline and hypersaline environments represented by saline lakes and other water systems as well as saline soils, are widely distributed on the Earth (Oren 2002). Such organisms, called halophiles, are found in all three domains of life: they are widespread in the bacterial and archaeal kingdoms and eukaryotic halophilic microorganisms, such as fungi and algae, are also known (Gunde-Cimerman et al. 2009).

The groups containing halophilic representatives seldom include solely halophiles and only a few phylogenetically consistent groups are composed entirely of halophiles and many genera, families and orders show different salt requirements and tolerance (Oren 2002, 2008). On the basis of the hypersaline conditions needed for growth, halophiles are classified as slight, moderate and extreme halophiles (requiring 2–5, 5–20 or 20–30 % NaCl, respectively). On these basis of a good and simple operative definition suggested by Oren, the halophiles grow optimally at salt concentrations ≥50 g/l and tolerate at least 100 g/l salt (Oren 2008). Moreover, halotolerants are able to grow at moderate salt concentrations, even though they grow best in the absence of NaCl (Ventosa et al. 1998).

In Euryarchaeota the most important salt-requiring microorganisms were found within the archaeal order of *Halobacteriales* and among the *Methanothermea*, in the order *Methanosarcinales*. No halophiles have yet been identified within the *Crenarchaeota* kingdom. The Bacteria domain contains many types of halophilic and halotolerant microorganisms, widespread over a large number of phylogenetic subgroups (for a review see Ventosa et al. 1998) including *Proteobacteria*, *Cyanobacteria*, the *Cytophaga-Flavobacterium* branch, the *Spirochaetes*, and the *Actinomycetes*. Within Gram-positive Bacteria (*Firmicutes*), halophiles are found both in the aerobic (*Bacillus* and related organisms) and in the anaerobic branches. Halophiles are scarcely present in Eukarya. In fact, the only eukaryal microorganism of importance, but almost ubiquitously present in high-salt environments, is the green alga *Dunaliella* (Oren 2005).

A cellulose-utilizing, extremely halophilic bacterium was first reported by Bolobova et al. (1992). An obligate anaerobic organism named *Halocella cellulolytica* is able to utilize cellulose as a sole carbon source. Another work has shown that many cellulose-utilizing extremely halophilic Archaea are present in subsurface salt formation (Vreeland et al. 1998). A preliminary work on extracellular hydrolytic enzymes of halophilic microorganisms from subterranean rock salt revealed the presence of cellulases and xylanases. The isolated strains producing these enzyme activities are Gram-negative rods and can grow at 120 °C. These microorganisms are unable to thrive in the presence of various antibiotics such as

neomicin, penicillin, anysomicin and erythromycin, and are tolerant to salt concentrations of up to 3 M NaCl (Cojoc et al. 2009).

The purification and properties of two new halotolerant xylanases with stability and activity in NaCl concentrations in the range 0–5 M from the extremely halophilic archaeon *Halorhabdus utahensis* have been reported (Wainø and Ingvorsen 2003; Wejse et al. 2003). This microorganism was isolated from sediment of Great Salt Lake of Utah, USA and grows with 27 % w/v NaCl and only a few carbohydrates.

In comparison with the thermophilic and the alkaliphilic extremophiles, halophilic microorganisms have found relatively few biotechnological applications yet. Nevertheless, these microorganisms produce unique enzymes stable and active in conditions in which their "conventional" counterparts could not be functional (e.g. high salinity, low water activities and presence of organic solvents) (Litchfield 2011). Moreover, the recent availability of new halophiles genome sequences will allow the identification of novel (hemi)cellulolytic strains useful for biotechnological purposes.

6.1.3 Alkaliphiles

Over the years, an increasing number of alkaliphilic microorganisms and related enzymes have been extensively investigated and exploited for industrial applications. Various definitions for the alkaliphiles have been used. Generally this term is applied to the microorganisms that grow optimally at pH $\geq$ 9.0–9.5. The bacteria that have their optimum growth at pH 7.0, and that can also tolerate pH 9.0 but cannot thrive at pH higher than 10.0, are defined alkali-tolerant. The extreme alkaliphiles can be further subdivided into facultative alkaliphiles, which show optimal growth at 10.0 or above but can grow well at neutral pH, and obligate alkaliphiles that optimally growth at pH $\geq$ 10.0 but not below 8.5–9.0.

A number of cellulolytic and xylanolytic microorganisms that thrive at high pH have been isolated from a variety of natural environments such as geothermal areas, carbonate laden soils, soda deserts and soda lakes. (Hemi)cellulolytic bacteria growing at pH $>$ 6.5 have also been isolated from additional sources such as kraft pulp, pulp and paper industry wastes, decomposing organic matter, insect intestinal tract, plant sources, soils, and even from neutral environments where they are found coexisting with neutrophilic microorganisms.

The group of the alkaliphiles exhibit a wide taxonomical diversity ranging from eubacteria belonging to genera *Bacillus, Micrococcus*, *Pseudomonas*, and *Streptomyces* to archaea such as *Natronobacterium* spp.. A huge number of xylanolytic and cellulolytic alkaliphiles known so far belongs to *Bacillus* spp and *Bacillus*-like genera (Subramaniyan and Prema 2000). These bacteria were also isolated from neutral soils but numerous species come from more alkaline environments. The occurrence of alkaliphiles in conventional ecosystems represents a peculiarity of this group of microorganisms with respect to hyperthermophiles and psychrophiles

adapted and distributed predominantly in selected environments. Presumably, alkaline microenvironments in the soil allow the growth of these extremophiles (Horikoshi 2006).

Several (hemi)cellulolytic bacteria belonging to *Bacillus* sp. (Horikoshi et al. 1984; Shikata et al. 1990; Nakamura et al. 1993; Blanco et al. 1995; Ito 1997; Subramaniyan et al. 1997; Takahashi et al. 2000; Kim et al. 2005), *Gracilibacillus* sp.strain TSCPVG (Giridhar and Chandra 2010), and *B. sphaericus* JS1 (Singh et al. 2004), have been isolated from soil. Generally, most of the alkaliphilic *Bacillus* spp. are facultative alkaliphiles and are distributed in a wider range of soil types compared with obligate alkaliphilic one. For example, the *Paenibacillus* strain Q8 isolated from the acid mine drainage of Carnoulès (France) is even able to grow under both acidic (pH 5.0) and alkaline (pH 9.0) conditions. Function-based screening of a Q8 DNA-library allowed the detection of three clones with xylan-degrading activity that was confirmed and measured in the crude extracts of Q8 grown in liquid medium (Delavat et al. 2012).

The majority of the truly alkalophilic species have been isolated from specific environments such as alkaline soda lakes characterized by the most stable conditions (e.g., pH > 11.5 and sodium bicarbonate/carbonate, and chloride concentrations ranging from about 5 to >15 % w/v). There, the surrounding flora is the possible source of allochthonous cellulose. The majority of the alkaliphiles isolated herein grows in the presence of high salinity and are among the best sources of enzymes with good potential for application in bioethanol production processes. Due to the high levels of halophilicity, halostability and, in several cases, thermostability, these biocatalysts can be added to plant biomasses directly after different types of pretreatment. Thus, the need for costly pH and temperature readjustment or solvent removal before the saccharification process can be completely avoided. Several (hemi)cellulolytic bacteria such as *Amphibacillus xylanus* (Niimura et al. 1987), *Bacillus* sp. (Gessesse and Gashe 1997; Gessesse 1998; Shah et al. 1999; Aygan and Arikan 2008; Roy and Belaluddin 2004; Zhang et al. 2012), *Micrococcus* sp.AR-135 (Gessesse and Mamo 1998), *B. halodurans* S7 (Mamo et al. 2006), *C. alkalicellum* (Zhilina et al. 2005) have been reported.

Another interesting source for alkaliphilic (hemi)cellulolytic bacteria is the intestinal tract of herbivorous insects. Here, different microorganisms carry on the degradation of the biomass facilitating insect phytophagy. Different parts of the intestinal tract show different pHs ranging from slightly acidic values in the foregut to neutral values in the midgut. However, in most larvae midgut high alkaline pH of 10–12 are detected. As reported by Anand et al. (2009) several xylanolytic and cellulolytic isolates were obtained from the digestive tract of *Bombix mori* larvae. In particular, isolates of *Aeromonas* sp, *B. circulans*, *Citrobacter freundii*, *Serratia liquefaciens*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Enterobacter* sp. were endowed with cellulase activities being able to digest both amorphous and crystalline cellulose while *B. circulans, Aeromonas sp.* and *S. liquefaciens* also showed xylanase activity. Moreover, species of the genus *Micrococcus* have been found in termites and have been reported to display CMCase and xylanase activity (Saxena et al. 1991). Species of the genera *Bacillus*

are predominant in the termite gut contents according to Wenzel et al. (2002) where *Comamonas* species are also found (Kudo 2009). *Micrococcus paraoxydans* has been isolated from the mosquito *Culex quinquefasciatus* midgut (Pidiyar et al. 2004). *Pseudomonas* spp isolates were found in the gut of *Holotrichia parallela* larvae (Huang et al. 2012) and of the ground beetle *Poecilus chalcites* (Lehman et al. 2008). Finally, the (hemi)cellulolytic *Promicromonospora pachnodae*, which can produce xylanase and endoglucanase activities under both aerobic and anaerobic conditions, was recovered from the hindgut of *Pachnoda marginata* (Cazemier et al. 2003).

Numerous (hemi)cellulolytic alkaliphiles that are able to utilize several ligno-cellulosic biomasses including agro-residues as carbon sources, have also been isolated from other various environments. As reported by Sanghi et al. (2010) the xylanolytic *B. subtilis* ASH 7414 isolated in enrichments from soil sample at pH 7.0–11.0 can utilize wheat bran as carbon source. This strain was exploited for the production of high levels of xylanase (8,964 U/g dry wheat bran) in solid state fermentation. High titer of xylanase were also reached by *B. pumilus* SV-85S, isolated from soil at moderate alkaline pHs and grown under submerged fermentation (SmF). Substrates such as wheat bran, rice straw, wheat straw, soybean flakes, rice bran, sugarcane bagasse, saw dust, ground nut shells, have been found to support the bacterial growth and xylanase production with the highest enzyme amounts obtained with wheat bran, probably beacause of its high xylan content (Nagar et al. 2010). The alkalo-thermo-anaero-bacterium, *Tepidimicrobium xylanilyticum* strain BT14, isolated from soil, has been reported to grow on corn hulls at pH 9.0 and 60 °C under anaerobic conditions (Paripok et al. 2010). Saccharification experiments on pretreated plant biomasses carried out with crude extracts of this bacterium, revealed a marked release of reducing sugars from substrates such as corn cob, rice straw, rice bran, and sugarcane bagasse. These cells also bind to Avicel, xylan, and corn hull and produce a cellulolytic and xylanolytic enzyme complex. Evidence of a cohesin-like domain sequences in the genome of *T. xylanilyticum* BT14 could indicate the presence of a cellulosome. Finally, the ability of the strain to grown on corn hull in anaerobic conditions at pH 9.0 and 60 °C, to produce ethanol and acetate, makes the microorganism exploitable for bioethanol production.

6.2 Extremophilic Enzymes

Hyperthermophilic enzymes are produced naturally by microbes living at temperatures higher than 80 °C. Because extreme heat and very low pH in the current processes for the pretreatment of lignocellulose are known to produce toxic by-products such as furfural (Heer et al. 2009; Mamman et al. 2008), adjustment to less extreme conditions with supplementation of a thermophilic and acidophilic enzyme may be suitable for improving the current pretreatment processes for ethanol production (Miller and Blum 2010). This paragraph summarizes the

state-of-the-art on extremozymes that mediate saccharification of cellulose and hemicellulose of relevance to biomass processing. In particular, 'primary' cellulolytic enzymes are defined as those that contain a catalytic domain and carbohydrate-binding domains (CBM), while 'secondary' enzymes lack a carbohydrate binding domain and/or multiple catalytic domains (Blumer-Schuette et al. 2010). Typically, hyperthermophiles have secondary cellulolytic enzymes whose optimum temperatures and thermal stability are superior to enzymes from thermophilic microorganisms. This is important for robust technological processes for the degradation of polysaccharides and, therefore, attracted interest.

6.2.1 Cellulases

Enzymatic deconstruction of crystalline cellulose can be achieved by three activities: cellobiohydrolase (exocellulase, E.C. 3.2.1.91 and E.C. 3.2.1.−), endoglucanase (endocellulase, E.C. 3.2.1.4), both acting on cellulose, and β-glucosidase (E.C. 3.2.1.21). A short survey of enzymes involved in the cellulose hydrolysis is reported in Table 6.2.

Cellobiohydrolases catalyse the release of cellobiose from either the non-reducing end or the reducing end of cellulose, depending on the enzyme. They belong to GH (glycoside hydrolase) families 5, 6, 7, 9 and 48. A hyperthermophilic cellobiohydrolase was isolated from *Thermotoga* sp. strain FjSS3-B.1 with an optimal temperature for its activity of 105 °C and an half-life of 70 min at 108 °C, making it one of the most thermostable cellobiohydrolases currently known (Ruttersmith and Daniel 1991).

Endoglucanases catalyse the endohydrolysis of (1,4)-β-D-glucosidic linkages in cellulose. They belong to families GH 5, 10, 12, 16, 18, 19, 26, 44, 45, 48, 51, 74 and 124. Several thermophilic bacteria contain 'free-acting' endoglucanases that are not part of a cellulosome complex (for a review see Blumer-Schuette et al. 2008). These include *C. saccharolyticus* (Rainey et al. 1994) and one of the most thermophilic cellulose-degrading organism known to date, *A. thermophilum* (Svetlichnyi et al. 1990). The cellulases of these two hyperthermophiles are multi-domain and multi-functional (VanFossen et al. 2008; Gibbs et al. 2000). They contain CBMs of different families, often duplicated, and in some cases two catalytic domains of different function and/or activity. For example, the *C. saccharolyticus* genome encodes a putative bifunctional cellulase CelB (Csac1078), which is composed of a N-terminal endoglucanase catalytic domain of family GH10, a triplet of CBM3, and a C-terminal exocellulase catalytic domain of family GH5. CelA of the same organism (Csac1076) has a similar domain arrangement: a GH9 endocellulase domain, a triplet of CBM3s, and a GH48 exocellulase (Te'o et al. 1995).

The genomes of many hyperthermophilic microorganisms encode enzymes that are, or appear to be, related to cellulose conversion, but most lack CBMs and/or multiple catalytic domains. For example, the bacterium *T. maritima,* although not growing on cellulose, shows in its genome several β-1,4-glucanases (Cel5A,

Table 6.2 Survey of thermophilic enzymes involved in the hydrolysis of cellulose

Organism	Enzyme	Name	GH family	EC number	Temp opt (°C)	pH opt.	References
C. saccharolyticus	Cellulase	CelB	GH10/GH5	3.2.1.4	80	4.7–5.5	Te'o et al. (1995)
	Cellulase	CelA	GH9/GH48	3.2.1.4	80	6.0	Te'o et al. (1995)
P. furiosus	endo-1.4-glucanase	EglA	GH12	3.2.1.4	100	7.0	Bauer et al. (1999)
	β-glicosidase	CelB	GH1	3.2.1.4	102–105	5.0	Pouwels et al. (2000)
T. maritima	Exoglucanase	cellulose II	n.d.	3.2.1.91	95	6.0–7.5	Bronnenmeier et al. (1995)
	β-1.4-glucanase	Cel5A	GH5	3.2.1.4	80	6.5	Bauer et al. (1999)
S. solfataricus	Endoglucanase	CelS-SSO2534	GH12	3.2.1.4	65	5.8	Limauro et al. (2001)
	Endoglucanase	SSO1949	GH12	3.2.1.4	80	1.8	Huang et al. (2005)
	β-glycosidase	LacS	GH1	3.2.1.21	95	6.5	Pouwels et al. (2000)
	aryl-β-glucosidase/ β-xylosidase	SSO1353	GH116	3.2.1.21	65	5.5	Cobucci-Ponzano et al. (2010)
A. acidocaldarius	Endoglucanase	Cel9A	GH9	3.2.1.4	70	5.5	Eckert et al. (2002)
	Endoglucanase	CelB	GH51	3.2.1.4	80	4	Eckert and Schneider (2003)
A. thermophilum	Endoglucanase	CelA	GH9/GH48	3.2.1.4	85–95	5–6	Zverlov et al. (1998)

Cel5B, Cel12A, Cel12B) that lack CBMs. Similarly, the archaeon *P. furiosus*, which grows optimally near 100 °C but does not grow on cellulose or xylan, contains an endo-1,4-glucanase (EglA) which is mostly active on cellooligosaccharides and CMC but not on insoluble cellulose (Bauer et al. 1999). Also the archaeon *S. solfataricus* possesses genes for the degradation of *β*-linked polysaccharides belonging to GH12 that have been characterized as endoglucanases. CelS (ORF SSO2534) was similar to CelB from *Thermotoga* species and EglA from *P. furiosus* (Limauro et al. 2001). SSO1949, which is similar to the GH12 enzyme Cel12A from *R. marinus* (Crennell et al. 2002; Huang et al. 2005), was extremely acidophilic and thermophilic, with optimum activity at pH 1.8 and T 80 °C. The adaptation of SSO1949 to hot and acidic environment, makes it a good candidate for the exploitation in the pretreatment of lignocellulosic biomass.

An exoglucanase, called cellulase II, was also reported from *T. maritima*. The enzyme has maximal activity at 95 °C, with a half-life of 30 min at that temperature and acted on crystalline cellulose (Bronnenmeier et al. 1995). Among thermophilic endoglucananses, Cel9A from *A. acidocaldarius* (GH9), has an optimum temperature of 70 °C at pH 5.5 and its primary role seems to be the degradation of short, soluble oligosaccharides imported into the cell (Eckert et al. 2002). In addition CelB, also from *A. acidocaldarius,* was expressed during growth on oat spelt xylan, birchwood xylan, CMC and cellobiose and is a cell-associated enzyme (Eckert and Schneider 2003). CelB had an optimal pH of 4.0, an optimal temperature of 80 °C and maintained its stability from pH 1.0 to 7.0. This endoglucanase, together with CelF from *Fibrobacter succinogenes* (Eckert and Schneider 2003), belongs to GH51, which actually consists largely of α-L-arabinofuranosidases (EC 3.2.1.55) (for more details see Miller and Blum 2010).

As described above, the order *Clostridiales* utilize crystalline cellulose, as well as hemicellulose, as growth substrates. The thermostable cellulase CelA from *A. thermophilum* contains an N-terminal GH9 domain, a triplet of CBMs and a C-terminal GH48 domain (Zverlov et al. 1998). Multiple CBMs and both endo- and exoacting domains are required for the efficient hydrolysis of the crystalline substrate (for a review see Blumer-Schuette et al. 2008).

