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Preface

In line with the current focus on a sustainable economy, bioethanol and other biofuels have received tremendous attention, making many headlines. Being produced in steadily growing volumes has made it necessary to consider production of biofuels from renewable raw materials that are not currently used. Therefore, the production of biofuels is at the gateway of moving from traditional raw materials to others such as lignocellulosic materials. However, such a transfer requires new production processes that are economically feasible. This volume addresses and discusses the current status of biofuels, covering aspects from enabling technologies to different technology and processes options, as well as economical and policy perspectives. It represents a timely and comprehensive overview.

In itself, the field bioethanol and other biofuels receive great current interest; however, development in this area will also pave the way for a breakthrough within industrial biotechnology (defined as the application of biotechnology for the processing and production of chemicals, material, and energy). The technology development driven by the interest in biofuels will lead to experiences most valuable for introduction of other industrial biotechnology processes. In parallel, scientific developments in the post-genomic era and achievements in systems biology will allow the necessary development and fine-tuning of the biological catalyst. In light of this, the development of biofuel processes, presented in this volume, can be seen in a much larger context.

I want to sincerely thank all authors that have contributed to the volume for their dedicated effort and their excellent contribution. I hope that you as a reader will enjoy the volume.

Kongens Lyngby, August 2007 Lisbeth Olsson

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Fueling Industrial Biotechnology Growth with Bioethanol

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Abstract Industrial biotechnology is the conversion of biomass via biocatalysis, microbial fermentation, or cell culture to produce chemicals, materials, and/or energy. Industrial biotechnology processes aim to be cost-competitive, environmentally favorable, and self-sustaining compared to their petrochemical equivalents. Common to all processes for the production of energy, commodity, added value, or fine chemicals is that raw materials comprise the most significant cost fraction, particularly as operating efficiencies increase through practice and improving technologies. Today, crude petroleum represents the dominant raw material for the energy and chemical sectors worldwide. Within the last 5 years petroleum prices, stability, and supply have increased, decreased, and been threatened, respectively, driving a renewed interest across academic, government, and corporate centers to utilize biomass as an alternative raw material. Specifically, bio-based ethanol as an alternative biofuel has emerged as the single largest biotechnology commodity, with close to 46 billion L produced worldwide in 2005. Bioethanol is a leading example of how systems biology tools have significantly enhanced metabolic engineering, inverse metabolic engineering, and protein and enzyme engineering strategies. This enhancement stems from method development for measurement, analysis, and data integration of functional genomics, including the transcriptome, proteome, metabolome,

and fluxome. This review will show that future industrial biotechnology process development will benefit tremendously from the precedent set by bioethanol – that enabling technologies (e.g., systems biology tools) coupled with favorable economic and sociopolitical driving forces do yield profitable, sustainable, and environmentally responsible processes. Biofuel will continue to be the keystone of any industrial biotechnology-based economy whereby biorefineries leverage common raw materials and unit operations to integrate diverse processes to produce demand-driven product portfolios.

Keywords Bioethanol · Biofuels · Biorefinery · Metabolic engineering · Systems biology

1 Introduction

1.1 Industrial Biotechnology

The term "industrial biotechnology" first widely appeared in the literature in the early 1980s when genetic engineering, propelled by recombinant DNA technology, was searching for applications beyond health care and medical biotechnology [1, 2]. Today, industrial biotechnology represents a well-defined field with strong academic, government, and corporate representation. Formally, industrial biotechnology is the bioconversion, either via microbial fermentation, cell culture, or biocatalysis, of organic raw materials extracted from biomass or their derivatives to chemicals, materials, and/or energy. Biomass is the result of photosynthetic carbon fixation by plants to form organic polymers that may be enzymatically or chemically digested to carbohydrate, protein, and lipid monomers. Industrial biotechnology, often referred to as white biotechnology [3], aims to provide cost-competitive, environmentally friendly, self-sufficient alternatives to existing or newly proposed petrochemical processes. Processes that exploit industrial biotechnology have recently garnered increasing global attention with traditional petrochemical processing under scrutiny due to increasing raw material costs, environmental constraints, and decreasing self-sufficiency.

Industrial biotechnology has experienced unprecedented growth with bio-based production processes representing 5% of the total chemical production sales volume. By 2010, several studies have estimated that the total fraction will increase to 20%, representing $310 billion of a projected total sales volume of $1600 billion. Industrial biotechnology will continue to capture significant sales volume percentages in the arenas of basic chemicals and commodities (2 to 15%), specialty or added-value chemicals (2 to 20%), and polymers (1 to 15%). However, the greatest percentage gain is likely to occur in the fine chemical market (16 to 60%), where industrial biotechnology platforms enable complex chemistries that are presently produced via synthetic or combinatorial routes [4]. Furthermore, indus-

trial biotechnology is enabling new products, particularly novel therapeutic agents such as polyketides and specialty chemicals not previously identified, such as the diverse polyunsaturated fatty acids and biopolymers produced by microalgae [5].

The significant increases in fundamental research and development, and commercialization at industrial scales of biotechnological processes may be attributed to several key observations. These observations may be classified and discussed in the context of four broader themes:

1. Petroleum economics in terms of raw material price, stability, and availability
2. Significant technical and scientific achievement within the fields of enzyme/protein engineering, metabolic engineering, systems biology, process life-cycle analysis, and process integration
3. Environmental awareness and preservation
4. National energy self-sufficiency and security

Within each of these categories there have been several identifiable and quantitative drivers fueling the application of industrial biotechnology to processes previously exclusive to the petrochemical industry.

Figure 1 presents four categories that any industrial biotechnology process must consider and evaluate prior to development. These areas include process economics, environmental impact, public perception and policy support, and sustainability and self-sufficiency. Figure 2 provides a more focused schematic overview of how modern industrial biotechnology process development has evolved with the integration of *x-ome* data.

This review aims to support the hypothesis that industrial biotechnology has benefited from bio-based ethanol production, and that fundamental tools developed previously, but applied in bioethanol development will be applied to future processes. Bioethanol in many cases has served as an industrial proof-of-concept for many biotechnology approaches. In particular, the tools and analysis developed are enabling the vision of a future biorefinery – an integrated process platform that converts biomass-derived feed streams to a diversified portfolio of product streams, adjusted according to market demands – to become a reality. Similar to the existing model of a petrochemical refinery, biorefineries will allocate renewable and sustainable raw materials to a diverse array of products, produced by environmentally favorable and cost-effective bioconversions.

1.1.1 Market Drivers

The four categories referred to in Fig. 1 are highly interconnected, and there is significant debate as to the ranking of these categories in terms of priority and impact. Process economics are often estimated by quantitative modeling that

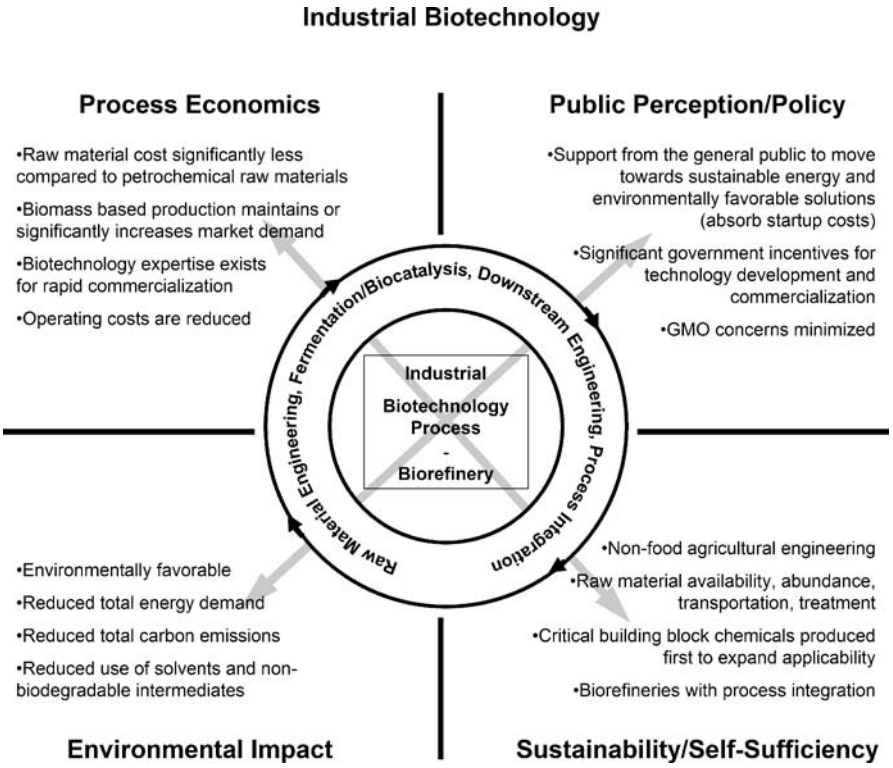


Fig. 1 Industrial Biotechnology. There are many sectors that drive an industrial biotechnology process. The figure schematically depicts the different sectors, and emphasizes the high degree of connectivity and influence that one sector can have on any other. At the core of industrial biotechnology and biorefineries is the technology that enables these processes, such as raw material engineering, fermentation/biocatalysis, downstream engineering, and process integration. Assuming that the technology is robust and mature enough to support process development, then commercialization is dependent upon clearly defining process economics, environmental impact, degree of sustainability/self-sufficiency, and the public perception and policy surrounding the process. Although weighting is not assigned to any one sector as this can depend on many factors, in general, it is expected that within each sector there will be significant driving forces present to make the industrial biotechnology process preferred to the petrochemical equivalent. By no means are the examples listed within each sector exhaustive

includes major process costs (both operating and capital) and process value, dictated by the product's estimated market price, demand growth rate, market share, and any competitive advantages that may exist. For commercialization it is reasonable to assume that process economics must be favorable before any further effort can continue on considering and improving public perception and environmental impact. Sustainability and self-sufficiency are perhaps less well defined, noting that many of the issues considered, in-

cluding raw material availability and the potential for process integration into a biorefinery, are likely to be discussed in the context of process economics. It is included in Fig. 1 and discussed as a separate category simply because of the recent focus it has received in the background of significantly increasing petroleum and feedstock prices. Self-sufficiency and sustainability may actually trump process economics in cases where issues such as national security play a role, for example dependence on a foreign state for significant fractions of energy.

We will aim to provide a brief overview of the four major categories (see Fig. 1), with particular attention paid to the economic drivers propelling bioethanol development. In fact, we will demonstrate that those economic drivers are not exclusive to bioethanol, but rather, serve as catalysts for the petrochemical industry's rapid adoption of industrial biotechnology as a platform for producing existing and future products.

1.1.2 Industrial Systems Biology

Functional genomics is the quantitative collection, analysis, and integration of whole genome scale data sets that enable biologically relevant and often predictive mathematical models to be constructed. With genome sequences becoming readily available for production organisms, bioethanol process development has been a benefactor of the scientific achievements in functional genomics, particularly in the areas of transcriptome analysis, proteomics, and fluxomics. Such developments today encompass a systems biology toolbox that may be further exploited for bioethanol and other industrial biotechnology processes.

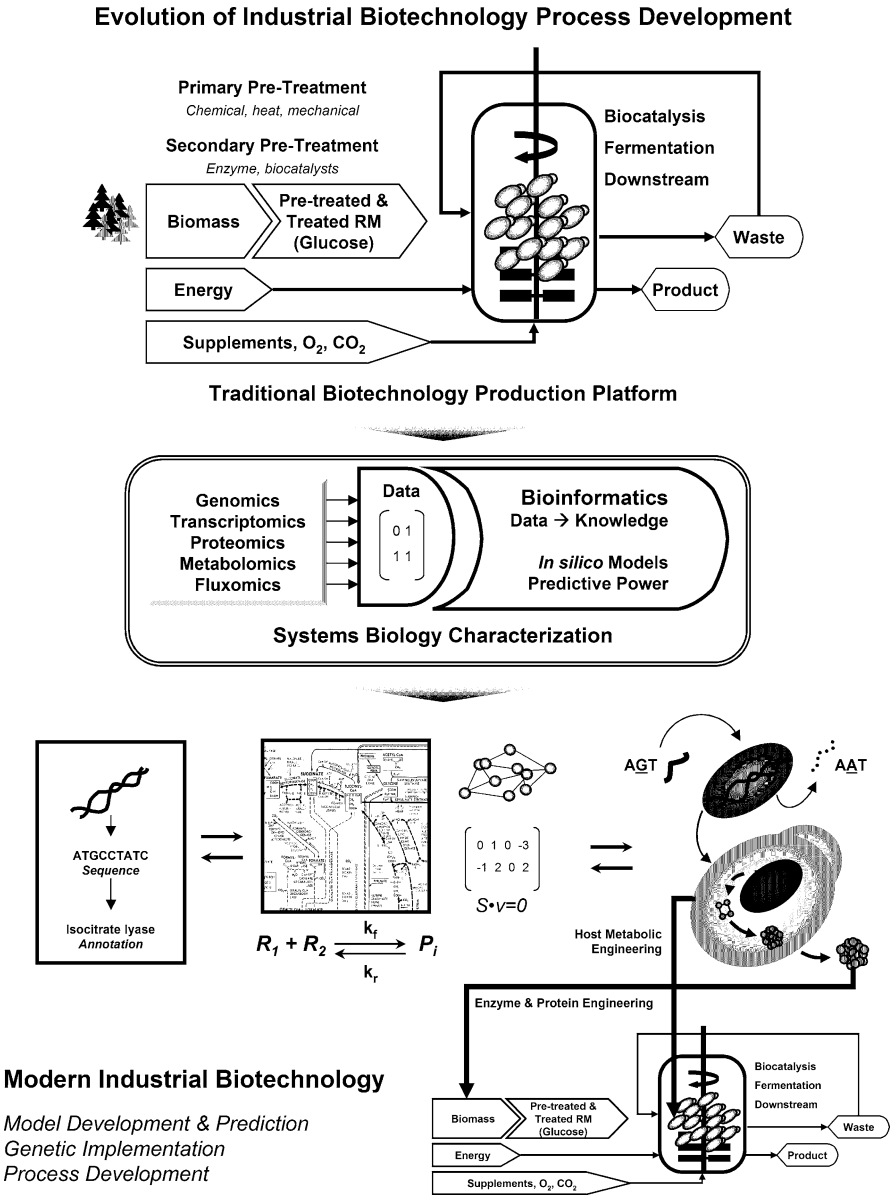
This volume will present a diverse collection of technical contributions that aim to provide insight and an update to the state of the art in bioethanol biotechnology. This introductory chapter and the subsequent chapters will be focused on the upstream bioprocess developments and challenges. Topics will include pretreatment of lignocelluloses, enzymatic hydrolysis, enzyme engineering, and metabolic engineering of production hosts including *S. cerevisiae*, *Pichia stipitis*, and *Zymomonas mobilis*. The examples cited in this chapter of systems biology tools will draw examples from numerous fermentation organisms; however, with focus on *S. cerevisiae*.

S. cerevisiae today is the preferred bioethanol production host primarily as a result of proven industrial process robustness and exceptional physiological and *x-omics* characterization [6–9]. The *S. cerevisiae* genome sequence, consisting of 6604 total open reading frames (4437 verified; 1343 uncharacterized; 834 dubious) [10], was first made publicly available in 1996 largely through André Goffeau's coordination of the European yeast research community [11]. Soon thereafter, in 1997 and 1998, the first cDNA spotted microarray exploring metabolic gene regulation, and the first commercial

Fig. 2 Evolution of Industrial Biotechnology Process Development. A modern industrial biotechnology process is composed of five major unit operations: raw material treatment, biocatalysis, fermentation, downstream engineering, and process integration. Raw material sources must first be selected based on a set of criteria that includes overall energy balance, availability, abundance, transportation costs, sustainability, self-sufficiency, and associated agricultural costs. Raw materials are then fed through primary and/or secondary treatment unit operations. Primary treatment may include chemical, heat, and/or mechanical force for hydrolysis of lignocelluloses and other relevant biomass materials. Secondary treatment will often include biocatalysis and enzyme treatment to further hydrolyze to monosaccharides. Monosaccharides are then converted to an end-of-fermentation product that is further converted and purified via downstream processing. Throughout this process there are numerous opportunities for energy, water, and intermediate recycling, along with waste management. These various operations are often lumped together and referred to as process integration. The traditional biotechnology platform, prior to the availability of genome sequences for many production organisms, was based on traditional random mutagenesis, selection, and conventional biochemical engineering solutions. This typically included selection of a high-producing host and then phenomenological optimization of fermentation processes. However, *x-ome* scale characterization and subsequent bioinformatics has enabled predictive solutions to be engineered not only to production organisms, but has also impacted biomass/biofuel crop engineering and enzyme and biocatalyst engineering. This improved approach enables a higher experimental probability of success through in silico prediction. The result is a modern platform for industrial biotechnology process development ►

platform (Affymetrix) microarray data exploring mitotic cell regulation were reported, respectively [12, 13]. The genome sequence coupled with extensive annotation based on fundamental biochemistry, peer-review literature, and available transcription data enabled publication of the first genome-scale metabolic model for *S. cerevisiae* in 2003 [14]. The genome-scale metabolic model represents an integration of extensive amounts of data into an annotated, defined, and uniform format permitting simulations of engineered genotypes to elicit desired phenotypes [14, 15].

Strain development has classically been dominated by random mutagenesis, largely by chemical mutagens and radiation, of a production host followed by screening and selection in controlled environments for a desired phenotype. Although this methodology has endured tremendous success, particularly in the areas of amino acid production (L-glutamate, L-lysine) [16–19], antibiotics (penicillin) [20, 21], and vitamins (L-ascorbic acid) [22], it has largely been end-product driven with minimal mechanistic understanding. Today, with the exponential increase in genome sequences of existing and future production hosts, coupled with tools from bioinformatics that enable integration and interrogation of *x-omic* data sets, it is possible to identify high-probability, targeted, genetic strategies to increase yield, titer, and/or productivity [23–25]. It is also now possible to perform inverse metabolic engineering, where previously successful production systems may be *x-omically* characterized to elucidate key metabolic path-



ways and control points for future rounds of targeted metabolic engineering [26]. In both *forward* and *reverse* metabolic engineering, systems level models and simulations are accelerating bio-based process development, resulting in reduced time to commercialization with significantly less resource commitment.

1.1.3
Perspectives on Biorefinery Development

Today, industrial biotechnologists are no longer discussing whether a single product can be produced via biotechnology, but are rather considering diverse portfolios of products leveraging expertise and resources. The various systems biology toolboxes applied to bioethanol production are now being exploited to develop integrated processes that will form "biorefineries". The concept of the biorefinery was first defined in 1999, when it was postulated that lignocellulosic raw materials may be converted to numerous biocommodities via integrated unit processes, and offer competitive performance to traditional petrochemical refineries [27]. Several chapters in this volume will more closely examine the biorefinery as a model and platform for future bio-based processes in terms of policy issues and process integration.

If the biorefinery platform model is to evolve from academic conception to industrial reality it will require two key components. First, the economic and socio-political landscape must support and warrant the significant financial investment, favorable legislative policy, and consumer-driven demand that will be required. Second, the advances and tools developed within systems biology for metabolic engineering must be successfully applied in commercial environments. Bioethanol is the first industrial biotechnology product to demonstrate that if these two elements are co-supported, then numerous bio-based processes can be developed and integrated into a biomass economy.

2
Market Drivers

2.1
Raw Material Drivers: Petroleum Economics

According to the *Chemical & Engineering News* survey of the top 50 chemical manufacturing corporations worldwide, including traditional petroleum companies with a chemical manufacturing business segment, the combined total sales in 2005 was $665.6 billion, representing a 15% increase since 2004. The global scope of the sector is reaffirmed by the distribution of total sales across the USA, Europe, and Japan, of 29.3% ($195.3 billion), 45.1% ($300.5 billion), and 12.8% ($86.9 billion), respectively, representing the top three geographical chemical sectors. For the previous 5 years (2000–2004), Dow Chemical (Midland, MI, USA), BASF (Ludwigshafen, Germany), and DuPont (Wilmington, DE, USA) have been the three largest chemical companies (based on total sales); however, in 2005 DuPont was reduced to the sixth position with Royal Dutch Shell (The Hague, The Netherlands), ExxonMo-

bil (Iving, TX, USA), and TOTAL (Courbevoie, France) moving into the third, fourth, and fifth positions, respectively [28]. The fact that these three companies are traditional petroleum refiners is a direct reflection of the increasing crude petroleum prices resulting in significant price increases in chemical raw material feedstocks. For example, a 2005 corporate press release from DuPont noted the impact that increasing raw material prices have on the chemical industry:

Higher crude oil prices have a global impact on fuel and feedstock costs. Higher prices for natural gas have an especially severe impact on costs for the North American chemical industry, which is highly dependent on natural gas as feedstock, while the rest of the world relies more heavily on oil derivatives. A $10 increase in the price of a barrel of oil increases variable costs to the US chemical industry by about $2.6 billion per year. (This includes fuel, power, and feedstock costs.) For natural gas a $1 increase per mmbtu increases variable costs to the US chemical industry by $3.7 billion per year [29]

Dow Chemical and BASF were able to retain their top positions in large part to the fact that they have a substantial hydrocarbon and energy business sector (i.e., Dow is world leader in olefins and aromatics), and a major oil and gas production business sector, respectively, complementing their chemical business under increasing crude petroleum prices [30, 31].

Between January 1978 and January 2001, the average world cost of petroleum increased 69% from $13/bbl[1] to $22/bbl, while between January 2001 and January 2006, the cost increased 150% from $22/bbl to $55/bbl [32]. Significant increases in crude petroleum prices were fueled by increased energy demands, and limitations or threats to supply. Global energy consumption is projected to increase by 57% from 435 trillion MJ in 2002 to 681 trillion MJ in 2025 [33]. Global petroleum demand in 2004 was 82 million bbl/day, with a projected increase to 111 million bbl/day in 2025 [34]. Furthermore, energy demands will continue to increase significantly as the emerging economies of China, India, and Russia continue to rapidly expand, reporting 2005 GDP growth rates of 9.9, 7.6, and 6.4%, respectively, compared to the average world GDP growth rate of 4.7% [35].

Is the recent price increase in crude petroleum fueling the renewed interest in industrial biotechnology as a means of developing sustainable, cost-effective, and environmentally favorable processes? Numerous peer-review literature reports have appeared outlining the role that industrial biotechnology can likely serve to exploit the benefits previously mentioned [5, 36–38]. However, perhaps more convincing, is a review of the 2005 corporate annual reports of the six largest petrochemical manufacturing companies, where five of the six clearly note that increasing prices of raw materials and feedstocks have required industrial biotechnology to become a core position of their

[1] The unit *bbl* is an abbreviation for barrel, a common unit of measurement for petroleum, equivalent to 42 US gallons or approximately 159 L.

business units (quoted directly from the 2005 annual report of the indicated company):

- Royal Dutch Shell: *Shell is the world's largest marketer of biofuels and a leading developer of advanced biofuels technologies. During 2005, we entered a partnership with CHOREN Industries GmbH which will work towards the construction of the world's first commercial facility to convert biomass into high quality synthetic biofuel. This is in addition to our existing partnership with Iogen which is producing cellulose ethanol in Canada from plant waste. We are now working with Iogen and Volkswagen on a joint study to assess the economic feasibility of producing cellulose ethanol in Germany. These advanced biofuels can be used in today's cars and can cut carbon dioxide emissions by* 90% *compared with conventional fuels* [39].
- DuPont: *In 2005, DuPont announced the creation of its newest Technology Platform, DuPont Bio-Based Materials. However, the first revolutionary products have already entered the marketplace. DuPont™ Sorona® is an innovative new polymer made with 1,3-propanediol (PDO). While PDO is currently made using a petroleum-based process, DuPont developed a way to make PDO from corn – a renewable resource – instead of petroleum. DuPont partnered with UK-based Tate & Lyle PLC to build the world's largest aerobic fermentation facility to produce Bio-PDO™ from corn. The facility, under construction in Loudon, Tennessee, will begin operation in late 2006. Production of Bio-PDO™ will consume 30 to 40 percent less energy per pound than petroleum-based PDO. So the production of 100 million pounds of bio-based material at the Loudon plant will save the equivalent of 10 million gallons of gasoline annually* [40].
- Dow Chemical Company: *2005 sales rose to a new high of $46.3 billion; however, feedstock and energy costs were approximately $4 billion – representing almost* 10% *of total sales* [30].
- BASF: *We have combined the important technology-driven issues of the future in five growth clusters: energy management, raw material change, nanotechnology, plant biotechnology and white (industrial biotechnology)… By expanding white biotechnology, we aim to use our expertise in the areas of enzyme catalysis and fermentative manufacturing processes to develop new products and processes outside the current key areas of fine chemicals and intermediates* [31].
- TOTAL: *TOTAL's efforts to expand its activities in the field of renewable energies are in line with our desire to prepare for the future of energy and to foster sustainable development. As regards biofuels, TOTAL already produces* 170 000 *metric tons per year of ETBE and our long-term diester supply contracts will be increasing strongly in the coming years, in conjunction with the food-processing industry. The Group is also launching research programs on second-generation biofuels. Within a few years, in-*

dustry should be able to produce biofuels from a wide variety of biomass sources [41].

In the United States in 2004, approximately 6–8% of all petroleum supplies were consumed by the chemical industry for manufacturing, primarily in the form of natural gas, naptha, and refinery gases (ethane, propane, and butane). The largest consumer of petroleum, as a sector, are transportation fuels forcing chemical companies to compete for refined petroleum products. Although academic centers and government research offices have long advocated and supported research in industrial biotechnology, commercialization will only be possible with the financial commitment of industry, beginning with the largest petrochemical manufacturing companies.

When a new industrial process is brought online, irrespective of the product, scale, or technology, there are inefficiencies and short-term operating and capital costs associated with start-up. As the process matures, and efficiencies are gained or introduced via subsequent process upgrades, the raw material cost as a percentage of the total operating cost increases across the life-time of the product, until it represents the largest cost fraction. This is particularly true for commodities and large volume products where profit margins are generally much lower relative to fine chemicals or pharmaceuticals. Hence, fine chemicals and pharmaceuticals have witnessed the largest penetration of industrial biotechnology [4, 5]. Therefore, the decision to develop bio-based process alternatives is highly dependent on the raw material cost, which in most cases is petroleum vs. biomass, either on a per mass or energy unit basis.

Petroleum products refined from crude oil are generally classified into three primary categories: (1) transportation fuels, (2) finished non-fuel products, and (3) feed stocks for further petrochemical processing [32]. In 2005 more than 75% of all petroleum was converted and sold as fuel, either in the form of gasoline (57.9%) or diesel (24.3%). Of this 75%, approximately 66% is dedicated towards transportation fuels. Interestingly, the average total refiner volume of petroleum products sold to end users in the USA between 2001 and 2005 saw a relatively small increase ($\sim$ 5.2%). However, the price of motor gasoline during the same time period increased 77.3%, confirming that demand continues to outpace supply [34]. It is no surprise therefore that transportation fuels have been the largest focus for commodity industrial biotechnology.

2.2 Transportation Fuel Alternatives – Bioethanol

Bioethanol was introduced into the transportation fuel supply chain as early as the 1970s with the introduction of the PROÁLCOOL program by the Brazilian government in an original effort to stabilize the international price of

sugarcane, which was highly sensitive to subsidies by other domestic producers. In 1979, the Brazilian government strengthened the program by sponsoring development of a fleet of ethanol-fueled vehicles [42]. Although the history of bioethanol in Brazil is quite tumultuous with significant government sponsorship, tax incentives, and subsidies, Brazil has emerged as the second largest producer of bioethanol (4.3 billion gallons/year in 2005) requiring 25% ethanol blends in transportation fuel, and has become energy self-sufficient by supplementing internal petroleum supplies and refining capacity with bioethanol production [43]. In 2005, total Brazilian petroleum production was estimated at 2 million bbl/day with consumption estimated at 1.6 million bbl/day, in contrast to the USA which produced 7.6 billion bbl/day, yet consumed 20 billion bbl/day [35].

Bioethanol may serve both as an additive or complete replacement for petroleum-derived transportation fuels, particularly gasoline in spark ignition (SI) engines. The volumetric energy fraction of ethanol is approximately 66% that of gasoline, suggesting a one-third reduction in the total kilometers per volume of ethanol consumed. However, review of the comparative physical chemistry data provides insight into why ethanol combustion results in a 15% higher efficiency [44]. Ethanol (C_2H_5OH, 34.7 wt % oxygen) is a partially oxidized fuel compared to gasoline (C_4-C_{12}, 0 wt % oxygen), resulting in a lower stoichiometric air-to-fuel ratio. Therefore, a larger mass or volume of ethanol compared to gasoline is required to yield the same caloric output from combustion. However, ethanol also has a higher octane number, permitting the fuel to be burned at a higher compression ratio (defined as the ratio of the volume between the piston and cylinder head before and after a compression stroke). A higher compression ratio results in higher power output, efficiency, and consequently favorable fuel economy [45]. Compared to gasoline, there is only a 20–25% reduction in kilometer efficiency [44]. Furthermore, as a result of the significantly higher latent heat of vaporization for ethanol (1177 kJ/kg compared to 348 kJ/kg at 60 °C) there is an effective engine cooling and leaner operation. This leads to significant reductions in $CO_{(g)}$ and $NO_{x,(g)}$ emissions, with 85% ethanol blends of gasoline (referred to as E-85) yielding $NO_{x,(g)}$ emission reductions of 20% compared to pure gasoline. However, the emission of reactive aldehydes, including acetaldehyde and formaldehyde, is increased [46, 47]. Several studies on the effect of ethanol–gasoline blends (up to 60% ethanol) on engine performance and exhaust emissions have suggested that proper fine tuning of engine parameters can lead to excellent performance with significantly reduced hydrocarbon and $CO_{(g)}$ emissions [46–48].

In 1990 the USA enacted the Clear Air Act Amendments, mandating that oxygenated additives (methyl-tertiary-butyl ether, MTBE; ethyl-tertiary-butyl ester, ETBE; or ethanol) be included at 2 wt % oxygen to decrease hazardous emissions. In 1999, 21 million tons of MTBE were produced globally, primarily in the USA, although Europe produced approximately 3.3 million tons.

In the USA, it is among the most frequently found groundwater contaminants with over 400 000 underground storage tanks identified to be leaking by the US Environmental Protection Agency (EPA) since 1988 [49]. Although there is still debate in the public health community as to the toxicity level and health risk that MTBE human consumption poses, a number of US states have banned the use of MTBE as a fuel additive. Furthermore, many European nations, including the UK, Germany, and Switzerland have identified MTBE-contaminated sites and are transitioning to ethanol enrichment [50, 51]. As a result, ethanol has been the favored fuel additive for increasing oxygenation.

In August 2005, the Energy Policy Act (EPACT) was enacted into US law creating the national Renewable Fuels Standard (RFS). The RFS calls for 15.1 billion L of renewable fuels (primarily ethanol but may include alternative fuels such as biodiesel) to be used by 2006, increasing by 2.6 billion L/year until 2011 when a final volume of 28.4 billion L will be called for in 2012 [43].

The USA is neither alone nor first with actively passing legislation that requires and promotes the integration of biofuels into the transportation economy. As previously discussed, Brazil presently requires a 25% ethanol blend of all gasoline, and continues to provide preferential tax treatment for ethanol producers and consumers. Argentina is requiring a 5% ethanol blend over the next 5 years. Thailand requires that all gasoline sold in Bangkok must be composed of 10% ethanol. India is requiring 5% ethanol gasoline blends. Canada has provided tax benefits for ethanol producers and consumers since 1992 [43].

The European Union (EU) has also taken an aggressive stance in reshaping its transportation fuel and energy supply chain, in addition to promoting industrial biotechnology as a sustainable and cost-effective alternative to petrochemical processes. In December 2005, the EU adopted the Biomass and Biofuels Action Plan. This plan encompasses more then 20 specific action items, including creation of the Biofuels Technology Platform with the explicit purpose of advancing research into the use of forestry, agricultural, and woody crops for energy purposes. In February 2006, the EU adopted the Strategy for Biofuels, which set out three objectives: (1) to promote biofuels in both the EU and developing countries, (2) to prepare for large-scale use of biofuels by improving their cost-competitiveness and increasing research into second-generation fuels, and (3) to support developing countries where biofuel production could stimulate sustainable economic growth [52].

Furthermore, the EU has established quantitative targets for incorporation of biofuels into a broader and emerging bio-based economy. The EU transport sector accounts for more than 30% of the total energy consumption, with a 98% dependency on fossil fuels. There is also significant pressure for the EU to comply with the Kyoto Protocol, an agreement under the United Nations Framework Convention on Climate Change, ratified by 160 countries to significantly reduce greenhouse gas emissions, specifically $CO_{2,(g)}$. The EU has failed to meet the Kyoto targets with 90% of the increases in $CO_{2,(g)}$ emissions

between 1990 and 2010 attributable to transportation fuel usage. Therefore, significant reform in transportation fuel usage is required. There are three specific legislative actions in place [53]:

- Promotion of renewable energy-based electricity generation from 14% in 1997 to 21% by 2010 for the EU 25 (22.1% for EU 15) (Directive 2001/77/EC)
- Promotion of biofuels for transport applications by replacing diesel and petrol to the level of 5.75% by 2010 (Directive 2003/30 EC) accompanied by detaxation of biofuels
- Promotion of cogeneration of heat and electricity (Directive 2004/8/EC)

It is clear, however, that the EU is not meeting the objectives set forth. Specifically, the current production of liquid biofuels in the EU is 2 million tons of oil equivalent (Mtoe), less than 1% of the market. The EU policy target for 2010 was 18 Mtoe in the transportation sector alone. Although it is unlikely the target will be met, it should be noted that between 4 and 13% of the total agricultural land in the EU would be required to meet the above target. Therefore, through the creation of the various plans and platforms highlighted before, the EU has established, "An ambitious and realistic vision for 2030 is that up to one-fourth of the EU's transport fuel needs could be meet by clean and CO_2-efficient biofuels" [53]. Although it remains to be seen whether the appropriate resources will be allocated to meet this goal, it is certainly clear that industrial biotechnology, in particular the concept of a bio-based economy with biorefineries at its core, has taken center stage in the EU meeting its energy needs and environmental targets.

2.3 Meeting Bioethanol Demand

During the same time period that the cost of crude petroleum rose 150%, from January 2001 to 2005, the total number of bioethanol refineries in the USA increased from 56 to 81, with total production capacity increasing from 6.6 billion L/year to 13.8 billion L/year. Within the last year, from January 2005 to 2006, the total number of refineries increased to 95 and output further increased to 14.3 billion L/year, a 1500% increase since 2001. Total world production in 2005 was 46 billion L, with the USA and Brazil representing a combined 70% of the world's production. It should be further noted that by the end of 2005, 29 ethanol refineries and nine expansions of existing refineries were under construction, with a combined annual capacity of 5.7 billion L. If you consider all of the US ethanol production capacity currently on-line, under expansion, and under construction, then the projected capacity is approximately 24 billion L – approximately 85% of that required by the RFS by 2012 [43].

In the USA, the raw material of choice for bioethanol production is corn. Approximately 13% of the US corn crop is dedicated to ethanol production,

third only to livestock feed and exports [43]. In Brazil, however, the raw material of choice is sugarcane. With over 100 countries producing sugarcane, no one has yet to match Brazil's cost structure and supply chain. In mid-2005, the sugar production costs in the three lowest countries were estimated to be \$145/metric ton in Brazil, \$185/metric ton in Australia, and \$195/metric ton in Thailand. About 25% of worldwide sugar production is at \$200–250/metric ton, above which the figure escalates to \$400/metric ton and higher. Sugarcane is a highly efficient crop for producing biomass, representing the highest biomass per growing area of any major commercial crop, including corn. This is a result of sugarcane's ability to incorporate C_3 and C_4 compounds in its photosynthetic pathway, while most plants only incorporate C_3 compounds. Brazilian ethanol is most likely the cheapest in the world, with an estimated production cost of between \$0.19 and \$0.21/L in 2005. For this reason Brazil is not only looking to expand its ethanol production capacity, but to further expand into biorefineries [54].

Brazil's sugarcane production is unique, and not representative of the general challenge almost all other nations face when determining which raw material source is preferred. Raw material utilization for bioethanol and biotechnology processes in general represents a significant challenge and opportunity for research and development. The US Department of Agriculture and Department of Energy estimate that the resources exist to produce over 1 billion tonnes of biomass annually, representing approximately 30% displacement of current fossil fuel usage (302 billion L) [55]. Biomass is composed of cellulose (40–50%), hemicellulose (25–35%) and lignin (15–20%) [56]. Significant effort in the fields of non-food agricultural engineering, enzyme catalysis of cellulose and hemicellulose, and hexose and pentose fermentation will be required to extrapolate the full energetic value of lignocellulose.

Figure 2 schematically shows how research in the aforementioned areas is integrated into bioethanol process development, specifically focusing on the secondary pretreatment of feedstocks and microbial metabolic engineering. In both examples, the application of systems biology to the metabolic engineering framework can yield improved products, either in the form of enzymes or microbial platforms. We will further explore how scientific and technical achievements in the fields of metabolic engineering and systems biology as applied to the afore mentioned areas and others, driven by industrial biotechnology and demand for bioethanol, will improve bioethanol process development.

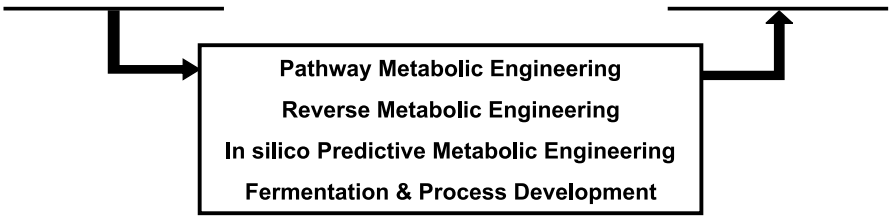
3
Industrial Systems Biology: X-*omics*

Systems biology is the quantitative characterization of genetic, transcription, protein, metabolic, signaling and other informational pathway responses to

a clearly defined perturbation of a biological system. More specifically, the perturbation may take the form of a genetic, biochemical, or environmental stimulus. At the core of systems biology is the transformation of quantitative, typically large-scale data sets, into in silico models that provide both interpretation and prediction. Systems biology has emerged as a tool applied in different fields, including metabolic engineering, to what many consider to be an independent discipline of study and research [57]. Table 1 provides an overview of commonly used industrial biotechnology strategies, focused on metabolic engineering with specific examples taken from applications

Table 1 Overview of commonly used industrial biotechnology strategies

Industrial biotechnology strategy	Examples of application to bioethanol production
Intermediates/impurities may be translated to marketable co-products to improve overall process economics.	A case study considering the co-production of ethanol and succinic acid suggests significant cost reduction (sales price of ethanol decreases from \$0.51 to \$0.42/gal.). Pilot plant confirmation pending [115, 116].
Existing metabolic pathways may be optimized/enhanced to increase (or decrease) product (or waste by-product) titer, yield, or productivity.	In silico aided metabolic engineering of *S. cerevisiae* lead to a 40% reduction in glycerol formation and 3% increase in ethanol yield in vivo [154].
Non-native host organism metabolic pathways may be introduced to increase (or decrease) product (or intermediate) yield and/or productivity.	Natural ethanol producing bacterium *Zymomonas mobilis* metabolically engineered to ferment xylose and arabinose as preferred carbon sources via introduction/expression of *E. coli* pathway genes [6, 155].
Alternative, more abundant, and more cost-effective carbon sources coupled with metabolic engineering may lead to higher yields, productivity, or cost-savings.	Xylose ($C_5H_{10}O_5$, significant fraction of lignocelluloses) utilization by *S. cerevisiae* investigated and optimized via introduction of a *Piromyces* sp. xylose isomerase (*XylA*). Further xylose metabolic structural genes were overexpressed. Xylose consumption of 0.9–1.1 g g-biomass^{-1} h^{-1}, demonstrated in vivo [156–159].



to bioethanol production. The examples cited exploit toolboxes developed within systems biology.

Therefore, we refer here to *industrial systems biology*, defined as the application of experimental or numerical methods developed from systems biology to improve bioprocess development in terms of final product titer, yield, or productivity, or process robustness and efficiency. In most cases, industrial systems biology has been product- or process-specific; however, there are emerging examples of successful commercialization of stand-alone systems biology tools and products for broad application [58].

Recent advances in high-throughput experimental techniques have resulted in rapid accumulation of a wide range of *x-omics* data of various forms (Fig. 3), providing a foundation for in-depth understanding of biological processes [59–62]. How to integrate, interpret, and apply these data is an area of active research. Bioinformatics has become a well-established and recognized interdisciplinary field. To date, large data sets of transcriptomes, metabolomes, and to lesser degrees proteomes and fluxomes, for multiple organisms have been acquired. Resources are being applied to integrating the various data sets for in silico simulations and creating relevant models that represent in vivo physiological conditions of host cells responding to environmental stimuli. Even though our ability to analyze these *x-omic* (see "Glossary") data in a truly integrated manner is limited, new targets for strain improvement can be identified from these global data [63–69].

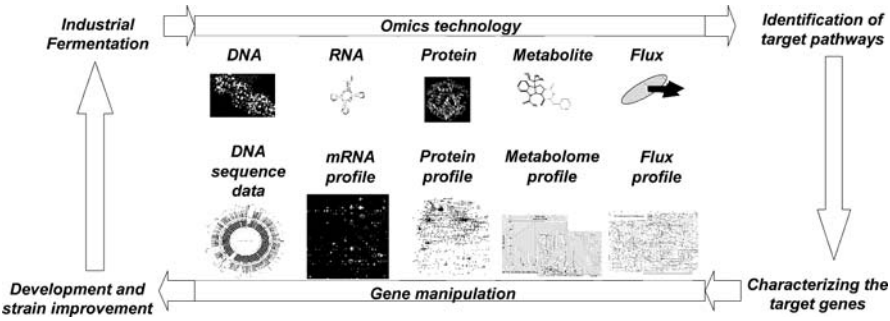


Fig. 3 Systems Biology Applied to Metabolic Engineering. Successful construction of recombinant microorganisms with enhanced production of bioproducts is significantly powered by high-throughput *x-omics* research. First, integration of the data provided by *x-omics* technology leads to the identification of target pathways and metabolic engineering strategies. Then, there is characterization of the candidate genes for improving the desired metabolic activity. Often the strategy involves deletion, overexpression, or modulation of more then one gene, and not necessarily in the same metabolic or signaling pathway. This is often a function of the multilayer regulatory cascade present in microorganisms. Gene manipulations are then carried out, and the resulting strain is characterized via fermentation and supporting analytical methods. This development cycle is then iterated to generate improved strains, as dictated by process requirements and resource availability

X-*omic* Glossary

Industrial systems biology: The application of numerical or experimental methods developed as a result of individual or combined *x-ome* analysis to bioprocess development. Bioprocess development encompasses strain or expression system improvements in terms of final product titer, yield, or productivity, or improvements in process robustness and efficiency.

Forward metabolic engineering: Defined as targeted metabolic engineering, it represents the linear progression from modeling to target gene identification to strain construction and characterization. Inherit in this strategy is specific and hypothesis-driven genetic manipulations.

Reverse metabolic engineering: Also defined as inverse metabolic engineering, a host strain constructed via random or directed mutagenesis, and/or evolution, is examined via systems biology tools to determine the genetic perturbation(s) that lead to the desired phenotype.

X-omics: A general term for referring to collection and analysis of any global data set whereby any type of informational pathway with reference back to the cell's genome is investigated. By definition, *x-omic* analysis and data collection requires the whole cell genetic sequence, preferably, annotated. *X-omics* may also be considered synonymous with *functional genomics*.

Genomics: The comprehensive study of the interactions and functional dynamics of whole sets of genes and their products.

Transcriptomics: The genome-wide study of mRNA expression levels in one or a population of biological cells for a given set of environmental conditions.

Proteomics: The large-scale analysis of the structure and function of proteins as well as of protein–protein interactions in a cell.

Metabolomics: The measurement of all metabolites to access the complete metabolic response of an organism to an environmental stimulus or genetic modification.

Fluxomics: The study of the complete set of fluxes that are measured or calculated in a given metabolic reaction network.

Metagenomics: The study of the genomes and associated *x-omes* in organisms recovered from the environment as opposed to laboratory cultures. Organisms recovered from the environment are often difficult to culture in controlled laboratory conditions, but may reveal interesting characteristics accessible through functional genomics.

On the basis of functional genomics data, transcriptomics and proteomics have helped us understand how microorganisms transcribe and translate their genetic information into functional proteins catalyzing heavily regulated networks of reactions to form complete pathways. Metabolomics coupled with flux measurements has provided both kinetic characterization and steady-state snapshots of how key metabolites are distributed throughout the metabolic network. These data have afforded metabolic engineers the capability to a priori evaluate large spaces of genetic engineering strategies, and following strain construction, have elucidated mechanistic understanding for future rounds of metabolic engineering.

A sampling of recent developments and applications in the field of systems biology will be discussed in relation to improving the productivity of bioethanol. Examples will be provided on single *x-ome* approaches and combined analysis of these *x-ome* data for the development of improved strains and enhancement of metabolic engineering strategies.

3.1 Genomics

Advances in the fields of genomics and metagenomics have dramatically revised our view of microbial biodiversity and the potential for biotechnological applications. In the last decade the revolution in computer processor speeds, memory storage capability, and expanding networks has made possible the large scale sequencing of genomes and management of large integrated databases over the Internet. Since the first microorganism, *Haemophilus influenzae*, was sequenced in 1995, genome sequencing initiatives have resulted in over 300 sequenced organisms, including 27 archaeal, 337 bacterial, and 41 eukaryotic genomes. As of July 2006, more than 1500 prokaryotic and eukaryotic genome sequencing projects are underway [70, 71]. The genome sequences of *Escherichia coli* and *Saccharomyces cerevisiae* were not only among the first to be published, but were also the first of wide relevance for the production of industrial biochemicals, including bioethanol. Given that the genome of a particular microorganism, following annotation, provides the theoretical enzyme reaction set, it serves as a preferred starting point for engineering metabolic pathways that will lead to significantly improved titer, yield, productivity, and performance of a microorganism [62].

Annotated genomes certainly compliment experimental designs; however, the design space that can be considered by visual inspection or classical hypothesis driven experimentation is limited given the high degree of connectivity of the metabolic network. Modifying a given enzyme or metabolite pool is likely to elicit a multilayered regulatory response that not only mitigates the original perturbation, but will shift the equilibrium of other enzymes, metabolite pools, or signalling pathways. To a large extent, this is why random mutagenesis approaches have been favored over targeted approaches, until recently. The first genome-scale in silico metabolic network model for *E. coli* was made available in 2000 and was among the first to demonstrate consistency between modeling predictions and in vivo physiology [72, 73]. Specifically, the model was used to explore the relationship between acetate, succinate, and oxygen uptake rates when attempting to maximize growth rate, to confirm the hypothesis that *E. coli* under acetate and succinate carbon limitations regulates its metabolic network to maximize growth rate. For industrial biotechnology process development, it is often desirable to shift carbon flux from biomass to product formation, thereby maximizing the yield of product on substrate.

The first eukaryotic genome-scale metabolic model was reported in *S. cerevisiae* in 2003 based on its annotated genome sequence and a thorough examination of online pathway databases, biochemistry textbooks, and journal publications [74]. This genome-scale in silico model, by using a relatively simple synthetic medium, could predict 88% of the growth phenotypes correctly, indicating that this model in many cases can predict cellular behavior. In one step further, Duarte et al. (2004) [74] used the *S. cerevisiae* genome-scale metabolic network constructed by Förster et al. (2003) [75] to generate a phenotypic phase plane (PhPP) analysis that describes yeast's metabolic states at various levels of glucose and oxygen availability. Examination of the *S. cerevisiae* PhPP has led to the identification of two lines of optimality: LO_{growth}, which represents optimal biomass production during aerobic, glucose-limited growth, and $LO_{ethanol}$, which corresponds to both maximal ethanol production and optimal growth during microaerobic conditions. The predictions of the *S. cerevisiae* PhPP and genome scale model were compared to independent experimental data, and the results showed strong agreement between the computed and measured specific growth rates, uptake rates, and secretion rates. Thus, genome-scale in silico models can be used to systematically reconcile existing data available for *S. cerevisiae*, particularly now that yeast resources, databases, and tools for global analysis of genomic data have been expanded and made publicly available, such as the *Saccharomyces* Genome Database [70, 71].

Another major challenge of current biotechnology, especially in the lignocellulose-to-ethanol process, is to identify novel biocatalysts and enzymes for enzymatic hydrolysis from the genomes of organisms and metagenomic libraries. A large number of protein sequences deduced from the genomes of industrial microorganisms have no assigned function, as they exhibit low (or no) homology to the enzymes and/or proteins functionally characterized thus far [76]. The demand for identification of novel biomass-degrading enzymes as well as for heterologous protein production at higher efficiencies and reduced costs has catalyzed an interest in elucidating the genomic sequence of *Trichoderma reesei* – the most prolific producer of biomass-degrading enzymes. Diener et al. (2004) [77] has described the creation of a *T. reesei* strain QM6A large-insert BAC (bacterial artificial chromosome) library and its subsequent analysis, which was successfully used to identify both biomass degradation and secretion related genes. These data revealed the utility of a BAC library for use in assembly of the *T. reesei* genome and isolation of genomic sequences of industrial interest.

Even though the above study represents a direct application of sequencing technology for identification of novel biomass-degrading enzymes, it is also often the case to use such high-throughput experimental techniques to elucidate mechanistic understanding of enzymes derived from random, natural selective pressures. The research of Foreman et al. (2003) [78] using *T. reesei* RL-P37, a strain that has been selected for improved production of

cellulolytic enzymes [79], is such an example. The mutation(s) that improved cellulase production concurrently improved the inducible expression of ancillary genes that do not have a known function in cellulose degradation. These results suggest significant regulatory points of convergence across the spectrum of cellular processes involved in carbon sensing, signal transduction, and transcriptional regulation. These findings will likely have significant implications for the design of industrial processes for commercial production of biomass-degrading enzymes.

In conclusion, the vastly improved computational capability integrated with large-scale miniaturization of biochemical techniques such as BAC, PCR, and microarray chips has delivered significant amounts of genomic data to researchers all over the world [80]. This availability of data has led to an explosion of genome analysis leading to many new discoveries and tools that are not possible in exclusively wet-lab experiments.

It is evident from the above applications of genomics coupled to in silico modeling that industrial biotechnology, and especially bioethanol production, can benefit from this technology platform both in the identification of metabolic engineering target genes to improve yields, titer, and productivity, and in the discovery of novel enzymatic catalysts. This is further reinforced by the various case studies to be presented in subsequent chapters, including the role genomics has played in the identification of thermostable cellulases, metabolic engineering for pentose and xylose utilization in *S. cerevisiae* and *Pichia stipitis*, development of ethanologenic bacteria, and development of *Z. mobilis* for bioethanol production.

3.2 Transcriptomics

Following the release and annotation of a genome, the next logical step is to evaluate the messenger RNA expression level on a whole genome scale, referred to as transcriptome analysis. Targeted metabolic engineering relies heavily on the assumption that a genetic perturbation – gene deletion, constitutive overexpression, regulated induction, or modulation – will confer a metabolic flux response. This stems from the central dogma of biology: DNA is transcribed to RNA and subsequently translated to polypeptides that give rise to phenotype. Prior to transcriptome analysis, genes were assumed to be expressed followed by post-translational regulation, with little understanding of interactions across gene loci [81]. In fact, transcriptome profiling of reference strains has provided a first approximation as to which pathways are active and, equally important, inactive, assuming that up-regulated gene expression leads to up-regulated pathway activity. It has since been shown that this is not always true – elevated mRNA levels do not always translate to elevated protein levels or activity. It has also provided significant insight into alternative modes of regulation, such as transcription factor-mediated

as opposed to post-translational regulation. This has permitted narrowing of the experimental space that metabolic engineers need to consider, and made available new strategies to consider. Additionally, transcriptome profiling provides a quantitative in vivo assessment of several key metrics following a genetic perturbation relative to a reference case: (1) what is the net change in mRNA expression levels of the targeted gene(s), (2) what is the net change in mRNA expression levels of non-targeted gene(s), and (3) what is the net change in mRNA expression levels of either reference or constructed strains under specific environmental conditions. These questions aim to isolate which genes and pathways may serve as targets and/or explanations for observed or induced phenotypes. Measurement of the transcriptome, via readily available microarray technology, has evolved into a routinely measured data set for many industrially relevant organisms, including *E. coli* and *S. cerevisiae*, and is playing a central role in both *forward* and *reverse* metabolic engineering [63, 82, 83].

Among the first applications of transcriptome measurements with industrial relevance to bioethanol production was establishing the baseline response of *S. cerevisiae* to diverse carbon substrates and medium compositions – essential for optimizing strains to given feedstocks and vice versa. Steady-state chemostat cultures were used to measure transcriptome responses under glucose, ethanol, ammonium, phosphate, and sulfate limitations [84]. Results suggested that genes related to high-affinity glucose uptake, the TCA cycle, and oxidative phosphorylation were up-regulated in glucose-limiting conditions, while genes involved in gluconeogenesis and nitrogen catabolite repression were up-regulated in ethanol-grown cells [84]. In a similar but earlier study, transcriptome measurements were performed of *S. cerevisiae* grown using glucose-limited chemostats coupled with nitrogen, phosphorus, and sulfur limitations [85]. In total, 1881 transcripts (31% of the total 6084 different open reading frames probed) were significantly up- or down-regulated between at least two conditions, and a total of 51 genes demonstrated a >tenfold higher or lower expression within a given condition [85]. The transcriptome profiles under each condition have provided genetic motifs that may be recognized and regulated by transcription factors. These may be used in metabolic engineering strategies that could cater to a specific growth medium composition.

With the experimental mechanics of collecting transcriptome data becoming common place, attention and focus is now placed on data analysis methods and integration with other *x-ome* data sets. It has become abundantly clear that transcriptome data alone, unless used for the purposes of environmental screening or quality control (i.e., confirming that an engineered genotype is producing the corresponding transcription profile), provides limited biological insight. Several efforts have emerged coupling transcriptome with metabolome and fluxome data [86–89]. For example, elementary flux modes for three carbon substrates (glucose, ethanol, and galactose) were deter-

mined using the catabolic reactions from the genome-scale metabolic model of *S. cerevisiae*, and then used for gene deletion phenotype analysis. Control-effective fluxes were used to predict transcript ratios of metabolic genes for growth under each substrate, resulting in a high correlation between the theoretical and experimental expression levels of 38 genes when ethanol and glucose media were considered [90]. This example demonstrates that incorporating transcriptional functionality and regulation into metabolic networks for in silico predictions provides both more biologically representative models and a means of bridging transcriptome and fluxome data.

In another example, the topology of the genome-scale metabolic model constructed for *S. cerevisiae* is examined by correlating transcriptional data with metabolism. Specifically, an algorithm was developed enabling the identification of metabolites around which the most significant transcriptional changes occur (referred to as reporter metabolites) [91]. Due to the highly connected and integrated nature of metabolism, genetic or environmental perturbations introduced at a given genetic locus will affect specific metabolites and then propagate throughout the metabolic network. Using transcriptome experimental data, a priori predictions of which metabolites are likely to be affected can be made, and serve as rational targets for additional inspection and metabolic engineering [91]. This algorithm has been recently extended to include reporter reactions, whereby transcriptional data is correlated with the metabolic reactions of the reconstructed *S. cerevisiae* genome-scale metabolic network model to identify those reactions around which a genetic or environmental perturbation conferring transcriptional changes cluster [92].

As more genomes continue to become available, and microarray technology continues to become more accessible with cost-effective customizable DNA microarrays now available, transcriptome data will continue to increase. Bioinformatics for data handling, integration of transcriptome with other *x-ome* data, and the development of various network models that rely on transcriptome data for biological interpretation will continue to develop. From an industrial biotechnology perspective transcriptome measurements and analysis have played a large role in reverse metabolic engineering; transcriptional surveying of a strain constructed either via random mutagenesis or directed evolution [63, 82, 83, 93]. For example, lysine production via *C. glutamicum* has undergone transcriptome and fluxome measurements to elucidate greater than 50 years of traditional metabolic engineering (random mutagenesis), providing new targets for improved strategies [94–96]. This effort, applied to other industrial biotechnology processes, is likely to intensify.

3.3 Proteomics

Proteomics is the quantitative study of all proteins expressed in a cell under defined conditions. Proteomics represents one of the more challeng-

ing *x-omes* given that analytical methods enabling measurement of all proteins with the sensitivity, accuracy, and precision required have only recently been developed [62, 72]. Rapid advances in protein analytical technologies, fueled by the addition of mass spectrometry (MS), liquid chromatography (LC), sequence databases, and data handling methods, have made it possible for protein chemists to identify and examine the expression of many proteins resolvable by 2DE (two-dimensional gel electrophoresis). The possibility for large-scale protein studies seemed attainable [97]. It was in this context that in 1994, at the first 2DE meeting in Siena, Italy, the term "proteome" was coined [98]. Methods employed in proteomics have since gone on to include two-dimensional differential gel-electropheresis (DiGE), multidimensional protein identification technology (MuDPiT), isotope-coded affinity tag technology (ICAT), and quantitative proteome analysis based on MS–MS spetra and a multiplexed set of chemical reagents referred to as iTRAQ [99]. Although still slowly emerging, there are clear examples of where proteome analysis has resulted in strain improvement and successful metabolic engineering strategies [62, 100].

In line with industrial biotechnology applications, results of 2DE analysis can identify targets for strain improvement, such as target gene deletions [101] or co-expression for product enhancement [102]. Proteome analysis may also improve the design and control of industrial fermentation processes. In such a study, the dynamics of the *E. coli* proteome were recorded during an industrial fermentation process with and without induction of recombinant antibody synthesis [103]. The recombinant antibody fragment CD18 $F(ab')_2$ was developed as a biopharmaceutical for the treatment of acute myocardial infarction. Proteomic analysis of the above fermentation process suggested co-expression of Phage shock protein A (PspA) with a recombinant antibody fragment in *E. coli* resulted in improved yields. Further investigation is required to understand why PspA addition resulted in improved yield [104]. Another example, more relevant to bulk chemical manufacturing, is the metabolic engineering of *E. coli* to produce the biodegradable and biocompatible thermoplastic polymer, poly-(3-hydroxybutyrate), often referred to as PHB, which has numerous applications including serving as a primary feedstock for synthesis of enantiomerically pure chemicals. Specifically, the proteome of the metabolically engineered *E. coli* XL-1 Blue for PHB intracellular accumulation was compared to the reference strain, noting that PHB accumulation is not observed in the reference strain. It was revealed that 2-keto-3-deoxy-6-phosphogluconate adolase (Eda) plays a pivotal role in supplying glycerol-3-phosphate and pyruvate to further increase the flux to acetyl-CoA. A larger acetyl-CoA and NADPH demand is consistent with cells that produce a large amount of PHB. These conclusions were based on identification of protein spots on 2DE using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry [105].

Among the most recent examples of proteomics applied to industrial biotechnology process development is the recent reporting of the complete proteome of *Mannheimia succiniciproducens* [100]. *M. succiniciproducens* MBEL55E is a capnophilic Gram-negative bacterium isolated from bovine rumen, which produces large amounts of succinic acid under anaerobic conditions (0.68 g-succinic acid/g-glucose), and was first reported in 2002 [106]. Succinic acid is a C_4 organic acid, traditionally produced via petrochemical conversion of maleic anhydride. It promises to be a strategic building block chemical to be produced by industrial biotechnology, due to its use as the primary feedstock in the synthesis of key products including butanediol, tetrahydrofuran, γ-butyrolactone, and poly-amides [107, 108]. Numerous groups are exploring production of succinic acid in different host organisms, including *E. coli* [109], *Anaerobiospirillum succiniciproducens* [110], *Actinobacillus succinogenes* [110, 111], *Aspergillus niger*, and *Saccharomyces cerevisiae*. In *M. succiniciproducens* using 2DE coupled with MS–MS identification and characterization lead to identification of 200 proteins, with 129 proteins from the whole cell proteome, 48 proteins from the membrane proteome, and 30 proteins from the secreted proteome. Characterization of cell growth and metabolite levels in conjunction with proteome measurements during the transition from exponential to stationary growth was carried out.

Two interesting conclusions could be drawn from such analysis that was not possible a priori. First, a gene locus previously annotated as the succinate dehydrogenase subunit A (*sdhA*) is likely to be the fumarate reductase subunit A (*frdA*), based on comparative proteome analysis supported by physiological data. Second, two novel enzymes were identified as likely metabolic engineering targets for future improvements in succinic acid production. PutA and OadA are enzymes responsible for acetate formation and conversion of oxaloacetate to pyruvate, respectively, and their deletion is likely to induce higher flux towards succinic acid through minimization of byproduct formation [100]. This is a clear example of where proteome measurement and analysis not only provided novel information for future metabolic engineering strategies, but also served as a quality-control check for two critical assumptions: (i) that genome annotation is error-free, and (ii) that mRNA expression directly correlates with protein expression and activity.

As discussed previously, acquisition of large bodies of genomic sequences has prompted development and application of tools such as cDNA/oligonucleotide microarrays, which in turn has made possible global analysis of cellular processes. As powerful as this approach is proving to be, much of the regulation of physiological processes occurs post-transcriptionally. Thus, measurement of mRNA levels provides an incomplete picture of cellular activity and regulatory control points that may yield themselves as preferred metabolic engineering targets. Methods and techniques developed to measure the global expression, localization, and interaction of proteins fall within the domain of proteomics. By integrating various data sources with known

biological function about individual genes and proteins, one starts uncovering underlying mechanisms leading to the creation and analysis of static and dynamic models of regulatory networks and pathways.

A recent study has shown the value of this union of data as an experimental strategy to gain insights into cellular physiology [87]. In this study, both transcriptional and proteomic data were collected from *S. cerevisiae* and all of the known components of the galactose induction pathway were systematically perturbed. The different data were integrated into a mathematical model that included enzymatic reactions, membrane transport, transcriptional activation, protein activation, and protein inhibition. The model predicted previously unknown intra-pathway interactions, and inter-pathway interactions of the galactose induction pathway and other cellular processes. Several of these predictions were then verified experimentally [87]. The galactose signaling pathway is of particular industrial relevance as one of the classical and best-understood promoter and induction systems used for protein expression. This example further highlights that even such an extensively studied pathway will manifest new mechanisms for control and manipulation using *x-omic* approaches.

Related directly to bioethanol process development, several groups are evaluating proteomes of production organisms under defined environments that are of immediate industrial relevance. For example, Salusjarv et al. (2003) performed a proteome analysis of metabolically engineered *S. cerevisiae* strains cultured on xylose as compared to glucose under aerobic and anaerobic carbon-limited chemostats [113]. Lignocellulosic feedstocks are abundant and renewable; however, are also composed of xylose – the most abundant pentose sugar in hemicellulose, hardwoods, and crop residues, and the second most abundant monosaccharide after glucose [114]. *S. cerevisiae* fails to consume pentose sugars efficiently, compared to glucose, and therefore significant research has occurred in metabolically engineering such strains (see Sect. 3.5 for further discussion). Proteome analysis of xylose fermentations revealed 22 proteins that were found in significantly higher concentrations relative to glucose fermentations. Such proteins included alcohol dehydrogenase 2 (Adh2p), acetaldehyde dehydrogenases 4 and 6 (Ald4p and Ald6p), and DL-glycerol-3-phosphatase (Gpp1p) [113]. As will be revealed in the fluxome discussion, this protein expression profile is indicative of the redirection of metabolic fluxes believed to occur under xylose fermentation. Proteome analysis bridges the gap between genetic engineering, transcription profiles, and observed metabolism by identifying that over- or underexpression of specific proteins (i.e., enzymes) are pushing targeted (or untargeted) metabolic fluxes in desired (or undesired) directions.

Proteomics is a rapidly developing area of research, whereby new technologies are often developed and validated in model systems such as *S. cerevisiae*. Compared with genomics, however, proteomics is still limited because it is strongly biased towards highly abundant proteins and, therefore, does not

yet provide the genome-wide coverage obtained by other *x-ome* technologies. Additionally, the proteome world is possibly the most complex of all *x-omes* because of its highly dynamic nature and complexity resulting from splice-variants, isoforms, and protein post-translational modifications. For some proteins, in excess of 1000 variants have been described [104]. It is evident that there is an ongoing need for improvement in (quantitative) proteomics technologies, whereby yeast will likely have its role again as the benchmark model system. Proteomics, largely absent in bioethanol development, is at the infancy of finding key roles in industrial products. Those products are likely to be targeted as co-products for bioethanol-based biorefineries. Succinic acid has already been considered as a potential added value co-product that could diversify the product portfolio of a biorefinery where the high-volume, low-value product will be bioethanol [115, 116].

3.4 Metabolomics

In the post-genomic era, increasing efforts have been made to quantitatively describe the relationship between the genome and phenotype of cells. At the interface between the environment and DNA-encoded processes, metabolite levels are quantitative phenotypic indicators that provide an important complement to the measurements of mRNA and proteins when studying cellular function. In the same way as for proteomics, where mRNA expression is often assumed to correlate linearly with protein expression and further correlate with protein activity, the false pretence of a one-to-one relationship between all gene expression and metabolite formation exists. In fact, metabolite levels may be viewed as the final result of a complex integration of gene expression, RNA translation, post-translational modification, enzyme activity, and pathway regulation [117]. Metabolomics is a burgeoning field producing volumes of data that, like other *x-omic* data, brings together analytical technology, genomics, bioinformatics, and model construction, and lies at the core of the systems biology agenda [118]. The general idea of metabolomics was first defined several years ago in the field of microbiology [119], and its importance in plant science was pointed out soon thereafter [120]. Today, metabolomics is also a powerful tool in drug discovery and development, especially for the identification of drug metabolites and biomarkers for organ-specific toxicities [121, 122]. Industrial biotechnology has also begun to benefit from integration of metabolomics into the systems biology framework. In metabolic engineering, quantitative metabolomics, by assigning function and confirming in silico pathways, could provide a measure of changes in regulatory driving forces and elucidate the impact of changes in enzyme activities on fluxes [123].

Panagiotou and colleagues performed a thorough examination of the metabolome (amino and non-amino acids of the pyruvate, glycine, serine,

threonine, phenylalanine, tyrosine, tryptophan, histidine, glutamine, glutamate and dibasic acid metabolism, and the TCA cycle) of *Fusarium oxysporum*, a promising microorganism for bioethanol production, in different physiological states [124–127]. They demonstrated that in spite of the diversity of mechanisms in fungi that regulate the flux of intracellular amino acids, the production of amino acids was closely correlated with the oxygen supply and growth medium, while less so to the cultivation phase [126]. By investigating the profile of several intracellular metabolites during cultivations on glucose and cellulose, metabolic limitations in *F. oxysporum* that determine the reduced growth rate of this organism compared to other filamentous fungi could be pinpointed [125, 127]. For example, one of the major drawbacks on the glucose-to-ethanol conversion by *F. oxysporum* is the formation of significant amounts of acetic acid as a by-product. A systematic metabolome analysis suggested that the γ-aminobutyric acid (GABA) shunt is active under anaerobic conditions [125]. This led to the suggestion that the high production of acetic acid, which indicates NAD(P)H regeneration, may be associated with a GABA shunt activation since such pathways act as sinks for excess NAD(P)H, e.g., when the TCA cycle is inhibited [128]. Also, a determination of the sugar phosphorylated profiles under aerobic and anaerobic cultivations in order to improve the understanding of slow arabinose fermentation by *F. oxysporum* [126] was performed. The identification of key metabolites in *F. oxysporum* cultivations uncovered the activation of novel pathways and possible bottlenecks of others, offering specific genetic targets for improved fermentation performance (overexpression of phosphoglucomutase, transaldolase/transketolase).

Metabolomics has not only been used as a tool for identification of targets for metabolic engineering as described above, but also as an all-encompassing approach to understanding total, yet fundamental, changes occurring after subtle genetic perturbations. For example, key intermediates in the pentose phosphate pathway (PPP) and the Entner–Doudoroff pathway (EMP) pathway were analyzed to gain further insight into the metabolism of laboratory and industrial *S. cerevisiae* strains [129]. The results verified that the profiles of metabolites are indicative of the reference genetic background of the strains and engineered genotype. Devantier et al. (2005) have investigated the influence of very high gravity simultaneous saccharification and fermentation process conditions on yeast cellular metabolism [130]. Laboratory and industrial *S. cerevisiae* strains were cultured mimicking fermentation conditions commonly found in the fuel ethanol industry. Concurrently, GC–MS metabolite profiling was performed to determine if there was a metabolic stress response under defined conditions. Metabolite profiling and multivariate data analysis was demonstrated by the ability to distinguish strains and fermentation conditions based on intra- and extracellular metabolites. Furthermore, the increased energy consumption of stressed cells was reflected in increased intracellular concentrations of pyruvate and related metabolites.

Consequently Villas-Boas and coworkers (2005) used the metabolite profile of *S. cerevisiae* during very high gravity ethanol fermentation [130] to elucidate un-described metabolic pathways [131]. They proposed a de novo pathway for glycine catabolism and glyoxylate biosynthesis in recombinant *S. cerevisiae* strains, demonstrating the great potential of coupling metabolomics and isotope labeling analysis for pathway reconstructions.

A recent literature review of the applications of metabolome data from microorganisms was summarized by Wang et al. (2006), and included comparative metabolite studies, fermentation control, metabolic control analysis and flux studies, and integration of metabolomics for strain improvement [132]. Clearly, metabolomics will have a strong impact on industrial biotechnology in the coming years as one of the corner stones of the systems biology toolbox being applied to metabolic engineering for bioethanol strain improvement.

3.5 Fluxomics

A metabolic flux is defined as a quantitative measurement of the rate of conversion of reactants to products, where rate may be defined as the mass or concentration per unit time of reactant consumption and product formation. For metabolic engineers, flux analysis represents a critical determinant of whether a given strategy has succeeded in re-directing flux from undesired to target products. In the classic textbook, *Metabolic engineering: principles and methodologies*, the authors note: "The combination of analytical methods to quantify fluxes and their control with molecular biology techniques to implement suggested genetic modifications is the essence of metabolic engineering" [133]. There is a significant body of literature describing methods for metabolic flux estimation and measurement, from single, clearly defined, and experimentally determined stoichiometric reactions or sets of reactions for extracellular metabolites, to the more recent in silico estimation of intracellular metabolites based upon feeding of isotope-labeled substrates. For more in-depth reviews of methods employed, including their advantages and limitations, please refer to the following references: [133–137]. The focus of this review will be to explore how recent fluxomic data has contributed to improved metabolic engineering strategies for bioethanol production. Furthermore, it will reveal that the fluxomic methods developed and data presented thus far have directly contributed to improvements in other industrial biotechnology process development efforts.