β-Glucosidases complete the degradation of cellulose by acting on soluble cello-oligosaccharides from the terminal non-reducing *β*-D-glucosyl residues. These widespread enzymes belong to families GH 1, 3, 5, 9, 30 and 116. Two hyperthermophilic *β*-glucosidases, belonging to family GH1, from *P. furiosus* and *S. solfataricus* were reported, namely CelB and LacS, respectively. CelB shows an optimal temperature of 102–105 °C and an optimal pH of 5.0, instead LacS shows an optimal temperature of 95 °C and an optimal pH of 6.5 (Pouwels et al. 2000).

Recently, in *S. solfataricus* an aryl-*β*-glucosidase/*β*-xylosidase (ORF SSO1353), that was grouped in the new GH116 family, was identified and characterized (Cobucci-Ponzano et al. 2010). The *sso1353* gene lies downstream of genes encoding endoglucanases, and, in *S. solfataricus*, this gene arrangement occurs twice. Presumably, this *β*-glycosidase activity is involved, in combination with the secreted endoglucanase, in the degradation of exogenous glucans used as carbon energy source as mentioned above (Cobucci-Ponzano et al. 2010).

6.2.2 Hemicellulases

Xylans are the most abundant class of hemicelluloses, with glucuronoarabinoxylan being the main target for enzymatic saccharification for renewable bio-feedstock production. Glucuronoarabinoxylan (e.g. from corn stover) is composed of a β-(1,4)-linked D-xylose polymer backbone (xylan) with L-arabinose and glucuronic acid side chains (Templeton et al. 2010). Extensive acetylation may occur and the L-arabinose side chains can be esterified with ferulic acid. Heterogeneity of glucuronoarabinoxylan requires six distinct enzyme activities for complete saccharification: endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.131), acetylxylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73). The complete description of these enzymatic activities goes beyond the aims of this book (for a review see Jordan et al. 2012), here, a short survey of enzymes from thermophilic source involved in the degradation of several hemicelluloses is reported in Table 6.3.

Many industrially relevant endoxylanase genes have been cloned from a wide array of bacteria (for a review see Collins et al. 2005). The majority of these enzymes are classified into GH10 and GH11 families. Of the extremophilic xylanases, the thermophilic, alkaliphilic and acidophilic ones have been extensively studied, while cold-adapted xylanases have been much less investigated.

A family 10 xylanase, XynA from *Thermotoga* sp. is one of the most thermostable xylanases reported to date with an apparent optimum temperature for activity of 105 °C (Simpson et al. 1991). While less frequent, GH11 thermophilic xylanases have also been isolated, with those from *Caldicellulosiruptor* sp. Rt69B.1, *Dictyoglomus thermophilum*, and *Bacillus* strain D3 being the most thoroughly investigated. In addition a number of xylanases producing hyperthermophilic archaea have also been recently reported, including *Thermococcus zilligii*, *P. furiosus*, *Pyrodictium abyssi* and a number of *Thermofilum* strains (for a review see Collins et al. 2005 and references therein). A xylanase activity (ORF SSO1354) was isolated from a strain of *S. solfataricus* capable of utilizing oat spelt xylan as the sole carbon source (Cannio et al. 2004; Maurelli et al. 2008; Girfoglio et al. 2012). The enzyme is most active at 95 °C at pH 4.0, with a half-life of 53 min at that temperature making it suitable, like SSO1949 mentioned above, to contribute toward glucose production from lignocellulosic biomass in the bioethanol industry.

Even though cold-temperature environments are the most abundant on earth, only a small number of psychrophilic xylanases have been identified in bacteria, such as *Pseudoalteromonas haloplanktis* TAH3a, *Flavobacterium frigidarium* sp. nov. and *Clostridium* strain PXYL1 (see Collins et al. 2005). The first report of a xylanase produced by an alkaliphilic microorganism was as early as 1973 for a xylanase from *Bacillus* sp. C-59-2 (Horikoshi and Atsukawa 1973). Since this initial finding a number of xylanases have been isolated from various acidophilic and alkaliphilic microorganisms, including GH10 and GH11 xylanases from a

Table 6.3 Survey of thermophilic (hemi)cellulolytic enzymes

Organism	Enzyme	EC number	Name	GH family	Temp opt (°C)	pH opt.	References
Bacillus sp. AR-009	Xylanase	3.2.1.8	XylB	10	70–75	9.0–10.0	Gessesse (1998)
C. cellulovorans	Xylanase	3.2.1.8	XynA	10	70	6.0	Sunna et al. (2000)
G.s stearothermophilus	Xylan 1,4-β-D-xylosidase	3.2.1.8		3	70	6.0	Nanmori et al. (1990)
G. stearothermophilus T-6	Xylanase	3.2.1.8	XynA	10	75	6.5	Khasin et al. (1993)
	α-D-glucuronidase	3.2.1.139	AguA	67	65	5.5	Zaide et al. (2001)
S. solfataricus	Xylanase	3.2.1.8	SSO1354	10	95	4.0	Cannio et al. (2004)
	β-D-xylosidase/ α-L-arabinosidase	3.2.1.37 3.2.1.55	XarS	3	80	6.5	Morana et al. (2007)
T. ethanolicus	β-D-xylosidase/ α-L-arabinosidase	3.2.1.37 3.2.1.55	XarB	3	93	5.8-6.0	Shao and Wiegel (1992)
T.maritima	Xylanase	3.2.1.8	XynA	10	105	6.2	Simpson et al. (1991)
	Xylan 1,4-β-D-xylosidase	3.2.1.37		3	95	6.1	Xue and Shao (2004)
	α-D-glucuronidase	3.2.1.131 3.2.1.139	AguA	67	85	6.3	Ruile et al. (1997)

number of *Bacillus* sp. and *Acidobacterium* sp. Quite unexpectedly, many of the xylanases from alkaliphiles showed pH optima in the near neutral region, but relatively high activity was retained in alkaline conditions. One the most alkaliphilic xylanases reported to date is XylB from *Bacillus* sp. AR-009, which has a pH optimum of pH 9.0–10.0 (Collins et al. 2005).

Xylan 1,4-β-D-xylosidases (EC 3.2.1.37), catalysing the hydrolysis of single D-xylose units from the non-reducing end of xylo-oligosaccharides, have been classified in CAZy under families GH1, 3, 30, 39, 43, 51, 52, 54, 116 and 120. At present, the preponderance of characterized extremophilic β-xylosidases belongs to GH43; instead, α-arabinofuranosidases (EC 3.2.1.55) classified in CAZy under GH3, 43, 51, 54 and 62, catalyse the hydrolysis of terminal non-reducing L-arabinose side chains from the xylan backbone, such as the GH43 enzyme from *R. marinus* (Gomes et al. 2000). Interestingly, the gene *xarS* (ORF SSO3032) from *S. solfataricus*, belonging to GH3, encodes a bifunctional enzyme with both β-D-xylosidase and α-L-arabinosidase activities. The optimal conditions for both activities are 80 °C and pH 6.5. Oat spelt xylan that was converted to xylobiose and xylotriose by *S. solfataricus* SSO1354 xylanase (see above) was further converted to xylose after addition of XarS (Morana et al. 2007).

α-Glucuronidases (EC 3.2.1.131), hydrolysing the 1,2-linked glucuronosyl side chains from xylan (de Wet and Prior 2004), are categorized as GH67 and remove only the glucuronosyl group that is attached to the terminal residue at the non-reducing end of xylo-oligosaccharides. The α-glucuronidase from *T. maritima* is the enzyme with the highest reported temperature optimum (85 °C) (Ruile et al. 1997).

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Chapter 7
The Alcohol Fermentation Step: The Most Common Ethanologenic Microorganisms Among Yeasts, Bacteria and Filamentous Fungi

Parameswaran Binod, Raveendran Sindhu and Ashok Pandey

Abstract Ethanol fermentation using the hydrolysate obtained after the saccharification of biomass is the last step in lignocellulosic bioethanol production process. The hydrolysate contains large amount of fermentable sugars that can be directly used by the ethanologenic microorganisms. Yeast is the most commonly and widely used microorganism for commercial ethanol production due to its some special characteristics such as fast growth rates, efficient glucose repression, efficient ethanol production, and a tolerance for environmental stresses, like high ethanol concentration and low oxygen levels. In addition to yeast, there are several other fungi and bacteria that can produce ethanol under various fermentation conditions. This chapter describes the most common wild-type microorganisms used for the fermentative production of ethanol.

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7.1 Introduction

With the ever increasing demand for energy and the fast depleting petroleum resources, there is an increased interest in alternative fuels, especially liquid transportation fuels. The use of lignocellulosic biomass for the production of biofuels, especially bioethanol, will be unavoidable if the fossil fuels are to be replaced by renewable and sustainable alternatives. Ethanol accounts for majority of biofuels worldwide and its production from lignocellulosic biomass through biological route seems very attractive and sustainable due to several reasons, among which the renewable and ubiquitous nature of biomass and its non-competitiveness with food crops, and the higher reduction in greenhouse gas emission.

There are a limited number of microorganisms which ferment carbohydrates, mainly pentose sugars or hexose sugars, into alcohols. The major bacterial strains producing ethanol include *Clostridium acetobutylicum, Klebsiella pneumoniae, Leuconostoc mesenteroides, Sarcina ventriculi, Zymomonas mobilis*. Several fungal species are also reported to be the producer of ethanol. These include *Aspergillus oryzae, Endomyces lactis, Kloeckera* sp., *Kluyreromyees fragilis, Mucor* sp., *Neurospora crassa, Rhizopus* sp., *Saccharomyces beticus, S. cerevisiae, S. elltpsoideus, S. oviformis, S. saki,Torula* sp., *Trichosporium cutaneum*.

The major characteristics of an organism to be used in ethanol production are the ability to give a high yield of ethanol, to produce it with a high productivity and to withstand high ethanol concentration. In addition, the organism should possess the ability to utilize multiple sugars as well as that to tolerate inhibitors that are usually present in the hydrolysate obtained after pretreatment and enzymatic saccharification. It should also possess the ability to tolerate temperature and low pH, in order to minimize the risk of contamination. From an industrial point of view, high temperature tolerant strains are preferred so as to eliminate the other contaminating mesophilic microbes by increasing the fermentation temperature which in turn reduces the step of sterilization and thus the process become more cost-effective. Simultaneous hydrolysis and fermentation (SSF) is the main route to produce lignocellulosic ethanol. It consists on the use of a unique reactor in which enzymatic hydrolysis and fermentation of the obtained sugars by the ethanologenic microorganisms, mainly yeasts, are carried out. For a successful SSF process, temperature and pH values should be modulated with the aim to optimize the operative conditions of both enzymatic hydrolysis and fermentation, without negatively affecting both the yield of sugars release and ethanol production.

7.2 Fermentative Production of Ethanol

Fermentation is the term used to describe any process for the production of a product by means of the mass culture of a microorganism. In simple way, it is a chemical change brought on by the action of microorganisms. The two key

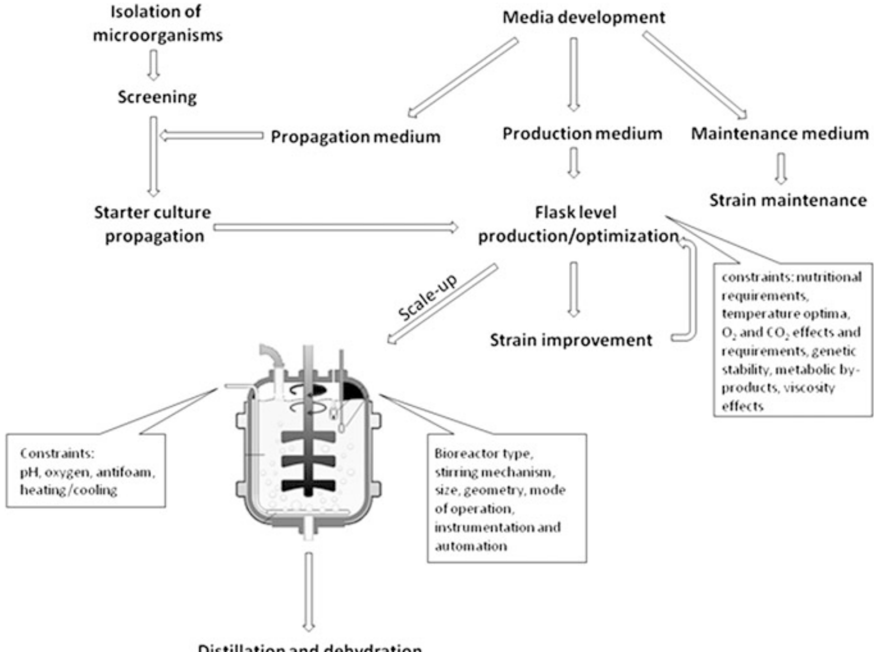

Fig. 7.1 Operation units in ethanol fermentation

components in the fermentation process are the microorganism and substrate. Control of the process, absence of contaminations, high fermentation rate and yield are the major factors which determine the total fermentation efficiency. The major steps in ethanol production process are shown in Fig. 7.1. The fermentation technique in lignocellulosic biomass to ethanol process is the same as that of conventional fermentation except the source of carbon is from the biomass.

Most of industrial ethanol fermentations are carried out by submerged fermentation (SmF), where a supply of oxygen is essential. SmF can be operated in batch culture, fed-batch culture, perfusion batch culture, and continuous culture.

As far as lignocellulosic bioethanol production is concerned, two main routes can be followed for ethanol production, namely separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Fig. 7.2). In SHF the bioconversion of lignocellulose takes place in two separate reactors, thus separating the saccharification and the fermentation processes. In this process each step can be conducted at optimal conditions of pH and temperature. The major steps involved in SHF are pretreatment, hydrolysis, and fermentation. Both pretreatment and hydrolysis are very crucial for obtaining fermentable sugars. The major aim of pretreatment is to separate cellulose and hemicelluloses from lignin. Pretreatment can be performed by physical, chemical, and biological means and each method has its own advantageous and disadvantageous. Chemical method is the most preferred way of pretreatment as it is very easy to perform.

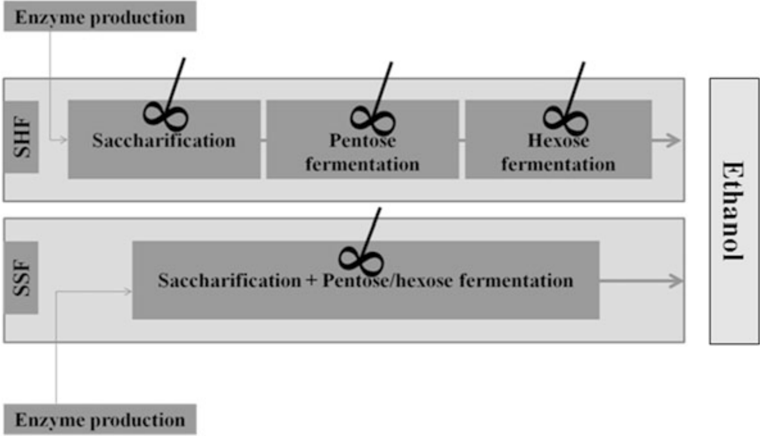

Fig. 7.2 Main modes of fuel ethanol production

The major drawback in chemical method is the formation of inhibitors and generation of waste chemical effluents. The formation of inhibitors adversely affect during fermentation. Microbe can tolerate inhibitors up to a certain concentrations beyond that it dies. To avoid that it needs to detoxify the pretreated as well as hydrolyzed liquor before fermentation. The inhibitors that affect fermentation include acetic acid, formic acid, levulinic acid, furfural, hydroxymethyl furfural, phenol, and vanillin. The effect of furfural on cultivation of yeast has been studied well. Among known effects for batch cultivations are decreased ethanol production rate and specific growth rate. The mode in which furfural inhibit yeast metabolism is not completely known, but it has been suggested that it inhibit central enzymes in glycolysis. In addition, enzymes coupled to the citric acid cycle and ethanol formation (e.g., alcohol dehydrogenase and aldehyde dehydrogenase) have also been suggested (Taherzadeh 2000).

Studies on SSF process has shown a potential for high rates and high ethanol yields from lignocellulosic materials. SSF allows performing the enzymatic hydrolysis of polysaccharides components of lignocelluloses, together with the fermentation, using a unique reactor. This results in the decrease of inhibition effects of the end-product on the enzymatic hydrolysis and an immediate availability of fermentable sugars. On the other side, the main drawback is the need to find favorable conditions (e.g., temperature and pH) for both the enzymatic hydrolysis and the fermentation and the difficulty to recycle the fermenting organism and the enzymes. Techno-economical analyses have shown that SSF is a much more competitive process in comparison to SHF. In fact, the use of a unique bioreactor results in a strong reduction of investment and operational costs.

A number of yeast and several bacterial strains have been studied for ethanol production under SSF.

7.2.1 Up-Stream Operations in Ethanol Fermentation

All the operations before starting the fermentation are generally called up-stream operations such as sterilization of reactor, preparation and sterilization of culture media, preparation and growth of suitable inoculums of microbial strains. All the process of up-stream operations is important for a successful fermentation among them media preparation and fermentation parameters play important roles. In the case of lignocellulosic ethanol production, the pretreatment of biomass is a key step to assure availability of polysaccharides to be converted into fermentable sugars.

The reaction environment should contain source for energy, water, carbon sources, nitrogen sources, vitamins, minerals, buffers, chelating factors, air, and antifoaming agents. Pretreated lignocellulose not always guarantees all these supplies thus pushing to the need of adding other components which can assure ethanologenic microorganism's growth and fermentation.

The culture medium should produce the desired product at a faster rate, and low yield of undesired products. Thus, the type and the amount of nutrient components of a medium are critical.

In the case of SSF, where enzymes are directly added for the hydrolysis of the polysaccharides, fermentable sugars, both pentoses and hexoses, are prompt available to be fermented, differently from SHF where an accumulation of sugars results in the inhibition of the enzymes involved in the hydrolysis and a decrease of the sugars production rate as a consequence. Carbon serves as a major energy source for the organisms.

The product formation depends on the rate at which the carbon source is metabolized and main product of fermentation depends on the type of carbon source used. Carbon enters the pathways of energy yielding respiratory mechanism. The carbon sources for fermentation can be simple or complex carbohydrates, organic acids, proteins, peptides, amino acids, oils, fats, and hydrocarbons. Many microorganisms can use a single organic compound to supply both carbon and energy needs.

Followed by carbon, nitrogen is the next most plentiful substance used in the fermentation media. Few microbes can utilize nitrogen as the energy source. It occurs in the organic compounds of the cell and also as reduced form in amino acids. The commonly used nitrogen sources in the fermentation media are yeast extract, ammonium salts, and urea. Other nitrogen sources include amino acids, proteins, sulfite waste liquor, corn steep liquor, and molasses. Nitrogen sources are added in the SSF reactor to assure the growth of the ethanologenic microorganisms.

For instance, yeast extract is generally added to SSF process, thus assuring a proper amount of nitrogen. Minerals supply the essential elements required for the cells during their cultivation. The essential minerals for all media include calcium, chlorine, magnesium, phosphorous, potassium, and sulfur. Other minerals like copper, cobalt, iron, manganese, molybdenum, and zinc are required in trace

amounts. The trace elements may contribute to both primary and secondary metabolite production. The specific concentration of the different minerals depends upon the type of microorganism being used. The functions of trace elements include coenzyme functions to catalyze many reactions, vitamin synthesis, and cell wall support (Vogel and Todaro 1996). Primary metabolite function is not very sensitive to trace element composition while secondary metabolite production is sensitive to trace element concentration.

Oxygen is normally present at very low levels in commercial-scale ethanol fermentations. In practice, the process cannot be completely anaerobic because oxygen is required for production of unsaturated fatty acids that are essential for yeast growth and ethanol production. It is generally recommended to avoid yeast stress factors such as high temperatures, high osmotic pressure, high sodium and other ionic concentrations, and high concentration of organic acids. Prevention of bacterial contaminants is critical in successful ethanol fermentation.

Besides nutrition, the yeast dose rate also affects on the total performance and it must be optimized for cost-effective performance. For instance, a higher dose rate results in a faster start of fermentation, which helps the control of contamination.

Saccharomyces cerevisiae is the most favored organism for ethanol production from hexoses while *Pichia stipitis* and *Candida shehatae* are yeasts capable of fermenting both hexose and pentose sugars to ethanol (Parekh and Wayman 1986). Bacteria belonging to the species *Clostridia* and *Zymomonas*, and fungi such as *Fusarium* spp. have been investigated for ethanol production. The success of fermentation depends on the nature of the lignocellulosic biomass, thus the effect of the pretreatment on its structure. The parameters like temperature, pH, degree of agitation, oxygen concentration must be monitored throughout the fermentation process, so that any deviation from the optimum conditions can be corrected by a control system.

In the case of SSF, the effects of enzymes and biomass loading should be even studied to optimize the process, particularly the saccharification from which fermentable sugars arise.

It is worth noting that several yeast growth inhibitors produced during the pretreatment can negatively affect the fermentation process.

7.2.2 Down-Stream Operations

The major down-stream operations in ethanol fermentation involve distillation. After distillation yield 95 % ethanol known as rectified spirit. It is not possible to remove the remaining water from rectified spirit by straight distillation, as ethanol forms a constant boiling mixture with water at this concentration known as azeotrope. In order to extract water from ethanol, it is necessary to use some dehydrants which are capable of separating water from ethanol. A simple dehydrant is the unslaked lime which is added to rectified spirit and left overnight for

complete reaction. The mix is then distilled in a fractionating column to get absolute alcohol. This process is mainly used in small-scale processes.

Dehydration by molecular sieve is another approach used in industry. In this technique the rectified spirit is superheated with steam in feed super-heater. It is then passed to one of the pair of molecular sieve beds for several minutes. On a time basis, the flow of the rectified spirit vapor is switched to the alternate bed of the pair. A portion of the anhydrous ethanol vapor leaving the fresh adsorption bed is used to regenerate the loaded bed.

The advantages of molecular sieve technology are the simplicity of the process and the fact that it is very easy to automate the process, reducing the labor. The process is inert and there is no use of chemicals. The desiccant material has very long life span. A properly designed molecular sieve can dehydrate 160− proof ethanol to more than 190+ proof ethanol and near theoretical recovery of ethanol is possible.

7.3 Ethanologenic Microbes

The ethanologenic microorganisms should satisfy a specific criterion for isolation which includes utilization of a cheap media for growth. It should convert the substrate into the product rapidly and the product should be easily recovered from the culture medium.

Efficiency or yield, throughput and consistency are the major objectives for selecting the organism for any fermentation processes. Several techniques were employed for isolating and screening of ethanologenic microorganisms from various sources. This includes the liquid culture method and solid culture method. The liquid culture is carried out in shake flasks containing liquid culture medium while the solid culture is carried out in solid culture medium containing a substrate. A number of high-throughput screening methods have been proposed by Qi et al. 2011. After isolating microbes from various sources, they can be cultured either in liquid or solid medium, such as nutrient agar, which contains the desired carbon containing feedstock as well as the other nutrients required for microbial growth, such as ammonia, salts, and trace metals. Ethanol produced by microbes is excreted into the extracellular culture medium. The secreted ethanol can then be detected and quantified by any suitable means such as GC or HPLC. It is highly desirable to employ a detection method that can at least partially quantify the ethanol produced by each individual microbe or the microbes in an individual microbial colony. As such, a preferred screening method is one which is applied in a solid phase screen, in which a very large number of individual microbial colonies can be easily separated. This offers a much higher throughput compared to a solely liquid phase screening in which samples must be manually separated and measured in liquid assay. A colorimetric assay method was developed by Fotheringham et al. (2009) where the assay solution contains alcohol oxidase, peroxide, and a peroxide co-substrate. The oxidase reaction upon alcohol produces hydrogen peroxide

which reacts with a second enzyme such as Horseradish Peroxidase, in the presence of a peroxidase co-substrate which is responsible for generating the color.

Yeast is the most commonly used microorganism for ethanol production by fermentation. There are certain unique properties of yeasts that make them outstanding for ethanol production. Some of these properties are: fast growth rates, efficient glucose repression, efficient ethanol production, and a tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels. Of the different types of yeast, *S. cerevisiae* is the industrially important yeast for alcohol fermentation, even if it is able to ferment only hexose sugars.

One of the best opportunities to further reduce the cost of cellulosic bioethanol is to enhance the sugar recovery from lignocellulose. This includes the exploitation of the hemicelluloses portion of biomass, mainly made of pentose sugars. Therefore, microorganisms which could ferment other sugars such as xylose, mannose, arabinose, or galactose are required for an economically viable conversion from lignocellulose to ethanol.

Besides *S. cerevisiae*, other examples of yeasts used for ethanol production are *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Candida spp*., *Pichia spp* that are able to ferment even pentose sugars.

Yeast can grow aerobically as well as anaerobically. Aerobic conditions favors yeast cell production, which is not of interest to ethanol producers. However, growth during anaerobic condition is very marginal and major reaction is conversion of sugar to ethanol for energy production. For growth and multiplication, yeast requires utilizable organic carbon (sugars), nitrogen source, and various organic and inorganic trace growth factors. During the conversion of sugar to ethanol, energy is produced, which is utilized by cells for different functions. In addition to yeast, a large number of bacteria are capable of ethanol production, but most of them produce other end products like butanol, isopropyl alcohol, acetic acid, formic acid, arabitol, glycerol, acetone, methane, etc., as well as ethanol. Bacteria that produce ethanol as the major product (i.e., a minimum of 1 mol ethanol produced per mol of glucose utilized) are shown in Table 7.1.

The major wild microbes used in fermentation processes are described below.

7.3.1 Yeast

7.3.1.1 *Saccharomyces* sp

Worldwide, nearly all ethanol production is accomplished using a single genus and species of yeast, namely *Saccharomyces cerevisiae*. Specific strains producing ethanol from sugarcane juice and molasses and also from beet juice and molasses have been reported and marketed also for commercial production. Yeast has been so far shown able to produce ethanol from hexose sugars obtained from lignocelluloses saccharification. Recently, high performance yeast strains have been selected and commercialized for dry grind corn ethanol production utilizing batch fermentation

Table 7.1 Major ethanologenic bacteria

Bacteria	Mmol ethanol produced per mmol glucose metabolized
Clostridium sporogenes	Up to 4.15
Clostridium indolis	1.96
Clostridium sphenoides	1.8
Clostridium sordelli	1.7
Zymomonas mobilis	1.9
Zymomonas mobilis ssp. Pomaceas	1.7
Spirochaeta aurantia	1.5
Spirochaeta stenostrepta	0.84
Spirochaeta litoralis	1.1
Erwinia amylovora	1.2
Leuconostoc mesenteroides	1.1
Streptococcus lactis	1.0
Sarcina ventriculi (syn. *Zymosarcina*)	1.0

processes. Some yeast strains ferment faster or are able to convert substrate to ethanol with increased yields. Several inducers and stress factors also affect the yeast growth and ethanol production. Genetically enhanced microorganisms for ethanol production are in various stages of development (described in Chap. 8).

An optimal process for fermentation uses a broth containing *S. cerevisiae* supplemented with 22 % (w/v) sugar, 1 % (w/v) of each of ammonium sulfate and potassium dihydrogen phosphate, and fermented at pH 5.0 and 30 °C (Junior et al. 2009). Under such conditions a typical strain of *S. cerevisiae* is capable of producing 46.1 g ethanol/l broth (Maziar 2010). Cane molasses conditioned with EDTA, ferrocyanide or zeolites, and fermented under similar conditions have been shown to enhance ethanol production (Ergun et al., 1997). Further, addition of minimal concentrations of hops acids to the fermentation broth has been shown to prevent bacterial growth and thus enhances ethanol yields (Maye 2006). Fermentation using immobilized yeast and broth supplemented with Mg, Zn, Cu or Capantothenate has also been shown to increase fermentation efficiency by almost 20 % (Nikolic et al. 2009). Ethanol production using steam pretreated barley straw with low enzyme loadings and low yeast concentration was evaluated by Linde et al. (2007). The highest ethanol yield and ethanol concentration of 82 % and 15.5 g/l, respectively, were obtained with 5 % solid loading, enzyme loading of 20 FPU/g and with 5 g/l of yeast. It was observed that with increase in solid loading and decrease of enzyme loading, there is a reduction in ethanol yield. Ethanol production using hydrothermal pretreated wheat straw by thermo-tolerant flocculating *S. cerevisiae* was recently evaluated by Ruiz et al. 2012. The study revealed that ethanol concentration was affected by enzyme loading and biomass loading. Maximum ethanol concentration of 14.84 g/l was obtained at 45 °C, with 3 % biomass loading and 30 FPU of enzyme loading.

Rodrigues et al. (2011) evaluated cashew apple bagasse as a potential substrate for bioethanol production using yeast. The fermentation of the hydrolyzate by *S. cerevisiae* resulted in ethanol concentration and productivity of 5.6 g/l and 1.41 g/l/h, respectively. Simultaneous saccharification and fermentation (SSF) of steam exploded citrus peel waste to ethanol by *S. cerevisiae* was reported by Wilkins et al. (2007). Steam explosion removed D-limonene, an inhibitor present in citrus peel waste. The highest ethanol concentrations were obtained when the initial pH of citrus peel was adjusted to 6.0.

Choi et al. (2012) reported bioethanol production from coffee residue by *S. cerevisiae*, achieving ethanol concentration and yield (based on sugar content) of 15.3 g/l and 87.2 %, respectively.

The previous studies show that sodium ion concentration has significant effects on ethanol production by *S. cerevisiae* and there is interactive effect between calcium and magnesium. The optimum sodium concentration was found to be 930 mg/l (Soyuduru et al. 2009) and increase in sodium concentration decreased ethanol production due to its negative effect on glycolysis as well as due to competitive inhibition of potassium uptake leading to depletion of potassium in the cell and increased level of sodium.

7.3.1.2 *Schizosaccharomyces* sp

Schizosaccharomyces is a genus of fission yeasts, able to ferment xylose to ethanol under microaerophilic or oxygen limited conditions. The studies carried out by Lastick et al. (1990) revealed that simultaneous fermentation and isomerization of xylose (SFIX) allows the total fermentation of xylose in a single step. SFIX provides a significant improvement for fermentation of xylose to ethanol since it is faster and more tolerant to higher concentrations of xylose and ethanol.

7.3.1.3 *Kluveromyces* sp

Direct fermentation of D-xylose to ethanol using *Kluveromyces marxianus* SUB-80-S was reported by Margaritis and Bajpai (1982). The strain produced ethanol under aerobic conditions in a medium containing 20 g/l xylose. The ethanol concentration and yield were 5.6 g/l and 0.28 g of ethanol/g of xylose after 48 h of incubation. Ethanol production from poplar and eucalyptus biomass by simultaneous saccharification and fermentation using thermo-tolerant yeast strain *K. marxianus* CECT 10875 was evaluated by Ballesteros et al. (2004). The results indicated that it is possible to reach SSF yields in the range of 50–72 % of the maximum theoretical SSF yield, in 72–82 h. Maximum ethanol contents from 16 to 19 g/l were obtained in fermentation media, depending on the material tested. The use of thermo-tolerant strains at high process temperatures (42 °C) will minimize the risk of contamination comparable with other fermenting yeasts. This allows for working under non-sterile conditions which is very favorable for

process scale up. Tomás-Pejo et al. (2009) developed a simultaneous saccharification and fermentation fed–batch process for bioethanol production by the thermo-tolerant strain *Kluveromyces marxianus* CECT 10875. The ethanol yield was 36.2 g/l which is 20 % more ethanol yield when compared with batch SSF. Garcia-Aparicio (2011) reported an economic process for high ethanol yield from steam exploded barley straw by *K. marxianus* CECT 10875. The ethanol concentration was 4 % (w/v) with a substrate loading of 15 %, after 72 h of fermentation.

Toyoda and Ohtaguchi (2008) reported ethanol production by *K. lactis* NBRC 1903 using cheese whey as lactose source. The study revealed that dissolved oxygen level has a key role for ethanol production in *K. lactis* NBRC 1903. The ethanol yield in batch culture was 63.7 g/l after 24 h of incubation. Ethanol production using switch grass in SSF with thermo-tolerant yeast strain, *K. marxianus* IMB3 was reported by Pessani et al. (2011), achieving ethanol concentration and yield of 22.5 g/l and 86 %, respectively, after 168 h of incubation. The coproduction of ethanol and polygalacturonase by *K. marxianus* in a pilot scale batch fermenter, using yeast extract-glucose-sugar beet molasses medium (SBM), was reported by Serrat et al. (2004). The ethanol productivity was 1.94 g/l/h and the fermentation efficiency was 95.1 %. Ethanol production using steam exploded and liquid hot water pretreated poplar (*Populus nigra*) by SSF was evaluated using *K. marxianus* CECT 10875 by Negro et al. (2003). The results indicate that fermentation using steam exploded pretreated poplar gave better SSF yield of 60 % of theoretical when compared to liquid hot water pretreated poplar.

7.3.1.4 *Candida* sp

The conversion of wood sugars to ethanol has been limited to the hexoses because xylose was not fermentable; however, xylose is a major component of lignocellulosic residues. Most xylose-metabolizing yeasts do not produce ethanol. Most of the yeasts can grow on xylose under aerobic conditions, but very few of them will ferment xylose.

One of the first examples regards *Candida tropicalis,* which is capable of fermenting xylose under oxygen limited conditions in the presence of increasing concentrations of polyethylene glycol (Hagerdal et al. 1985).

Jeffries and Alexander (2012) produced ethanol from xylose using *C. shehatae* grown under continuous and fed-batch conditions. The concentration of ethanol produced is proportional to the vigor, viability, and growth rate of the starting culture. This group has developed a two-phase process for ethanol production. In the first phase, a continuous culture was used to generate a vigorous cell suspension and in the second phase, fed-batch fermentation was carried out by pumping in a concentrated sugar feed under semi-aerobic conditions. The cells adapt to oxygen limitation by synthesizing alcohol dehydrogenase (ADH) and ferment the xylose rapidly to ethanol. For the cost-effective production of bioethanol, the yeast strain should be able to convert both glucose and xylose at

elevated temperature. Tanimura et al. (2012) isolated a novel yeast strain *C. shehatae* which is capable of ethanolic fermentation at elevated temperature. The ethanol production yield was 71.6 % in SX medium (3 % xylose and 0.67 % YNB (Yease Nitrogen Base) without amino acid) after 24 h of incubation at 37 °C. This strain produced ethanol even from rice straw and it was found to be superior to *S. cerevisiae* for producing ethanol from lignocellulosic biomass.

In a study carried out by Watanabe et al. (2010) using respiratory deficient *C. glabrata,* higher ethanol production ability was observed in SSF. High temperature (45 °C) and agitation (150 rpm) are advantageous for ethanol production from insoluble feed stock using SSF. Nakayama et al. (2008) reported *C. krusei* IA-1 producing 55 g/l of ethanol from 150 g/l of glucose. The study revealed that *C. krusei* can be used as a potential alternative to *S. cerevisiae* for cost-effective production of ethanol.

Dahiya and Vij (2012) reported ethanol production from whey using different strains of immobilized *Candida* species, *C. inconspicua* W16, and *C. xylopsoci* W 23. *C. inconspicua* W16 was shown to be more efficient in ethanol (3.03 % v/v) production from whey when it is immobilized. *Candida tropicalis* can convert xylose to ethanol under aerobic conditions and the ethanol production is accelerated by aeration. In order to convert xylose to ethanol under aerobic conditions, it is necessary to have active Embden Meyerhoff and pentose phosphate pathways which are not repressed by air under the conditions employed.

Alexander et al. (1988) evaluated continuous xylose fermentation by *C. shehatae* in a two-stage reactor. This can overcome the major factor preventing continuous production of ethanol in batch culture. The steady influx of fresh cells and continuous removal of spent cells helps minimize loss of fermentative activity due to anaerobiosis and exposure to high levels of ethanol concentration. The final ethanol yield was 37 g/l in two-stage while in batch it was 0.38 g/l.

7.3.1.5 *Pachysolen* sp

Saharan and Sharma (2010) investigated the role of trehalose in ethanol induced oxidative condition in *Pachysolen tannophilus.* It was observed that there was a marked increase in trehalose content after ethanol stress. In addition there was an increase in protein carbonyl content, Reactive Oxygen Species (ROS) generation and lipid peroxidation and there was a decrease in reduced and total glutathione. This study revealed the protective role of trehalose in oxidative stress conditions generated by ethanol. In a study conducted by Kruse and Schuger (1996) by employing batch, fed-batch, and continuous cultivation of *Pachysolen tannophilus* on various substrates under aerobic, anaerobic, and microaerobic conditions in stirred tank reactor it was observed that under anaerobic conditions low cell biomass and low amount of ethanol were formed. Highest ethanol was produced under microaerobic conditions.

7.3.1.6 *Pichia* sp

Among the pentose fermenting organisms, *P. stipitis* has been shown to have most promise for industrial applications (Agbogbo et al. 2006). For example, the hemicellulosic hydrolysates of *Prosopis juliflora* (18.24 g sugar/l broth) when fermented with *P. stipitis* produced 7.13 g/l ethanol (Gupta et al. 2009). Detoxified xylose rich hydrolysate of *L. camara* when fermented with *P. stipitis* 3498 at pH 5.0 and 30 C for 36 h resulted 0.33 g alcohol/g lignocellulose used (Kuhad et al. 2010). In yet another example, the detoxified water hyacinth hemicellulose acid hydrolysate (rich in pentose sugars) fermented with *P. stipitis* NCIM-3497 at pH 6.0 and 30 C resulted in 0.425 g ethanol/g lignocellulose.