Prior to discussion, it should be noted that although the flux data is growing to include measurement and estimation of many fluxes, at present, the reconstructed metabolic network used in flux estimation represents only a fraction (less than 5%) of the genome-scale metabolic network. For example, the genome-scale metabolic model for *S. cerevisiae* contains a total of 708 open reading frames, 584 metabolites, and 1175 reactions [14], while recent work

describing the flux network of xylose fermenting *S. cerevisiae* strains was based on a total of 40 measured fluxes (17 measured fluxes and 23 determined by sum fractional labeling using [1-^{13}C] glucose with 99% abundance) that were then applied to a reconstructed metabolic network model consisting of 37 irreversible reactions, seven reversible reactions, and 24 balanced metabolites [138]. Although flux analysis has rapidly been upgraded to *fluxome*, and continuing expansion of intracellular and extracellular metabolome measurement capabilities will enable more flux determinations, the large majority of fluxes have yet to be experimentally determined.

There have been several examples where flux measurements and analysis has significantly contributed to bioethanol strain development, particularly with respect to engineering xylose- and pentose-consuming fermentations. Native *S. cerevisiae* is incapable of metabolizing xylose and has therefore been an area of very active research and metabolic engineering [114]. For example, Grotkjær et al. (2005) compared the flux profile of two recombinant *S. cerevisiae* strains, TMB3001 and CPB.CR4, both expressing xylose reductase (XR) and xylitol dehydrogenase (XDH) from *P. stipitis*, and the native xylulokinase (XK). CPB.CR4 included a *GDH1* deletion and *GDH2* being put under a *PGK* promoter [138]. Expression of XR, XDH, and XK led to highly inefficient xylose utilization due to a cofactor imbalance, where excess NADH must be regenerated via xylitol production resulting in reduced ethanol yield. Therefore, metabolic engineering of the ammonium assimilation through deletion of the NADPH-dependent glutamate dehydrogenase (*GDH1*) and overexpression of the NADH-dependent glutamate dehydrogenase (*GDH2*) resulted in a 16% higher ethanol yield due to a 44% xylitol reduction [138, 139]. Using a reverse metabolic engineering approach, metabolic flux analysis was used to characterize the intracellular fluxes for both strains based on experimental data of anaerobic continuous cultivations using a growth-limited feed of ^{13}C-labeled glucose, confirming that XR activity shifted from being mostly NADPH to partly NADH dependent in the CPB.CR4 strain. Furthermore, the analysis revealed, unexpectedly, activation of the glyoxylate cycle in CPB.CR4, generating the question of whether glyxoylate cycle activation may be preferred for ethanol yield. It was only through flux measurements and analysis that the distribution of carbon believed to have been altered via targeted genetics could be confirmed.

In a separate example, again addressing the issue of redox balance resulting from xylose fermentation, metabolic flux analysis was used to predict a priori that activation of the phosphoketolase pathway (PKP), which leads to the net re-oxidation of one NADH per xylose converted to ethanol, would be preferred [140]. The PKP converts xylose-5-P to acetyl-P and glyceraldehyde-3-P, enabling the maximum theoretical yield of 0.51 g-ethanol/g-xylose without affecting the NADPH/NADH consumption ratio of the XR reaction. A functional PKP was reconstructed in strain TMB3001c and the ethanol yield was increased by 25% due to minimization of xylitol formation; how-

ever, metabolic flux analysis predicted that only about 30% of the optimum flux required to eliminate xylitol and glycerol accumulation was present. Further overexpression of PKP; however, lead to increased acetate and a reduced xylose consumption rate, prompting the investigators to overexpress the acetaldehyde dehydrogenase, *ald6*. This reduced acetate formation, and produced a strain with 20% higher ethanol yield and a 40% higher xylose consumption rate compared to the reference strain [141]. Metabolic flux analysis served two purposes: (i) determination a priori of preferred metabolic engineering targets, and (ii) experimental confirmation of carbon flux distributions, neither possible based on visual inspection of biochemical pathways. For a more in-depth review of the recent advances in metabolic engineering of xylose fermentation in *S. cerevisiae*, specifically focusing on the introduction of a xylose isomerase from *Piromyces* sp. as a critical milestone in xylose substrate utilization for ethanol production, refer to chapters co-authored by Antonius J.A. van Maris and Bärbel Hahn-Hägerdal in this volume.

Fluxome analysis is developing at an accelerated pace, particularly in two areas that will have direct impact on strain development for industrial biotechnology applications. First, significant effort is being dedicated towards developing bioinformatic tools that enable integration of experimentally or in silico-determined flux values with other *x-ome* data. For example, experimentally determined flux values have recently been used as a quality control check of previously generated *E. coli* genome-scale metabolic models, whereby reaction constituents, direction, or stoichiometry have been revised to reflect in vivo observations [141]. In addition to bridging fluxomics with genomics, integration of transcriptome and fluxome data was also previously discussed under transcriptomics.

The second area of rapid progress is the experimental determination of fluxes. Presently, most isotope labeling experiments are performed in well-controlled stirred tank bioreactors, often at working volumes ranging from 0.1 to 1.0 L, and many times in a continuous culture mode to ensure both growth and isotopic steady state (i.e., 1-[^{13}C]-glucose feed). These systems, while reliable, are low-throughput and costly to sustain, particularly the large volumes of isotope-labeled substrate required to reach isotopic steady state (generally five residence volumes). Numerous groups are working on enabling high-throughput flux measurements in small-volume (i.e., 1–100 mL) culture systems. For example, Fischer et al. (2003) reported the development of a novel methodology for ^{13}C-constrained flux balancing applied to data of *E. coli* cultures fed [U-^{13}C]-glucose and [1-^{13}C]-glucose in shake flasks and 1-mL deep-well microtiter plates [142]. There was excellent agreement of flux values with conventional and comprehensive isotopmer metabolic models [142, 143]. In another example, Yang et al. (2006) developed a novel method for metabolic flux studies of central carbon metabolism based exclusively on online measurement of carbon dioxide evolved from shake flask cultures of *Corynebacterium glutamicum* for improving lysine production.

This method, referred to as respirometric ^{13}C flux analysis, was experimentally validated in cultures supplemented with [1-^{13}C]-, [6-^{13}C]-, and [1,6-^{13}C]-glucose, and successfully resolved the major fluxes of central carbon metabolism [144, 145]. These examples are on the forefront of enabling high-throughput flux analysis and measurement to become common place among bioprocess development efforts.

3.6 Industrial Systems Biology and Bioethanol

The development of functional genomics has provided new tools and approaches for understanding, mapping, modeling, and manipulating cells. Therefore, the metabolic engineering goal of identifying genes that confer a particular phenotype is conceptually and methodologically congruent with central issues in functional genomics. Functional genomics will not only elucidate what the genes do but will also help determine when, where, and how they are expressed as an organized system. The combination of genetics and a wide variety of *x-omics* data (transcriptomics, proteomics, metabolomics, fluxomics) can also be applied directly in metabolic engineering to identify new targets for improved phenotype. Table 2 provides a summary of genome, transcriptome, proteome, and fluxome data applied to industrial biotechnology process development. However, for industrial systems biology to be further applied in experiments and development efforts, the quality and range of the different *x-omics* data should be comparable. The implementation of high-throughput, easy to use, platform technologies will be critical in bringing these tools to broad applicability in bioprocess development.

A final point worth touching upon is that industrial partners have often cited that many of the *x-omes*, particularly the younger disciplines (e.g., proteomics), while providing academically interesting research, have not translated into methods or approaches with industrial impact and value. This is a fair assessment, but one that is changing. The momentum of bioethanol development, and consequently other industrial biotechnology-produced products (e.g., 1-3-propandiol), is driving manufacturers to develop better processes with higher yields, titer, productivity, robustness, and efficiencies. The margins and areas for improvement are narrowing, and can only be met with innovative approaches and strategies that may be yet undiscovered. *X-ome* analysis and data is providing the innovation by developing data sets and tools that are beginning to answer fundamental questions (i.e., Is this pathway's regulation transcriptional or translational? Is carbon being lost through the citric acid cycle or pentose phosphate pathway?). But perhaps more important, industrial systems biology is leading to new questions not previously considered. In the struggle of how to handle and what do with all this data will emerge the questions that lead to novel and yet unrealized metabolic engineering strategies. Furthermore, these methods are data driven. Even if

Table 2 Examples of *x-omes* applied to industrial biotechnology

X-ome	Microorganism	Product	Description	Refs.
Genome	*Saccharomyces cerevisiae*	Ethanol	Phenotypic Phase Plane analysis for the identification of optimality for both ethanol production and growth	[74]
	Trichoderma reseei	Cellulases	Construction of Bacterial Artificial Chromosome library for the identification of biomass degradation and secretion related enzymes	[77]
Tran-scriptome	*Saccharomyces cerevisiae*	Ethanol	The baseline response to diverse carbon substrates and medium compositions was established	[84]
Proteome	*Escherichia coli*	Poly-3-hydroxy-butyrate (PHB)	Protein spots on 2DE revealed the large demand of the cell for acetyl-CoA and NADPH for the high production of PHB	[105]
	Manheimia succiniciproducens	Succinic acid	2DE coupled with MS-MS identified two enzymes (PutA and OadA) as metabolic engineering targets for increasing the final titres	[100]
	Saccharomyces cerevisiae	Ethanol	22 proteins were found in higher concentrations in xylose cultivations compared to glucose and three enzymes were targeted for further improvement of the process	[113]
Meta-bolome	*Saccharomyces cerevisiae*	Ethanol	A de novo pathway for glycine metabolism and glyoxylate biosynthesis was proposed during very high gravity fermentations	[130, 131]
	Fusarium oxysporum	Ethanol	Coupling the formation of the major by-product acetic acid with the activation of the GABA shunt suggested novel targets for improved fermentation performance	[125]
Fluxome	*Saccharomyces cerevisiae*	Ethanol	Determination a priori of preferred metabolic engineering targets and experimental confirmation of carbon flux distributions-both not possible based on visual inspection of biochemical pathways	[138, 140]

manipulation, analysis, and interpretation of the data are not clear, biologically and statistically high quality data are required to drive development. Industry requires this innovation to remain sustainable, and, therefore, must support industrial systems biology in its infancy and development.

4 Future Perspectives and Outlook

The focus of this review has been twofold. First, to present a summary of the economic and socio-political landscape that has fueled the resurgence in bioethanol as a biofuel, and consequently, the general adoption of industrial biotechnology as a cost-effective, sustainable, and preferred alternative to traditional petrochemical processing. Second, to offer the hypothesis that significant scientific achievements in metabolic engineering and systems biology have been applied to bioethanol and other chemical products for successful commercialization, suggesting a graduation of the field to *industrial systems biology*. If we revert back to Fig. 1, we have focused most of our attention on process economics, with some indirect discussion of environmental impact and sustainability/self-sufficiency. Within the discussion of industrial systems biology we have focused only on the upstream bioprocessing steps schematically shown in Fig. 2, namely, systems biology used for enhancement of metabolic engineering. An area that we have not discussed, but is addressed in the chapter co-authored by Warren E. Mabee in this volume, and suggested in Fig. 1, is public perception and policy.

In the July 2006 issue of *Nature Biotechnology* there were a series of editorials and commentaries written exploring bioethanol as a biofuel [146–150]. Amongst these articles was a discussion of the various public perception issues facing bioethanol, ranging from statements of support, such as, "At least three major factors – rapidly increasing atmospheric CO_2 levels, dwindling fossil fuel reserves and their rising costs – suggest that we now need to accelerate research plans to make greater use of plant-based biomass for energy production and as a chemical feedstock as part of a sustainable energy economy" [149], to highly critical statements, such as, "At present, ethanol is not price competitive by any stretch of the imagination – even with the absurd and decidedly anti-free-market 54-cent per gallon tariff Washington imposes upon Brazilian ethanol" [147]. Both in the scientific peer-review and mainstream literature, there is still debate as to whether bioethanol for biofuel makes sense. This debate has prompted the development of new methods for analysis of whether bioethanol is economically feasible, and more importantly, sustainable over the long-term. A general classification often used to evaluate process feasibility is life-cycle analysis.

Life-cycle energy analysis is a methodology used to answer the bottom-line question: is more energy contained in the fuel than is used in the

production of that fuel? Life-cycle energy analysis, much like the tools employed in functional genomics, is a systems approach to evaluate all aspects of the production process from feedstock processing, availability, and transportation, to opportunities for recycling of energy and mass pre-, during, and post-production [151–153]. Life-cycle energy analysis, unlike earlier approaches, has suggested that process integration, energy recycling, and careful selection of raw materials and unit operations can yield bioethanol processes that are energetically favorable [151–153]. Consequently, biorefineries are viewed as a natural extension of bioethanol production facilities given the opportunities for integration, recycling, and production of higher value chemicals.

Holistic approaches that take a systems level approach must be refined, improved, and presented to policy makers and key stakeholders to finally put an end to the question – does bioethanol make sense? The question should be revised to ask under which conditions does bioethanol makes sense, and what is required to commercialize those conditions. Bioethanol, biorefineries, and industrial biotechnology will not be successful and expand to novel areas if we do not focus on *engineering* public perception and policy. Bioethanol has become a commercial vehicle for *industrial* systems biology applied to *industrial* biotechnology. Now, it needs to become the commercial vehicle for reaching public, government, and corporate support, and again, it will take systems level data to get there.

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Pretreatment of Lignocellulosic Materials for Efficient Bioethanol Production

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Abstract Second-generation bioethanol produced from various lignocellulosic materials, such as wood, agricultural or forest residues, has the potential to be a valuable substitute for, or a complement to, gasoline. One of the crucial steps in the ethanol production is the hydrolysis of the hemicellulose and cellulose to monomer sugars. The most promising method for hydrolysis of cellulose to glucose is by use of enzymes, i.e. cellulases. However, in order to make the raw material accessible to the enzymes some kind of pretreatment is necessary. During the last few years a large number of pretreatment methods have been developed, comprising methods working at low pH, i.e. acid based, medium pH (without addition of catalysts), and high pH, i.e. with a base as catalyst. Many methods have been shown to result in high sugar yields, above 90% of theoretical for agricultural residues, especially for corn stover. For more recalcitrant materials, e.g. softwood, acid hydrolysis and steam pretreatment with acid catalyst seem to be the methods that can be used to obtain high sugar and ethanol yields. However, for more accurate comparison of different pretreatment methods it is necessary to improve the assessment methods under real process conditions. The whole process must be considered when a performance evaluation is to be made, as the various pretreatment methods give different types of materials. (Hemicellulose sugars can be obtained either in the liquid as monomer or oligomer sugars, or in the solid material to various extents; lignin can be either in the liquid or remain in the solid part; the composition and amount/concentration of possible inhibitory compounds also vary.) This will affect how the enzymatic hydrolysis should be performed

(e.g. with or without hemicellulases), how the lignin is recovered and also the use of the lignin co-product.

Keywords Assessment · Enzymatic hydrolysis · Lignocellulose · Pretreatment · Review

1 Introduction

Replacement of gasoline by liquid fuels produced from renewable sources is a high-priority goal in many countries worldwide. It is driven by the aims of a secure and sustainable energy supply and a desire to diminish the greenhouse effect. The transportation sector in the European Union (EU) is totally dependent on imported fossil fuels, and thus extremely vulnerable to market disturbances. It is also the sector responsible for the main part of the increase in CO_2 emissions. The use of biofuels in the EU is encouraged by a Directive that set a target of 2% substitution of gasoline and diesel with biofuels in 2005 on an energy basis, which should have increased to 5.75% by 2010 [1].

Bioethanol is projected to be one of the dominating renewable biofuels in the transportation sector within the coming 20 years, and has already been introduced on a large scale in Brazil, the USA and some European countries. The advantages of bioethanol are that it can be produced from a variety of raw materials, it is non-toxic and is easily introduced into the existing infrastructure, either as a low blend with gasoline (e.g. E5 and E10) or used in flexi-fuel vehicles at a high concentration (e.g. E85) or as a neat fuel in dedicated engines. However, almost all bioethanol today is produced from sugar- or starch-based agricultural crops, using so-called first-generation technologies. Although this ethanol is produced at a competitive cost, the raw material supply will not be sufficient to meet the increasing demand for fuel ethanol, and also the reduction of greenhouse gases resulting from the use of sugar- or starch-based ethanol is not as high as desirable.

One of the most promising options to meet this challenge is the production of bioethanol from lignocellulose feedstocks, such as agricultural residues (e.g. wheat straw, sugar cane bagasse, corn stover) and forest residues (e.g. sawdust, thinning rests), as well as from dedicated crops (salix, switch grass) using second-generation technologies. These raw materials are sufficiently abundant and generate very low net greenhouse gas emissions, reducing environmental impacts.

However, to compete with gasoline the production cost must be substantially lowered. Today, raw material and enzyme production are two of the main contributors to the overall production cost [2, 3]. Efficient use of the whole crop is required, i.e. high overall yield of ethanol produced by hydrolysis and fermentation of the carbohydrate fraction (hemicellulose and cellulose), as well as a high yield of the main co-product (lignin). However,

producing monomer sugars from cellulose and hemicellulose at high yields is far more difficult than deriving sugars from sugar- or starch-containing crops, e.g. sugar cane or corn.

Ethanol production from lignocellulosic materials comprises the following main steps: hydrolysis of hemicellulose; hydrolysis of cellulose; fermentation; separation of lignin residue; recovery and concentration of ethanol; and wastewater handling. Figure 1 shows a simplified process flowsheet for ethanol production from lignocellulosic materials based on enzymatic hydrolysis. Some of the most important factors to reduce the production cost are: an efficient utilization of the raw material by having high ethanol yields, high productivity, high ethanol concentration in the distillation feed, and also by employing process integration in order to reduce capital cost and energy demand. Part of the lignin can be burnt to provide heat and electricity for the process, and the surplus is sold as a co-product for heat and power applications, which will increase the energy efficiency of the whole system. It is thus necessary to minimize the internal energy demand and to maximize the production of the solid fuel. The two conversion steps can be considered key processes: hydrolysis of the hemicellulose and cellulose to sugars, and fermentation of all the sugars.

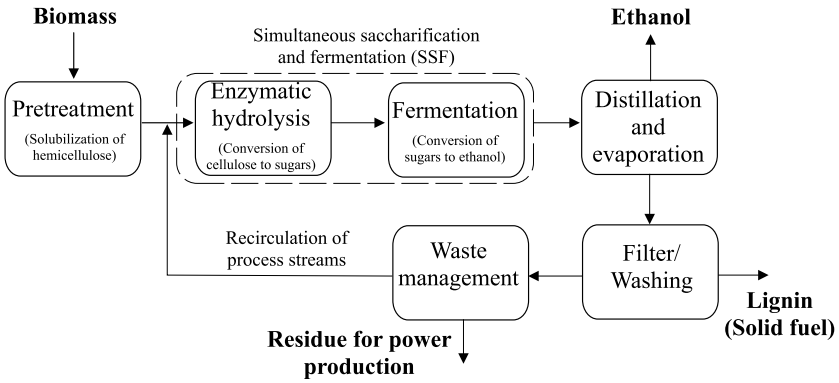

Fig. 1 Simplified flowsheet for ethanol production from biomass

The enzymatic process is regarded as the most attractive way to degrade cellulose to glucose [4–6]. However, enzyme-catalysed conversion of cellulose to glucose is very slow unless the biomass has been subjected to some form of pretreatment, as native cellulose is well protected by a matrix of hemicellulose and lignin. Pretreatment of the raw material is perhaps the single most crucial step as it has a large impact on all the other steps in the process, e.g. enzymatic hydrolysis, fermentation, downstream processing and wastewater handling, in terms of digestibility of the cellulose, fermentation toxicity, stirring power requirements, energy demand in the downstream processes and wastewater treatment demands.

An effective pretreatment should have a number of features. It has to:

- Result in high recovery of *all* carbohydrates.
- Result in high digestibility of the cellulose in the subsequent enzymatic hydrolysis.
- Produce no or very limited amounts of sugar and lignin degradation products [7]. The pretreatment liquid should be possible to ferment without detoxification.
- Result in high solids concentration as well as high concentrations of liberated sugars in the liquid fraction.
- Have a low energy demand or be performed in a way so that the energy can be reused in other process steps as secondary heat.
- Have a low capital and operational cost.

Additional positive features would be if hemicellulose sugars were obtained in the liquid as monomer sugars, as this would help to avoid the use of hemicellulases, and/or if the lignin—without being oxidized—was separated from the cellulose, as this would alleviate the unproductive binding of cellulases on lignin in the enzymatic hydrolysis step.

This chapter will focus on pretreatment of lignocellulosic raw materials. Some of the most common methods that have been investigated will be presented and to some extent compared for various raw materials.

2 Assessment of Pretreatment

Evaluation of various pretreatment conditions and the effect on key variables, such as the overall yield of sugars or ethanol, needs to be assessed in an easy way to be able to judge the result. In several studies on pretreatment of biomass the "severity factor" has been used for comparing pretreatment results. Although it does not provide an accurate measure of the severity it can be used for rough estimates [8, 9]. The severity correlation describes the severity of the pretreatment as a function of treatment time (minutes) and temperature (°C), $T_{ref} = 100\,°C$.

$$\log(\mathrm{Ro}) = \log\left(t \cdot \exp\left(\frac{(T - T_{ref})}{14.75}\right)\right) . \tag{1}$$

When pretreatment is performed under acidic conditions (e.g. by impregnation with H_2SO_4), the effect of pH can be taken into consideration by the combined severity [10] defined as:

$$\text{Combined severity (CS)} = \log(\mathrm{Ro}) - \mathrm{pH} \tag{2}$$

It is well known that more severe conditions during pretreatment will cause greater degradation of hemicellulose sugars [11–13]. A high severity in the

pretreatment is nevertheless required to enhance the enzymatic digestibility of cellulose [14]. The ideal pretreatment would hydrolyse the hemicellulose to its monomer sugars without further degradation. It would also cause an increase of the pore size and reduce cellulose crystallinity to enhance the enzymatic digestibility of the cellulose fibres. However, these two effects are not reached at the same pretreatment severity, at least not using current technologies.

Assessment of pretreatment is usually done by using some of (or a combination of) the following methods:

1. Analysis of the content of sugars liberated during pretreatment to the liquid as a combination of monomer and oligomer sugars, as well as analysis of the carbohydrate content of the water-insoluble solids (WIS). This gives the total recovery of carbohydrates in the pretreatment step.
2. Enzymatic hydrolysis (EH) of the WIS, either washed or non-washed.
3. Fermentation of the pretreatment liquid to assess inhibition of the fermenting microorganism.
4. Simultaneous saccharification and fermentation (SSF) of either the whole slurry or the washed WIS.

The enzymatic hydrolysis (in 1 and 4) is performed using cellulases, i.e. a mixture of various cellobiohydrolases and endoglucanases supplemented with β-glucosidase. The latter is not a cellulase as it only cleaves cellobiose into two glucose molecules. It has, however, a very important role in hydrolysis since cellobiose is an end-product inhibitor of many cellulases [15, 16]. On the other hand, β-glucosidase is also inhibited by glucose [17]. Since the enzymes are inhibited by the end products, the build-up of any of these products affects the cellulose hydrolysis negatively. The maximum cellulase activity for most fungus-derived cellulases and β-glucosidase occurs at $50 \pm 5\,^{\circ}\mathrm{C}$ and a pH of 4.0–5.0. However, the optimal conditions for enzymatic hydrolysis change with the hydrolysis residence time [18] and are also dependent on the source of the enzymes.

The enzymatic hydrolysis for assessment of pretreatment can be performed using various conditions (substrate concentration, enzyme dosage, temperature, stirring speed etc.). A common way is to use washed material at 2 wt % WIS, or alternatively at 1 wt % cellulose, to avoid end-product inhibition [19]. This could be seen as the maximum achievable digestibility or glucose yield. However, it does not reflect the pretreatment efficiency in terms of avoiding formation of compounds that are inhibitory to the cellulases. In a full-scale process it is crucial to reach high sugar and ethanol concentrations in order to decrease the energy demand in the downstream processes. To increase the sugar concentration during large-scale operation, it is assumed that the whole slurry after pretreatment would be used without introducing separation steps, which would dilute the process stream. Furthermore, the overall substrate loading in enzymatic hydrolysis would probably need to be above

10 wt % WIS to meet the energy requirement for ethanol recovery. To mimic a situation that will be more similar to final process conditions, the enzymatic hydrolysis can be performed using the whole slurry from the pretreatment diluted to various WIS concentrations, e.g. 10 wt %. In this case also the effect of inhibitors is assessed. However, due to the higher concentration of sugars the enzymes will also suffer from end-product inhibition.

To assess the effect of possible inhibitors acting on the microorganism used for fermentation of the sugars released in the enzymatic hydrolysis, method 2 is most often combined with method 3. The overall ethanol yield depends not only on the sugar yield, but also on the fermentability of the solution. Inhibition is influenced by the concentration of the soluble substances released during pretreatment, present in the original raw material, e.g. acetic acid, or formed in the pretreatment step. Some of the substances present in the slurry are furfural and 5-hydroxymethylfurfural (HMF), which are the result of degradation of pentoses and hexoses, respectively. Furfural may react further to yield formic acid, or it may polymerize. HMF can be converted to formic acid and levulinic acid. In some pretreatments lignin degradation products are also formed. The concentrations of these and all other inhibitory substances in the fermentation step depend on the configuration of the preceding process steps. At ethanol concentrations below 4 to 5 wt % the energy demand increases rapidly with decreasing ethanol concentration. It is thus important to evaluate the fermentability of the concentrated pretreatment hydrolysates. The fermentability test is usually performed on the liquid obtained from the pretreatment, either directly or diluted to a concentration corresponding to what is expected to be suitable in a final process.

Another option for evaluation of the pretreatment step is to perform SSF either on the whole slurry diluted to a suitable WIS concentration or on the washed water-insoluble solid material, in both cases at a WIS around 5% or higher. In this case the glucose produced is immediately consumed by the fermenting microorganism, e.g. *Saccharomyces cerevisiae*, which removes the end-product inhibition of glucose and cellobiose. SSF adds information about the pretreatment efficiency, since SSF usually gives a higher overall ethanol yield than separate enzymatic hydrolysis and fermentation (SHF) due to conversion by the microorganism of some compounds that are inhibitory to the enzymes to less inhibitory compounds [20]. Also in the assessment by SSF the conditions may vary, e.g. substrate concentration, enzyme dosage, concentration of microorganism etc.

It has to be added that variations between different laboratories in configurations and conditions used for assessment of the pretreatment make it very difficult to compare various pretreatment methods unless they are assessed in exactly the same way. Even so, the conclusions may be incorrect as the conditions used may be unfavourable to a specific method. For instance, the use of hemicellulases in the enzymatic hydrolysis, instead of

only cellulases, will be beneficial to pretreatment methods that result in large amounts of oligomer hemicellulose sugars, as will be discussed in the results section.

It is our opinion that the assessment of pretreatment has to be performed in a more rigorous way. The standard enzymatic hydrolysis at low substrate concentration may well be used to assess the maximum digestibility. However, in this case both cellulases and hemicellulases are needed. The "real" assessment should be performed by optimizing the conditions for all subsequent process steps under more realistic process conditions, taking into account the special features of the pretreated material, and then comparing the production cost for the various alternatives.

3 Pretreatment Methods

A multitude of different pretreatment methods have been suggested during the past few decades. They can loosely be divided into different categories: physical (e.g. milling, grinding and irradiation), chemical (e.g. alkali, dilute acid, oxidizing agents and organic solvents), physicochemical (e.g. steam pretreatment/autohydrolysis, hydrothermolysis and wet oxidation) and biological, or combinations of these. In general, it is difficult to place the methods into one category only.

A rough classification of the pretreatment methods can also be made according to the following:

- Acid-based methods, i.e. pretreatment at low pH, result in hydrolysis of the hemicellulose to monomer sugars and minimize the need for hemicellulases.
- Methods working close to neutral conditions, e.g. steam pretreatment and hydrothermolysis, solubilize most of the hemicellulose due to the acids released from the hemicellulose, e.g. acetic acid, but do not usually result in total conversion to monomer sugars. This thus requires hemicellulases acting on soluble oligomer fractions of the hemicellulose.
- Alkaline methods leave a part of the hemicellulose, or in the case of ammonia fibre explosion (AFEX), almost all hemicellulose in the solid fraction. This then requires hemicellulases acting both on solid and on dissolved hemicellulose. An alternative is to perform an acid hydrolysis of this fraction, for instance after removal of the cellulose by enzymatic hydrolysis.

This affects, of course, not only the method that should be used for assessment of the pretreatment but also the cost of the overall hydrolysis of the carbohydrates.

3.1 Physical Methods

Enzymatic hydrolysis can be facilitated by chipping, milling and grinding the biomass into a fine powder to increase the surface area of the cellulose. In most cases the power consumption is forbiddingly high to reach high digestibility in the enzymatic hydrolysis. It can be even higher than the theoretical energy content that is available in the biomass [21]. However, physical treatment in an extruder combined with heating and addition of chemicals could be an interesting option [22]. Another method that has been suggested is irradiation of cellulose by gamma rays, which cleaves the β-1,4-glycosidic bonds, thus giving a larger surface area and a lower crystallinity [23]. This method is, however, far too expensive to be used in a full-scale process. It is also doubtful that it can be used in combination with technologies supposed to be environmentally friendly.

3.2 Chemical Methods

The regular and cross-linked cellulose chains form a very efficient barrier against penetration of the enzymes into the fibres. Swelling of the pores can be achieved by alkaline pretreatment through soaking of the material in an alkaline solution, such as NaOH, and then heating it for a certain time. The swelling causes an increase in the internal surface area, and a decrease in the degree of polymerization and crystallinity. Usually a major fraction of the lignin is solubilized together with some of the hemicellulose. A rather large fraction of the hemicellulose sugars are usually recovered as oligomers. Alkaline pretreatment breaks the bonds between lignin and carbohydrates and disrupts the lignin structure, which makes the carbohydrates more accessible to enzymatic attack. As it acts mainly by delignification, it is more effective on agricultural residues and herbaceous crops than on wood materials, as these materials in general contain less lignin. For softwood species, which contain a large amount of lignin, a small or no effect has been observed. Pretreatment using lime instead of sodium hydroxide is another alkaline method, especially suited for agricultural residues, e.g. corn stover, or hardwood materials, such as poplar [24, 25].

Dilute acid pretreatment is performed by soaking the material in dilute acid solution and then heating to temperatures between 140 and 200 °C for a certain time (from several minutes up to an hour). Sulphuric acid, at concentrations usually below 4 wt %, has been of most interest in such studies as it is inexpensive and effective. The hemicellulose is hydrolysed and the main part is usually obtained as monomer sugars. It has been shown that materials that have been subjected to acid hydrolysis may be harder to ferment because of the presence of toxic substances [26–28].

Another approach is to use an organic or aqueous–organic solvent mixture with addition of an inorganic acid catalyst (H_2SO_4 or HCl), which is used to break the internal lignin and hemicellulose bonds. These methods are usually referred to as organosolv processes [29]. In these cases the hydrolysed lignin is dissolved and recovered in the organophilic phase. It is important to thoroughly wash the material prior to enzymatic hydrolysis and fermentation, as the solvents may act as inhibitors. Solvents that are used are typically methanol, ethanol, acetone, ethylene glycol, triethylene glycol and phenol. Some of these substances are explosive and highly inflammable and thus difficult to handle.

3.3 Physicochemical Methods

This category includes methods in between, or a mixture of, purely physical and chemical methods. Steam pretreatment is one of the most widely used methods for pretreatment of lignocellulosics. This pretreatment method used to be called steam explosion, since it was believed that an "explosive" action on the fibres was necessary to render a material suitable for hydrolysis. It has been shown that it is more likely that the effect of steam pretreatment is due to acid hydrolysis of the hemicellulose, which is the reason why some cellulosic materials are easier than others to break down [30, 31]. In particular, agricultural residues and some types of hardwood contain organic acids, which act as catalysts for the hemicellulose hydrolysis. Using steam pretreatment the raw material is usually treated with high-pressure saturated steam at a temperature typically between 160 and 240 °C (corresponding to a pressure between 6 and 34 bar), which is maintained for several seconds to a few minutes, after which the pressure is released. During pretreatment some of the raw material, predominantly hemicellulose, is solubilized and found in the liquid phase as oligomeric and monomeric sugars. The cellulose in the solid phase then becomes more accessible to the enzymes. It is in some cases difficult to find conditions that result in high yields of both hexose and pentose sugars, and at the same time also create a cellulose fraction which is easy to hydrolyse to glucose. This may call for steam pretreatment using two steps, where hemicellulose sugars are recovered at lower severity, while the cellulose fraction is subjected to pretreatment at higher severity.

Steam pretreatment can be improved by using an acid catalyst, such as H_2SO_4 or SO_2. The acid increases the recovery of hemicellulosic sugars, and also improves the enzymatic hydrolysis of the solid residue. The use of an acid catalyst in steam pretreatment results in an action similar to dilute acid hydrolysis but with less liquid involved. It is especially important to use an acid catalyst for softwood, since softwood in general is more difficult to degrade.

Steam pretreatment with addition of a catalyst is the pretreatment method for hydrolysis and enzymatic digestibility improvement that is closest to

commercialization. It has been widely tested in pilot-scale equipment, for example, in the NREL pilot plant in Golden, CO (USA) [32] and in the SEKAB pilot plant in Örnsköldsvik (Sweden) [33], and is also used in a demonstration-scale ethanol plant at Iogen in Ottawa (Canada) [34].

Hydrothermolysis, or liquid hot-water (LHW) treatment, involves treatment in water at high temperature. This method is similar to steam pretreatment, but lower temperatures and lower dry matter (DM) content are used, and thus more poly- and oligosaccharides are recovered [35, 36]. A catalyst, such as an acid, can be added, making the method similar to dilute acid pretreatment. Since the water content is much higher than in steam pretreatment, the resulting sugar solution is more diluted and thus causes the downstream processes to be more energy demanding. In the range 1–10 wt % DM virtually no difference in ethanol yield was found when bagasse was treated at 220 °C, after which SSF was performed using *S. cerevisiae* [37].

Wet oxidation pretreatment involves the treatment of the biomass with water and air, or oxygen, at temperatures above 120 °C, sometimes with the addition of an alkali catalyst. This method is suited to materials with low lignin content, since the yield has been shown to decrease with increased lignin content, and since a large fraction of the lignin is oxidized and solubilized [38]. As with many other delignification methods, the lignin cannot be used as a solid fuel, which considerably reduces the income from by-products in large-scale production. As discussed in the "Process Economics" chapter, it is extremely important to recover as much as possible of the lignin fraction (Sassner et al., in this volume).

Ammonia fibre explosion (AFEX) is also an alkaline method which, similarly to the steam pretreatment process, operates at high pressures. The biomass is treated with liquid ammonia for about 10–60 min at moderate temperatures (below 100 °C) and high pressure (above 3 MPa) [39, 40]. Up to 2 kg of ammonia is used per kg of dry biomass. The ammonia is recycled after pretreatment by reducing the pressure, as ammonia is very volatile at atmospheric pressure. During pretreatment only a small amount of the solid material is solubilized, i.e. almost no hemicellulose or lignin is removed. The hemicellulose is degraded to oligomer sugars and deacetylated [41], which is a probable reason for the hemicellulose not becoming soluble. However, the structure of the material is changed, resulting in increased water holding capacity and higher digestibility. Like the other alkaline pretreatment methods AFEX performs best on agricultural waste, but has not proven to be efficient on wood due to its higher lignin content [42, 43]. According to Sun et al. the AFEX process does not produce inhibitors that may affect downstream biological processes [44].

Another type of process utilizing ammonia is the ammonia recycle percolation (ARP) method [45, 46]. In the process aqueous ammonia (10–15 wt %) passes through biomass at elevated temperatures (150–170 °C), after which the ammonia is recovered. ARP is an efficient delignification method for

hardwood and agricultural residues, but is somewhat less effective for softwood.

3.4 Biological Methods

Biological pretreatment can be performed by applying lignin-degrading microorganisms, such as white- and soft-rot fungi, to the lignocellulose materials [44, 47]. The method is considered to be environmentally friendly and energy saving as it is performed at low temperature and needs no use of chemicals. However, the rate of biological pretreatment processes is far too low for industrial use, and some material is lost as these microorganisms to some extent also consume hemicellulose and cellulose, or lignin [42]. Nevertheless, the method could be used as a first step followed by some of the other types of pretreatment methods.

4 Results from Pretreatment Studies

There is a vast range of materials suitable for the production of ethanol. They can, somewhat arbitrarily, be put into three categories: agricultural, hardwood and softwood materials. It must be emphasized that it is not always possible to transfer the results from one type of material to another. During the last three decades, many types of materials using various pretreatment methods have been studied. Some hardwood materials, such as poplar, salix or aspen, have been frequently used in various investigations [48–52]. Other

Table 1 Typical composition of various lignocellulosic materials (% of dry material) and theoretical ethanol yield (L/ton DM) based on available carbohydrates (given as anhydrous sugars)

	Spruce	Douglas fir	Pine	Corn stover
Glucose	45.0	44.0	43.3	36.8
Mannose	13.5	12.2	10.7	–
Xylose	6.6	2.4	5.3	22.2
Arabinose	1.2	1.1	1.6	5.5
Galactose	1.6	3.5	2.9	2.9
Lignin	27.9	30.0	28.3	23.1
Other[a]	4.2	6.8	7.9	9.5
Ethanol from hexoses	425	422	403	280
Ethanol from pentoses	57	25	49	200

[a] Ash, extractives, protein etc.

materials examined have been straw [53–58], sugar cane bagasse [59–61] and olive tree wood [62], to mention a few.

In this summary some lignocellulosic materials were chosen for a more in-depth discussion. The materials that are discussed are an agricultural residue (corn stover) for which the hemicellulose is mainly composed of the pentose sugar xylose (about 22% xylose, 5.5% arabinose and 3% galactose; all as anhydro-sugars) and a softwood (spruce) where the hemicellulose mainly consists of the hexose sugar mannose (about 12–13% mannose, 5% xylose, 2% galactose and 2% arabinose; all as anhydro-sugars). Table 1 shows the composition of these materials as well as the theoretical amount of ethanol that can be produced from the hexose and the pentose fractions. It is clear that in some cases it is very important to utilize not only the hexose fraction, but also the pentose part of the material.

4.1 Corn Stover

The pretreatment of corn stover has been investigated in a large number of studies, as it is an abundant agricultural residue, primarily in the USA but also in Europe. In an extensive study undertaken in the USA, where the same batch of corn stover was pretreated using various pretreatment methods (acid hydrolysis by dilute acid, AFEX, ARP, lime treatment and LHW treatment) and then subjected to standard evaluation techniques, the yields of sugars were found to be more or less the same [63]. This study is commonly referred to as the CAFI study. Total sugar yields—after pretreatment followed by enzymatic hydrolysis—of around 90% or more were reached (see Table 2).

Teymouri et al. pretreated corn stover using AFEX [40], which resulted in 96% glucose yield and about 78% xylose yield after enzymatic hydrolysis of washed material, corresponding to a glucan concentration of 1 wt % after 168 h with 15 FPU/g cellulose Spezyme CP loading. The ammonia loading was 1 : 1 (equal amounts, by weight, of ammonia and dry corn stover) and the maximum sugar yield was obtained at 37 wt % moisture in the raw material.

Continuous ammonia pretreatment (ARP) can be used either by itself or preceded by percolation with hot water, in order to hydrolyse the hemicellulose under milder conditions and thus prevent hemicellulose loss. Kim et al. [64] performed low-liquid ARP and reported a glucan yield in enzymatic hydrolysis of 88% using Spezyme CP at an activity of 15 FPU/g glucan. The glucan recovery following APR was, however, not reported. Kim and Lee [65] also performed a two-step percolation using water in the first step and ammonia in the second. This resulted in 83% xylose recovery after pretreatment and 85% glucan yield in enzymatic hydrolysis, again using a Spezyme CP loading of 15 FPU/g glucan.

Several other types of alkaline solutions have been used for pretreatment of corn stover. Kaar and Holtzapple [66] used alkali pretreatment with lime to

Table 2 Summary of studies on corn stover

Method	Catalyst	Time (min) & temp. (°C)	Dry matter (wt%)	Enzymatic hydrolysis conditions	Glucose yield[a] (%)	Xylose yield[a] (%)	Refs.
AFEX	Conc NH_3	5, 90	63	1% glucan, washed, 50 °C, 15 FPU/g cellulose	96.0	77.7	[39]
ARP	NH_3	10, 170	23[d]	1% glucan, washed, 50 °C, 15 FPU/g cellulose	90.0	41.1[b]	[63]
Alkali	$Ca(OH)_2$	4 weeks, 55	na	1% glucan, washed, 50 °C, 15 FPU/g cellulose	92.0	52.8[b]	[66]
Dilute acid hydrolysis-1	H_2SO_4 0.49%	20, 160	5	1% glucan, washed, 50 °C, 15 FPU/g cellulose	91.6	91.2	[69]
Dilute acid hydrolysis-2	H_2SO_4 5%	90, 120	10	3% solids, 50 °C, 15 FPU/g solids, 72 h	54.6	100[c]	[68]
Steam-1	H_2SO_4	5, 190	9.5	5% solids, washed, 50 °C, 25 FPU/g solids, 48 h	73.6	61.0	[71]
Steam-2	SO_2	5, 190	35	2% solids, washed, 40 °C, 15 FPU/g solids, 96 h	90.0	84.0	[72]
Liquid hot water	Water	15, 190	16	1% glucan, washed, 50 °C, 15 FPU/g cellulose	85.2	26.3[b]	[70]
Wet oxidation	O_2, Na_2CO_3	15, 195	6	2% solids, washed, 50 °C, 25 FPU/g solids, 24 h	74.0	53.7[c]	[74]

[a] Based on monomer sugar obtained in the hydrolysate after pretreatment and in the enzymatic hydrolysis of pretreated material.
[b] A large fraction of xylose was released in oligometric form.
If this is taken into consideration the xylose yield increases to 76.4% for Alkali, up to 90% for ARP and 81.7 for Liquid hot water.
[c] This value is for xylose + arabinose as the sum was presented in the reference.
[d] Based on a liquid throughput of 3.3 mL of liquid per g of corn stover.

facilitate enzymatic hydrolysis. Pretreatment using 0.075 g $Ca(OH)_2$ per g dry biomass resulted in 88 and 87% yields in enzymatic hydrolysis for glucose and xylose, respectively, after 7 days hydrolysis. However, a rather high enzyme loading of 25 FPU/g dry biomass was used (which is about 42–50 FPU/g cellulose, assuming that the cellulose content in the pretreated material is about 50–60% of the total). At an enzyme loading of 23 FPU/g cellulose, which is more in the same range as that used for the data in Table 2, the glucose and xylose yields dropped to 60 and 47%, respectively. Higher yields were achieved when lower pretreatment temperatures (55 °C) and longer residence times (4 weeks) were used [67] (see Table 2). The pretreatment was in this case performed in an excess of lime, 0.5 g per g raw material with aeration, although only about 0.08 g lime was consumed per g raw material.

Varga et al. [68] reached high sugar yields with alkaline pretreatment using 10 wt % NaOH at 120 °C for 60 min. At these conditions more than 95% of the lignin and about 88% of the hemicellulose was removed from the solid material. After enzymatic hydrolysis, at 50 °C for 48 h, of the solid material diluted to 2 wt % WIS and using 25 FPU/g DM, the overall amount of released sugars reached 63.7 g per 100 g corn stover, which corresponds to a yield of about 82% of the theoretical based on the amount of hemicellulose and cellulose present in the raw material. This high overall sugar yield was also obtained using a considerably lower and, from an economic standpoint, more feasible alkali concentration of 0.5 wt % NaOH, after pretreatment at 120 °C for 180 min.

Dilute acid pretreatment is probably one of the most investigated pretreatment methods. The addition of acid enhances the yield of hemicellulose sugars significantly. Acids are also good catalysts during pretreatment. Kálmán et al. [69] used dilute sulphuric acid pretreatment and obtained a 55% overall glucose yield after enzymatic hydrolysis with 15 FPU/g dry biomass. Lloyd and Wyman [70] optimized the conditions for pretreatment of corn stover after soaking in H_2SO_4 at a dry matter content of 5 wt % solids and a H_2SO_4 concentration of 0.49 wt %. The pretreatment was performed in a reactor with indirect heating. The highest overall sugar yield, i.e. considering both glucose and xylose, was obtained for pretreatment at 160 °C for 20 min resulting in 91.6 and 91.2% glucose and xylose yield, respectively. The high liquid to solid ratio is very beneficial to prevent hemicellulose sugar degradation. They also reported 100% xylose solubilization in the pretreatment.

LHW pretreatment with controlled pH (the pH-adjusting chemical was not reported) has been performed by Mosier et al. [71]. In this study an enzyme loading of 15 FPU/g cellulose was used in the hydrolysis. An overall glucose yield of 91% and an overall xylose yield of 82% were obtained after 48 h hydrolysis.

Varga et al. [72] investigated steam pretreatment with H_2SO_4, i.e. by using direct steam at a higher dry matter content. The highest overall sugar yield (glucose, xylose and arabinose), 56.1 g/g raw material corresponding to 73%

of the theoretical, was obtained after pretreatment at 190 °C for 5 min with 2 wt % H_2SO_4 and enzymatic hydrolysis of 5 wt % solids using an enzyme loading of 25 FPU/g dry matter. At these conditions the overall glucose yield was about 74%.

Other acid catalysts have a similar effect on the hydrolysis of various materials. Öhgren et al. [73] also performed steam pretreatment, but instead of H_2SO_4 used SO_2 as acid catalyst at a concentration of about 2–3 wt % and at a higher dry matter content of 35 wt %. The highest overall sugar yield, 90% of theoretical for glucose and 84% for xylose, was obtained for pretreatment at 190 °C for 5 min.

In a study where the dry matter content was higher, i.e. 40 wt %, steam pretreatment of corn stover after impregnation with SO_2 was evaluated [74]. Pretreatment of SO_2-impregnated corn stover, with a dry matter content of 40 wt %, at 200 °C for 5 min resulted in a glucose yield of 92% of the theoretical and a xylose yield of 66%. The maximum xylose yield was 84%, obtained with pretreatment at 190 °C for 5 min. Under these conditions the glucose yield was 90%.

High enzymatic conversion of cellulose in enzymatic hydrolysis can also be achieved by wet oxidation [75]. The recovery of cellulose after wet oxidation of corn stover at 6 wt % WIS with 2 g/L Na_2CO_3 and 12 bar O_2 as catalysts, and at 195 °C for 15 min, was 85.1%. The enzymatic conversion was 83.4% and the overall glucose yield was 74% after enzymatic hydrolysis at 50 °C using 25 FPU/g dry pretreated corn stover. A decrease in enzyme activity to 5 FPU/g dry pretreated material decreased the overall yield to 63.4%. The overall yield of hemicellulose sugars was about 54%, which indicates a rather high degradation.

This shows that rather high sugar yields from corn stover can be obtained using a variety of pretreatment methods. Thus, it can be concluded that corn stover is easily hydrolysed using the enzymatic process. The overall sugar yields can come close to what is theoretically possible.

However, in the comparison shown in Table 2, hydrolysis was performed on washed material in most cases, and at low solids concentrations. In a full-scale process, the whole slurry from pretreatment would probably be used and at high solids concentrations. The hydrolysis yield alone is not an indicator of a successful pretreatment. The fermentability of the liquid fraction after pretreatment is, for example, an equally important parameter. Also, the concentration of sugars after hydrolysis must be high enough to result in an acceptable ethanol concentration. The duration of enzymatic hydrolysis required to reach the desired yield is another important factor as regards process economics, since longer reaction times imply larger reaction vessels for a certain production capacity.

Several studies have indeed been performed, both on SHF and SSF, at higher dry matter levels, but these are more difficult to compare. Varga et al. [76] performed SSF of wet-oxidized corn stover at high dry matter con-

tent, up to 15 wt % DM. However the wet oxidation was performed at 6 wt % DM under the following conditions: 2 g/L Na_2CO_3, 12 bar O_2, 195 °C, 15 min. The liquid was then removed and the concentrated pretreated solids were added back to a smaller amount of the liquid in a fed batch SSF. The highest yield in the SSF (performed at 30 °C using baker's yeast and 30 FPU/g dry pretreated stover for 120 h) was 83% of the theoretical based on the glucose content in the pretreated material. Considering that the recovery of cellulose in the pretreatment was 86%, the overall ethanol yield was 71% of the theoretical based on the glucose content in the raw material. A decrease of the enzyme loading in the SSF to 15 FPU/g dry pretreated corn stover resulted in a decrease in the overall ethanol yield to 63% of the theoretical.

Öhgren et al. (2006) [77] performed SSF on the whole slurry from corn stover, at 11 wt % WIS, after pretreatment of corn stover impregnated with 2 wt % SO_2 at 200 °C for 5 min. The SSF was performed as fed batch at 35 °C for 96 h using 5 g/L of a xylose-utilizing yeast, TMB3400, cultivated on pretreatment liquid and a cellulase loading of 15 FPU/g WIS. The overall ethanol yield was 92% of the theoretical, based on the glucose content in the raw material, and 59% based on the content of both glucose and xylose. The ethanol concentration was 36.8 g/L. However, a major part of the xylose was still left in the broth. The conclusions were that the cultivated yeast was tolerant to the inhibitors present in the pretreated slurry, but that it is necessary to develop a better feeding strategy to ferment all xylose.

The method of assessment of the pretreatment is crucial. In a study performed by Öhgren et al. (2007) [78], steam pretreatment of corn stover was assessed by enzymatic hydrolysis using ordinary cellulases supplemented with xylanases and also after partial removal of lignin. The pretreatment was performed either without any impregnation or with impregnation by SO_2. The conditions and overall sugar yields after pretreatment and enzymatic hydrolysis are given in Table 3.

The addition of small amounts of xylanases had a major impact on the sugar yield. The overall glucose yield after enzymatic hydrolysis increased from around 83% to near theoretical and the xylose yield from 71 to 74%, based on the content in the raw material for the pretreatment with catalyst. For the less severe pretreatment using auto-hydrolysis (i.e. 190 °C, 5 min, no catalyst), the addition of xylanases had an even higher effect resulting in an increase of the overall xylose yield from 74.6 to 85% of theoretical. The glucose yield increased even more from 69 to 94%. It should be noted that the addition of xylanases had a higher effect on the improvement of cellulose hydrolysis than on the increase of hemicellulose sugars.

It should be emphasized that the assessment with addition of xylanases was performed under pretreatment conditions that were optimized based on assessment with cellulases only in previous studies. Assessment with addition of xylanases during the optimization of the pretreatment might have resulted in less severe pretreatment conditions.

Table 3 Overall sugar yields as % of theoretical based on content in raw material after enzymatic hydrolysis of steam-pretreated corn stover, with and without impregnation with SO_2

Pretreatment conditions	Enzymatic hydrolysis using only cellulases[a]		Enzymatic hydrolysis using cellulases[a] with addition of xylanases[b]	
	Glucose	Xylose	Glucose	Xylose
170 °C, 9 min + SO_2	62.2	67.0	70.7	70.6
190 °C, 5 min + SO_2	83.2	70.5	96.0	73.9
190 °C, 5 min, no catalyst	69.3	74.6	93.8	85.3

[a] Celluclast 1.5L and Novozyme 188 (both from Novozymes A/S, Bagsaerd, Denmark)
[b] Multifect xylanase (from Genencor Int., Rochester, NY, USA)
Total amount of protein equal in all enzymatic hydrolyses

In order to compare pretreatment methods the whole optimized process, including process integration and the preparation and use of co-products, which may differ between different pretreatment methods, has to be assessed. These data are not available, not even for corn stover, which is one of the most investigated materials.

Eggeman et al. [79] investigated the pretreatment cost in ethanol production from corn stover for the five different pretreatment methods included in the CAFI study described above. The pretreatment design was based on experimental data [80] from the various research groups in the CAFI study, and was implemented in the Aspen Plus model for a full-scale bioethanol plant previously developed by NREL [81]. The model was based on a corn stover feed rate of 2000 dry metric tons per day. The process configuration was based on pretreatment, SSF, ethanol recovery and internal production of heat and electricity from the syrup and solid residue from the process. The process configuration was identical for all processes except for the pretreatment step. The dilute acid pretreatment process resulted in the lowest ethanol production cost, 0.26 US$ per litre for the base case alternative where oligomers released in the pretreatment and hydrolysis steps were not considered for ethanol production. However, it should be emphasized that a fairer comparison would require optimization of each process alternative taking into consideration the specific features of the pretreatment method used.

4.2 Softwood Species

Numerous pretreatment investigations have been carried out using agricultural residues and hardwoods. Softwood, on the other hand, has not been as thoroughly investigated. Table 4 presents a list of studies using softwood

Table 4 Pretreatment investigations using various softwoods as raw material

Substrate	Pretreatment conditions Catalyst	Temp. (°C)	Time	Refs.
Pine	0.5–12% SO_2	182–248	0.5–18 min	[81]
Spruce/pine	1–6% SO_2	190–230	2–15 min	[82]
Spruce	0.5–4.4% H_2SO_4	180–240	2–20 min	[89]
Pine	0.4% H_2SO_4	201–231	125–305 s	[87]
Spruce	0.5–5% H_2SO_4	190–220	50–250 s	[88]
Pine	4.5% SO_2	175–215	4.5–7.5 min	[11]
Spruce	3% SO_2	180–220	2–10 min	[84]
Spruce	0.5–1% H_2SO_4	180–220	2–10 min	[85]
Spruce	0.5% H_2SO_4/3% SO_2	180–220	2–10 min	[86]
Mixed[a]	H_2SO_4, pH 2–4	185–198	30–60 min	[28]

[a] Mixture of spruce, pine and fir

as raw material. As with any type of lignocellulosic starting material it is very difficult to compare the yields from the different investigations. The pretreatment step is usually evaluated using subsequent enzymatic hydrolysis; different substrate and enzyme concentrations in this step result in overall yields that are difficult to compare. Yields are often reported for a single step and occasionally no description is given for the yield calculations, which makes comparisons even more difficult. However, investigations on steam pretreatment and dilute-acid pretreatment by Clark et al. [82], Stenberg et al. [83], Tengborg et al. [84] and Söderström et al. [85–87] were all performed in a fairly similar fashion.

One of the most extensive investigations on the softwood *Pinus radiata* has been performed by Clark and Mackie [82]. They used steam pretreatment covering a temperature range of 148–248 °C, residence times of 0.5–18 min and catalyst concentrations of 0.5–12 wt % SO_2 (w/w dry wood). The pretreatment was assessed by enzymatic hydrolysis of washed solid material at 2 wt % WIS and 20 FPU/g WIS. The sugar yield increased with the impregnation concentration of SO_2, up to a concentration of 3 wt %. The optimal temperature and residence time, 215 °C and 3 min, were the same for the different concentrations of SO_2. The highest sugar yield after steam pretreatment and enzymatic hydrolysis was 57–60 g/100 g original dry wood (ODW), corresponding to 80–84% of the theoretically obtainable yield. Enzymatic hydrolysis improved with more severe pretreatment conditions, which also decreased the amount of carbohydrates in the solids. No investigations of the fermentability and the formation of by-products were reported.

Stenberg et al. and Tengborg et al. investigated steam pretreatment of softwood including impregnation with either SO_2 or H_2SO_4 in two different

studies [83, 84]. In the SO_2 study [83] mixed softwood (*Picea abies, Pinus sylvestris*) was used. The temperature range studied was 190–230 °C, with residence times of 2–15 min and SO_2 concentrations of 1–6% (w/w) WIS. The pretreatment was assessed by enzymatic hydrolysis on 2 wt % WIS of washed material with a cellulase activity of 15 FPU/g WIS and a β-glucosidase activity of 22 IU/g WIS. Fermentation of the liquid after pretreatment was performed with a yeast cell concentration of 9 g DM/L. Increasing severity resulted in the release of more sugars during pretreatment as well as a lower fibre yield. The yield in the enzymatic hydrolysis showed an optimum at medium severity, while the overall sugar yield increased with severity in the range studied. The fermentability decreased with increasing temperature. The optimal conditions were at a severity factor around 4.0 (215 °C, 3 min) with an SO_2 concentration of 3.5 wt %.

In the study with H_2SO_4-impregnated spruce [84], pretreatment was performed in a temperature range of 180–240 °C, with residence times of 1–20 min and H_2SO_4 concentrations of 0.5–4.4 wt %. Evaluation of the pretreatment was performed in the same way as in the SO_2 study. The yield of hexoses indicated that the optimal combined severity (2.3–2.7) for mannose was lower than that for glucose (2.9–3.4). Degradation of sugar increased with harsher pretreatment conditions. Enzymatic hydrolysis showed an optimum glucose yield in the same range of combined severity as that for pretreatment (2.9–3.4). Fermentation of the liquids after pretreatment showed that material pretreated at a combined severity higher than 3.4 was not fermentable. The highest overall yield of fermentable sugars, 35 g/100 g DM (70%), was obtained at 225 °C, 5 min and 0.5 wt % H_2SO_4.

Nguyen et al. studied the pretreatment of Douglas fir and Ponderosa pine [88]. Impregnation with 0.4 wt % H_2SO_4 was used and pretreatment was performed at temperatures from 201 to 231 °C and residence times of 125–305 s. The pretreatment was assayed with enzymatic hydrolysis, SSF and determination of the fermentability. Enzymatic hydrolysis was performed at a solids concentration corresponding to 1 wt % cellulose, and at an enzyme activity of 60 FPU/g cellulose. The overall glucose yield could only be calculated in one case (corresponding to pretreatment conditions of 212 °C and 105 s), resulting in a yield of 80% of the theoretical. The fermentability test showed that samples treated at 230 °C did not ferment, while some of those treated at 215 °C fermented poorly.

Schwald et al. [89] investigated SO_2 impregnation prior to steam pretreatment of Black spruce. Pretreatment was performed at temperatures of 190–220 °C, residence times of 50–250 s and an SO_2 concentration of 0.5–5 wt % (dry wood basis). Alkali treatment and H_2O_2 treatment after steam pretreatment were included in the study. The effects of pretreatment and post-treatment were evaluated with enzymatic hydrolysis of washed solid material at 2 wt % DM and 15 FPU/g substrate. The highest overall sugar yield was 50 g/100 g ODW, but no theoretical yield can be calculated, as the com-

position of the raw material was not given. Oligomers, which may have been present in the liquid, were not included in the sugar yield. The alkali extraction decreased the efficiency of enzymatic hydrolysis, while treatment with H_2O_2 improved it. Sulphur dioxide had a positive effect on enzymatic hydrolysis up to a concentration of 3.5 wt %. However, the yield in the enzymatic hydrolysis step alone is not a good measure of the overall yield, since loss of sugars in the pretreatment step must be taken into account. By-products were determined, but no fermentation was performed.

In the Lignol organosolv process softwood has successfully been treated, yielding a material that is susceptible to enzymatic hydrolysis and simultaneous saccharification and fermentation [28]. Ethanol (40–60%) was used with H_2SO_4 as catalyst at elevated temperatures (around 200 °C) to extract most of the lignin, which was recovered as a precipitate. The enzymatic hydrolysis yield was higher than 90%. However, the concentration of solid material (spruce/pine/fir) during hydrolysis was only 2%, which may be too low to see the effects of possible inhibitors. The overall sugar yield was not presented.

4.3 Two-Stage Pretreatment

From the investigations presented above it has been concluded that the maximum yields of mannose, the main hemicellulose sugar in softwood, and of glucose are not obtained at the same degree of severity. The optimal yield of mannose is obtained at a lower severity than that required for maximum digestibility of the cellulose in the subsequent enzymatic hydrolysis step. This suggests two-stage steam pretreatment, in which the first stage is performed at low severity to hydrolyse the hemicellulose, and a second stage, at a higher degree of severity, in which the solid material from the first step is pretreated again [82].

Although there are several studies on two-stage acid hydrolysis of softwood, the number of studies on two-stage steam pretreatment is scarcer. Söderström et al. [85–87] performed a thorough investigation on the two-stage pretreatment of spruce using either SO_2 impregnation or H_2SO_4 impregnation in both steps, as well as H_2SO_4 in the first stage and SO_2 in the second. The highest sugar yields were achieved for two-step pretreatment with either SO_2 impregnation or H_2SO_4 impregnation in both steps (see Fig. 2). A wide range of pretreatment conditions resulted in similar sugar yields of about 50 g per 100 g raw material.

The highest sugar yield was 51.7 g per 100 g, corresponding to 80% of the theoretical, obtained for pretreatment conditions of 190 °C for 2 min and 210 °C for 5 min. This yield (in %) is slightly lower than that reported by Nguyen et al. [14]. However, the amount of sugar obtained expressed as grams per 100 g dry raw material is higher. Ngyuen et al. stated that they obtained a sugar yield of 82%, which, in their case, corresponds to 46 g/100 g dry raw

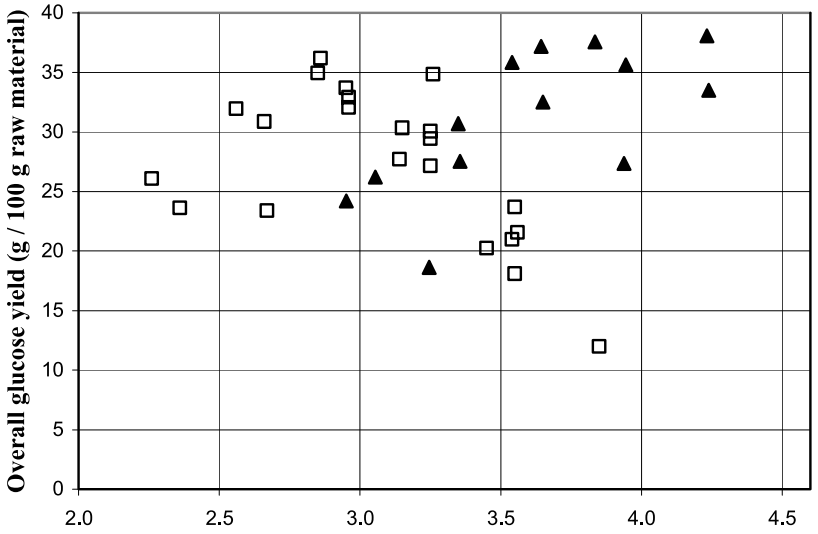

Fig. 2 Overall glucose yield

material. They used a cellulase activity of 60 FPU/g cellulose, which is more than twice the amount that was used in the study shown in Fig. 2. The maximum overall sugar yield obtained with two-step pretreatment using H_2SO_4 in both stages was only slightly lower, 77% of theoretical.

Besides overall sugar yield it is also of importance to investigate the fermentability of the pretreated materials. Impregnation with dilute H_2SO_4 followed by pretreatment at a high combined severity (i.e. high temperature and/or long residence time) resulted in materials that were not fermentable. Impregnation with SO_2, however, was successful in creating fermentable materials for all investigated pretreatment severities.

The two-step pretreatment results in a higher ethanol yield than does the one-step pretreatment, and it has also the advantage of lower requirement of enzymes and water in the SSF step. Major drawbacks are, however, the higher capital cost and the higher energy consumption. In a study by Wingren et al. [90] the overall ethanol production cost was shown to be very much dependent on the way the two pretreatment steps are performed, especially if the pressure is released or not between the steps, and also on the dry matter (WIS) content in the second step. The lowest cost estimated for the two-stage process, 3.90 SEK/L, which was about 6% lower than that for the one-stage process, requires a high ethanol yield, high concentration of WIS in the filter cake between the steps, and that the sugars being fed to the second step will not become degraded. The higher yield has been demonstrated experimentally, but the two other assumptions still need to be verified on the pilot scale.

5 Conclusions

In conclusion, a large number of pretreatment methods have been investigated and developed during the last 10 years, resulting in high recovery of sugars and rather high overall ethanol yields. However, most of the results were obtained in studies using batch-operating equipment on a rather small scale. Enzymatic hydrolysis has also, in most cases, been assessed at low substrate concentration.

One problem with the data produced so far is the difficulty in comparing methods, as the assessment is performed in different ways. In most cases the pretreatment is not assessed under realistic process conditions. The whole process must be considered as the various pretreatment methods give different types of materials: hemicellulose sugars can be obtained either in the liquid as monomer or oligomer sugars, or in the solid material to various extents; lignin can be either in the liquid or remain in the solid; the composition and amount/concentration of possible inhibitory compounds also vary. This will affect how the enzymatic hydrolysis should be performed (e.g. with or without hemicellulases), how the lignin is recovered and also the use of the lignin co-product.

For agricultural residues a large number of pretreatment methods result in high sugar yields while for wood, and especially softwood, the number of feasible methods is smaller. Acid hydrolysis and steam pretreatment with acid catalyst seem to be the methods that can be used for all types of raw materials, but the drawback is the high equipment cost and the formation of inhibitors. This requires further improvement and also a better integration with the enzymatic hydrolysis development, as improved enzyme mixtures may lead to less severe pretreatment conditions and thereby lower cost and reduce formation of inhibitory compounds.

To verify the technology the next step is to implement all of these improvements in a pilot-scale process with all steps integrated into a continuous pilot plant. This will provide better data for assessment and for scale-up to a demo- or full-scale process. It will also give better information on how various pretreatment conditions will affect all the other process steps, i.e. enzymatic hydrolysis, fermentation, downstream processing and wastewater treatment, as well as product and co-product quality.

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Substrate Pretreatment: The Key to Effective Enzymatic Hydrolysis of Lignocellulosics?

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Abstract Although the structure and function of cellulase systems continue to be the subject of intense research, it is widely acknowledged that the rate and extent of the cellulolytic hydrolysis of lignocellulosic substrates is influenced not only by the effectiveness of the enzymes but also by the chemical, physical and morphological characteristics of the heterogeneous lignocellulosic substrates. Although strategies such as site-directed mutagenesis or directed evolution have been successfully employed to improve cellulase properties such as binding affinity, catalytic activity and thermostability, complementary goals that we and other groups have studied have been the determination of which substrate characteristics are responsible for limiting hydrolysis and the development of pretreatment methods that maximize substrate accessibility to the cellulase complex. Over the last few years we have looked at the various lignocellulosic substrate characteristics

at the fiber, fibril and microfibril level that have been modified during pretreatment and subsequent hydrolysis. The initial characteristics of the woody biomass and the effect of subsequent pretreatment play a significant role on the development of substrate properties, which in turn govern the efficacy of enzymatic hydrolysis. Focusing particularly on steam pretreatment, this review examines the influence that pretreatment conditions have on substrate characteristics such as lignin and hemicellulose content, crystallinity, degree of polymerization and specific surface, and the resulting implications for effective hydrolysis by cellulases.

Keywords Biomass · Cellulose · Cellulases · Hemicellulose · Hydrolysis · Lignin · Steam pretreatment

1 Background

There have been several recent reviews [1–7] that have considered the various enzymatic factors that influence the efficiency of hydrolysis of lignocellulosic substrates. However, it is apparent that the physical and chemical nature of lignocellulosic substrates imparted by different pretreatment procedures are just as complex and influential as the enzyme systems used to breakdown the various components that comprise a lignocellulosic substrate into fermentable monosaccharides and other industrially relevant chemical compounds. Despite intensive research over the last 30 years or so, obtaining the rapid, complete and efficient conversion of cellulosic substrates by enzymatic hydrolysis remains a challenging goal. Up until about 5 or 6 years ago, various technoeconomic models had indicated that the enzyme production step of the overall biomass-to-ethanol process was one of the most expensive. Recent efforts by some of the world's leading industrial enzyme manufacturers have resulted in an approximate 20- to 30-fold reduction in the cost of cellulases utilized for the hydrolysis of pretreated corn stover [8]. However, it is acknowledged that the nature of the substrate and pretreatment method used continue to influence the effectiveness of the enzyme mix employed [9]. The significant decreases in the cost of the enzyme hydrolysis step have highlighted how the cost and nature of the biomass feedstock and the pretreatment method used to enhance both overall product recovery and enzymatic hydrolysis of the cellulosic and hemicellulosic components are significant technical and economic considerations.

Typically, after an initial rapid phase, the hydrolysis rate decreases rapidly during the saccharification process, resulting in lower glucose yields and longer processing times and, in most cases, the accumulation of a recalcitrant residue due to incomplete hydrolysis of the substrate. When a typical progress curve for enzymatic hydrolysis of cellulose is plotted, the reaction rate usually remains constant during the first few hours. However, the reaction rate eventually slows down and it has been suggested that the decrease in reaction rate

can be attributed to both enzyme- and substrate-related factors [2–4, 6]. Various substrate-related factors that affect hydrolysis include: how the presence of extraneous materials such as lignin and hemicellulose impede the action of cellulases, the influence of cellulose crystallinity and degree of polymerization (DP), and the amount of accessible surface area available to react with cellulases [2]. Enzyme-related factors that have been studied include: shear or thermally induced deactivation [10] occurring during mixing or exposure to high temperatures, the separation of enzyme components by the physical characteristics of the substrate resulting in a loss of synergism [11], as well as product inhibition due to an accumulation of cellobiose and glucose in the reaction medium. It is known that both enzyme- and substrate-related factors influence the efficiency of enzymatic hydrolysis [2]. However, depending on the nature of the substrate and pretreatment used, one factor could be more influential than another.

As mentioned earlier, an effective pretreatment method should be cheap (both capital and operating costs), effective on a wide range of lignocellulosic materials, require minimum preparation/handling steps prior to pretreatment, ensure recovery of all of the lignocellulosic components in a useable form, and provide a cellulosic stream that can be efficiently hydrolyzed with low concentrations of enzymes. With regard to the latter requirement, it would be beneficial if the pretreatment process could degrade the cell-wall structure by reducing the cellulose crystallinity, DP and particle size, while removing hemicellulose and lignin and increasing pore volume such that the cellulosic and hemicellulosic surface area available to the enzymes is greatly increased. However, as will be discussed in more detail, no one currently available pretreatment process can provide all of these desired outcomes on all lignocellulosic materials and it is the nature of the compromised conditions that will be described in this review.