Canilha et al. (2010) evaluated hemicellulosic hydrolyzate from sugarcane bagasse for ethanol production by *Pichia stipitis* DSM 3651. Fermentation was carried out by supplementing yeast extract and malt extract at 3 g/l level and peptone 5 g/l level, respectively. It was observed that detoxification of hemicellulosic hydrolyzate by changing the pH and using active charcoal improved bioconversion of hemicelluloses into ethanol. The fermentation yields with detoxified and non- detoxified hydrolyzate were 0.30 g/g and 0.20 g/g, respectively. The effect of various process parameters affecting ethanol production from rice straw hemicellulosic hydrolyzate by *P. stipitis* NRRL Y-7124 was evaluated by Silva et al. 2010. Parameters like initial xylose concentration, agitation, and aeration were evaluated. Initial xylose concentration of 50 g/l was found to be optimum while increase in aeration and agitation caused a deviation in yeast metabolism from ethanol to biomass production. Under optimized conditions a process efficiency of 72.5 % was achieved. Shupe and Liu 2012 studied the effect of agitation rate on ethanol production from sugar maple hydrolyzate by *P. stipitis*. It was reported that the highest ethanol yield (29.7 g/l) was observed when the air flow rate was set at 100 cm^3 and agitation rate at 150 rpm. Increasing or decreasing the agitation rate in the range 300–350 rpm resulted in a decline in ethanol production. An improved method for ethanol production from undetoxified hemicellulosic hydrolyzate from steam exploded corn stover was evaluated using *P. stipitis* CBS 5776 by Yong et al. 2012. It was observed that domestication of *P. stipitis* improved sugar consumption and ethanol yield by increasing the ratio of hydrolyzate in the medium. The ethanol yield was 80 % and the sugar consumption was 90 %.

7.3.2 Bacteria

7.3.2.1 *Clostridium* sp

The ability of *Clostridium beijerinckii* in acetone butanol ethanol (ABE) fermentation using degermed corn was reported by Campos et al. (2002). Batch fermentation resulted in 8.93 g/l of total ABE production as compared with 24.80 g/l of total ABE when supplemented with P2 medium nutrients. Several

studies report the cost-effective production of ethanol using filter paper, corn steep liquor, cysteine HCl, magnesium chloride and ferrous sulfate and these nutrients play an important role in growth as well as ethanol production by *Clostridium* sp.

7.3.2.2 *Zymomonas* sp

Lawford and Rousseau (1997) reported ethanol production by *Zymomonas* using corn steep liquor as a cost-effective medium. 1 % (v/v) corn steep liquor was found to be optimum and sugar to ethanol conversion efficiency as well as product recovery were 98 % and 100 %, respectively. Immobilized *Z. mobilis* showed high productivity and conversion compared to free cells (Davison and Scott 1988). The theoretical ethanol yield was reported as 97 % under incubation temperature 30 °C and pH 5.0. Ethanol production from starch hydrolyzates using *Z. mobilis* and glucoamylase entrapped in polyvinyl alcohol hydrogel was carried out by Rebros et al. (2009). Ethanol productivity increased 2.1 times with immobilized glucoamylase compared to free enzyme—free microorganism system.

7.3.2.3 *Thermanaerobacter* sp

Lacis and Lawford (1991) studied the potential of *Thermanerobacter ethanolicus* for ethanol yield form glucose or xylose. It was observed that the ethanol yield depend on the cultivation time and growth rate. The highest ethanol yield (0.42 g/g) was attained at low growth rates. Thermophilic ethanol production by thermophilic bacterium *Thermanerobacter* BG1L1 in a continuous reactor was investigated by Georgieva et al. (2008) using wet exploded wheat straw. Fermentation was carried out in a fluidized bed reactor at 70 °C. The ethanol yield using non-detoxified hydrolysate was 0.39–0.42 g/g. This study revealed the potential of *Thermanaerobacter* using fluidized bed reactor for anaerobic ethanol fermentation.

7.3.3 Filamentous fungi

Several fungal species are also reported as a producer of ethanol. The studies carried out on various fungal species for ethanol production are described in the following section.

7.3.3.1 *Fusarium* sp

Joshi and Verma (1990) evaluated ethanol production from wood hydrolysate by *Fusarium oxysporum*. Ethanol production at pH 5.5 and 30 C after 96 h of fermentation was of 12.3 g/l and 11.7 g/l by *F. oxysporum* strain D-140 and

NCIM-1072, respectively. The ethanol production in presence of yeast extract and minerals was 13.2 g/l after 108 h of incubation.

Brewer's spent grain is an attractive low cost feed stock for bioethanol production. Xiros and Christakopoulos (2009) evaluated bioethanol production by *Fusarium oxysporum* by submerged fermentation adopting a consolidated bioprocess strategy. Effects of various process parameters affecting ethanol production were evaluated. Hydrolysis seems to be the bottleneck while the bioethanol yield of 109 g kg^{-1} of dry material by *F. oxysporum* was achieved which constitute 60 % of theoretical yield making the process economically feasible for commercial application. *F. oxysporum* has the ability to ferment xylose which is present in Brewer's spent grain. The effect of initial sugar concentration and aeration rate affects the fermentation performance of *F. oxysporum*. The SSF of cellulose by *F. oxysporum* was investigated by Panagiotou et al. (2005). It was found that *F. oxysporum* grows with a maximum specific growth rate of 0.023 h^{-1} on cellulose at aerobic conditions and that it can produce ethanol with a volumetric productivity of 0.044 g/l/h under anaerobic conditions. Ruiz et al. (2007) evaluated ethanol production from lignocellulosic residues by *F. oxysporum*, achieving an ethanol yield of 0.28 g/g from a 50 % xylose/50 % glucose mixture. The fermentation efficiency was lower but its ability for SSF is a potential advantage.

7.3.3.2 Aspergillus sp

Pushalkar and Rao (1998) reported a cellulolytic fungus *Aspergillus terreus* which showed an additional property of fermenting glucose, other hexoses, pentoses, and disaccharides to ethanol. Of the various carbon sources tested, glucose yielded maximum ethanol (2.46 % w/v). The ethanol values and the theoretical yields produced by *A. terreus* with glucose and cellobiose were comparable to or higher than that reported by other fungal species.

7.3.3.3 Mucor sp

Sues et al. (2005) identified *Mucor indicus* as a potential ethanol producing strain capable of growing aerobically as well as anaerobically on different pentoses and hexoses with yield and productivity same as that of *S. cerevisiae*. Asachi et al. (2011) developed a cost-effective medium for ethanol production using the fungal extract of *M. indicus* biomass which is a by-product of fermentation showed improved ethanol production. Yeast extract in the fermentation medium was replaced with fungal extract of *M. indicus*. The ethanol yield and productivity were 0.46 g/g and 0.69 g/l h, respectively. Ethanol production was higher during aerobic growth on glucose under non-oxygen limiting conditions.

7.3.3.4 Neurospora sp

Dogaris et al. (2012) reported bioethanol production from dilute acid pretreated sweet sorghum bagasse using *Neurospora crassa*. The study revealed that the bioconversion ability of *N. crassa* was superior to *S. cerevisiae*, while their mixed cultures have negative impact on ethanol production.

7.4 Conclusions

Lignocellulosic biomass offers as excellent raw material for ethanol production. There occurs several technological challenges in lignocellulosic biomass to ethanol conversion process and the major challenge in fermentation process is the selection of suitable microorganism. The formation of inhibitors during the pretreatment and hydrolysis stages limits its application and hence ethanologenic organisms capable of tolerating these inhibitors are necessary. Exploration and exploitation of wild and extreme environmental niches may provide novel ethanologenic microorganisms with higher inhibitor tolerance. The search for new ethanologenic microorganisms as well as the improvement in the techniques of fermentation may help in the advancement of cost-effective production of lignocellulosic ethanol.

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Chapter 8
Other Ethanologenic Microorganisms

Eulogio Castro

Abstract The economical production of ethanol from lignocellulosic materials needs the conversion not only of glucose, which is the sugar of preference of the best performing ethanologenic microorganisms, but also of the rest of sugars found in the fermentation broth, derived from pretreatment and enzymatic steps. This chapter summarizes recent work directed to that objective, by using different modification techniques of microorganisms. After considering the main metabolic pathways for pentoses, the second most abundant kind of fermentable sugars, a review of such modifications taking either *Escherichia coli* or *Saccharomyces cerevisiae* as a basis is presented. Although *E. coli* and *S. cerevisiae* are the most studied microorganisms through a wide range of techniques, other microorganisms are also being subject of study with the same purpose, and are briefly described at the end of this chapter.

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8.1 Introduction

Lignocellulosic materials are mainly composed of cellulose, hemicelluloses, lignin, extractives, and ashes. It has been claimed that they are the most promising sugar source for biofuel production mainly due to their renewable nature and the lack of competition with food or feeding applications. But in contrast to starch or raw materials containing sucrose such as corn or sugarcane, the generation of fermentable sugars from the homogeneous (cellulose) or heterogeneous (hemicelluloses) polysaccharides of lignocellulose need an intense, energy demanding pretreatment step. Cellulose forms the major part of lignocellulosic materials and hence, once the pretreatment and enzymatic hydrolysis steps have been performed, the sugar broth is constituted by glucose as the major sugar. Nevertheless, the fraction of hemicellulose can account for up to 30 % depending on the material, making sugars derived from hemicellulose very important in economic terms; sometimes their transformation into ethanol becomes the threshold of profitability. Among these sugars, xylose is the most abundant, after glucose. Arabinose and cellobiose are also present in significant proportions in certain lignocellulosic materials. Converting xylose and other sugars, in addition to glucose, into ethanol would reduce the overall production costs and make the process a real alternative to fossil fuels from an economic point of view. Additionally, the pretreatment step is also responsible for the presence of inhibitor compounds which may hinder the fermentation process. To overcome these drawbacks, the ideal ethanologenic microorganism should be able to efficiently produce ethanol from different kinds of sugars and be resistant to the presence of both inhibitors and ethanol. There are a number of microorganisms able to naturally ferment a wide variety of sugars, including glucose, xylose, arabinose, and others but, unfortunately, those microorganisms do not perform the same when a mixture of sugars is present. Instead, they assimilate in such a way that one of the sugars is preferable to the others, and this particular one may act in some way to repress the others, thereby reducing the overall capacity of the microorganisms to transform all the sugars. *Escherichia coli* is one of those microorganisms able to ferment a wide range of sugars and it is the focus of a very intense investigation centered on trying to improve ethanolic fermentation results.

Another strategy to take full advantage of the different sugars issued from the pretreatment of lignocellulose consists in using metabolic engineering to modify microorganisms that ferment glucose with good results, and to repeat this behavior with other sugars. Recombinant DNA technology and evolutionary engineering techniques are being assayed in an attempt to improve both ethanol yield and productivities. In this particular field, *Saccharomyces cerevisiae* is the main candidate to modification.

As an alternative to use several steps to convert pretreated lignocellulosic materials into ethanol, the process can also be addressed by combining all of them in a single one. This is the fundamental of the so-called Consolidated Bioprocessing (CBP), which can be advantageous from an economic point of view

because enzyme production, saccharification, and fermentation are conducted in a single vessel. In CBP, both cellulosic and hemicellulosic materials should be simultaneously fermented. This kind of process requires a highly engineered microorganism able to produce effective hydrolyzing enzymes, for high ethanol titer and productivities, using both hexoses and pentoses from a high solid pre-treated material. In addition, it is recommended that this special microorganism exhibits a high resistance to ethanol, fermentation inhibitors, and stressful environments (Hasunuma and Kondo 2012).

This chapter summarizes the main strategies aimed at taking full advantage of all sugar fractions obtained from lignocellulosic materials for ethanol production through the use of modified microorganisms. First, it presents a brief introduction to pentose metabolism so that the main points for modification in microorganisms can be identified. Then, these main points are described based on either *E. coli* or *S. cerevisiae*. Finally, the chapter presents a concise review of recent results dealing with ethanol production from lignocellulosic materials using other modified microorganisms.

8.2 Pentose Metabolic Pathways

Figure 8.1 shows a simplified diagram of the main sugars, other than glucose, found in lignocellulosic materials hydrolysates, D-xylose, and L-arabinose (Hahn-Hägerdal et al. 2007).

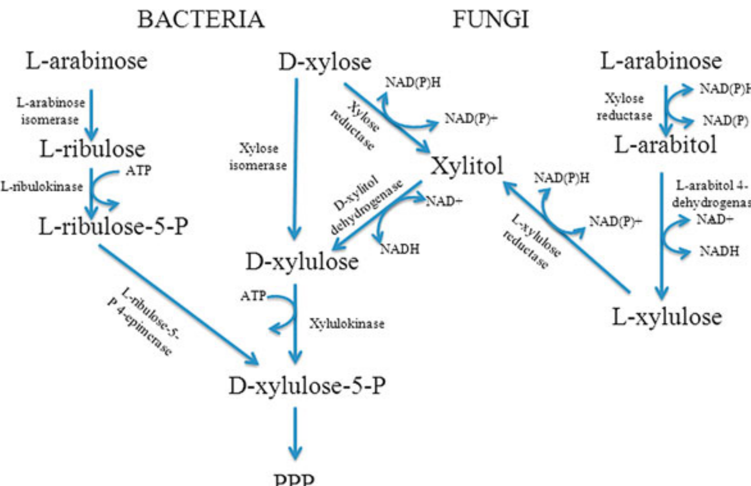

Fig. 8.1 Simplified metabolism of D-xylose and L-arabinose in bacteria and fungi (Hahn-Hägerdal et al. 2007)

The biochemical route for xylose metabolism is the pentose phosphate pathway (PPP), which is present in all cellular organisms, and can be described as a two-step process (Jeffries 2006): conversion of D-glucose 6P into D-ribulose 5P (oxidative phase) and further conversion (non-oxidative phase) into several compounds including D-xylulose 5P, which is the way in which D-xylose enters the PPP. To point out one main difference, the conversion of D-xylose into D-xylulose occurs in bacteria under the action of xylose isomerase, while in yeasts, the process includes reduction and oxidation mediated by the enzymes xylose reductase and xylitol dehydrogenase (Fig. 8.1), with xylitol as an intermediate compound.

Concerning L-arabinose, its metabolism is more complex, and requires more enzymatic reactions for the transformation into compounds entering the PPP (Hahn-Hägerdal et al. 2007). Nevertheless, the amount of this sugar in lignocelluloses is in general much lower than that of D-xylose, drawing little attention on the arabinose metabolism pathway.

The modification strategies implemented in the processes regarding how microorganisms can get a better use of sugars contained in lignocellulosic materials and improve ethanol yield include several metabolic and evolutionary engineering techniques and advanced genomics, transcriptomics, proteomics, metabolomics techniques, as reported by Gírio et al. (2010). Considering the metabolic pathways depicted in Fig. 8.1, an intense research work has been developed in recent years through the introduction of foreign genes, the elimination of competitive pathways, the disruption of byproducts formation (Jarboe et al. 2007), and the study of the redox imbalance from xylose reductase and xylitol dehydrogenase, xylulokinase, among others (Chu and Lee 2007).

8.3 *Escherichia coli*-Based Modifications

The nonpathogenic, Gram + species of bacterium *E. coli* ferments a wide range of monomeric sugars, including all those present into hemicellulosic and cellulosic hydrolysates: xylose, arabinose, glucose, galactose, mannose, and also uronic acids, such as glucuronate, that is obtained from the hydrolysis of some lignocellulosic materials (Alterthum and Ingram 1989). However, the cultivation of wild-type *E. coli* under fermentative conditions produces a variety of fermentative products: lactate, succinate, acetate, formate, hydrogen, carbon dioxide, and small amounts of ethanol. Furthermore, for several decades *E. coli* has been the workhorse for the development of genetic and molecular biology tools, including modifications for the production of ethanol through metabolic engineering, i.e., the improvement of cellular activities by manipulations of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology (Bailey 1991). Therefore, for more than two decades, this microorganism has been the target for metabolic modifications and it has been studied for the production of ethanol (Jarboe et al. 2007; Orencio-Trejo et al. 2010). With the advent of the system and synthetic biology tools, a new generation of *E. coli* strains are being metabolically engineered

for the production of fuel ethanol and the so-called, advanced biofuels including, but not limited to: 1-propanol, *n*-butanol, isobutanol, isopentanol, pinene, farnesane, bisabolane, fatty-acid methyl esters, fatty-acid ethyl esters, fatty alcohols, polyketide-derived biofuels, aromatic alcohols, and alkenes and alkanes from short (C5) to long carbon chain (up to C20) (Dellomonaco et al. 2011; Huffer et al. 2012; Peralta-Yahya et al. 2012; Rodríguez-Moya and Gonzalez 2010).

The introduction of foreign genes, using plasmids or cloned into the *E. coli* chromosome, the elimination of competitive pathways, the adaptive evolution, carbon flux distribution, and the disruption of byproducts formation have been the main strategies used to improve sugar utilization, ethanol and inhibitors tolerance, ethanol yields on carbon consumed, and specific and volumetric ethanol productivity (Ingram et al. 1999; Jarboe et al. 2007; Orencio-Trejo et al. 2010). But some drawbacks are still highlighted for this microorganism, such as the low tolerance toward ethanol and the narrow and neutral working pH (6–8) (Gírio et al. 2010). However, several metabolically engineered strains had shown ethanol yields above 95 % of the theoretical maximum, either using pure sugars, sugar mixtures (pentoses and hexoses), and actual lignocellulosic hydrolysates containing acetate (Fernández-Sandoval et al. 2012; Geddes et al. 2011).

In the late 1980s, *E. coli* was used to drive the expression of the *Zymomonas mobilis* genes that encode pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhII*) (Ingram and Conway 1998), allowing the production of ethanol instead of organic acids. *E. coli* K011, developed by Ingram and coworkers (USA Patent 5,000,000), constitutes one of the breakthroughs in *E. coli* toward the development of ethanologenic bacteria by means of metabolic engineering. The development consisted in: (i) the chromosomal integration of genes encoding *pdc* and *adhII* from *Z. mobilis* under the control of the pyruvate formate lyase promoter (this promoter drives a strong transcription under fermentative conditions); (ii) deletion of one subunit of the fumarate reductase (to avoid the formation of succinate under fermentative conditions); (iii) and selection of variants (through adaptive evolution in plates with a high concentration on the antibiotic marker used for the integration of *pdc* and *adhII*) showing high pyruvate decarboxylase and alcohol dehydrogenase enzymatic activities. The main results attained with *E. coli* K011 under different operational conditions are summarized in Table 8.1, and Fig. 8.2 shows common modifications performed into wild-type *E. coli* strains to attain ethanologenic strains.