1.1 Summary of Pretreatment Processes

Pretreatment strategies have generally been categorized into biological, physical and chemical processes, or a combination of these approaches.

Biological pretreatments typically utilize wood degrading fungi (soft, brown and white rot) to modify the chemical composition of the lignocellulosic feedstock. Generally, soft and brown rot fungi primarily degrade the hemicellulose while imparting minor modifications to lignin. White-rot fungi can more actively attack the lignin component [12]. Although there has been a fair amount of work done in this area, the primary application has been as a biopulping option for the pulp and paper industry rather than as a pretreatment for bioenergy applications. In addition to the requirements for careful control of growth conditions and for large amounts of space to perform biological treatments, a major disadvantage of biological/fungal treatments is the

typical residence time of 10–14 days. For these reasons, biological pretreatments are considered to be less attractive commercially.

Physical pretreatments involve the breakdown of the biomass feedstock into smaller particles that are more amenable to subsequent enzymatic hydrolysis. Physical treatments such as hammer- and ball-milling [13–16] have been shown to improve hydrolysis yields by disrupting cellulose crystallinity and by increasing the specific surface area of the feedstock, rendering them more accessible to attack by cellulases. One of the advantages of physical pretreatment is that it is relatively insensitive to the physical and chemical characteristics of the biomass employed. However, the physical pretreatment processes are energetically demanding and do not result in lignin removal. Lignin has been shown to restrict access and inhibit cellulases [17, 18]. Furthermore, physical pretreatments have yet to be shown to be economically viable at a commercial scale.

Most of the chemical pretreatments that have been assessed to date (typically acid and alkali based) have had the primary goal of enhancing enzyme accessibility to the cellulose by solubilizing the hemicellulose and lignin, and to a lesser degree decreasing the DP and crystallinity of the cellulosic component. Pretreatments that reduce cellulose crystallinity include mild swelling agents such as NaOH, hydrazine and anhydrous ammonia, and extreme swelling agents such as sulfuric acid, hydrochloric acids, cupram, cuen, and cadoxen [19]. Treatments that reduce the lignin content of the substrate include organosolv pulping with various solvents including ethanol, glycerol and ethylene glycol.

Typically, all chemical pulping processes in commercial use today involve the removal of lignin to produce pulp for various paper products. Although these processes could be considered as potential pretreatment methods, they are optimized to maintain the fiber/strength integrity of the pulp, not to increase accessibility to the cellulose. The relatively high value of pulp (at the time of writing, approximately US$730 per tonne of northern bleached softwood Kraft pulp in Europe according to the PIX Pulp Benchmark Index) can justify the high capital and operating costs of chemical pulping, while lower-value biofuels must seek cheaper pretreatment alternatives. Despite these apparent drawbacks, various groups have looked at modified pulping processes as potential pretreatment methods, most likely since these pulping processes produce readily hydrolyzable substrates. For example, in a Kraft pulping process NaOH and Na_2S are combined in an aqueous liquor to cook wood chips under elevated pressures, followed by a pressure-release defibration step. The resulting Kraft pulps have been shown to be receptive to subsequent hydrolysis by cellulases [16], most likely because of the combination of chemical dissolution of lignin and a decrease in average particle size that occurs during physical defibration.

Pretreatments that combine both chemical and physical processes are referred to as physiochemical processes. These pretreatment methods have

received the most attention in recent years and are the major focus of this review. In particular, steam pretreatment has received significant attention for its suitability in generating easily hydrolyzable substrates from lignocellulosic biomass. However, several aspects that affect the viability of the overall process will be discussed in more detail later in this review, including the handling and preparation of the feedstock prior to the pretreatment step, the need to minimize processing costs, and the need to maximize the value of coproducts derived from the hemicellulose and lignin streams. For example, if a pretreatment method has a requirement for very fine, uniform feedstock with a particle size of less than 10 mm, this will have a significant impact on the overall economic viability of the overall process because of the energy requirements to produce this fine material [20, 21]. Similarly, although acid-based pretreatment processes have been shown to be effective on a range of lignocellulosic substrates, downstream costs including the need for alkaline neutralization chemicals such as $CaOH_2$ [22], must be considered. At the same time alkaline-based pretreatment methods such as lime, ammonia freeze explosion (AFEX), and ammonia recycle percolation (ARP) processes can effectively reduce the lignin content of agricultural crops such as wheat straw and corn stover, but have a much more difficult time processing recalcitrant substrates such as softwoods.

To summarize this general introduction, it is unlikely that one pretreatment process will be declared a "winner" as each method has its inherent advantages/disadvantages. However, as discussed in more detail below, steam pretreatment is one method that is effective on a range of lignocellulosic substrates and, through companies such as Masonite, has been shown to work effectively at a commercial scale.

1.2 Steam Pretreatment of Biomass

Over the past 20 years, our group has looked at steam pretreatment (SP) with regard to its suitability for pretreating a range of lignocellulosic substrates, the subsequent ease of enzymatic hydrolysis of the cellulosic stream and the recovery of most of the hemicellulose sugars and lignin in a useable form. SP is an attractive pretreatment process as it makes limited use of chemicals, requires relatively low levels of energy and, depending on the conditions employed, results in the recovery of most of the original cellulose- and hemicellulose-derived carbohydrates in a fermentable form [23–28]. As will be discussed in more detail, SP disrupts the lignin barrier [29] and facilitates access of cellulases to the cellulose fibers [30]. Previous work has shown that SO_2-catalyzed SP is an effective pretreatment for softwood [26–34], hardwood [35–37] and agricultural residues [22] and that impregnation of SO_2 prior to pretreatment results in lower treatment temperatures and shorter reaction times, thereby improving hemicellulose recovery and reducing the

formation of sugar degradation products [23]. It has also been shown that SO_2 impregnation prior to SP enhanced the carbohydrate hydrolysis rate by increasing the accessibility of cell walls via the formation of fractures and the removal of hemicellulose during the steaming of the substrate [28], while reducing the DP of the oligomers and increasing the proportion of monomers

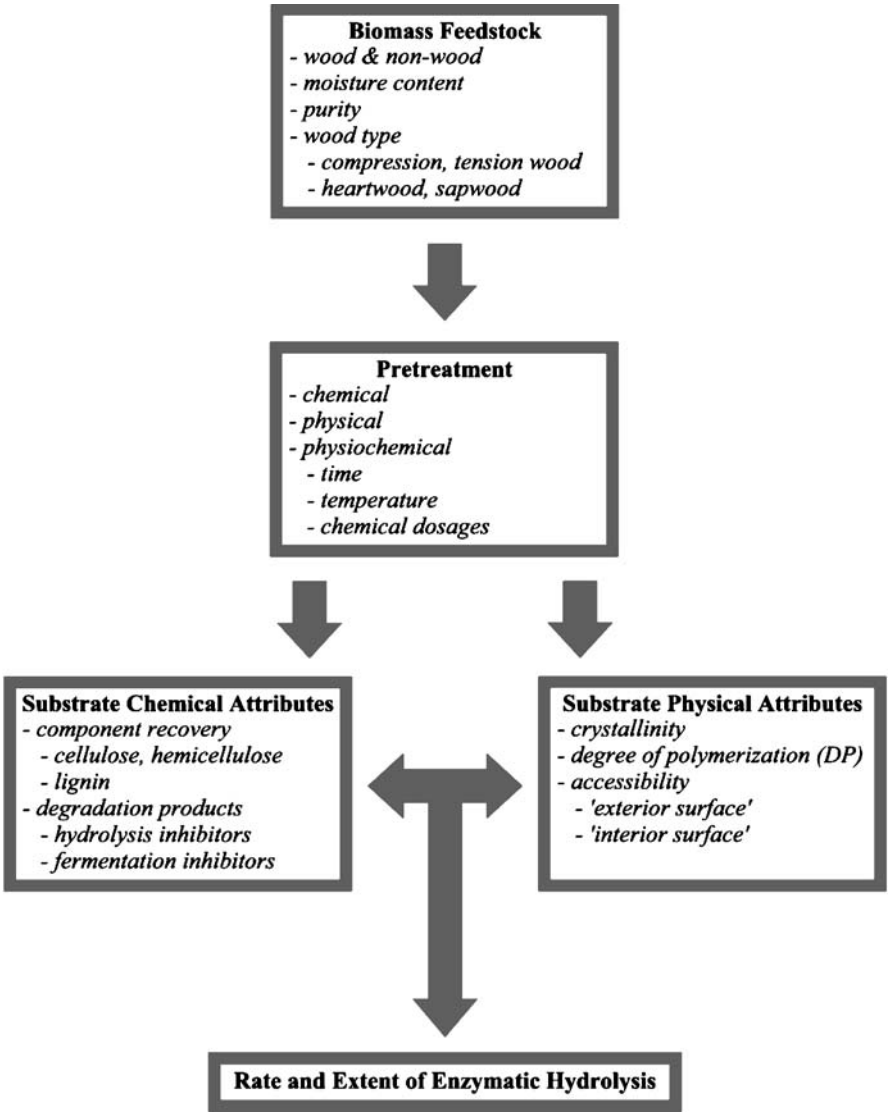

Fig. 1 Interrelated factors that govern the ease of hydrolysis of lignocellulosic substrates pretreated for bioconversion to ethanol

in the water-soluble stream [31, 38–40]. The "severity" of SP can be summarized by a single factor called R_o ($R_o = t \exp(T - 100)/14.75$) which links the effects of time (t, min) and temperature (T, °C) [41]. Due to the high temperature and acidic conditions employed during the SP process, depending on the "severity" (temperature, time, pressure, catalyst dosage) of the treatment, a portion of the hemicellulose-derived sugars and solubilized lignin fragments can be degraded or transformed into compounds such as furfural and 5-hydroxymethylfurfural (5-HMF); aliphatic acids, such as acetic, formic, and levulinic acid; and phenolic compounds [42]. It is known that these compounds can inhibit both downstream hydrolysis by cellulases [43] and fermentation of the liberated sugars to ethanol [44]. Therefore, compromised SP conditions have to be defined that provide an easily hydrolyzable cellulosic substrate, good recovery of the hemicellulose-derived sugars, ideally in a monomeric form, while minimizing the formation of inhibitors. Ideally, a reactive lignin stream, with a higher economic application than its intrinsic fuel value should also be obtained.

It is apparent that the nature of the substrate and the pretreatment method used has at least as much influence on the ease of enzymatic hydrolysis as does the nature and efficiency of the enzyme system used to conduct hydrolysis. As illustrated in Fig. 1, the efficiency of enzymatic hydrolysis of a given lignocellulosic substrate is the result of interplay of various factors. Although it is evident that substrates such as agricultural residues are generally less recalcitrant than softwood residues, it is recognized that enzyme- or substrate-related factors that govern effective hydrolysis can be controlled to an appreciable extent by the type and conditions of the pretreatment employed.

In the sections below we will describe how pretreatment, specifically SP, influences the characteristics of the substrate and the subsequent recovery of the cellulose, hemicellulose and lignin components. Recent progress in elucidating the role of substrate properties such as crystallinity, DP, pore volume, and available surface area in enzymatic hydrolysis will also be discussed using wood pulps as "model substrates". The final section offers concluding remarks and outlines the remaining challenges associated with understanding the progress of enzymatic hydrolysis during the bioconversion process.

2
Substrate Characteristics of Steam-Pretreated Wood

By selectively choosing pretreatment conditions it should be possible to create substrates with characteristics (hemicellulose and lignin content, particle size, available surface area etc.) that greatly enhance subsequent enzyme-mediated hydrolysis. For example we can adjust the time, temperature and SO_2 concentration of steam pretreatment with consequential effects on overall product recovery, ease of hydrolysis, etc. In addition to adjusting pretreat-

ment parameters, it has been shown that the inherent physical properties of the biomass can be used to anticipate the performance of the pretreated substrate in subsequent hydrolysis experiments [45–47]. The particle size, purity, moisture content, and the internal variations in the biomass feedstock, such as the presence of compression or tension wood can all have significant effects on the efficiency of pretreatment. As mentioned earlier, a reduction in the substrate particle size prior to pretreatment is a common practice used in some pretreatment processes [22]. However, size reduction through milling or grinding requires a substantial input of energy, and adds significantly to the total cost of the pretreatment [47]. Therefore, in the case of woody biomass, it is desirable to utilize a biomass particle size that can be produced economically at a large scale with existing equipment, such as the wood chips used in the pulp and paper industry [48].

For the operation of a large-scale bioconversion process a suitable wood chip size should be selected based on a compromise between the energy/cost of producing the chips and the subsequent effectiveness of the pretreatment and product recovery. Some of our previous work has shown that chip size and moisture content have a profound effect on the ease of hydrolyzability of the resulting substrate [46]. For example, by increasing the chip size of Douglas-fir (*Pseudotsuga menziesii* (Mirb.)) from 0.42 mm^2 to 5 cm^2 prior to SO_2-catalyzed steam pretreatment, greater recovery of the solids and a reduction in the production of inhibitors could be observed. This could be attributed to a decrease in the "relative severity" of pretreatment undergone by the larger chip at equivalent pretreatment conditions. It was also shown that the lignin present in the larger chips (5×5 cm) experienced less condensation and was therefore more amenable to subsequent alkaline peroxide delignification. This material from the larger chips consequently exhibited a 10–15% increase in hydrolysis yield over that obtained with the material originating from the smaller chips. Similarly, by increasing the moisture content of the chips from 12 to 30% an improved recovery of glucose and hemicellulose-derived sugars could be achieved. The increased recovery of sugars by raising the moisture content of the chips could be explained by a similar mechanism as it was observed with the increase in chip size. This was due to the additional moisture adsorbing the heat applied during steam pretreatment, resulting in a decrease in the severity of the treatment undergone by the chips.

In addition to the inherent variations in the properties of incoming biomass, pretreatment schemes must also deal with the fluctuating "purity" of the lignocellulosic substrate, as woody biomass can be expected to contain "contaminants" such as bark, needles, leaves, branches, etc, that differ significantly in their chemical composition from "white wood" [49]. It is likely that future wood-based bioconversion facilities would involve large amounts of biomass being sent to a chipping unit without careful control of debarking or branch removal. Similarly, it is unlikely that higher-value wood chips will be used

extensively in commercial bioconversion facilities due to competition from traditional pulp and paper mills. Therefore a bioethanol facility's feedstocks are likely to be either coppiced whole plants such as willow or, in the short term, residues from saw and pulp mills such as sawdust, shavings and hogfuel that contain significant amounts of bark, ash and lipophilic extractives. Tree thinnings such as branches have been pretreated using dilute acid during investigations assessing their viability as a biomass feedstock for bioconversion [50], where a two-stage pretreatment was required to hydrolyze 50% of the cellulose while the remaining cellulose was readily hydrolyzed by cellulases.

In similar work, utilizing feedstocks with high bark content, the liquid stream from the steam pretreatment (SP) of a Douglas-fir chip furnish containing 30% bark (SP-DFB) was shown to contain lower amounts of total sugars, furfural and 5-hydroxymethylfurfural compared to the liquid stream (prehydrolyzate) isolated from the pure whitewood (SP-DF) [51]. Although the concentration of lipophilic extractives increased in the bark-laden water-soluble stream, subsequent fermentation by *Saccharomyces cerevisiae* resulted in a complete utilization of the hexose sugars within 48 h with comparable ethanol yields regardless of whether bark was present or not. Although these results looked encouraging, with regard to the "robustness" of the overall SP process, the solid fraction of the pretreated Douglas-fir containing bark showed significant differences when compared to the whitewood. The addition of 30% bark to a Douglas-fir chip furnish prior to SP resulted in a significant increase in the lignin detected in the water-insoluble fraction. The SP-DFB solids fraction also contained 56% lignin compared to only 44% lignin in the case of the pure whitewood (SP-DF) sample. Consequently, the lignin content decreased to only 18% for the SP-DFB fraction as compared to 9% in the case of SP-DF upon subsequent alkaline peroxide delignification [52]. Although the alkaline peroxide delignified SP-DFB had a higher lignin and phenolic extractive content, it resulted in a similar hydrolysis yield to the SP-DF fraction, most likely due to the removal of surface lignin during the alkaline peroxide delignification stage [53]. Although hydrolysis was not performed in the absence of the delignification step, it can be expected that, due to its higher lignin content, the SP-DFB fraction would be more resistant to hydrolysis than was the SP-DF substrate. It is apparent that, during SP of lignocellulosic feedstocks such as Douglas-fir, the inherent properties of the wood have a significant effect on the downstream partitioning of the cellulose, hemicellulose and lignin components.

3 Substrate Lignin

Lignin is an aromatic network polymer composed of phenylpropane units [53]. It is generally accepted that lignin is the "glue" that binds cellulose and

hemicellulose, imparting both rigidity and moisture resistance to the lignocellulosic structure. Lignin has also been implicated as an inhibitor of cellulases; therefore, many of the pretreatment methods currently being explored have tried to decrease the lignin content of the solid substrate while minimizing the degradation of carbohydrates [22]. The amount of lignin that must be dealt with by a particular pretreatment and subsequent hydrolysis depends on the source of biomass. For example, corn fiber has a low lignin content of 7% (w/w) [23] compared to 30% (w/w) in the case of a softwood such as Douglas-fir [51]. In addition to the amount of lignin present in a biomass feedstock, the type of lignin differs between agricultural residues, hardwoods and softwoods [54]. Grasses and agricultural residues contain primarily *p*-hyroxyphenyl units while hardwoods and softwoods contain greater amounts of syringyl and guaiacyl subunits, respectively [54]. Softwoods lignin also exhibits a greater degree of cross-linking due to an extra linking site provided by the presence of only a single methoxyl group on the guaiacyl aromatic ring [54]. Another factor that must be considered is the existence of lignin carbohydrate complexes (LCCs) that consist of lignin linked to carbohydrates through bonds such as ester, ether or ketal [55]. LCCs have been shown to be particularly problematic for hydrolysis processes, as access to the carbohydrate fraction is restricted by the attached lignin, therefore pretreatment processes should either expand the pore structure of the substrate or remove the lignin outright [56].

3.1 The Effects of Pretreatment on Lignin Content

Pretreatment methods such as solvent extraction (organosolv pulping) [57] or ammonia fiber explosion treatment (AFEX) [58] either modify or remove lignin, while a large proportion of lignin remains intact in the solid phase after SO_2-catalyzed steam pretreatment [59] or dilute acid pretreatment [60]. Since lignin is intertwined amongst cellulose and hemicellulose, varying the pretreatment method or conditions employed to improve cellulose or hemicellulose yields will undoubtedly affect the lignin content [57, 59]. For example, it has been shown that the amount of lignin in the solid fraction of the substrate increases as the severity of SP is increased. Based on 13 severity factors used during SO_2-catalyzed SP of corn fiber, it was shown that the amount of lignin in the solid phase increased as the severity of the pretreatment was raised [59]. The lignin was most likely concentrated in the solid fraction due to the solubilization and degradation of the carbohydrates as the severity was raised [59]. It should be noted that the Klason method that is commonly used to estimate the lignin content of the pretreated substrate can result in artificially high values for lignin, as sugar degradation products and entrapped low molecular weight phenolics can also be measured as "lignin". For example, it was shown that the Klason lignin contents of steam-pretreated aspen

at a series of severities ranged from 6–30%. However, the supposedly corresponding methoxyl groups in the samples were only in the 0.8–7.7% range. This possible elevation of lignin values should be taken into consideration when determining Klason lignin content of a steam-pretreated substrate [61]. Realizing that it is difficult to reduce the amount of substrate lignin by fine-tuning SP process conditions, various researchers have explored methods of "post-treatment" to reduce the lignin content of steam-pretreated substrates.

In the past [62–64], we have tried to enhance the removal of lignin from steam-pretreated substrates, and consequentially increase the rate and extent of hydrolysis by cellulases, by applying several chemical post-treatments (Table 1). Oxygen–alkali and hydrogen peroxide post-treatments removed similar amounts of lignin and thus improved hydrolysis. However, of note, we also showed [64] that the removal of only 7% of the lignin from a steam-pretreated Douglas-fir substrate using a cold NaOH treatment resulted in a 30% improvement in hydrolysis yields, indicating that, in addition to the amount of lignin, the location of lignin is also an important factor affecting hydrolysis. Palonen et al. [65] have applied an alternative delignification method employing laccase enzymes in combination with mediators to steam-pretreated softwood, resulting in a slight release of aromatics into the system (delignification was not measured) and a corresponding 21% increase in hydrolysis yield. These researchers also showed that the oxidation of lignin surfaces by the application of laccases in the absence of mediators, as shown by others with pulp fibers [66, 67], also resulted in a 13% improvement in hydrolysis yield. These results suggest that the modification of lignin surfaces may also play a role in reducing its inhibitory effect on hydrolysis, perhaps affecting non-productive binding of cellulases to lignin. Since most of the studies have been concerned with altering lignin content by affecting the pretreatment conditions or applying post-treatments, there have only been

Table 1 Various chemical post-treatments applied to steam-pretreated Douglas-fir wood chips[a] to improve subsequent hydrolysis by cellulases

Treatment	Lignin removal (%)	Hydrolysis improvement[b] (%)	Refs.
1% H_2O_2, pH 11.5, 2% solids	90	45	[62]
Pressurized O_2, 15% NaOH, 5% solids	84	55	[63]
1% NaOH (cold), 4% solids	7	30	[64]

[a] Douglas-fir substrate prepared by steam pretreatment at 195 °C, 4.5% SO_2, 4.5 min

[b] Improvement in hydrolysis yield after 100 h hydrolysis reaction using 20 FPU cellulase/g cellulose in substrate supplemented at a ratio of 1 : 2 with β-glucosidase at a 2% solids (w/v) in 50 mM acetate buffer pH 4.8, 45 °C and shaking at 200 rpm

limited studies linking the various chemical structures in lignin to changes in hydrolytic activity of cellulases.

3.2 The Effects of Substrate Lignin on Enzymatic Hydrolysis

The lignin content and type of lignin has a significant effect on the hydrolysis of various cellulosic substrates as lignin acts as both a physical barrier, restricting access of cellulases to cellulose [68], and as an attractant to cellulases, resulting in non-productive binding [69, 70]. It has been shown that the chemical and physical structure of lignin plays a significant role in determining the magnitude of inhibition it contributes to hydrolysis, and the structure of lignin is heavily dependent on the conditions of the substrate pretreatment. However, some general observations can be made for substrates treated by specific pretreatments. The main chemical bond linking lignin subunits is the β-*O*-4 aryl ether bond [53, 54]. As a result, previous work that has examined the structure of lignin in pretreated substrates has mainly observed changes in β-*O*-4 aryl ethers and the resulting increase in free phenolic groups that occur after β-*O*-4 cleavage. During SO_2-catalyzed steam pretreatment, lignin tends to undergo decreases in both β-5 and β-*O*-4 aryl ethers [71, 72]. Due to the addition of SO_2, acid-catalyzed condensation reactions also occur, which are manifested by an increase in the number of aromatics substituted at the C6 [73–75]. It has also been shown that steam pretreatment performed at higher severity results in greater reductions in β-*O*-4 structures, resulting in more depolymerized lignin and a higher amount of free phenolic groups. Organosolv lignin from a mixed hardwood exhibited significantly lower amounts of β-*O*-4 structures than did steam-pretreated lignin from both yellow poplar and aspen [75, 76], which is indicative of the greater degree of delignification that occurs during the organosolv process.

Early work showed that exposure of cellulases to soluble lignin obtained from an alkaline organosolv process resulted in reduced hydrolytic activity [77]. Converse et al. [74] reported that the adsorption of cellulases to lignin resulted in decreases in the rate of enzymatic hydrolysis. There have been limited studies investigating the effects of specific lignin functionalities on cellulase activity, however, these studies have concluded that the likelihood of lignin binding cellulases can be linked directly to the presence of specific functional groups. This work is complicated by the fact that subtle changes in pretreatment conditions can result in significant changes in lignin structure [75, 76]. Sewalt et al. [78] added powdered lignins to ideal substrates in order to study the effects of lignin structure on cellulose hydrolysis. In the presence of a filter paper substrate and 1.5 mg/mL of lignin, cellulases exhibited reductions in activity of up to 60%. The inhibition by lignin was only moderately remedied by increasing the cellulase loading from 5 to 50 FPU/g cellulose, thus indicating that the inhibition resulted from a binding of cellulase to the

substrate. It should be noted that these studies added insoluble lignin to the reaction with filter paper, thus the particle sizes of the added lignin should also be considered. The authors concluded that the 6.3% phenolic group content measured in the organosolv lignin compared to 4.3% obtained for the steam-exploded lignin was most likely responsible for the increase in the inhibitory effect of the organosolv lignin. To test this further, the phenolic groups on the added lignin were blocked by hydroxypropylation, which resulted in a virtual elimination of the inhibitory effect of the added lignin.

Sewalt et al. [78] also incubated cellulases with lignin in the absence of substrate, which resulted in a 10–30% decrease in the protein content in the liquid phase, indicating a precipitation with lignin. The authors surmised that the enzyme was bound to lignin, resulting in its deactivation. However, the binding was strictly due to the presence of phenolic groups that mediated the addition of the enzyme to quinone methide groups on lignin. In a recent study, Berlin et al. [18] compared organosolv lignin isolated in the ethanol-soluble stream to the pulp residual lignin isolated by digest by cellulases. Both lignin preparations contained low amounts of β-*O*-4 and β-5 linkages, indicative of their extensive delignification. The dissolved lignin contained 19% more phenolic hydroxyl groups than the isolated residual lignin; however, the residual lignin contained 46% more aliphatic hydroxyl groups and 67% more carboxylic groups. Not surprisingly, the residual lignin was also found to be more condensed than the ethanol-soluble lignin. The incubation of cellulases with the ethanol-soluble lignin, with its higher phenolic content, resulted in a decrease in hydrolytic activity to a greater degree than the enzymatically liberated residual lignin sample. Unlike Sewalt et al, Berlin et al. attributed the difference in the inhibitory effects between the two lignin preparations to the lower amount of carboxylic groups and aliphatic hydroxyl groups of the ethanol-soluble lignin. This may have resulted in a more hydrophobic lignin preparation that was more amenable to hydrophobic interactions with cellulases. Unlike previous studies, the particle size of the lignin preparations was considered; however, the amount of cellulases that may have bound to the lignin preparations was not measured. The most likely explanation for the results is that a combination of increased lignin phenolic groups and increased hydrophobicity was responsible for the inhibition of cellulases by the various lignin preparations.

There has also been strong evidence [70, 79] supporting the role of hydrophobic interactions in the non-productive binding of cellulases to lignin. Multiple studies [70, 80, 81] have shown that the addition of the surfactant Tween, to cellulolytic hydrolysis improved hydrolysis yields. Similarly, the addition of agents such as BSA (bovine serum albumin) [69, 78], gelatin, and PEG (polyethylene glycol) [78] have also reduced the inhibition of cellulases by lignin. It seems safe to assume that during the hydrolysis of lignocellulosic substrates, the addition of a hydrophobic compound or surfactant to compete with the cellulase proteins for adsorption sites on lignin would re-

sult in a reduction in non-productive binding and an increase in hydrolysis performance [79, 80]. The surfactant may also facilitate the desorption of cellulases that have bound to lignin, similar to the enhancement in cellulase desorption observed during the hydrolysis of pure cellulose substrates in the presence of non-ionic surfactants. Overall, it is apparent that the surfactant, added protein or compound possessing both a hydrophobic and hydrophilic component, aids in reducing the adsorption of cellulases to lignin thereby improving the hydrolysis performance.

Considering the detrimental effect of lignin–enzyme interactions on hydrolysis performance, Berlin et al. [82] introduced a novel approach to enzyme improvement involving a reduction in the affinity of enzymes for lignin rather than an alteration of the substrate. It was shown that naturally occurring enzymes with similar catalytic activities tested on "model" substrates such as Avicel and Sigmacell may differ in their interaction with lignin, which may therefore affect performance on the native substrate [82]. As mentioned earlier, Berlin et al. [18] investigated enzyme–lignin interactions, and isolated and characterized two lignin preparations from softwood using ethanol organosolv pretreatment. After testing seven different cellulase preparations, three different xylanase preparations and one β-glucosidase preparation, it was shown that the various cellulases differed by up to 3.5-fold in their inhibition by lignin, while the xylanases showed less variability. Moreover, β-glucosidase was least affected by lignin. The authors concluded that the selection or engineering of so-called "weak-lignin-binding enzymes" in the future will offer an alternative means of enzyme improvement [82]. Overall, it has been demonstrated that the presence of lignin presents a significant obstacle during hydrolysis. However, early work [83] has also shown that hemicellulose removal during pretreatment also results in significant improvements in hydrolysis performance. Hemicelluloses differ significantly from lignin, since their recovery is quite sensitive to changes in processing conditions, and their hydrolysis can potentially be used to fortify recovered sugars to increase ethanol yields in subsequent fermentation [59].

4 Substrate Hemicelluloses

Lignocellulosic biomass feedstocks are composed of various types of hemicelluloses. A recent review by Saha [84] detailed hemicellulose structure, enzymatic saccharification, fermentation and the production of potentially valuable products from a hemicellulose hydrolyzate. Hemicelluloses are heterogeneous, branched polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. Hardwood hemicelluloses are composed mainly of xylan [54]. Xylans from many plant materials are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked

β-D-glucopyranose units. Besides xylose, xylan may contain arabinose, glucuronic acid or its 4-*O*-methyl ether, and acetic, ferulic, and *p*-coumaric acids. The frequency and composition of branches depends on the source of xylan [54]. Softwood hemicelluloses consist of mostly galactoglucomannans, with a linear or branched chain of 1,4-linked glucose or mannose units. Other softwood hemicelluloses include arabinoglucuronoxylan, arabinogalactan and others [54]. Within the plant cell wall architecture, hemicelluloses are thought to coat the cellulose-fibrils resulting in a reduced accessibility of the cellulose-fibrils [83]. Therefore, enzymatic hydrolysis of the hemicelluloses is essential to facilitate complete cellulose degradation. Given the diversity of xylan and mannan structures, a variety of hemicellulases such as endo/exoxylanases, arabinosidases, acetylesterase, glucoronisidases, and mannanases should be required to degrade hemicellulose. Therefore, a pretreatment such as SO_2-catalyzed steam pretreatment, which degrades a significant amount of hemicellulose to monomeric sugars, would be invaluable for their potential utilization in fuel and/or bioproduct production.

4.1 The Effect of Pretreatment on Hemicellulose Content

The removal of hemicellulose undoubtedly imparts substantial modifications to the structure and accessibility of lignocellulosic substrates. Ideally, the pretreatment should fractionate the cellulose, hemicellulose and lignin so that cellulases can react with pure cellulose. However to obtain the greatest value from biomass feedstocks, it will be necessary to recover all of the components in an exploitable form [22]. Most of the pretreatments that are currently being advocated for their potential application in the bioconversion process employ "optimal" treatment conditions that maximize the recovery of cellulose. However, these conditions differ from those that target maximum lignin and hemicellulose yields [85]. Of the three main components of lignocellulose, hemicelluloses have been shown to be the most sensitive to changes in pretreatment conditions [59]. Steam pretreatment at high severity (higher temperature, residence time and catalyst concentration) is required to maximize cellulose and lignin yields, while a significant portion of the hemicellulose is destroyed at these conditions [86]. Hemicellulose degradation products such as furfural and hydroxymethyl furfural inhibit subsequent fermentation [87]. Therefore, pretreatment conditions are frequently tailored considering the compromise between separating the lignin and hemicellulose components from cellulose while concurrently maximizing the recovery of all the available carbohydrates. Each pretreatment method approaches the recovery of hemicellulose in a distinct manner, as diverse pretreatment methods all have varying effects on the hemicellulose fraction.

Depending on the nature of the pretreatment, either a solid fraction, or combined solid and liquid fractions are generated for subsequent sacchari-

fication processes [22]. For example, a pretreatment such as AFEX produces only a solid fraction [88] while pretreatments at an acidic pH such as dilute acid, steam pretreatment and wet oxidation produce a combined liquid and solid stream [22]. Alkaline pretreatment methods such as AFEX modify and/or remove lignin and leave both hemicellulose and cellulose intact [22]. Acidic pretreatments, such as SO_2-catalyzed steam pretreatment or dilute acid pretreatments, depending on the acid catalyst employed, produce a liquid fraction that is composed mainly of hemicellulose, either in monomeric or oligomeric form [59].

The tendency for hemicellulose to be relegated to either the liquid or solid fraction is highly dependent on the severity of the pretreatment (time, temperature and amount of catalyst). In the case of aspen wood (*Populus tremuloides* (Michx.)), it was shown that varying the steam pretreatment conditions followed by alkaline peroxide bleaching of the solid fraction resulted in large variations in the cellulose and hemicellulose content of the solid fraction. Increasing the severity of pretreatment decreased the cellulose and hemicellulose molecular weight. For example, it was shown that at low severity the solid fraction contained a xylan content of 7% and a cellulose molecular weight of 900 000, while at higher severity the xylose content and cellulose molecular weight in the solid fraction were reduced to 1% and < 40 000, respectively. Without the alkaline peroxide treatment, the xylose content in the solid fraction varied from 12.4 to 2.5% as the severity of the steam pretreatment was raised from $\log_{10} R_o$ 2.76 (180.3 °C, 2 min) to $\log_{10} R_o$ 3.62 (189.1 °C, 10 min) [59]. Similarly, during SO_2-catalyzed steam pretreatment of corn stover, the solid fraction obtained from pretreatment at 190 °C for 5 min while varying the catalyst dosage from 0 to 3% SO_2 was composed of almost identical amounts of glucan (56%); however, the amount of xylan differed from 18 to 10%, respectively [89]. Similar results were reported by Boussaid et al. [39] while treating Douglas-fir wood chips as pretreatment at low severity (175 °C, 7.5 min, and 4.5% SO_2) resulted in the solubilization of 87% of the hemicellulose component into the water-soluble stream with 80% of the recovered sugars in monomeric form. However, the recovery of hemicellulose was reduced to 43% upon increasing the pretreatment severity (215 °C, 2.38% of SO_2, 2.38 min) with a concomitant increase in the production of sugar degradation products as furfural and hydroxymethylfurfural, which compromised subsequent fermentation to ethanol by *Saccharomyces cerevisiae*.

Later work by Shevchenko et al. [90] showed that the hemicelluloses solubilized into the liquid stream during the steam pretreatment of Douglas-fir wood chips that remained in oligomeric form could be further processed by an acid-catalyzed post-hydrolysis step in order to recover the remaining hemicellulose in monomeric form. Furthermore, it was shown that the partial oxidation undergone by the added SO_2 catalyst during steam pretreatment to sulfuric acid provided an acid concentration sufficient to depolymerize the remaining

oligomeric hemicelluloses with only minimal production of fermentation inhibitors. These results show that the steam pretreatment process is capable of recovering a large proportion of the feedstock hemicelluloses in a monomeric form, depending on the pretreatment conditions employed. Although process modifications to improve hemicellulose recovery decrease the production of inhibitory compounds and increase total sugar concentrations, which aid in subsequent fermentation, ultimately, it is the hydrolysis of the solid cellulosic substrate that provides the majority of the glucose for ethanol production. Increasing the total recovery of sugars while minimizing inhibitor production must be weighed against the negative effect of significant amounts of substrate hemicellulose on the ease of hydrolyzability of the substrate.

4.2 The Effect of Substrate Hemicellulose Content on Hydrolysis

Compared to studies on lignin content, studies evaluating the effect of hemicellulose content on the rate and extent of hydrolysis of lignocellulosics have been far less frequent. The lack of studies in this area is most likely due to the sensitivity of the hemicellulose component to the pretreatment conditions. However, it has been widely accepted that, similarly to lignin, hemicellulose acts as a barrier within the lignocellulosic matrix restricting access of cellulases to cellulose [11, 91, 92]. In the case of steam-pretreated substrates, strong evidence supporting the role of hemicellulose in cellulose hydrolysis has been shown on numerous occasions, as increasing solubilization of hemicellulose during pretreatment has facilitated subsequent hydrolysis by cellulases [26, 34, 93]. As discussed earlier, the pretreatment process is a balance between maximizing recovery and removal of hemicelluloses from the solid fraction, while minimizing degradation of hemicellulose sugars to fermentation inhibitors. However, especially in the case of softwood substrates, it is challenging to obtain the conditions that minimize hemicellulose degradation while solubilizing sufficient hemicellulose sugars in the solid component to promote the complete degradation of cellulose in subsequent hydrolysis. This point was studied in depth by Boussaid et al. and Wu et al. [34, 94] who concluded that during steam pretreatment of Douglas-fir wood chips, higher severities resulted in a near complete solubilization of the hemicellulose with a concomitant increase in enzyme digestibility of the cellulose component; however, this was accompanied by the production of fermentation inhibitors. Due to these factors, researchers utilizing SO_2-catalyzed steam explosion pretreatments have focused on employing a medium severity pretreatment, which improves the enzymatic hydrolyzability of the solids and results in the recovery of the majority of hemicellulose in a monomeric form within the water-soluble stream [23, 95]. Although pretreatment and hydrolysis studies have strongly suggested that hemicellulose hinders the hydrolysis of lignocellulosic substrates, further evidence has been presented in studies that have

employed cellulases in combination with hemicellulases to hydrolyze pretreated substrates.

In nature, woody lignocellulosics are degraded by microorganisms such as white-rot fungi, which are capable of degrading the entire wood structure, utilizing the combined activities of a host of enzyme such as cellulases, hemicellulases, pectinases, lignin peroxidases, manganese peroxidases, and laccases [12]. Therefore, it is to be expected that the presence of peripheral materials such as hemicellulose and lignin would be an obstruction when applying cellulases exclusively for the hydrolysis of lignocellulosic substrates. Indeed, recent work by Berlin et al. compared the hydrolysis performance of seven fungal cellulase preparations from both *Trichoderma* and *Penicillium* on steam-pretreated softwoods and organosolv-pretreated softwood and hardwood substrates, only to find that the differences in performance among the enzyme preparations was heavily dependent on the level of β-glucosidase supplementation [96, 97]. Consequently the β-glucosidase added to the hydrolysis reaction also contained xylanases, which according to the authors, most likely facilitated the cellulose hydrolysis by removing xylan, thus improving accessibility of the substrate to cellulases. Similar results have been shown when combining cellulase with xylanase for treating pulps used in papermaking, as xylanases have been shown to act synergistically with cellulases to improve paper properties. In the case of Douglas-fir Kraft pulp fibers, cellulase–xylanase combinations were shown to improve fiber/paper strength properties [98], most likely by improving accessibility to cellulases by the removal of xylan and its substituents as well as any associated low molecular weight lignin fragments by the xylanases [99]. Likewise, cellulases have been shown to improve the accessibility of xylanases to softwood Kraft pulps as mild cellulase pretreatments increased the apparent median pore size of the pulp to facilitate subsequent prebleaching by xylanases [100].

From the discussion thus far it is apparent that the pretreatment methods currently being advocated for their potential application in bioconversion processes have a significant effect on the proportions of lignin cellulose and hemicellulose within the substrate, while the presence of hemicellulose and lignin plays a significant role in affecting the ease of enzymatic digestibility of lignocellulosics. However, the effects of lignin and hemicellulose are only part of the "hydrolysis puzzle", as it is likely that changes in the primary components of lignocellulose also have significant effects on the physical and chemical characteristics of the substrate.

5
Physical Properties Affecting the Hydrolysis of Substrates by Cellulases

The assignment of specific substrate factors that render a substrate recalcitrant to cellulase hydrolysis is a controversial subject. Crystallinity, DP and

specific surface area have all been thought to undergo significant changes during pretreatment, consequently influencing subsequent hydrolysis [2]. The original work prior to the 1990s, focusing on the physical characterization of substrates, has been presented in previous reviews [2, 7]. More recently, our group and others have published work correlating the physical properties of wood pulp fibers employed in papermaking to their hydrolyzability, as cellulases have been explored as a potential means to improve both the drainage of recycled pulps and to enhance pulp fiber properties [101]. Admittedly, pulps are not identical to substrates pretreated specifically for subsequent hydrolysis by cellulases. However, it should be noted that, similarly to pretreated substrates, pulp fibers also represent a lignocellulosic matrix and that the general principles gained from examining the physical properties of pulp fibers that affect hydrolysis can be cautiously extrapolated to substrates pretreated for bioconversion.

5.1 Specific Surface Area

As has been demonstrated previously with pretreated substrates [30], the rates and extents of hydrolysis of pulp fibers have also been directly correlated to their initial specific surface area. This is not surprising, since the very existence of a substrate pretreatment step stems from the necessity to increase the accessibility of reaction sites on substrates to cellulases, as lignocellulosic substrates such as wood possess limited reactive surface area available to cellulases prior to pretreatment. Coincidentally, both the chemical and mechanical pulping processes that have been applied to produce pulp for the formation of paper also result in an increase in accessibility to cellulases compared to the starting lignocellulosic furnish. During pulping, wood chips are subjected to either physical or physiochemical action, resulting in the breakdown of the lignocellulosic matrix into fiber cells [48]. Consequently, the breakdown of the wood yields fibers with various physical attributes such as length, coarseness, width, kink and curl [102, 103]. Surface area of pulp fibers can be divided into exterior surface area affected mainly by fiber length and width, or interior surface area, which is governed by the size of the lumen and the number of fiber pores and cracks. The varying fiber lengths and widths produced during pulping can be viewed in a similar manner as the array of particle sizes produced during the pretreatment of lignocellulosic substrates for bioconversion. The specific surface area of a mixture of particles is inversely proportional to their average diameter, therefore, a smaller average particle size results in an increase in surface area. Indeed, cellulases have been shown to act on the surface of pulp fibers, resulting in a "peeling effect" [104]. Therefore, smaller particle sizes with a greater amount of specific surface area would be expected to hydrolyze at a faster rate.

In an investigation assessing the hydrolysis of Douglas-fir Kraft and mechanical pulps, Mooney et al. showed that at equal lignin contents, the "fines" (small particles) of a delignified mechanical pulp were hydrolyzed faster than the longer fibers (large particles) of the Kraft pulp [17]. When each fiber length fraction was hydrolyzed separately, it was shown that the isolated long fiber fraction hydrolyzed slower and consequently adsorbed a lower amount of cellulases than the whole pulp [17]. The increased hydrolysis rate of the whole pulp was attributed to the greater amount of specific surface available for the adsorption of cellulases provided by the pulp fines and short fibers. Although it is apparent that particle size has a significant effect on cellulose hydrolyzability, it has also been shown that the fiber delamination and enhanced swelling that results from mechanical treatment of Kraft and recycled pulp fibers has a greater effect on hydrolysis by cellulases than does a decrease in particle size [105]. Since recycled pulps originate from fiber sources that undergo irreversible changes in their structure upon drying [106], their swelling properties must be regenerated by employing a mechanical treatment referred to as "refining" or "beating". The swelling properties of pulps can be measured using the water retention value measurement [107]. After beating, the pulp sample usually drains at an inadequate rate to be used on a high-speed paper machine. Consequently, cellulases have been shown to improve the drainage of recycled pulps. Oksanen et al. [108] applied separate EG1, EG2 and CBH1 cellulase components to pulps during each recycling round. As each pulp was beaten after recycling, the water retention value (WRV) increased and the pulp became more responsive to cellulases, especially EG1 and EG2. Although the particle size and swelling properties of pulps have been shown to be related to the ease of hydrolysis of lignocellulosic substrates, it has been shown that a greater amount of information related to the action of cellulases can be obtained from measurements of the pores or "interior" surface area of pulp fibers available for penetration by cellulases.

Direct correlations have been found between the initial pore volume or interior surface area of lignocellulosic substrates and their extent of hydrolysis [30, 83, 90]. It has been proposed that the efficacy of cellulose hydrolysis is enhanced when the pores of the substrate are large enough to accommodate both large and small enzyme components to maintain the synergistic action of the cellulase enzyme system [11]. From extensive studies, Grethlein et al. [83] and others [109–111] have found that the rate-limiting pore size for the hydrolysis of lignocellulosic substrates was 5.1 nm, thus the solute exclusion technique utilizing molecular probes in this size range has been shown to be effective for assessing the pore volume of substrates. Mooney et al. [68] utilized dextran molecular probes in the solute exclusion method to measure the pore volume of refiner mechanical pulp (RMP), sulfonated RMP, sodium chlorite delignified RMP and Kraft pulp from Douglas-fir to assess the ease of these pulps to subsequent hydrolysis by cellulases. As mentioned earlier, the delignification of the RMP resulted in a greater rate and extent of hydrolysis

than the Kraft pulp sample, which may be attributed to the smaller particle size of the RMP. The sulfonation of the RMP dramatically increased swelling. Unlike delignification, however, this did not translate into either enhanced access to the 5.1 nm probe or hydrolysis performance. The most feasible explanation for these results is that the lignin content of the sulfonated pulp (30.9%) inhibited hydrolysis, regardless of the greater swelling of the pores, thus demonstrating the detrimental effect of substrate lignin on hydrolysis as mentioned earlier.

Since it is well known that the pore volumes of pulps undergo significant reductions upon drying [106], Esteghlalian et al. [112] innovatively applied the Simons' stain technique to measure changes in pore volume imparted by air, oven and freeze drying prior to enzymatic hydrolysis. As expected, drying significantly reduced the number of larger pores in the pulp sample, which most likely restricted the access of the fibers to cellulases and thus decreased hydrolysis rate over 12 h [112]. Although the specific surface area of the substrate provided by decreased particle size and increased swelling and pore volume plays a significant role in facilitating hydrolysis by cellulases, the interconnecting role of other substrate factors such as crystallinity and DP should also be considered.

5.2 Cellulose Crystallinity and Degree of Polymerization

There have been only limited studies assessing the significance of initial cellulose crystallinity and DP of lignocellulosic substrates with regard to subsequent substrate hydrolysis by cellulases; however, the importance of these factors has been the subject of considerable debate [113, 114]. It has been suggested that amorphous cellulose is hydrolyzed, initially resulting in an accumulation of crystalline cellulose rendering the substrate increasingly recalcitrant as the hydrolysis progresses [113, 114]. Most studies that have established a correlation between crystallinity and hydrolysis have utilized substrates of relatively pure cellulose, which most likely do not represent the heterogeneous lignocellulosic substrate encountered during the hydrolysis of substrates pretreated for bioconversion [115–117]. Furthermore, to demonstrate the effect of crystallinity on hydrolysis, these studies frequently utilize physical treatments such as ball milling [116] or gamma irradiation [118] to alter the initial substrate crystallinity, which can also result in increases in specific surface area. As a result, in previous work both crystallinity and specific surface area of pure cellulose substrates have been combined into models predicting the rate and extent of hydrolysis [119]. As for crystallinity, it is difficult to assess the effects of DP exclusively, since altered DP can be associated with crystallinity or accessible surface area. Nevertheless, there have been a few studies investigating the effects of the crystallinity and DP of chemical pulps on their hydrolysis by cellulases.

Employing unbeaten, beaten and recycled softwood pulps as substrates to assess various substrate characteristics that influence the enzymatic hydrolysis of cellulose, Nahzad et al. [105] showed that although the pulps possessed similar crystallinity, the beaten pulp hydrolyzed more readily than the unbeaten pulp without any appreciable changes in crystallinity occurring during the hydrolysis. Similar results have been found by Ramos et al. during the treatment of fully bleached eucalyptus Kraft pulp [104] and by Mansfield et al. during combined cellulase–xylanase treatment of Douglas-fir Kraft pulps [99]. Nahzad et al. [105] also showed that the initial DP of the pulps did not play a role in affecting subsequent hydrolysis; however, the DP was decreased by 2/3 during the hydrolysis period and the polydispersities of all the hydrolyzed pulps were quite similar. Mansfield et al. [99] did not observe appreciable changes in cellulose DP during hydrolysis, however, it should be noted that they employed low cellulase loadings to impart subtle modifications to the pulp fiber. It is evident from the literature presented here that attributing the ease of enzymatic digestibility of a given substrate to initial crystallinity or DP is a dubious task, compared to studies that have tied the ease of hydrolysis of substrates to their initial surface area. However, pore volume determinations require a significant investment in time to obtain reproducible results. Also, it is likely that the pores in a lignocellulosic substrate will have irregular shapes, thus affecting the accuracy and precision of the measurement [120]. Another drawback is that the method does not measure the areas in pores that are larger than the size of the probe, which would provide the easiest access for cellulases. Investigations into the substrate physical factors that affect hydrolysis should be aided by the continual evolution of analytical techniques such as thermoporosimetry [121] and high resolution fiber quality analysis [122], which may be capable of dealing with the diversity of pretreated lignocellulosic substrates produced for subsequent hydrolysis and fermentation in the bioconversion process.

6 Conclusions

In this review we suggested that, although the properties of the cellulase enzyme complex has a significant effect on how effectively a lignocellulosic material will be hydrolyzed, it is the biomass pretreatment and the intrinsic structure/composition of the substrate itself that are primarily responsible for its subsequent hydrolysis by cellulases. It is apparent that in sequential series of events, the conditions employed in the chosen pretreatment will affect various substrate characteristics, which in turn govern the susceptibility of the substrate to hydrolysis by cellulases and the subsequent fermentation of the released sugars. Choosing the appropriate pretreatment for a particular biomass feedstock is frequently a compromise between minimizing the

degradation of the hemicellulose and cellulose components while maximizing the ease of hydrolysis of the cellulosic substrate. The digestibility of pretreated lignocellulosic substrates is further complicated by the lignin–hemicellulose matrix in which cellulose is tightly embedded. Pretreatment conditions can be tailored to create either solid or solid/liquid substrates with varying levels of cellulose, hemicellulose and lignin. It is apparent that lignin affects enzymatic hydrolysis by blocking cellulose and by chemical interactions facilitated by its hydrophobic surface properties and various functional groups. The role of hemicellulose is less obvious although there is good evidence to support the action of hemicellulose as a barrier restricting access to cellulases. In the past, many investigators have attributed the enhanced enzymatic hydrolysis performance of a particular pretreatment to changes in the proportion of the lignin, hemicellulose and cellulose in the substrate. However, it is important to advance this conclusion one step further as it is likely that decreases in lignin and hemicellulose content that occur as a result of pretreatment also affect the physical properties of the cellulosic component, such as its crystallinity, the degree of polymerization and the surface area of the substrate accessible to cellulases.

Various studies conducted with different cellulase systems and a range of cellulosic substrates all indicate that it is ultimately the "accessibility" of the cellulose fraction to the enzyme system that determines how fast (reaction rate) and how far (% conversion) the hydrolysis reaction can proceed [112]. In work conducted with either wood pulps or substrates pretreated for bioconversion we and other groups have shown that accessibility is a property that describes the static environment encountered by the cellulase complex when it is combined with the substrate, and its action is governed by the intrinsic pore size distribution, degree of swelling and other gross and detailed substrate characteristics. As enzymatic hydrolysis commences, the situation becomes dynamic, as substrate attributes begin to change due to cellulose hydrolysis and the hydrolysis rate decreases. Some workers have reported decreases in accessible surface area as hydrolysis proceeds without appreciable changes in crystallinity [99, 105, 123], while others have reported decreases in crystallinity and increases in accessible surface area during hydrolysis [124]. The discrepancy in results regarding the decrease in the hydrolysis rate of pretreated substrates can most likely be attributed to variations in the substrates being studied and the techniques used for measurement of substrate properties. Furthermore, as cellulose is hydrolyzed, the lignin and hemicellulose that accumulate in the hydrolysis residue can potentially restrict access to cellulases and decrease the hydrolysis rate. Therefore, pretreatments should aim to produce a readily hydrolyzable substrate by increasing accessibility to cellulases and limiting the negative effects of hemicellulose and lignin on hydrolysis, while maximizing the total carbohydrate recovery.

However, it is important to recognize that studies which try to optimize pretreatment (as assessed by product recovery) need to be performed in

parallel with measurements of key substrate characteristics in order to associate specific aspects of pretreatment to substrate attributes that facilitate subsequent hydrolysis by cellulases. This emphasizes the significance of the pretreatment since the effectiveness of pretreatment affects both the upstream selection of biomass, the efficiency of recovery of the overall cellulose, hemicellulose and lignin components, and the chemical and morphological characteristics of the resulting cellulosic component, which governs downstream hydrolysis and fermentation.

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Progress and Challenges in Enzyme Development for Biomass Utilization

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Abstract Enzymes play a critical role in the conversion of lignocellulosic waste into fuels and chemicals, but the high cost of these enzymes presents a significant barrier to commercialization. In the simplest terms, the cost is a function of the large amount of enzyme protein required to break down polymeric sugars in cellulose and hemicellulose to fermentable monomers. In the past 6 years, significant effort has been expended to reduce the cost by focusing on improving the efficiency of known enzymes, identification of new, more active enzymes, creating enzyme mixes optimized for selected pretreated substrates, and minimization of enzyme production costs. Here we describe advances in enzyme technology for use in the production of biofuels and the challenges that remain.

Keywords Biomass · Enzymes · Hydrolysis

1 Introduction

The utilization of lignocellulose for the production of fuels and chemicals has the potential to change the world economically, socially, and environmentally. Today roughly 87% of the energy used in the world is derived from non-renewable sources such as oil, natural gas, and coal, with total energy consumption increasing at approximately 4% per annum. About 28% of that energy consumption is in the form of liquid transportation fuels, derived almost entirely from petroleum [1]. The long-term cost of continued use of these finite fuel sources can already be seen in increased conflict over their control and distribution, climate change linked to increased greenhouse gas emissions, and increasing prices, all of which negatively impact people around the world every day. Lignocellulosic biomass, in the form of plant materials such as grasses, woods, and crop residues, offers a renewable, geographically distributed, greenhouse-gas neutral source of sugars that can be converted to ethanol or other liquid fuels via microbial fermentation.

In the past 30 years, ethanol produced from corn starch and sugarcane has been established as an economically viable supplement to gasoline. In the USA over the past 5 years, production has increased from 175 million gallons per year to nearly 4.5 billion gallons last year, and is growing at more than 25% per year. In the near future, the use of sugar and starch as feedstocks for fuel production is expected to face increasing competition with their direct use as food and animal feed, impacting both availability and price. Current estimates suggest that in the USA, starch-based ethanol output will reach a maximum of between 12 and 15 billion gallons per year [2]. To significantly impact the use of petroleum in the USA, which uses approximately 140 billion gallons of gasoline per year, additional sources of fermentable sugar for ethanol production will be required.

Lignocellulosic biomass has the potential to become a major source of these fermentable sugars in the future. It is estimated that in the USA alone, more than one billion tons per year of biomass could be sustainably harvested in the form of crop and forestry residues, replacing as much as 30% of the total US gasoline consumption [3].

To turn the prospect of replacing a significant proportion of the current liquid fuels into reality, the conversion of lignocellulose to ethanol must become less expensive in both operating cost and capital investment. Current estimates suggest that the cost of producing cellulosic ethanol is $1.80/gallon or higher, or almost twice as high as the cost of producing ethanol from starch [4]. Part of this high cost results from a significantly higher estimated capital investment for the construction of cellulosic plants compared to starch-based production facilities. Cellulose-to-ethanol plants in current design scenarios require more unit operations, must be larger to accommodate more dilute sugar streams, and in some cases require acid-resistant

construction materials, which in sum are projected to increase the investment more than fourfold relative to current dry milling starch-based ethanol plants (from $1.10/gallon installed capacity to $4.70/gal) [4]. On the operating cost side, equipment replacement may be more frequent due to processing materials that are more abrasive than seed, enzyme cost will be significantly higher due to the increased complexity of the substrate and higher enzyme dosage required to release the sugars, and higher water consumption may be required to remove compounds that interfere with the hydrolysis and fermentation processes.

Starch is present in plants as an energy source for growing seeds, while lignocellulose is present as a structural cell wall component to give the plant rigidity; therefore it should be no surprise that the latter is much more resistant to enzymatic attack. On a protein weight basis, it takes anywhere from 40–100 times more enzyme to break down cellulose than starch, yet the cost of enzyme production is not substantially different (Novozymes, unpublished data).

In 2001, Novozymes was awarded a research subcontract by the US Department of Energy with the goal of reducing the cost of cellulases for ethanol production from biomass. This effort, called the Cellulase Cost Reduction Project, was administered by the National Renewable Energy Laboratory (NREL), with Novozymes providing expertise for enzyme improvement and production, and NREL contributing expertise in biomass pretreatment and enzyme evaluation. The stated goal of the project was to achieve a tenfold reduction in the cost of enzymes for the conversion of acid pretreated corn stover to ethanol in laboratory-scale testing. At the beginning of this work, the cost of providing a commercial cellulase preparation for the conversion of 80% of the cellulose in acid pretreated corn stover to fermentable glucose was estimated to be $5.40/gallon ethanol produced. During the course of the contract, significant advances were made in improving the efficiency of the cellulases, increasing the yield in production, and reducing the cost of production. In addition, work focusing on other enzyme activities required for effective enzymatic hydrolysis of lignocellulosic substrates other than acid pretreated corn stover was successfully conducted. In this manuscript, we highlight some of those efforts that have contributed to making enzymes for lignocellulose hydrolysis more affordable.

2
Lignocellulosic Biomass to Ethanol Process Overview

While possible variations in the process of converting lignocellulosic biomass to ethanol are virtually endless, it can most simply be described as the integration of five unit operations: (1) desizing, (2) thermochemical pretreatment, (3) enzymatic hydrolysis, (4) fermentation, and (5) ethanol recovery

(Fig. 1). In the first step of the process, the delivered biomass must be made uniform in size to facilitate handling and transport via conveyor or screw drive and to provide a more consistent surface-to-mass ratio for thermochemical pretreatment. The pretreatment step is typically a short- (minutes) to long-term (hours) exposure to extremes of temperature (150–200 C), pH (<2 or >10) and pressure (2–5 atm) and may additionally involve a rapid pressure release that facilitates chemical infiltration and fiber explosion. Ideally, pretreatment produces a disrupted, hydrated substrate that is accessible to enzymatic attack, but avoids both the production of sugar degradation products and fermentation inhibitors. As discussed below, some pretreatments solubilize hemicellulose to oligomeric and/or monomeric sugars comprised largely of pentoses that can be fermented independently or together with the glucose released from the cellulose fraction. In the next step, the pH is adjusted and enzymes are added to initiate cellulose hydrolysis to fermentable sugars. With pretreatments that do not solubilize the hemicellulose fraction, additional enzymes may be required to hydrolyze the hemicellulose

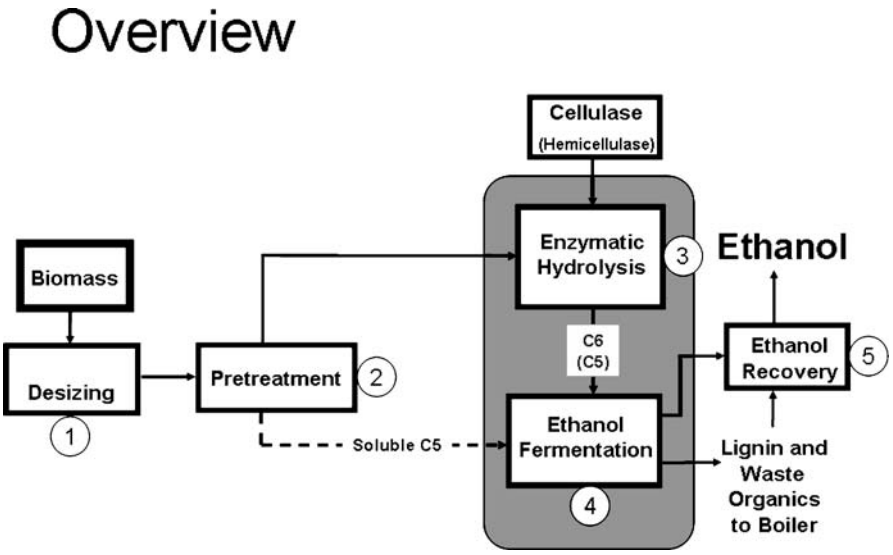

Fig. 1 Five-step process for the conversion of biomass to ethanol. *Step 1* The biomass is physically reduced in size by milling or chopping to increase surface area and uniformity. *Step 2* Some form of thermochemical pretreatment consisting of exposure to high pressure, temperature and extremes of pH is conducted to destroy the plant cell wall and expose the sugar polymers to the liquid phase. *Step 3* Enzymatic hydrolysis using a complex mix of glycosyl hydrolases to convert sugar polymers to monomeric sugars. *Step 4* Fermentation of the monomeric sugars to ethanol by addition of a fermentation organism. *Step 5* Ethanol recovery from the fermentation using distillation or some other separation technology. *C6* refers to glucose derived from cellulose hydrolysis, while *C5* refers to pentose sugars (mainly xylose) derived from hemicellulose

polymer. Hydrolysis typically is performed at pH 5 and 50 °C for 24–120 h, followed by addition of a fermentation organism to begin production of ethanol. In many cases (as described below) fermentation is initiated long before hydrolysis has completed, since both the extent and speed of ethanol production can often be increased by combining the hydrolysis and fermentation steps. In the final step, ethanol is recovered via distillation, and remaining organic waste is burned for production of heat and/or power.

2.1 Minimizing Yield Loss and Cost

The key to developing an economically viable biorefinery is to employ a holistic approach that integrates the unit steps, maximizing the yield at each, while minimizing both capital and operating costs. At each step of the process, from pretreatment to fermentation, effort must be made to minimize any loss in potential ethanol production. In the example in Fig. 2, the production of degraded sugars during pretreatment, incomplete cellulose or hemicellulose

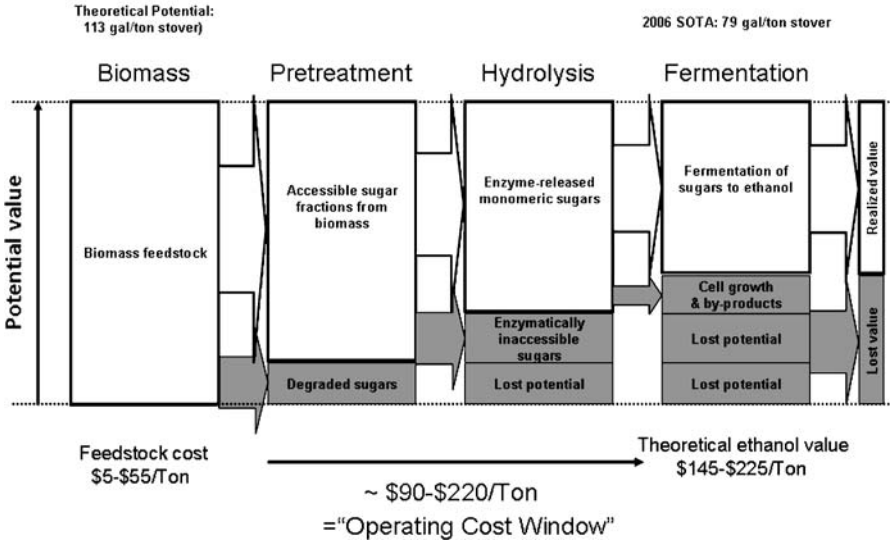

Fig. 2 Defining the operating cost window. These calculations utilized bone-dry corn stover and assumed the only sugar polymers used to produce ethanol are cellulose (40%) and xylan (25%). Ethanol yield was calculated according to the yield calculator from the US Department of Energy [5]. The theoretical ethanol value is based on $2/gallon selling price. 2006 SOTA is a current state-of-the-art scenario for conversion of cellulose (74% of theoretical) and xylan (64% of theoretical) to ethanol to yield 79 gallons of ethanol per bone-dry ton of corn stover. The value of any products other than ethanol, such as excess heat or power, is not included. For reference, corn grain at 72% starch has a theoretical yield of 124 gallons/ton

conversion to fermentable sugars during hydrolysis, and fermentation losses due to sugar consumption by the yeast all contribute to lost value in the conversion. If biomass feedstock such as corn stover, purchased at $5/ton, could be converted with perfect efficiency to its theoretical potential of 113 gallons of ethanol per ton of stover with an ethanol selling price of $2/gallon, the value of the ethanol would be ~$225/ton, creating an "operating cost window" for depreciation of capital, operation, and profit of ~$220/ton [5]. Losses in any unit step that reduces the overall yield will reduce the value per ton, whether the losses result from reduced enzyme hydrolysis, poor fermentability of the hydrolyzate sugars, or reduced fermentation yield. It is also important to note that maximizing the conversion of the two most abundant sugars, glucose and xylose, is important to viable economics. If only cellulose is utilized with no conversion of hemicellulose, the theoretical yield drops 39% to 69 gallons/ton, reducing the cost window to ~ $135/ton. Unless the xylose is utilized to produce something of equal or higher value, it is unlikely that such a process could be viable. Similarly, a pretreatment selected on the basis of a reduced capital cost for installed equipment, but increasing the required enzyme dosage, may reduce the operating cost window significantly.

3 Impact of Process Steps on Enzyme Dosage and Cost

The amount and types of enzymes required for the saccharification of cellulose and hemicellulose are strongly dependent on the biomass being hydrolyzed and the type and severity of pretreatment. Ultimately the selection of biomass feedstock will be based on local availability and economy of supply. In the early stages of commercial development, feedstocks with the greatest potential for demonstrating economic viability of an integrated process are likely to be developed first. These likely will include processing residues that are already available at processing plants such as corn fiber, soybean hulls, sugarcane bagasse, wood waste, and paper mill waste. The selection of both desizing and pretreatment processes may also be strongly influenced by local economics, especially with regard to co-location with existing wood, coal, or municipal solid waste-burning power plants, where inexpensive power and steam are available. With a diversity of potential substrates, different thermochemical pretreatments will be utilized to balance accessibility to enzymatic attack with destruction of valuable sugars. Variations in the severity of the pretreatment (pretreatment severity is defined as the combined effect of temperature, acidity, and duration of treatment) must also be varied to maximize both sugar and fermentation compatibility. For example, an acid pretreatment, run at high temperature, high pressure and for long periods of time is considered more severe than a neutral pH water pretreatment run under the same temperature and pressure conditions. A low severity pretreat-

ment will solubilize less of the hemicellulose fraction, increasing the amount of hemicellulase enzymes required, but may also reduce the production of by-products toxic to the fermentation, increasing the ethanol yield from the fermentation.

3.1 Impact of Substrate Selection on Enzyme Cost

The principal components of biomass are: cellulose (~ 30–50%), hemicellulose (~ 20–30%) and lignin (~ 20–30%); with minor components of starch, protein and oils. The exact composition of each biomass varies depending both on the plant species and the plant tissue utilized. Table 1 shows a variety of substrates in an effort to illustrate the variability of the composition of different substrates. In addition to the variability seen between plant species, work at the US National Renewable Energy Laboratory has demonstrated that even within a single plant species there is considerable variability in composition [6]. Using near infrared spectroscopy, they showed that the total sugar content contributed by cellulose and hemicellulose varied from 45 to 68% of dry mass between 1061 samples of corn stover. Lignin content, which has a direct impact on enzymatic digestibility, varied between 12 and 20%. These differences can be attributed to the genetic background of the corn variety, environmental factors such as weather, location, and pest invasion, and differences in farming practices.

The substrate characteristics that have been shown to impact the rate of hydrolysis include accessibility, degree of cellulose crystallinity, and the type and distribution of lignin [8]. The presence of lignin in a cellulose–cellulase

Table 1 Composition of representative biomass samples

Samples	Variety	% Mass		
		Total lignin	Cellulose	Hemicellulose
Monterey Pine	*Pinus radiata*	25.9	41.7	20.5
Hybrid Poplar	DN-34	24	40	22
Sugarcane bagasse	*Gramineae saccharum* var. 65-7052	24	43	25
Corn stover	*Zea mays*	18	35	22
Switchgrass	Alamo	18	31	24
Wheat straw	Thunderbird	17	33	23
Barley straw	*Hordeum vulgare* sp.	14	40	19
Rice straw	*Oryza sativa* sp.	10	39	15

Source: [7]

reaction is hypothesized to decrease the quantity of the enzyme associated with the cellulose due to nonspecific adsorption of the enzyme to lignin [9] and steric hindrance [10]. Steric hindrance occurs when lignin encapsulates the cellulose and makes it less accessible to enzyme attack [11]. Each of the factors summarized above are known to effect enzyme action and no single parameter correlates absolutely with enzymatic digestibility. The variation in composition of a given biomass requires some tailoring in the conversion method.

3.2 Impact of Pretreatment Selection

For an industrial process to be economically viable, enzymatic breakdown of lignocellulose to fermentable sugars must occur as quickly as possible, preferably in hours or days. No known enzyme or mix of enzymes are able to accomplish this feat on crude biomass. To make lignocellulose more amenable to breakdown, a wide array of thermochemical pretreatments have been devised [12]. Pretreatment has been described as the second most expensive unit cost in the conversion of lignocellulose to ethanol using enzymatic hydrolysis [13], after feedstock cost. A wide variety of pretreatments have been extensively described, including comminution [14], steam explosion [15], ammonia fiber explosion [16], and acid [17] or alkaline treatments [18] with different chemicals [19]. It is not our intent to review these sundry pretreatments, but only to indicate how they differ in terms of their impact on downstream enzymatic hydrolysis.

Pretreatments vary from extremely acidic to quite alkaline, modifying the composition of the biomass and making it more accessible to the enzymes. For example, acidic pretreatments will hydrolyze the majority of the hemicellulose while largely leaving the cellulose and lignin intact [20, 21]. Dilute acid (0.5–1.0% sulfuric) at moderate temperatures (140–190 °C) hydrolyzes most of the hemicellulose to soluble pentose sugars (both monomers and oligomers), with a concomitant increase in the efficiency of enzymatic cellulose digestion [22]. Although very little lignin is solubilized, the lignin is disrupted or redistributed in such a way that enzymatic digestion is enhanced [23]. Alkaline pretreatments typically solubilize less of the hemicellulose and lignin than acidic pretreatments, but modify or redistribute the lignin [24, 25]. Alkaline pretreatments therefore require enzymes that hydrolyze both cellulose and hemicellulose. Pretreatments differ not only in the degree of hemicellulose depolymerization, but also in the formation of compounds such as furfurals, acetate, and other chemicals that may be deleterious to the fermentative organism [26]. The effect of inhibitors released during pretreatment can also inhibit enzyme activity [27, 28]. An ideal pretreatment would be inexpensive both in capital and operating costs, create cellulose and hemicellulose substrates that require low enzyme dosages to release the

monomeric sugars, generate no hazardous waste, and produce a sugar stream that is fermentable without detoxification. Alterations in the type and severity of pretreatment can have a profound impact on enzyme dosage required for hydrolysis, and therefore on the cost of enzymatic hydrolysis.