Although *E. coli* K011 produced ethanol with specific rates as high as those of yeasts (*S. cerevisiae*), its ethanol tolerance was lower than that of the yeasts, hence metabolic evolution was applied for improving ethanol tolerance and ethanol production, leading to *E. coli* strain LY01 (Yomano et al. 1998; Jarboe et al. 2007). Nevertheless, the performance of both K011 and LY01 relied on costly nutritional supplementation which is not justified when producing biofuels. To address this issue, a new medium was developed (AM1, Martínez et al. 2007) along with a new development of homo-ethanologenic strains, such as LY168 (Yomano et al. 2009). This strain contained a deletion of the methylglyoxal synthase gene (*mgsA*) that resulted in the co-metabolism of pentose and hexose sugars to ethanol and was

Table 8.1 Relevant aspects of modified *E. coli*-derived research

Strain	Main results	References
E. coli LY01	Ethanol yield up to 0.47 was achieved from 90 g xylose/L	Yomano et al. (1998)
E. coli FBR5	Study on ethanol production from xylose in batch reactors. Maximum xylose concentration, 250 g/L; max ethanol concentration produced 43.5 g/L; max ethanol yield 0.50 g g^{-1} xylose	Qureshi et al. (2006) Saha et al. (2011)
	Undetoxified wheat straw hydrolysate. Yield of total sugars from wheat straw (150 g/L) is 86 g/L	
E. coli K011	First report of a metabolic engineered bacteria for the production of ethanol	USA Patent 5,000,000
	Corn fiber hydrolysates (90 g/L sugars) added with LB broth components or mixture of sugars (100 g/L), ethanol yields were 80–88 %, respectively, and global volumetric productivities of 0.38 and 0.66 g/L h ethanol were observed, without xylose being completely consumed	Ingram et al. (1991) Dien et al. (1997) Leite et al. (2000)
	Sweet whey (58 g/L sugars) without supplements, reaching 38 % of the theoretical ethanol yield. When the sweet whey was supplemented with yeast extract and a trace metals mixture, the ethanol yield increased to 100 %	Jin et al. (2012) Yasuda et al. (2012)
	The xylose consumption capability of *E. coli* KO11 was almost totally inhibited by the presence of both degradation products and ethanol in AFEX™ treated corn stover hydrolysate	
	The combination of simultaneous saccharification and fermentation and pentose fermentation resulted in 144 mg ethanol per g untreated substrate	
Recombinant from *E. coli* MG1655	First reported work on successful cellobiose and xylose co-metabolism	Vinuselvi and Lee (2012)
E. coli MM160	Hexose and pentose sugars from phosphoric acid pretreated sugarcane bagasse were co-fermented (SScF) to ethanol in a single vessel, eliminating process steps for solid–liquid separation and sugar cleanup. Ethanol yield was 0.21 g ethanol/g bagasse dry weight	Geddes et al. (2011)
E. coli MM170	Improved results by serial transfers of MM160 in hemicellulose hydrolysates and by liquefaction plus simultaneous saccharification and co-fermentation process (L + SScF). Ethanol yield up to 0.27 g g^{-1} bagasse (dry weight)	Nieves et al. (2011)

able to utilize hemicellulose sugars from hydrolysates as carbon source. In order to increase the substrate range, further modifications to LY168 resulted in the strain LY180 (Miller et al. 2009). LY180 was then subjected to serial transfers in hemicellulose hydrolysates obtained from the steam explosion pretreatment of sugarcane bagasse using dilute sulfuric acid (and later dilute phosphoric acid) to increase the tolerance of the strain to the fermentation inhibitors resulting from

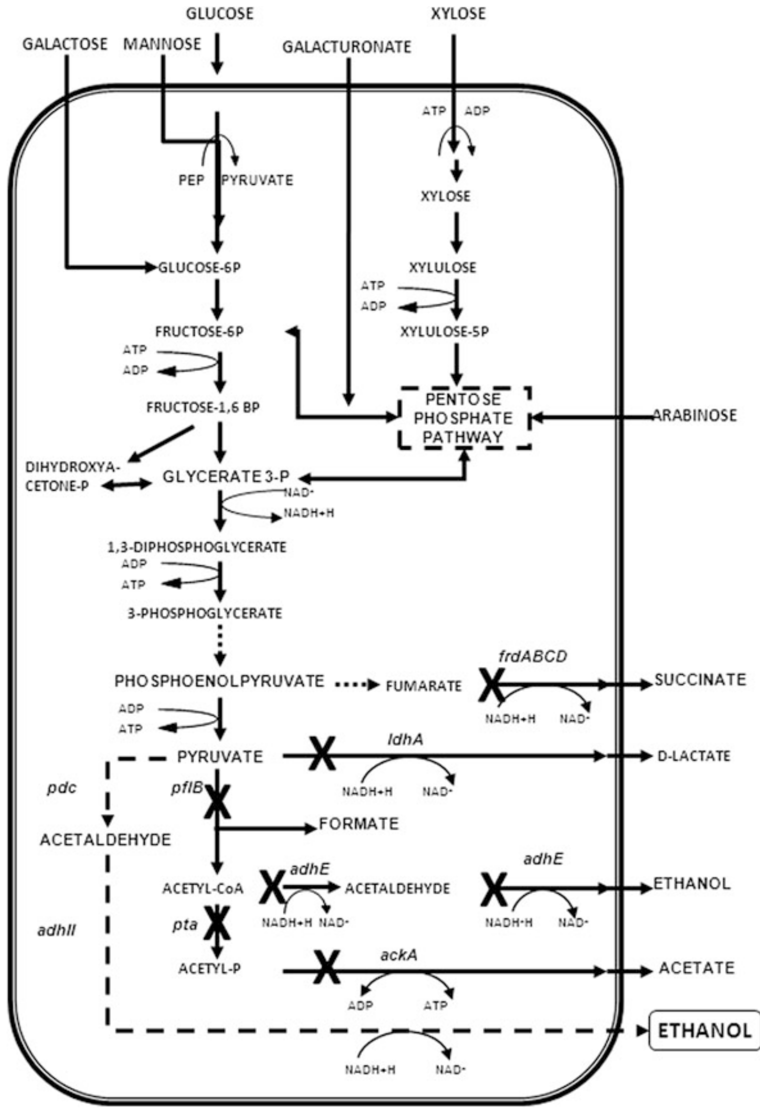

Fig. 8.2 Fermentation pathways in wild-type *E. coli* and common modifications in metabolically engineered ethanologenic strains. Genes encoding enzymes are indicated by *italics*, the "X" sign indicate deleted genes to reduce byproducts. Ethanol production pathway (*pdc* and *adhB*) from *Z. mobilis* is shown by *dashed arrows*. *adhE* alcohol dehydrogenase, *frdABCD* fumarate reductase, *ldhA* lactate dehydrogenase, *pflB* pyruvate formate lyase, *pta* phosphotransacetylase, *ackA* acetate kinase A, *pdc* pyruvate decarboxylase from *Z. mobilis*, *adhB* alcohol dehydrogenase B from *Z. mobilis*

pretreatment. The inhibitor tolerant strain obtained (MM160) was able to ferment slurries of dilute phosphoric acid pretreated sugarcane bagasse with ethanol yields of over 0.20 g/g (Geddes et al. 2011). Additional serial transfers of MM160 in hemicellulose hydrolysates resulted in a superior strain (MM170) with an improved yield of 0.27 g/g (Nieves et al. 2011).

Vinuselvi and Lee (2012) assayed a combination of genetic and evolutionary engineering strategies for improving simultaneous utilization of cellobiose and xylose. The recombinant *E. coli* was capable of utilizing around 6 g/L of cellobiose and 2 g/L of xylose in approximately 36 h, whereas wild-type *E. coli* was unable to utilize xylose completely in the presence of 6 g/L of glucose even after 75 h.

Recent reports have demonstrated the development of a new generation of homo-ethanologenic strains that can grow and produce ethanol efficiently in the presence of acetate (Fernández-Sandoval et al. 2012); ferment cellobiose and glucose mixtures simultaneously (Muñoz-Gutiérrez et al. 2012); produce cellulases and xylanases allowing the direct fermentation of pretreated corn stover cellulose into ethanol (Ryu and Karim 2011); ferment alginate from brown macroalgae into ethanol (Wargacki et al. 2012); ferment pectin-rich lignocellulosic biomass, specifically sugar beet pulp, into ethanol (Edwards et al. 2011).

Concerning the transformation of actual hydrolysates into ethanol, some authors have determined the performance of different *E. coli* strains. Thus, the effects of both ethanol and degradation products have been identified as the main reasons for the loss of fermentability of *E. coli* K011 for transforming sugars contained in corn stover Ammonia Fiber Explosion (AFEX) pretreated xylose hydrolysate into ethanol (Jin et al. 2012). However, using a new generation of metabolic engineered strains (*E. coli* MM160 and MM170), Geddes et al. (2011) and Nieves et al. (2011) have studied the fermentation of sugarcane bagasse slurries, obtained by phosphoric acid pretreatment, into ethanol. After applying simultaneous saccharification and fermentation, with a previous liquefaction step, these researchers have demonstrated a yield of 340 l of ethanol per dry metric ton of untreated bagasse. Furthermore, the fermentation of these slurries has been scaled up to 80-L fermentors (Nieves et al. (2011), and Ingram and coworkers in partnership with Buckeye Technologies Inc. are developing, for the first time, a biorefinery demonstration plant in Perry, Florida, USA, with the aim to "produce up to 400 gal of fuel ethanol (and 2,000 pounds of organic acids for biopolymers) each day", using lignocellulosic biomass and the *E. coli* strains described in this paragraph.

Some studies addressed the behavior of modified-ethanologenic *E. coli* strains in salt-rich media, because hydrolysates from agricultural residues are frequently rich in salts, which can exert an inhibitory effect on the growth of the bacteria. For example, Qureshi et al. (2006) determined that ethanologenic *E. coli* FBR5 could tolerate up to 40 g/L of NaCl, although the microorganism exhibited some inhibition for concentrations above 10 g/L. Saha et al. (2011) successfully used recombinant *E. coli* FBR5 on hydrolysates from sulfuric acid pretreated wheat straw. 41.1 g ethanol/L were produced in 168 h at pH 7.0 and 35 °C. The authors

claim that this is the first report showing greater than 4 % ethanol production from lignocellulose by the strain.

It is also worth noting that *E. coli*, which had long been considered incapable of utilizing glycerol as raw material for ethanol production, was recently metabolically engineered by overexpressing the aldehyde dehydrogenase and deleting the lactate dehydrogenase genes, which resulted in a high yield of ethanol (20.7 g/L), associated with a productivity of 0.22 g/L/h (Durnin et al. 2009). Further studies with glycerol have allowed proposing the use of this residual and inexpensive chemical from the biodiesel industry as a platform for the production of several biofuels and chemical with the concomitant development of *E. coli* metabolical engineered strains (Dellomonaco et al. 2011; Rodríguez-Moya and Gonzalez 2010).

8.4 *Saccharomyces cerevisiae*-Based Modifications

Because *S. cerevisiae* is one of the best glucose-fermenting microorganisms but it lacks the ability to ferment xylose, several strategies have been identified to enable it to utilize xylose. These strategies correspond to the two pathways depicted in Fig. 8.1. The first one (reductive-oxidative pathway) is moduled by xylose reductase (XR), to catalyze the transformation of xylose to xylitol, and xylitol dehydrogenase (XDH), which catalyzes further conversion to xylulose. The second pathway implies the use of xylose isomerase to directly convert xylose to xylulose, which in turn enters the pentose phosphate pathway (Parachin et al. 2011) by means of the action of xylulokinase (XK).

It is worth mentioning that even if *S. cerevisiae* possesses the genes encoding the enzymes XR, XDH, and XK, it is not able to ferment xylose even when the genes were overexpressed, Hahn-Hägerdal et al. (2007). So a big research effort in the last four decades has been devoted to express those genes into *S. cerevisiae* so that it is able to efficiently utilize xylose.

It has previously been suggested that xylose is taken up by both high- and low-affinity systems of glucose transporters (Fig. 8.3), but the uptake is increased in the presence of low glucose concentrations (Olofsson et al. 2008).

One of the first attempts to get *S. cerevisiae* utilizing xylose was based on the fact that it can ferment xylulose. For this reason, it was thought that expressing xylose-isomerase (XI) would be a promising modification, and the strain TBM3050, carrying XI from *Thermus thermophiles* was produced (strain TMB3050). As the yeast *Pichia stipitis* was known for its ability to ferment xylose with minimal amounts of xylitol as coproduct, it was selected for the first successful xylose-utilizing of *S. cerevisiae*, which was produced by expressing *P. stipitis* genes encoding XR and XDH. The ethanol yields were however low, and xylitol was also produced, which was attributed to the need of a high activity of both XR and XDH.

A complete description of xylose consumption process by different *S. cerevisiae* strains, including ethanol and xylitol yields anaerobic and oxygen-limited batch

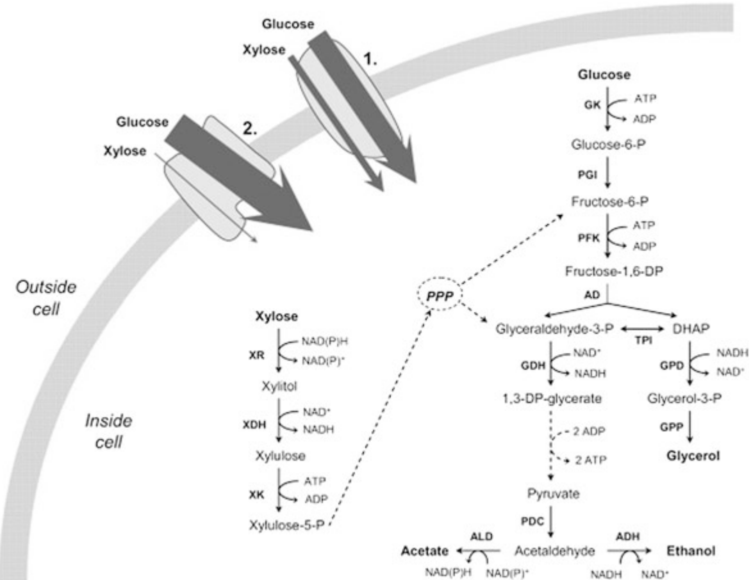

Fig. 8.3 Simplified scheme of sugar transport and metabolism in *S. cerevisiae* (Olofsson et al. 2008). 1. Low- and intermediate-affinity hexose transporters. 2. High-affinity hexose transporters. Abbreviations: *PPP* pentose phosphate pathway, *XR* xylose reductase, *XDH* xylitol dehydrogenase, *XK* xylulokinase, *GK* glucokinase, *PGI* phosphoglucose isomerase, *PFK* phosphofructokinase, *AD* aldolase, *TPI* triose phosphate isomerase, *GDH* glyceraldehyde-3-P dehydrogenase, *GPD* glycerol-3-P dehydrogenase, *GPP* glycerol-3-phosphatase, *PDC* pyruvate decarboxylase, *ALD* acetaldehyde dehydrogenase, *ADH* alcohol dehydrogenase

cultures which can be found in Hahn-Hägerdal et al. (2007), and in Chu and Lee (2007). Although much work has been dedicated to lab scale study on pentose-fermenting *S. cerevisiae*, there are still some issues to be addressed, especially those related with industrial uses, as pointed out by Matsushika et al. (2009).

A review of more recent work on modified *S. cerevisiae* for ethanol production from xylose or pentoses in general is presented below (Table 8.2).

Recently, Liu and Hu (2010) reported on a *S. cerevisiae* strain via combined approaches of recombinant DNA technology, chemical mutagenesis, and evolutionary adaptation for an efficient xylose utilization and ethanol fermentation. A haploid derivative of an industrial-fermenting strain was first engineered to express the genes from *P. stipitis* encoding XR and XDH, and XK. Then, the recombinant strain was submitted to ethyl-methanesulfonate mutagenesis followed by adaptive evolution, resulting in a single isolate with improved xylose utilization characteristics.

Although large research efforts have been devoted toward modifying *S. cerevisiae* to allow xylose consumption and ethanol production, a number of issues have limited the success of the process, including poor xylose uptake, cofactor imbalance, insufficiency in the pentose phosphate pathway, deregulation of the ethanologenic

Table 8.2 Some recent results derived from research on modified *S. cerevisiae*

Yeast (strain)	Modifying strategy	Main results	References
ADAP8	Serial transfer in xylose-containing minimal medium under aerobic conditions	Ethanol yield 0.48 g g^{-1} complex in medium containing yeast extract, peptone, and borate	Madhavan et al. (2009)
LEK513	Recombinant DNA technology, chemical mutagenesis, and evolutionary adaptation	95 % xylose consumed; 11 % more ethanol in oxygen-limited conditions than in aerobic fermentation	Liu and Hu (2010)
H131-A3	Overexpression of the *Piromyces* xylose isomerase gene (XYLA), *P. stipitis* xylulose kinase (XYL3), and genes of the non-oxidative pentose phosphate pathway (PPP)	anaerobic growth rate (0.203 h^{-1}) and xylose consumption rate (1.866 g g^{-1} h^{-1}) along with high ethanol conversion yield (0.41 g g^{-1})	Zhou et al. (2012)
S.cerevisiae MN8140	Codisplaying endoxylanase, β-xylosidase, and β-glucosidase; expression of xylose reductase and xylitol dehydrogenase from *P. stipitis* and xylulokinase from *S. cerevisiae*	Ethanol yield 0.41 g g^{-1} (82 % theoretical). 8.2 g ethanol/l after 72 h fermentation	Sakamoto et al. (2012)
S. cerevisiae	Evolutionary engineering strategy: repeated batch cultivation with repeated cycles of consecutive growth in three media with different compositions	Fermentation of mixtures of glucose, xylose, and arabinose. Ethanol yield (0.43 g g^{-1} of total sugar). No formation of the side products xylitol and arabinitol	Wisselink et al. (2009)
S. cerevisiae BY4741-S1A, -S1A1, -S1A2, -S1A3, and -S2A3	directed evolution of the *Piromyces* sp. xylose isomerase (encoded by xylA) three rounds of mutagenesis and growth-based screening	10- to 30-fold- and 3- to 10-fold-higher rates than previously reported engineered strains for xylose isomerase pathways and oxidoreductase pathways, respectively	Lee et al. (2012)
TMB 3420	Randomly mutagenized xylose reductase	The ethanol productivity from xylose in TMB 3420 was increased $\approx$40 times compared to that of the parent strain	Runquist et al. (2010)
Y5	Newly developed strain in the lab (patent no. ZL200810222897.7, CGMCC2660, China general microbiological culture collection center)	The fermentation of hydrolysate of non-detoxified steam-exploded corn stover resulted in ethanol concentration of 44.55 g/L, (94.5 % of the theoretical yield)	Li et al. (2011)
S. cerevisiae ZU-10	Expression of xylose reductase (XR) and xylitol dehydrogenase (XDH) from the *P. stipitis* genes XYL1 and XYL2, respectively, and overexpresses the homologous XKS1 gene encoding xylulokinase (XK)	Ethanol concentration of 31.1 g/L (ethanol yield on fermentable sugars of 0.41 g g^{-1}) within 72 h in batch fermentation of detoxified corn stover hydrolysate with immobilized cells	Zhao and Xia (2010)

enzymes, and specially the regulation of metabolism in the eukaryotic yeasts, less known than that of bacteria (Hahn-Hägerdal et al. 2007). Efficient utilization of xylose appears to require complex global changes in cellular processes (Liu and Hu 2010). The need for novel tools and approaches to overcome the major remaining difficulties, like engineering simultaneous, exogenous sugar metabolism has also been emphasized. Beyond catabolic pathways, the focus must shift toward non-traditional aspects of cellular engineering such as host molecular transport capability, catabolite sensing, and stress response mechanisms (Young et al. 2010).