As an example, a comparison of enzyme digestibility was made at Novozymes between alkaline pretreated and acid pretreated corn stover using the same enzyme mix composed of *T. reesei* cellulase (Novozymes' Celluclast 1.5 L) supplemented with cellobiase (Novozym 188) (Fig. 3). For the alkaline pretreatment, two conditions of severity were supplied for analysis, while for the acid pretreated material, one sample was washed exhaustively with water to remove solubles, while the other was simply adjusted to pH 5 with base. Although the samples contained the same cellulose content, large differences were seen in the enzyme loading required to hydrolyze the cellulose, with only the cellulose in the washed acid pretreated material being hydrolyzed to completion. The unwashed acid pretreated material was more

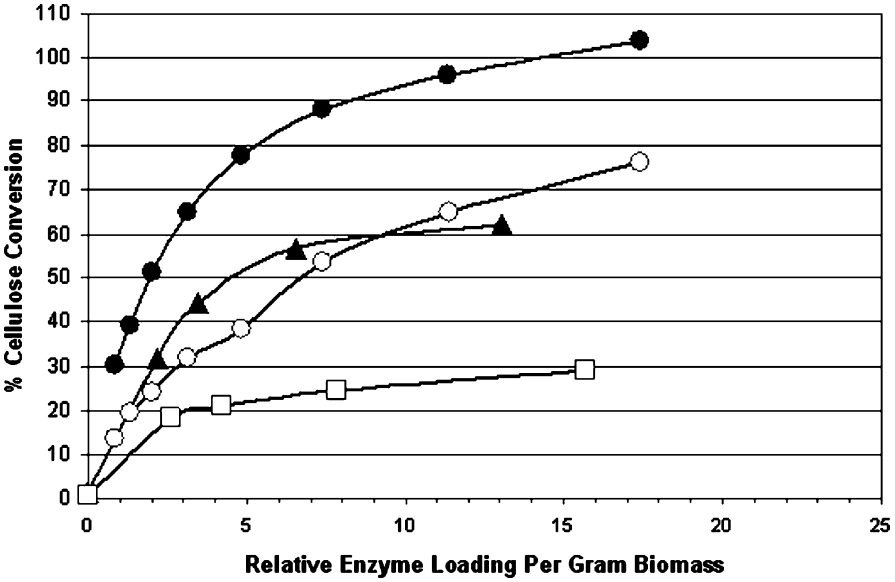

Fig. 3 Enzymatic digestibility of acid and alkaline pretreated corn stover, washed and unwashed. Comparison of the enzymatic digestion of washed (●) and unwashed (○) acid pretreated corn stover, and two (▲, □) severities of an alkaline pretreated corn stover using a mixture of Celluclast 1.5 L and Novozym 188 at various enzyme loadings. Pretreated corn stover was supplied by NREL and others. Acid pretreated corn stover was washed with water until the supernatant reached pH 5. Cellulose content was estimated from compositions provided by biomass suppliers. Enzymatic hydrolysis was conducted with the same enzyme mix in 50 g assays containing 13.5% dry solids at 50 °C for 168 h. Calculation of approximate conversion was based on the amount of glucose released as a percentage of the theoretical yield from cellulose

resistant to hydrolysis, likely due to the presence of inhibitors that block enzyme action. While the cellulose in the unwashed stover was hydrolyzed to a greater extent with increasing enzyme dose, both of the alkaline pretreatments show a plateau in cellulose hydrolysis, likely due to steric hindrance by unremoved hemicellulose or lignin. Addition of hemicellulase activities can improve the cellulose digestion in these cases, but at an increased cost for the additional activities. Our enzyme mix was optimized for an acid pretreatment, and better enzyme mixes for both alkaline and acid pretreatments could and should be possible.

3.3 The Impact of Process Integration on Enzyme Requirements

The process steps of pretreatment, hydrolysis, and fermentation need to be viewed holistically to maximize ethanol yield and overall process cost. As discussed previously, different pretreatments produce different substrates for enzyme action, impacting both the required mix of enzymes, the dosage of those enzymes, and the cost of the hydrolysis step. Similarly, the selection of the fermentation organism determines the pH and temperature optima of the fermentation step, which can affect enzyme performance and loading since hydrolysis and fermentation are often combined at some stage of the hydrolysis in a single reactor.

The enzymatic hydrolysis can either be done separately from the fermentation (SHF, separate hydrolysis and fermentation) or in combination with the fermentation (SSF, simultaneous saccharification and fermentation). In SHF, hydrolysis is allowed to proceed to a point of completion at reaction conditions optimal for enzyme action, which today for *T. reesei* enzymes is 50 °C and pH 5, then the temperature is lowered to allow survival of the fermentation organism (typically 30–40 °C) and the pH is adjusted upwards to pH 5.5–7. The primary drawback to the SHF process is a reduced rate of hydrolysis caused by product inhibition of the enzymes by the released monomeric and oligomeric sugars. The SSF process for producing ethanol is capable of improved hydrolysis rates, yields, and product concentrations compared to SHF because of the continuous removal of the reaction end products (the sugars) by the yeast, preventing competitive inhibition of some of the component enzymes, provided the temperature and pH required for fermentation does not drastically slow enzyme action. Ideally we will see organisms and enzymes developed that have similar growth and reaction optima, allowing optimal growth and enzyme action to occur in a single vessel. Currently, compromises in either or both must be made in the process design since there is a 10–20 °C gap in temperature optima and a 0.5–2 pH unit gap in pH optima.

In hybrid hydrolysis and fermentation (HHF), the hydrolysis and fermentation are temporally separated to optimize the combined rate of hydrolysis and fermentation. Hydrolysis is allowed to proceed to a point at which glucose

release slows significantly, then the temperature is dropped, the pH increased, and fermentation is initiated by addition of the organism. The development of an economically viable process depends on optimizing the timing of the shift from hydrolysis to fermentation, and is dependent on the enzymes, the organism, and all the factors that contribute to process cost, such as feedstock cost, hydrolysis/fermentation residence time, solids loading, and capital investment.

It has been established that digestibility of a biomass substrate is highly dependent upon the type of pretreatment, enzyme efficiency, and dosage. Recent results indicate that mixing is also an important parameter in integrating pretreatment and hydrolysis [29]. In Fig. 4, acid pretreated corn stover (PCS) and hot water autolysed wheat straw (HWS) were hydrolyzed at Novozymes with a Celluclast/Novozym 188 mix at the same loadings using two different types of mixing: shake flask orbital mixing versus tumbling (lift and drop). While the PCS, a well-pretreated substrate whose cellulose can be wholly hy-

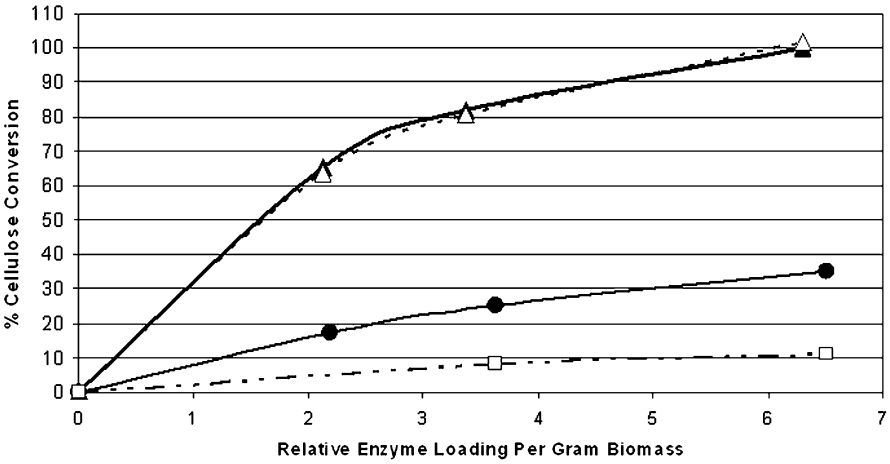

Fig. 4 Comparison of the impact of shake flask orbital mixing versus tumbling on enzymatic hydrolysis. Acid pretreated corn stover (PCS, supplied by the NREL) was washed prior to use. Pretreated wheat straw (HWS) was produced by wet autoclaving at 132 °C for 30 h as estimated by application of the Arrhenius equation to the data of Garrote (1999) so as to produce minimally pretreated biomass. Residual dry weights were determined as per NREL laboratory analytical procedure (LAP) 012 (NREL procedures can be found at [46]). Cellulose content was estimated from published values (HWS) [47], limit enzymatic hydrolysis, and carbohydrate compositional analysis (PCS). Hydrolysis was performed essentially as per NREL protocol LAP 009 (72 h, pH 5, 50 °C), using either flasks in a rotary shaker at 150 rpm (shaker) or in sealed tubes tumbling free in a rolling tub (tumbling). Analysis of resulting hydrolysis sugars was performed according to NREL protocol LAP 13–15. Calculation of approximate conversion was based on the amount of cellobiose and glucose released as a percentage of the theoretical yield from cellulose. PCS tumbling (▲), PCS in a shaker (△), HWS tumbling (●), and HWS in a shaker (□)

drolyzed, shows no difference in either the rate or extent of hydrolysis, the poorly pretreated HWS shows a dramatic improvement in hydrolysis from the more disruptive tumble mixing as compared to orbital mixing. Although conversion is fairly low for the HWS compared to the PCS, we saw both an increase in the rate and endpoint conversion of cellulose to glucose with the tumble mixing. These results indicate that the type and vigor of mixing during hydrolysis may allow less severe pretreatments to be implemented, with the potential to decrease both capital and operating costs during pretreatment. In addition, this type of vigorous mixing may allow higher solids levels during pretreatment and hydrolysis, resulting in a more concentrated sugar stream and higher ethanol titers from the fermentation. This has the potential to reduce operating costs in energy consumption used for ethanol distillation. In addition, utilization of higher solids increases plant throughput, reducing the total capital investment required.

4
Enzyme Discovery: Catalytic Efficiency and Productivity

There are numerous organisms that rely on biomass degradation for their survival, often existing in the natural environment as a complex consortia of fungi, bacteria, and protozoa, working synergistically to decay the plant cell wall. All of these organisms are potential sources of enzyme discovery, but current commercial products for biomass treatment are derived from fungi because these organisms produce a complex mix of enzymes at high productivity and catalytic efficiency, both of which are required for low-cost enzyme supply. Unlike most bacteria, which express complexes of many carbohydrate-degrading activities arrayed on molecular scaffolds physically attached to the bacterial cell wall, fungal cellulases are typically secreted into the growth medium, allowing cost-efficient separation of the active enzymes in a liquid form suitable for delivery to a hydrolysis reactor.

4.1
T. reesei Cellulases: The Current Industry Standard

The most widespread, commercial enzyme products currently available for biomass hydrolysis are produced by submerged fermentation of the saprophytic mesophilic fungus *T. reesei* [30]. This organism, first isolated over 60 years ago from decaying cotton tents during World War II [31] is a prolific producer of secreted cellulases. Since its initial isolation, numerous mutants have been isolated that increase the productivity of the strain by over 20-fold [28, 32, 33]. Three enzymes classes form the core of the *T. reesei* cellulase system: exoglucanases comprised of two primary cellobiohydrolases, a number of endoglucanases, and β-glucosidases (Fig. 5). There are two types of

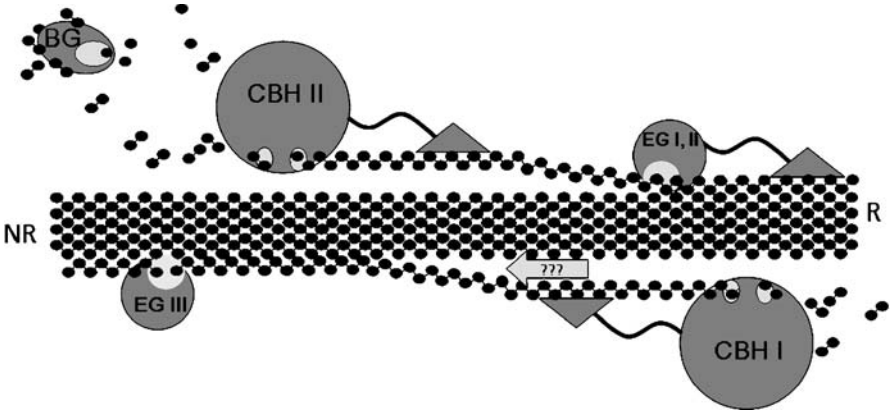

Fig. 5 Schematic of the primary *T. reesei* enzymes involved in hydrolysis of cellulose. Cellulose is represented as stacked chains of *black circles* with reducing (*R*) and non-reducing (*NR*) ends indicated. There are two major cellobiohydrolases that attack the cellulose chain ends processively from the reducing (*CBH I*) and non-reducing (*CBH II*) ends of the chain, releasing the glucose disaccharide cellobiose. In addition, there are three major endoglucanases depicted (*EGI*, *II*, and *III*) that attack the cellulose chain randomly, and two β-glucosidases (*BG*) that hydrolyze cellobiose released by the CBHs to glucose. *Triangles* represent cellulose binding motifs, and the *arrow* represents an additional hypothetical protein components that may assist in cellulase action by disrupting the cellulose crystal structure

cellobiohydrolases, CBH I and CBH II, that constitute roughly 60% and 20% of the secreted protein mix and are critical to the efficient hydrolysis of cellulose [34]. The CBH I and II hydrolyze the cellulose chain processively from the reducing and non-reducing ends of cellulose chains, respectively, releasing the glucose disaccharide cellobiose. Endoglucanases (EG I-IV) constitute roughly 15% of the secreted protein and hydrolyze β-1,4 linkages within the cellulose chains, creating new reducing and non-reducing ends that can then be attacked by the CBHs. β-Glucosidases (BGL I and II), constituting roughly 0.5% of the secreted protein mix, and hydrolyze cellobiose and some other short-chain cellodextrins into glucose.

4.2 Searching for Synergy

The primary factor in the high cost of enzymes for biomass hydrolysis is simply the amount of enzyme that must be used. Compared to starch hydrolysis, 40- to 100-fold more enzyme protein is required to produce an equivalent amount of ethanol (Novozymes data). It was recognized very early on that efficient cellulose hydrolysis requires a complex, interacting collection of enzymes during initial characterization of the *T. reesei* cellulase system [35]. To significantly reduce the amount of these enzymes requires that either more

efficient component enzymes are identified or that additional enzymes can be added that reduce the total enzyme loading. Synergy, the ability of two or more enzymes to work simultaneously more effectively than in succession, was first described in cellulases more than 30 years ago when describing the action of CBH I and EG activities [36]. In this case, the synergy can be mechanistically explained by the production of new cellulose ends by the action of the endoglucanase, creating new sites of exoglucanase attack by the CBH. Similarly, studies of the observed synergism between CBH I and CBH II from *Humicola insolens*, revealed that this CBH II, although capable of acting processively from non-reducing chain ends, does also cleave the cellulose chains in an endo fashion [37]. To drive enzyme loading down, we needed to search for similar synergistic enzyme pairs that could complement the preferred *T. reesei* cellulase system.

4.2.1 β-Glucosidase

An "efficient" cellulase system requires sufficient β-glucosidase (BG) to hydrolyze cellobiose produced by the action of the CBHs to prevent their product inhibition [38]. The addition of BG to a complex cellulase mix such as the Novozymes Celluclast 1.5 L dramatically improves the extent and, during the later stages of hydrolysis, the rate of cellulose saccharification. This is re-

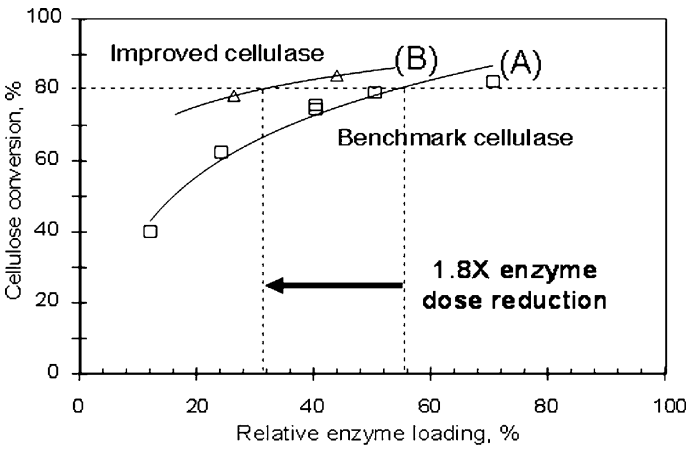

Fig. 6 Improvement of PCS-hydrolyzing cellulases by increasing levels of β-glucosidase (BG) activity. Comparison of *T. reesei* cellulase preparations, with (*B*) and without (*A*) supplementation with purified *A. oryzae* BG, in the hydrolysis of cellulose present in acid pretreated corn stover demonstrates a significant benefit in reducing the amount of enzyme required. Addition of small amounts of BG, present as a few percent of total protein, allowed hydrolysis of 80% of the cellulose to glucose with an enzyme protein dosage 1.8-fold lower that the unsupplemented cellulase

flected in Fig. 6, where the *T. reesei* strain used to produce Celluclast 1.5 L was compared to the same strain expressing *Aspergillus oryzae* BG in hydrolysis assays. Due to relief of the product inhibition at high solids loadings (13.5% w/w in this example), the amount of total enzyme protein required to hydrolyze 80% of the cellulose to glucose was reduced by nearly twofold. At this solids loading, the beneficial effect of BG addition was saturated when it reached ~ 5% of the total enzyme protein, but higher solids would require higher BG levels or a more active BG.

4.2.2 Glycosyl Hydrolase Family 61

In nature, microbes can efficiently degrade biomass by secreting an array of synergistic enzymes, including cellulases, often from numerous microbes intermingled in their growth. In an effort to identify new proteins that could work synergistically with those secreted by *T. reesei*, we conducted mixing experiments by supplementing Celluclast 1.5 L with broth from a wide array of cellulolytic fungi grown under cellulase-inducing conditions. By comparing the saccharification of acid pretreated corn stover using equal protein loadings of either Celluclast alone or mixtures of Celluclast with these broths, fungi secreting components that could work synergistically with the *T. reesei* cellulases could be detected. In Fig. 7, an example of such an experiment shows that a mixture of *T. reesei* broth and *Thielavia terrestris* broth has the same level of hydrolyzing activity as twice as much *T. reesei* or *T. terrestris* broth alone. These results suggested that some activity present in the *T. terrestris* broth was working in synergy with the cellulases present in *T. reesei* broth to more efficiently degrade the cellulose in the corn stover.

In order to identify the protein or proteins responsible for the observed synergism with the *T. reesei* cellulases, the *T. terrestris* broth was fractionated and individual fractions were assayed for synergism similarly. Fractions displaying synergism were separated on one- and two-dimensional polyacrylamide gels, individual proteins were isolated by removal from the gels and subjected to sequencing by tandem mass spectrometry. Several independent chromatographic fractions contained proteins with homology to glycosyl hydrolase family 61A, a protein previously identified in a number of cellulolytic fungi. When purified to homogeneity, a number of these proteins were demonstrated to significantly enhance the activity of the *T. reesei* cellulases in synergism assays. Inclusion of these proteins at less than 5% of the total enzyme dose in some cases could reduce the required cellulase loading by as much as twofold. These results suggested that the GH61 family proteins were the major components responsible for the enhancement of Celluclast 1.5 L activity by crude *T. terrestris* fermentation broth.

We also tested the cellulase-enhancing effect of GH61 proteins on a variety of other lignocellulosic substrates from a variety of pretreatments when

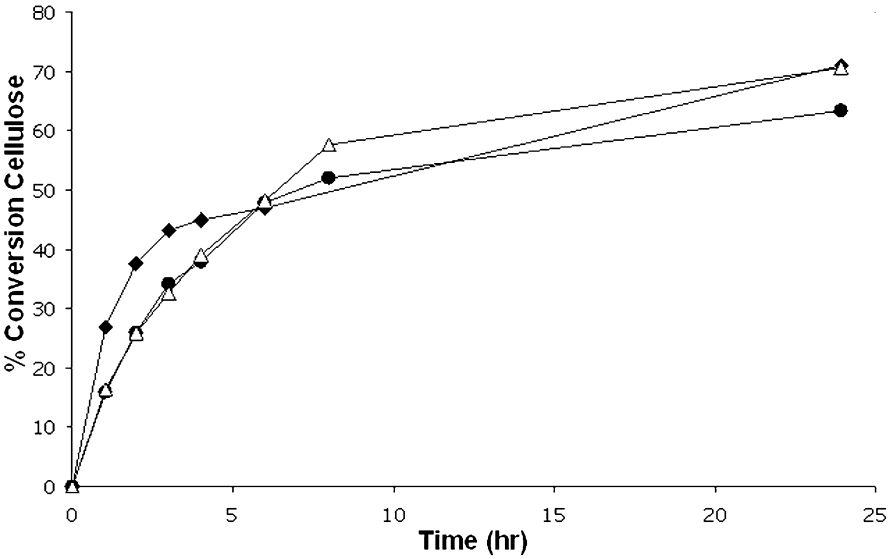

Fig. 7 Synergy between the cellulases of *T. terrestris* and *T. reesei*. Hydrolysis of PCS at 50 °C using cellulase-induced broth samples of *T. reesei* (◆), *T. terrestris* (●), or a 1 : 1 mix of the two broths at one-half the enzyme loading (△). The 1 : 1 mixture of the two cellulase preparations performed as well as the individual system dosed at *twice* as much as the *T. reesei* cellulolytic system alone, indicating a significant synergism between the two systems

combined with *T. reesei* cellulases. Those GH61 proteins capable of enhancing hydrolysis of acid pretreated corn stover also enhanced hydrolysis of other substrates, although they differed in their effectiveness by varying amounts. None of the GH61 proteins were able to enhance the hydrolysis of pure cellulose in the form of filter paper. This lack of enhancement was also shown with other pure cellulose substrates such as Avicel, phosphoric-acid swollen cellulose, and carboxymethyl cellulose.

The GH61 proteins by themselves showed no significant detectable hydrolytic activity on PCS or any other lignocellulosic substrate tested, indicating that the enhancement was not likely to be the result of any intrinsic endo- or exoglucanase activity of the GH61 proteins. The hydrolytic activity of several GH61 proteins was tested on a variety of model cellulose and hemicellulose substrates, but little or no activity was found. These results suggest that the enhancement of cellulolytic activity by GH61 is limited to substrates containing other cell wall-derived material such as lignin or hemicellulose, although there is no clear correlation between the proportions of these materials and the degree of enhancement observed. These findings could be of significant interest for not only the elucidation of the physiological functions of the GH61 protein family, but also the development of a viable enzymatic system to convert biomass to simple platform sugars.

Several of the GH61 genes were transformed into *T. reesei*, resulting in transformants expressing GH61 at various levels, depending on the number of inserts and site of integration. Fermentation broths produced by these transformants were assayed for PCS hydrolysis at various protein loadings to assess their improvement in specific performance relative to control strains not expressing non-native GH61 proteins. The results confirmed that certain GH61 proteins expressed at relatively low levels are capable of significantly enhancing the hydrolysis of cellulose in PCS. For example, expression of *T. terrestris* GH61B in *T. reesei* allows for a reduction in protein loading of 1.4-fold to reach 90% conversion of cellulose to glucose in 120 h. The protein loading reduction made possible by GH61 addition becomes more pronounced at longer incubation times and higher levels of hydrolysis, and higher solids loadings.

4.2.3 Synergistic Hemicellulases

Development of improved enzymes for the hydrolysis of the other major carbohydrate polymer present in lignocellulosic biomass is also of commercial interest, particularly to those utilizing neutral or alkaline pretreatments that leave much of the hemicellulose intact. To develop these enzymes, an industrial residue of the wheat starch industry was used as a model substrate. In Europe, wheat is one of the major substrates for production of fuel ethanol. Processing of wheat starch for glucose results in a by-product stream (vinasse) consisting mainly of the wheat endosperm cell wall material and leftover yeast cells following the fermentation of the starch. The hemicellulose by-product is approximately 33 wt % carbohydrates of which approximately 66 wt % is arabinoxylan. Arabinoxylans consist of a linear backbone of β-1,4-linked D-xylopyranosyl units that are partially substituted with arabinofuranosyls. The major portion of the arabinoxylan in industrial wheat fermentation residues is water-soluble [39], the water-insoluble arabinoxylan is quantitatively more abundant in cell walls isolated directly from unprocessed wheat endosperm [40]. Arabinoxylans are hydrolyzed to monosaccharides by acid treatment or by enzymatic hydrolysis. The enzymatic hydrolysis is usually preferred because it allows for a more specific and controlled modification and fewer undesirable by-products, making it more suitable for microbial fermentation using organisms that can metabolize both xylose and arabinose [41].

The enzymatic degradation of arabinoxylans requires both side-group cleaving and depolymerizing enzymes. Cleavage of the side chains requires the action of several accessory enzyme activities, including α-L-arabinofuranosidases, α-glucuronidases, ferulic acid esterase, and acetylesterases [41, 42]. Depolymerization requires endo-1,4-β-xylanases that result in unbranched xylooligosaccharides, including xylotriose and xylobiose, and

β-xylosidases that cleave xylobiose and attack the non-reducing ends of short chain xylooligosaccharides to liberate xylose [41].

The hydrolysis of arabinoxylan is critical for improved utilization of wheat hemicellulose in the ethanol industry. Three Novozymes cellulolytic and hemicellulolytic enzyme preparations, Celluclast 1.5 L, Ultraflo L, and Viscozyme L were tested in various combinations for their ability to liberate arabinose and xylose from water-soluble wheat arabinoxylan. The substrate was medium viscosity water-soluble wheat arabinoxylan from Megazyme (Bray). The three different enzymes were evaluated individually and also in 50 : 50 combinations to look for possible synergistic effects. Reactions were carried out at pH 5 and 50 °C followed by analysis of arabinose, galactose, glucose, xylose, xylobiose, and xylotriose by high-performance anion exchange chromatography (HPAEC) [43]. The molecular weight and distribution of water-soluble wheat arabinoxylan and hydrolyzates were determined by high-performance size exclusion chromatography (HPSEC).

In those reactions containing the individual enzyme preparations, the levels of arabinose and xylose increased with increasing enzyme dosage and time. Ultraflo L was superior to Celluclast 1.5 L and Viscozyme L in releasing the arabinose from the water-soluble wheat arabinoxylan, meaning that Ultraflo L must contain a significant amount of α-L-arabinofuranosidase. Celluclast 1.5 L was the best enzyme preparation for liberating xylose, resulting in 26 wt % of the available xylose. Ultraflo L released 16 wt % while Viscozyme L released less than 1.5 wt %. In a mixture of 50 : 50 Celluclast 1.5 L and Ultraflo L there was no interaction among the arabinose-releasing side activities since the same amount of arabinose was obtained as when the two individual enzyme preparations were used and then the arabinose total was combined. The Viscozyme L preparation exhibited a weak antagonistic effect with Ultraflo L and Celluclast 1.5 L since the amount of arabinose actually decreased compared to that observed with the individual enzyme preparations. The results indicated that the arabinose-releasing side activities of Viscozyme L had the same activity as those demonstrated by Ultraflo L and Celluclast 1.5 L. Another possibile but less likely explanation is the Viscozyme L contained α-L-arabinofuranosidase inhibitors [43]. The 50 : 50 mixture of Celluclast 1.5 L and Ultraflo L produced an increase in the release of xylose compared with the sum of the individual enzyme preparations (Fig. 8). The mixture released 59 wt % of the available xylose, which was 32 wt % more than the theoretical addition of the individual enzyme preparations alone. Combination of Ultraflo L and Viscozyme L showed no such synergism, but incubation of Celluclast 1.5 L and Viscozyme L showed a weak synergistic effect in liberating some of the xylose from the wheat arabinoxylan.

To further examine the synergistic affect between Celluclast 1.5 L and Ultraflo L the amounts of xylobiose and xylotriose released during enzymatic hydrolysis were quantified using HPAEC for both individual and combined enzyme preparations. During the initial stage of incubation, Celluclast 1.5 L

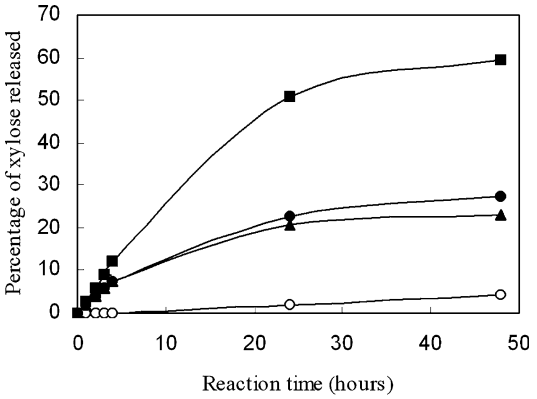

Fig. 8 Synergy between Ultraflo L and Celluclast 1.5 L. Enzyme preparations were from Novozymes (Bagsvæd, Denmark). Weight percent of xylose released from water-soluble wheat arabinoxylan after treatment with: ▲ 5 wt % Celluclast 1.5 L, ○ 5 wt % Ultraflo L, and ■ 10 wt % mix of Ultraflo L and Celluclast 1.5 L (50 : 50 mixture) for 48 h at 50 °C. ● represents the sum of Celluclast 1.5 L and Ultraflo activities, without cooperativity [43].

liberated small amounts of both xylobiose and xylotriose, indicating the presence of endo-1,4-β-xylanase activities. As hydrolysis continued, the released xylobiose and xylotriose was hydrolyzed to xylose, indicating the Celluclast 1.5 L contained one or more β-xylosidase activities.

Ultraflo L treatment resulted in continual liberation of both xylobiose and xylotriose. Ultraflo L showed a low release of free xylose indicating one or more endo-1,4-β-xylanase activities, but little β-xylosidase activity. The synergistic effect between Celluclast 1.5 L and Ultraflo L in releasing xylose is therefore likely to be a result of the action of α-L-arabinofuranosidase and endo-1,4-β-xylanase activities present in Ultraflo L and the β-xylosidase present in Celluclast 1.5 L [43].

Since a strong synergistic effect was observed with a 50 : 50 combination of Celluclast 1.5 L and Ultraflo L for the breakdown of arabinoxylan, a second study was conducted to look for similar effects and viscosity reduction in the fermentation residue, vinasse. The effects of enzyme dosage, optimal temperature, and pH were examined in hydrolysis of whole vinasse, vinasse supernatant, and washed vinasse sediment that was provided by Tate & Lyle, Amylum UK (Greenwich, UK). On whole vinasse, the enzyme-catalyzed release of arabinose and xylose by the 50 : 50 combination of Ultraflo L and Celluclast 1.5 L decreased as the substrate concentration of the vinasse increased. The monosaccharide release also decreased when the substrate concentration of the vinasse increased. Release of arabinose and xylose from the vinasse sediment was very low. The release of arabinose from the whole vinasse varied from 40–50 g arabinose per kilogram vinasse DM while xylose release was between 75–100 g xylose per kilogram vinasse DM after a 24 h hydrolysis. The

Ultraflo L:Celluclast 1.5 L mixture released 53–75 g arabinose and 75–115 g of xylose per kilogram of vinasse DM after a 24 h hydrolysis [44].

Significant viscosity reduction was obtained by enzyme-catalyzed degradation of arabinoxylans present in the fermentation residue stream, vinasse. However, there was limited hydrolysis of the insoluble arabinoxylans in the vinasse sediment. The efficiency of enzymatic degradation of the arabinoxylan in vinasse was dependent on enzyme dosing and substrate dry matter [44].

In an effort to narrow down the specific activities involved in the previous studies, the β-xylosidase from Celluclast 1.5 L was purified and used as a supplement to Ultraflo L enzyme preparation. When dosed at 0.25 g β-xylosidase protein per kilogram of arabinoxylan along with Ultraflo L, this enzyme mix released the same or more xylose as the enzyme mix consisting of 50 : 50 Ultraflo L and Celluclast 1.5 L (Fig. 9).

In order to determine the optimal enzyme mix for the hydrolysis of vinasse arabinoxylan, several recombinant enzymes were made and tested in various combinations. Genes were cloned and expressed in the fungal host *A. oryzae*. Based on our studies the optimal enzyme mix for vinasse hydrolysis consists of α-L-arabinofuranosidase from *Meripilus giganteus*, α-L-arabinofuranosidase II from *Humicola insolens*, and *T. reesei* β-xylosidase. A mixture of 25 : 25 : 50 of α-L-arabinofuranosidase from *M. giganteus*, α-L-arabinofuranosidase from *H. insolens* and β-xylosidase from *T. reesei* was determined to be optimal for maximizing arabinoxylan hydrolysis. The success of this work in identifying and exploiting synergism between hemicellulase component activities is currently being applied to other relevant lignocellulosic substrates that differ significantly in their hemicellulose composition.

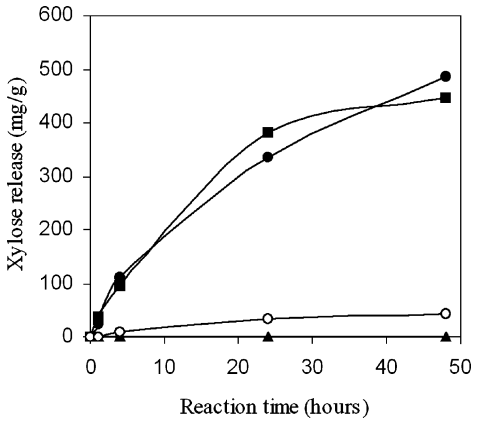

Fig. 9 Xylose released from water-soluble wheat arabinoxylan after treatment with: ▲ 0.25 g β-xylosidase protein kg^{-1} arabinoxylan, ○ 5 wt % Ultraflo L, ● 5 wt % Ultraflo L + 0.25 g β-xylosidase protein kg^{-1} arabinoxylan, and ■ 10 wt % Celluclast 1.5 L/Ultraflo L (50 : 50 mixture) for 48 h at pH 5 and 50 °C [48]. © 2006, with permission from Elsevier

5
Producing Enzymes Economically

There is arguably no other industrial enzyme application that poses a greater challenge to the enzyme producer than supplying cost-effective enzymes for biomass utilization. The high enzyme loading required, combined with the low value of the final product, in the form of ethanol, requires not only that the enzymes be as efficient as possible, but that the cost of producing them be as low as possible. To this end, significant effort has been expended over the past 6 years to increase the productivity of the fungal strains used to produce the enzymes, to reduce the cost of the enzyme fermentation process by reducing the cost of carbon and nitrogen sources for the fermentations, and to reduce the complexity of enzyme recovery and formulation.

Improving the host by classical mutagenesis is one way of developing a host strain with improved total protein production and improved activities. This approach has a long and successful history. Montenecourt and Eveleigh [32] isolated RutC30, one of the best existing *Trichoderma* cellulase mutants, using a combination of ultraviolet irradiation and nitrosomethyl guanidine (NTG). Recently, Toyama, et al. [45] demonstrated a method to screen for increased cellulase production using growth through an overlay of cellulose substrate (Avicel) in Petri plates. In an effort to increase total cellulase productivity, a combination of these methods were utilized on the *T. reesei* strain currently used to produce Celluclast 1.5 L. Chemical mutagenesis was used to generate mutants that were screened using the method of Toyama [45] with minor changes. Briefly, mutagenized spores were suspended in an agar medium, poured into a plate and allowed to harden. The spore-containing layer was then covered with a top layer of agar containing washed, acid pretreated corn stover (PCS) as the sole carbon source. Colonies growing through the PCS layer fastest were isolated and used in a secondary screening. In this, spores from selected fast-growing colonies were inoculated into shake flasks containing cellulase-inducing media. After 5 days of growth, broth samples were tested by robotic assay for production of reducing sugars from hydrolysis of PCS. Total protein assays were then conducted, and mutants expressing elevated cellulase and/or total protein were then re-grown in 2-L fermentors. Broth from the fermentors was then analyzed again in PCS hydrolysis assays and for total protein. Some mutants were identified as having improved PCS hydrolysis and increased total protein secretion compared with the control. Top strains isolated in this manner showed significant increases in protein production and secreted cellulase activity.

Another method of improving a cellulase productivity is through increasing the expression of target proteins using genetic engineering. In many cases the total cost of supplying a heterologous mix of enzymes can be reduced by creating a single expression host expressing not only the native cellulases

and hemicellulases, but expressing additional components, such as the BG and GH61 proteins, without negatively impacting the expression of the native proteins. The introduction of multiple genes into a single host is no easy feat. A significant amount of work was done to identify strong promoters, to identify a number of selectable markers, and to develop a successful transformation technique that allows for co-transformation of multiple transgenes. These technological improvements have allowed us to rapidly and efficiently investigate the effect of introducing various enzymes into the *T. reesei* cellulase mix.

In addition to controlling gene expression transcriptionally, by utilizing promoters of different strengths, we have focused on enhancing individual protein yield by optimizing protein secretion. One example is the replacement of the *A. oryzae* BG signal sequence with a signal peptide from *H. insolens* Cel45A EG, which improved the BG secretion in *T. reesei* by two- to threefold relative to the unfused gene (Fig. 10).

As previously mentioned, several GH61 proteins result in a "boost" in PCS hydrolysis when supplemented to Celluclast 1.5 L. In addition, our studies show that increased levels of β-glucosidase are required in our *Trichoderma* host. Therefore, numerous co-transformations of *T. reesei* with various GH61s, *A. oryzae* β-glucosidase, and other genes of interest were carried out. Those transformants expressing both a GH61 and the β-glucosidase were then screened in PCS hydrolysis assays in order to identify the top strains in true performance assays. Those strains demonstrating the best perform-

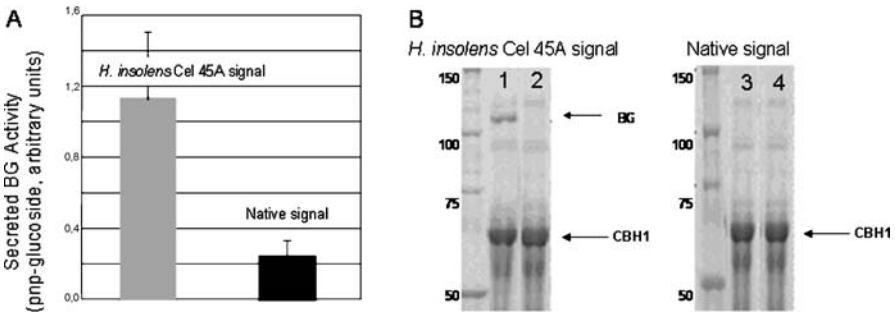

Fig. 10 Signal peptide effect on β-glucosidase (BG) secretion in *T. reesei*. *T. reesei* strains were genetically modified to heterologously express *A. oryzae* BG, using either the native *A. oryzae* signal peptide or the *H. insolens* Cel45A signal peptide. **a** Relative BG activity measured in the secreted fraction, using 4-nitrophenyl β-D-glucopyranoside at pH 5. **b** SDS-PAGE of secreted proteins from the two *T. reesei* strains. *Lane 1* BG expression utilizing the *H. insolens* Cel45A signal sequence. *Lane 2* parent of strain used to generate the strain in lane 1 (untransformed). *Lane 3* BG expression utilizing native signal sequence. *Lane 4* parent of strain used to generate the strain in lane 3. The positions of molecular weight markers are labeled and the positions of *A. oryzae* BG and *T. reesei* CBHI are designated by *arrows*

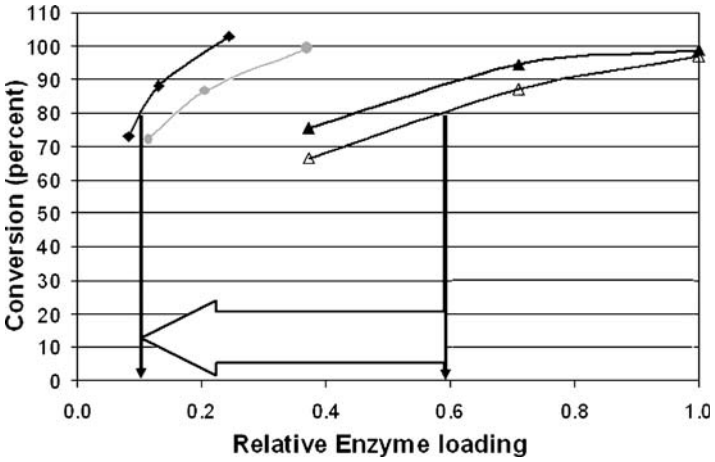

Fig. 11 Stepwise improvements in enzyme performance in hydrolysis of PCS. Relative enzyme protein loading is plotted vs. percent cellulose conversion. Celluclast 1.5 L supplemented with 1% w/w Novozym 188 (Novozymes' BG product) at 38 °C (△) and 50 °C (▲). The Celluclast 1.5 L strain expressing a recombinant BG (●), and the Celluclast 1.5 L strain expressing a recombinant BG, a GH61 protein, and two additional heterologous proteins (◆) were tested to determine the enzyme protein loading required to reach 80% of the theoretical cellulose hydrolysis using acid pretreated corn stover in 168 h. The final *T. reesei* strain produced a cellulase mix roughly sixfold more efficient than the Celluclast 1.5 L supplemented with 1% w/w Novozym 188

ance in PCS hydrolysis were then fermented in 2-L bioreactors and retested in PCS hydrolysis assays. Eventually, a single strain was identified exhibiting improved hydrolysis from our original strains and high total protein production (Fig. 11).

5.1 Reduced Enzyme Recovery

The total production cost for cellulosic ethanol must still be substantially reduced to enable large scale commercialization, and at least a portion of this reduction must come from enzyme cost. Realistically, enzyme cost targets in the range of $0.30/gallon at the commercial scale should be achievable in the near future by avoidance of transportation and formulation costs. In such a scenario, on-site or near-site enzyme production is essential, where enzymes are produced using reduced-cost feedstocks, transported short distances, and not stored for extended periods of time. The least expensive alternative in this situation involves the direct use of whole fermentation broth (including cell mass) to circumvent expensive cell removal and enzyme formulation steps. To investigate this possibility, we compared the use of whole fermentation broth and cell-free broth as catalysts for PCS hydro-

lysis in microtiter-scale reactions at 50 °C, pH 5.0, for up to 120 h. The results of this study strongly suggest that both preparations, dosed at equal volumes, give comparable yields of reducing sugars from PCS, suggesting that costly cell removal may not be required.

6 Conclusions

The development of cost-effective enzymes for the widespread utilization of lignocellulosic biomass will require continued research and development to be successfully deployed. Although great progress has been made in identifying new enzyme mixes with improved catalytic efficiency, improvements in enzyme yield, and improved enzyme production economics, much work remains. There are thousands of organisms involved in the natural decomposition of plant material in our biosphere, and only a fraction of those have been isolated or investigated. Since these organisms work collectively to degrade biomass, better enzymes, with greater synergies, will be uncovered with additional work. Future efforts will also likely require the use of directed evolution techniques to collectively optimize enzymes to perform under conditions more compatible with the fermentation organisms used to produce ethanol and other products. In the short term, there are also great strides to be made in the area of process integration. Here, closely coupling the steps of pretreatment, hydrolysis, and fermentation has the potential to significantly increase overall process efficiency and reduce cost.

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Thermostable Enzymes in Lignocellulose Hydrolysis

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Abstract Thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosic substrates; higher specific activity decreasing the amount of enzymes, enhanced stability allowing improved hydrolysis performance and increased flexibility with respect to process configurations, all leading to improvement of the overall economy of the process. New thermostable cellulase mixtures were composed of cloned fungal enzymes for hydrolysis experiments. Three thermostable cellulases, identified as the most promising enzymes in their categories (cellobiohydrolase, endoglucanase and β-glucosidase), were cloned and produced in *Trichoderma reesei* and mixed to compose a novel mixture of thermostable cellulases. Thermostable xylanase was added to enzyme preparations used on substrates containing residual hemicellulose. The new optimised thermostable enzyme mixtures were evaluated in high temperature hydrolysis experiments on technical steam pretreated raw materials: spruce and corn stover. The hydrolysis temperature could be increased by about 10–15 °C, as compared with present commercial *Trichoderma* enzymes. The same degree of hydrolysis, about 90% of theoretical, measured as individual sugars, could be obtained with the thermostable enzymes at 60 °C as with the commercial enzymes at 45 °C. Clearly more efficient hydrolysis per assayed FPU unit or per amount of cellobiohydrolase I protein used was

obtained. The maximum FPU activity of the novel enzyme mixture was about 25% higher at the optimum temperature at 65 °C, as compared with the highest activity of the commercial reference enzyme at 60 °C. The results provide a promising basis to produce and formulate improved enzyme products. These products can have high temperature stability in process conditions in the range of 55–60 °C (with present industrial products at 45–50 °C) and clearly improved specific activity, essentially decreasing the protein dosage required for an efficient hydrolysis of lignocellulosic substrates. New types of process configurations based on thermostable enzymes are discussed.

Keywords Thermostable · Cellulases · Cellobiohydrolase · Endoglucanase · β-Glucosidase · Lignocellulose · Hydrolysis

1 Introduction

The present challenge is to substantially increase the production and use of biofuels for the transport sector. In order to reach the future goals of substituting fossil based fuels, it will be necessary to promote the transition towards second generation biofuels. These can be produced from a wider range of feedstock, including lignocellulosic raw materials. Biomass resources can be broadly categorised as agricultural or forestry-based, including secondary sources derived from agro- and wood industries, waste sources and municipal solid wastes. Fuels from lignocellulosic biomass have a higher potential to reduce greenhouse gas emissions, and hence are an important means to fulfil the CO_2 emissions targets, as compared with first generation biofuels. Lignocellulosic raw materials comprise an abundant source of carbohydrates (cellulose and hemicellulose) for a variety of biofuels, including bioethanol. The conversion technologies of lignocellulosic raw materials are, however, more complex and need novel enzyme systems and advanced fermentation technologies. The rate-limiting step in the conversion of cellulose to fuels is hydrolysis, especially the initial attack on the highly ordered, insoluble structure of crystalline cellulose. In spite of recent achievements, further developments are still needed to improve the overall economy of the lignocellulose-to-ethanol process. These novel conversion techniques would also be applicable for the production of other sugar platform-based chemicals.

2 Enzymatic Hydrolysis of Cellulose

Plant cellulose exists in a highly crystalline form. In addition, it is associated with hemicellulose and surrounded by lignin, which may also be covalently bound to hemicellulose. Pretreatments aim at increasing the surface area of cellulose by either removing lignin or solubilising hemicellulose, disrupting

the crystallinity and/or by increasing the pore volume. Hydrolysis of cellulose requires the co-operation of three classes of cellulolytic enzymes, namely cellobiohydrolases (CBH, EC 3.2.1.91), endo-β-1,4-glucanases (EG, EC 3.2.1.4) and β-glucosidases (BG, EC 3.2.1.21). The CAZY (carbohydrate active enzymes) [16] classification system collates glycosyl hydrolase (GH) enzymes into families according to sequence similarity, which have been shown to reflect shared structural features. Most of the initial cellulase work was concentrated on the biochemistry, genetics and process development of the mesophilic fungus *Trichoderma reesei*. This fungus is one of the most powerful secretors of extracellular proteins. It is industrially used for the production of various homologous and heterologous proteins. Also several thermostable enzymes have been expressed in this host, as reviewed by Bergquist et al. [8].

Trichoderma reesei produces several cellulases which act synergistically in the degradation of cellulose. Eight major cellulase genes have so far been identified from the *T. reesei* genome; two cellobiohydrolases (CBH I and II, i.e. Cel7A and Cel6A), and six endoglucanases (EG I–VI, i.e. Cel7B, Cel5a, Cel12A, Cel45A, Cel61A, Cel74A) [24]. All known *T. reesei* cellulases, with one exception (Cel12A), have a two-modular structure. They consist of a catalytic module and a carbohydrate binding module (CBM) connected with a linker region. Cel7A (CBHI) is the major cellulose produced by *T. reesei*; it has been reported to hydrolyse solid cellulose and constitutes about 60% of the cellulases expressed [51, 73]. It has been shown that Cel7A hydrolyses the cellulose chain from the reducing end and it is believed that the chain is hydrolysed processively [3, 19, 55]. Cel6A, on the other hand, preferably hydrolyses the cellulose chain from the non-reducing end [55, 73]. It constitutes about 10–15% of total cellulase proteins [51]. The Cel7B is the major endoglucanase, forming about 6–10% of total *T. reesei* cellulase [51, 73]. It has activity against solid and soluble substrates, such as CMC, as well as against xylan, PASC and glucomannans [71]. Also the endoglucanase Cel5A is reported to have activity against solid (Avicel, BMCC) and soluble (CMC, mannan) substrates [31, 38, 44], but not on xylans. This enzyme comprises about 1–10% of the total cellulases in *T. reesei* [51, 73]. The minor endoglucanases Cel12A, Cel61A and Cel45A are reported to hydrolyse solid (Avicel, filter paper) and soluble (CMC, glucomannan) substrates with diverse specific activities. Two β-glucosidase encoding genes from *T. reesei* have been cloned [2, 74]. Cel74A [24] is a xyloglucanase having also endoglucanase activity against β-glucan and CMC [28].

3 Thermostable Cellulases

Thermostable enzymes are gaining wide industrial and biotechnical interest due to the fact that they are more stable and thus generally better suited

for harsh process conditions. The concept of thermostability is, however, not very clear, and the thermostability is a relative term. The enzymatic activity is known to increase with increasing temperature up to the temperature where inactivation starts to occur [25]. Thermostability is usually defined as the retention of activity after heating at a chosen temperature for a prolonged period. The drawback is that it only measures how well an enzyme tolerates high temperature and does not take into consideration the number of variables affecting this measurement. The most appropriate way to express thermostability is to measure the half-life of enzyme activity at elevated temperatures. Thermostable enzymes are produced both by thermophilic and mesophilic organisms. Although thermophilic microorganisms are a potential source for thermostable enzymes, the majority of industrial thermostable enzymes originate from mesophilic organisms. Thermophilic bacteria have, however, received considerable attention as sources of highly active and thermostable enzymes.

Thermostable enzymes in the hydrolysis of lignocellulosic materials have several potential advantages: higher specific activity (decreasing the amount of enzyme needed), higher stability (allowing elongated hydrolysis times) and increased flexibility for the process configurations. The two first characteristics would expectedly improve the overall performance of the enzymatic hydrolysis even at the range of conventional enzymes active at around 50 °C. Thus, carrying out the hydrolysis at higher temperature would ultimately lead to improved performance, i.e. decreased enzyme dosage and reduced hydrolysis time and, thus, potentially decreased hydrolysis costs. Thermostable enzymes would expectedly also allow hydrolysis at higher consistency due to lower viscosity at elevated temperatures and allow more flexibility in the process configurations. The characteristics of thermostable cellulases are reviewed in Table 1. The enzymes are categorised as endo- or exoglucanases, based on the information available.

Several hyperthermostable cellulolytic enzymes have been isolated from various thermophilic bacteria including the anaerobic *Thermotoga* [11, 14, 21], *Anaerocellum thermophilum* [82] and *Rhodothermus* strains [34]. Significant research efforts have been invested in the thermophilic bacterial cellulosome systems of Clostridia (reviewed by [17]). Concepts for the direct conversion of lignocellulose into ethanol using clostridial co-culture process have been studied [33]. In addition, thermostable ascomycete cellulases have been characterised [30, 37, 57]. Several mesophilic or moderately thermophilic fungal strains are also known to produce thermostable enzymes. These enzymes are stable and active at temperatures that are essentially higher that the optimum temperatures for the growth of the microorganism [65]. Some filamentous fungi produce cellulases that retain relatively high cellulose-degrading activity at elevated temperatures, particularly those from the species *Talaromyces emersonii* [27, 50, 78], *Thermoascus aurantiacus* [26, 59, 70], *Chaetomium thermophilum* [48], *Myceliophthora ther-*

Table 1 Thermostable cellulases

Organisms	Enzymes	Characteristics of enzymes				Refs.
		MW (SDS PAGE) (kDa)	pH optimum	T optimum (°C)	Stability	
Acidothermus cellulolyticus	Endoglucanase I	57.420–74.580	5.0	83	Inactivated at 110 °C	[18, 32, 67]
Anaerocellum thermophilum	Endoglucanase	230	5–6	95–100	Half-life 40 min at 100 °C	[82]
Bacillus sp. KSM-S237	Endoglucanase	86	8.6–9.0	45	30% of activity remained after 10 min at 100 °C	[29]
Caldocellum saccharolyticum	Endoglucanase	na			na	[76]
Caldocellulosiruptor saccharolyticus	Endoglucanases Exoglucanases	na	7.0	68–70	na	[7, 76]
Chaetomium thermophilum	Endoglucanase	68	4.0	60	Stable at 60 °C > 60 min, half-life 7 min at 90 °C	[42]
Cladosporium sp.	Endoglucanase Exoglucanase	na	4–6	60	Stable at 60 °C for 24 h	[1]
Clostridium stercorarium	Endoglucanase	100	6.0–6.5	90	Stable for several days	[13]
Clostridium stercorarium	Exoglucanase	87	5–6	75	Stable at 70 °C for 3 days	[12]
Clostridium thermocellum	Endoglucanase	83	6.6	70	33% of activity remained after 50 h at 60 °C	[22]
Clostridium thermocellum	Endoglucanase	76	7.0	70	50% of activity remained after 48 h at 60 °C	[61]
Melanocarpus albomyces	Endoglucanase	20	6–7	70	70% of activity remained after 60 min at 80 °C	[47]
Rhodothermus marinus	Endoglucanase	49	7.0	95	50% of activity remained after 3.5 h at 100 °C, 80% after 16 h at 90 °C	[34]

na: not available

Table 1 (continued)

Organisms	Enzymes	Characteristics of enzymes				Refs.
		MW (SDS PAGE) (kDa)	pH optimum	T optimum (°C)	Stability	
Rhizomucor pusillus	Endoglucanase	na	5.5	60	Stable at 60 °C for 84 h	[68]
Sporotrichum sp.	Endoglucanase	33	4.5–5	70	Stable at 70 °C for 30 min	[35]
Streptomyces sp.	Exoglucanase	44	4	60	30% of activity remained after 10 min at 100 °C	[57]
Streptomyces sp.	Endoglucanase	26	4	60	20% of activity remained after 10 min at 100 °C	[57]
Talaromyces emersonii	Exoglucanase (CBH IA)	66	3.6	78	Half-life 34 min at 80 °C	[78]
Thermoascus aurantiacus	Endoglucanase		4.5	75	Half-life 98 h at 70 °C and 4.1 h at 75 °C	[26]
Thermomonospora curvata	Endoglucanase	100	6.0–6.5	70–73	Stable for 48 h at 60 °C, half-life 30 min at 80 °C	[41]
Thermotoga sp.	Exoglucanase	36	6.8–7.8	100–105	Half-life 70 min at 108 °C, 2 min at 117 °C	[64]
Thermotoga maritima	Endoglucanase	27	6.0–7.5	95	Half-life 2 h at 95 °C	[14]
Thermotoga neapolitana	Endoglucanase (CelA)	29	6.0	95	Half-life > 240 min at 100 °C	[11]
	Endoglucanase (CelB)	30	6.0–6.6	106		

na: not available

mophila [63], *Thielavia terrestris* and *Corynascus thermophilus* [45]. Thermophilic β-glucosidases have been obtained from e.g. *Aureobasidium* sp. [66], *Chaetomium thermophila* [79], *Talaromyces emersonii* [15], *Thermoascus aurantiacus* [23, 26, 59, 70] and *Thermomyces lanuginosa* [40]. The literature data shows that a number of enzymes are stable at temperatures around 70 °C for elongated periods, but the data does not allow comparison of the properties under similar conditions.

4 Process Concepts

The enzymatic hydrolysis of the pretreated raw material and the fermentation of the hydrolysed sugars can be performed separately or simultaneously, commonly referred to as SHF (separate hydrolysis and fermentation) or as SSF (simultaneous saccharification and fermentation). The SSF process configuration has been generally considered more favourable for reducing the ethanol production costs [72, 81]. The hydrolysis rate in the separate hydrolysis is strongly inhibited by the accumulation of the end products, cellobiose and glucose [60]. In the simultaneous hydrolysis and fermentation, the end product inhibition is alleviated by the continuous removal of glucose by the fermenting organism. In the separate hydrolysis and fermentation the most severe end product inhibition caused by cellobiose has been overcome by adding an adequately high amount of β-glucosidase. For the same reason, the enzyme dosage needed is obviously lower in the SSF. Other claimed advantages of the SSF are the lower risk of contamination and reduction of investment costs by combined reactors. The low concentration of free glucose and the presence of ethanol make it more difficult for contaminating microorganisms to take over the fermentation and decrease the ethanol yield. The drawback of the SSF is that the conditions, i.e. the pH and temperature of the hydrolysis and fermentation, are suboptimal in a combined process. The optimal temperature for the enzymatic hydrolysis is clearly higher than that of the presently used fermenting organisms. Another drawback of the SSF is the difficulty in optimising the fermentation of techniques, i.e. by running continuous fermentation or recirculating and reusing the yeast due to the presence of the solid residues from the hydrolysis.

To improve the overall process economics and to achieve a faster hydrolysis rate by using thermostable enzymes, various modifications of the present process configurations can be considered (Fig. 1). After the pretreatment, the temperature of the substrate is high, and is reduced to achieve the operating temperature in the following process stages. In the traditional SSF, the temperature is about 35 °C. In a separate hydrolysis and fermentation process, the first total hydrolysis stage is carried out at about 45–50 °C with the present commercial enzymes, or above 60 °C with novel thermostable

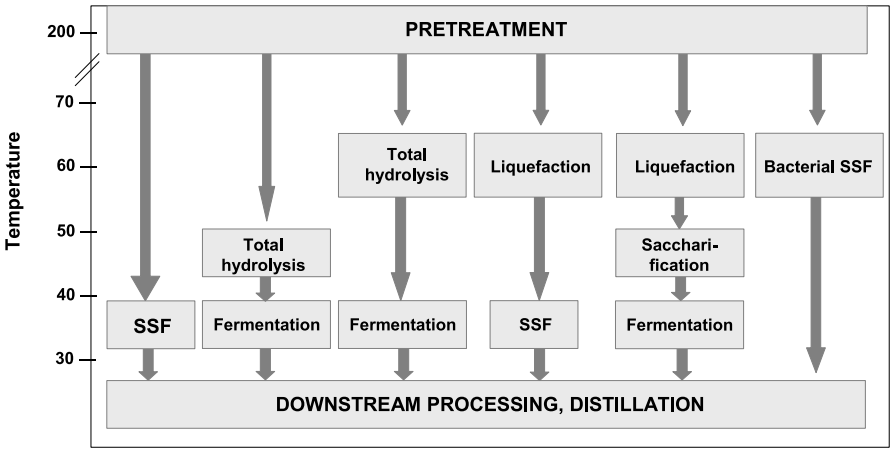

Fig. 1 Configurations of hydrolysis and fermentation processes at different temperatures using thermostable enzymes

enzymes. Other options include a partial prehydrolysis at higher temperatures, denoted as liquefaction, where the viscosity of the substrate is decreased using a chosen composition of thermostable cellulases based on one or several enzymes. The liquefaction stage, i.e. an enzymatic treatment improving the rheological properties (improved flowability, reduced viscosity) of the slurry, can significantly improve the mixing properties of the substrate slurry [83]. This partial hydrolysis can be carried out even with the limited number of thermostable cellulolytic and hemicellulolytic enzymes available. Using a set of thermoactive enzymes in the prehydrolysis, it was possible to reduce the viscosity and increase the sugar formation [83]. The high viscosity is a consequence of a high initial substrate consistency, needed to achieve a high final sugar and ethanol concentration and to decrease the distillation costs [69]. With a theoretical ethanol yield of 25–30% of the raw material, the raw material consistency should be at least 15% (d.w.) to reach an ethanol concentration of 4–5%. Some of the technical obstacles related to high consistency can thus be overcome by a rapid decrease of viscosity. After a liquefying partial hydrolysis, the saccharification stage using a complete or complementary set of hydrolytic enzymes, either simultaneously or separately from the fermentation (SSF or SHF), can be carried out. A separate hydrolysis stage (SHF) can be carried out at elevated temperatures with the complete set of hydrolytic thermostable enzymes needed for a chosen substrate. Finally, thermostable enzymes could be supplemented to bacterial fermentations using anaerobic, ethanol producing strains, such as Clostridia, to improve their conversion rate of cellulosic substrates into sugars (SSF or consolidated bioprocessing). Thus, new thermostable enzymes would allow the design of more flexible process con-

figurations, based on the availability of novel thermostable lignocellulolytic enzymes.

The performance of chosen thermostable cellulolytic enzymes with present commercial fungal enzymes was compared in this paper. The reference enzyme preparations contain the whole set of cellulolytic enzymes, i.e. cellobiohydrolases and endoglucanases, as well as several hemicellulolytic activities and β-glucosidases. These enzymes work at temperatures up to about 45 °C in long-term hydrolysis conditions and up to 50 °C in short-term conditions. New enzyme compositions were designed and tested in the hydrolysis of various steam pretreated raw materials.

5 Evaluation of Novel Thermophilic Enzymes; Materials and Methods

Enzymes

The thermostable enzyme preparations were kindly provided by ROAL, Finland. The genes encoding three thermostable enzymes: cellobiohydrolase (CBH/Cel7A) from *Thermoascus aurantiacus*, fused with the *T. reesei* CBHI cellulose binding domain (CBM), endoglucanase (EG/Cel45A) from *Acremonium thermophilum* and a xylanase and β-glucosidase (BG/Cel3A) from *T. aurantiacus* were inserted under the control of a strong *T. reesei cbh1* promoter and transformed into a host strain where all the major cellulase genes were deleted (phenotype CBHI/Cel7A – CBHII – Cel6A – EGI/Cel7B – EGII/Cel5A). Fermenter supernatants produced at pilot scale were used to produce various mixtures of the thermostable enzymes. The background activities in the deletion strains were measured. The composition of the mixture of the three thermostable enzymes was optimised based on the average cellulase composition of *T. reesei*. The enzyme components were mixed in different ratios and the total cellulase activity of the mixtures was measured at 50 °C (as FPU mL^{-1}) and used as the basis of enzyme dosing. In addition, a family 10 thermostable xylanase from *T. aurantiacus*, cloned and expressed in the *T. reesei* deletion strain, was added to some preparations to ensure complete hydrolysis.

Celluclast 1.5 L FG (Novozymes, Denmark) and Econase CE (ABEnzymes, Finland), eventually supplemented with BG from Novozym 188 (Novozymes, Denmark) were used as reference enzymes. The standard enzyme dosage was 10 FPU g^{-1} cellulose for Celluclast 1.5 L FG, supplemented with additional BG (100–500 nkat g^{-1} cellulose). Assuming an average 50% cellulose content of the lignocellulose substrates, the enzyme dosage was thus 5 FPU g^{-1} substrate. For the hydrolysis experiments at elevated temperatures, higher dosage of Celluclast (22 FPU g^{-1} cellulose) was used.

The total cellulolytic activity used as a basis for dosing of the enzyme mixtures was evaluated using the FPU activity, measured against Whatman no. 1 filter paper [36]. The EG activity was assayed using hydroxyl ethyl cel-

lulose as substrate [36]. The CBH activity was determined by using 4-methyl umbelliferyl-β-D-lactoside as substrate, estimating the effect of EGs by carrying out the assay with or without 20 mM cellobiose in the reaction [6]. The xylanase activity was assayed using birchwood glucuronoxylan as substrate [4] and that of BG using *p*-nitrophenyl-β-D-glucopyranosidase as substrate [5]. Protein was assayed according Lowry et al. [43]. All the enzyme activity assays were carried out at pH 5.

Substrates

The substrates used were steam pretreated, washed spruce solid fraction kindly provided by Guido Zacchi at the Lund University, Sweden, and steam pretreated corn stover kindly provided by Francesco Zimbardi at ENEA, Italy. The solid fraction of spruce substrate after the pretreatment was separated from the liquid fraction by filtration, washed and used in the hydrolysis experiments. The composition of the fibre fractions of the substrates is presented in Table 2. In addition to the insoluble fibre fraction, the corn stover substrate contained significant amounts, about 17% (d.w.) of solubilised mono- and oligosaccharides, solubilised mainly from hemicelluloses. Based on secondary analytical enzymatic hydrolysis and HPLC analysis, the carbohydrates in the soluble fraction consisted of xylose (74%), arabinose (15%), galactose (5%) and glucose (6%). Comparative hydrolysis experiments were carried out using crystalline cellulose (Avicel, Sigma).

Table 2 Composition of substrates used in the hydrolysis experiments

	Spruce	Corn stover
Pretreatment conditions	215 °C, 4 min	195 °C, 5 min
Catalysts	SO_2 impregnation	No
Composition of solid fraction(%)		
• Glucan	50.8	56.0
• Xylan	0.11	9.3
• Mannan	0.16	bdl
• Lignin and others	49	35

bdl below detection limit

Hydrolytic Properties of *T. reesei* Enzymes at High Temperatures

The hydrolysis experiments were carried out at a substrate consistency of 10 g L^{-1} in 50 mM sodium acetate, pH 5, in a volume of 100 mL, and incubated in shake flasks with shaking (200 rpm) at different temperatures from 55 °C to 70 °C. Duplicate shake flasks were sampled (5 mL sample) at 2 h, 4 h, 6 h, 24 h, 48 h and 72 h from the start of the hydrolysis. Possible evaporation was checked by weighing and corrected when necessary by adding a correspond-

ing amount of water. The release of hydrolysis products was followed during the hydrolysis.

Hydrolytic Properties of Thermostable Enzyme Mixtures

The performance of the thermostable enzyme mixtures was studied in hydrolysis experiment in test tubes (5 mL). Enzyme mixtures were dosed on the basis of FPU activity to the substrates (10 g L^{-1} dry matter) suspended in 50 mM sodium acetate, pH 5. The standard enzyme dosage was 10 FPU g^{-1} cellulose. Triplicate samples were incubated with mixing at 35 °C, 45 °C, 55 °C or 60 °C for 24 h, 48 h or 72 h. Reference samples with inactivated enzymes and corresponding substrates were also prepared.

Chemical Analysis

The release of hydrolysis products was measured as reducing sugars assayed by the DNS method using glucose as standard [10]. The results were corrected by taking into account the blank samples containing corresponding amounts of inactivated enzymes and substrate. The mono- and oligosaccharides formed were also analysed by high-performance anion-exchange chromatography on a Dionex 4500i series chromatograph with pulsed amperometric detection (HPAEC-PAD), as described earlier [75].

6
Composition of the Thermophilic Enzyme Mixtures

The tested thermostable fungal enzymes, classified as cellobiohydrolases (CBHs) or endoglucanases (EGs) based on the activity determinations, were chosen by preliminary screening and characterisation. Several thermostable CBHs from various thermophilic organisms were purified and characterised (Voutilainen et al, manuscript in preparation). The gene of the most potential CBH isolated from *Thermoascus aurantiacus* was fused with the *T. reesei* CBHI cellulose binding domain (CBM). In addition, an EG from *Acremonium thermophilum*, a β-glucosidase and a xylanase from *T. aurantiacus* were used to compose the thermostable mixtures. Fermenter supernatants produced in pilot scale were used to obtain the thermostable cellulase mixtures. The optimal ratio of EG to CBH amount (measured as protein of the enzyme mixtures) was determined on the basis of FPU activity of the preparations. The highest FPU activity was obtained by an EG to CBH protein ratio of 3 : 10, which corresponded well to the respective ratio of the native *T. reesei* enzymes. This ratio also gave the highest sugar yields in the hydrolysis of the steam pretreated corn stover substrate (results not shown) and was used as the standard basis for various mixtures. Three different mixtures were used in this work, differing with respect to the xylanase activity (Table 3). The xylanase-free preparation (TM 1) was first used for the spruce substrate

Table 3 Activity ratios of the thermostable enzyme mixtures (TM) used in the hydrolysis experiments

Enzyme mixture	EG : CBH (nkat : nkat)	BG : CBH (nkat : nkat)	XYL : CBH (nkat : nkat)
TM 1	0.53	3.5	0
TM 2	0.53	3.5	17.3
TM 3	0.53	3.5	8.8

The enzymes were composed of a thermostable cellobiohydrolase (CBH), endoglucanase (EG) and β-glucosidase (BG) (mixture TM 1) supplemented either with high (TM 2) or low (TM 3) amounts of xylanase (XYL). The activities of the enzyme mixtures are expressed as the ratio of the added key activity (EG, BG or XYL) to the CBH activity of the enzyme mixture

and the xylanase-containing preparations (TM 2 and TM 3) for the corn stover substrate. As it has frequently been observed that xylanases enhance the hydrolysis of lignocellulosic substrates containing even low amounts of residual xylan [9], preparations with xylanase activity were later used for both substrates.

7 Performance of Commercial Fungal Preparations at Elevated Temperatures

The activities of commercial reference preparations were first measured at higher temperatures in order to evaluate their general performance and to estimate the role of the background activities originating from the production strain. The hydrolysis of the pretreated spruce substrate by the commercial preparations (with and without added β-glucosidase, BG) at various temperatures from 50 to 70 °C was estimated during the first 24 h of the hydrolysis. The native *Trichoderma* cellulases and the *Aspergillus* BG were rapidly inactivated during the first 2 h of hydrolysis of the pretreated spruce substrate at temperatures above 60 °C (Fig. 2). The hydrolysis ceased after 24 h at 60 °C and after 48 h at 55 °C (results not shown). As expected, the effect of the added BG on the sugar yield was significant. The relative inactivation of BG was more pronounced even at 60 °C (Fig. 2b). The hydrolytic effect of the rather high loading (about 20 FPU g^{-1} cellulose) of *T. reesei* and *Aspergillus* enzymes was obviously due to the initial stage of hydrolysis during which the enzymes remained active. The hydrolysis yield of sugars from spruce during the first 2 h was 15% of the theoretical maximum at 70 °C, 22% at 65 °C and 33% at 60 °C. There were indications that the temperature optimum of the commercial *T. reesei* enzymes in the hydrolysis of the pretreated spruce substrate was about 5 °C lower than on pure cellulose (results not shown).