Direct evolution is one of the strategies to improve the performance of *S. cerevisiae* for utilizing all sugars derived from pretreatment of LCM. Using pine hydrolysates, Hawkins and Doran-Peterson (2011) proposed a combination of adaptation by inoculation first into a solids loading of 7 % 'w/v for 24 h, followed by a 10 % v/v inoculum into 17.5 % w/v solids. Under such conditions, the final strain (AJP50) produced ethanol at more than 80 % of the maximum theoretical yield after 72 h of fermentation, and more than 90 % of the maximum theoretical yield after 120 h of fermentation. This improvement in comparison with results by the starting industrial strain (XR122n) was attributed to the ability of AJP50 to rapidly convert furfural and hydroxymethylfurfural to their less-toxic alcohol derivatives.

In a recent work, Zhou et al. (2012) described the metabolic engineering of a *S. cerevisiae* strain, including overexpression of the *Piromyces* xylose isomerase gene (XYLA), *P. stipitis* xylulose kinase (XYL3) and genes of the non-oxidative pentose phosphate pathway (PPP). The engineered strain, named H131-A3, was used to initialize a three-stage process of evolutionary engineering, first through aerobic and anaerobic sequential batch cultivation followed by growth in a xylose-limited chemostat. These authors claimed that the evolved strain H131-A3-ALCS produced the best result on xylose utilization and ethanol production by *S. cerevisiae* reported to-date.

Concerning the application of recombinant *S. cerevisiae* on lignocellulose hydrolysates, several strategies have also been applied. For example, the fermentability of ammonia-pretreated corn stover detoxified hydrolysate was significantly improved by using immobilized cells of recombinant *S. cerevisiae* in Ca-alginate (Zhao and Xia 2010). The composition of the detoxified and concentrated hydrolysate included 72 g/L xylose and 14.3 g/L arabinose and the ethanol yield based on fermentable sugars was 0.41 g/g within 72 h in batch fermentation.

Strain *S. cerevisiae* Y5 is a newly lab-developed patented microorganism. The strain has the ability to metabolize furfural, tolerate fermentation inhibitors, and efficiently metabolize glucose to produce ethanol. It was used for ethanol production from enzymatic hydrolysates of non-detoxified steam-exploded corn stover, with and without a nitrogen source, and decreasing inoculum size (Li et al. 2011). Results showed that ethanol yields as high as 94.5 % of the theoretical yield were obtained after 24 h, with an inoculum size of 10 % (v/v) and nitrogen source (corn steep liquor) of 40 mL/L.

8.5 Other Modified Microorganisms

In addition to *E. coli* and *S. cerevisiae*, a number of other microorganisms have also been extensively studied for ethanol production from lignocellulosic materials. Some examples of these recent studies are summarized in Table 8.3.

Zymomonas mobilis is a bacterium that can ferment certain sugars to ethanol via the Entner–Doudoroff (ED), glyceraldehyde-3-phosphate-to-pyruvate (GP), and pyruvate-to-ethanol (PE) pathways. It has been proposed as a promising alternative to other fermenting microorganisms as ethanol yields as high as 97 % of the theoretical maximum along with high production rates have been reported (Hayashi et al. 2011). This advantageous behavior is a consequence of a favorable energy balance from the ED pathway, compared to Embden–Meyerhof–Parnas pathway used by both *E. coli* and *S. cerevisiae* (Gírio et al. 2010).

However, *Z. mobilis* exhibits a relatively low tolerance to inhibitors generated during the conversion process. This fact, along with the lack of the complete pentose metabolism pathway necessary for fermentation of lignocellulosic hydrolysates (Davis et al. 2005), are the reasons for several engineering works on this microorganism trying to improve ethanol yields. The introduction of the xylose metabolism pathway has been one of the main strategies, although with limited results (Chandel et al. 2011). In another study, a threefold higher ethanol concentration was produced when a fragment from *Enterobacter cloacae* conferring cellulase activity was cloned in *Z. mobilis* (Vasan et al. 2011) in comparison with results with the native *E. cloacae*.

Bacillus subtilis is also a promising microorganism known for producing ethanol from lignocellulose, as it has xylose isomerase and xylulokinase for metabolizing xylose. However, the wild type lacks a specific xylose transporter and hence it is not feasible to use xylose as a sole carbon source. In an attempt to improve xylose transportation, the arabinose: H+ symporter, AraE protein from *B. subtilis* was expressed in *B. subtilis* 168 (Park et al. 2012). *B. subtilis* has also been engineered to produce ethanol and depolymerize cellulose (Romero et al. 2007; You et al. 2011).

Clostridium acetobutylicum is an anaerobic bacterium that is known for its excellent capacity to produce ABE (acetone, butanol, and ethanol) solvents. Nevertheless, it shows inefficient pentose consumption when fermenting sugar mixtures. As a strategy to overcome this fact, a predicted glcG gene, encoding enzyme II of the D-glucose phosphoenolpyruvate-dependent phosphotransferase system (PTS), was first disrupted in the ABE-producing model strain *C. acetobutylicum* ATCC 824, which resulted in a greatly improved D-xylose and L-arabinose consumption in the presence of D-glucose. Further overexpression of the xylose pathway resulted in an engineered strain (824glcG-TBA) that was able to efficiently conferment mixtures of D-glucose, D-xylose, and L-arabinose, with better results than the results tied to the wild-type strain (Xiao et al. 2011).

Thermoanaerobacterium saccharolyticum is a potential catalyst for lignocellulose conversion that can naturally hydrolyze xylan and ferment all

Table 8.3 Some recent results related to the use of modified microorganisms for ethanolic fermentation

Microorganism (strain)	Modification strategy	Main result	References
B. subtilis JY123	Expression of arabinose:H+ symporter, AraE protein from B. subtilis	Efficient transport of D-xylose	Park et al. (2012)
T. saccharolyticum	Encoding urease genes	The engineered strain hydrolyzed urea, producing a high ethanol titer (54 g/L)	Shaw et al. (2012)
		2.5-fold reduction in cellulase loading compared with using S. *cerevisiae* at 37 °C	Shaw et al. (2008)
Z. mobilis	A fragment conferring cellulase activity from which threefold was higher than the amount obtained with the original *Enterobacter cloacae* isolate was cloned in *E. coli*, and then the cellulose gene was introduced into *Z. mobilis*	Using carboxymethyl cellulose and 4 % NaOH pretreated bagasse as substrates, the recombinant strain produced 5.5 % and 4 % (V/V) ethanol, respectively	Vasan et al. (2011)
C. acetobutylicum ATCC 824	Integration of glcG disruption and genetic overexpression of the xylose pathway	24 % higher ABE solvent titer and a 5 % higher yield (0.28 g g^{-1}) compared to those of the wild-type strain	Xiao et al. (2011)
K. pneumoniae GEM167	γ-irradiation mutant Then overexpression of *Z. mobilis pdc* and *adhII* genes encoding pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (Adh)	Ethanol titer of 21.5 g/l, with a productivity of 0.93 g/l/h Improved to 25.0 g/l in the second mutant	Oh et al. (2011)

monosaccharides and disaccharides found in lignocellulosic materials. Encoding the urease gene resulted in one of the highest titers reported in this microorganism. In addition, it is evident that the use of urea instead of ammonium salts can be advantageous here because of the lower cost (Shaw et al. 2012). Ethanologenic strains of *T. saccharolyticum* have been obtained through metabolical engineering and adaptive evolution and 37 g/L of ethanol were produced using simultaneous saccharification and fermentation from Avicel (Shaw et al. 2008). Lynd and coworkers from Dartmouth College claim that this is one of the best microorganism to develop CBP.

Klebsiella pneumoniae is a Gram-negative bacterium that has been described as capable of fermenting glycerol, a by-product of biodiesel. Due to the large amount

of glycerol generated (equivalent to approximately 10 % of biodiesel produced) and its need to be treated before discharge, a great interest has emerged regarding the use of glycerol as raw material for the production of industrially valuable materials like ethanol. The estimated cost of ethanol produced from glycerol, considering both the feedstock demand and operational costs, is 40 % less than the estimated cost of production from corn-derived sugars (which is seen as an additional advantage). Oh et al. (2011) have isolated a γ-irradiated mutant strain of *K. pneumoniae* (GEM167) exhibiting high production of ethanol from glycerol. Ethanol production level was improved to 25.0 g/L upon overexpression of *Z. mobilis pdc* and *adhII* genes encoding pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (Adh), respectively in the mutant strain GEM167.

8.6 Conclusions

The conversion of sugars contained in lignocellulosic materials into ethanol is a promising way for partially substituting fossil fuels. As a consequence of the pretreatment step in the bioconversion process, a mixture of hexose and pentose sugars, together with sugar degradation and inhibitory products, is produced and this must be considered in the subsequent hydrolysis and fermentation steps. No matter what the process configuration is, the corresponding microorganism should produce ethanol with both high yields and productivity, with low medium and operational requirements, and should at the same time be tolerant of ethanol and resistant to inhibitors. Unfortunately, such an ideal ethanologenic microorganism is not available at the moment. Nevertheless, a great deal of research is being devoted to modify, adapt, and engineer yeasts and bacteria for such a purpose. Recombinant DNA technology and evolutionary engineering techniques, direct evolution, introduction of foreign genes, elimination of competitive pathways, disruption of byproducts, and formation are some of the many strategies being assayed that should lead to ethanol production from lignocellulosic materials at an industrial scale in the near future.

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Chapter 9
Consolidated Bioprocessing for Improving Cellulosic Ethanol Production

Antonella Amore, Simona Giacobbe and Vincenza Faraco

Abstract Consolidated bioprocessing (CBP) is a potential system for reducing costs of production of bioethanol from lignocelluloses, combining hydrolysis and fermentation into a unique reactor, and exploiting the ability of engineered microorganisms to perform both the reactions. CBP would represent a breakthrough for low-cost biomass processing, due to economic benefits of process integration and, mainly, avoiding the high costs of the cellulolytic enzymes. This chapter discusses the progresses achieved in the development of both the CBP category I and category II, where CBP category I deals with engineering of a cellulase producer to make it ethanologenic and category II consists in engineering an ethanologenic microorganism to render it cellulolytic.

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9.1 Introduction

The extensive exploitation of fossil sources has been causing increasing concern both on security of their supply and alarm over greenhouse gas emission and global warming. Being produced in huge amounts all over the world, lignocellulose represents the best alternative to fossil sources as feedstock for production of energy and fuels. Bioethanol production from lignocelluloses requires several steps such as the pre-treatment, for lignin removal and cellulose/hemicelluloses accessibility, the hydrolysis of the polysaccharides to achieve both hexoses and pentose fermentable sugars, the fermentation step for conversion of sugars into ethanol.

One of the major problems in producing ethanol from lignocellulosic biomass is the high production costs, particularly those associated with the hydrolysis step.

Consolidated bioprocessing (CBP) has been so far recognized as a potential system to reduce the cost of biomass processing, by combining the main events required for lignocellulose conversion into one reactor, particularly the hydrolysis of the polysaccharides and the subsequent fermentation of the hexoses/pentose sugars. However, no microorganism shows this combined ability, thus the development of "CBP microorganisms" is required by using the genetic engineering.

There are two main routes to perform CBP: category I CBP is based on the engineering of a cellulase producing microorganism to make it able to ferment sugars, while category II CBP aims at engineering ethanologenic microorganisms to make them cellulolytic.

Filamentous fungi like *Trichoderma, Aspergillus*, *Rhizopus,* and *Fusarium* have been identified as good candidates for CBP category I, *Trichoderma reesei* being the best candidate due to the high level of cellulase activity production, the deep knowledge of its physiology, and the availability of genetic manipulation tools.

As far as CBP category II is concerned, bacteria and yeasts are the best candidates. *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are the most investigated among the yeasts, while *Escherichia coli* and *Zymomonas mobilis* are the most interesting bacteria for CBP type II. Tools for genetic manipulation of these microorganisms have been so far developed and definition of growth conditions for concomitant cellulose hydrolysis and ethanologenesis results to be easier for them than for fungi.

This chapter reviews the recent developments in production of CBP microorganisms, showing the advantages and disadvantages of this process and the challenges to be faced.

9.2 Potential of Fungi as CBP Microorganisms

Amore and Faraco (2012) recently reported an overview of the state-of-the-art of CBP I Category.

Some filamentous fungi belonging to the genera *Trichoderma, Aspergillus, Rhizopus,* and *Fusarium* have been reported to possess the ability to directly ferment cellulose to ethanol, even if with very low yield or high concentration of by-products. This conversion ability is thought to depend on two metabolic routes, one route involving the production of cellulases to degrade cellulose to soluble sugars under aerobic conditions, the other producing ethanol and other by-products, such as acetic acid, under anaerobic conditions.

T. reesei stands out as a good CBP I candidate, because of the extensive knowledge of its physiology and the wide range of tools for its genetic manipulation. It has been so far reported as the main producer of cellulases and it has been shown to possess all the metabolic pathways necessary to fully utilize the lignocellulose sugars for production of ethanol, as reported by Xu et al. (2009). It can produce ethanol in the range 0.2–4.8 g L^{-1}, where the maximum yield is achieved by fermenting glucose. Nevertheless, some difficulties have to be faced to make this fungus an effective CBP microorganism, such as the low ethanol yield and productivity, the low ethanol tolerance and the high energy required for mixing during fermentation, due to its morphology. Moreover, the obligate aerobe nature of *T. reesei* hinders its growth without oxygen, this being an important issue to be faced in order to develop an ethanologenic *T. reesei* strain.

Several manuscripts have so far reported the potential of *Aspergillus spp*. for (hemi)cellulases production at industrial scale. *Aspergillus* species are the major agents of (hemi)cellulose decomposition and thus possess the capability to produce a broad range of (hemi)cellulolytic enzymes. Most of *Aspergilli* have been shown to be able to produce ethanol from glucose. For instance, *A. terreus* has been demonstrated able to ferment fructose and mannose, with a yield of 2.16 % (w/v) (85 % conversion) and 1.98 % (w/v) (78 % conversion), respectively (Pushalkar and Rao 1998).

However, the main limitations of a cellulose CBP based on *Aspergillus spp*. consist of the very low ethanol yields that these microorganisms exhibit in comparison with *S. cerevisiae*, and formation of the by-product lactate.

Fusarium spp. has been shown to be a great cellulase and hemicellulase producer. Moreover, the ability of this species to convert biomass directly to ethanol was shown more than 20 years ago for the first time. The growth, substrate consumption, product and by-product formation of *F. oxysporum* in a minimal glucose-based medium in aerobic, anaerobic, and oxygen-limited batch cultivations have been investigated (Ruiz et al. 2007; Panagiotu et al. 2005). The highest ethanol yield—1.66 mol ethanol/mol of glucose, corresponding to 80 % of the theoretical maximum yield—was achieved under anaerobic conditions. *F. oxysporum* wild-type strain F3 was able to grow at a maximum specific growth rate of 0.023 h^{-1} on cellulose in aerobic conditions, and to produce ethanol with a yield

of 0.35 g/g cellulose under anaerobic conditions. However, for this specie, the low yield of lignocellulosic hydrolysis is the major bottleneck that does not make it a microorganism ready to be used in CBP process.

R. oryzae has been generally reported able to produce ethanol, but lactic acid has been reported to be an abundant by-product. For example, Fujio et al. (1985) reported growth of *R. koji* on uncooked cassava starch with a maximum productivity of ethanol of 2.3 g ethanol/Lh, which corresponds to the 50 % of the general ethanol yield of a common yeast. *R. javanicus* and *R. oryzae* have been shown to produce a maximum of 33 g L^{-1} and 32 g L^{-1} of ethanol from glucose, respectively (Skory et al. 1997; Stevenson et al. 2002). To the best of our knowledge, few studies have been so far reported on the production and characterization of cellulases by *Rhizopus* spp.. In fact, in comparison with other filamentous fungi, *Rhizopus* genome contains a very low number of Glycoside hydrolases (GHs) coding genes.

Thus, even if there are several fungi potentially involved in CBP process, many efforts are still needed for developing a suitable CBP type I process which allows high yield of ethanol from lignocellulose raw materials.

9.3 Potential of Yeasts as CBP Microorganisms

9.3.1 Saccharomyces cerevisiae

The well-known ethanol producing yeast *Saccharomyces cerevisiae* has been long employed for the industrial production of ethanol from hexose sugars (Nissen et al. 2000; Van Dijken et al. 2000; Kuyper et al. 2004) and it has been the most exploited host for developing a CBP. *S. cerevisiae* plays a major role in applied research due to its capacity to produce ethanol and carbon dioxide from sugars with high productivity, titer and yield. Different process operation modes have been used for ethanol production by *S. cerevisiae*: Simultaneous Saccharification and Fermentation processes (SSF) (Jung et al. 2013), Simultaneous Saccharification and Co-Fermentation (SSCF) (Jin et al. 2012; Ohgren et al. 2006; Olofsson et al. 2010), Separate Hydrolysis and Fermentation (SHF) (Tomas-Pejò et al. 2008), and Pre-saccharification and Simultaneous Saccharification and Fermentation (PSSF) (Moreno et al. 2012).

S. cerevisiae has many positive traits making it a suitable host for CBP, such as the high ethanol yields ($\sim$0.45–0.50 g EtOH/g glucose) and productivity from glucose (around $\sim$2–5 g EtOHL-1 h-1), its GRAS (Generally Recognized As Safe) status, the ability to grow at low pHs, the tolerance toward osmotic stress and inhibitors, robustness in industrial fermentation and amenability to genetic manipulations (Li and Elledge 2007; Shao et al. 2009; Gibson 2009; la Grange et al. 2010). Moreover, it produces several glucanase activities (Farkas et al. 1973; Larriba et al. 1995), such as a cell wall-bound endo-β-1,3-glucanase, encoded by

BLG2 (Mrsa et al. 1993), extracellular exo-β-1,3-glucanases EGX1 (BGL1) and EGX2 (Nebreda et al. 1986; Kuranda and Robbins 1987; Van Rensburg et al. 1997) with β-1,3- and β-1,6-activities (Nombela et al. 1988), an intracellular sporulation specific exo-β-1,3-glucanase (encoded by SSG1 or SPR1; San Segundo et al. 1993; Muthukumar et al. 1993), but no glucanase with β-1,4-activity.