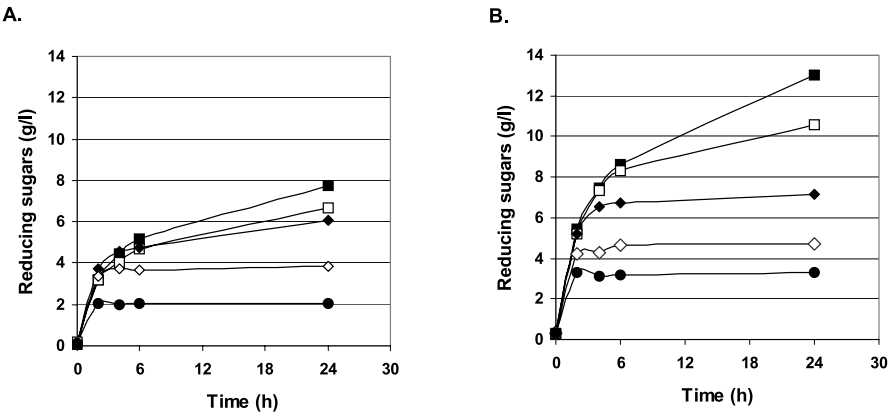

Fig. 2 Hydrolysis of washed, steam pretreated spruce substrate (cellulose content 18.3 g L^{-1}) with Celluclast 1.5 L FG alone (**A**) or supplemented with Novozym 188 (**B**) at various temperatures at pH 5. The dosage of Celluclast was 22 FPU g^{-1} cellulose and the Novozym 188 β-glucosidase 550 nkat g^{-1} cellulose. ■50 °C, □55 °C, ♦60 °C, ◇65 °C and • 70 °C

8
Evaluation of New Thermostable Enzyme Mixtures

Mixtures of selected thermostable enzymes (Table 2) were first evaluated for their hydrolytic efficiency by measuring the FPU activities at different temperatures (Fig. 3). The temperature optima of the new thermostable mixtures in the FPU activity assay were 5–10 °C higher than those of the commercial enzyme mixtures when a relatively short reaction time (60 min) in this assay was used. The relative FPU activity was set at the value of 100 at the reference point at 50 °C. The maximum FPU activity of the novel enzyme mixture was about 25% higher at the optimum temperature at 65 °C as compared with the highest activity of the commercial reference enzyme at 60 °C. As could be expected, at lower temperature (35 °C), corresponding to the fermentation temperature of traditional yeasts in a simultaneous saccharification and fermentation process (SSF), the FPU activities of the thermostable preparations were slightly lower than those of the commercial *T. reesei* enzymes.

The thermostable enzyme mixture without added xylanase activity (TM 1) was evaluated on pure cellulose (Avicel) and compared with the commercial enzyme preparations (Celluclast supplemented with β-glucosidase) at 45 °C, 55 °C and 60 °C in a 48 h hydrolysis (Fig. 4). On pure cellulose, the mixture of thermostable enzymes gave nearly similar hydrolysis results at 60 °C as the *T. reesei* enzymes at 45 °C, i.e. thus enabling an increase of temperature of about 15 °C. At 60 °C, the hydrolysis yield of Avicel was about three- to four-fold better with the thermostable enzymes than with the commercial fungal enzymes. The highest hydrolysis yield was about 90% of the theoretical.

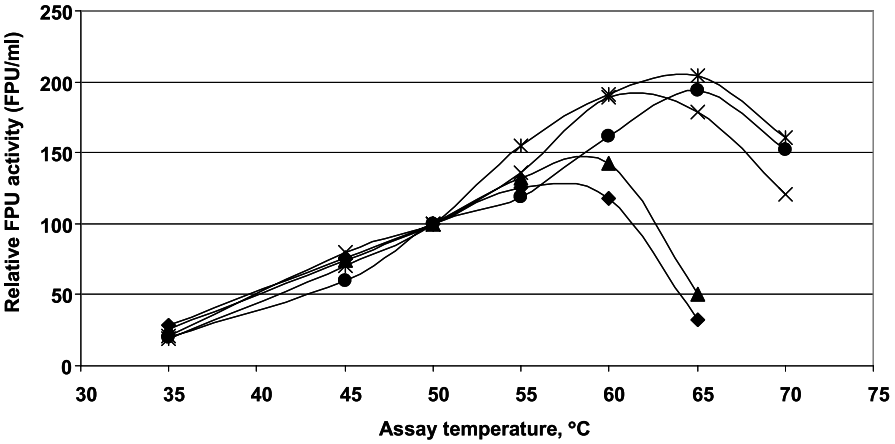

Fig. 3 Temperature optimae of cellulase activity (FPU) of the thermostable enzyme mixtures and of the commercial enzyme preparations (Econase or Celluclast supplemented with Novozym 188). ♦ Econase + Novozym 188, ▲ Celluclast + Novozym 188, ∗ TM 1, × TM 2, ● TM 3

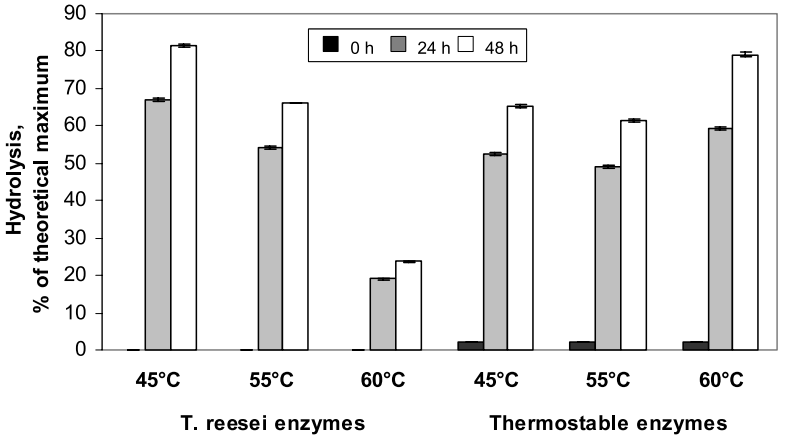

Fig. 4 Hydrolysis of cellulose (Avicel, 10 mg mL^{-1}, *left*) with Celluclast and the thermostable enzyme mixture (TM 1) at 45, 55 and 60 °C. Hydrolysis yield was measured as reducing sugars. Enzyme dosages: Celluclast 10 FPU g^{-1} cellulose, supplemented with 100 nkat Novozym 188 g^{-1} substrate (total activity 12 FPU g^{-1}); thermostable enzyme 10 FPU g^{-1} cellulose. Hydrolysis time 72 h at pH 5, triplicates with mixing. ■0 h, ▩24 h, □48 h

On the spruce substrate, the thermostable enzyme mixture resulted in an even more significant improvement in the performance at higher hydrolysis temperature as compared with the commercial enzymes. Thus, the hydrolysis yield was about threefold better at 55 °C and about fivefold better at 60 °C

using the thermostable enzyme mixture (Fig. 5). The hydrolysis was, however, also decreased with the thermoenzyme mixture at 60 °C. When comparing the hydrolytic performance of the commercial enzymes by increasing the temperature from 45 °C to 60 °C on Avicel and on spruce, it can be observed that the increased hydrolysis temperature decreased the performance on the natural lignocellulose substrate significantly more: from 70–10% on spruce, as compared with 90–30% on Avicel within 48 h. Obviously, the spruce substrate, even washed, contained compounds that, with increasing temperature, inhibited or inactivated not only the *T. reesei* enzymes, but also the thermostable enzymes.

High temperature enzyme mixtures suitable for hemicellulose-containing raw materials were evaluated in the hydrolysis of steam pretreated corn stover substrate (Fig. 6). With this raw material, the hydrolysis by the thermostable enzyme mixture at 45 °C was better than with the commercial preparation. The hydrolysis was still efficient at 55 °C and only slightly decreased at 60 °C with the thermostable enzyme mixture. The relative decrease of the hydrolytic performance of both enzyme preparations was less pronounced on the corn stover substrate than with the spruce substrate at elevated temperatures. Based on HPLC analysis (Table 4) of the corn stover hydrolysates, the yield of glucose was around 90–95% of the theoretical after 72 h. The corresponding yield of xylose was about 80–90% at temperatures up to 60 °C. The hydrolysis yields of the minor monosaccharide sugars, arabinose and galactose, were not significantly improved by the thermophilic enzyme mixtures, indicating the absence of corresponding thermostable enzymes, i.e. arabinosidases and galactanases in the mixtures. In the hydrolysis of the

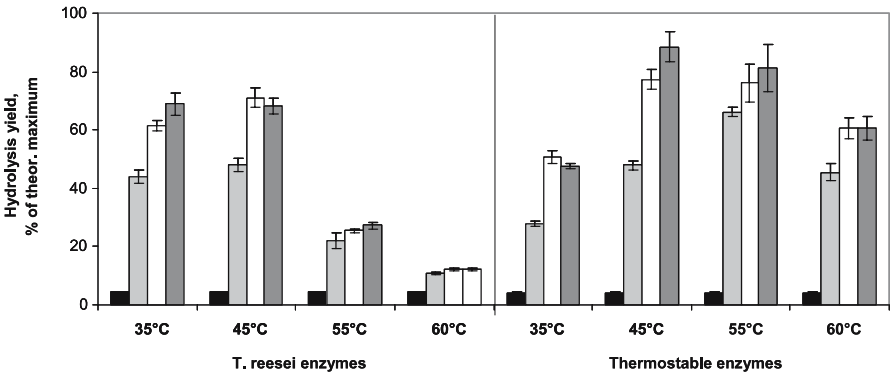

Fig. 5 Hydrolysis of pretreated washed spruce (10 mg mL^{-1}) with Celluclast and the thermostable enzyme mixture (TM 3) at temperatures from 35 to 60 °C. Hydrolysis yield was measured as reducing sugars. Enzyme dosages: Celluclast 5 FPU g^{-1} substrate, supplemented with 100 nkat Novozym 188 g^{-1} substrate; thermostable enzyme 5 FPU g^{-1} substrate. Hydrolysis time 72 h at pH 5, triplicates with mixing. ■0 h, ▩24 h, □48 h and ▨72 h

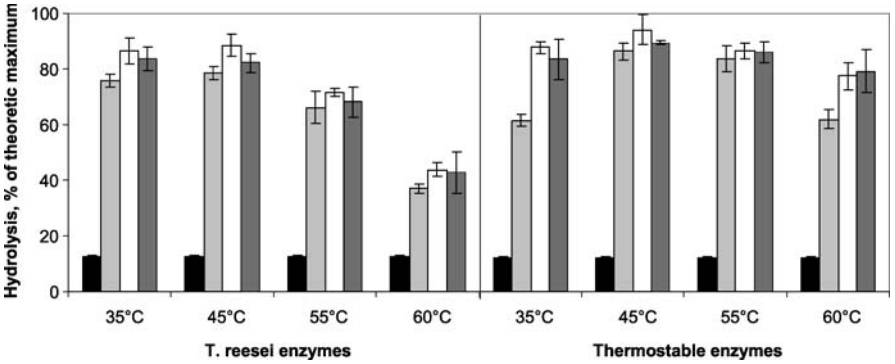

Fig. 6 Hydrolysis of pretreated corn stover (10 mg mL^{-1}) with Celluclast and the thermostable enzyme mixture (TM 3) at temperatures from 35 to 60 °C. Hydrolysis yield was measured as reducing sugars. Enzyme dosages: Celluclast 5 FPU g^{-1} substrate, supplemented with 100 nkat Novozym 188 g^{-1} substrate; thermostable enzyme 5 FPU g^{-1} substrate. Hydrolysis time 72 h at pH 5, triplicates with mixing. ■0 h, ▩24 h, □48 h and ▨72 h

Table 4 Sugars released from steam pretreated spruce and corn stover (% of the initial sugar component in the substrate), analysed by HPLC

Enzymes	Hydrolysis temp. (°C)	Sugars released from spruce % of theoretical Glucose	Sugars released from corn stover % of theoretical Glucose	Xylose	Arabinose	Galactose
Commercial enzymes (Celluclast + Novozym 188)	35	76	76	80	25	9
	45	75	83	81	25	13
	55	26	67	74	20	11
	60	9	28	50	6	4
Thermostable mixture (TM 3)	35	51	95	84	31	12
	45	95	90	84	36	15
	55	82	96	97	31	8
	60	56	81	85	22	2

Enzyme dosage was for reference enzymes: Celluclast 5 FPU g^{-1} substrate supplemented with 100 nkat g^{-1} Novozym 188; and for thermostable enzyme (TM 3) 5 FPU g^{-1} substrate. Hydrolysis time 72 h at pH 5, triplicates with mixing. Release of xylose, mannose and arabinose from spruce substrate was below the reliable detection limit (less than 0.1% of the substrate)

spruce substrate (Fig. 5, Table 4), only glucose was released. The individual sugar analyses corresponded well with the measured values of the reducing sugars.

9
Performance of the Thermostable Enzymes at Lower Temperatures

The performance of the thermostable enzymes at a lower temperature, the 35 °C commonly used in SSF, was compared. The *T. reesei* deletion strains produced only low amounts of background cellulase activities, mainly due to the presence of native EGIII (Cel12A) and EGV (Cel45A). However, the deletion strains used for the production of thermoenzymes produced some hemicellulases. For practical use, any mesophilic background activity enhancing the hydrolysis can be considered useful, but in order to evaluate the performance of the thermophilic enzymes, the level of remaining background activities was evaluated. The FPU activity in the background was negligible and the endoglucanase activity was very low as compared to the commercial preparations. Most of the endoglucanase activity, 85–90%, was inactivated during the thermal treatment at 60 °C, pH 6.5 for 2 h. Obviously, the EGV activity was the most stable remaining activity. Thus, the background activities originating from the *T. reesei* deletion strains had only a minor contribution to the total hydrolysis above 65 °C.

The actual hydrolysis performance of the new thermostable enzyme mixtures on various pretreated lignocellulose substrates (spruce and corn stover) at 35 °C showed some variations as compared with the *T. reesei* enzymes: on

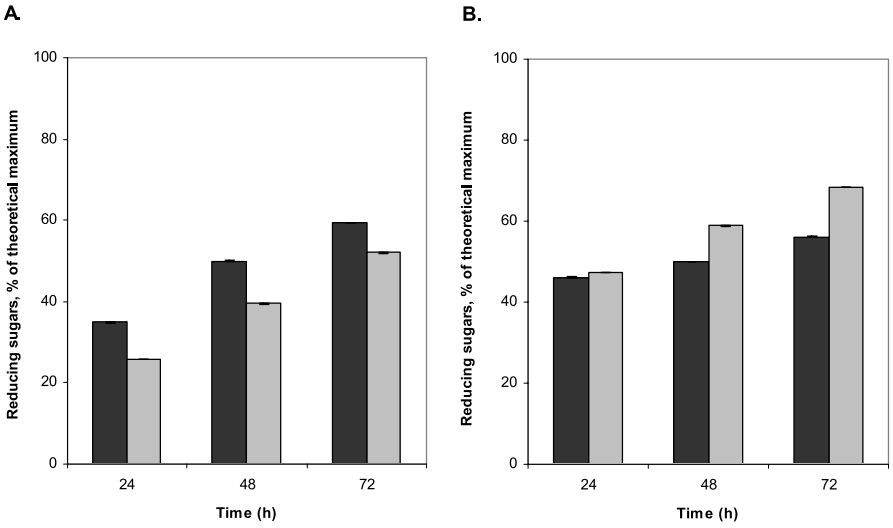

Fig. 7 Hydrolysis of steam pretreated washed spruce (**a**) and unwashed corn stover (**b**) by Celluclast (■) and (▩) the thermostable enzymes (TM 1 for spruce and TM 2 for corn stover) at 35 °C. Enzyme dosages: Celluclast 5 FPU g^{-1} substrate, supplemented with 100 nkat Novozym 188 g^{-1} substrate; thermostable enzymes 5 FPU g^{-1} substrate, substrate concentration 10 g L^{-1}, hydrolysis time 72 h at pH 5, triplicates with mixing

spruce, the sugar yield obtained by the thermophilic enzymes was generally lower and on corn stover higher than with the commercial *T. reesei* enzymes (Fig. 7). The result was the same, irrespective of the presence of the thermoxylanase in the preparation (TM 1 in Fig. 7 or TM 3 in Fig. 5). Thus, with this substrate the relatively lower cellulase activity at 35 °C is obviously the reason for the poorer hydrolysis at the lower temperature. In contrast, on the xylan-containing substrate, corn stover, the additional xylanase activity in the thermostable enzyme mixture had a more profound effect. The total xylanase activity was somewhat higher in the thermostable preparation, emphasising the importance of hemicellulases in the hydrolysis of substrates containing residual xylans. Further research would be needed to study in detail the structural differences of both cellulose and hemicellulose in the two substrates and their impact on the performance of the enzyme patterns used.

10 Discussion

When designing novel thermostable enzyme systems, the structural features of the substrates determine the number of enzymes needed for total hydrolysis. The crystallinity of cellulose, the available surface area and the distribution of lignin and hemicellulose are the major substrate-related factors limiting the hydrolysis rate of cellulose. An efficient pretreatment is the most straightforward solution for improving the hydrolysis rate and decreasing the amount of enzymes needed. Using various pretreatment techniques, either most of the hemicellulose or lignin is removed. It has been observed that the removal of hemicellulose has a direct correlation with the efficiency of the hydrolysis [56]. Even low amounts of residual xylan can limit the extent and rate of the hydrolysis. This can be overcome by addition of suitable hemicellulases, especially xylanases, to substrates with high original xylan content. Usually, the xylanase activity in commercial *T. reesei* preparations has been adequately high to overcome this limitation on xylan-containing substrates. Lignin content and distribution has also been proposed to be a substrate-related factor that affects the efficiency of enzymatic hydrolysis [49]. The close association of lignin and cellulose may prevent swelling of the fibrous substrate and result in limited accessibility of enzymes. The role of lignin or lignin-derived compounds in destabilising or deactivating enzymes is obviously also crucial. High temperature and pressure during the pretreatment result in a variety of soluble inhibitors for the enzymes and the yeast. In this work, it was observed that the inhibition of cellulases on lignin-containing substrates was increased at higher temperatures (Figs. 4 and 5).

The optimal cellulase composition varies depending on the substrate used but usually, the major cellulases comprise two cellobiohydrolases (about 60–70% of total protein), and two major and several minor endoglucanases

(about 25% of the total protein). Various models and mechanisms for the synergistic action of cellulases have been proposed. These studies have focused on the *T. reesei* exo–exo synergism [31, 53, 77, 80] or on the endo–exo synergism [39, 46, 52, 80]. The key role of β-glucosidase in a separate hydrolysis process has been clearly demonstrated, and is due to the end product inhibition of especially cellobiohydrolases caused by cellobiose [20, 54]. In *T. reesei* this activity is partly mycelium-bound and obviously limits the enzyme performance in commercial *T. reesei* preparations. Therefore, β-glucosidase is usually supplemented, generally originating from *Aspergillus niger*. Interestingly, in this work it was shown that just three major thermostable cellulases, i.e. one cellobiohydrolase and one endoglucanase supplemented by β-glucosidase, used in a preliminarily optimised ratio were able to produce a hydrolysis yield comparable with that obtained with the whole set of cellulolytic and accessory enzymes present in the commercial *T. reesei* preparations. Further research would be necessary to clarify the detailed mechanisms of these enzymes. Although the endo–exo synergism was obviously efficient enough to result in a high sugar yield, it could be further improved by optimising the thermostable cellulase components. The optimal ratio of the major enzymes was shown to be close to that of *T. reesei*. In this work, the individual thermostable cellulases were preliminarily screened based on their activity profiles and not based on their synergistic action. Therefore, the hydrolysis result can be considered extremely promising. Previously, thermostable enzymes from different organisms have not been combined to form new efficient mixtures. Expectedly, further optimisation, as well as supplementation of other synergistically acting enzymes would further improve the hydrolytic efficiency. In the present work, only thermostable xylanase was added to the mixture of the three cellulases (endoglucanase, cellobiohydrolase and β-glucosidase).

Previously, thermostable enzymes have only been studied as individually added proteins to improve the performance of the cellulases from *T. reesei* [62]. The *T. reesei* cellulase system is rapidly inactivated at temperatures above 45 °C, and the optimal temperature is generally considered to be below 45 °C on substrates requiring longer hydrolysis times, e.g. due to higher substrate consistency. Crude culture filtrates from various moderately thermophilic fungi (*C. thermophilum, T. terrestris, T. aurantiacus, C. thermophilus, M. thermophila*) were added on the protein basis to a commercial *T. reesei* preparation. Obviously, due to the relatively high proportion of *T. reesei* enzymes in the mixture, and the consequent inactivation of these enzymes at elevated temperatures, no improvement of the hydrolysis at higher temperatures could be observed. The main advantage was expected to be due to more active endoglucanases or due to a improved improved ratio of endoglucanase and cellobiohydrolase in the crude fermentation broth. In addition, unidentified enzyme activities in the preparations may also have caused some effects.

In this work, the individual cloned thermostable enzymes were produced with a *T. reesei* strain where the four genes encoding the major cellulases, i.e. Cel7A, Cel6A, Cel7B and Cel5A, had been deleted. Thus, only the minor endoglucanases Cel12A, Cel61A and Cel45A, as well as xylanases and other accessory enzymes, were present in the *T. reesei* background. In addition, most of these activities were inactivated in a thermal treatment. Only the Cel45A was somewhat more resistant to thermal inactivation and retained most activity at higher temperatures. Thus, the hydrolysis results were non-disputably obtained due the cloned thermostable enzymes, and the background activities were negligible. This was also clear from the hydrolysis experiments with the commercial *T. reesei* enzymes, showing clearly a decreased performance at temperatures of 50 °C or above.

In addition to improved performance in the hydrolysis of lignocellulosic substrates, thermophilic enzymes allow the design of more flexible process configurations. Traditionally, *T. reesei* enzymes are used either in a separate hydrolysis and fermentation process (SHF) or in a simultaneous saccharification and fermentation (SSF) process. It is commonly stated that the major advantage of the SHF is that both process steps (hydrolysis and fermentation) can be run under optimal conditions. Typically, hydrolysis of the SHF is carried out at around 45–50 °C at pH 5, and the fermentation at 35 °C at a lower pH. The SSF, on the other hand is usually carried out at 35 °C at pH 4.5–5. A more efficient hydrolysis is expected to take place at higher temperatures. In this work, using thermostable enzymes, it was indeed possible to obtain about 10 °C higher operation temperature than with the present commercial *T. reesei* enzyme preparations. The applicable hydrolysis temperature could be 60–65 °C for the hydrolysis of corn stover substrate and about 55 °C for spruce substrate. The hydrolysis rates at 55 °C were higher than those of the commercial enzymes at 45 °C. The enzymatic hydrolysis at higher temperatures would potentially reduce the reaction time and the enzyme loading.

It can be concluded that the cloned thermostable enzymes in preliminarily optimised preparations clearly demonstrate that the hydrolysis of lignocellulosic raw materials can be further improved, leading to potential savings in the hydrolysis costs. Previously, it has been shown that the costs of cellulases can be radically decreased, e.g. by improving the specific activity, by omitting the downstream processing of enzyme production or by improving the production process by other means. A mixture of only four thermostable enzymes was shown to be superior to the present commercial *T. reesei* preparations, which are comprised of at least ten enzymes acting synergistically on cellulose and on other components of the lignocellulosic substrates under optimal conditions. Further supplementation of other cellulases or accessory enzymes would expectedly further improve the hydrolysis result and the overall economy of the process.

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Metabolic Engineering for Pentose Utilization in *Saccharomyces cerevisiae*

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Abstract The introduction of pentose utilization pathways in baker's yeast *Saccharomyces cerevisiae* is summarized together with metabolic engineering strategies to improve

ethanolic pentose fermentation. Bacterial and fungal xylose and arabinose pathways have been expressed in *S. cerevisiae* but do not generally convey significant ethanolic fermentation traits to this yeast. A large number of rational metabolic engineering strategies directed among others toward sugar transport, initial pentose conversion, the pentose phosphate pathway, and the cellular redox metabolism have been exploited. The directed metabolic engineering approach has often been combined with random approaches including adaptation, mutagenesis, and hybridization. The knowledge gained about pentose fermentation in *S. cerevisiae* is primarily limited to genetically and physiologically well-characterized laboratory strains. The translation of this knowledge to strains performing in an industrial context is discussed.

Keywords Arabinose · Ethanol · Fermentation · Lignocellulose · Xylose · Yeast

Abbreviations

G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
mRNA	Messenger RNA
PPP	Pentose phosphate pathway
RKI	Ribose-5-phosphate ketol-isomerase
RPE	Ribulose-5-phosphate 3-epimerase
TAL	Transaldolase
TKL	Transketolase
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulokinase
XR	Xylose reductase

1 Introduction

When in the late 1970s it was discovered independently in two laboratories in North America [1, 2] that baker's yeast *Saccharomyces cerevisiae* could ferment the pentose sugar xylulose to ethanol, it was proclaimed that the development of recombinant xylose-fermenting strains of *S. cerevisiae* was a task that would be efficiently solved within a couple of years. Still, more than 25 years later, only a limited number of industrial *S. cerevisiae* strains that ferment pentose sugars have been generated [3–9]. Furthermore, there are relatively few studies on the performance of these strains under industrial conditions in lignocellulosic hydrolysates [6, 10–14]. The difficulty in developing efficient pentose-fermenting *S. cerevisiae* strains is no doubt that the regulation of metabolism in the eukaryotic yeasts is much less understood than that of, for example, the prokaryotic bacterium *Escherichia coli*. Consequently, the research on pentose-fermenting strains of *S. cerevisiae* has had the spin-off effect of generating more knowledge on the metabolism of this species, not least in relation to other yeasts.

The rationale for developing pentose-utilizing *S. cerevisiae* strains relies on the fact that this yeast has been used for the industrial production of ethanol and carbon dioxide as long as human history has been recorded. Presently, *S. cerevisiae* forms the basis for the world's largest fermentation industry producing beer, wine, potable and industrial ethanol, and baker's yeast. In addition, this organism serves as a eukaryotic model organism with an intensely studied cell biology and arrays of genetic engineering tools [15]. However, the most important reason for developing pentose-fermenting *S. cerevisiae* is the fact that such strains can be integrated into existing ethanol plants already using this yeast. Two independent investigations have estimated that integrated approaches to the production of lignocellulosic ethanol will reduce the production cost by nearly 20% [16, 17].

This chapter summarizes the metabolic engineering approaches taken to develop pentose-fermenting strains of *S. cerevisiae*. Different engineering strategies and their physiological context are described below, and the respective fermentation results from each study are chronologically summarized in Tables 1–4. Metabolic engineering for arabinose utilization is reported separately, since engineering L-arabinose utilization in *S. cerevisiae* has only recently been addressed. As will be detailed below, the fermentation of pentose sugars is governed by carbon catabolite repression and by reoxidation of reduced cofactors. Fermentation results of recombinant *S. cerevisiae* strains have therefore been summarized in relation to batch (Tables 1 and 2) and continuous culture (Tables 3 and 4), and in relation to anaerobic (Tables 1 and 3), oxygen-limited (Table 2), and aerobic conditions (Table 4). Moreover, the data have been organized in relation to the respective control strain to highlight the relative improvement of a particular engineering strategy. Studies that do not use the four aforementioned experimental conditions, or for which information on fermentation parameters is insufficient, have been omitted.

2 Xylose

Since *S. cerevisiae* cannot utilize xylose, but does utilize and ferment its isomer D-xylulose [1, 2], the obvious first step to allow xylose metabolism is to introduce a heterologous pathway converting xylose to xylulose. Over the years, several approaches have been explored to express a pentose utilization pathway from naturally pentose-utilizing bacteria and fungi in *S. cerevisiae*. Figure 1 summarizes the initial pathways for D-xylose utilization in bacteria and fungi.

Table 1 Xylose consumption rates (g xylose/g biomass h), ethanol yields (g ethanol/g sugar), and xylitol yields (g xylitol/g xylose) in anaerobic batch cultures with glucose and xylose or only xylose by recombinant *S. cerevisiae* strains. Defined mineral medium was used if other medium is not indicated

Strain	Relevant genotype/phenotype	Sugar composition	Xylose cons. rate	Ethanol yield	Xylitol yield	Ref. to strain	Ref. to ferm. data
H1693	*XYL1*, *XYL2*	50 g/l xyl	0.09	0.04	0.47	[100]	[100]
H1691	*XYL1*, *XYL2*, *XKS1*	50 g/l xyl	0.20	0.12	0.41	[100]	[100]
TMB3001	*XYL1*, *XYL2*, *XKS1*	50 g/l glu + 50 g/l xyl	0.06	0.23	0.16[b]	[79]	[4]
A4	*XYL1*, *XYL2*, *XKS1*	50 g/l glu + 50 g/l xyl	0.21	0.27	0.27[b]	[4]	[4]
A6	*XYL1*, *XYL2*, *XKS1*	50 g/l glu + 50 g/l xyl	0.14	0.27	0.32[b]	[4]	[4]
TMB3399	*XYL1*, *XYL2*, *XKS1* introduced in USM21	20 g/l xyl	NR	0.05	0.59	[5]	[5]
TMB3400	Xylose-growing strain isolated after chemical mutagenesis of TMB3399	20 g/l xyl	NR	0.18	0.25	[5]	[5]
C1	Xylose-growing strain evolved from TMB3001	10 g/l xyl	0.56	0.24	0.32	[131]	[131]
H2674 (control)	*XYL1*, *XYL2*, *XKS1*	50 g/l xyl	0.07	0.14	0.56	[115]	[115]
H2673 (GPD1)	*XYL1*, *XYL2*, *XKS1*, *GPD1* overexpression	50 g/l xyl	0.06	0.17	0.49	[115]	[115]
H2723 (Δ*zwf1*)	*XYL1*, *XYL2*, *XKS1*, Δ*zwf1*	50 g/l xyl	0.05	0.18	0.29	[115]	[115]
H2684 (*GPD1*Δ*zwf1*)	*XYL1*, *XYL2*, *XKS1*, *GPD1* overexpression, Δ*zwf1*	50 g/l xyl	0.06	0.31	0.35	[115]	[115]
TMB3001	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 50 g/l xyl	0.39	0.33	0.48	[79]	[121]
CPB.CR1 (Δ*gdh1*)	*XYL1*, *XYL2*, *XKS1*, Δ*gdh1*	20 g/l glu + 50 g/l xyl	0.28	0.16	0.21	[121]	[121]
CPB.CR4 (Δ*gdh1 GDH2*)	*XYL1*, *XYL2*, *XKS1*, Δ*gdh1 GDH2*	20 g/l glu + 50 g/l xyl	0.45	0.39	0.26	[121]	[121]
CPB.CR5 (Δ*gdh1* GS-GOGAT)	*XYL1*, *XYL2*, *XKS1*, Δ*gdh1* GS-GOGAT	20 g/l glu + 50 g/l xyl	0.39	0.28	0.52	[121]	[121]
TMB3001	*XYL1*, *XYL2*, *XKS1*	50 g/l glu + 50 g/l xyl	NR	0.33[1]	0.30	[79]	[7]

Table 1 (continued)

Strain	Relevant genotype/phenotype	Sugar composition	Xylose cons. rate	Ethanol yield	Xylitol yield	Ref. to strain	Ref. to ferm. data
C1	Xylose-growing strain evolved from TMB3001	50 g/l glu + 50 g/l xyl	NR	0.32[c]	0.24	[131]	[7]
C5	Xylose-growing strain evolved from TMB3001	50 g/l glu + 50 g/l xyl	NR	0.34[c]	0.28	[131]	[7]
F12	Xylose pathway introduced in F	50 g/l glu + 50 g/l xyl	NR	0.26[c]	0.40	[7]	[7]
A4	Xylose pathway introduced in A	50 g/l glu + 50 g/l xyl	NR	0.24	0.41	[4]	[7]
BH42	Xylose-growing strain obtained by breeding	50 g/l glu + 50 g/l xyl	NR	0.28[c]	0.36	[7][1]	[7]
TMB3399	*XYL1, XYL2, XKS1* introduced in USM21	50 g/l glu + 50 g/l xyl	NR	0.23[c]	0.39	[5]	[7]
TMB3400	Xylose-growing strain isolated after chemical mutagenesis of TMB3399	50 g/l glu + 50 g/l xyl	NR	0.24[c]	0.41	[5]	[7]
RWB202-AFX	*XI, evolved isolate*	20 g/l xyl	0.21[b]	0.42	0.02[b]	[92]	[92]
RWB217	*XI, XK, ΔGRE3,* overexpressed PPP	20 g/l glu + 20 g/l xyl	0.67[b]	0.43	0.006[b]	[97]	[97]
RWB217	*XI, XK, ΔGRE3,* overexpressed PPP	20 g/l xyl	NR	0.43	0.003[b]	[97]	[97]
RWB218	*XI, XK, ΔGRE3,* overexpressed PPP, selected for enhanced glucose uptake	20 g/l glu + 20 g/l xyl	0.59[b]	0.40	0.003[b]	[146]	[146]
RWB218	*XI, XK, ΔGRE3,* overexpressed PPP, selected for enhanced glucose uptake	20 g/l xyl	NR	0.41	0.001[b]	[146]	[146]
H2490-4	*XYL1, XYL2, XKS1 evolved isolate*	45 g/l xyl[a]	0.58	0.14[b]	0.82	[110]	[110]
R267H	*XYL1, XYL2*, mutated XR (R267H)	5 g/l glu + 15 g/l xyl	NR	0.43	0.15	[89]	[89]

[1] Spencer-Martins, personal communication

Table 1 (continued)

Strain	Relevant genotype/phenotype	Sugar composition	Xylose cons. rate	Ethanol yield	Xylitol yield	Ref. to strain	Ref. to ferm. data
TMB3001	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 50 g/l xyl	0.21	0.15 [c]	0.59 [d]	[79]	[54]
TMB3260	*XYL1*, *XYL2*, *XKS1*, high XR activity	20 g/l glu + 50 g/l xyl	0.22	0.19 [c]	0.48 [d]	[93]	[54]
TMB3062	*XYL1*, *XYL2*, *XKS1*, XR, and XDH on plasmid	20 g/l glu + 50 g/l xyl	0.14	0.29 [c]	0.22 [d]	[54]	[54]
TMB3056	*XYL1*, *XYL2*, *XKS1*, Δ*GRE3*, XR, and XDH on plasmid	20 g/l glu + 50 g/l xyl	0.11	0.24 [c]	0.22 [d]	[42]	[54]
TMB3057	*XYL1*, *XYL2*, *XKS1*, Δ*GRE3*, overexpressed PPP, XR, and XDH on plasmid	20 g/l glu + 50 g/l xyl	0.25	0.27 [c]	0.28 [d]	[42]	[54]

NR: not reported

[a] Batch culture by pulsing a chemostat culture

[b] Calculated from reference

[c] Ethanol yield on xylose

[d] Calculated after glucose depletion

Table 2 Xylose consumption rates (g xylose/g biomass h), ethanol yields (g ethanol/g xylose), and xylitol yields (g xylitol/g xylose) in controlled oxygen-limited batch cultures with xylose by recombinant *S. cerevisiae* strains. Defined mineral medium was used if other medium is not indicated

Strain	Relevant genotype/phenotype	Conditions	Xylose cons. rate	Ethanol yield	Xylitol yield	Ref. to strain	Ref. to ferm. data
1400(pLNH32)	*XYL1*, *XYL2*, *XKS1*	YEPX, 50 g/l xylose, 47 h	NR	0.33[a]	0.10[a]	[101]	[101]
TMB3001	*XYL1*, *XYL2*, *XKS1*	50 g/l xylose, 70 h	0.15	0.31	0.29	[79]	[114]
TMB3255	*XYL1*, *XYL2*, *XKS1*, Δ*zwf1*	50 g/l xylose, 70 h	0.02	0.41	0.05	[114]	[114]
TMB3008	*XYL1*, *XYL2*, *XKS1*, Δ*gnd1*	50 g/l xylose, 70 h	0.08	0.38	0.13	[114]	[114]
TMB3250	*XYL1*, *XYL2*, *XKS1*, 100% PGI	50 g/l xylose, 70 h	0.10	0.30	0.30	[114]	[114]
TMB3251	*XYL1*, *XYL2*, *XKS1*, 10% PGI	50 g/l xylose, 70 h	0.07	0.34	0.21	[114]	[114]
TMB3256	*XYL1*, *XYL2*, *XKS1*, *ZWF1* w/o promoter	50 g/l xylose, 70 h	0.06	0.36	0.13	[116]	[116]
TMB3037	*XYL1*, *XYL2*, *XKS1*, YRP13-*ZWF1*	50 g/l xylose, 70 h	0.11	0.34	0.19	[116]	[116]
TMB3260	*XYL1*, *XYL2*, *XKS1*, high XR activity	50 g/l xylose, 70 h	0.25	0.30	0.13	[93]	[93]
TMB3261	*XYL1*, *XYL2*, *XKS1*, high XR activity, Δ*zwf1*	50 g/l xylose, 60–70 h	0.32	0.34	0.08	[93]	[93]
TMB3270	*XYL1*, *XYL2*, *XKS1*, mutated *XR* (K270M)	50 g/l xylose, 60–70 h	0.16	0.36	0.17	[88]	[88]
TMB3271	*XYL1*, *XYL2*, *XKS1*, mutated *XR1* (K270M), 2 copies	50 g/l xylose, 60–70 h	0.23	0.31	0.09	[88]	[88]
TMB3253 control strain for TMB3254	*XYL1*, *XYL2*, *XKS1*,	50 g/l xylose, 40 h	0.16	0.28	0.34	[116]	[116]
TMB3254	*XYL1*, *XYL2*, *XKS1*, overproducing transhydrogenase	50 g/l xylose, 40 h	0.16	0.28	0.30	[116]	[116]
TMB3001c	*XYL1*, *XYL2*, *XKS1*	50 g/l glucose + 50 g/l xylose	0.12	0.29	0.33	[79]	[128]

Table 2 (continued)

Strain	Relevant genotype/phenotype	Conditions	Xylose cons. rate	Ethanol yield	Xylitol yield	Ref. to strain	Ref. to ferm. data
TMB3001c-p6XFP/p4PTA/p5EHADH2	TMB3001c expressing phosphoketolase, phosphotransacetylase, and acetaldehyde dehydrogenase	50 g/l glucose + 50 g/l xylose	0.07	0.12	0.30	[128]	[128]
TMB 3001	*XYL1*, *XYL2*, *XKS1*	10 g/l xylose, 72 h	0.026	0.21	0.44	[98]	[98]
TMB 3120	*XYL1*, *XYL2*, *XKS1*, Δ*GRE3*	10 g/l xylose, 72 h	0.031	0.09	0.46	[98]	[98]
TMB 3050	*T.th XI*, *XK*, Δ*GRE3*, overexpressed PPP	50 g/l xyl	0.002	0.29	0.23	[42]	[42]
MT8-1/Xyl	*XYL1*, *XYL2*, *XKS1*	50 g/l xylose + casamino acids, 72 h	NR	0.37	0.04[a]	[14]	[14]

NR: not reported

[a] Calculated from reference

Table 3 Xylose consumption rates (g xylose/g biomass h), ethanol yields (g ethanol/g sugar), and xylitol yields (g xylitol/g xylose) in anaerobic chemostat cultures with glucose and xylose by recombinant *S. cerevisiae* strains. Defined mineral medium was used if other medium is not indicated

Strain	Relevant genotype/phenotype	Conditions	Dil. rate	Xylose cons. rate	Ethanol yield	Xylitol yield	Ref. to strain	Ref. to ferm. data
TMB3001	*XYL1*, *XYL2*, *XKS1*	10 g/l glu + 10 g/l xyl, 0.06 h^{-1}	0.06	0.14	0.29/0.35 [a]	0.43	[79]	[79]
TMB 3001	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 20 g/l xyl	0.12	0.29	0.31	0.37	[79]	[90]
TMB3026	*XYL1*, *XYL2*, *XKS1*, overexpressed PPP	20 g/l glu + 20 g/l xyl	0.12	0.29	0.34	0.38	[113]	[90]
TMB3001	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 20 g/l xyl, 0.06 h^{-1}	0.06	0.23	0.30	0.43	[79]	[90]
TMB3255	*XYL1*, *XYL2*, *XKS1*, Δ*zwf1*	20 g/l glu + 20 g/l xyl, 0.06 h^{-1}	0.06	0.12	0.39	0.13	[114]	[114]
TMB3260	*XYL1*, *XYL2*, *XKS1*, high XR activity	20 g/l glu + 20 g/l xyl, 0.06 h^{-1}	0.06	0.34	0.29	0.42	[93]	[93]
TMB3261	*XYL1*, *XYL2*, *XKS1*, high XR activity, Δ*zwf1*	20 g/l glu + 20 g/l xyl, 0.06 h^{-1}	0.06	0.13	0.37	0.13	[93]	[93]
TMB 3001	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 20 g/l xyl, 0.06 h^{-1}	0.06	0.23	0.30	0.43	[79]	[90]
TMB3026	*XYL1*, *XYL2*, *XKS1*, upregulated non-oxid. PPP	20 g/l glu + 20 g/l xyl, 0.06 h^{-1}	0.06	0.23	0.33	0.43	[113]	[90]
TMB3001	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 20 g/l xyl	0.12	0.29	0.31	0.37	[79]	[96]
TMB3120	*XYL1*, *XYL2*, *XKS1*, Δ*GRE3*	20 g/l glu + 20 g/l xyl	0.12	0.40	0.37	0.19	[96]	[96]
CPB.CR1	*XYL1*, *XYL2*, *XKS1*, Δ*gdh1*	20 g/l glu + 50 g/l xyl	0.05	0.25	0.31	0.59	[121]	[121]
CBP.CR4	*XYL1*, *XYL2*, *XKS1*, Δ*gdh1 GDH2*	20 g/l glu + 50 g/l xyl	0.05	0.31	0.34	0.47	[121]	[121]
CPB.CR5	*XYL1*, *XYL2*, *XKS1*, Δ*gdh1* GS-GOGAT	20 g/l glu + 50 g/l xyl	0.05	0.34	0.33	0.44	[121]	[121]
H2490	*XYL1*, *XYL2*, *XKS1*	3 g/l glu + 27 g/l xyl	0.047	0.46	0.28	0.49	[147]	[147]
CEN.PK	Control for RWB202	20 g/l glu + 10 g/l xyl	0.10	0.11	0.39	0.36	[43]	[43]
RWB202	XI	20 g/l glu + 10 g/l xyl	0.10	0.73	0.39	0.07 [b]	[43]	[43]
TMB3400	Xylose-growing strain isolated after chemical mutagenesis of TMB3399	10 g/l glu + 10 g/l xyl, 0.06 h^{-1}	0.06	0.22	0.35	0.19	[5]	[148]

Table 3 (continued)

Strain	Relevant genotype/phenotype	Conditions	Dil. rate	Xylose cons. rate	Ethanol yield	Xylitol yield	Ref. to strain	Ref. to ferm. data
TMB3001	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 50 g/l xyl	0.05	0.31	0.28	0.56	[79]	[125]
CPB.CR2	*XYL1*, *XYL2*, *XKS1*, Δ*mig1*	20 g/l glu + 50 g/l xyl	0.05	0.39	0.32	0.45	[125]	[125]
CPB.MBH2	*XYL1*, *XYL2*, *XKS1*, Δ*mig1* Δ*mig2*	20 g/l glu + 50 g/l xyl	0.05	0.34	0.29	0.59	[125]	[125]
TMB3001	*XYL1*, *XYL2*, *XKS1*	10 g/l glu + 10 g/l xyl	0.05	0.14	0.31[b]	0.36[b]	[79]	[109]
C1	*XYL1*, *XYL2*, *XKS1*, xylose-growing strain evolved from TMB3001	10 g/l glu + 10 g/l xyl	0.05	0.31	0.27[b]	0.35[b]	[131]	[109]
TMB3001	*XYL1*, *XYL2*, *XKS1*	2.5 g/l glu + 13 g/l xyl	0.058	NR	0.34[b]	0.44[b]	[79]	[149]
CBP.CR4	*XYL1*, *XYL2*, *XKS1*, Δ*gdh1 GDH2*	2.5 g/l glu + 13 g/l xyl	0.058	NR	0.37[b]	0.23[b]	[121]	[149]
TMB3260	*XYL1*, *XYL2*, *XKS1*, high XR activity	10 g/l glu + 10 g/l xyl, 0.06 h^{-1}	0.06	0.19	0.36	0.58	[93]	[88]
TMB3271	*XYL1*, *XYL2*, *XKS1*, mutant XR (K270M), high activity	10 g/l glu + 10 g/l xyl, 0.06 h^{-1}	0.06	0.16	0.40	0.44	[88]	[88]
CPBCB5	*XYL1*, *XYL2*, *XKS1*	20 g/l glu + 50 g/l xyl	0.05	NR	0.29	0.21	[120]	[120]
CPBCB4	*XYL1*, *XYL2*, *XKS1*, *gapN*	20 g/l glu + 50 g/l xyl	0.05	NR	0.36	0.14	[120]	[120]

NR: not reported

[a] Measured and corrected by closing the DR balance

[b] Calculated from reference

Table 4 Xylose consumption rates (g xylose/g biomass h), and biomass and CO_2 formation (g/l) in aerobic chemostat cultures with glucose and xylose by recombinant *S. cerevisiae* strains. Defined mineral medium was used if other medium is not indicated

Strain	Relevant genotype/phenotype	Conditions	Dil. rate	Xylose cons. rate	Biomass (g/l)	CO_2 (g/l)	Ref. to strain	Ref. to ferm. data
TMB3399	*XYL1*, *XYL2*, *XKS1*, introduced in USM21	10 g/l glu + 10 g/l xyl	0.10	0.085	7.4	11.1	[5]	[91]
TMB3400	Xylose-growing strain isolated after chemical mutagenesis of TMB3399	10 g/l glu + 10 g/l xyl	0.10	0.097	8.1	12.6	[5]	[91]
TMB3400	Xylose-growing strain isolated after chemical mutagenesis of TMB3399	20 g/l xyl	0.10	0.254	5.4	8.8	[5]	[91]
H2490	*XYL1*, *XYL2*, *XKS1*	3 g/l glu + 27 g/l xyl,	0.047	0.108	2.5	n/a	[147]	[147]
RWB02	XI	5 g/l glu + 2.5 g/l xyl	0.10	0.33	3.0[a]	3.9[a]	[43]	[43]
TMB3001	*XYL1*, *XYL2*, *XKS1*	10 g/l glu + 10 g/l xyl	0.05	0.032	6.5	11.7	[79]	[109]
C1	Xylose-growing strain evolved from TMB3001	10 g/l glu + 10 g/l xyl	0.05	0.048	10.0	14.0	[131]	[109]
C1	Xylose-growing strain evolved from TMB3001	20 g/l xyl	0.05	0.116	8.7	13.9	[131]	[109]
C5	Xylose-growing strain evolved from TMB3001	20 g/l xyl	0.05	0.128	7.8	14.0	[131]	[109]
F12	*XYL1*, *XYL2*, *XKS1*, introduced in F	10 g/l glu + 10 g/l xyl	0.10	0.068	9.4	13.2	[7]	[132]
BH42	*XYL1*, *XYL2*, *XKS1*, xylose-growing strain obtained by breeding	10 g/l glu + 10 g/l xyl	0.10	0.068	11.5	12.9	[7]	[132]
H2490-4	*XYL1*, *XYL2*, *XKS1* evolved isolate	30 g/l xyl	0.05	0.116[a]	5	4.4[a]	[110]	[110]

[a] Calculated from reference

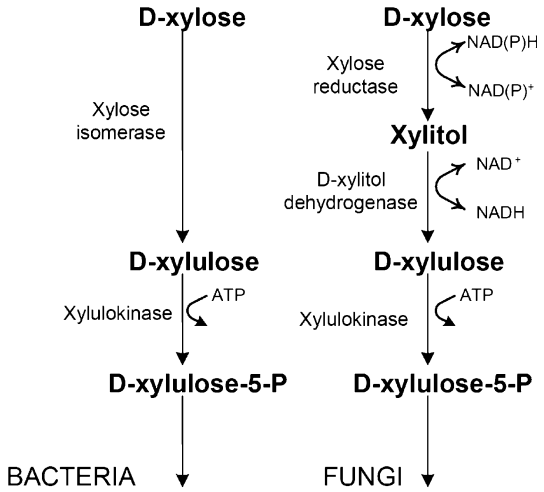

Fig. 1 The initial xylose utilization pathways in bacteria and fungi

2.1 Xylose Utilization Pathways

In naturally xylose-utilizing bacteria, D-xylose is isomerized to D-xylulose [18] by xylose isomerase (XI). Xylulose is then phosphorylated to xylulose 5-phosphate [19], which is an intermediate of the pentose phosphate pathway (PPP). A similar pathway has been found in an anaerobic fungus [20]; however, most naturally xylose-utilizing fungi contain a more complex pathway consisting of reduction–oxidation reactions involving the cofactors NAD(P)H and $NAD(P)^+$ (Fig. 1). Xylose is reduced to xylitol [21–23] by a NAD(P)H-dependent xylose reductase (XR), and xylitol is then oxidized to D-xylulose by a NAD^+-dependent xylitol dehydrogenase (XDH) [22, 24, 25]. As in bacteria, xylulose is phosphorylated to D-xylulose 5-phosphate by a xylulokinase (XK) [26, 27]. Despite the inability of *S. cerevisiae* to utilize xylose, the genes encoding the reductive–oxidative xylose pathway enzymes XR, XDH, and XK are present in its genome [26, 28, 29]; however, they are expressed at too low levels to allow xylose utilization. Even when the genes were overexpressed, no growth on xylose could be detected [30]. Neither was it possible through adaptation protocols to upregulate the expression of these genes to levels high enough to allow significant xylose fermentation [31].

2.2 Expression of XI in *S. cerevisiae*

The observation that most xylose-utilizing fungi produce considerable amounts of xylitol from xylose, and that only species containing also the

NADH-dependent XR activity are capable of producing ethanol from it, suggested that the different cofactor preferences of XR and XDH limit ethanolic xylose fermentation by yeast [21, 32]. Since *S. cerevisiae* ferments xylulose [1, 2], it was suggested that xylose fermentation could be easily achieved by heterologous expression of an XI [32, 33]. Indeed, xylose was fermented to ethanol when extracellular XI was added to the medium [33]. This enzyme, with activity not only for xylose but also for glucose, is industrially used for the production of high-fructose corn syrup (HFCS) [18] to convert starch-derived glucose into the sweeter sugar fructose to reduce the sugar demand in the food industry. Heterologous expression of bacterial XI genes in *S. cerevisiae* proved to be challenging, and for many years no actively expressed enzyme was reported [34–39]. The first functionally expressed XI in *S. cerevisiae* [40] originated from the bacterium *Thermus thermophilus* [41]. It was later shown that the low activity of the bacterial XIs in yeast could be partially related to intracellular precipitation [39], and it was suggested that the rigid nature of the thermotolerant *T. thermophilus* XI aided correct folding of the protein in *S. cerevisiae*. However, the activity of this enzyme at 30 °C was too low to allow xylose fermentation. Still, when combined with other genetic modifications, aerobic growth on xylose was demonstrated by *S. cerevisiae* carrying the *T. thermophilus* XI [42] (strain TMB3050, Table 2).

More recently, an XI from the obligate anaerobe rumen fungus *Piromyces* [20] was expressed in *S. cerevisiae* with an activity of about 1 U/mg protein at 30 °C [43] (strain RWB202, Tables 3 and 4). Later, bacterial XIs with high sequence similarity to the *Piromyces* XI, such as those from *Bacteroides thetaiotaomicron* [44] and *Xanthomonas campestris* [45], were also expressed in *S. cerevisiae*, but the activity of these enzymes in *S. cerevisiae* was lower than that of the *Piromyces* XI. Despite the relatively high activity of *Piromyces* XI in *S. cerevisiae*, the expression of this enzyme alone did only allow very slow growth on xylose [43], suggesting that the conversion of xylose to xylulose does not alone control the xylose metabolism in *S. cerevisiae* [42]. This observation may also set in a new light the failures of early trials for heterologous XI expression where, in many cases, functional XI expression was only assayed as growth on xylose [35, 37].

2.3 Expression of XR and XDH in *S. cerevisiae*

The first xylose-utilizing strains of *S. cerevisiae* were generated by expressing the *Pichia stipitis* genes *XYL1* [23] and *XYL2* [24], encoding XR and XDH, respectively [46–48]. *P. stipitis* was chosen as the source of the heterologously expressed enzymes because it produces ethanol from xylose with theoretical yield, albeit only under well-controlled oxygen limitation [47, 49, 50], while most other naturally xylose-fermenting yeasts produce considerable amounts of the by-product xylitol [50]. Xylitol formation is a consequence

of the inability of the cell to oxidize reduced cofactors in the absence of oxygen [32]. Contrary to XRs from most xylose-utilizing yeasts, XRs from *P. stipitis, Pachysolen tannophilus*, and *Candida shehatae* can use not only NADPH but also NADH as a cofactor [21], which permits recirculation of the cofactors between the first two steps of the xylose pathway (Fig. 1).

Nevertheless, the first *S. cerevisiae* strains expressing the *P. stipitis* XR and XDH produced xylitol, and the ethanol yield from xylose was low [47, 48]. This was ascribed to the preference for NADPH over NADH of the XR [23]. Much research has been devoted to developing metabolic engineering strategies to improve xylose fermentation by XR- and XDH-carrying strains, often guided by the early suggestions to express either a strictly NADH-specific XR activity [32] or to express a transhydrogenase activity [21]. Both approaches are further discussed in the following sections together with other metabolic engineering strategies. Kinetic modeling estimated that the conversion of xylose to xylulose required a ratio of 1:10 of the initial XR and XDH activities [51], which has been experimentally supported by several independent investigations [51–54]. The higher level of XDH is necessary to "pull" the xylose toward central metabolism [55], especially since the equilibrium of the XDH reaction favors xylitol formation [56]. In addition, it has more recently been found that efficient xylose metabolism requires high activity of both XR and XDH [54, 57].

3
Arabinose

3.1
Arabinose Utilization Pathways

Lignocellulosic raw materials contain much less L-arabinose than D-xylose, and solving the problem of xylose fermentation has been prioritized. The relative amounts of the sugars strongly depend on the raw material. For example, corn stover contains of 19% xylan and 3% arabinan, whereas wheat bran contains 19% xylan and 15% arabinan [58]. As a consequence, L-arabinose-utilizing strains of *S. cerevisiae* have been developed only recently. Furthermore, the conversion of L-arabinose into intermediates of the PPP requires more enzymatic reactions than the conversion of xylose (Fig. 2). In many bacteria, such as *E. coli*, L-arabinose is utilized via L-arabinose isomerase (AraA), L-ribulokinase (AraB), and L-ribulose-5-phosphate 4-epimerase (AraD) [59]. Xylulose-5-phosphate is then further metabolized via the PPP. Enzymatic activities of alternative bacterial arabinose and xylose utilization pathways have also been described [60–62].

The fungal arabinose utilization pathway consists of four alternating reduction–oxidation reactions (Fig. 2), where L-arabinose is converted to

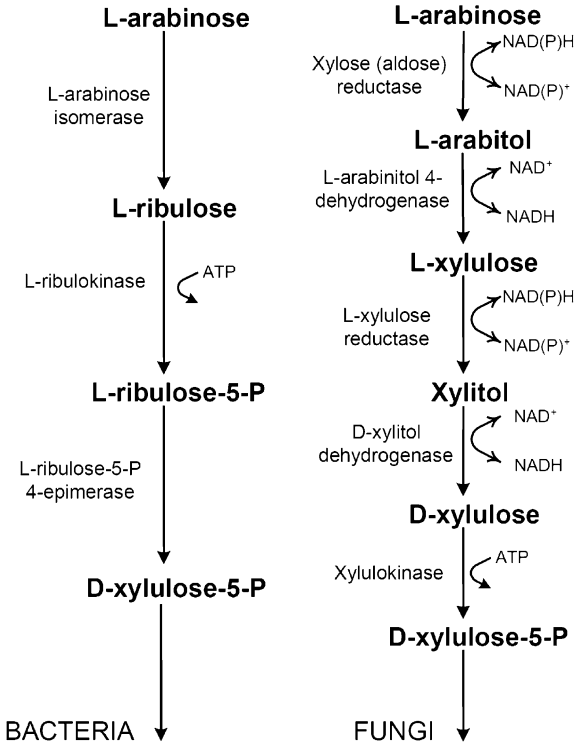

Fig. 2 The initial L-arabinose utilization pathways in bacteria and fungi

D-xylitol via L-arabi(ni)tol and L-xylulose [23, 63–65]. D-Xylitol is then further metabolized by XDH and XK, resulting in the PPP intermediate D-xylulose-5-phosphate. The first two complete fungal arabinose utilization pathways were recently kinetically characterized for *Candida arabinofermentans* PYCC 5603^T and *Pichia guilliermondii* PYCC 3012 [65]. The fungal xylose and arabinose utilization pathways share the enzymes XR, XDH, and XK, since XR also reduces L-arabinose [65–68]. Indeed, all arabinose-utilizing yeast and fungi also utilize xylose, whereas not all xylose-growing yeasts utilize arabinose [66, 69]. Similar to the fungal xylose pathway, the cofactors of the enzymes in the fungal arabinose pathway cannot be regenerated within the pathway but require oxygen or an external electron acceptor for regeneration (Fig. 2).

3.2 Engineering Arabinose Utilization in *S. cerevisiae*

The first attempt to introduce an L-arabinose utilization pathway in *S. cerevisiae* by heterologous expression of the complete *E. coli* L-arabinose pathway

did not result in appreciable arabinose utilization [70], most likely due to the absence of functional expression of the L-arabinose isomerase. It was only when the *E. coli araA* gene encoding the L-arabinose isomerase was substituted by the corresponding *Bacillus subtilis* gene that a functional arabinose pathway was established in *S. cerevisiae* [71]. Similar to the use of the heterologous XI pathway, other genetic modifications in addition to the new L-arabinose isomerase were required for the recombinant strain to grow on L-arabinose as sole carbon source [71]: an additional copy of the galactose permease (*Gal2*), which also transports arabinose [72], and an unspecified adaptation for growth on arabinose [71].

The fungal L-arabinose utilization pathway has also been introduced in *S. cerevisiae*, combining enzymes from *P. stipitis* and from the filamentous fungus *Trichoderma reesei*. The enzymes were actively expressed; however, neither appreciable growth on L-arabinose nor significant ethanolic fermentation was observed [73]. The dysfunction of the fungal arabinose pathway with respect to ethanolic fermentation parallels the inability of the naturally arabinose-growing yeasts to ferment L-arabinose to ethanol [50, 69]. Instead, these yeasts often produce L-arabitol from L-arabinose (Fig. 2) [65, 66, 69]. Minute ethanolic fermentation has been observed for six yeast species, *C. arabinofermentans, P. guilliermondii, C. auringiensis, C. succiphila, Ambrosiozyma monospora*, and *Candida* sp. YB-2248, but only in rich medium [65, 69]. Rich media may contain other fermentable sugars as well as undefined electron acceptors that serve to regenerate reduced cofactors [32, 74–76], which appears necessary for ethanolic arabinose fermentation to occur via the fungal pathway. Also, the presence of low amounts of oxygen aids cofactor regeneration [50, 77].

4 Improving Ethanolic Fermentation by Pentose-Utilizing *S. cerevisiae*

It soon became evident that the mere introduction of pentose utilization pathways in *S. cerevisiae* was not enough to render the recombinant strains traits for efficient ethanol fermentation [43] (strain RWB202, Tables 3 and 4), [47, 48, 78, 79] (strain TMB3001, Tables 1–3). A number of metabolic engineering strategies to enhance ethanolic xylose (and arabinose) fermentation in *S. cerevisiae* have been explored, the most important of which will be discussed below. The initial xylose utilization pathway, the cellular redox metabolism, and the flux of central carbon metabolism have been the main targets of these engineering strategies. Figure 3 highlights the metabolic reactions that have been engineered to improve ethanolic xylose fermentation by *S. cerevisiae*.

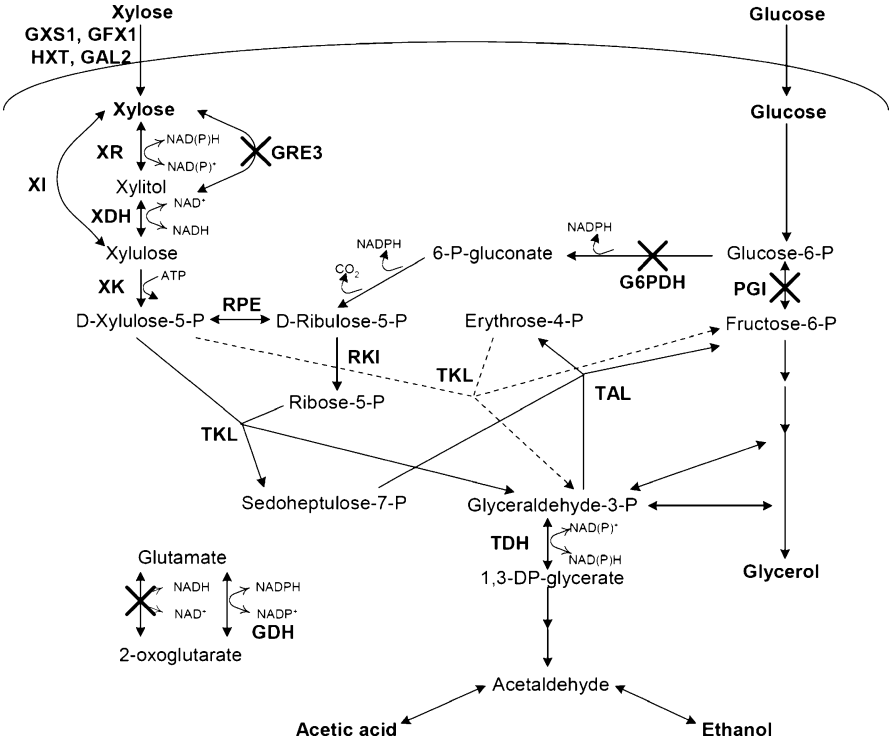

Fig. 3 Simplified illustration of metabolic steps engineered for improved xylose fermentation. The identified enzymes have been overexpressed; crossed pathways indicate deleted enzymes

4.1 Sugar Transport

The transport of pentose sugars in *S. cerevisiae* occurs through hexose transporters [80, 81], albeit with an affinity one to two orders of magnitude lower than for hexose sugars [47, 82]. Therefore, pentose transport was early considered a rate-controlling step for ethanolic pentose fermentation [47]. Nevertheless, a metabolic control analysis study demonstrated that transport controlled xylose conversion only in strains with high XR activity, and only at low xylose concentrations [83]. Few reports exist on expression of pentose transporters in pentose-utilizing *S. cerevisiae* strains [80, 84]. The most effective approach has been the overexpression of the galactose permease *Gal2* in recombinant arabinose-fermenting *S. cerevisiae* [71]. This is in part due to the difficulty in actively expressing heterologous membrane proteins. In a recent breakthrough, the first active heterologous expression of a glucose/xylose facilitated diffusion transporter and a glucose/xylose symporter from *Candida intermedia* [85] in *S. cerevisiae* [86] was reported. So far the

fermentation performance of these strains has not been reported. Nevertheless, the expression of heterologous xylose transporters opens up new metabolic engineering strategies to further increase the rate of xylose utilization in xylose-fermenting *S. cerevisiae* strains.

4.2 Improving the Conversion of Xylose to Xylulose

4.2.1 Cofactor Dependence

The production of xylitol and L-arabitol during pentose consumption by natural as well as recombinant pentose-utilizing yeasts has been rationalized with the difference in cofactor preferences between the enzymes in the initial pentose utilization pathways. XR from *P. stipitis* preferentially uses NADPH, but can also use NADH as a cofactor [23], whereas XDH exclusively uses NAD^+ [24, 46]. This may result in excess NADH formation and lack of NAD^+, since yeasts do not harbor a transhydrogenase enzyme that would allow direct conversion of $NADP^+$ to NAD^+ [32]. Numerous investigations have supported this metabolic model; external electron acceptors, which are reduced by NADH-dependent enzymes in *S. cerevisiae*, reduce xylitol formation [32, 74–76]. Xylitol formation in recombinant *S. cerevisiae* was also reduced by changing the kinetic properties of the enzymes involved by expressing a fusion protein of XR and XDH [87], or by expressing mutated XR with altered cofactor affinity [88, 89] (strains TMB3270, TMB3271, R267H, Tables 1–3). However, significantly increased ethanolic xylose fermentation as a result of such engineering strategies has been less frequently reported [89].

4.2.2 Activity of Initial Pentose Pathway Enzymes

In the first generation of recombinant xylose-utilizing *S. cerevisiae* strains the activity of the enzyme(s) converting xylose to xylulose has been insufficient to support ethanolic fermentation of xylose [42, 43, 54] (strain RWB202, Tables 3 and 4). For example, overexpression of the nonoxidative PPP improved xylose fermentation only when the XR and XDH activities were enhanced [54, 90] (strain TMB3057, Table 1, vs strain TMB3026, Table 3), indicating that when the flux through central metabolism was high, the control of xylose metabolism was in the steps converting xylose to xylulose [42] (strain TMB3050, Table 2). Invariably, increased XR and XDH activities have been observed in mutant *S. cerevisiae* strains with improved xylose utilization [31, 57, 91]. Similarly, high activity of *Piromyces* XI allowed higher xylose fermentation rates than the lower bacterial XI activity [42, 92] (strain RWB202-AFX, Table 1; strain TMB3050, Table 2). Also, in recombi-

nant arabinose-utilizing *S. cerevisiae* strains, enhanced levels of the arabinose isomerase significantly improved arabinose fermentation [8].

The fact that not only the cofactor specificities, but also the relative activities of XR and XDH affect xylitol formation suggests that the redox model for xylitol formation [32] may have to be reevaluated. Not only the cofactor preferences of the enzymes involved but also, equally importantly, the levels of the XR and XDH activities affect xylitol formation during xylose fermentation [54]. A plain increase in XR and/or XDH activity, allowing an increased flux through the initial pathway, significantly reduces xylitol excretion [53, 54, 93] (strain TMB3062, Table 1; strain TMB3061, Table 2).

4.2.3 *GRE3* Deletion

Natural *S. cerevisiae* strains reduce xylose to xylitol with an endogenous xylose (aldose) reductase encoded by the *GRE3* gene [28]. Xylitol strongly inhibits the activity of XI [94], and therefore deleting the *GRE3* gene improves efficient xylose utilization in XI-expressing *S. cerevisiae* strains [95]. Improved ethanol yields at the expense of reduced xylitol yields were indeed observed for XI-carrying strains [96, 97] (strain RWB217, Table 1). Furthermore, the *GRE3* deletion decreased xylitol formation also in strains carrying XR and XDH, albeit only under continuous fermentation [98] (strain TMB3120, Tables 2 and 3). However, the aldose reductase endoded by *GRE3* belongs to a group of generally stress-induced proteins [28] and the deletion of it reduces the growth by 30% [96]. This limits the usefulness of *GRE3* deletion in strains aimed at industrial applications.

4.3 Xylulokinase

The *S. cerevisiae* genome contains the gene *XKS1* coding for XK [26, 27], but the XK activity in wild-type *S. cerevisiae* is too low to support ethanolic xylose fermentation in strains engineered with a xylose pathway [26, 99, 100]. It is only when additional copies of *XKS1* are expressed that recombinant xylose-utilizing *S. cerevisiae* produce ethanol from xylose [79] (strain TMB3001, Tables 1–4), [100] (strain H1691, Table 1), [101] (strain 1400 (pLNH32), Table 2; strain H2490, Tables 3 and 4). However, nonphysiological or unregulated kinase activity may cause a metabolic disorder [102]. It was indeed experimentally demonstrated that only fine-tuned overexpression of *XKS1* in *S. cerevisiae* led to improved xylose fermentation to ethanol [103, 104]. Similarly, it was shown that arabinose-utilizing recombinant *S. cerevisiae* strains expressed a mutated L-ribulokinase gene with lower specific activity, indicating that a low kinase activity had been selected as advantageous for arabinose utilization [71].

4.4 Pentose Phosphate Pathway

In contrast to the energy-conserving function of glycolysis, the main metabolic function of the PPP is to provide anabolic intermediates such as ribulose 5-phosphate, erythrose 4-phosphate, and NADPH for biosynthesis and cell growth. The flux through the nonoxidative PPP in *S. cerevisiae* was found to be much lower than in other yeasts [105], which was later confirmed by metabolome analysis [106]. The low PPP activity in *S. cerevisiae* is sometimes interpreted to be a result of the domestication of *S. cerevisiae* by prolonged selection for carbon dioxide and ethanol production from hexose sugars. However, PPP activity is a crucial part of pentose metabolism, since it is virtually the only way to introduce xylulose into the central metabolism. It was early pointed out that the PPP activity may limit xylose metabolism in *S. cerevisiae* [47, 107], which was further supported when excretion of PPP intermediates was observed in xylulose- and xylose-metabolizing *S. cerevisiae* [43, 108].

The insufficient flux through the nonoxidative PPP in *S. cerevisiae* has been indirectly confirmed in several genome-scale and enzymatic analyses of mutant strains with improved xylose metabolism, where invariably either the transaldolase (*TAL1)* or the transketolase (*TKL1*) genes, or both, have been found to be upregulated [57, 71, 91, 109–111]. Directly, the importance of the flux through the PPP has been confirmed by the superior pentose utilization and ethanolic fermentation by strains in which the enzymes of the nonoxidative PPP have been overexpressed. An early attempt to overexpress *P. stipitis* transketolase in xylose-metabolizing *S. cerevisiae* was not successful [112], whereas overexpression of the endogenous *S. cerevisiae* transaldolase (*TAL1*) resulted in improved growth on xylose [78]. Later, the overexpression of all four nonoxidative PPP genes, including not only *TAL1* and *TKL1* but also ribulose-5-phosphate 4-epimerase (*RPE1*) and ribulokinase (*RKI1*), was shown to improve xylulose consumption by *S. cerevisiae* [90, 113] (strain TMB3026, Table 3). Moreover, the improvement resulting from the overexpression of the four genes was higher than when each gene was overexpressed alone [90]. The simultaneous overexpression of the whole nonoxidative PPP, together with *GRE3* deletion, allowed growth on xylose in a strain carrying a bacterial XI [42] (strain TMB3050, Table 2). The usefulness of this combination of modifications was confirmed when it allowed aerobic and anaerobic growth on xylose in a strain carrying the *Piromyces* XI [97] (strain RWB217, Table 1). PPP overexpression also allowed superior xylose fermentation rates in combination with high levels of XR and XDH [42, 54] (cf. strains TMB3057, TMB3056, and TMB3062, Table 1 and Fig. 5).

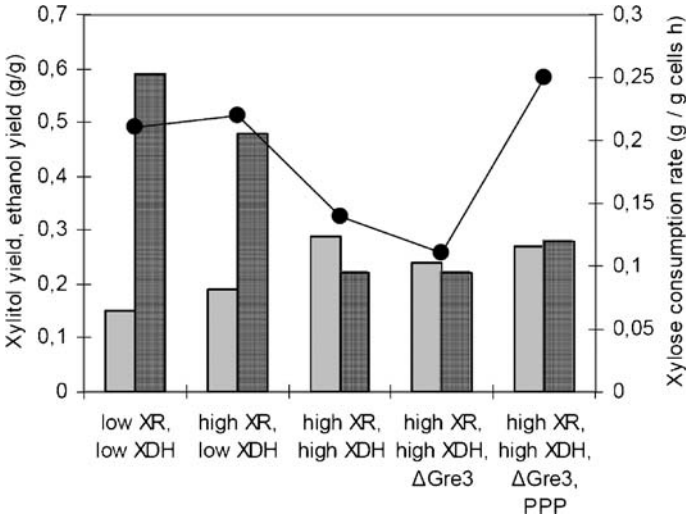

Fig. 4 Xylitol yield (*patterned columns*), ethanol yield (*solid columns*), and xylose consumption rate (*line*) in strains carrying low or high XR and/or XDH activities, *GRE3* deletion, and/or overexpression of PPP [54]

4.5 Engineering the Redox Metabolism of the Cell

Reducing xylitol formation has been a major challenge in xylose fermentation by recombinant *S. cerevisiae* carrying the *P. stipitis* xylose pathway enzymes XR and XDH. Xylitol formation has primarily been ascribed to the difference in cofactor requirements of the two enzymes, so that the intracellular concentration of NAD^+ controls the amount of xylitol being converted to xylulose [21, 32, 47, 74–76]. However, xylitol formation during ethanolic xylose fermentation also depends on the strain background, i.e., the metabolism of the host cell, since for example some strains of *P. stipitis* do not produce xylitol [47, 49, 50]. Thus, engineering the redox metabolism of the *S. cerevisiae* host has been given great attention where the aim primarily has been to manipulate the intracellular concentrations and fluxes of cofactors to minimize xylitol formation.

4.5.1 Oxidative PPP

The *P. stipitis* XR, which converts xylose to xylitol, prefers the cofactor NADPH over NADH by a factor of approximately 100 [23]. In yeast, NADPH is primarily formed in the oxidative PPP converting glucose-6-phosphate to ribulose-5-phosphate. Therefore, genes coding for enzymes in the oxidative PPP were deleted in order to decrease NADPH concentration in the cell and

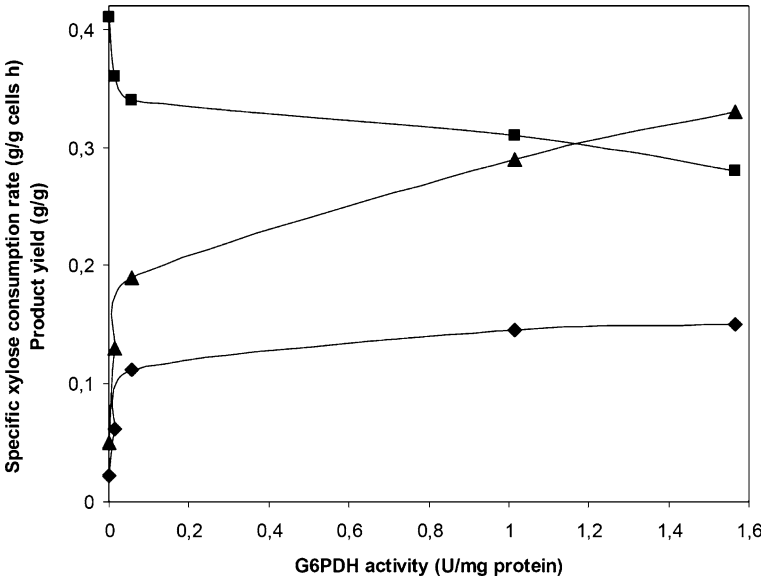

Fig. 5 Specific xylose consumption rate (♦), ethanol yield (■), and xylitol yield (▲) as a function of G6PDH activity

thus force XR to use NADH instead of NADPH, which was demonstrated by the deletion of *ZWF1*, coding for glucose-6-phosphate dehydrogenase (G6PDH) [114] (strain TMB3255, Table 2), [115] (strain H2723, Table 1). Increased ethanol formation at the expense of not only xylitol formation but also the xylose consumption rate was observed [114] (strains TMB3001 and TMB3255, Tables 2 and 3). In a follow-up study, the G6PDH activity was instead fine-tuned, which enabled the design of strains with increased ethanol yield and reasonable xylose consumption rate [116] (strains TMB3256 and TMB3037, Table 2, Fig. 4). However, in an industrial context, it is worth noticing that the *ZWF1* deletion increases the sensitivity toward lignocellulose hydrolysates, possibly due to the limited intracellular NADPH concentration, which is important for inhibitor tolerance [116, 117].

4.5.2 Transhydrogenase and Redox Enzymes

The problem of cofactor regeneration has also been addressed by engineering reactions distant from the xylose utilization pathway, as demonstrated by different approaches to introduce a transhydrogenase function in *S. cerevisiae*. Heterologous expression of a bacterial transhydrogenase [118] in *S. cerevisiae* carrying XR and XDH reduced xylitol formation, but also increased glycerol, rather than ethanol formation [116] (strain TMB3254, Table 2), indicating

that the transhydrogenase reaction did not proceed in the direction favorable for ethanolic xylose fermentation [116, 118].

Intracellular cofactor concentrations have also been altered, introducing in *S. cerevisiae* the $NAD(P)^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Kluyveromyces lactis* [119] (strain H2673, Table 1). When the *ZWF1* gene was simultaneously deleted, the expression of GAPDH improved ethanol formation [115] (strain H2684, Table 1). Similarly, when a $NAD(P)^+$-dependent nonphosphorylating GAPDH from *Streptococcus mutans* was overexpressed in an XR–XDH–XK-carrying strain, increased ethanol formation was observed [120] (strain CPBCB4, Table 3). The result suggested that less carbon was lost as carbon dioxide when NADPH was formed outside the oxidative PPP and that NAD^+ consumption in the lower glycolysis was simultaneously reduced.

Engineering the ammonium assimilation pathway [121] has also been used to modify the intracellular cofactor concentrations. Based on the assumption that NADH would be used for ammonium assimilation to generate NAD^+ for the XDH reaction, the NADPH-dependent glutamate dehydrogenase gene *GDH1* was deleted, and an NADH-dependent isoenzyme (*GDH2*) was overexpressed. Reduced xylitol formation and higher ethanol formation were observed [121] (strain CPB.CR4, Tables 1 and 3). Alternatively, the GS-GOGAT complex coded by the genes *GLT1* and *GLN1* was overexpressed, which only affected xylose fermentation in a continuous fermentation setup [121] (strain CPB.CR5, Tables 1 and 3).

4.6 Glycolytic Flux

In addition to the transport flux and the flux through the initial pentose-converting enzymes, the "pulling" effect [55] of the flux through enzymatic reactions downstream of xylitol, as well as through glycolysis, appears to be equally important for ethanolic pentose fermentation. It was early recognized that the presence of glucose during xylose fermentation enhanced the glycolytic activity [122–124]. Furthermore, it was recently shown that no xylitol was formed in the glucose–xylose coconsumption phase during xylose fermentation with recombinant *S. cerevisiae* in mineral medium [54], nor in lignocellulose hydrolysates which contain hexose sugars [6, 12, 14].

4.7 Other Modifications

Transcription factors involved in glucose repression have also been modified in order to affect ethanolic xylose fermentation. The gene *MIG1*, or both *MIG1* and *MIG2*, were deleted in an XR–XDH–XK-carrying strain of *S. cerevisiae* [125] to generate strains which were constantly glucose de-repressed

during glucose–xylose cofermentation. This engineering strategy had little effect on ethanol formation. It rather led to increased xylitol formation [125] (strains CPB.CR2 and CPB.MBH2, Table 3). Similarly, when truncated versions of the *MIG1* gene were expressed in xylose-utilizing strains of *S. cerevisiae*, growth and ethanol formation were only marginally affected [126]. The bacterial phosphoketolase pathway, which converts xylulose-5-phosphate directly to glyceraldehyde-3-phosphate and acetyl-P, has also been introduced in *S. cerevisiae* to enhance ethanolic xylose fermentation [127, 128]. The xylitol yield decreased without any increase in the ethanol yield [128] (strain TMB3001c-p6XFP/p4PTA/p5EHADH2, Table 2). In contrast, heterologous expression of a bacterial hemoglobin gene to render the cells a more oxidized state in oxygen-limited conditions was successful [129]. Improved ethanolic xylose fermentation was observed. This strategy is, however, only applicable in oxygenated cultures [129].

4.8 Random Methods

Random methods such as mutagenesis, adaptation, hybridization, and evolutionary engineering [130] have been employed to obtain improved xylose-utilizing [5, 42, 110, 131] (strains TMB3400, C1, C5, BH42, RWB218, RWB202-AFX, H2490-4, Tables 1, 3, and 4) and arabinose-utilizing [71] *S. cerevisiae* strains. Some of the resultant strains have been analyzed in order to identify molecular traits related to the improved ethanolic fermentation of pentose sugars. High-throughput technologies, such as transcription analysis [71, 91, 109, 132], enzyme and metabolite analysis [110], and proteome analysis [57], have been used. In many cases, the mutations and alterations observed in mutant strains are the same as have been earlier rationally engineered, confirming previous knowledge and hypotheses about control and regulation of pentose metabolism. So far, no report exists where completely novel information would have been obtained from high-throughput molecular analyses. Thus, the investigations have mainly served to confirm and demonstrate the validity of the technologies.

5 Industrial Pentose-Fermenting Strains

Metabolic engineering strategies for pentose fermentation are developed to finally generate strains that ferment pentose sugars to ethanol under industrial conditions, which may include suboptimal pH and an array of compounds which inhibit cellular metabolism. Industrial strains of *S. cerevisiae*, including baker's yeast, generally out-compete most other microorganisms with regard to the properties required in industrial ethanol production [9, 10, 133–135],

including ethanol productivity, ethanol tolerance, lignocellulose hydrolysate tolerance, and tolerance to low pH [136].

5.1 Inhibitor Tolerance

Many compounds that result from the pretreatment and heating of the lignocellulosic material, e.g., furfural and hydroxymethylfurfural, are severely inhibitory to most microorganisms [117, 137]. Detoxification procedures for lignocellulose hydrolysates are under development [134]; however, the practical large-scale use of detoxification is technically complex and adds cost to the fermentation process [138]. Similarly to inhibitors, low pH is required in the industrial context both for the function of cellulases and for avoiding bacterial infections, which is a frequently occurring problem in fermentation plants [139].

S. cerevisiae is the most robust microorganism among those with potential for efficient pentose fermentation, but differences between different *S. cerevisiae* strains are considerable. Laboratory yeast strains have been selected with regard to properties such as biomass yield and stability under well-defined conditions [140], whereas industrial isolates have been naturally selected for tolerance to industrial conditions. Although laboratory yeast strains are useful to evaluate metabolic engineering strategies and to compare cellular physiology, these strains do not possess the robustness that is required in the industrial context. Several investigations have shown that laboratory *S. cerevisiae* strains are generally less tolerant to lignocellulose hydrolysates than more robust industrial strains [7, 141, 142]. In addition, tolerance and robustness varies between different industrial strains [7]. Whereas some industrial strains require detoxification of the hydrolysate for efficient fermentation [6], others are able to ferment undetoxified hydrolysates [12, 13, 143]. However, it is important to note that hydrolysates prepared with different methods and from different raw materials contain significantly different concentrations of inhibitors as well as of the fermentable sugars, as detailed elsewhere in this volume (pretreatment and hydrolysis). Therefore, the hydrolysate to be used has to be taken into account when selecting a strain for a fermentation process [144].

5.2 Strain Stability

In addition to tolerance and robustness, strain stability is a prerequisite when designing yeast strains for industrial use. Strains carrying multicopy plasmids are generally not applicable in industry due to their instability [123, 145]. Multicopy plasmids require auxotrophic or antibiotic resistance markers to be retained in the cell, both of which are not ap-

plicable in industrial media containing complex nutrients and being used in large volumes. Thus, chromosomal integration is necessary for any genes to be introduced in industrially applied yeast strains. Ideally this requires sufficient specific activity of the introduced heterologous enzymes, so that single-copy integration supplies enough activity for metabolic function. Multiple chromosomal integration has also been utilized to generate stable pentose-fermenting strains with high activity of the enzymes introduced [6–8].

Metabolic engineering strategies applied on industrial strains have been limited to the introduction of the initial xylose and arabinose utilization pathways [4, 5, 8, 101]. Only the XR–XDH pathway has been developed in industrial *S. cerevisiae* strains [4, 5, 101] (strains A4 and A6, Table 1; strain F, Tables 1 and 4; strain TMB3400, Tables 1, 3 and 4; strain 1400(pLNH32), Table 2). No chromosomally integrated XI constructs have been reported. XI expression in *S. cerevisiae* seems to require a multicopy expression system to provide sufficient enzyme activity for xylose growth and fermentation [43]. Due to the difficulty of applying complex metabolic engineering strategies in industrial strains, procedures for random strain improvement have been relied upon to improve xylose utilization.

5.3 Fermentation of Hydrolysates

Reports on xylose fermentation by recombinant strains in industrial substrates are relatively few [6, 10, 12–14]. Laboratory strains are usually not viable in toxic lignocellulose hydrolysates, and strains with industrial background must be used. In general, xylose fermentation in hydrolysates occurs more slowly than in laboratory media even by industrial strains. For example, the rate of xylose fermentation of strain TMB3400 in dilute-acid spruce hydrolysate was an order of magnitude lower than that in mineral medium [54]. Another general observation is that very little xylitol is produced when xylose in lignocellulose hydrolysates is fermented by XR- and XDH-carrying industrial strains. This is most likely due to the presence of external electron acceptors in industrial media [74–76, 144], paradoxically removing the problem of xylitol formation, which has been considered the main drawback of the XR- and XDH-based metabolic engineering strategy.

6 Conclusion and Future Outlook

In conclusion, the domestication of *S. cerevisiae* for carbon dioxide and ethanol formation from hexose sugars has led to the fact that the metabolism of hexose and pentose sugars in this yeast are fundamentally different. As

evidenced by genome-scale transcriptome and proteome analyses of numerous recombinant pentose-utilizing *S. cerevisiae* strains, the difference is not only limited to the initial sugar conversion pathways, but also comprises the central metabolism and the glycolytic pathway. The major future challenge remains to translate the knowledge acquired from laboratory strains to industrial production strains.

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Development of Efficient Xylose Fermentation in *Saccharomyces cerevisiae*: Xylose Isomerase as a Key Component

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Abstract Metabolic engineering of *Saccharomyces cerevisiae* for ethanol production from D-xylose, an abundant sugar in plant biomass hydrolysates, has been pursued vigorously for the past 15 years. Whereas wild-type *S. cerevisiae* cannot ferment D-xylose, the keto-isomer D-xylulose can be metabolised slowly. Conversion of D-xylose into D-xylulose is therefore crucial in metabolic engineering of xylose fermentation by *S. cerevisiae*. Expression of heterologous xylose reductase and xylitol dehydrogenase does enable D-xylose utilisation, but intrinsic redox constraints of this pathway result in undesirable byproduct formation in the absence of oxygen. In contrast, expression of xylose isomerase (XI, EC 5.3.1.5), which directly interconverts D-xylose and D-xylulose, does not have these constraints. However, several problems with the functional expression of various bacterial and Archaeal XI genes have precluded successful use of XI in yeast metabolic engineering. This changed with the discovery of a fungal XI gene in *Piromyces* sp. E2, expression of which led to high XI activities in *S. cerevisiae*. When combined with over-expression of the genes of the non-oxidative pentose phosphate pathway of *S. cerevisiae*, the resulting strain grew anaerobically on D-xylose with a doubling time of ca. 8 h, with the same ethanol yield as on glucose. Additional evolutionary engineering was used to improve the fermentation kinetics of mixed-substrate utilisation, resulting in efficient D-xylose utilisation in synthetic media. Although industrial pilot experiments have already demonstrated high ethanol yields from the D-xylose present in plant biomass hydrolysates, strain robustness, especially with respect to tolerance to inhibitors present in hydrolysates, can still be further improved.

1 Introduction

1.1 *Saccharomyces cerevisiae* and Fermentation of Lignocellulosic Hydrolysates

The worldwide annual ethanol production via microbial fermentation amounted to ca. 40 Mt in 2005 (according to the Renewable Fuel Association; www.ethanolrfa.org) and is rapidly growing. Although bacteria such as *Zymomonas mobilis* and engineered *Escherichia coli* strains are capable of homoethanolic fermentation of sugars [17], the yeast *Saccharomyces cerevisiae* remains the organism of choice for large-scale industrial production of ethanol. Factors contributing to the popularity of *S. cerevisiae* as an industrial ethanol producer include its high ethanol tolerance, its ability to grow under strictly anaerobic conditions and – an important characteristic distinguishing it from prokaryotic organisms – its insensitivity to bacteriophage contaminations. Moreover, *S. cerevisiae* grows well at low pH, reducing problems with contamination of industrial processes with, for example, lactic acid bacteria.

Global concern about carbon dioxide emissions and climate change, depletion of oil reserves and geopolitical issues all contribute to a drive to increase the production of ethanol as a renewable transport fuel (see the contribution of Otero et al. in this volume). Presently, ethanol is exclusively produced from the starch or the sucrose fraction of a small number of (edible) agricultural

crops such as corn, sugar cane, sugar beet and grain. To expand the feedstock range for large-scale ethanol production and to improve productivity, it is of vital importance to enable efficient ethanol production from agricultural residues and other low-value sources of carbohydrates. Feedstocks such as corn stover, bagasse, wheat straw, non-recyclable paper or dedicated crops such as switchgrass represent an enormous potential in terms of available carbohydrates. However, instead of starch and sucrose, the carbohydrates in these feedstocks consist of a complex matrix of cellulose, hemicellulose, pectin and lignin [69].

The use of lignocellulosic raw materials for ethanol production poses a number of major challenges compared to the use of conventional starch- or sucrose-based feedstocks:

(i) Release of monomeric sugars from lignocellulosic biomass requires a mix of physicochemical (extreme pH, high temperature, high pressure) and enzymic polysaccharide (hydrolases) treatments [19, 37].
(ii) The resulting lignocellulose hydrolysates contain a wide variety of compounds that may inhibit the fermentation process. These compounds are either formed during the pretreatment process (e.g. furfural and hydroxymethylfurfural) or are biomass constituents that are released during hydrolysis (e.g. acetate, formate) [31, 37, 49, 54].
(iii) Whereas starch- and sucrose-based feedstocks yield hexoses upon hydrolysis, lignocellulosic biomass, and in particular its hemicellulose fraction, also contains large amounts of the pentose sugars D-xylose and L-arabinose. D-Xylose, generally the most abundant pentose, comprises up to 25% of the total sugar content in some hydrolysates [24, 46, 69].

Whereas *S. cerevisiae* spp. can rapidly ferment hexose sugars such as glucose, fructose, mannose and galactose, they cannot grow on nor ferment D-xylose or L-arabinose [7, 69]. Given the importance of xylose fermentation for the efficient production of ethanol from lignocellulosic biomass [24, 46, 69], it is not surprising that introduction and optimisation of heterologous pathways for xylose fermentation into *S. cerevisiae* has long been a hot topic in metabolic engineering of yeast.

Interestingly, it has long been known that *S. cerevisiae* is able to slowly metabolise the pentose sugar D-xylulose [30, 71]. This keto-isomer of xylose is phosphorylated to D-xylulose-5-phosphate by xylulokinase (*XKS1*, [57]) and subsequently metabolised via the non-oxidative part of the pentose phosphate pathway and glycolysis. It is therefore logical that strategies for converting D-xylose into D-xylulose are an exhaustively studied topic in the quest for alcoholic fermentation of D-xylose by *S. cerevisiae*. These strategies will be briefly discussed in Sects. 1.2–1.4.

1.2 Introduction of Heterologous Genes Encoding Xylose Reductase and Xylitol Dehydrogenase: Redox Restrictions

In contrast to *S. cerevisiae*, many yeast species are capable of utilising xylose as the sole carbon and energy source for respiratory growth. However, only few of these yeasts are capable of fermenting xylose to ethanol under oxygen-limited conditions, such as for instance *Pichia stipitis* and *Pachysolen tannophilus* [65].

Maybe not surprisingly, xylose-metabolising yeasts have predominantly been isolated from wood-related environments. The pathway for D-xylose metabolism used by these yeasts to convert D-xylose to D-xylulose was first described in 1955 [25] and involves a two-step conversion that involves two oxidoreductases (Fig. 1): xylose reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9). The xylose reductase has a strong preference for NADPH, whereas the subsequent oxidation of xylitol via xylitol dehydrogenase produces NADH (Table 1).

Clearly, this difference in cofactor specificity can result in redox imbalance. To generate the NADPH for the xylose reductase reaction, part of the D-xylose carbon must be directed through the oxidative pentose phosphate pathway (involving the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reactions). While this results in a loss of some carbon as CO_2,

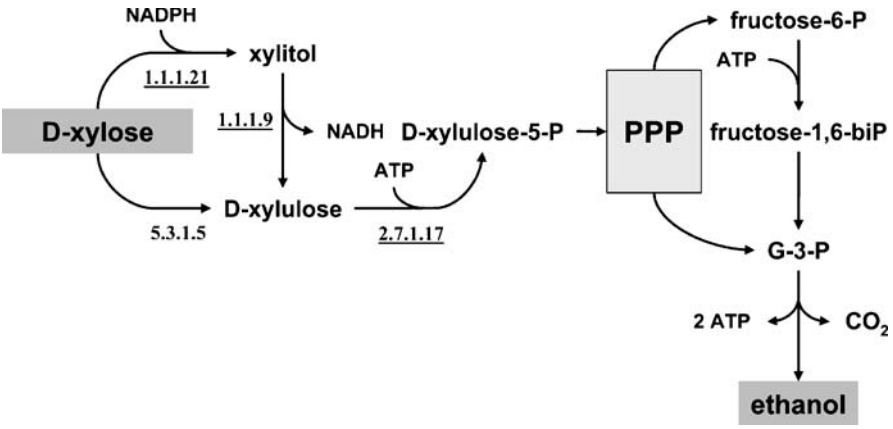

Fig. 1 D-Xylose catabolism in (metabolically engineered) *S. cerevisiae* strains. *Underlined* EC numbers represent enzymes/steps present in wild-type *S. cerevisiae* metabolism. The gene names corresponding to the enzymes are given in parentheses: 1.1.1.21, aldose/xylose reductase (*GRE3*/*xyl1*); 1.1.1.9, xylitol dehydrogenase (*XYL2*/*xyl2*); 2.7.1.17, xylulokinase (*XKS1*/*xyl3*); 5.3.1.5, xylose isomerase (*xylA*). *G-3-P* glyceraldehyde-3-phosphate, *PPP* pentose phosphate pathway

Table 1 NADPH-linked and NADH-linked xylose reductase activities in batch cultures of various D-xylose-assimilating yeasts

Organism	CBS no.	Specific activity NADH	NADPH	Ratio	Xylose fermentation[a]
Candida tenuis	615	2	130	0.02	–
	2226	7	320	0.02	–
	2885	0[b]	100	0	–
	4113	60	120	0.5	+
	4285	305	670	0.5	+
	4434	0[b]	485	0	–
	4435	340	670	0.5	+
	4604	0[b]	365	0	–
Candida shehatae	5813	210	480	0.4	+
Candida utilis	621	0[b]	75	0	–

Cells were harvested at mid-exponential growth phase. Enzyme activities are expressed as nmol(mg protein)$^{-1}$ min^{-1}. Data taken from Bruinenberg et al. (1984) [15]

[a] Results obtained in a fermentation test using a Durham vial

[b] Not detectable

– No gas production, ethanol less than 0.3 g L^{-1}

+ Gas production, ethanol higher than 5.0 g L^{-1}

which goes at the expense of the ethanol yield on D-xylose, it enables the efficient regeneration of NADPH [16, 32, 45, 69].

However, the cells have to take additional measures to reoxidise the excess NADH generated in the xylitol dehydrogenase reaction. In the presence of oxygen, this excess NADH can be reoxidised by respiration. This will require accurate dosage of oxygen to prevent full respiration of D-xylose. Such accurate control is difficult to envisage in large-scale processes for ethanol production, which should preferably involve a minimum of aeration to reduce costs.

Under anaerobic conditions, reoxidation of excess NADH can be accomplished via the production of compounds that are more reduced than D-xylose, such as xylitol and/or glycerol. The production of xylitol occurs via xylose reductases, which have a dual co-enzyme specificity and thereby can also use NADH, or alternatively via other aspecific reductases. As this mechanism involves the consumption of one D-xylose for each NADH generated, it has a tremendously negative impact on the ethanol yield from D-xylose [45]. Glycerol production is a well-known redox sink during hexose fermentation and especially under anaerobic conditions, but requires both carbon and ATP [67].

The preference of xylose reductase for NADPH is not only species- but also strain-dependent (Table 1). The in vivo ratio of NADPH over NADH utilisation by xylose reductase and the redox balance requirements determine the

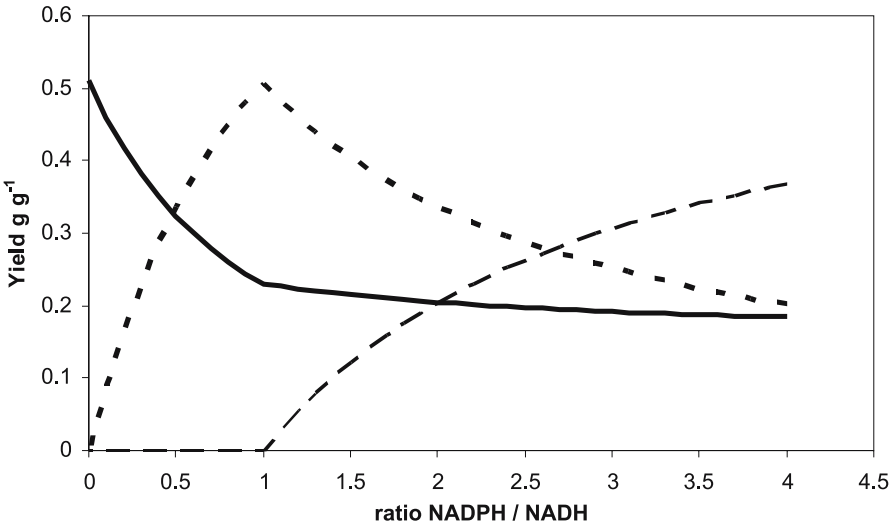

Fig. 2 Calculated ethanol (–), xylitol (- - -) and glycerol (– – –) yields during anaerobic catabolism of D-xylose as a function of the ratio of the fluxes via NADPH-linked and NADH-linked xylose reductase calculated from Eqs. 1, 2 and 3. Assumed is that (ATP-using) glycerol formation does not occur below a ratio of 1. In other words, NADH is preferentially shuttled into xylitol formation instead of glycerol formation. Above a ratio of 1 there is a stoichiometric necessity for an alternative redox sink such as glycerol formation. At a ratio of 4.0 the ATP yield is zero. Figure from van Maris et al. 2006 [69]

requirement for NADH sinks such as xylitol and glycerol (Fig. 2) in anaerobic cultures [14, 69]. When this NADPH/NADH ratio equals zero, xylose reductase only uses NADH and thereby consumes all NADH produced in the xylitol dehydrogenase reaction. Since in addition no regeneration of NADPH is required for the xylose reductase reaction, redox-balanced xylose metabolism will occur according to Eq. 1:

$$\text{Ratio} = 0: \quad 6 \text{ xylose} \rightarrow 10 \text{ ethanol} + 10\ CO_2 + 10 \text{ ATP}\,. \tag{1}$$

At a ratio of one (Eq. 2), one out of every two D-xylose molecules can be further metabolised to ethanol, whereas the other is reduced to xylitol to maintain NADH balance. In addition, some carbon has to be redirected for the generation of NADPH, resulting in the formation of only 9 mol of ethanol from 12 mol of D-xylose (45% of the theoretical yield). Following these redox-balance considerations, catabolism via a xylose reductase with a NADPH/NADH-utilisation ratio of one will follow:

$$\text{Ratio} = 1: \quad 12 \text{ xylose} \rightarrow 9 \text{ ethanol} + 12\ CO_2 + 9 \text{ ATP} + 6 \text{ xylitol}\,. \tag{2}$$

At ratios above one, NADH-dependent xylitol formation cannot compensate for the production of NADH in the xylitol dehydrogenase reaction and glycerol formation becomes essential for redox balancing [32, 45, 69]. When the xylose

reductase solely uses NADPH (an infinite NADPH/NADH ratio) this would result in the formation of only 0.5 mol ethanol per mol of xylose fermented.

$$\text{Ratio} = \infty: \quad 6 \text{ xylose} + 3 \text{ ATP} \rightarrow 3 \text{ ethanol} + 6 \text{ glycerol} + 6 \text{ CO}_2 . \qquad (3)$$

Despite these inherent redox restrictions and ensuing loss of ethanol yield on D-xylose, the expression of xylose reductase and xylitol dehydrogenase has long been the most successful strategy to enable D-xylose consumption by *S. cerevisiae* (elsewhere in this volume, and [29, 32, 33, 39, 63]). Although attempts have been made to change the cofactor specificity of xylose reductase, fermentation properties of a *S. cerevisiae* strain containing this gene are not available [55]. Similarly, expression of a transhydrogenase in *S. cerevisiae*, with the aim of converting excess NADH into NADPH, did not result in reduced byproduct formation [51]. The latter result is perhaps not altogether surprising as, with NADPH/$NADP^+$ ratios generally being higher than NADH/NAD^+ ratios [51], reduction of $NADP^+$ with NADH is thermodynamically unfavourable.

Despite the inherent redox constraints of *S. cerevisiae* strains based on the xylose reductase/xylitol dehydrogenase strategy, this strategy has resulted in many important insights into the kinetics of D-xylose metabolism by engineered *S. cerevisiae* strains. These findings include the benefits of overexpression of xylulokinase [29, 56], the side role of the *S. cerevisiae* aldose reductase (Gre3) (besides the heterologous dual specificity xylose reductases) in xylitol formation [66], the role of the enzymes of the non-oxidative part of the pentose phosphate pathway [34, 43], characterisation of D-xylose transport [27, 62] and many studies on the inhibitor tolerance/sensitivity of D-xylose-consuming strains [54]. The latter will be especially crucial for successful application of D-xylose-consuming *S. cerevisiae* strains for ethanol production from lignocellulosic hydrolysates (see Sect. 7).

1.3 Native D-Xylose-Metabolising Enzymes in *S. cerevisiae*

Although *S. cerevisiae* cannot grow on D-xylose as the sole carbon source, its genome does contain genes that code for a non-specific NADH-dependent aldose reductase (*GRE3*) and for a xylitol dehydrogenase (*XYL2*). It has been shown that over-expression of these native *S. cerevisiae* genes using endogenous promoters enabled a specific growth rate of 0.01 h^{-1} on D-xylose in shake flasks [64]. However, in these shake-flask cultures this engineered yeast strain converted D-xylose into xylitol with a yield of 55%. Under anaerobic conditions, precluding respiratory NAD^+ regeneration, the strain overexpressing the endogenous enzymes was unable to utilise D-xylose [64].

In addition to this metabolic engineering approach, the presence of endogenous genes for D-xylose-converting enzymes has been used in recent experiments by Attfield and Bell (2006), describing a non-recombinant *S. cere-*

visiae strain that grows on D-xylose as the sole carbon source in aerobic shake flask cultures. In their study a combination of population genetics and evolutionary engineering [5, 60] resulted in an increase in growth rate from extremely low, barely measurable growth rates to a specific growth rate of around 0.12 h^{-1} (a doubling time of less than 6 h) over a period of 1400 days. Apparently, this *S. cerevisiae* strain had evolved in such a way that the very low "background" xylose reductase and xylitol dehydrogenase activities, which were previously described as insufficient for growth on D-xylose [8], increased to levels that did enable growth. Indeed, subsequent analysis of the evolved strain showed that xylose reductase activity had increased fourfold and the xylitol dehydrogenase activity 80-fold relative to the parental strain. The actual genes that underwent mutation have not yet been characterised. Although this very interesting study underlines the tremendous potential of evolutionary approaches, the selection procedure inevitably resulted in a yeast strain displaying the characteristics of redox imbalance, such as xylitol production.

1.4 One-Step Conversion of D-Xylose into D-Xylulose via Xylose Isomerase

In view of the intrinsic redox restrictions associated with the combined introduction of xylose reductase and xylitol dehydrogenase into *S. cerevisiae*, it is relevant to explore alternative metabolic engineering strategies. As will be discussed below, expression of heterologous genes for xylose isomerase (an enzyme that does not naturally occur in *S. cerevisiae*) offers such an alternative [14]. In the following sections, we will briefly discuss the properties and taxonomic distribution of xylose isomerases. This will be followed by a brief overview of previous attempts at functional expression of xylose isomerases in *S. cerevisiae*. We will then discuss how, in the past few years, fast progress has been made due to the discovery of a new, fungal xylose isomerase gene. Finally, we will discuss the status of the xylose isomerase strategy with regard to full-scale industrial application.

2 Xylose Isomerase: Properties and Occurrence

Xylose isomerase (XI, D-xylose ketol isomerase, EC 5.3.1.5) catalyses the reversible isomerisation of D-xylose to D-xylulose. This enzyme has been the subject of much applied research because it also catalyses the isomerisation of D-glucose and D-fructose. In this role of "glucose isomerase", xylose isomerase is applied on a huge scale for the production of high-fructose corn syrup and continues to be one of the most abundantly applied industrial enzymes. The high-fructose syrup application has led to intensive screening and protein engineering studies, with increased activity and stability of XIs at el-

evated temperature as a priority target [11, 23]. For excellent reviews on the molecular and industrial aspects of XI, the reader is referred to a number of specialised reviews [4, 11, 12].

In the context of the present paper, several characteristics of XIs are noteworthy. First and foremost, and in contrast to the xylose reductase/xylitol dehydrogenase pathway, the XI reaction does not involve pyridine nucleotide cofactors. As this will entirely circumvent the cofactor regeneration challenges associated with the xylose reductase/xylose dehydrogenase pathway, functional expression of a XI in *S. cerevisiae* has long been regarded the most promising approach to engineering *S. cerevisiae* for alcoholic fermentation of D-xylose [14].

XIs generally require divalent cations, but the specificity of the metal requirement is strongly dependent on the source of the enzyme, with many enzymes requiring Co^{2+}, but others Mn^{2+} or Mg^{2+} [11]. Although *S. cerevisiae* has been demonstrated to accumulate cobalt intracellularly [18], it is not clear whether this metal is available in the cytosol or sequestered in, for example, the vacuole. Other aspects with potential relevance for yeast metabolic engineering include the high temperature optimum (60–80 °C) and the relatively high pH optimum (7.0–9.0) of many of the XIs that have been characterised [11]. As *S. cerevisiae* is a mesophilic micro-organism with a cytosolic pH slightly below 7, intracellular expression of heterologous structural genes for XIs may not always lead to optimal activity.

Even in the pre-genomics era, it was clear that XIs are widespread among prokaryotic micro-organisms, and also occur in several plants [11]. Figure 3 shows a phylogenetic tree of XI gene sequences based on an October 2006 GenBank database search. This phylogenetic tree gives a good indication of the diversity of XI genes and the phylogenetic relationships between sequences from related organisms. With respect to eukaryotes, the tree contains four sequenced XI sequences from the plants *Hordeum vulgare*, *Arabidopsis thaliana*, *Oryza sativa* and *Medicago truncatula*, which cluster together (Fig. 3). The phylogenetic tree contains only one other eukaryotic XI sequence, namely that of the anaerobic fungus *Piromyces* sp. E2 [28]. Interestingly, this eukaryotic XI sequence clusters with those of the prokaryotic phylum Bacteroidetes, which has led to the suggestion that the fungus may have acquired XI via horizontal gene transfer [28], as previously suggested for other enzymes in anaerobic fungi [20].

3 Expression of Xylose Isomerases in *S. cerevisiae*: a Long and Winding Road

The expression of a cofactor-independent, heterologous XI is the solution for bypassing the intrinsic redox constraints of the XR/XDH approach. Suc-

cessful implementation, however, requires an in vivo activity of XI similar to that of key glycolytic enzymes such as hexokinase and phosphofructokinase. In practice, this corresponds to an activity, under physiological conditions, of 0.5–1.0 μmol D-xylose converted per milligram soluble cell protein per minute [68]. The apparent simplicity of this objective turned out to be deceptive. In fact, studies on the functional expression of heterologous structural genes for XI in *S. cerevisiae* now spans roughly two decades.

Expression in *S. cerevisiae* of the *E. coli xylA* gene (which clusters with the XI genes from other Proteobacteria, Fig. 3), resulted in no [13] or very low in vitro XI activities [59]. Sarthy et al. (1987) showed that, while the *E. coli* XylA protein was produced in *S. cerevisiae*, its specific activity was three orders of magnitude below that of XylA protein produced in *E. coli* [59]. Improper protein folding, sub-optimal intracellular pH, post-translational modification, inter- or intramolecular disulfide bridge formation and a lack of specific cofactors or metal ions in *S. cerevisiae* were mentioned as possible causes [59]. However, no single factor was identified that could explain the low activity, and attempts to increase *E. coli* XI expression levels in *S. cerevisiae* were unsuccessful [59]. Subsequently, attempts were made to express XI-encoding genes from other prokaryotic phyla. Attempts to express XI genes from *Clostridium thermosulfurogenes* [48], *Bacillus subtilis* or *Actinoplanes missouriensis* [1], which originate from different prokaryotic phyla (Fig. 3), also failed to result in the production of an active XI enzyme in *S. cerevisiae*.

The first study that achieved significant activities of a heterologous XI enzyme in *S. cerevisiae* was based on expression of the XI gene from the thermophile *Thermus thermophilus* [70]. Indeed, an enzyme activity of up to 1.0 μmol(mg protein)$^{-1}$ min^{-1} was found in cell extracts of the engineered *S. cerevisiae* strain. However, this activity was assayed at the optimum temperature for activity of the *T. thermophilus* XI of 85 °C, which is not compatible with yeast growth or survival. At 30 °C, the optimum temperature for growth of *S. cerevisiae*, activity was only 0.04 μmol (mg protein)$^{-1}$ min^{-1} [70]. Although subsequent random mutagenesis resulted in variants of the *T. thermophilus* XI with improved temperature characteristics [26, 47], in vivo enzyme activities of the *T. thermophilus* XI in *S. cerevisiae* strains remained too low to sustain rapid anaerobic growth on D-xylose ([35], see Sect. 5).

A breakthrough came with the discovery of a XI in an unicellular eukaryote, the anaerobic fungus *Piromyces* sp. E2 [28]. Expression of this *Piromyces xylA* gene in *S. cerevisiae* resulted in high enzyme activities (up to 1.1 μmol(mg protein)$^{-1}$ min^{-1} at 30 °C [42].

The molecular basis for the high functional expression levels obtained with the *Piromyces* xylA gene remains unclear. We have recently expressed the XI sequence from *Bacteroides thetaiotaomicron* into *S. cerevisiae*. This prokaryotic sequence is 83% identical and 88% similar to the *Piromyces* xylA gene. *S. cerevisiae* strains expressing this prokaryotic XI can utilise D-xylose, albeit

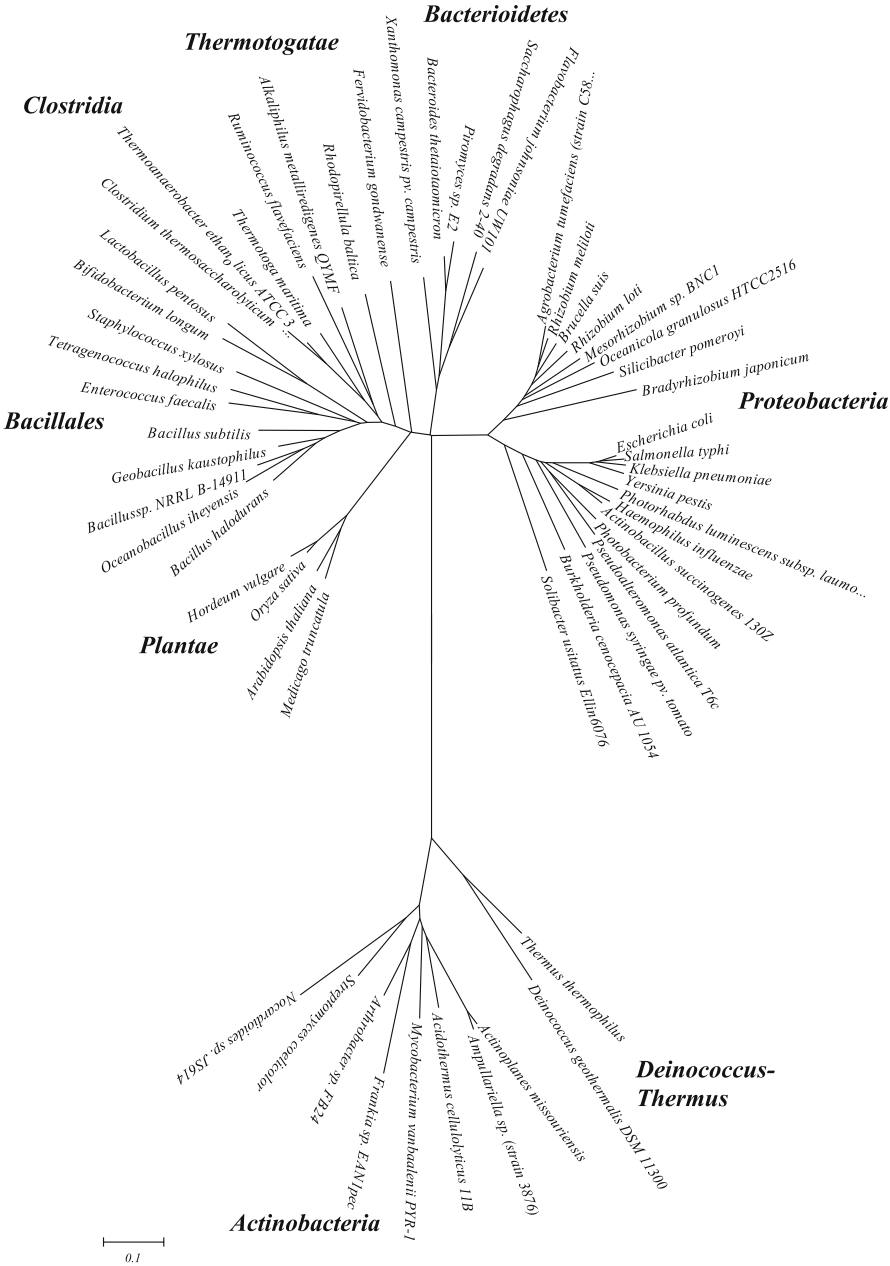

Fig. 3 Phylogram based on the aminoacid sequences of a selection of the xylose isomerases present in BRENDA and GenBank [10, 61]. The tree is the consensus of 500 bootstrap repetitions and unrooted. The *bar* indicates ten substitutions per 100 amino acid residues. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [41]

at a somewhat lower rate than similar strains expressing the *Piromyces* xylA gene (A.A. Winkler et al. unpublished). This indicates that its probable evolutionary history (horizontal gene transfer followed by evolutionary adaptation to a eukaryotic host) may not be the sole factor in the successful expression of the *Piromyces* enzyme.

In terms of GC content and codon usage, the *Piromyces* xylA gene appears to have favourable characteristics for expression in *S. cerevisiae*. At 45%, its GC content is much closer to that of *S. cerevisiae* (39%), than that of, for example, the *T. thermophilus* gene (little over 64% GC). Also the high codon bias index of the *Piromyces* gene for expression in *S. cerevisiae* (0.642 versus – 0.018 for the *T. thermophilus* gene) may contribute to its efficient expression. Future structure–function studies will likely identify critical factors for high-level functional expression in yeast, in the *S. cerevisiae* genome as well as in the sequence of heterologous XI genes. However, while of great scientific interest, innovation in D-xylose fermentation is no longer dependent on such research, as the availability of the *Piromyces* xylA gene has paved the way for metabolic engineering of *S. cerevisiae* for anaerobic fermentation of D-xylose to ethanol. Recent progress in this area will be discussed in the following paragraphs.

4 Characterisation of Yeast Strains with High-Level Functional Expression of a Fungal Xylose Isomerase

Expression of the *Piromyces* sp. E2 XylA gene under control of a strong, constitutive *TPI1* promoter on a 2μ-based plasmid (pAKX002) in the haploid laboratory strain *S. cerevisiae* CEN.PK resulted in XI activities ranging from 0.33 to 1.1 μmol (mg protein)$^{-1}$ min^{-1} in cell extracts [42]. These activities are similar to those of key enzymes of alcoholic fermentation in glucose-fermenting cultures [68]. Apparently, conditions in the cytosol of *S. cerevisiae* do not preclude accurate folding of the fungal XI, as has previously been reported for the *Streptomyces rubiginosus* XI [21]. In addition, in contrast to the previously expressed XI from *T. thermophilus*, the *Piromyces* XI yielded the above-mentioned activities at a temperature of 30 °C.

Although the high XI activities found in XylA-expressing *S. cerevisiae* strains provided an excellent starting point for further strain development, they did not as such enable a high specific rate of D-xylose fermentation. In fact, the specific growth rate in aerobic cultures on 20 g L^{-1} D-xylose as the sole carbon source was only 0.005 h^{-1} (Fig. 4). A similar very low specific growth rate was found in earlier engineered *S. cerevisiae* strains that expressed the *P. stipitis* xylose reductase and xylitol dehydrogenase genes [38, 39]. The low rate of D-xylose conversion in strains with a high XI activity suggested that D-xylose consumption was either controlled by D-xylose transport or by reactions downstream from D-xylulose.

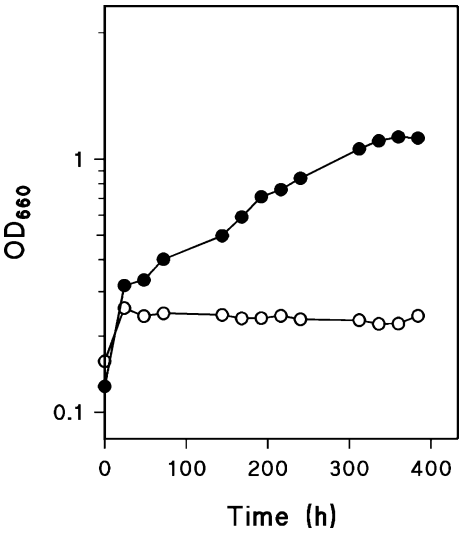

Fig. 4 Growth of *S. cerevisiae* RWB 202 (●) (CEN.PK 113-5D with pAKX002), expressing *Piromyces* xylose isomerase, and the reference strain CEN.PK113-7D (○) in shake-flask cultures on synthetic medium with 20 g L^{-1} D-xylose as the sole carbon source. Data from Kuyper et al. 2003 [42]

Since the low specific growth rates of the Piromyces XylA-expressing strains on D-xylose complicated studies in batch cultures, initial studies on D-xylose consumption kinetics and product formation were performed in anaerobic chemostat cultures grown on glucose–xylose mixtures. Anaerobic chemostat cultivations on glucose alone demonstrated that expression of a heterologous XI did not interfere with product formation during growth on glucose [42]. However, when D-xylose was also included in the medium of the anaerobic glucose-limited chemostat cultures, a significant effect of XylA expression was observed. With 20% of the added D-xylose being consumed, a significant increase of the ethanol yield on consumed glucose was observed (from 0.40 g g^{-1} to 0.44 g g^{-1}). Although no labeling studies were performed, it stands to reason that this ethanol was produced from the consumed D-xylose.

Interestingly, these anaerobic chemostat cultures of the XylA-expressing strains excreted significant amounts of D-xylulose. At a specific D-xylose consumption rate of 0.73 mmol (g biomass)$^{-1}$ h^{-1} this yeast excreted D-xylulose at a rate of 0.20 mmol (g biomass)$^{-1}$ h^{-1} (corresponding to 30% of consumed D-xylose), which suggested that reactions downstream of D-xylulose were rate-controlling. Moreover, small amounts of xylitol were produced in these cultivations, suggesting involvement of a non-specific aldose reductase such as encoded by *GRE3* [66]. This information on D-xylulose and xylitol production was used in subsequent metabolic engineering attempts to improve the D-xylose consumption rate and to minimise xylitol formation.

5 Metabolic Engineering for Improved Xylose-Isomerase Based D-Xylose Utilisation

Metabolic engineering is defined as the improvement of cellular activities by manipulation of enzymic, transport and regulatory functions of the cell with the use of recombinant DNA technology [6]. After the successful expression of a XI in *S. cerevisiae* [42], reactions downstream of D-xylulose and the, presumably Gre3-dependent, formation of xylitol were identified as priority targets (see previous section).

As it is unlikely that the high capacity of glycolysis in *S. cerevisiae* would limit D-xylose fermentation rates; limitations in D-xylose fermentation are likely to reside either in the reaction catalysed by xylulokinase or in one of the four reactions of the non-oxidative pentose phosphate pathway. Modulating the flux through a certain pathway by up-modulation of single enzymes often has little effect, as can be shown by metabolic control analysis [50]. Hence, it was decided to simultaneously increase the levels of all five enzymes. To this end, the *S. cerevisiae* structural genes encoding xylulokinase (*XKS1*), ribulose-5-phosphate epimerase (*RPE1*), transketolase (*TKL1*), transaldolase (*TAL1*) and ribulose-5-phosphate isomerase (*RPI1*) were over-expressed together with the *Piromyces* sp. E2 XylA gene [43]. Since the non-specific aldose reductase encoded by *GRE3* had previously been implicated in xylitol formation by *S. cerevisiae*, this gene was also deleted in the engineered strain [45, 66].

Research on pentose metabolism in *S. cerevisiae* is increasingly impeded by the fact that key biochemical intermediates can no longer be purchased commercially [35, 43]. While this precluded enzyme-activity assays for several of the over-expressed genes, mRNA analysis indicated that over-expression, either from strong constitutive promoters inserted in front of chromosomal genes or from plasmid-borne expression cassettes, was successful.

Remarkably, the *S. cerevisiae* strain (RWB 217) harbouring the six over-expressions and single deletion was directly capable of anaerobic growth on D-xylose as the sole carbon source at a growth rate of $0.09\,h^{-1}$ [43]. Starting with a low-density inoculum, this strain consumed $20\,g\,L^{-1}$ of D-xylose within 40 h, with an ethanol yield on D-xylose of $0.43\,g\,g^{-1}$. This ethanol yield, which is lower than the theoretical yield of $0.51\,g\,g^{-1}$ due to the formation of biomass and glycerol, was virtually identical to the ethanol yield found on glucose in exponentially growing, anaerobic *S. cerevisiae* cultures. Deletion of *GRE3* reduced xylitol production to trace amounts (0.4 mM from $20\,g\,L^{-1}$ D-xylose), indicating that alternative D-xylose- or D-xylulose reducing enzymes were active at very low rates in this *S. cerevisiae* background. In the engineered strain, D-xylulose no longer accumulated in the broth, indicating that limitations downstream of D-xylulose had been successfully eliminated.

In an independent study, Karhumaa et al. (2005) expressed the XI gene from *T. thermophilus* together with the same combination of pentose phosphate pathway enzymes [35]. In these strains the specific activity of XI was 0.008–0.017 μmol (mg protein)$^{-1}$ min^{-1} at 30 °C. In contrast to the efficient anaerobic growth of the above-described *S. cerevisiae* expressing the *Piromyces* sp. E2 XI, D-xylose consumption by the *T. thermophylus* XI-containing strain (TMB 3045) was not observed under aerobic conditions. After additional se-

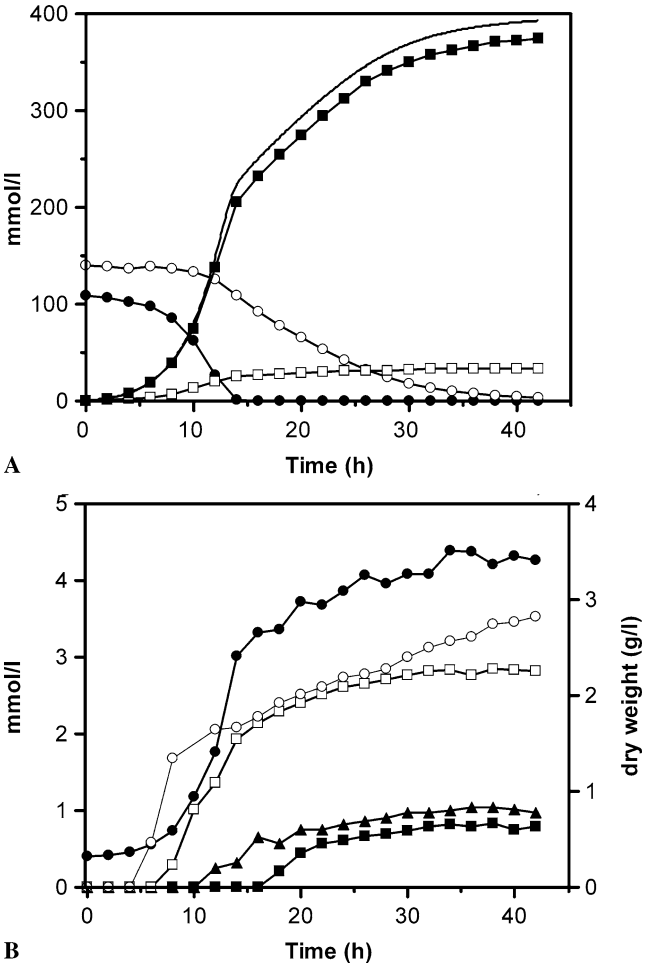

Fig. 5 Anaerobic growth of strain RWB 217 in fermenters on synthetic medium with 20 g L^{-1} glucose and 20 g L^{-1} D-xylose as the carbon source; duplicate experiments differed by less than 5%. **a** Glucose (●), D-xylose (○), ethanol (■), glycerol (□) and cumulative CO_2 produced per litre as deduced from gas analysis (–). **b** dry weight (●), acetate (○), xylitol (■), lactate (□) and succinate (▲). Data from Kuyper et al. 2005 [43]

lection, a strain capable of aerobic growth on D-xylose at a maximum specific growth rate of 0.045 h^{-1} was isolated (TBM 3050). Confusingly, although the abstract claims anaerobic production of ethanol, the experimental description and results section describe the production of 0.29 g ethanol (g D-xylose)$^{-1}$ at a rate of 2.4 mg (g biomass)$^{-1}$ h^{-1} under oxygen-limited conditions [35]. The ethanol production rates, are more than 400-fold lower than observed in the *Piromyces* XylA-based strain [35, 42]. This observation, combined with the interesting observation that TMB 3045 and TMB 3050 display almost identical specific growth rates on D-xylulose, indicates the importance of high-level functional expression of XI for efficient D-xylose fermentation.

In lignocellulosic hydrolysates, D-xylose is generally the second most abundant sugar, with glucose accounting for the majority of the fermentable sugar [24, 46, 69]. Rapid consumption of glucose–xylose mixtures – either sequential or simultaneous – is therefore crucial for successful industrial implementation. When the metabolically engineered strain RWB 217 (described above) was grown in anaerobic batch cultures on mixtures of 20 g L^{-1} glucose and 20 g L^{-1} D-xylose (Fig. 5), sequential utilisation was observed. Although both sugars were consumed within 40 h, D-xylose consumption only commenced when the glucose concentration dropped below 4 g L^{-1}. Instead of increasing exponentially, as anticipated based on the kinetics of D-xylose consumption in D-xylose-only cultures, the specific rate of D-xylose consumption decreased over time. Clearly, the kinetics of D-xylose consumption by cells grown in the presence of glucose were sub-optimal. This challenge was addressed by evolutionary engineering.

6
Evolutionary Engineering for Improved Xylose-Isomerase-Based D-Xylose Utilisation

6.1
Evolutionary Engineering of D-Xylose-Consuming *S. cerevisiae* for Improved Mixed Substrate Utilisation

The sub-optimal kinetics of mixed-substrate utilisation by the genetically engineered XylA-expressing strain RWB 217 [43] suggested a low affinity (q_{max}/K_s) for D-xylose. Soon after the invention of the chemostat it was already established that prolonged cultivation in nutrient-limited chemostats leads to selection of spontaneous mutants with an improved affinity for the growth-limiting nutrient [52, 53]. This principle, which has since been demonstrated for many micro-organisms and nutrients [40, 58, 72, 73] was applied to improve the affinity of *S. cerevisiae* RWB 217 for D-xylose [44].

Indeed, during prolonged anaerobic D-xylose-limited chemostat cultivation at a dilution rate of 0.06 h^{-1}, the residual D-xylose concentration decreased

threefold, indicating that cells with improved affinity for D-xylose were selected for [44]. After 1000 h (85 generations) of this directed evolution in chemostat cultures, single-colony isolates were tested for batch growth on a mixture of glucose and D-xylose. Although the fermentation kinetics of some of these single-cell lines, as evaluated by carbon dioxide production profiles, were already drastically improved relative to the parental strain (Fig. 6), the D-xylose phase remained slower than anticipated based on batch cultivation on D-xylose alone. A further 85 generations of chemostat cultivation resulted in only marginal improvement of the D-xylose consumption characteristics.

To select for further improvement of D-xylose fermentation kinetics, an additional evolutionary engineering strategy was applied, which involved sequential anaerobic batch cultivation on glucose–xylose mixtures [44]. To maximise the number of generations that the cells grow on D-xylose, the D-xylose concentration in the cultures was raised to 90 g L^{-1}, with a glucose concentration of 20 g L^{-1}. After 20 cycles, the evolved culture was capable of complete anaerobic conversion of a mixture of 20 g L^{-1} glucose and 20 g L^{-1} D-xylose in about 20 h, with an inoculum size of 5% (v/v) [44].

Characterisation of the resulting strain RWB 218 (derived from single colony isolate) showed that D-xylose consumption followed the consumption of glucose rapidly (Fig. 7). The D-xylose consumption rate observed in these cultures was 0.9 g D-xylose (g dry weight)$^{-1}$ h^{-1}. This evolved XI-based strain, in contrast to strains based on xylose reductase and xylitol dehydrogenase, produced only 0.45 mM of xylitol, indicating that redox imbalance does

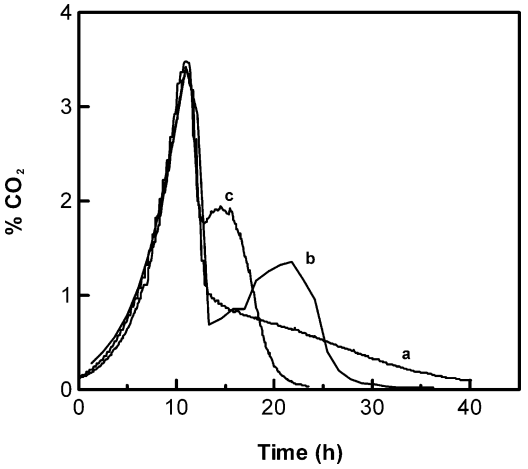

Fig. 6 CO_2 production profiles, per litre culture, as measured in off gas of anaerobic fermenter batch cultures with 20 g L^{-1} glucose and D-xylose each. Profiles have been aligned on the glucose consumption peak to eliminate variations in initial biomass. *a* RWB 217, *b* culture after chemostat selection, *c* RWB 218. Initial biomass concentrations were 0.20 ± 0.05 g L^{-1}. Data from Kuyper et al. 2005 [44]

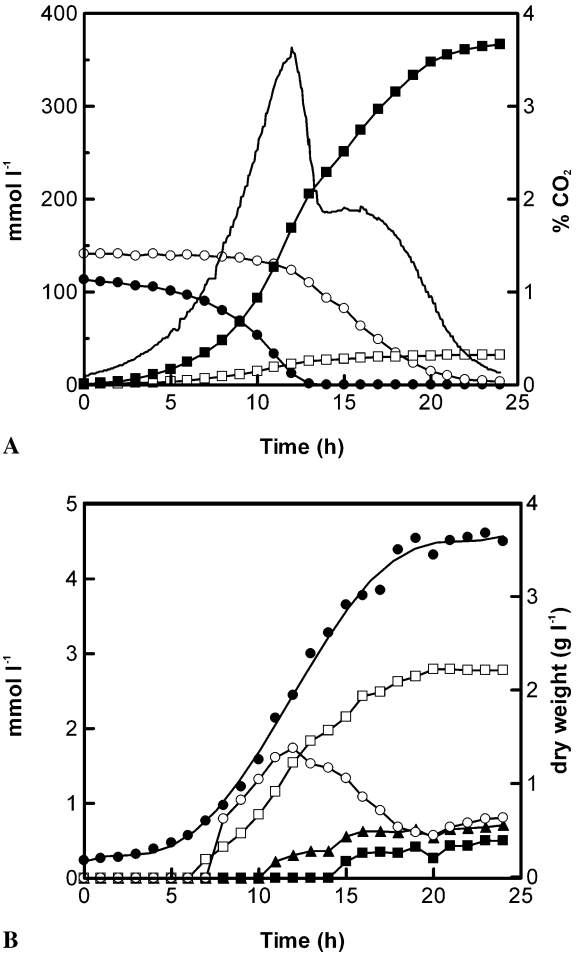

Fig. 7 Typical graph of anaerobic growth of strain RWB 218 in fermenters on synthetic medium with 20 g L^{-1} glucose and D-xylose each as the carbon source, duplicate experiments differed by less than 5%. **a** Glucose (●), D-xylose (○), ethanol (■), glycerol (□) and % CO_2 measured in off gas per litre culture (–). **b** Dry weight (●), acetate (○), xylitol (■), D-lactate (□) and succinate (▲). Initial biomass concentration was 0.17 g L^{-1}. Data from Kuyper et al. 2005 [44]

not occur during alcoholic fermentation of D-xylose. The ethanol yield on total sugar in batch cultures co-fermenting glucose and D-xylose was typically 0.40 g g^{-1}, which is identical to the ethanol yield that would be achieved in glucose-grown cultures in a similar set-up. Even when tested in more concentrated sugar mixtures (100 g L^{-1} glucose and 25 g L^{-1} D-xylose), resembling an industrial situation, this strain consumed both sugars within 24 h, starting with 1.1 g L^{-1} yeast dry weight as the inoculum [44].

With evolutionary engineering as a proven tool for obtaining (yeast) strains with improved properties, a full understanding of the underlying molecular changes becomes the next challenge. In an attempt to unravel the changes between the original metabolically engineered and the subsequently evolved *Piromyces* XI-based strains, anaerobic chemostat cultivations on D-xylose as the sole carbon source were used as the basis for transcriptome analysis with Affymetrix DNA arrays (J.T. Pronk, unpublished data). The most striking observation amongst the genes with a changed transcript level was the representation of various members of the hexose transport family, including *HXT1*, *HXT2* and *HXT4*. Interestingly, *HXT1* and *HXT4* have been associated with D-xylose transport in previous studies [27, 62]. To investigate whether the improved fermentation characteristics were indeed due to changes in sugar transport, zero trans-influx assays were performed using both the strain that was only metabolically engineered and the subsequently evolved strain [44]. The D-xylose uptake kinetics obtained for the metabolically engineered strain (K_m 132 mM, V_{max} 15.8 mmol (g dry weight)$^{-1}$ h^{-1}) were in agreement with other studies [22, 39]. Strikingly, the D-xylose uptake kinetics of the evolved strain had changed drastically, with a 25% reduction in the K_m (to 99 mM) and a twofold increase of V_{max} to 32 mmol (g dry weight)$^{-1}$ h^{-1}.

6.2 Evolutionary Engineering of *S. cerevisiae* only Containing Fungal Xylose Isomerase

After the proof of principle of XI expression in *S. cerevisiae*, not only metabolic engineering, but also evolutionary engineering was applied to improve the rate of D-xylose utilisation of a strain solely over-expressing XI [44]. Since improvement of the aerobic consumption rate was initially the target of this selection experiment, serial transfer in a shake flask was chosen as the cultivation condition of this evolution run. Indeed, after 30 serial transfers, the specific growth rate of this culture improved drastically (24-fold) from 0.005 h^{-1} to 0.12 h^{-1} (Fig. 8). However, a strain isolated from this selection experiment was not yet capable of anaerobic growth. Therefore, an additional ten selection rounds were performed in oxygen-limited batch cultures, finally followed by ten cycles in an anaerobic sequencing batch reactor. From this culture a single colony was isolated (named RWB 202-AFX, for anaerobic fermentation of D-xylose based on strain RWB 202) and used for further characterisation of the end product of this evolutionary engineering.

It was shown that only the expression of a XI, followed by evolutionary engineering for anaerobic growth, can also result in a *S. cerevisiae* strain that can grow on 2% D-xylose as the sole carbon source, with a growth rate of 0.03 h^{-1} in anaerobic batch fermentations [45]. However, although this strain displayed a good ethanol yield on D-xylose (0.42 g g^{-1}) and very low production of xylitol (2.8 mM), the obtained growth rate, and therefore ethanol

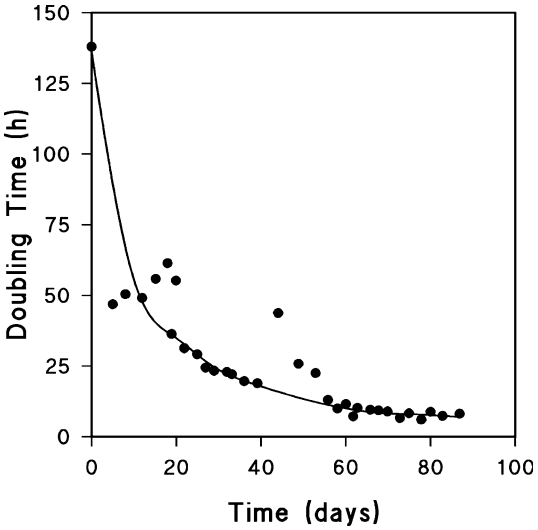

Fig. 8 Doubling time during serial transfer of *S. cerevisiae* RWB 202 in shake-flask cultures on synthetic medium with xylose. Each data point represents the doubling time of a single serial-transfer flask estimated from the OD_{660} measured at inoculation and at the time of the next transfer. Occasional transfer of cultures after they had reached stationary phase probably accounts for the unexpectedly high estimated doubling times in some of the cultures. Data from Kuyper et al. 2004 [45]

production rate, were insufficient to allow economically viable industrial application. During these batch cultivations, small amounts of D-xylulose (up to 8 mM) were still excreted into the broth, indicating that evolutionary engineering alone did not fully overcome the metabolic limitations downstream of this metabolite. This result indicates that although evolutionary engineering is a very powerful tool, it has limitations and, in this case, the combination of knowledge-based metabolic engineering (Sect. 5) combined with evolutionary engineering (Sect. 6.1) resulted in more desirable attributes and higher ethanol production rates.

7
Towards Industrial Application: Fermentation Trials with Xylose-Isomerase-Expressing *S. cerevisiae*

7.1
From the Laboratory to the Real World: Strains and Media

Successful expression of XI in *S. cerevisiae* enabled further engineering for high-yield production of ethanol from D-xylose under anaerobic conditions. D-Xylose fermentation rates reported for *S. cerevisiae* strains based on the

Piromyces sp. E2 XI were, in principle, sufficiently high for industrial implementation. However, the studies on these strains that have hitherto been cited in this review were all performed under "academic" conditions. These involved the use of defined synthetic media controlled at pH 5.0 and, perhaps most importantly, the absence of inhibitors that are characteristic for real-life plant biomass hydrolysates [31, 37, 49, 54].

The *S. cerevisiae* strains expressing the *Piromyces* sp. E2 XI are based on the *S. cerevisiae* CEN.PK platform. Interestingly, preliminary tests showed that the parental strain CEN.PK113-7D demonstrated an almost similar performance in industrial-grade molasses compared with industrial bakers' yeast strains. Moreover, deletion of the *GRE3* gene (which encodes a non-specific aldose reductase, [66]) was not detrimental for performance in molasses-based industrial fermentations (W. de Laat, unpublished data). Therefore, trials to test the glucose/xylose fermenting strain *S. cerevisiae* RWB 218 [44] were initiated in both wheat straw and corn stover hydrolysates. Results from these fermentation trials will be briefly discussed below.

7.2 Batch Fermentation of Wheat Straw Hydrolysate

Wheat straw is an abundant lignocellulosic crop residue with potential as a feedstock for ethanol production, especially in Canada and Europe. Wheat straw hydrolysate was therefore selected as one of the fermentation feedstocks for evaluating the fermentation characteristics of *S. cerevisiae* RWB 218 under industrially relevant conditions (W. de Laat, unpublished data). Wheat straw was pretreated using steam explosion (Sunopta, Canada). The pulp thus obtained was then hydrolysed enzymically at pH 5.0 with cellulases and hemicellulases, yielding a hydrolysate that contained 50 g L^{-1} glucose, 20 g L^{-1} D-xylose, 6 g L^{-1} arabinose and 6 g L^{-1} of disaccharides (cellobiose, melibiose, maltose and sucrose, indicated as DP-2 in Table 2). The hydrolysate, which

Table 2 Characteristics of a batch fermentation of the D-xylose fermenting strain RWB 218 on wheat straw hydrolysate with 0.4 g L^{-1} ammoniumphosphate as the only nutrient addition

Time (h)	Total sugar (g L^{-1})	DP2 (g L^{-1})	Glucose (g L^{-1})	Xylose (g L^{-1})	Ethanol (g L^{-1})	Y_{se} ($g_{ethanol}/g_{total\ sugar}$)
0	75.2	6.7	47.8	20.7	0.0	–
20	16.7	5.1	0.4	11.1	30.0	0.47
55	5.8	3.0	0.5	2.3	38.1	0.51

The biomass was inoculated to a starting dry weight of 1.5 g L^{-1}. The sugar fraction indicated by DP2 includes amongst others cellobiose, melibiose, maltose and sucrose

also contained 3 g L^{-1} acetic acid and 0.3 g L^{-1} of lactic acid, was supplemented with 0.4 g L^{-1} of $(NH_4)_2PO_4$ as a combined source of nitrogen and phosphate. Fermentations were run at 32 °C, with an initial pH of 4.8.