However, this yeast has several shortcomings in terms of CBP-processing organism such as the inability to grow on complex substrates like lignocellulose, to hydrolyze polysaccharides (cellulose and hemicellulose) and to ferment pentose sugars. Thus, one of the main challenges to exploit *S. cerevisiae* as a CBP organism is to confer it the ability to hydrolyze the insoluble polysaccharides present in lignocellulosic biomass into monomeric sugars.

Heterologous expression of cellulases in *S. cerevisiae* has been an object of investigation for long time (Van Rensburg et al. 1998; Fujita et al. 2002, 2003) and there are several works concerning the expression of cellulase encoding genes in this yeast (Van Zyl et al. 2007, 2011; Ilmen et al. 2011; Den Haan et al. 2007a).

As reported in Table 9.1 where cellulase components expressed in *S. cerevisiae* are listed, cellobiohydrolases (CBH) and endoglucanases (EG) have been successfully expressed in *S. cerevisiae*, EG production being much more successful than CBH production (Den Haan et al. 2007a, b; Hong et al. 2003; Takada et al. 1998). Penttilä et al. (1988) expressed CBH genes from *T. reesei* in *S. cerevisiae*, achieving a yield of 100 and 2 mg L^{-1} for CBHII and CBHI, respectively. Recently, Den Haan et al. (2007a) reported the expression of four fungal individual CBH genes including two from *T. reesei* (*cbh1* and *cbh2*), one from *Aspergillus Niger* (*cbhB*), and one from *Phanerochaete chrysosporium* (*cbh1–4*). The activity expression level of the *cbh2* from *T. reesei* was significantly higher than the other CBHs, correlating well with the results of Penttilä et al. (1988). However, all studies demonstrated that the expression of fungal CBH genes in *S. cerevisiae* is too low to allow an efficient CBP and that *S. cerevisiae* is still unable to convert crystalline cellulose into fermentable sugars.

β-glucosidases (BGL) production in *S. cerevisiae* has been also reported by Pentillä et al. (1984). However, only one out of the five BGL genes from *Aspergillus* studied in this work was found to be expressed in the yeast, and even at a low level. Van Rooyen et al. (2005) showed the ability to ferment cellobiose by a recombinant *S. cerevisiae* strain possessing BGL encoding genes. In particular, the recombinant *S. cerevisiae* strain secreting BGL1 from *Saccharomycopsis fibuligera* (Y294[SFI]) was identified as the best strain with an ethanol yield of 0.41 g per gram of cellobiose consumed, which correspond to 89 % of maximum yield of the wild- type strain when it was grown on glucose. Cho et al. (1999) showed that during SSF experiments with a *S. cerevisiae* strain producing a BGL and an enzyme with both exo- and endo- cellulase activities, the need of additional cellulases decreases. The co-expression and surface display of EG II and CBHII from *T. reesei* and BGL from *Aspergillus aculeatus* resulted in a strain able to convert phosphoric acid swollen cellulose (PASC) to ethanol with a yield of 3 g L^{-1} from 10 g L^{-1} PASC in 40 h (Fujita et al. 2004). A *S. cerevisiae* strain co-expressing endoglucanase (EG) from *T. reesei* and BGL from *Saccharomycopsis fibuligera*

Table 9.1 Cellulases expressed in *S. cerevisiae*

Enzyme	Organism	References
CBHI	*Trichoderma reesei*	Penttila et al. (1988), Den Haan et al. (2007a)
CBHB	*Aspergillus niger*	Den Haan et al. (2007a)
CBH1–4	*Phanerochaete chrysosporium*	Den Haan et al. (2007a), Van Rensburg et al. (1998)
CBHI	*Thermoascus aurantiacus*	Hong et al. (2003)
CBHI	*Aspergillus aculeatus*	Takada et al. (1998)
CBH II	*Trichoderma reesei*	Penttila et al. (1988), Den Haan et al. (2007a), Fujita et al. (2004)
EGII	*Trichoderma reesei*	Fujita et al. (2002)
eg1	*Thermoascus aurantiacus*	Hong et al. (2003)
END1	*Butyrivibrio fibrisolvens*	Van Rensburg et al. (1997)
Endo/exo bifunctional enzyme	*Bacillus circulans*	Cho et al. (1999)
BEG1	*Bacillus subtilis*	Van Rensburg et al. (1997)
BGLI	*Aspergillus aculeatus*	Takada et al. (1998)
BGLI	*Saccharomycopsis fibuligera*	Den Haan et al. (2007b)
BGL	*Bacillus circulans*	Cho et al. (1999)
BGLI	*Endomyces fibuliger*	Van Rensburg et al. (1998)
CEL1	*Ruminococcus flavefaciens*	Van Rensburg et al. (1997)
BGL	*Aspergillus niger*	Pentillä et al. 1984)

was also produced, showing the ability to grow on and convert PASC to ethanol with a yield of 1 g L^{-1} from 1 g L^{-1} PASC (van Zyl et al. 2011). In order to optimize cellulase expression levels, different cellulase expression cassettes were integrated into *S. cerevisiae* chromosome in one step; the obtained strain expressed BGL, EG, and CBH and was shown able to hydrolyze PASC with a yield of 7.5 g L^{-1} ethanol in 72 h of fermentation at 37 °C (Yamada et al. 2011).

Several studies have been performed to develop *S. cerevisiae* strains able to ferment hemicellulose (Kuyper et al. 2005; Hahn-Hägerdal et al. 2001; Katahira et al. 2004). Hemicellulolytic enzymes, such as β-xylanase, β-xylosidase, and auxiliary enzymes, such as β-glucuronidase and arabinofuranosidase, have also been successfully produced in *S. cerevisiae* (Table 9.2) (La Grange et al. 1996, 1997, 2000; Crous et al. 1996; Ho et al., 1999). Sakamoto et al. (2012) showed that co-displaying genes encoding for endoxylanase from *T. reesei*, β-xylosidase from *Aspergillus oryzae* and BGL from *A. aculeatus* on the surface of xylose-utilizing *S. cerevisiae* cells, an ethanol titer of 8.2 g L^{-1} after 72 h of rice straw fermentation can be achieved. Furthermore, laboratory and industrial yeast strains have also been engineered to co-ferment D-xylose and L-arabinose (Becker and Boles 2003; Karhumaa et al. 2006) and to co-ferment xylose and cellobiose (Cho et al. 1999). In particular, Karhumaa et al. (2006) described genetical engineering of *S. cerevisiae* strains to co-ferment the pentose sugars D-xylose and L-arabinose, showing

Table 9.2 Hemicellulases expressed in *S. cerevisiae*

Enzyme	Organism	References
XYN2	*Trichoderma reesei*	La Grange et al. (1996)
XYNB	*Bacillus pumilus*	La Grange et al. (1997)
XYLA	*Aspergillus oryzae*	Katahira et al. (2004)
ABFB	*Aspergillus niger*	Crous et al. (1996)

that the co-utilization of arabinose together with xylose significantly reduced arabitol yield and increased ethanol yield from both xylose and arabinose.

Recently, the cellodextrin transporter of *Neurospora crassa* was expressed in *S. cerevisiae*, resulting in a strain able to grow on cellobiose (Galazka et al. 2010). Ha et al. (2011) reported that xylose fermenting strain, that also produces cellodextrin transporter, shows inhibition of xylose utilization by glucose. The strain expressing both cellodextrin transporter and BGL co-fermented cellobiose and xylose simultaneously and exhibited improved ethanol yield. In particular this resulting strain consumed 40 g L^{-1} of cellobiose within 24 h, producing 16.8 g L^{-1} of ethanol.

The expression of lactose permease encoding gene from *Kluyveromyces lactis* (*lac1*) facilitates the transport of cellobiose in *S. cerevisiae*, as shown by Sadie et al. (2011). Moreover, they demonstrated that a *S. cerevisiae* strain co-expressing *lac1* gene and *Clostridium stercorarium* cellobiose phosphorylase coding gene was able to grow on cellobiose as only carbon source.

Most of the cellulases produced in *S. cerevisiae* are non-complex or free enzymes, differently from the intricate hydrolytic enzyme complex—named cellulosome—that cellulolytic anaerobes use for biomass degradation (Devaux 2004; Doi 2008; Doi et al. 1998, 2003; Fierobe et al. 1999, 2005, 2008). Therefore, another approach to increase the hydrolytic activity of yeasts is to mimic the cellulosome complex. For instance, Lilly et al. (2009) reported the expression of *Clostridium cellulolyticum* mini-cellulosome on *S. cerevisiae* cells surface. The functionality of the minicellulosome was demonstrated by several experiments. Particularly, they demonstrated a two-fold increase of endoglucanase enzyme activity in *S. cerevisiae* cells expressing the minicellulosome compared with the wild-type strain.

Tsai et al. (2009) studied the in vitro assembly of a tri-functional mini-cellulosome containing an EG, an exoglucanase, and a BGL, thus obtaining a yeast able to hydrolyze cellulose and ferment PASC to ethanol. The levels of ethanol production and PASC hydrolysis were directly correlated with the number of cellulases docked on the cell surface and the maximum yield of ethanol was of 3.5 g L^{-1} (0.49 g ethanol/g sugar consumed) after 48 h, corresponding to 95 % of the theoretical ethanol yield.

A similar approach was followed by Wen et al. (2010) who engineered *S. cerevisiae* strains with uni, bi, and trifunctional minicellulosomes. These minicellulosomes consisted of a miniscaffold containing a cellulose-binding domain, three cohesion modules, and up to three types of cellulases. It was shown that the

recombinant yeast cells displaying the trifunctional minicellulosome had the ability to breakdown and ferment PASC to ethanol with a titer of 1.8 g L^{-1}.

Although several studies have reported coexpression of multiple genes in *S. cerevisiae* strains, many issues remain. In particular, the main challenges to be met are the poor growth of the engineered strains, the low yields, and titers of ethanol and limited substrate range. Moreover, these strains are often sensitive to the inhibitors present in lignocellulose biomasses and also have low ethanol tolerance.

9.3.2 *Kluyveromyces marxianus*

Kluyveromyces spp. yeasts are currently mainly used as an alternative to *S. cerevisiae* (Pecota and Da Silva 2005; Wolf et al. 2003). *Kluyveromyces marxianus* is a facultative fermentative and Crabtree-negative yeast (van Dijken et al. 1993), firstly described in 1888 (Lodder and Kreger-van Rij 1952). However, *K. lactis* was chosen by the scientific community as the model organism for the *Kluyveromyces* genus, probably due to the fact that strains belonging to the yeast species *K. marxianus* were isolated from a great variety of habitats, which results in a high metabolic diversity and a substantial degree of intraspecific polymorphisms that also represents one of the advantages of *K. marxianus*.

When compared to *K. lactis, K. marxianus* shows interesting properties that make it a potential candidate for a wide range of biotechnological applications, such as CBP. These properties include its GRAS status, its ability to grow on a broad variety of substrates and at high temperatures, its high specific growth rates (Singh et al. 1998), and its propension to produce ethanol even in the presence of sugar excess (Rouwenhorst et al. 1988; Steensma et al. 1988; Bellaver et al. 2004). Moreover, *K. marxianus* possesses the natural ability to excrete enzymes. This is a desired property for cost-efficient downstream processing of low- and medium-value enzymes (Hensing et al. 1994). Examples of *K. marxianus* application in industrial field include the production of ethanol (Singh et al. 1998; Kourkoutas et al. 2002), cell protein (Kim et al. 1998; Grba et al. 2002; Schultz et al. 2006), enzymes, such as inulinase β-galactosidase, β-glucosidase, and polygalacturonases (Rouwenhorst et al. 1988; Hensing et al. 1994, 1995; Passador-Gurgel et al. 1996; Rajoka and Shahid 2003; Rajoka et al. 2004), pectinase (Cruz-Guerrero et al. 1999). It is worth noting its use as baker's yeast (Caballero et al. 1995), and as a host for heterologous protein production (Swinkels et al. 1993; van den Berg et al. 1990). Physiological studies of this yeast started in the 1990s, focusing the attention on the regulation of respiration, fermentation, and on the so-called Crabtree-effect (van Urk et al. 1990; Verduyn et al. 1992). It was then shown that *K. marxianus* presents a strong Crabtree-negative character, since no ethanol production was observed after a glucose pulse applied to respiring cells, in contrast to what is commonly observed in *S. cerevisiae* (Kiers et al. 1998; Bellaver et al. 2004). Different process operation modes have been used for ethanol production by *K. marxianus*: batch cultures with elevated substrate concentrations (Grubb and

Mawson 1993; Barron et al. 1996), fed-batch systems (Ferrari et al. 1994; Gough et al. 1998; Love et al. 1996), continuous system (Love et al. 1998), membrane recycle bioreactors (Tin and Mawson 1993), two-stage fermentation (Hack et al. 1994; Banat et al. 1996), extractive fed-batch cultures (Jones et al. 1993), immobilization with β-galactosidase (Hahn-Hägerdal 1985), cells calcium-alginate immobilization (Bajpai and Margaritis 1987a, b; Marwaha et al. 1988; Nolan et al. 1994; Riordan et al. 1996; Barron et al. 1996; Brady et al. 1996, 1997a, b, 1998; Ferguson et al. 1998; Gough and Mchale 1998), immobilization of cells in poly(vinylalcohol) cryogel beads (Gough et al. 1998), or in Kissiris (a mineral glass foam derived from lava) (Nigam et al. 1997; Love et al. 1996, 1998).

K. marxianus has been also used in SSF processes with added hydrolytic enzymes (Barron et al. 1995b, 1996, 1997; Boyle et al. 1997; Nilsson et al. 1995; Ballesteros et al. 2002a, b, 2004; Kádár et al. 2004), cloning heterologous cellulase genes (Hong et al. 2007) or developing mixed cultures (Ward et al. 1995).

Ethanol production at elevated temperatures by *K. marxianus* has received much attention.

K. marxianus was shown to ferment carbohydrates to ethanol at temperatures above 40 °C with an efficiency in the range of 85–90 %. Moreover, it was shown to possess a maximum growth temperature in the range of 47–52 °C (Anderson et al. 1986; Hughes et al. 1984; Banat et al. 1992). Lower ethanol tolerance was observed when *K. marxianus* was compared to *S. cerevisiae*, and this was correlated with the activity of the plasma membrane ATPase (Rosa and Sa-Correia 1992; Fernanda and Sa-Correia 1992).

Hacking et al. (1984) screened *K. marxianus* strains for their ability to ferment glucose to ethanol at high temperatures. *Kluyveromyces* strains were shown more thermotolerant than *Saccharomyces*, which in turn can produce higher ethanol yields.

Sakanaka et al. (1996) reported protoplasts fusion of a thermotolerant strain of *K. marxianus* with a high ethanol producing strain of *S. cerevisiae*. The ethanol fermentation ability of fusants was as same as that of *K. marxianus* parental cells at 30 °C and was best at 43 °C. However, the amount of ethanol produced by fusants at 43 °C was about 3.0 % in 60 h, which was inferior to that at 30 °C. Moreover, the thermostability of the fusants was found to be lower than for either of the parental cells.

Schwan and Rose (1994) studied the effect of medium composition on ethanol production. They reported that growth and ethanol production in media containing galactose was not as high as in the presence of glucose as the carbon source and also the endopolygalacturonase (PG) secretion was lowered, while Duvnjak et al. (1987) found that galactose was a better carbon source for ethanol production than glucose; however, the strains employed in both works were different.

Xylose has also been described as a suitable carbon source for ethanol production by *K. marxianus* strains (Margaritis and Bajpai 1982). In particular, Margaritis and Bajpai (1982) reported a *K. marxianus* strain able to ferment xylose with an ethanol yield of 0.28 g per gram of d-xylose, which represents 55 % of theoretical yield.

K. marxianus strains used for SSF experiments at 42–45 °C in the presence of cellulases gave good results (Abdel-Banat et al. 2010a; Ballesteros et al. 2004; Boyle et al. 1997; Gough et al. 1996; Krishna et al. 2001; Lark et al. 1997; Oliva et al. 2004; Tómas-Pejó et al. 2009). In particular, Boyle et al. (1997) described ethanol production by *K. marxianus* strain in a SSF system at 45 °C; the ethanol yields amounted to 20 g ethanol from 100 g of straw.

Several heterologous genes have been expressed in *K. marxianus* using antibiotic resistant genes or auxotrophic marker genes (Pecota and Da Silva 2005).

Hong et al. (2007) reported the expression of thermostable cellulase genes encoding EG, BGL, and cellobiohydrolase in *K. marxianus* strain, resulting in a strain able to grow in synthetic medium containing cellobiose or carboxymethylcellulose (CMC) as the single carbon source. However, this strain was not able to ferment cellulosic material (CMC), probably due to a feedback inhibition of endoglucanase activity (Bok et al. 1998; Mosolova et al. 1993) or to a cell growth inhibition caused by CM-glucose (Rasmussen et al. 1988).

Recently, Yanase et al. (2010) showed the ability of a recombinant *K. marxianus* strain to convert cellulosic materials to ethanol. This strain was genetically engineered with a *T. reesei* endoglucanase (EG) and a *A. aculeatus* β-glucosidase (BGL) displayed on the cell surface, thus being able to convert a cellulosic β-glucan to ethanol directly. The maximum production of ethanol was achieved at 48 °C with an ethanol yield of 0.47 g per gram of β-glucan consumed, corresponding to 92.2 % of the theoretical yield; while in glucose fermentation, the wild-type strain of *K. marxianus* showed the highest production of ethanol at 40 °C. The recombinant strain was also able to produce ethanol from cellobiose with a yield of 43.4 g L^{-1} ethanol from 10 % cellobiose at 45 °C. Recombinant strains of this study have higher EG activity than previous strains (Hong et al. 2007). In particular, the high-BGL activity strain completely consumed cellobiose after 12 h of fermentation between 45 °C and 48 °C. In the case of cellobiose fermentation at elevated temperatures, glucose fermentation ability seems to be very important because of its reduction at above 48 °C and high-BGL activity in yeast.

Recently, Serrat et al. (2004) studied the combination of environmental and nutritional variables on ethanol and endopolygalacturonase co-production by *K. marxianus*. The authors reported the optimal conditions of simultaneous ethanol and endopolygalacturonase production achieving an ethanol yield of 47.6 g L^{-1}, corresponding to an increase of 22 % compared to the concentration obtained in suboptimal conditions. Recently, Yuan et al. (2011) reported for *K. marxianus* a CBP strategy that integrates inulinase production, saccharification of inulin contained in Jerusalem artichoke tubers, and ethanol production from sugars achieving 71 g L^{-1} ethanol in 48 h. Moreover, they demonstrated that *K. marxianus* possesses a distinctive superiority in the thermotolerance and utilization of inulin-type oligosaccharides reserved in Jerusalem artichoke tubers in comparison to *S. cerevisiae* (Yuan et al. 2011). For instance, *K. marxianus* was able to ferment Jerusalem artichoke tubers flour without any nutrients addition, achieving 90 % of theoretical ethanol yield at 40 °C, while *S. cerevisiae* produced 79.7 % of theoretical ethanol yield at 40 °C.

Besides *S. cerevisiae* and *K. marxianus*, several other yeasts are able to produce ethanol, as reported below.

9.3.3 Other Yeasts

Some native yeasts such as *Pichia stipitis* could be used in place of *S. cerevisiae* due to its ability to utilize xylose and hexoses (Jeffries 1996). In particular, *P. stipitis* (du Preez 1986) can utilize glucose, galactose, mannose, cellulose, and xylan as carbon source to produce ethanol. *P. stipitis* is superior to all other yeast species for ethanol production from xylose and it can convert xylose to ethanol at a high yield without xylitol formation. However, this native yeast has low ethanol tolerance. *P. stipitis* genome sequence has been recently published (http://www.jgi.doe.gov/pichia) (Jeffries 2007), showing several genes coding for enzymes involved in lignocellulose conversion such as α-glucosidase, xylanase, β-mannosidase, endo-1,4-β-glucanase, and exo-1,3-β-glucosidase, but low amounts of these enzymes are produced (Jeffries 2007). *P. stipitis* mainly produces β-glucosidases which degrade cellobiose to glucose, in turn fermented to ethanol. However, the ethanol production rate from glucose is at least five times lower than that obtained with *S. cerevisiae* (Jeffries 2007; Chandrakant 1998). *P. stipitis* is able to growth in the presence of L-arabinose as carbon source, while it does not show the ability to use polymeric cellulose as a carbon source (Nigam 2002). Xylose utilization by *P. stipitis* is regulated both by end-product repression by ethanol as well as by catabolite repression by glucose. Several glycoside hydrolases have been successfully expressed in *P. stipitis* in order to increase its capability to degrade polysaccharides. For instance, endoglucanase from *C. thermocellum* was produced in *P. stipitis* (Piotek et al. 1998). Den Haan and Van Zyl (2003) enhanced the xylanolytic ability of *P. stipitis* by co-expressing both xylanase of *T. reesei* and *Aspergillus kawachii* and xylosidase of *Aspergillus niger* encoding genes. The resulting strains were shown able to grow on medium containing on medium birchwood glucuronoxylan as sole carbohydrate source.

P. stipitis has an ethanol yield in the range of 33–57 g L^{-1}, however, 30 g L^{-1} is known as a critical concentration above which cells can not grow at 30 °C (du Preez 1987; Slinger et al. 1982). Although mutant strains of *P. stipitis* with increased ethanol tolerance were recently isolated, *P. stipitis* is not a very potent ethanol producer (Watanabe et al. 2011) and its maximum ethanol productivity is around 0.9 g L^{-1}/h (Jeffries 1996).

Hansenula polymorpha (*Pichia angusta*) is a thermotolerant methylotrophic yeast able to ferment a wide range of soluble sugars such as glucose, cellobiose, and xylose to ethanol (Ryabova 2003). It is worth noting that it has an optimal growth temperature of 37 °C or even higher up to 48–50 °C (Cabeca-Silva and Madiera-Lopes 1984; van Uden 1984). Moreover, it is resistant to both metals and

oxidative stress (Blazhenko et al. 2006; Mannazzu et al. 1998 and 2000). *H. polymorpha* is more ethanol tolerant than *P. stipitis* but more susceptible than *S. cerevisiae* (Ryabova et al. 2003). Several genes have been successfully expressed in *H. polymorpha* such as a thermostable endoglucanase and endoglucanases from *A. aculeatus* and *Humicola insolens* (Müller et al. 1998; Papendieck et al. 2002).

Since *H. polymorpha* cannot metabolize starchy materials or xylans, amylolytic and xylanolytic enzymes have been expressed in this yeast. For instance, both an alpha-amylase and a glucoamylase encoding genes from the yeast *Schwanniomyces occidentalis* have been expressed in *H. polymorpha*. *xyn2* gene encoding endoxylanase from *T. reseei* has also been successfully expressed in *H. polymorpha* and the co-expression of a *β*-xylosidase encoding gene from *A. niger* and the *xyn2* gene led to the production of transformants able to grow and perform alcoholic fermentation on a minimal medium supplemented with birchwood xylan as a sole carbon source (Voronovsky 2009).

In order to increase *H. polymorpha* fermenting ability, a pyruvate decarboxylase gene (*pdc1*) from *K. lactis* was expressed, resulting in increased ethanol production from xylose. Besides that, the introduction of multiple copies of the *H. polymorpha pdc1* gene showed a 20-fold increase in pyruvate decarboxylase activity and up to a 3-fold increase of ethanol production (Ishchuk 2008).

To improve both high-temperature resistance and fermentation ability of *H. polymorpha*, strains carrying deletion of acid trehalase gene (*ath1*) and overexpressing genes coding for heat-shock proteins were constructed. The recombinant strains have up to 12-fold increased tolerance to heat-shock treatment and to 5.8-fold improvement of ethanol production from xylose at 50 °C, even if the maximum ethanol concentration achieved from xylose was only 0.9 g L^{-1} (Ishchuk 2009).

Moreover, both its ethanol yield from xylose and ethanol resistance were increased through expression of *S. cerevisiae mpr1* gene encoding N-acetyltransferase. *H. polymorpha* recombinant strains harboring 1-3 copies of the *mpr1* gene showed enhanced tolerance to L-azetidine-2-carboxylic acid and ethanol (Ishchuk 2010).

Another candidate for CBP II is the thermotolerant yeast *Issatchenkia orientalis*. The *I. orientalis* MF-121 strain is a multistress-tolerant yeast, acid tolerant, salt tolerant, ethanol tolerant, and also thermotolerant. However, this yeast does not possess any genes coding for cellulase activity. Thus, recently it has been engineered with a *β*-glucosidase gene from *A. aculeatus* (Kitagawa 2010). The transformant strain produced 29 g L^{-1} of ethanol in 72 h at 40 °C during SSF in medium containing 100 g l^{-1} of microcrystalline cellulose and a commercial cellulase. Kwon et al. (2011) have recently isolated a new *I. orientalis* strain showing high ability to tolerate lignocellulosic inhibitory compounds and able to produce ethanol with a theoretical yield of 85 % per g of glucose at 42 °C.

9.4 Potential of Bacteria as CBP Microorganisms

9.4.1 Escherichia coli

Among bacteria, *E. coli* represents the main candidate for CBP II category since it is able to metabolize a wide spectrum of sugars and it is a well-know microorganism to be genetically engineered.

As reported by Ingram et al. (1987, 1998), in wild-type *E. coli*, pyruvate is converted to formate and acetyl-CoA, the latter being subsequently reduced to acetaldehyde by an aldehyde dehydrogenase and then to ethanol by an alcohol dehydrogenase, via Embden–Meyerhof–Parnas (EMP) pathway. Both these steps require NADH and the conversion of an equal amount of acetyl-CoA to acetate to maintain redox balance. Therefore, in native *E. coli*, only half of the available pyruvate is converted to ethanol differently from other ethanologenic microorganisms, like *Z. mobilis*, which produce one mole of ethanol for every mole of pyruvate thanks to the presence of a pyruvate decarboxylase.

Many efforts have been done to improve *E. coli* ethanol production yields, firstly the expression of the *Z. mobilis* pyruvate decarboxylase, achieving the *E. coli* KO11 strain which produces an amount of ethanol comparable to that by *Z. mobilis* which produces in fact the highest ethanol yields among Gram-negative bacteria (Ohta et al. 1991a). *E. coli* KO11 is able to utilize both mannitol and glucose. For instance, when the strain was cultured in *L. japonica* hydrolysate, mainly composed of mannitol and glucose, supplemented with Luria–Bertani medium and hydrolytic enzymes, a yield of 0.4 g ethanol per g of carbohydrate was obtained (Kim et al. 1998) .

However, *E. coli* strain KO11 still requires the addition of costly enzymes, such as cellulases, hemicellulases, pectinases to degrade the biomass into fermentable monomeric sugars and it is not able to ferment cellobiose which is known to have inhibitory effects on cellulose degradation (Holtzapple et al. 1990). Edwards et al. (2011) sequentially engineered *E. coli* KO11 strain with the *Klebsiella oxytoca* cellobiose phosphotransferase genes (*casAB*), a pectate lyase (*pelE*) from *Erwinia chrysanthemi*, the Sec-dependent pathway *out* genes from *E. chrysanthemi* and the oligogalacturonide lyase (*ogl*) from *E. chrysanthemi* to produce the strains LY40A, JP07, JP07C, and JP08C, respectively, which possess significant cellobiase activity, for ethanol production from pectin-rich lignocellulosic biomass. Particularly, *E. coli* strain LY40A produced 45 % more ethanol from model sugars than KO11, due to its capability to ferment cellobiose. JP07, JP07C, and JP08C produced concentrations of ethanol that were higher than those produced by KO11 (with an increase of around 15 %) but lower than those produced by LY40A.

The ethanologenic recombinant *E. coli* strain (FBR5) was created with the goal to produce ethanol from both hexoses and pentoses sugars, generated from pretreatment and hydrolysis of lignocellulosic biomass, such as glucose, xylose,513 arabinose, and galactose. The bacterium is stable without antibiotics and can tolerate ethanol up to 50 g L^{-1}, with a yield of around 45 g ethanol per L (Saha

and Cotta 2012) For instance, it has been shown to produce 40–50 g L^{-1} ethanol from 100 g L^{-1} xylose in batch reactors (Qureshi et al. 2012).

Several hydrolytic enzymes have been cloned into *E. coli* ethanologenic strains, but the main obstacle to an effective conversion of cellulose and hemicellulose biomass into fermentable sugars is the limited ability of the microorganism to secrete proteins which results in the accumulation of recombinant enzyme in the periplasmatic area.

However, most of the available studies so far reported describe the use of *E. coli* only as a suitable host of cellulase coding genes from different sources, for cellulase overproduction and characterization.

Among hydrolytic enzymes cloned in *E. coli*, a family 45 glycoside hydrolase from *Fibrobacter succinogenes* S85 (Park et al. 2007), CelC from *Salmonella typhimurium* UR (Yoo et al. 2004), Cel48Y, a noncellulosomic family 48 cellulase from *Clostridium thermocellum* (Berger et al. 2007) have been successfully produced.

Seven different bacterial genes, all producing cell-associated endoglucanase activity were expressed in *E.coli* KO11 strain by Wood et al. (1997). They demonstrated that *E. coli* KO11 can be used to produce recombinant endoglucanase as a co-product with ethanol. A production of 3,200 IU of recombinant *E. chrysanthemi* EGZ/L endoglucanase, equivalent to over 13 % of the commercial endoglucanase activity added to ferment crystalline cellulose, was achieved, demonstrating a new approach to reduce the amount of fungal cellulase required for the conversion of cellulose into ethanol. Amore et al. (2012) cloned a GH family 12 cellulase from a *Streptomyces* sp. G12 strain into *E. coli*, while recently, the first report on the cloning and expression of a GH family 43 β-xylosidase gene from thermophilic fungi in *E. coli* has been published by Teng et al. (2011) who cloned and extracellularly expressed a novel β-xylosidase gene (designated as PtXyl43) from thermophilic fungus *Paecilomyces thermophila.*

The secretion of the soluble cellulases/hemicellulases is the major prerequisite for CBP, thus many efforts have been done to increase secretion level of recombinant glycosyl hydrolases in *E. coli*.

Cellulase from *Bacillus subtilis* was cloned into *E. coli* and detectable extracellular secretion was achieved due to the presence of the signal peptide of the *B. subtilis* cellulase that might have specificity toward native protein secretion system in *E. coli* (Hinchliffe et al. 1984), while Lam et al. (1997) demonstrated an efficient secretion of an exoglucanase from *Cellulomonas fimi,* when fused to *ompA* sequence and expressed under a weak promoter (PlacUV5). Zhou et al. (1999) enabled *E. coli* to secrete more than 50 % of the recombinant Cel5Z from *E. chrysanthemi* by reconstructing the type II secretion system, encoded by the genes from *E. chrysanthemi*. In fact, at least three different types of protein secretion systems have been identified in Gram-negative bacteria, type II being the most widely used for protein secretion (la Grange et al. 2010).

Despite the extensive research performed to optimize *E. coli* recombinant cellulase production and reduce the addition of external cellulases, the recombinant strains so far obtained are still unable to directly grow on lignocellulosic

biomass, mainly due to the complex cocktail of enzyme needed for the complete lignocelluloses conversion into fermentable sugars. As a consequence, co-culture strategies have also been developed, to avoid the expression of multiple heterologous genes within a single cell.

For instance, Bokinsky et al. (2011) obtained the conversion of pretreated switchgrass through a co-culture consisting of *E. coli* strains capable of expressing either the cellulolytic or hemicellulolytic enzyme complexes, while Singh et al. (1998) developed a binary culture for efficient xylan conversion into fermentable sugars with two strains of *E. coli,* expressing a different combination of the six major xylanases.

However, the lack of a complete knowledge of the mechanism of glycosyl hydrolases limits the potential of *E. coli* as CBP II category microorganism. Metagenomic libraries represent an important tool for isolation of new genes which can be properly expressed in *E. coli,* thus overcoming the difficulty in design of suitable cellulase/hemicellulase expression system.

9.4.2 Zymomonas mobilis

Zymomonas mobilis is well known for its remarkably high ethanol production rate and tolerance to the toxicity of the final product (Lee 1980). It is able to ferment sugars at low pH and shows high resistance to the inhibitors produced during lignocellulosic pretreatment. Differently from *E. coli*, it ferments only glucose and fructose through the Entner–Doudoroff (ED) pathway but it has been successfully engineered to ferment also xylose and arabinose, by introduction of seven different genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transaldolase, and transketolase (Zhang 1995a, 1995b; Deanda 1996).

Several strains of *Z. mobilis* possess extracellular glycosyl hydrolases active toward carboxymethylcellulose, suggesting the potential of *Z. mobilis* as a suitable host for cellulase recombinant expression.

Many manuscripts about ethanol production by SSF of *Z. mobilis* have been reported (Soleimani et al. 2012; Eklund and Zacchi 1995; Park et al. 1993; Yamada et al. 2002). Soleimani et al. (2012) reported an ethanol production of around 14.49 g L^{-1} (w/v) in the basal medium, which increased up to 86.26 g L^{-1} (v/v) in optimized nutritional conditions.

Few examples of cellulase recombinant expression in *Z. mobilis* are available. For instance, *Erwinia chrysanthemi cel5Z* was successfully expressed with a yield of 1,000 IU/L (Brestic-Goachet et al. (1989); *Acetobacter xylinum* cellulase gene and *Bacillus subtilis* endo-β-1,4-glucanase gene transfer into *Z. mobilis* has been reported by Okamoto et al. (1994) and Yoon et al. (2007), respectively. In most of the cases, the recombinant endoglucanases could not be secreted, mainly because of the protective outer membrane.

Expression of multiple cellulolytic enzymes is required for biomass conversion. Thus, Linger et al. (2010) expressed two cellulolytic enzymes, E1 and GH12 from *Acidothermus cellulolyticus*, in *Z. mobilis*. However, the lack of a secretion signal in their genes resulted in the localization of 96 % of GH12 activity within the cytoplasm, which decreased to 26 % when the *phoC* secretion signal was added.

9.4.3 Other Bacteria

Together with *E. coli*, *Klebsiella oxytoca* is one of the main promising Gram-negative bacteria for CBP II category. It is able to metabolize a wide spread of monomeric sugars present in the lignocellulose and it is known to possess four fermentative pathways: the pyruvate formate-lyase pathway, the lactic acid pathway, the succinate pathway, and the butanediol pathway (Ohta et al. 1991b).

K. oxytoca strain M5A1 has been engineered by introducing *Z. mobilis* genes for ethanol production, thus achieving the strain P2 able to utilize cellobiose and cellothriose for ethanol production. Maximum theoretical yields from glycolysis and fermentation were 0.51 g of ethanol per g of glucose, 0.536 g of ethanol per g of cellobiose, and 0.56 g of ethanol per g of cellulose (Wood and Ingram 1992).

It has been shown that the time taken by *K. oxytoca* P2 to produce up to about 33 g L^{-1} ethanol was much less than for any other organism investigated, including ethanol-tolerant strains of *Saccharomyces pastorianus*, *K. marxianus,* and *Z. mobilis*. However, *K. oxytoca* produces slightly less ethanol (maximum 36 g L^{-1}) than these organisms, reflecting its lower ethanol tolerance. Thus, co-cultures of *K. oxytoca* P2 with *S. pastorianus*, *K. marxianus,* or *Z. mobilis* have been developed demonstrating that the combination of *K. Oxytoca* P2 with thermotolerant yeasts or *Z. mobilis* is a good system for SSF (Golias et al. 2002).

Several cellulases have been expressed in *K. oxyotica* strains, in order to reduce the amount of commercial cellulase to be used in SSF. As a first attempt, a thermostable cellulase gene from *Clostridium thermocellum* has been expressed in *K. oxyotica* P2 strain by Wood and Ingram (1992), resulting in the hydrolysis of amorphous cellulose into cellobiose.

To improve *K. oxyotica* P2 strain SSF ability, Zouh et al. (2001) expressed *Erwinia chrysanthemi* genes *celY* and *celZ* encoding endoglucanases. Both were secreted with a total production level of 20,000 Units of CMCase activity per liter. Moreover, an increase in ethanol production up to 22 % more than the unmodified strain was achieved.

The *Z. mobilis* ethanol fermenting genes *pdc* and *adh* II were also cloned into three facultative anaerobic, Gram-negative cellulolytic bacteria, namely *Enterobacter cloacae* JV, *Proteus mirabilis* JV, and *Erwinia chrysanthemi* (Piriya et al. 2012). The latter was shown to be the best system for ethanol production in SSF experiments, due to both its higher tolerance to ethanol and its higher cellulase production.

Bacillus subtilis represents another candidate for CBP, being well known from the metabolic, biochemical, and physiological points of view. Moreover, it possesses polysaccharides degrading enzymes and it is able to produce secretory proteins. Romero et al. (2007) have produced an ethanologenic *B. subtilis* (strain BS36) through homologous recombination, disrupting the native *ldh* gene by chromosomal insertion of *pdc* and *adhB* genes from *Z. mobilis*, put under the control of the *ldh* native promoter, and inactivating the *alsS* gene involved in butanediol production.

9.5 Conclusions

This chapter discusses the advantages of consolidated bioprocessing as an alternative strategy to simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) techniques for reducing the cost of cellulosic ethanol production.

Both yeasts and bacteria are good candidate for CBP type II. However, the limited ability of these microorganisms to produce cellulolytic enzymes in sufficient quantity and quality for lignocellulose degradation is the main drawback in CBP type II process, thus pushing the interest toward filamentous fungi as CBP type I organisms, due to the ability of fungi to produce high amounts of cellulolytic enzymes. Development of new genetic tools for engineering filamentous fungi is needed to increase the ethanol yield and titers which are still low, mainly due to by-product formation and the slow rates of fermentation.

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