When batch cultures on the wheat straw hydrolysates were inoculated with 1.5 g L^{-1} of *S. cerevisiae* RWB 218, most of the available sugars were converted within 55 h (Table 2). The yield of ethanol on the consumed sugars was very high and, towards the end of the fermentation, even approached the theoretical maximum yield of 0.51 g g^{-1}. This very high apparent yield might partially be caused by the additional hydrolysis of some oligosaccharides or by the presence of other sugars that were not identified in the analyses. Xylitol formation was not observed.

Even when a much lower initial biomass concentration of 0.1 g L^{-1} was used, *S. cerevisiae* RWB 218 reached the same degree of conversion in 80 h. Addition of vitamins, trace elements and/or the anaerobic growth factors Tween-80 and ergosterol [2, 3] did not result in a faster fermentation. This demonstrates the modest nutritional requirements of *S. cerevisiae* during fermentation of hydrolysates of lignocellulosic materials, which often contain very low levels of nutrients required for microbial growth.

7.3 Fed-Batch Fermentation of Corn Stover Hydrolysate

Corn stover is another potentially interesting feedstock for ethanol production, especially in the USA. The fermentation characteristics of *S. cerevisiae* RWB 218 on corn stover hydrolysate were tested under industrially relevant fed-batch conditions (W. de Laat, unpublished data). The corn stover pulp obtained after steam explosion (190 °C, 5 min, ENEA, Italy) was diluted with water to 150 g L^{-1} dry matter and subsequently hydrolysed with 10 g cellulase protein (kg hydrolysate dry matter)$^{-1}$ (GC220, Genencor, 96 h at 50 °C). After filtration, the resulting sugar solution contained 40 g L^{-1} glucose, 9 g L^{-1} D-xylose and 4 g L^{-1} acetic acid.

Fermentation experiments were initiated by a 32 h batch phase on molasses medium (containing 100 g L^{-1} sucrose, pH 4.8, 32 °C) in a volume of 200 mL. Subsequently, 455 mL of corn stover hydrolysate was added during a 16 h fed-batch phase. During the fed-batch phase, glucose was almost completely consumed. However, only 40% of the D-xylose fed to the culture was consumed during this phase (Fig. 9). After 16 h, the fed-batch phase was terminated and the culture was allowed to consume accumulated sugars. Conversion was complete after 35 h. At a biomass concentration of 1.0–1.5 g L^{-1}, this corresponded to a D-xylose fermentation rate of 0.5 mmol g^{-1} h^{-1} during this latter phase. The overall ethanol yield on total sugars was 0.46 g g^{-1}, which corresponds to 90% of the theoretical maximum yield on glucose and D-xylose. Consistent with the wheat straw hydrolysate fermentations, xylitol formation was not observed.

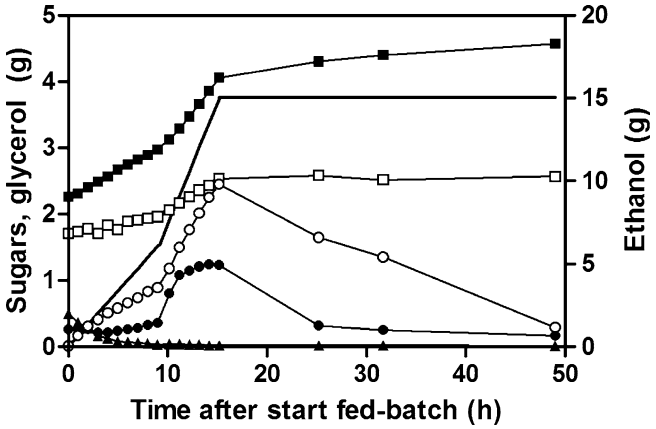

Fig. 9 Profiles of sugars and metabolites in an anaerobic corn stover hydrolysate fed-batch fermentation by *S. cerevisiae* RWB 218. *Symbols* indicate amounts of the following compounds present in the fermenter: glucose (●), D-xylose (○), ethanol (■), glycerol (□), fructose (▲) and cumulative added D-xylose (–). The experiments were initiated by a 32 h batch phase on molasses medium (containing 100 g L^{-1} sucrose, pH 4.8, 32 °C) in a volume of 200 mL. Subsequently, 455 mL of corn stover hydrolysate (containing 40 g L^{-1} glucose, 9 g L^{-1} D-xylose and 4 g L^{-1} acetic acid) was added during a 16 h fed-batch phase

8 Outlook

Functional expression in *S. cerevisiae* of a highly active fungal XI has paved the way for metabolic engineering of this yeast towards high-yield, rapid production of ethanol from D-xylose under fully anaerobic conditions. On theoretical grounds, this XI-based approach is superior to the extensively studied xylose reductase/xylitol dehydrogenase strategy. While considerable experimental proof to substantiate this statement has been obtained under "academic" conditions, a next important challenge is to do the same under industrial conditions. While the first experiments in real-life plant biomass hydrolysates are quite promising, there remains plenty of scope for integrating the D-xylose-fermentation genotype with other metabolic and process-engineering strategies for further increased robustness under process conditions.

In addition to D-xylose, plant biomass hydrolysates contain several other potentially fermentable substrates that cannot be converted by wild-type *S. cerevisiae* strains [69]. While these compounds often represent only a few percent of the potentially fermentable carbon, they can have a decisive impact on economical competitiveness and sustainability of high-yield, high-volume processes such as fuel ethanol production. Functional integration of a highly efficient D-xylose fermentation pathway with pathways that are under devel-

opment (e.g. arabinose [9, 36]) or under consideration (e.g. rhamnose [69]) therefore presents an additional challenge in metabolic engineering for efficient fermentation of plant biomass hydrolysates. We are convinced that creative integration of metabolic engineering, evolutionary engineering and process design can result in rapid breakthroughs in these areas.

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Consolidated Bioprocessing for Bioethanol Production Using *Saccharomyces cerevisiae*

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Abstract Consolidated bioprocessing (CBP) of lignocellulose to bioethanol refers to the combining of the four biological events required for this conversion process (production of saccharolytic enzymes, hydrolysis of the polysaccharides present in pretreated biomass, fermentation of hexose sugars, and fermentation of pentose sugars) in one reactor. CBP is gaining increasing recognition as a potential breakthrough for low-cost biomass processing. Although no natural microorganism exhibits all the features desired for CBP, a number of microorganisms, both bacteria and fungi, possess some of the desirable properties. This review focuses on progress made toward the development of baker's yeast (*Saccharomyces cerevisiae*) for CBP. The current status of saccharolytic enzyme (cellulases and hemicellulases) expression in *S. cerevisiae* to complement its natural fermentative ability is highlighted. Attention is also devoted to the challenges ahead to integrate all required enzymatic activities in an industrial *S. cerevisiae* strain(s) and the need for molecular and selection strategies pursuant to developing a yeast capable of CBP.

Keywords Consolidated bioprocessing · Cellulolytic yeast · One-step bioethanol production · *Saccharomyces cerevisiae*

1 Introduction

Biomass is the only foreseeable renewable feedstock for sustainable production of biofuels. The main technological impediment to more widespread utilization of this resource is the lack of low-cost technologies to overcome the recalcitrance of the cellulosic structure [1]. Four biological events occur during conversion of lignocellulose to ethanol via processes featuring enzymatic hydrolysis: production of saccharolytic enzyme (cellulases and hemicellulases), hydrolysis of the polysaccharides present in pretreated biomass, fermentation of hexose sugars, and fermentation of pentose sugars [2]. The hydrolysis and fermentation steps have been combined in simultaneous saccharification and fermentation (SSF) of hexoses and simultaneous saccharification and cofermentation (SSCF) of both hexoses and pentoses schemes. The ultimate objective would be a one-step "consolidated" bioprocessing (CBP) of lignocellulose to bioethanol, where all four of these steps occur in one reactor and are mediated by a single microorganism or microbial consortium able to ferment pretreated biomass without added saccharolytic enzymes (Fig. 1).

CBP is gaining increasing recognition as a potential breakthrough for low-cost biomass processing. A fourfold reduction in the cost of biological processing and a twofold reduction in the cost of processing overall is projected when a mature CBP process is substituted for an advanced SSCF process featuring cellulase costing US $0.10 per gallon ethanol [3]. The US Department of Energy (DOE) Biomass Program multiyear technical plan states: "Making the leap from technology that can compete in niche or marginal markets for fuels and products also requires expanding the array of possible concepts and strategies for processing biomass. Concepts such as consolidated bioprocessing ... offer new possibilities for leapfrog improvements in yield and cost." [4]. The detailed analysis of mature biomass conversion processes by Greene et al. [5] finds CBP to be responsible for the largest cost reduction of all R&D-driven improvements incorporated into mature technology scenarios featuring projected ethanol selling prices of less than US $0.70 per gallon. Finally, a recent report entitled *Breaking the Biological Barriers to Cellulosic Ethanol* states: "CBP is widely considered to be the ultimate low-cost configuration for cellulose hydrolysis and fermentation." [6].

In addition to being desirable, recent studies of naturally occurring cellulolytic microorganisms provide increasing indications that CBP is feasible. Lu et al. [7] showed that cellulase-specific cellulose hydrolysis rates exhibited by growing cultures of *Clostridium thermocellum* exceed specific rates exhibited by the *Trichoderma reesei* cellulase system by approximately 20-fold, with a substantial part of this difference resulting from "enzyme–microbe synergy" involving enhanced effectiveness of cellulases acting as part of cellulose–enzyme–microbe complexes. Whereas cellulase synthesis

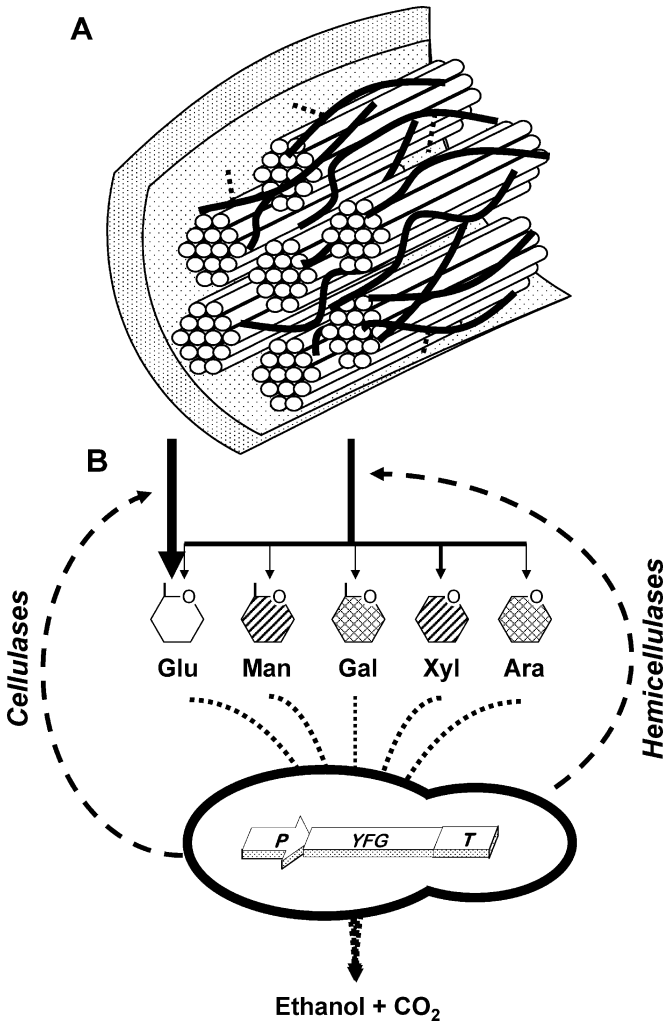


Fig. 1 Graphic illustration of **a** lignocellulose conversion to bioethanol in a single bioreactor by **b** a CBP microorganism. The enzymatic hydrolysis of the cellulose and hemicellulose fractions to fermentable hexoses and pentoses requires the production of both cellulases and hemicellulases (*dashed lines*), and the subsequent conversion of the hexoses and pentoses to ethanol requires the introduction of pentose fermenting pathways. The thickness of the *arrows* imitates the relative amounts of hexoses and pentoses released during hydrolysis of plant material

was thought to be a substantial metabolic burden for anaerobic microbes fermenting cellulose without added saccharolytic enzymes, *C. thermocellum* realizes cellulose-specific bioenergetic benefits that exceed the bioenergetic cost of cellulase synthesis [8]. These and other observations provide guid-

ance with respect to features that may be beneficial in the course of creating recombinant cellulolytic microbes, and also underscore the point that microbial cellulose utilization is differentiable from enzymatic hydrolysis from both fundamental and applied perspectives [1, 3].

Although no natural microorganism exhibits all the features desired for CBP, a number of microorganisms, both bacteria and fungi, possess some of the desirable properties. These microorganisms can broadly be divided into two groups: (1) native cellulolytic microorganisms that possess superior saccharolytic capabilities, but not necessarily product formation, and (2) recombinant cellulolytic microorganisms that naturally give high product yields, but into which saccharolytic systems need to be engineered [1, 9]. Examples of native cellulolytic microorganisms under consideration include anaerobic bacteria with highly efficient complexed saccharolytic systems, such as mesophilic and thermophilic *Clostridium* species [9, 10], and fungi that naturally produce a large repertoire of saccharolytic enzymes, such as *Fusarium oxysporum* [11] and a *Trichoderma* species [12]. However, the anaerobic bacteria produce a variety of fermentation products, limiting the ethanol yield, whereas the filamentous fungi are slow cellulose degraders and give low yields of ethanol [13]. Candidates considered as potential recombinant cellulolytic microorganisms into which saccharolytic systems have been engineered include the bacteria *Zymomonas mobilis* [14, 15], *Escherichia coli* [16, 17] and *Klebsiella oxytoca* [18, 19], and the yeast *Saccharomyces cerevisiae* and xylose-fermenting yeasts *Pachysolen tannophilus* [20], *Pichia stipitis*, and *Candida shehatae* [21].

While both native and recombinant cellulolytic microorganisms merit investigation, this review will focus on the well-known ethanol producing yeast *S. cerevisiae*, which has a long commercial history as microorganism of choice for beer, wine, baker's yeast, and commercial ethanol production. In particular, we address recent progress in heterologous cellulase expression pursuant to development of recombinant cellulose-fermenting yeast strains [22–25].

2
Baker's Yeast (*S. cerevisiae*) as a CBP Host

Despite the status of *S. cerevisiae* as a proven industrial microorganism, conferring the ability to rapidly convert pretreated cellulose to ethanol is a daunting proposition. Apart from essential traits, such as high ethanol yield and productivity, industrial strains need to concurrently ferment both hexoses and pentoses under robust industrial conditions that require minimum nutrient requirements and high ethanol and inhibitor tolerance. In addition, these strains also have to hydrolyze cellulosics and thus need to produce and secrete heterologous hydrolases at high enough levels to sustain hydrolysis and fermentation of cellulosics to ethanol (Table 1). Before contemplating these

challenges, it is worth considering the evolutionary development of *S. cerevisiae* as microorganism of choice for ethanol production.

Through the serendipitous duplication of its entire genome about 100 million years ago, followed by the further duplication of the alcohol dehydrogenase (*ADH*) genes < 80 million years ago, the *S. cerevisiae sensu stricto* yeast (comprised of 14 *Saccharomyces* species) adapted the "make–accumulate–consumption strategy" for ethanol production [27, 28]. This ability is largely attributed to its overriding glucose repression circuit that suppresses respiration of glucose and other C_6 sugars above 20–40 mM threshold concentrations in the presence of oxygen, a characteristic feature of Crabtree-positive yeasts [29]. This strategy provided the ancestor of *S. cerevisiae* with an advantage over its competitors because high ethanol levels (concentrations exceeding 4% v/v) are toxic to most other microbes. Once *S. cerevisiae* has colonized a niche by producing ethanol levels often exceeding 10% v/v from readily available hexoses, the produced ethanol is reconsumed if oxygen is present. These yeasts therefore developed two distinct alcohol dehydrogenase enzymes through the duplication of the *ADH* genes for the production and

Table 1 Features required from *S. cerevisiae* as successful CBP microorganism (modified from [2, 26])

Required traits	Suitability of currently available strains of *S. cerevisiae*
Essential traits:	
Ability to ferment hexoses and pentoses	Only hexoses by native industrial strains. Partial pentose utilization has been engineered in some laboratory and industrial strains
High ethanol yield and productivity	Most industrial strains
High ethanol and inhibitor tolerance	Most industrial strains
General robustness for industrial processes	Most industrial strains
High level of heterologous gene expression	Primarily multicopy expression in laboratory strains
High levels of secreted heterologous proteins	Laboratory and some industrial strains
Desirable traits:	
Concurrent fermentation of sugars	Manipulated laboratory and some industrial strains (maltose and glucose utilization)
GRAS status	Most laboratory and industrial strains
Recyclable	Most industrial strains
Minimum nutrient supplementation	Some industrial strains, particularly wine strains
Amendable to DNA manipulation, particularly DNA transformation	Laboratory and some industrial strains

subsequent utilization of the ethanol: Adh1 that is constitutively produced and is required for ethanol production, and Adh2 that is only induced in the absence of C_6 sugars and is necessary for ethanol consumption.

Regardless of the processes used for biomass hydrolysis, CBP-enabling microorganisms may encounter a variety of toxic compounds derived from biomass pretreatment and hydrolysis that could inhibit microbial growth, particularly in the presence of ethanol [30]. However, industrial strains of *S. cerevisiae* have been adapted to handle stress conditions, such as high ethanol and sugar concentrations (hence osmotolerance), in fermenting simple hexoses (glucose, fructose, galactose, and mannose) or disaccharide (sucrose and maltose) streams. It also has a natural hardiness against inhibitors and has the ability to grow at low oxygen levels. These features confer to *S. cerevisiae* a general robustness in industrial process conditions [28]. *S. cerevisiae* has proven itself as a robust ethanol producer in traditional large-scale processes, and therefore presents itself as platform organism for plant biomass conversion to products such as ethanol [2].

3 Engineering *S. cerevisiae* for Sugar Fermentation

The composition of plant biomass can vary substantially but all plant biomass is composed of four major polymeric compounds: cellulose (~ 33–51%), hemicellulose (~ 19–34%), pectin (~ 2–20%), and lignin (~ 20–30%) [16, 31]. Upon hydrolysis, plant biomass yields a variety of fermentable hexoses (glucose, 36–50%; mannose, 0.3–12%; galactose, 0.1–2.4%) and pentoses (xylose, 3.4–23%; arabinose, 1.1–4.5%). *S. cerevisiae* can ferment all the hexoses to ethanol, but not the pentoses, which can be a significant portion (25%) of, for example, sugarcane bagasse, a preferred feedstock for bioethanol production [32]. More than three decades of research have been devoted to the development of yeast for efficient xylose fermentation, initiating with the search for alternative yeasts, such as *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehatae*, and in the last two decades focusing on the genetic engineering of *S. cerevisiae* to utilize xylose and arabinose [33]. Please refer to the chapters by Hahn-Hägerdal et al. and van Maris et al. (in this volume) for detailed reviews of this topic [34, 35].

After several unsuccessful attempts to produce a functional bacterial xylose isomerase in *S. cerevisiae*, many groups focused for the last decade on efficient expression of fungal xylose utilizing genes and manipulating the pentose phosphate pathway to enhance xylose utilization and fermentation in *S. cerevisiae* [36]. These research efforts ensured steady but slow progress toward the development of xylose utilizing *S. cerevisiae* strains, and it was recent successes with the production of a functional *Piromyces* sp. xylose isomerase in recombinant *S. cerevisiae* that opened the way for efficient xylose

fermentation by *S. cerevisiae* at low oxygen levels [37, 38]. The main advantage of this approach is the circumvention of the redox imbalance problem created by expressing xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) fungal genes in *S. cerevisiae*. Recognizing the need for *S. cerevisiae* to ferment all hexoses and pentoses produced during enzymatic hydrolysis of wood, both laboratory and industrial *S. cerevisiae* platform strains have been developed that can utilize xylose [39] and later co-utilize xylose and arabinose [40].

Apart from monosaccharides, *S. cerevisiae* can utilize the disaccharides sucrose and maltose, and some *Saccharomyces* strains can also utilize melibiose and the trisaccharides maltotriose and raffinose [41, 42]. However, the major end products of cellulose hydrolysis are cellobiose and cellooligosaccharides, which cannot be utilized by *S. cerevisiae*. The heterologous expression of four different β-glucosidases in *S. cerevisiae* was evaluated and the β-glucosidase (BGL1) of *Saccharomycopsis fibuligera* was found to be produced at the highest activity levels [43]. Expression of the β-glucosidases encoding genes of *Candida wickerhamii*, *Aspergillus kawachii*, and *T. reesei* yielded activities at least one order of magnitude lower than that of *Saccharomycopsis fibuligera*. It was shown that multicopy expression of the *S. fibuligera BGL1* gene could enable growth on cellobiose as sole carbon source at a rate equivalent to that found on glucose [43, 44]. Recently, a *S. cerevisiae* strain was developed that could utilize both xylose and cellobiose [45].

Even with the introduction of pentose and cellobiose utilizing genes, *S. cerevisiae* strains preferentially utilize glucose before the other mono- and disaccharides. Deregulation of the strong glucose repression effect in *S. cerevisiae* would be required to allow cometabolism of sugars derived from plant biomass for high ethanol productivity. Disrupting both the *MIG1* and *MIG2* genes allowed cometabolism of glucose and sucrose [46], and similar strategies could be used to allow co-utilization of sugars released from plant biomass. Furthermore, simultaneous co-transport of glucose and xylose must be facilitated as the delayed utilization of xylose (in a recombinant xylose utilizing strain) is in part an effect of competition for the same glucose transporters in the absence of a xylose-specific transporter [37].

4 Expression of Cellulases in *S. cerevisiae*

The major requirement for *S. cerevisiae* as CBP yeast would be sufficient expression and production of extracellular saccharolytic enzymes [1]. In the context of creating such a CBP yeast, the first question researchers would like to answer is, "How much saccharolytic enzyme, particularly cellulase expression, is enough to enable CBP conversion of plant material to ethanol, and is that amount feasible in *S. cerevisiae*?" The obvious follow-up question is,

"How do we accomplish those levels of expression?" Recent analyses [9, 44] have approached the first question from a kinetic standpoint, balancing the demand for soluble products of cellulose hydrolysis (glucose) by cells with the production of those products by cellulase systems. Demand is a simple function of the growth rate and the cell yield: $\mu/Y_{X/S}$ = g glucose/g cells/h, while supply is just the cell-specific cellulase activity: g glucose/g cells/h. These relationships can be used to calculate a number of useful quantities, including the percentage of total cell protein that needs to be cellulase to achieve a particular growth rate on a cellulosic substrate. The relative levels of individual cellulase component expression can be calculated based on the known ratios of those components in native systems.

In the last two decades there have been several reports on the expression of cellulases in *S. cerevisiae*. Table 2 summarizes some of the results found to date. Most reports regarding the expression of cellulases and hemicellulases in yeast employed strong glycolytic (or other constitutively expressed) promoters to drive expression of the heterologous gene(s). Although the choice of promoter and leader sequences will undoubtedly have a great influence on expression levels attained, there are not enough data in the literature to suggest any general trends as to what are the best promoter and leader sequences to use when expressing cellulases and hemicellulases. Several researchers have sought to produce cellulases in an organism that would not yield interfering activities so as to gain insight into the mechanism of the original cellulolytic enzyme [99], whereas others have sought to enable the yeast to hydrolyze nonnative cellulolytic substrates [43, 59, 78, 102]. Although most of the cellulases that have been successfully produced in *S. cerevisiae* were of fungal origin, there are reports of successful bacterial cellulase production [76, 82].

Full enzymatic hydrolysis of crystalline cellulose requires three major types of enzymatic activity: (1) endoglucanases (1,4-β-D-glucan 4-glucanohydrolases; EC 3.2.1.4); (2) exoglucanases, including D-cellodextrinases (1,4-β-D-glucan glucanohydrolases; EC 3.2.1.74) and cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases; EC 3.2.1.91); and (3) β-glucosidases (β-glucoside glucohydrolases; EC 3.2.1.21) (Fig. 2a). Cellobiohydrolase (CBH) enzymes are key components for fungal cellulase systems, and their functional secretion is critical for allowing CBP. For example, CBHs make up ~ 80% of the total mass for the *T. reesei* system, and CBH1 plays a particularly important role, making up 60% of the total mass [103]. CBHs have been successfully produced and secreted by *S. cerevisiae* and were tested for activity on a variety of substrates ranging from small synthetic molecules to amorphous and crystalline forms of cellulose (Table 1). Some reports have shown decreased specific activity on certain substrates, presumably as a result of hyperglycosylation [47, 48]. However, in a recent study it was shown that the specific activity of a glycosylated heterologous CBH1 did not differ significantly from that of the native enzyme produced by *T. reesei* [49].

Table 2 Cellulase components expressed in *S. cerevisiae*

Organism & gene/enzyme	Titer (mg/L)	% cell protein	Substrate(s) activity was detected against (values indicate activity measured per L culture broth)	Specific activity (U/mg)	Refs.
CBHI					
Trichoderma reesei CBHI	2	1.5	MUC, AC	NR	[47]
	5	*0.123*	MUL, BMCC	0.26 (on BMCC)	[48]
	0.22	0.006	0.06 U/L (PASC), 0.06 U/L (BMCC)	0.22 (on PASC)	[49]
Aspergillus niger CBHB	NR	NR	0.035 U/L (AC), 0.03 U/L (BMCC)	NR	[49]
Phanerochaete chrysosporium CBH1–4	NR	NR	12 U/L, ~ 3.3 U/g DCW (BBG), 10 U/g DCW	NR	[50]
	NR	NR	(PNPC) 22 U/g DCW (AC)	NR	[51]
	NR	NR	18 U/g DCW (PNPC)	NR	[52]
	NR	NR	0.035 U/L (AC), 0.03 U/L (BMCC)	NR	[49]
Penicillium janthinellum CBH1	NR	NR	MUL	NR	[53]
Thermoascus aurantiacus CBHI	0.1	*0.002*	Avicel, AC, PNPC, PNPL	0.03, 0.04, 0.11, 0.29 (same order as activity)	[54]
Aspergillus aculeatus CBHI	7	*0.173*	Avicel, MUL	0.007 (Avicel)	[55]
Cellulomonas fimi cex	2.5	*0.03*	8 U/L, *~ 1.0 U/g DCW* (PNPC)	3 (on PNPC)	[56]
Cellulomonas fimi Exg (*cex*)	12.5	NR	45 U/L (PNPC)	3.6 (PNPC)	[57]
CBHII					
Trichoderma reesei CBHII	100	*2.6*	BBG, AC	NR	[47]
	10	*0.33*	24 U/L, 3 U/gDCW (AC)	0.7 (on AC)	[58]
	NR	NR	0.15 U/g DCW (AC)	NR	[59]
	NR	NR	0.14 U/L (AC), 0.09 U/L (BMCC)	NR	[49]
Agaricus bisporus CEL3	NR	NR	0.06 U/g DCW (AC), 0.033 U/g DCW (CC), 0.008 U/g DCW (BBG)	NR	[60]
EG					
Trichoderma reesei EGI	NR	0.5	CMC	15 (on CMC)	[61]
	10	*0.09*	MUC	NR	[62]
	0.66	0.25	BBG, lichenan, CMC, HEC, MUL, MUC	NR	[62]

Table 2 (continued)

Organism & gene/enzyme	Titer (mg/L)	% cell protein	Substrate(s) activity was detected against (values indicate activity measured per L culture broth)	Specific activity (U/mg)	Refs.
	5	*0.12*	72 U/g DCW (HEC)	60 (on HEC)	[63]
Trichoderma reesei EGII	NR	NR	3.64 U/g DCW (AC)	NR	[64]
Trichoderma reesei EGIII	NR	NR	BBG, lichenan, CMC, HEC	NR	[62]
Trichoderma reesei EGV	NR	NR	BBG, HEC	NR	[65]
Trichoderma reesei EGIV	NR	NR	BBG, AC, CMC	NR	[66]
Aspergillus niger eng1	*2.8*	*0.07*	574 U/L (CMC)	204 (on CMC)	[67]
Aspergillus aculeatus CMCase	NR	NR	0.5 U/L, ~ *0.06 U/g DCW* (CMC)	NR	[68]
Aspergillus aculeatus F1-CMCase	NR	NR	60 U/L (CMC)	NR	[69]
	NR	NR	CMC, IOSC	11 (on IOSC)	[70]
Cellulomonas fimi Eng (*cenA*)	*13*	NR	293 U/L (low viscosity CMC)	NR	[57]
Cellulomonas fimi CMCase	NR	NR	1600 U/L (CMC)	NR	[71]
Thermoascus aurantiacus eg1	1.5	*0.04*	107 U/mg total protein, ~ 535 U/L (CMC)	336 (on CMC)	[54]
Cryptococcus flavus CMC1	NR	NR	12 500 U/L, ~ 1, 390 U/g DCW (CMC)	NR	[72]
Clostridium thermocellum celA	NR	NR	280 U/L, 24 U/g DCW (CMC)	NR	[73]
Clostridium thermocellum EG (celA)	NR	NR	2000 U/g total protein (CMC)	NR	[74]
Butyrivibrio fibrisolvens END1	NR	NR	*22 U/g DCW* (AC)	NR	[51]
	NR	NR	*4.3 U/g DCW* (BBG)	NR	[52]
	NR	NR	1100 U/L, ~ *306 U/g DCW* (BBG)	NR	[50]
	NR	NR	3.460 U/L (CMC)	NR	[75]
	NR	NR	BBG	NR	[76]
Scopulariopsis brevicaulis EGI	NR	NR	109 U/L, ~ *12.1 U/g DCW* (CMC)	NR	[77]
Bacillus circulans Endo/ Exo bifunctional enzyme	NR	NR	300 U/L, ~ *33 U/g DCW* (CMC)	NR	[78]
Trichoderma longibrachiatum egl1	NR	NR	azo-BBG	NR	[79]
Bacillus subtilis endo-beta-1,3- 1,4-glucanase	NR	NR	33 600 000 U/L (BBG)	NR	[80]
	NR	NR	2.3 U/g total protein (BBG)	NR	[81]
Bacillus subtilis BEG1	NR	NR	BBG	NR	[76]

Table 2 (continued)

Organism & gene/enzyme	Titer (mg/L)	% cell protein	Substrate(s) activity was detected against (values indicate activity measured per L culture broth)	Specific activity (U/mg)	Refs.
Bacillus subtilis EG	NR	NR	1650 U/L (CMC)	NR	[82]
Thermoanaerobacter cellulolyticus Endoglucanase	NR	NR	26 U/L (CMC)	NR	[83]
Cellulomonas biazotea EG	NR	NR	167 U/L (CMC)	NR	[84]
Acidothermus cellulolyticus E1 beta-1,4-endo-glucanase	NR	NR	1 700 000 U/g total protein (MUC)	NR	[85]
Trichoderma longibrachiatum EG	NR	NR	azo-BBG	NR	[86]
Barley 1,3- 1,4-beta-glucanase	NR	NR	BBG	NR	[87]
BGL					
Kluyveromyces fragilis BGL	NR	15	PNPG, C2	64.4 (on PNPG)	[88]
Aspergillus aculeatus BGLI	NR	NR	BGL1 = 21.3 U/g DCW (PNPG)	NR	[64]
	1	*0.02*	IOSC	25 (on IOSC)	[55]
Saccharomycopsis fibuligera BGLI	*10*	*0.25*	PNPG, C2, C3, C4	43.3, 20.1, 26.2, 27.1 (as for activity)	[89]
Saccharomycopsis fibuligera BGLII	18.9	*0.47*	PNPG, C2, C3, C4	168, 0.8, 1.7, 1.5 (as for activity)	[89]
	NR	NR	115 000 U/L, ~ 12 800 U/g DCW (PNPG)	NR	[72]
	NR	NR	112 U/g DCW (PNPG)	NR	[43]
	NR	NR	19 U/g DCW (PNPG)	NR	[43]
Bacillus circulans BGL	NR	NR	450 U/L, ~ *50 U/g DCW* (PNPG)	NR	[78]
Endomyces fibuliger BGLI	NR	NR	2023 U/g DCW (C2)	NR	[51]
	NR	NR	172 U/g DCW (C2)	NR	[52]
Ruminococcus flavefaciens CEL1	NR	NR	5.46 U/g DCW (PNPC)	NR	[51]
Candida wickerhamii bglB	NR	NR	0.298 U/L (PNPG)	NR	[90]
Bacillus polymyxa bglA	NR	NR	2.3 U/mg total protein	NR	[91]

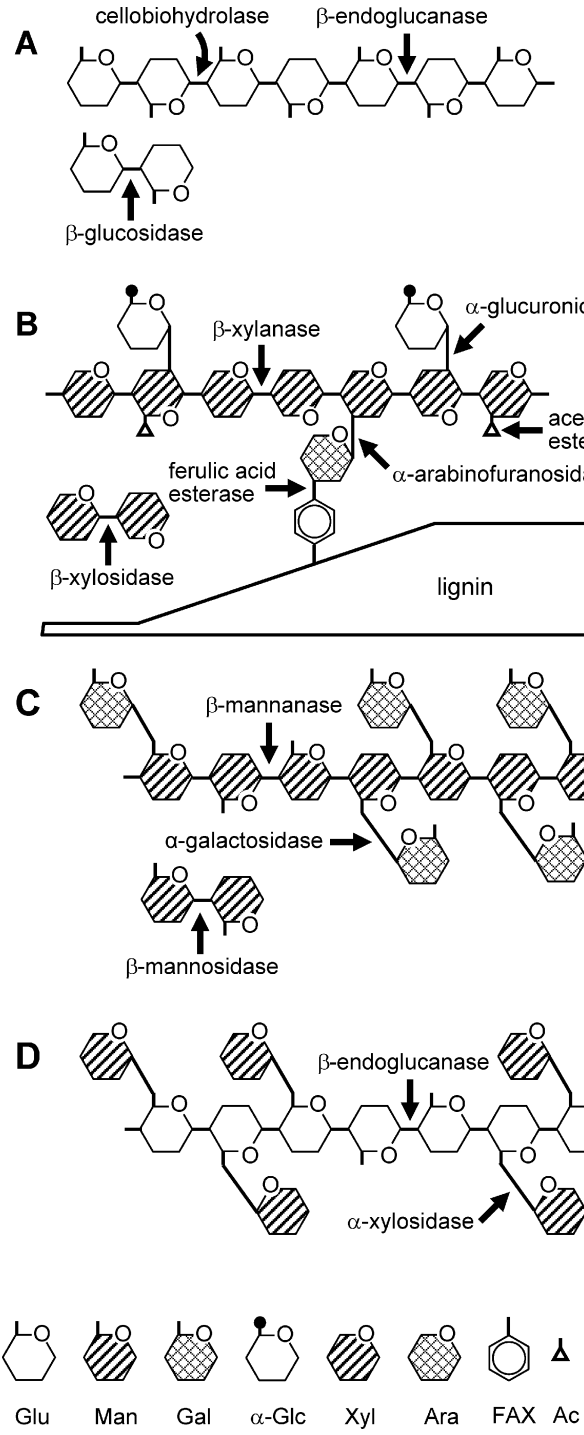
Table 2 (continued)

Organism & gene/enzyme	Titer (mg/L)	% cell protein	Substrate(s) activity was detected against (values indicate activity measured per L culture broth)	Specific activity (U/mg)	Refs.
Candida molischiana BGLN	NR	NR	48 U/L (PNPG)	NR	[92]
Cellulomonas biazotea Beta-glucosidase	NR	NR	2000 U/L (C2)	NR	[93]
Trichoderma reesei bgl 1	NR	NR	PNPG	NR	[94]
Bacillus circulans BGL	NR	NR	64 U/g DCW (PNPG)	NR	[95]
Candida pelliculosa BGL	NR	NR	17 500 U/L, $\sim$ *1950 U/g DCW* (PNPG)	NR	[96]
Aspergillus niger BGL	NR	NR	Xglu	NR	[97]
Kluyveromyces fragilis BGL	NR	NR	1700 U/g total protein (C2)	NR	[98]

U = micromole substrate released/min, NR = not reported; italics indicate calculation based on assumptions (0.45 g DCW/g glucose, 0.45 g protein/g DCW, 1.3×10^7 cells/mg DCW, 1 OD(600) = 0.57 g DCW/L).
CBH = cellobiohydrolase, EG = endoglucanase, BGL = beta-glucosidase, AC = amorphous cellulose, BMCC = bacterial microcrystalline cellulose, BBG = barley beta-glucan, CC = crystalline cellulose, IOSC = insoluble cellooligosaccharides, C2 = cellobiose, C3 = cellotriose, C4 = cellotetraose, PNPC = *p*-nitrophenol cellobioside, PNPL = *p*-nitrophenol lactoside, MUC = methylumbelliferyl cellobioside, MUL = methylumbelliferyl lactoside, Xglu = 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside

Reports of CBH production in yeast have also shown that a relatively low titer of secreted cellulase is found, although the range of reported values is quite large—0.002 to 1.5% of total cell protein. Coupled with the low specific activity of CBHs, CBH expression has been identified as a limiting factor for CBP using yeast [9]. However, in a recent report the amount of CBH1 required to enable growth on crystalline cellulose was determined and was found to be, in terms of total cellular protein, within the capacity of heterologous protein production in *S. cerevisiae*, i.e., between 1 and 10% of total cell protein [49, 104–106].

Fig. 2 Illustration of the complexity of cellulose and hemicellulose and the enzymes involved in their degradation. Cellulose (**a**) and hemicellulose structures for arabinoxylan (**b**), galactomannan (**c**) , and xyloglucan (**d**) depicting the different side chains present. Hexoses are distinguished from pentoses by the presence of a protruding line from the cyclic hexagon (pyranose ring), depicting the CH_2OH group. Hydrolase enzymes and the bonds targeted for cleavage in the four polysaccharide structures are indicated by *arrows* [100, 101] ►


A
cellobiohydrolase
β-endoglucanase
β-glucosidase
B
β-xylanase
α-glucuronidase
acetylxylan esterase
ferulic acid esterase
α-arabinofuranosidase
β-xylosidase
lignin
C
β-mannanase
α-galactosidase
β-mannosidase
D
β-endoglucanase
α-xylosidase
Glu
Man
Gal
α-Glc
Xyl
Ara
FAX
Ac

Fungal and bacterial endoglucanase (EG) production in *S. cerevisiae* have been by and large more successful than CBH production (Table 2). This is not surprising considering that EG enzymes usually have specific activities 2 to 3 orders of magnitude higher on synthetic and amorphous cellulose substrates, such as phosphoric acid swollen cellulose (PASC) and carboxymethyl cellulose (CMC), in comparison to CBHs. It is thus easier to measure the presence of even small amounts of heterologous EG compared to CBHs. Although secreted heterologous EGs were usually reported to be hyperglycosylated, this did not necessarily influence their specific activity negatively [61]. Sufficiency analysis shows that, assuming that a *T. reesei* system is reconstructed, even if all of the non-CBH cellulase system components were EG, it would still only need to make up $\sim 0.3\%$ of cell protein, well within the range of possibility for a *S. cerevisiae* secretion system. The successful expression of β-glucosidases in *S. cerevisiae* at sufficient levels to sustain growth on cellobiose as sole carbon source at a rate comparable to glucose suggests that BGL expression will not be a limiting step in cellulase system reconstruction [43, 44].

A number of studies have expressed multiple cellulase enzymes in attempts to recreate a fully cellulolytic, fermentative system [45, 59, 64, 78, 102]. Van Rensburg et al. [51] constructed a yeast capable of hydrolyzing numerous cellulosic substrates and growing on cellobiose, while Cho et al. [78] showed that decreased loadings of cellulase could be used for SSF experiments with their strain expressing a BGL enzyme and an enzyme with dual exo/endocellulase activity. Fujita et al. [59, 64] reported coexpression and surface display of cellulases in *S. cerevisiae*, and a recombinant strain displaying the *T. reesei* endoglucanase II, cellobiohydrolase II, and the *Aspergillus aculeatus* β-glucosidase 1 was built. High cell density suspensions of this strain were able to directly convert PASC to ethanol with a yield of approximately $3\,g\,L^{-1}$ from $10\,g\,L^{-1}$ within 40 h [59]. Den Haan et al. [102] reported growth on and direct conversion of PASC to ethanol by a *S. cerevisiae* strain coexpressing the *T. reesei EG1* and the *Saccharomycopsis fibuligera BGL1* (Fig. 3). Anaerobic growth ($0.03\,h^{-1}$) up to $0.27\,g\,L^{-1}$ dry cell weight was observed with this strain on medium containing $10\,g\,L^{-1}$ PASC as sole carbohydrate source with concomitant ethanol production of up to $1.0\,g\,L^{-1}$. As an exocellulase activity such as CBH is required for the successful hydrolysis of crystalline cellulose, it is postulated that the addition of successful, high-level expression of CBH to this strain will enable CBP of crystalline cellulose to ethanol.

5
Expression of Hemicellulases in *S. cerevisiae*

Hemicellulose refers to a number of heterogeneous structures, such as (arabino)xylan, galacto(gluco)mannan, and xyloglucan [107, 108]. These chem-

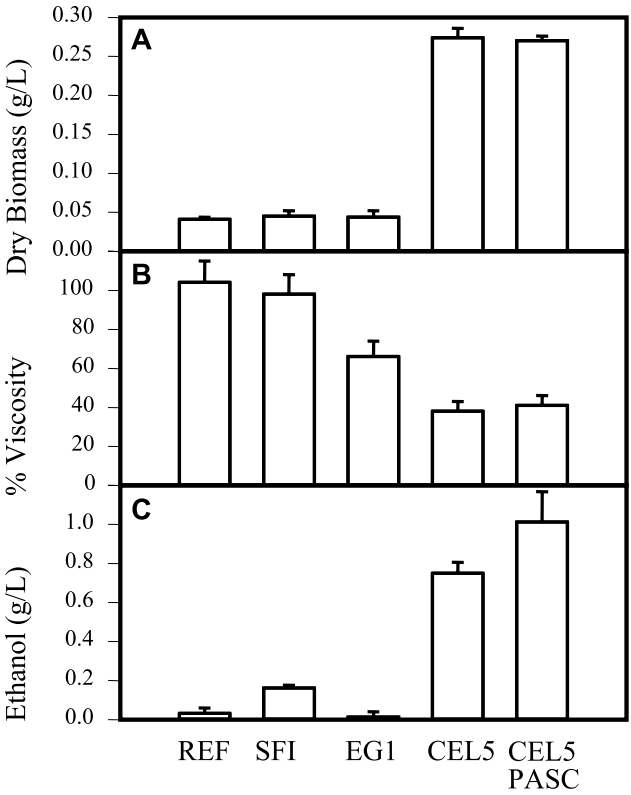


Fig. 3 Demonstration of amorphous cellulose conversion to bioethanol by recombinant *S. cerevisiae* Y294 strains. **a** Biomass production after 240 h in YP-PASC (10 g L^{-1} PASC) medium showing growth of the *BGL1*, *EG1* coexpressing strain Y294[CEL5] pregrown in YPD and Y294[CEL5] (PASC) strain pregrown in YP-PASC. A concomitant drop in **b** viscosity due to PASC degradation by *EG1* expressing strains and **c** ethanol production by the *BGL1*, *EG1* coexpressing strain is also shown [102]. REF = reference strain *S. cerevisiae* Y294 with no heterologous expression cassette; SFI = *S. cerevisiae* Y294 expressing the *Saccharomycopsis fibuligera BGL1* gene; EG1 = *S. cerevisiae* Y294 expressing the *T. reesei EG1* gene; CEL5 = *S. cerevisiae* Y294 coexpressing the *Saccharomycopsis fibuligera BGL1* and *T. reesei EG1* genes

ically diverse polymers are linked together through covalent and hydrogen bonds, as well as being intertwined. Although many pretreatments remove variable amounts of hemicellulosics, it remains imperative from an economic perspective that sugars contained in the hemicellulose fraction of lignocellulose are also converted to ethanol [9, 36]. The hydrolysis of xylans, the second most abundant sugar polymer in nature, and utilization of xylose, its main constituent, are therefore crucial in a viable CBP configuration.

The cross-linked and partially crystalline nature of the matrix offer great resistance to enzymatic hydrolysis. Furthermore, as the structure of xylan

is variable, involving not only linear β-1,4-linked chains of xylose, but also branched heteropolysaccharides; its degradation requires the synergistic action of a range of different enzymes [109–111]. To date, hydrolytic enzymes for the cleavage of almost all chemical bonds found in plant structures have been identified in microbial sources (Fig. 2). Hemicellulases, such as β-xylanases and β-mannanases, have drawn attention as they can help facilitate industrial processes such as bleaching in the pulp and paper industry.

There have been several reports of the successful expression of hemicellulases in *S. cerevisiae* (Table 3). Xylan hydrolyzing enzymes such as β-xylanase, β-xylosidase, and auxiliary enzymes such as α-glucuronidase and α-arabinofuranosidase have all been produced successfully in yeast [114, 120, 121, 128]. The heterologous production of mannanase and α-xylosidase, active against mannan and xyloglucan, respectively, was also reported [107, 124, 127].

Degradation of the β-1,4-xylan backbone requires the action of endo-β-1,4-xylanases (β-1,4-D-xylan xylanohydrolase EC 3.2.1.8) and β-xylosidases (β-1,4-D-xylan xylohydrolase EC 3.2.1.37) (Fig. 2a). cDNA copies of β-xylanase encoding genes cloned from the yeasts *Cryptococcus albidus* and *Aureobasidium pullulans* and the filamentous fungi *A. niger*, *A. kawachii*, and *T. reesei* have been expressed in *S. cerevisiae* under transcriptional control of glycolytic promoters [112–114, 116, 129]. Secreted active enzyme could be assayed in all cases. The cDNA copy of the *T. reesei* β-xylanase II (*xyn2*) was expressed in *S. cerevisiae* under the transcriptional control of the *PGK1* and *ADH2* promoters [114]. Efficient secretion of the heterologous β-xylanase was achieved by the native *T. reesei xyn2* secretion signal and the recombinant β-xylanase was 27 kDa in size. The molecular mass of the mature protein in *T. reesei* was found to be 21 kDa, with virtually no glycosylation. The extra molecular weight of the heterologous Xyn2 protein secreted by *S. cerevisiae* was shown to be the result of hyperglycosylation of the protein; however, the extra sugar moieties did not influence the activity of the enzyme.

The β-xylosidase encoding gene of *Bacillus pumilus* (*xynB*) was cloned from a genomic DNA library and expressed in *S. cerevisiae* under transcriptional control of the *S. cerevisiae ADH2* promoter [118]. To promote secretion of the enzyme, the gene was fused in reading frame with the *S. cerevisiae* mating pheromone α-factor (MFα1) secretion signal. Biologically active β-xylosidase was obtained, but remained mostly cell associated. When this fusion gene and *T. reesei xyn2* were coexpressed in *S. cerevisiae* under transcriptional control of the *S. cerevisiae ADH2* promoter, a 25% increase in the amount of reducing sugars released from birchwood xylan was obtained, compared to strains expressing β-xylanase alone. However, no xylose was produced from birchwood xylan, presumably due to very low β-xylosidase activity. A cDNA copy of the *A. niger* β-xylosidase encoding gene was subsequently cloned [115]. The mature protein encoding region was fused in reading frame with the *S. cerevisiae* MFα1 secretion signal to ensure secretion

Table 3 Hemicellulase components expressed in *S cerevisiae*

Organism & gene/enzyme	Titer (mg/L)	% cell protein	Substrate(s) activity was detected against (values indicate activity measured per L culture broth)	Specific activity (U/mg)	Refs.
Xylan degradation: ***β-Xylanase***					
Cryptococcus albidus XLN	NR	NR	1.3 U/mg protein (xylan)	NR	[112]
Aspergillus kawachii xynC	NR	NR	18 000 U/L (BG-xylan)	NR	[113]
Trichoderma reesei xyn2	NR	NR	72 000 U/L (BG-xylan)	NR	[114]
	NR	NR	51 600 U/L (BG-xylan) – coexpression	NR	[115]
Aureobasidium pullulans xynA	~ 13.1 mg/L	*1.6%*	26 200 U/L (BG-xylan)	2000 U/mg (native)	[116]
β-Xylosidase					
Trichoderma reesei bxl1	NR	NR	19.6 U/L (PNP-β-X), xylan, PNP-β-G, xylobiose	NR	[117]
Bacillus pumilus xynB	NR	NR	5.4 U/L (PNP-β-X)	NR	[118]
Aspergillus niger xlnD	NR	NR	318 U/L (PNP-β-X), xylobiose, xylotriose	NR	[115]
Aspergillus oryzae xylA	NR	NR	316 U/g DCW (PNP-β-X)	NR	[119]
α-Glucuronidase					
Aureobasidium pullulans aguA	0.1 mg/L	*0.013%*	5 U/L (ABIU, ATRU, ATEU)	135 U/mg (ATEU)	[120]
α-L-Arabinofuranosidase					
Aspergillus niger abfB	NR	NR	1400 U/L (PNPA)	NR	[121]
	117.3 mg/L	*5.2%*	678 U/L (PNPA)	5.78 U/mg	[122]
	NR	NR	25.7 U/L (PNPA)	NR	[123]
Trichoderma reesei abf1	NR	NR	205 U/L (PNPA), arabinoxylan	NR	[117]
Mannan degradation: ***β-Mannanase***					
Trichoderma reesei man1	150 μg/L	NR	132 U/L (LBG)	NR	[105]
Aspergillus aculeatus man1	118 mg/L	*5.04%*	31 260 U/L (LBG), INM	82 U/mg	[124]
Orpinomyces PC-2 manA	6 mg/L	*0.74%*	1150 U/L (LBG), INM	179 U/mg	[106]

Table 3 (continued)

Organism & gene/enzyme	Titer (mg/L)	% cell protein	Substrate(s) activity was detected against (values indicate activity measured per L culture broth)	Specific activity (U/mg)	Refs.
αGalactosidase					
Trichoderma reesei agl1	NR	NR	516 U/L (PNPαGal) PNPA, raffinose, melibiose, LBG, PGGM	NR	[125]
Trichoderma reesei agl2	NR	NR	20.8 U/L (PNPαGal) LBG, PGGM	NR	[125]
Trichoderma reesei agl3	NR	NR	1.32 U/L (PNPαGal) LBG, PGGM	NR	[125]
Xyloglucan degradation: ***Endo-β-1,4-glucanase***					
Aspergillus aculeatus	NR	NR	AZCL XG	NR	[126]
α-Xylosidase					
Arabidopsis thaliana AtXYL1	NR	NR	0.0006 U/g wet weight (EG digested xyloglucan)	NR	[127]

U = micromole substrate released/min, DCW = dry cell weight, NR = not reported; substrate used for activity determination is given in parentheses; italics indicate calculation based on assumptions (0.45 g DCW/g glucose, 0.45 g protein/g DCW, 1.3×10^7 cells/g DCW, 1 OD(600) = 0.57 g DCW/L).
BG-xylan = birchwood glucuronoxylan, PNP-β-X = *p*-nitrophenyl-β-D-xylopyranoside, AZCL-XG = azurine-dyed cross-linked xyloglucan, ABIU = aldobiouronic acid, ATRU = aldotriouronic acid, ATEU = aldotetraouronic acid, PNPA = *p*-nitrophenyl-α-L-arabinofuranoside, LBG = locust bean gum, INM = ivory nut mannan, PGGM = pinewood galactoglucomannan, PNPαGal = *p*-nitrophenyl-α-D-galactopyranoside

from *S. cerevisiae* and secreted β-xylosidase activity was obtained. When this fusion gene and *T. reesei xyn2* were coexpressed in *S. cerevisiae*, high levels of β-xylanase and β-xylosidase activity were obtained in autoselective strains grown in rich medium. Coproduction of these two enzymes allowed this recombinant *S. cerevisiae* strain to convert up to 46% of birchwood xylan to xylose [115].

Using a cell surface engineering system based on α-agglutinin, *S. cerevisiae* strains displaying the β-xylanase II separately or in combination with the β-xylosidase from *Aspergillus oryzae* on the cell surface were constructed [119, 130]. When xylan was incubated with high cell concentrations of these strains, HPLC analysis showed that xylose was the main product of the yeast strain codisplaying the β-xylanase and β-xylosidase, while xylobiose and xylotriose were detected as the main products of the yeast strain dis-

playing the β-xylanase. Subsequently, a xylan utilizing *S. cerevisiae* strain was constructed by introducing genes for xylose utilization, specifically, those encoding xylose reductase and xylitol dehydrogenase from *Pichia stipitis* and xylulokinase from *S. cerevisiae* into the strain codisplaying the β-xylanase and β-xylosidase. Ethanol was directly produced from birchwood xylan, and the yield in terms of grams of ethanol per gram of carbohydrate consumed was 0.30 g/g. This strain, though not able to completely degrade xylan and still suffering from the redox imbalance problem during xylose utilization, supports the potential of using *S. cerevisiae* in a CBP configuration for converting xylan to ethanol.

In order to achieve complete degradation of complex substituted xylans, a series of accessory or debranching enzymes are also needed, namely α-D-glucuronidases (EC 3.2.1), α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranosidase EC 3.2.1.55), and acetylesterases or acetyl xylan esterases (EC 3.1.1.6) [131]. Successful expression of an α-glucuronidase in *S. cerevisiae* was recently reported [120]. The secreted enzyme was active on aldouronic acids from aldobiuronic to aldopentauronic acid. The *T. reesei* α-arabinofuranosidase encoding gene, *abf1*, was expressed in *S. cerevisiae* and the resulting enzyme released L-arabinose from *p*-nitrophenyl-α-L-arabinofuranoside and arabinoxylans [117]. Successful expression of the gene encoding α-L-arabinofuranosidase B (*abfB*) from *A. niger* was also shown [121, 122].

The major hemicelluloses in softwoods are acetylated galactoglucomannans [107]. These consist of a backbone of β-1,4-linked mannose and glucose residues substituted with α-1,6-linked galactosyl side groups. Mannanase (endo-1,4-β-mannanase; mannan endo-1,4-β-mannosidase; EC 3.2.1.78) randomly hydrolyzes the 1,4-β-mannosidic bonds of the main chain of glucomannan and galactomannan (Fig. 2c). Endomannanases of *T. reesei* (*man1*), *Aspergillus aculeatus* (*man1*), and *Orpinomyces* PC-2 (*manA*) have all been expressed and secreted in *S. cerevisiae* [107, 108, 124]. The secreted enzymes showed activity toward locust bean gum and ivory nut mannan with the *A. aculeatus* enzyme exhibiting the highest titer and activity. The enzyme α-galactosidase (α-D-galactoside galactohydrolase) catalyzes hydrolysis of α-1,6-linked galactosyl residues from galacto(gluco)mannans and simple oligosaccharides such as raffinose and is required for the complete hydrolysis of galactomannan [125]. Three α-galactosidases, *agl1*, *agl2*, and *agl3*, from *T. reesei* were cloned and expressed in *S. cerevisiae*. The recombinant enzymes were able to hydrolyze raffinose, melibiose, and *p*-nitrophenyl-α-D-galactopyranoside and release galactose from galacto(gluco)mannan.

Xyloglucan is the main hemicellulosic polysaccharide present in the primary cell walls of dicotyledonous plants [127]. It consists of a linear 1,4-β-linked D-glucan backbone that carries α-D-xylosyl, β-D-galactosyl-1,2-α-D-xylosyl, and α-L-fucosyl-1,2-β-D-galactosyl-1,2-α-D-xylosyl side chains attached to the OH-6 of β-glucosyl residues. α-Xylosidase releases the unsubsti-

tuted side chain xylosyl residue attached to the backbone glucosyl residue situated farthest from the reducing end of the molecule (Fig. 2d). When a gene encoding the *Arabidopsis thaliana* α-xylosidase (*AtXYL1*) was expressed in *S. cerevisiae*, activity could be detected inside the cell [127]. A xyloglucan-specific endo-β-1,4-glucanase from *Aspergillus aculeatus* was isolated and expressed in yeast [126]. The recombinant enzyme was active in yeast, showing clearing zone formation on azurine-dyed cross-linked xyloglucan containing plates.

As the technologies of pentose sugar utilization and hemicellulase production in *S. cerevisiae* mature, integration of these processes and subsequent single-step processing of biomass hemicellulose to commodity products such as ethanol becomes ever more easily envisioned.

6 Selection for the Development of Superior CBP Yeasts

Den Haan et al. [49] calculated that a 20- to 120-fold improvement in CBH expression, as well as simultaneous high-level expression of other cellulase components, will be necessary for slow growth on crystalline cellulose. This calculation assumes a strain that can grow at 0.02 h^{-1} has a typical anaerobic yield of 0.1 g biomass/g substrate or an aerobic yield of 0.45 g biomass/g substrate, that the expressed cellulase has a specific activity which is the same as that of crude *T. reesei* cellulase on avicel (0.6 U/mg), and that CBH1 would make up the same fraction of total cellulase protein as in the *T. reesei* system [9]. While techniques for rational design of cellulases for improvement in expression level and potentially specific activity will be important to achieving this goal, techniques involving random natural and/or induced mutation will also play an important role. The well-established success of directed evolution techniques for enzymes and enzyme systems (e.g., see reviews in [132, 133]) can be transferred to engineering organisms for CBP, although this application does present unique challenges due to the lack of a good high-throughput screening technique for activity on insoluble cellulosic substrates. On the other hand, the natural connection between cellulase expression and growth on cellulose for CBP organisms makes whole cell selection-based strategies for improving cellulase production a powerful way to screen very large libraries of candidate cells, mimicking the evolutionary process found in nature.

An assumption for any selection-based improvement for CBP organisms is that mutations can result in increased cellulase activity expression. For total cellulase activity such mutations would increase either the per cell expression level (g cellulase/g cell) or the cellulase specific activity (U/mg cellulase). Mutagenesis and screening techniques have allowed researchers to isolate strains of *S. cerevisiae* with "super-secreting" phenotypes [134–136], and similar

techniques for the expression of individual cellulase components have been successful [137]. Also, random mutation has been used to change the properties of cellulase enzymes (e.g., [138–140]; a further review can be found in [141]), although to our knowledge enhanced overall specific activity of cellulase on insoluble substrates has not been demonstrated via directed evolution. However, the specific activity of a mixture of cellulases also depends on the relative amounts of cellulase components to achieve the highest degree of enzyme–enzyme synergy [142], as well as other parameters (as yet not elucidated) that determine enzyme–microbe synergy [6]. These features could be impacted by mutation and therefore lead to enhanced specific activity of cellulase systems expressed by recombinant cellulolytic CBP organisms.

Earlier in this review (Sect. 3) the relationship between cellulase activity and growth rate was examined from a whole-population perspective, using parameters that are averages for many cells. The relationship between growth rate and cellulase expression for an individual cell, especially a cell harboring mutations affecting cellulase expression, as compared to other cells in the population depends on the diffusion of the soluble reaction products from the point they are created at the cellulose surface to the point they are taken up by a particular cell. When a connection between growth rate and enzyme production can be established, selection in liquid culture—particularly continuous culture—has the potential to screen many cells. For example, if a continuous reactor had a cell concentration of 10^{10} cells/L and was operating at a dilution rate of 0.02 h^{-1}, then 10^8 cells/(L*h) would be screened, and a 100-h continuous culture would screen 10^{10} cells.

The power of this system has been recognized previously (see [143–145] for reviews) and demonstrated in many examples where the enzyme of interest is located intracellularly [146–153], including some cases where the limiting enzyme made up 25% of the total cellular protein after selection, an approximately fourfold increase in expression in both cases [154, 155]. In a very recent study, the authors were able to create a strain of *S. cerevisiae* capable of utilizing xylose as the sole carbon source with a 6-h doubling time without using recombinant genetic techniques—only using selection on xylose minimal media from a strain that could grow only very poorly initially [156]. For secreted enzymes (both cell-associated or extracellularly), far fewer studies have shown improvements via selection in liquid culture. Francis and Hansche [157] were able to isolate a mutant of *S. cerevisiae* in a well-mixed chemostat with 1.7-fold improvement in acid phosphatase activity, and Naki et al. [158] were able to isolate mutants of *Bacillus subtilis* with about fivefold increased secretion of protease by growing the cells in a hollow fiber apparatus, which physically separated cells, with bovine serum albumin as the limiting nitrogen source. Therefore, understanding the physical characteristics of the cell/enzyme/substrate system and the resulting magnitude of differences in growth rate between mutants is critical to applying selection to this system.

When cells are grown on solid media, with significant space between initial cell colonies, those cells that produce more or better cellulase will retain the products of their hydrolytic reactions, and will form larger colonies. This technique—selection by people judging the size of colonies—has the advantage of maintaining separation between cells. It has the disadvantage of limiting the number of cells that can be screened. It is hard to imagine how more than 10^9 cells (10^3 colonies/plate*10^6 plates) can be screened in a reasonable amount of time, even utilizing high-throughput approaches.

When cells are grown in well-mixed liquid culture, the situation is much different because the products of hydrolysis are free to diffuse. A schematic representation of some of the liquid culture cases relevant to recombinant cellulolytic CBP organisms is presented in Fig. 4. In case A, where cellulase enzymes are secreted away from the cell, cellulases with cellulose binding domains will diffuse to cellulose, bind to it, and release soluble hydrolysis products. In the final step of the overall hydrolysis reaction secreted β-glucosidase converts soluble glucose oligomers into glucose (an overview of fungal cellulase systems can be found in [1]). Lelieveld [159] predicted that in cases such as this, the limiting enzyme will form a gradient in the diffusion boundary layer around the cell, creating a gradient of the limiting nutrient as well. Such a gradient would provide a link between mutations conferring increased enzymatic activity and supply of the limiting nutrient to the cell. With respect to selecting CBP organisms, when a cell secretes a growth limiting cellulase that binds cellulose, it will not necessarily take up the products of the reaction preferentially compared to another cell in the population. Thus, increased activity of that cellulase cannot be selected for. The remaining question is whether the postulated gradient of β-glucosidase exists, and if so what is the effect of the glucose gradient (ΔA) on a mutant's growth rate compared to a parent strain producing less β-glucosidase. Fan et al. [160] used a 2-D reaction/diffusion model to predict that the differences in growth rates between mutants and parents in this case are too small to allow the mutant to outgrow the parent in a reasonable length of time.

Recombinant xylanases and cellulases can also be expressed as tethered enzymes [59, 119, 130] (Fig. 4, cases B and C). In the case where a cell does not bind to the cellulose substrate (case B) (e.g., cellulases with cellulose binding domains are not tethered to the cell surface), the limiting enzyme reaction is once again β-glucosidase conversion of cello-oligomers to glucose. The β-glucosidase enzyme is concentrated at the cell surface, setting up a larger gradient (ΔB) than in case A. However, in this case Fan et al. [160] found that unless the Monod constant (k_S) for the substrate was very low, this gradient would not be large enough to allow mutants to outgrow parents in liquid culture.

Case C presents the situation when cellulases are tethered to the surface and the cell binds to a substrate particle. In this case, the particle acts to trap the hydrolysis products, creating a substantial difference between the glucose

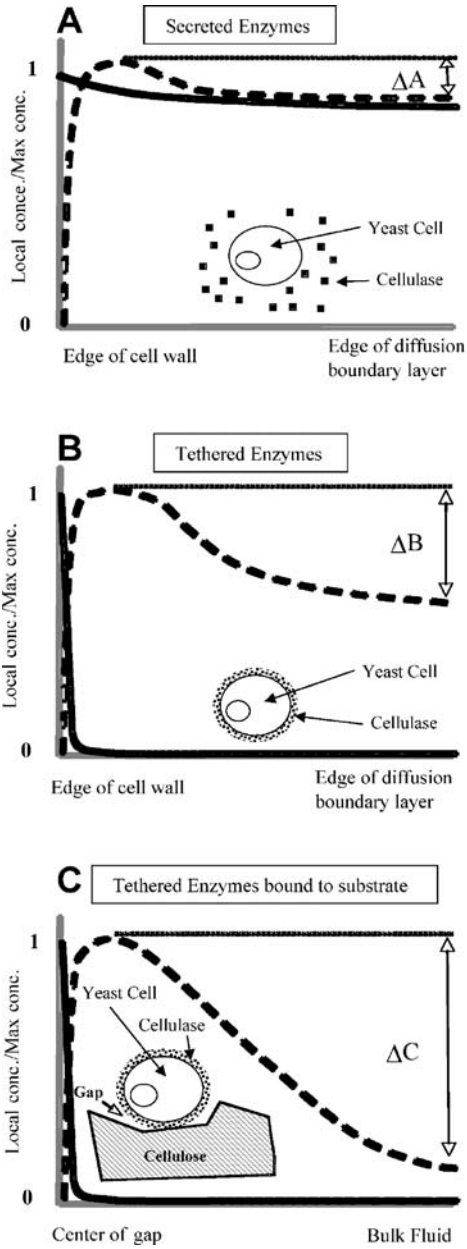


Fig. 4 Schematic representation of enzyme concentration (*solid line*) and hydrolysis product concentration (often glucose, *dashed line*) as a function of position for three cell/enzyme/substrate configurations. *Vertical arrows* show the difference between the local maximum and minimum for hydrolysis product concentrations, which would, in turn, determine the growth rate of a hydrolysis product limited cell. Depictions of cells, enzyme, and substrate illustrate the configuration being analyzed

concentration in this gap and the substrate and the bulk fluid. When this cell/enzyme/substrate relationship is operative, Fan et al. [160] predict that differences in enzyme expression level will lead to differences in growth rates between mutant and parent cells, and that this will allow selection-based population changes to occur in a reasonable amount of time. To date, the promise of selection for improving cellulase production by recombinant cellulolytic microorganisms has not been realized, and knowledge of the local concentration of glucose around such cells is limited to prediction. However, it is known that cellulose hydrolysis by naturally occurring cellulolytic microorganisms occurs much faster when mediated by cells adhering to the substrate as compared to nonadherent mutants [161]. It has been suggested that adherence confers a competitive advantage associated with first access to hydrolysis products.

7 Integration of Different Enzymatic Activities into a Single CBP Yeast and Transfer to Industrial Strains

Significant advances related to recombinant enzyme expression support the potential for *S. cerevisiae* as a CBP host. However, the challenge of integrating all the different aspects of enzymatic hydrolysis of cellulose and hemicellulose and subsequent fermentation of the sugars released to ethanol in a single reactor with a CBP should not be underestimated. A pertinent question often asked by critics is, "Would *S. cerevisiae* be able to simultaneously express multiple genes, while producing and secreting the different cellulases, hemicellulases, and pentose utilizing enzymes required?" Several studies demonstrate coexpression of multiple genes in *S. cerevisiae*, for example in the case of the expression of tethered cellulolytic and xylanolytic enzymes [59, 119], xylose and arabinose utilizing enzymes [40, 162], as well as xylose and cellooligosaccharide utilizing enzymes [45]. The expression and secretion of a variety of cellulases, amylases, and pectinase has also been demonstrated without adversely affecting yeast growth [51, 52].

However, the number of genes expressed is probably not as important a challenge as the need for high-level expression as well as the stress responses that may accompany such high-level expression. Factors that may impose unnecessary stress on the cell are (1) sequestering of transcription factors at highly expressed promoters used for heterologous gene expression, (2) impact of unfavorable codon bias on the translation of heterologous protein (can be overcome by the use of codon-optimized synthetic genes), and (3) improper folding of foreign proteins that can evoke the (4) unfolded protein response (UPR) and consequently the endoplasmic reticulum-associated protein degradation (ERAD) response [163]. Some of these effects may be exacerbated by (5) interrupted transport of foreign proteins through the se-

cretion pathway, or (6) accumulation of larger proteins at the cell wall due to low permeability [164]. The answer would thus not be simply overexpression of all the required genes to ensure a functional CBP yeast with the desirable enzymatic activities, but much more attention should also be devoted to the careful manipulation of the required enzyme activities and producing them at the right concentration to provide functionality without exerting too much unnecessary stress on the CBP yeast.

Essentially all work carried out thus far involving heterologous expression of saccharolytic enzymes in yeast has involved laboratory strains. Much of this work has to be transferred to industrial strains that provide the fermentation capacity and robustness desired for industrial processes. Different strategies have been used for the overexpression of multiple genes in industrial *S. cerevisiae* strains. High copy-number episomal YEp vectors, often using the two-micron autonomous replicating sequence (ARS), have been very helpful in demonstrating proof of concept in laboratory strains of *S. cerevisiae* [43, 51, 102, 115]. However, these vectors are usually mitotically unstable and require selection for the episomal plasmid, which often means using a defined medium that is not applicable to industrial uses [164]. The preferred route taken for industrial strains has been the use of integrative YIp vectors that facilitate direct integration of foreign expression cassettes into a target gene on the yeast genome [165, 166] or recycling dominant selectable markers for multiple integration [167–170]. Although these methods provide stable expression from the yeast genome and are amendable to industrial strains, the major drawback has been low expression levels and often not delivering high enough quantities of the required gene product.

Different approaches have been pursued in an attempted to combine the advantages of overexpression from multicopy plasmids with the stability of chromosomal integration, which is also applicable to industrial strains when dominant selectable markers are used. These include the use of repetitive chromosomal DNA sequences such as rDNA [171] and δ-sequences [172]. There are approximately 140–200 copies of rDNA existing in the haploid yeast genome; however, rDNA is located in the nucleolus, which may affect the accessibility to RNA polymerase II transcription. Also, the size of pMIRY (multiple integration into ribosomal DNA in yeast) vectors could determine the mitotic stability of these multiple integrations [173]. The δ-sequences are the long terminal repeats of *S cerevisiae* retrotransposon Ty. More than 400 copies of δ-sequences can exist either Ty associated or as sole sites in the haploid yeast genome [174]. δ-Integration thus makes it possible to integrate more copies of a gene of interest into the yeast genome than the conventional integration systems [78, 175]. Host strains and integrated gene size can significantly affect the transformation efficiency at δ-sequences; however, the transformation frequency can be 10- to 100-fold those obtained when transforming with vectors that target a single gene on the yeast genome [176].

Although the necessary tools exist for multiple and repeated integration of genes of interest into the genome of industrial strains to complement the required features for CBP (Table 1), a more strategic approach would be required to design a yeast that produces the required enzyme activities, yet retains the competence to still perform well under industrial conditions for the economic conversion of plant biomass to ethanol. Such an approach will most probably start by building on a platform industrial yeast that cometabolises hexoses and pentoses, and subsequently finding the right combination and level of expression for saccharolytic enzymes. This approach will inevitably use reiterated metabolic engineering and flux analysis, selection and mutagenesis strategies, and even strain breeding to allow the microorganism itself to overcome rate-limiting hurdles toward developing an efficient CBP yeast. Examples of such approaches in the past have been performed to enhance xylose fermentation in laboratory and industrial strains [33, 37, 39, 177].

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Development of Ethanologenic Bacteria

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Abstract The utilization of lignocellulosic biomass as a petroleum alternative faces many challenges. This work reviews recent progress in the engineering of *Escherichia coli* and *Klebsiella oxytoca* to produce ethanol from biomass with minimal nutritional supplemen-

tation. A combination of directed engineering and metabolic evolution has resulted in microbial biocatalysts that produce up to 45 g L^{-1} ethanol in 48 h in a simple mineral salts medium, and convert various lignocellulosic materials to ethanol. Mutations contributing to ethanologenesis are discussed. The ethanologenic biocatalyst design approach was applied to other commodity chemicals, including optically pure D(–)- and L(+)-lactic acid, succinate and L-alanine with similar success. This review also describes recent progress in growth medium development, the reduction of hemicellulose hydrolysate toxicity and reduction of the demand for fungal cellulases.

Keywords *Escherichia coli* · Ethanol · Hemicellulose hydrolysate · Lactic acid

1 Introduction

Increasing petroleum costs, together with our increasing dependency on crude oil imports, have provided an opportunity for bio-based fuels and chemicals to become economically competitive. With the development of new technologies, replacement of the current petroleum-based automotive fuels and petrochemicals and supplementation of the national energy supply with sustainable resources, such as plants and plant-derived materials, is now feasible. Currently, 65% of the oil consumed in the USA is imported. More than 211 billion gallons, or roughly half of the total US energy consumption, were burned as automotive fuel in 2005 [1]. Therefore, development of an alternative renewable transportation fuel, such as ethanol, will significantly reduce US imported oil dependency, contribute to preservation of finite natural resources, and improve the environment.

The use of sugar-derived ethanol as the chief component of automotive fuel was successfully implemented in Brazil nearly three decades ago. While the USA already has automobiles capable of utilizing ethanol blended with gasoline and the infrastructure required to distribute ethanol across the nation, ethanol production lags significantly behind the 168 billion gallon domestic fuel demand. In 2006, the USA produced approximately 4.9 billion gallons of fuel ethanol [2]. Lignocellulosic materials provide the opportunity to further expand ethanol production.

Lignocellulose is a complex substance that accounts for approximately 90% of the dry weight of plant material. It represents the most abundant renewable energy source in the world and is comprised of cell wall structural polymers (cellulose, hemicellulose, pectin, and lignin) (Fig. 1). Due to the complexity of lignocellulose and the biological limitations of existing biocatalysts, the current conceptual process designs for lignocellulose-based ethanol production are more complex than starch-based processes. The development of a microbial biocatalyst that is capable of metabolizing lignocellulose and all of the constitutive sugars will simplify the process and reduce the cost of ethanol production.

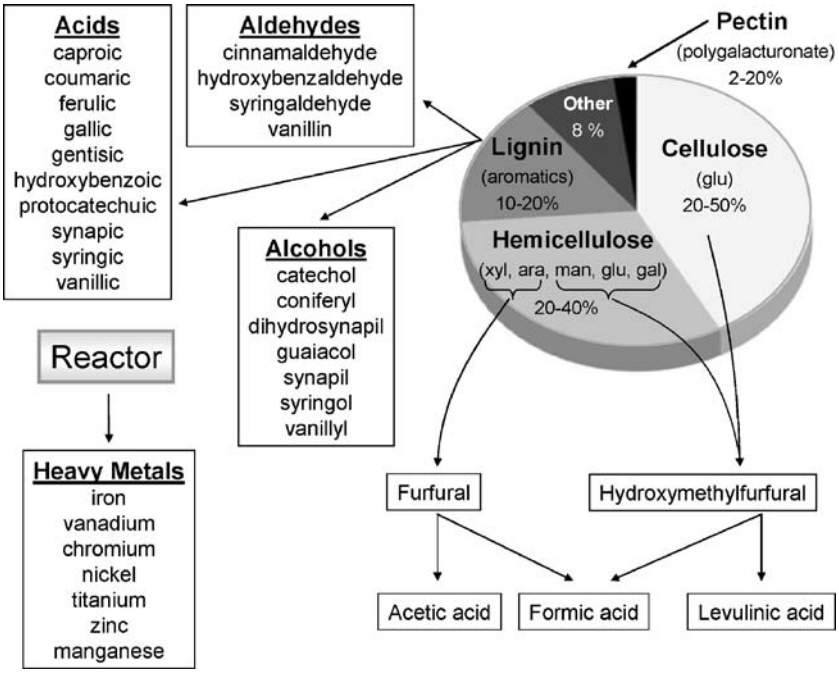

Fig. 1 Composition of lignocellulose and the toxins and inhibitors produced upon pretreatment. The average approximate lignocellulose composition is given as a percentage of total weight. Abbreviations: *xyl* xylose, *ara* arabinose, *man* mannose, *glu* glucose, *gal* galactose

The common bacterial ethanol production pathway, shown in Eq. 1 and Fig. 2, does not allow complete, balanced conversion of glucose to ethanol. In contrast, the homoethanol pathway, comprised of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), allows balanced production of two ethanol molecules per glucose. The homoethanol pathway is present in yeast, plants, and fungi, but is rare in prokaryotes and animals. Bacterial PDCs have a low pyruvate K_m relative to other pyruvate-utilizing enzymes, resulting in effective competition for the pyruvate pool [3]; K_m values are indicated for pyruvate-consuming reactions in Fig. 2.

$$\text{Native } \textit{E. coli} \text{ Glucose} \rightarrow \text{Ethanol} + \text{Acetate} + 2\ \text{Formate} \quad (1)$$

$$\text{Homoethanol Glucose} \rightarrow 2\ \text{Ethanol} + 2CO_2 \quad (2)$$

Recombinant expression of the *Zymomonas mobilis* homoethanol pathway in *E. coli* was first described nearly 20 years ago and has been previously reviewed [4–8]; this review will focus on progress made in the past 10 years. Additionally, this review will discuss advances in hemicellulose utilization and the application of the ethanologenic microbial biocatalyst design scheme to successful production of other commodity chemicals.

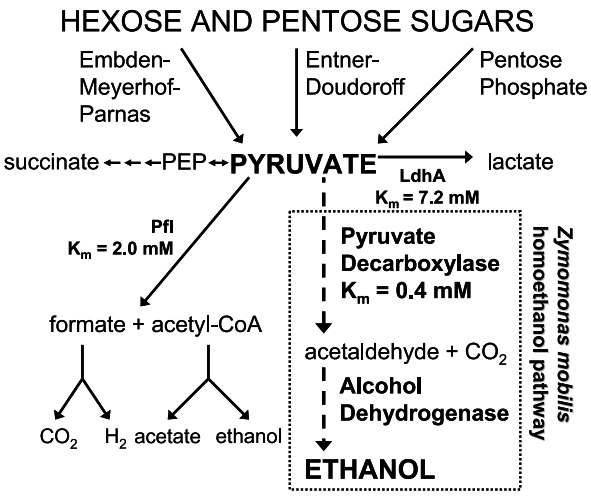

Fig. 2 Conversion of hexose and pentose sugars to ethanol by recombinant *E. coli* in conjunction with the *Z. mobilis* homoethanol pathway. Native *E. coli* reactions are depicted with a *solid arrow* (→), those from *Z. mobilis* with a *dashed arrow* (⇢)

2
Engineering and Performance of Ethanologenic *E. coli*

Historically, *Saccharomyces* has served as the main biocatalyst for commercial ethanol production. Considering that *Saccharomyces* and *Z. mobilis* are naturally ethanologenic, these organisms are obvious candidates for ethanol production. However, both organisms lack the native ability to utilize pentose sugars, the major component of the hemicellulose fraction of biomass [9, 10]. Though *E. coli* lacks the native ability to produce ethanol as the major fermentation product, it utilizes both hexose and pentose sugars [11] and the uronic acid constituents of pectin [12]. The breadth of carbohydrates metabolized, extensive background of knowledge, and ease of genetic manipulation made *E. coli* an obvious choice for metabolic engineering of a microbial biocatalyst for production of ethanol from lignocellulose.

2.1
Ethanologenic Biocatalysts KO11 and LY01

2.1.1
Engineering Scheme

The development of ethanologenic *E. coli* has included a combination of directed engineering and metabolic evolution; the overall scheme is summarized in Fig. 3. The *Z. mobilis* homoethanol pathway (PET operon) was introduced

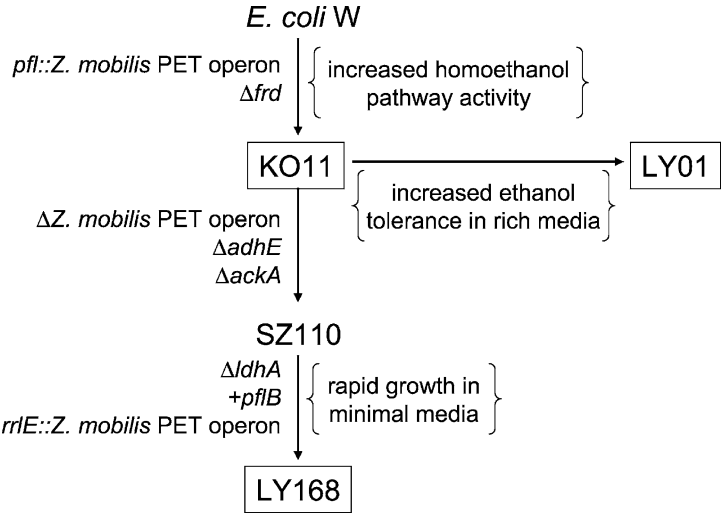

Fig. 3 Ethanologenic *E. coli* design summary. Our design of ethanologenic KO11, LY01, and LY168 has featured a combination of directed engineering, as indicated on the *left of each arrow*, and metabolic evolution, as indicated in {}. For clarity, only major directed metabolic mutations are indicated. KO11 was constructed from *E. coli* W by the introduction of *pdc* and *adhB* from *Z. mobilis* and deletion of *frd* to prevent succinate production. The *Z. mobilis* genes, along with *adhE* and *ackA*, were removed during conversion of KO11 to SZ110. Lactic-acid producing SZ110 was re-engineered to ethanologenic LY168 by removal of *ldhA,* reinsertion of the *Z. mobilis* genes and restoration of the native *pflB*. Please see the text for complete details on each strain

into *E. coli* in plasmids and these derivatives produced ethanol as the main fermentation product [13–16]. The PET operon was stably integrated into the chromosome at the *pfl* locus along with an antibiotic resistance marker; spontaneous mutants exhibiting high ADH activity and high antibiotic resistance were selected to ensure high PET activity. Side reactions that drain carbon away from ethanol were eliminated either by mutation (*frd* – succinate) or physiologically (differences in K_m for pyruvate) (Fig. 2). The resulting strain KO11 produced ethanol at a yield of 95% in complex media [17]. While it was originally reported that KO11 was derived from *E coli* B, it has recently been discovered that *E coli* W is the parental strain (Jarboe and Ingram, unpublished).

While the rate of ethanol production by KO11 is as high as yeast, the ethanol tolerance is lower than the commercially employed yeast strains. In complex media, KO11 shows a complete lack of growth in the presence of $35\,g\,L^{-1}$ ethanol and only 10% survival from 30 s of exposure to $100\,g\,L^{-1}$ ethanol [18]. Using strain KO11 as a starting point, mutant strains with significantly increased ethanol tolerance were isolated. The 3-month metabolic evolution consisted of alternating periods of selection in liquid media for in-

creased ethanol tolerance and selection on solid media for increased ethanol production. The final product of this evolution, strain LY01, was able to grow in the presence of 50 g L^{-1} ethanol and had greater than 80% survival from 30 s of exposure to 100 g L^{-1} ethanol. The method of metabolic evolution used to derive LY01 from KO11 has proved to be successful and has been applied to the design of other ethanologenic biocatalysts and to the production of other commodity products, as described in Sect. 5.

2.1.2 Utilized Substrates

The utility of KO11 for production of ethanol from biomass has been demonstrated with multiple substrates including, but not limited to, rice hulls [19], sugar cane bagasse [20], agricultural residues [20], *Pinus* sp. hydrolysate [21], corn cobs, hulls and AFEX-pretreated fibers [22, 23], orange peel [12], willow [24], pectin-rich beet pulp [25], sweet whey [26], brewery waste [27], and cotton gin waste [28]. The final ethanol titers and fermentation times for these substrates are presented in Table 1. Consistent with the robustness of the parental *E. coli* W, KO11 is relatively robust to changes in temperature and pH [29]. KO11 has also been the subject of an empirical kinetic model [24].

While similar ethanol yields are obtained from glucose and xylose, differences in transport mechanisms result in a lower ATP yield for xylose. Both KO11 and LY01 grow approximately 50% faster and produce three times as much ATP from glucose relative to xylose [30]. As expected, the expression

Table 1 Biomass utilization by ethanologenic *E. coli* KO11 and *K. oxytoca* P2

Organism	Biomass	Ethanol (g L^{-1})	Fermentation time (h)	% of theoretical yield	Refs.
E. coli KO11	Rice hulls	46	72	92	[19]
	Sugar cane bagasse	37	60	90	[20]
	Corn hulls and fibers	44	72	94	[20]
	Beet pulp	40	120	n/a	[25]
	Corn hulls	38	48	100	[22]
	Pinus sp (softwood)	35	48	100	[21]
	Orange peel	28	72	81	[12]
	Sweet whey	20	96	96	[26]
	Willow (hardwood)	4.5	14	n/a	[153]
	Brewery wastewater	15	84	n/a	[27]
K. oxytoca P2	Crystalline cellulose	43	96	76	[55]
	Mixed waste office paper	39	80	83	[54]
	Sugar cane bagasse	39	168	70	[56]

of xylose metabolic genes is increased during xylose growth relative to glucose growth. However, genes contributing to metabolism of other pentose sugars, such as arabinose, ribose and lyxose, also have increased expression during xylose growth, consistent with a relaxation of the cAMP–CRP control system [30].

2.1.3 Limitations and Challenges

Because dependence on nutritional supplementation increases the process cost, the ideal biocatalyst should produce high amounts of ethanol in simple mineral salts growth medium. While KO11 and LY01 both attained high ethanol yields and titers in rich media, these microbial biocatalysts perform poorly in minimal media. With nutritional supplementation, KO11 produced 45 g L^{-1} ethanol from 100 g L^{-1} glucose in 72 h; in minimal medium less than 30 g L^{-1} were produced in 96 h [31]. Results for LY01 were similarly disappointing: the final cell mass and ethanol titer attained in minimal medium were tenfold lower than in rich medium [32]. Considering that these strains were selected in rich media, this is not a surprising result. The low ethanol production by KO11 in minimal media has been attributed to suboptimal partitioning of pyruvate for biosynthesis [33, 34]. Low acetyl CoA and high NADH levels result in inhibition of citrate synthase, limiting the availability of 2-oxoglutarate for biosynthesis. 2-Oxoglutarate is required for the biosynthesis of many amino acids and is an important compound for osmotic tolerance. This proposed inhibition of citrate synthase was supported by the finding that expression of a NADH-insensitive citrate synthase from *Bacillus* increased the growth and ethanol production of KO11 by about 75% [33].

The ability of microbial biocatalysts to retain ethanologenicity over time without dependence on antibiotics is important for minimizing production costs. While instability of KO11 has been reported [35, 36], other reports have demonstrated maintenance of KO11 ethanologenicity for up to 27 days in continuous stirred tank and fluidized beds reactors [37].

In addition to the production of 48 g L^{-1} ethanol in rich media, KO11 also produced up to 192 mg L^{-1} of the undesirable co-product ethyl acetate. An esterase with ethyl acetate hydroylase activity (*estZ)* from *Pseudomonas putida* was introduced into KO11 and the presence of this enzyme reduced the ethyl acetate level to less than 20 mg L^{-1}, a level comparable to that of yeast fermentation [38].

2.2 Ethanologenic Biocatalyst, Strain LY168

To eliminate the dependence of KO11 and LY01 on costly nutritional supplementation, a new ethanologenic *E. coli* strain was constructed. The starting

strain SZ110, a derivative of KO11 modified for production of lactic acid in mineral salts medium (see Sect. 5.1), was re-engineered for ethanol production.

2.2.1 Conversion of SZ110 to LY168

Strain SZ110, a derivative of KO11, was engineered and metabolically evolved to produce lactic acid, as described in detail below [39]. Evolved derivatives of SZ110 produced D-lactate at 92% yield from 100 g L^{-1} glucose in inexpensive mineral salts media. Since this cheap and efficient utilization of large amounts of sugar is the desired biocatalyst behavior, strain SZ110 was chosen as the starting point for re-engineering of ethanologenic *E. coli*(Yomano et al., submitted). Conversion of this strain from lactic acid production to ethanol production involved several steps, beginning with deletion of the lactic acid production gene *ldhA*. The *Z. mobilis* PET operon, inserted at the *pfl* locus in KO11, was removed during engineering of SZ110 for lactic acid production by deletion of the entire *focA-pflB* region [39]. Since elimination of *ackA* and *adhE* prevents undesirable carbon loss, deletion of *pflB* is unnecessary and possibly limits acetyl-CoA levels. Therefore, the native *pfl* gene was restored in the re-engineered ethanologenic *E. coli*. To select for optimal integration of the *Z. mobilis* homoethanol pathway, a promoterless operon containing *pdc*, *adhA*, and *adhB* was randomly inserted by transposon.

Specific growth requirements of both the donor and recipient strains enabled direct functional selection in minimal medium without antibiotics. Candidate ethanologenic strains were enriched by serial transfers in mineral salts medium. One clone was selected and designated LY160. Further evolution of strain LY160 by serially subculturing into fresh mineral salts medium every 24 h for 32 days led to strain LY160im, an intermediate strain with continued improvement in performance. It was determined that the *Z. mobilis* ethanol pathway in LY160im was integrated within *rrlE*, a 23S ribosomal RNA subunit, concurrent with the direction of transcription. The complex regulation of ribosomal RNA transcription is reviewed in [40, 41]; the presence of two promoters results in high expression at high growth rates and basal expression at low growth rates and during stationary phase, making *rrlE* an excellent site for PET integration. The *Pseudomonas putida* short chain esterase *estZ* gene was also integrated into the microbial biocatalyst to lower ethyl acetate levels in the broth. The final strain was designated LY168.

2.2.2 Ethanol Production by LY168

LY168 produced 0.5 g ethanol per gram of xylose during growth in mineral salts medium with betaine, a value close to the theoretical maximum of

Table 2 Comparison of ethanologenesis from xylose

Organism	Xylose ($g\,L^{-1}$)	Medium	Ethanol ($g\,L^{-1}$)	Yield ($g\,g^{-1}$)	Refs.
E. coli LY168	90	Min	45.5	0.51	[a]
E. coli LY168	90	LB	45.3	0.50	[a]
E. coli KO11	90	LB	43.2	0.48	[a]
E. coli LY01	90	LB	42.4	0.47	[18]
E. coli FBR5 (pLOI297)	95	LB	41.5	0.44	[44]
E. coli KO11	90	Min	26.9	0.30	[a]
Non-recombinant *E. coli* SE2378	50	LB	20.5	0.41	[46]
K. oxytoca M5A1 (pLOI555)	100	LB	46.0	0.46	[17]
Z. mobilis CP4 (pZB5)	80 + 8 G	YE	36.6	0.42	[154]
Z. mobilis CP4 (pZB5)	60	YE	23.0	0.38	[154]
Z. mobilis CP4 (pZB5)	25	YE	11.0	0.44	[155]
Z. mobilis ZM4/Ac (pZB5)	60	LB	11.0	0.44	[156]
S. sp strain 1400 (pLNH32)	50	YEP	23.0	0.46	[157]
S. cerevisiae RE700A (pKDR)	50	YEP	23.0	0.46	[158]
S. cerevisiae RWB202-AFX	20	Synth	8.6	0.43	[159]
S. cerevisiae RWB217	20	Synth	8.7	0.44	[160]

LB yeast extract + tryptone
Min minerals + 1 mM betaine
YE yeast extract supplemented with phosphate
YEP supplemented with yeast extract and peptone
Synth minerals supplemented with a mixture of vitamins
8 G 8 g of glucose added per liter
[a] Yomano et al. 2007

0.51 (Yomano et al., submitted). As described in detail below, osmolyte stress in mineral salts media limits biocatalyst performance and betaine supplementation combats this stress [42]. The amount of ethanol produced by LY168 during 24-h fermentations in mineral salts medium with betaine or LB were equivalent. Thus, strain LY168 fulfills the goal of constructing a microbial biocatalyst that produces ethanol without dependence on costly nutritional supplements. As presented in Table 2, the LY168 ethanol yield from xylose is higher than any previously reported ethanologenic biocatalyst.

2.3 Other Recombinant Ethanologenic *E. coli* Strains

The same PET operon used in engineering of KO11 has also been used to construct a series of ethanologenic K12-derivatives, designated FBR for the Fermentation Biochemistry Research Unit. These strains were engineered with the goal of maximizing strain stability [43, 44]. The most recent strain in

this line, FBR5, produced ethanol from a variety of substrates at 86–92% of the theoretical yield [44]. Long-term stability of this strain was demonstrated by the maintenance of ethanol yields over 26 days of continuous culture on glucose or xylose [45]. However, the final ethanol concentration and yield from FBR5 in LB xylose are lower than LY168 in minimal medium (Table 2); additionally, these strains have the disadvantage of rich media dependence and contain plasmids.

2.4 Non-recombinant Ethanologenic *E. coli*

Recombinant expression of the *Z. mobilis* homoethanol pathway has been the cornerstone of *E. coli* ethanologenesis. However, recent progress has enabled ethanol production by a mutant *E. coli* strain lacking foreign genes [46]. Due to the inability to regenerate NAD^+ and maintain redox balance, wild-type *E. coli* is unable to grow anaerobically in the absence of both *ldhA* and *pflB* [47]. Chemical mutagenesis was used to isolate Δ*ldhA*/Δ*pflB* derivatives capable of anaerobic growth. The resulting strain SE2378 fermented glucose and xylose to ethanol with 82% yield. Further analysis of SE2378 revealed an essential mutation within the pyruvate dehydrogenase (PDH) operon. In native strains, pyruvate formate-lyase is primarily responsible for production of acetyl-CoA during anaerobic growth; PDH is reportedly inactive [48] or weakly active [49] under these conditions. The essential mutation in the *pdh* operon restored function during anaerobic growth and produced an additional NADH for each pyruvate. This additional NADH allowed the balanced production of 2 moles of ethanol per mole of glucose by a novel pathway not previously known in nature. The anaerobic specific growth rate of SE2378 was reduced approximately 50% relative to the parental strain in rich media and no growth was observed in glucose minimal media without acetate, glutamate, or corn steep liquor supplementation. Despite growth challenges, the maximum specific productivity of SE2378, 2.24 g ethanol h^{-1} g $cells^{-1}$, is comparable to KO11 and ethanol was produced from 50 g L^{-1} glucose and xylose at greater than 80% of the theoretical yield.

2.5 Ethanol Production in Organisms Other than *E. coli*

Like *E. coli*, *Klebsiella oxytoca* is able to metabolize a variety of biomass-derived monomeric sugars, but unlike *E. coli* it also has the native ability to transport and metabolize cellulose subunits cellobiose and cellotriose [50, 51]. The PET operon was expressed in *K. oxytoca* concurrent with the original *E. coli* work [52] and was later chromosomally integrated, resulting in strain P2 [51]. Ethanol production by *K. oxytoca* P2 from various substrates

has been reported (Table 1) [53–56]. *K. oxytoca* strain BW21, which was derived from strain P2 by elimination of the butanediol pathway, produces over 40 g L^{-1} ethanol in 48 h in OUM1 medium. OUM1 is a medium designed specifically for *K. oxytoca*, as described below [57].

When *Z. mobilis* was selected as the source of the PET operon, *Z. mobilis* and Gram-positive *Sarcina ventriculi* were the only known bacteria with PDC activity [58–61]. Since that time, PDC activity has been identified in other bacteria, including Gram-negative *Acetobacter pasteurianus* [62] and *Zymobacter palmae* [3]. The *Z. palmae* PDC has a higher specificity and lower pyruvate K_m than *Z. mobilis*, *S. ventriculi*, and *A. pasteurianus* [63]. The *S. ventriculi* PDC is different from the *Z. mobilis* enzyme but is highly related to the PDC found in fungi; its expression in *E. coli* requires the presence of accessory tRNA due to differences in codon usage [63]. In *A. pasteurianus*, PDC seems to have the unusual role of functioning in an aerobic pathway, contributing primarily to the conversion of pyruvate to acetaldehyde [62].

The robustness of Gram-positive organisms is appealing for industrial applications, but initial attempts to express the *Z. mobilis* homoethanol pathway in *Bacillus* and lactic acid bacteria had limited success [64–67]. However, the discovery of new PDC forms has enabled renewed engineering attempts. The PDCs from *S. ventriculi*, *A. pasteurianus*, and *Z. mobilis*, as well as *S. cerevisiae*, were each expressed in *Bacillus megaterium*, with the *S. ventriculi* PDC showing the highest activity. When coupled with ADH from *Geobacillus stearothermophilus*, the *S. ventriculi* PDC enabled *B. megaterium* to convert 13.2 g L^{-1} pyruvate to 3.3 g L^{-1} ethanol, a tenfold increase relative to strains lacking PDC [68]. The *S. ventriculi* PDC was also expressed in *Lactobacillus plantarum*, with production of up to 6 g L^{-1} ethanol from 40 g L^{-1} glucose [69]. Recent attempts to express the *Z. mobilis* pathway in *Corynebacterium glutamicum* have resulted in production of ethanol from glucose [70].

Considerable effort has been extended to engineering of *Z. mobilis* for improved ethanol production, as covered elsewhere in this volume. However, the ethanol titers attained by ethanologenic *E. coli* LY168 in minimal medium exceed published values for *Z. mobilis* in rich medium (Table 2).

3 Metabolic and Transcriptomic Changes Accompanying Ethanologenicity

Since the selection of spontaneous mutations during metabolic evolution has been a major component of the development of ethanologenic *E. coli*, many of the underlying changes contributing to ethanologenesis remain unidentified. Identification of these changes will aid in the development of biocatalysts with desired properties for production of other products.

3.1 Physiological Differences Conferring Ethanol Resistance to LY01

LY01 is a derivative of KO11 that was selected in rich medium for increased ethanol tolerance and yield [18]. As described above, LY01 had greater than 80% survival from brief exposure to 100 g L^{-1} ethanol, compared to only 10% survival for KO11 [18]. The transcriptomes of these two strains were compared in LB with glucose or xylose and with 0, 10, or 20 g L^{-1} ethanol [72]. Some 205 genes were differentially expressed in LY01 relative to KO11, as determined by the student's *t*-test; 49 of these genes were greater than twofold different in each comparison. Functional groups related to amino acid biosynthesis, cell processes, cell structure, central intermediary metabolism, and energy metabolism contained a high percentage of differentially expressed genes. Additionally, many stress-related genes, including those related to acid and osmotic stress, were differentially expressed.

Three major physiological differences between LY01 and KO11 were suggested by transcriptome data and supported by further analysis: increased glycine degradation, increased expression of genes related to betaine synthesis and uptake of protective osmolytes, and lack of FNR regulatory function [72]. Normally, FNR regulates the expression of genes required for fermentation and anaerobic respiration (reviewed in [73]). Glycine metabolism and expression of FNR-regulated genes both impact the availability and distribution of pyruvate. It is interesting to note that betaine synthesis genes are affected by FNR via ArcA [74, 75]. Thus, the increased ethanol tolerance of LY01 seems to be a combination of several physiological factors, particularly those related to pyruvate partitioning and osmotic protection.

4 Challenges for Ethanol Production

4.1 Cost Effective Growth Media

In order for ethanol production to be commercially feasible, the growth media cost should be kept at a minimum. In addition to engineering strains to require less nutritional supplementation, the design of simpler, and therefore cheaper, growth media is important for the expansion of bioethanol production.

AM1 [76] and NBS mineral salts media [77] are two simple mineral salts media developed in our laboratory. Both have been shown to support high levels of cell growth and ethanol production. AM1 is a derivative of NBS, with a 65% reduction in salts. With low total alkali (4.5 mM) and total salts (4.2 g L^{-1}), AM1 was able to support production of ethanol from xylose and lactate from glucose with average productivities of 18–19 mmol L^{-1} h^{-1}.

OUM1 medium contains corn steep liquor, mineral salts, and urea as sole nitrogen source; *K. oxytoca* BW21 produced over 40 g L^{-1} ethanol (0.47 g ethanol per gram glucose) in this medium within 48 h [57]. The use of urea as sole nitrogen source has the benefit of cost reduction while also reducing media acidification [78].

On-site preparation of crude yeast autolysate from spent yeast offers potential synergy between grain-based and lignocellulosic processes. Preparation of this autolysate, optimization of the resulting media, and ethanol production by KO11 were demonstrated by [31], with ethanol yields comparable to LB.

K. oxytoca is able to utilize urea as sole nitrogen source, where urea has roughly half the cost of ammonium on an equivalent nitrogen basis. Additionally, because urea metabolism does not contribute to media acidification [79], the use of urea reduces the cost of pH control. With the goal of reducing the nutrient cost of *K. oxytoca*-based ethanol production, optimized urea medium (OUM1) was developed. In addition to containing urea as the sole nitrogen source, OUM1 contains corn steep liquor, mineral salts, and glucose [57].

4.2 Osmolyte Stress Limits Performance in Mineral Salts Media

In order to attain the desired high product titers, biocatalysts must be supplied with high levels of sugars. These high sugar levels in turn create osmotic stress, which is compounded by the desire to use simple mineral salts media. Osmolytes such as trehalose, betaine, proline, and glutamate help bacteria maintain appropriate cell turgor and volume despite changes in extracellular osmolality; osmolyte uptake and synthesis are reviewed in [80]. Increased activity of the native trehalose synthesis pathway elevated the growth rate of *E. coli* W3110 in the presence of various osmotic stress agents [81], and betaine supplementation increased the production of D-lactic acid by *E. coli* SZ132 in NBS mineral salts media [42]. A combination of betaine supplementation and elevated trehalose synthesis increased the tolerance of W3110 to xylose, glucose, sodium lactate, and sodium chloride more than betaine supplementation or elevated trehalose synthesis alone [82].

As described above, the poor performance of ethanologenic *E. coli* strain KO11 in minimal media has been attributed to NADH-mediated inhibition of citrate synthase, limiting the availability of glutamate, a protective osmolyte [33]. Additionally, the increased performance of LY01 relative to KO11 can be partly attributed to increased osmolyte production and uptake [83]. NMR analysis confirmed that intracellular pools of glutamate, trehalose, and betaine are very low in KO11 during anaerobic growth relative to aerobic growth in the same medium [84]. Growth and ethanol production of KO11 was increased by supplementation with various osmolytes, demonstrating that the glutamate limitation is related to osmotic stress, not to a specific metabolic demand for glutamate [84].

4.3 Hemicellulose Hydrolysate Contains Inhibitors

While hemicellulose represents a large potential biomass source that is not presently utilized, pretreatment is required for depolymerization of its soluble components. Many depolymerization techniques are available, but research in this laboratory has focused on hydrolysis with dilute mineral acid at modest temperatures [85, 86]. Unfortunately, dilute acid hydrolysis produces toxins that negatively affect biocatalyst growth and metabolism (reviewed in [87]); many of these toxins are listed in Fig. 1. Recent work has focused on an increased understanding of the underlying mechanisms of toxicity and methods for toxicity quantification and reduction.

Furfural, a pentose sugar derivative, is present in hemicellulose hydrolysate at a concentration of 1–4 $g\,L^{-1}$ [88] but can inhibit *E. coli* growth at concentrations as low as 2.4 $g\,L^{-1}$ [89, 90]. While other aldehydes, such as 4-hydroxybenzaldehyde and syringaldehyde, are more toxic than furfural on a weight basis, the presence of furfural enhances the effect of other toxins [90]. Despite the observed toxicity, ethanologenic *E. coli* KO11 and LY01 and *K. oxytoca* P2 have demonstrated a native ability to transform furfural to furfuryl alcohol [91]; the size and substrate specificity of the LY01 furfural reductase suggests that it is a new type of alcohol-aldehyde oxidoreductase [92]. Strain LY01, which has higher ethanol tolerance than KO11, also has higher furfural tolerance: KO11 growth was completely inhibited by 3 $g\,L^{-1}$ furfural but LY01 was not, although growth was reduced by more than 50% [90]. Contrastingly, there is no difference in the syringaldehyde tolerance of the two strains [90].

The toxicity of representative alcohol, aldehyde, and acid components of hemicellulose hydrolysis were investigated and found to affect ethanologenic *E. coli* LY01 in various ways [90, 93, 94]. In all cases, toxicity was related to hydrophobicity. The organic acid data suggests that aliphatic and mononuclear acids both inhibit biocatalyst growth and ethanol production by collapsing ion gradients and increasing the internal anion concentration, and not by inhibiting central metabolic or energy pathways [93]. At least some inhibitors are present at sufficient concentrations to account for the observed growth inhibition: 9 $g\,L^{-1}$ of acetic acid in rich media inhibits LY01 growth by 50%, and acetic acid concentration in hydrolysate can exceed 10 $g\,L^{-1}$.

While all of the tested aldehydes did inhibit growth, only furfural had an impact on ethanol production [90]. Alcohols have a lower toxicity than aldehydes and acids and appeared to inhibit ethanol production primarily by inhibiting growth [94].

Total furan content is representative of total toxicity and can be estimated from UV spectra [95]. The adjustment of hydrolysate pH to 9–10 by the addition of $Ca(OH)_2$, a process known as overliming, is an effective method of hydrolysate toxicity reduction [96]. LY01 was able to produce less than 1 $g\,L^{-1}$

ethanol from hydrolysate adjusted only to pH 6.5–6.7 but produced 33 g L^{-1} ethanol from baggase hydrolysate that was overlimed to pH 11 [97].

4.4 Reducing the Requirement for Fungal Cellulases

Cellulose is organized into insoluble crystalline ribbons with extensive hydrogen bonds between strands [98, 99]. This structure is not easily hydrated and the fungal cellulase enzymes used for hydrolysis have low catalytic rates in comparison to other glycosidases. Thus, the cost of these enzymes is a major consideration in cellulose utilization [100]. An additional challenge is the feedback inhibition of cellulose hydrolysis by glucose and cellobiose, the products of the hydrolysis process. Simultaneous saccharification and fermentation (SSF), developed by Gulf Oil Company in 1976, combines cellulose saccharification and fermentation of the resultant glucose by *Saccharoymyces* in a single vessel [101, 102]. Recent work in this area has focused on reducing the supplemental cellulase demand by engineering the biocatalysts to produce recombinant cellulase enzymes.

Erwinia chysanthemi contains two endoglucanases, CelZ and CelY, which work synergistically to degrade amorphous cellulose and carboxymethyl cellulose [103]. In order to effectively reduce the demand for cellulase supplementation, CelZ and CelY need to be expressed at high levels and secreted by the biocatalyst. The use of a surrogate *Z. mobilis* promoter and addition of the *E. chrysanthemi out* secretion system resulted in high levels of CelZ expression in *E. coli* and *K. oxytoca* P2, with active glycan hydrolase representing approximately 5% of total cellular protein in both organisms [104, 105]. High endoglucanase activity from recombinantly expressed CelZ and CelY enabled *K. oxytoca* M5A1 to produce ethanol from amorphous cellulose without the addition of supplemental cellulase enzymes [106, 107].

As described above, the primary product of cellulose digestion by endoglucanase and cellobiohydrolase is cellobiose. Unfortunately, cellobiose is a potent inhibitor of these enzymes [108]. The ability to metabolize cellobiose is widespread in prokaryotes [108] and is desirable for biomass-utilizing strains. Ethanologenic *K. oxytoca* P2 has the native ability to transport and metabolize cellobiose, reducing the initial demand for supplemental β-glucosidase [50]. The *K. oxytoca* cellobiose-utilization operon *casAB* has been functionally expressed in *E. coli* KO11, enabling production of ethanol from cellobiose or, with the aid of commercial cellulase, from mixed-waste office paper [50, 109].

In the pursuit of a decreased supplemental cellulase demand, an alternative approach to biocatalyst engineering is the use of non-biological processes to improve cellulose hydrolysis. For example, the use of ultrasound during SSF resulted in a 20% increase in ethanol production from mixed-waste office paper by *K. oxytoca* P2 [110]. Additionally, fungal cellulase demand dur-

ing mixed waste office paper fermentation by P2 was reduced by recycling cellulase [54].

5
Application of Ethanol Design Scheme to Other Commodity Products

Petrochemicals and petroleum-based products such as plastics are widely integrated into our lifestyles and make a major, irreplaceable contribution to virtually all product areas. Increasing petroleum costs have provided an opportunity for a number of renewable bio-based chemicals or plastics, in addition to the bio-based fuels, to become economically competitive. However, full commercialization of renewable commodity chemicals to replace the currently exploited petrochemicals is critically tied to production cost. Therefore, development of low cost fermentation routes, increased microbial biocatalyst efficiency and productivity and increased final fermentation titer are desired.

5.1
Optically Pure D(–)- and L(+)-Lactic Acid

The use of polylactic acid (PLA) as a biodegradable carbohydrate-based plastic is rapidly expanding in many areas such as food packaging, drug delivery, textiles, medical implants, and cosmetics [111–114]. Both the physical properties and the rate of biodegradation can be controlled by adjusting the ratio of the blended enantiomers, D(–)-lactate and L(+)-lactate [115]. For decades, lactic acid bacteria have been used to produce optically pure D(–)- and L(+)-lactate. However, high costs due to the need for complex media and the inability to ferment a broad range of sugars have constrained the use of PLA to the manufacture of medical grade sutures and implants. Alternative biocatalysts from a variety of organisms are currently being investigated for efficient and inexpensive production of optically pure isomers [116–121].

Biocatalysts derived from *E. coli* K-12 had been previously engineered to produce D(–)-lactate but these were not able to metabolize 10% glucose or sucrose to completion in rich or minimal media [122–124]. The success and robustness of *E. coli* W derived-ethanologenic KO11 prompted the redirection of metabolism in this organism from ethanol to D(–)-lactate production [39]. Elimination of *adhE*, *ackA*, and the *Z. mobilis* homoethanol pathway from KO11 yielded strain SZ110 [39]. SZ110 was subjected to metabolic evolution in LB 100 g L^{-1} glucose [39], mineral salts medium with 100 g L^{-1} sucrose, and mineral salts medium with 100 g L^{-1} glucose, along with genetic manipulations to reduce co-product formation and remove foreign genes, to ultimately generate D(–)-lactate-producer SZ194 [125]. Replacement of the native SZ194

ldhA gene with the *Pediococcus acidilactici ldhL* gene and further metabolic evolution in mineral salts medium with glucose resulted in L(+)-lactate producer strain TG103 [121]. Both SZ194 and TG103 produced 1.2 M lactate from 12% glucose in mineral salts medium supplemented with 1 mM betaine. However, lactate optical purity decreased from 99.5% to 95% in the presence of betaine [42, 121]. This chiral impurity was associated with high glycolytic flux rates; spillover of carbon to lactic acid through the methylglyoxal pathway was the source of the contamination [126–128]. Elimination of the first committed enzyme of the methylglyoxal pathway (*mgsA*) restored the product optimal purity to close to 100% [121]. The resulting *E. coli* strains, TG114 and TG108, consistently produced high titers of greater than 99.9% chirally pure D(–)- and L(+)-lactate, respectively, from 12% glucose at greater than 95% of the theoretical yield [121]. The lactate titer, yield, and optical purity attained by TG114 and TG108 are the highest compared to other lactate-producing organisms and were achieved in simpler fermentation medium and condition, making these microbial biocatalysts some of the most efficient lactate producers.

5.2 Acetate and Pyruvate

During oxidative growth, roughly half of the sugar carbons can be diverted into cell mass and CO_2 [129, 130]. For this reason, bacterial production of commodity chemicals has traditionally focused on generating reduced end-products using anaerobic conditions, in order to minimize the loss of carbon as cell material or CO_2. Current biological production of acetate involves complex growth conditions consisting of two separate organisms: an initial fermentation of sugars to ethanol by *Saccharomyces* and subsequent oxidation to acetate by *Acetobacter* under aerobic conditions [131–133]. Strain TC36, an *E. coli* W3110 derivative, was engineered to merge aspects of both fermentative and oxidative metabolism for the production of acetate via a single microbial biocatalyst [77]. TC36 contains multiple chromosomal gene deletions to eliminate production of formate (*focA-pflB)*, succinate (*frdBC*), lactate (*ldhA*), and ethanol (*adhE*), to disrupt the tricarboxylic acid cycle (*sucA*) and, most notably, to inactivate oxidative phosphorylation (*atpFH*) in order to direct the flow of carbons from sugar to acetate with minimal carbon loss to other fermentation products, CO_2, and cell mass. A maximum of 878 mM acetate was produced by TC36 in mineral salts medium. Though this is a lower titer than that achieved during ethanol oxidation by *Acetobacter*, TC36 has a twofold higher production rate, can metabolize a wide range of sugars, and requires a simple, single step process in mineral salts medium.

Pyruvate is used as a food additive, nutriceutical, weight control supplement, and starting material for the production of amino acids and acetalde-

hyde [15, 134]. Pyruvate can be produced by either chemical or biological processes. Chemical synthesis from tartrate entails the use of toxic solvents, requires a great deal of energy, and is very costly [135]. Biological production involves two auxotrophic microorganisms that require costly nutritional supplements and strict regulation of media composition [134, 136], or an *E. coli* strain that produces pyruvate from glucose and acetate in complex medium [137]. More recently, a microbial biocatalyst has been developed for the efficient synthesis of pyruvate from sugar requiring only inexpensive mineral salts medium [138]. Strain TC36, an *E. coli* W3110 derivative described above, was used as a platform to generate the pyruvate producer TC44. This strain encompasses two additional chromosomal deletions, *ackA* and *poxB*, to allow pyruvate accumulation and eliminate acetate production. TC44 yields (0.75 g pyruvate per gram of glucose), titer (749 mM maximum) and production rate (1.2 g of pyruvate $L^{-1}\,h^{-1}$) in mineral salts medium were comparable to or better than the previously described biocatalysts, which required costly nutritional supplements and complex media [138]. This strain improves the cost of pyruvate production by reducing the costs of materials, process controls, product purification, and waste disposal.

5.3 Xylitol

Xylitol has recently been recognized as one of the top 12 value-added chemicals from biomass by the DOE [139]. This pentahydroxy sugar alcohol is commonly used to replace sucrose in food products and in toothpastes as a natural, non-nutritive sweetener that inhibits dental caries [140]. In addition, xylitol can serve as a valuable synthetic building block for derivatives intended for new polymer opportunities [139]. Production of xylitol, which typically involves hydrogenation of xylose derived from hemicellulose-xylan hydrolysates with an active catalyst such as nickel, ruthenium, or rhodium [139], is currently very limited. Numerous yeast strains have been developed that are capable of producing xylitol in complex medium [141–144]. Xylitol production (up to 237 $g\,L^{-1}$) by *Candidata tropicalis* has been optimized by growth in complex media containing urea and numerous expensive vitamin supplements [145]. More recently, strain PC09 was derived from *E. coli* W3110, which is capable of fermenting a broad range of sugars in mineral medium. PC09 can process glucose and xylose blends into xylitol by using an NAD(P)H-dependent xylose reductase from *Candida boidinii* (CbXR) to reduce xylose to xylitol, whereas glucose serves as the cell growth substrate and to regenerate the reducing equivalents [146]. Resting cells and controlled fermentations of PC09 produced 71 and 250 mM xylitol while consuming 15 and 150 mM glucose, respectively. In the controlled fermentations, approximately 25 mM xylulose was formed as co-product [146].

Because glucose was used to regenerate reducing equivalents and was not converted to xylitol, the xylitol yield was quantified in terms of a molar yield of reduced product formed per glucose consumed. In the case of zero growth, a maximum molar yield of 10–12 is expected; resting cells and controlled fermentations of PC09 had molar yields of 4.7 and 1.7, respectively. While the molar yield is relatively low compared to the theoretical maximum, this process could prove to be more economical after further optimization and metabolic engineering.

5.4 Succinate

Succinate, a natural *E. coli* fermentation product, can serve as substrate for the production of many compounds currently derived from petroleum [112]. Although there have been numerous reports of succinate production by *E. coli* and other biocatalysts (for example [147, 148]), these processes often involve undesirable nutritional supplementation, multiple steps and low product titers.

Due to our success, described above, in using a combination of directed engineering and metabolic evolution to design ethanol and lactic acid microbial biocatalysts, we have used a similar approach to develop a succinate-producing microbial biocatalyst that attains high product titers in simple mineral salts media [149]. Directed engineering consisted of elimination of the lactate, acetate, and ethanol-forming pathways (*ldhA*, *ackA*, *adhE*), leaving succinate production as the primary route of NADH oxidation. The poor growth and fermentation of the resulting strain in mineral salts media were improved by metabolic evolution. Further directed engineering (*focA*, *pflB*, *mgsA*) reduced co-product formation. The resulting microbial biocatalysts, KJ060 and KJ073 (*poxB)*, produced nearly 700 mM succinate from glucose with a molar yield of 1.2–1.6; the maximum theoretical molar yield is 1.71 (Jantama et al., unpublished results). KJ060 and KJ073 produced 250 and 183 mM acetate, 39 and 118 mM malate, 0 and 5 mM pyruvate, and 2 and 0 mM lactate as co-products.

5.5 L-Alanine

L-Alanine, used in the pharmaceutical industry [150] and as a food additive [151], is commercially produced by enzymatic decarboxylation of L-aspartic acid with either immobilized cells or cells suspensions [152]. However, recent attention has shifted to fermentative production [150, 151].

Given our success in producing microbial biocatalysts by a combination of directed engineering and metabolic evolution, we modified lactic-acid producing *E. coli* B derivative SZ194 for alanine production. The native *ldhA* gene was

replaced with the ribosome binding site, coding region, and transcriptional terminator of the thermostable alanine dehydrogenase *alaD* from *Geobacillus stearothermophilus* XL-65-6 (Zhang et al., unpublished results). While the initial microbial biocatalyst was capable of producing L-alanine as the primary fermentation product, long incubation times were required and the productivity was low. As with other microbial biocatalysts designed in our laboratory, metabolic evolution was used for strain improvement. The strain was further engineered to reduce co-product formation (*mgsA)* and increase the chiral purity (*dadX*). The final microbial biocatalyst, XZ132, produced near 1.3 M L-alanine from 12% glucose within 48 h, a yield greater than 95%, in AM1 mineral salts media.

6 Summary

E. coli has the capability of utilizing many different sugar substrates and produce a wide spectrum of fermentation products (Fig. 4). However, redirection of a microorganism's metabolism for the efficient production of a single compound is often far more complex than anticipated. The expression level of multiple genes, which may not be predictable, must be optimized for performance. Our success in generating microbial biocatalysts capable of pro-

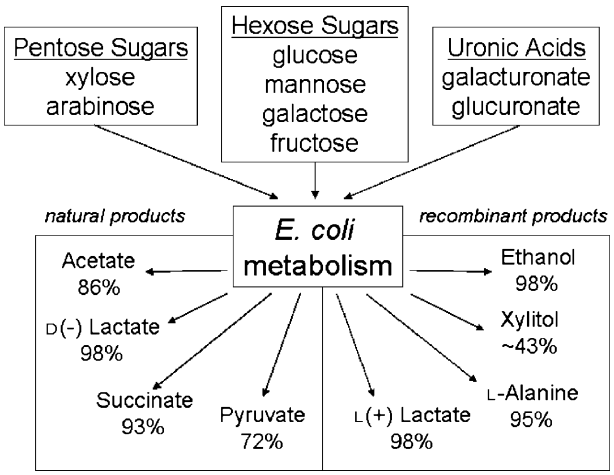

Fig. 4 Due to the plasticity of *E. coli*'s metabolism, a variety of sugars are converted to a wide spectrum of microbial products. Acetate, D(−)-lactate, succinate, and pyruvate are natural *E. coli* products; recombinant strains use genes from *Z. mobilis, C. boidinii, B. stearothermophilus* and *P. acidilactici* for production of ethanol, xylitol, L-alanine, and L(+)-lactate, respectively. The maximum percent of the theoretical yield are shown as reported in [77, 121, 138, 146] (Yomano et al. 2007)

ducing high titers of chemicals has been dependent on an approach that utilizes the organism's natural ability to evolve. Genetically engineered microorganisms require a period of time to adapt to the growth environment. This was accomplished by growing the microbial biocatalysts in the desired mineral salts medium with high sugar concentrations and allowing them to evolve in the new environment. This method has resulted in microbial biocatalysts proficient in production of ethanol and other commodity products, demonstrating that this approach can be applied to many different microbial biocatalysts to improve the overall efficiency and titer.

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Zymomonas mobilis for Fuel Ethanol and Higher Value Products

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Abstract High oil prices, increasing focus on renewable carbohydrate-based feedstocks for fuels and chemicals, and the recent publication of its genome sequence, have provided continuing stimulus for studies on *Zymomonas mobilis*. However, despite its apparent advantages of higher yields and faster specific rates when compared to yeasts, no commercial scale fermentations currently exist which use *Z. mobilis* for the manufacture of fuel ethanol. This may change with the recent announcement of a Dupont/Broin partnership to develop a process for conversion of lignocellulosic residues, such as corn stover, to fuel ethanol using recombinant strains of *Z. mobilis*. The research leading to the construction of these strains, and their fermentation characteristics, are described in the present review. The review also addresses opportunities offered by *Z. mobilis* for higher value products through its metabolic engineering and use of specific high activity enzymes.

Keywords Ethanol production · Glycose/Xylose fermentations · Higher value products · Lignocellulosics · Metabolic engineering · *Zymomonas mobilis*

1 Introduction

Zymomonas mobilis has attracted considerable interest over the past decades as a result of its unique metabolism and ability to rapidly and efficiently produce ethanol from simple sugars. An early paper by Millis [1] characterized the role which *Zymomonas* sp. play in causing cider sickness and a comprehensive review by Swings and DeLey [2] provided much of the background for the subsequent stimulus in research activity in the early 1980s which followed the first of the "oil price shocks". Further reviews over the ensuing decades [3–9] included extensive data on genetic and kinetic characterization of strains of *Zymomonas mobilis* capable of growing on an increasingly wide range of sugars. In a fine example of metabolic (pathway) engineering, recombinant strains of *Z. mobilis* were reported in 1995/6 from the National Renewable Energy Laboratory (NREL) Golden, CO, USA, that were capable of the efficient conversion to ethanol of the C5 sugars, xylose and arabinose present in lignocellulosic hydrolysates [10, 11]. Most recently, the reporting of the complete genome sequence of *Z. mobilis* ZM4 (ATCC 31821) [12] has opened up further potential for strain enhancement and for its use for higher value products.

Table 1 provides an outline of the key research milestones which have occurred for *Z. mobilis* over the past three decades with the present review focusing particularly on those developments which have been reported over the past 5–10 years.

Table 1 Zymomonas research milestones

Activity	Period	Refs.
Review of ethanologenic potential of *Z. mobilis*	Late 1970s	Swings & DeLey [2]
Kinetic confirmation of high rate, high ethanol yields	Early 1980s	Rogers et al. [13] Lee et al. [14]
Batch, continuous and cell recycle evaluations of various strains	Early 1980s	Lavers et al. [15] Lawford et al. [16] Doelle et al. [17]
Development of genetic engineering techniques for *Z. mobilis*	Early 1980s	Skotnicki et al. [18] Dally et al. [19] Drainas et al. [20]

Table 1 (continued)

Activity	Period	Refs.
Cloning of individual heterologous genes to extend substrate range beyond glucose, fructose and sucrose	Mid 1980s	Carey et al. [21] Goodman et al. [22] Strzelecki et al. [23] Su et al. [24]
Characterization of enzymes in the Entner–Doudoroff Pathway	Mid 1980s	Scopes et al. [25] Neale et al. [26, 27]
Cloning of genes to complete pathways for xylose/arabinose utilization	Mid 1990s	Zhang et al. [10] Deanda et al. [11]
Kinetic evaluation of rec strains using glucose/xylose/arabinose media	Late 1990s/ early 2000s	Joachimsthal et al. [28] Joachimsthal & Rogers [29] Lawford et al. [30–38] Mohagheghi et al. [39]
Evaluation of industrial lignocellulosic hydrolysates	Early 2000s	Lawford et al. [38, 40] Mohagheghi et al. [41]
Publication of complete genome sequence of *Z. mobilis* ZM4	2005	Seo et al. [12]
Metabolic engineering for efficient succinate production	2006	Kim et al. [42]
Dupont/Broin Partnership announced to develop *Zymomonas*-based process for ethanol from corn stover	October 2006	Industry report [43]

2
Development of Recombinant Strains of *Z. Mobilis*

2.1
Increased Substrate Range Through Expression of a Single Heterologous Gene

One of the possible disadvantages of *Z. mobilis* is that it has a limited carbon substrate range as it can only use the simple C6 sugars glucose, fructose and sucrose. As a result early studies on its genetic manipulation focused on extending its substrate range for ethanol production. Skotnicki et al. [18] first reported high frequency conjugal transfer of plasmids from *Escherichia coli* and *Pseudomonas aeruginosa*, and this was followed by expression of the *lac Z* gene and production of β-galactosidase in strains of *Z. mobilis* [21, 22]. However, the strain ZM6100 (RP1:Tn 951) derived from this work was shown to progressively lose all plasmid markers in batch culture under non-selective conditions. Subsequently a new strain, ZM6306, was developed in continuous culture which showed 100% stability for all plasmid markers when grown without selection pressure. Synthesis of β-galactosidase was induced

in continuous culture by addition of lactose resulting in increased ethanol production and unutilized galactose [23].

Further studies to extend the substrate range were reported which involved the cloning and expression of a *β-glucosidase* gene from *Xanthomonas albineans* [24] and *α-glucosidase* gene from a *Bacillus* sp [44], however enzyme expression levels were low.

2.2 Strain Construction for Utilization of C5 Sugars

An early attempt was made to construct a xylose-utilizing strain of *Z. mobilis* by Liu et al. [45, 46] involving expression of genes for xylose isomerase (XI), xylulokinase (XK) and the xylose transport protein from *X. albilineans* XA1-1. Although the recombinant strain was shown to possess both XI and XK activity, it was unable to grow on xylose as the sole carbon source. Subsequently, Feldmann et al. [47] constructed a recombinant strain of *Z. mobilis* ZM4 (pZY228) that expressed the *xyl*A and *xyl*B genes from *Klebsiella pneumoniae* for XI and XK, respectively, and the *tkt*A gene for transketolase (TKT) activity from *Escherischia coli*. However, this recombinant strain was also unable to grow on xylose.

On the basis of these earlier studies, Zhang et al. [10] constructed a recombinant strain that successfully converted xylose to ethanol by expression of a transaldolase (*talB*) gene from *E. coli* in addition to those expressing XI, XK and TKT activity. This recombinant strain encoded genes for enzymes both for xylose assimilation (XI, XK) and for completion of the pentose phosphate pathway (TKT, TAL) in *Z. mobilis*. The transformation of wild-type strains of *Z. mobilis* with the 14.4 kb expression vector (pZB5) was then shown to facilitate the efficient conversion of xylose to ethanol via a completed pentose phosphate pathway (Fig. 1).

This research at NREL was continued further by Deanda et al. [11] who successfully developed a strain capable of arabinose utilization. This recombinant strain harbored a plasmid (pZB206) expressing five heterologous genes from *E. coli* encoding L-arabinose isomerase (*ara*A), L-ribulokinase (*ara*B), L-ribulose-5-phosphate-4-epimerase (*ara*D), transaldolase (*tal*B) and transketolase (*tkt*A).

In related studies on the development of a xylose utilizing strain of *Z. mobilis*, De Graaf et al. [48] built on the earlier research by Feldmann et al. [47] and introduced a further plasmid (pZY228) into strain ZM4 (pXY228). This former plasmid harbored *tal*B from *E. coli* thereby facilitating expression of all the requisite additional enzymes in *Z. mobilis* for xylose assimilation and metabolism.

Although there were differences in their construction of these two recombinant strains, the metabolic pathway for both recombinant strains resulting from expression of *xyl*A, *xyl*B, *tkt*A and *tal*B was the same as shown pre-

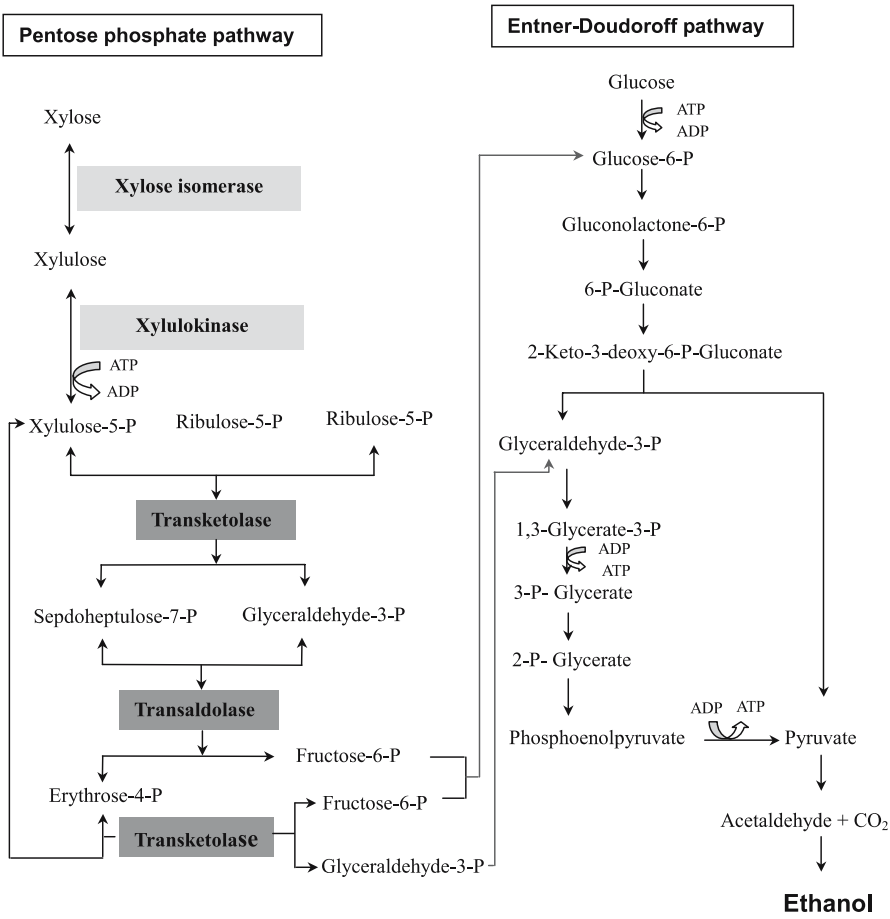

Fig. 1 Pathways for pentose and glucose metabolism (Entner–Doudoroff pathway) in genetically engineered *Z. mobilis* (after Zhang et al. [10]). The shaded enzymes indicate those which have been cloned into *Z. mobilis* from *E. coli*

viously in Fig. 1. Xylose enters the Entner–Doudoroff pathway via fructose-6-phosphate and glyceraldehyde-3-phosphate and is converted into ethanol. The following balance equations represent the metabolism of glucose and xylose by these recombinant xylose-metabolizing strains of *Z. mobilis*.

$$\text{Glucose} + \text{ADP} + \text{Pi} \rightarrow 2\text{Ethanol} + 2\text{CO}_2 + \text{ATP},$$

$$3\text{Xylose} + 3\text{ADP} + 3\text{Pi} \rightarrow 5\text{Ethanol} + 5\text{CO}_2 + 3\text{ATP},$$

Theoretical ethanol yield = 0.51 g ethanol/g sugar (glucose or xylose).

Further studies on recombinant strains created at NREL have involved the construction of integrant xylose-utilizing strains [36–38] and additionally an

integrant xylose/arabinose-utilizing strain designated AX101 [39]. This strain was produced using random insertion and site-specific insertion via homologous recombination.

The specific enzyme activities of the various xylose-utilizing recombinant strains have been determined as a means of identifying possible rate limitations. For the strains developed at NREL, the specific activity associated with XI was the lowest [49, 50]. However, based on calculation of the metabolic fluxes associated with the each enzyme introduced for xylose metabolism, De Graaf et al. [48] concluded for their strain that the flux associated with XK was significantly lower than that of others. This suggested that a metabolic bottleneck may exist in their strain, ZM4 (pZY228) (pZY557 *tal*), due to the low expression of xylulokinase.

Subsequent kinetic studies involving the over-expression of XK (Fig. 2) in an acetate-resistant mutant of the NREL-derived strain ZM4 (pZB5) showed no increase in the maximum specific growth rate or specific rate of xylose metabolism, although there was evidence of a small increase ($0.4\,g\,L^{-1}$) in production of xylitol for the over-expressing strain [51]. Further research on

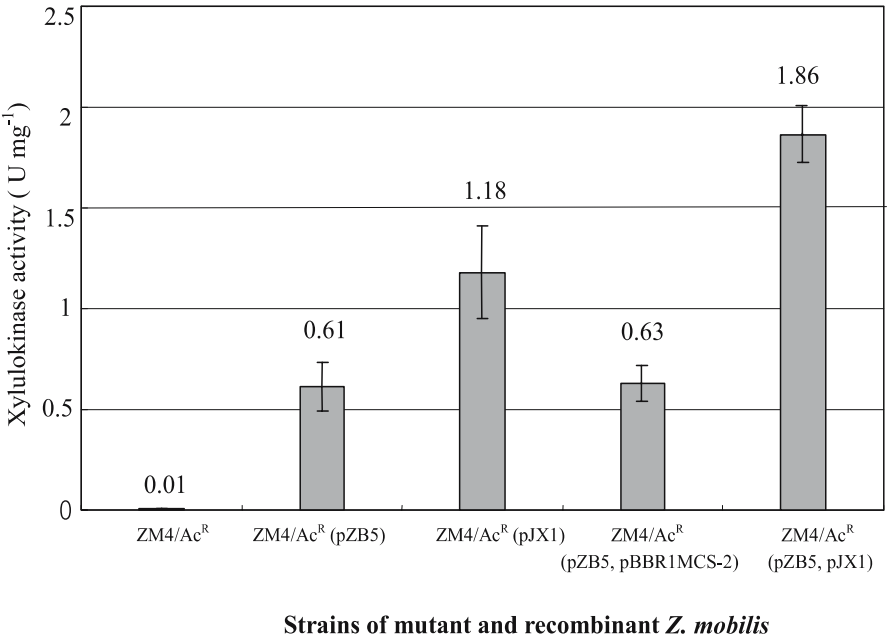

Fig. 2 Xylulokinase (XK) over-expression in acetate-resistant recombinant strains of *Z. mobilis* ZM4/Ac^R (pZB5). Both pZB5 and pJX1 carried genes from *E. coli* for XK expression in *Z. mobilis*. The plasmid pBBR1MCS-2 was based on a broad host range vector suitable for transformation of *Z. mobilis* and used to construct pJX1. Error bars show mean and standard deviation values from triplicate experiments

these recombinant strains is likely to focus on potential rate-limiting sites, as well as expression of heterologous enzymes from other microbial sources for increased ethanol tolerance.

2.3 NMR Analysis of Metabolic Characteristics of Recombinant Strains

The application of ^{13}C and ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy can provide information on both metabolic and energy status during cell growth through determination of the levels of various phosphorylated intermediates and energy rich compounds as shown in earlier studies on wild-type strains of *Z. mobilis* [48, 52–55].

More recent research with ^{31}P NMR has identified a less energized state of ZM4 (pZB5) when grown on xylose media [56, 57]. ^{31}P NMR studies have established that levels of nucleoside tri-phosphates (mostly ATP) and sugar phosphates were lower for growth on xylose compared to that on glucose, with this energy limitation resulting in a potential growth restriction. The presence of by-products identified as xylitol, acetate, lactate, acetoin and dihydroxyacetone by ^{13}C NMR spectroscopy and high-performance liquid chromatography may also result in some inhibition of growth. Further ^{31}P NMR studies [58] have shown that the addition of inhibitory concentrations of sodium acetate caused decreased levels of nucleotide tri-phosphates and sugar phosphates, together with increased cytoplasm acidification.

2.4 Kinetic Characteristics of Recombinant Strains

Detailed kinetic studies have been reported in the literature for several recombinant strains of *Z. mobilis* from NREL capable of utilizing both glucose and xylose. The initial evaluation by Zhang et al. [10] involved the batch culture growth of the strain CP4 (pZB5) on medium containing $25\,g\,L^{-1}$ glucose and $25\,g\,L^{-1}$ xylose. Batch and continuous culture studies on strain 39676 (pZB4L) were reported subsequently by Lawford et al. [31, 33, 34]. This strain was derived from the host ATCC 39676 transformed with a plasmid derived from pZB4. Final product values for $40\,g\,L^{-1}$ glucose/$40\,g\,L^{-1}$ xylose medium included $4.04\,g\,L^{-1}$ xylitol as well as $36.6\,g\,L^{-1}$ ethanol [49] although it should be noted that xylitol levels with this particular recombinant strain were unusually high. Further studies reported by Lawford and Rousseau [35] focused on kinetic and energetic evaluations of strain CP4 (pZB5) in batch and fed-batch fermentations. Kinetic characterization of the chromosomally integrated xylose/arabinose strain AX101 (derived from ATCC 39676) was also reported [37, 38].

To determine which of the strains was likely to be most suitable for larger scale ethanol production, a comparative evaluation in batch and continuous

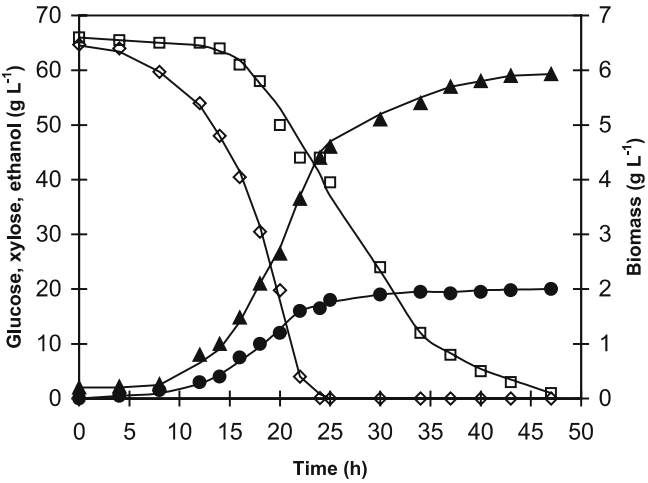

Fig. 3 Kinetics of ethanol production by *Z. mobilis* ZM4 (pZB5) in controlled batch culture on medium containing 65 g L^{-1} glucose and 65 g L^{-1} xylose (T = 30 °C, pH = 5.0). Symbols: ● biomass; ◊ glucose; □ xylose; ▲ ethanol

culture of strains CP4(pZB5) and ZM4(pZB5) was carried out by Joachimsthal et al. [28]. From the results it was found that ZM4(pZB5) was capable of converting a mixture of 65 g L^{-1} glucose and 65 g L^{-1} xylose to more than 60 g L^{-1} ethanol in 48 h in batch culture with an ethanol yield of 0.46 g g^{-1}, with this latter strain demonstrating superior specific sugar uptake and ethanol production rates. The results for ZM4(pZB5) are shown in Fig. 3 together with the values of comparative kinetic parameters in Table 2. Higher sugar concentrations (75 g L^{-1} each sugar) resulted in incomplete xylose utilization (80 h) presumably due to increasing ethanol inhibition of xylose assimilation/metabolism at ethanol concentrations of 65–70 g L^{-1}.

The results for continuous culture with ZM4 (pZB5) and medium containing 40 g L^{-1} glucose and 40 g L^{-1} xylose are shown in Fig. 4 [28]. While the concentration of glucose was close to zero at dilution rates up to D = 0.15 h^{-1}, increasing residual xylose at dilution rates higher than 0.08 h^{-1} indicated that the maximum volumetric rate of xylose uptake for the culture had been exceeded. The maintenance energy coefficient (m) under these conditions was estimated by extrapolation as 1.6 ± 0.2 g g^{-1} h^{-1} (within 95% confidence limits) based on linear regression analysis of the data from Fig. 4a for the maximum specific sugar uptake rate (glucose and xylose) vs. dilution rate (D) (Fig. 4b). A "true biomass yield" of 0.044 g g^{-1} was determined from the inverse of the gradient of this linear plot. For similar experimental conditions, closely related values were observed by Lawford and Rousseau for strain CP4 (pZB5) [34]. However, Lawford and Rousseau noted, when ob-

Table 2 Kinetic Comparison of *Z. mobilis* CP4 (pZB5) and ZM4 (pZB5) on glucose/xylose media ($T = 30\,^{\circ}C$, pH = 5.0). After Joachimsthal et al. [28]

	CP4(pZB5)		ZM4(pZB5)	
	Glucose/xylose (gL^{-1})			
Kinetic parameters	50/50	65/65	50/50	65/65
Max. specific rates				
Glucose/xylose				
μ_m (h^{-1})	0.28	0.27	0.26	0.20
$(q_s)_m$ ($gg^{-1}h^{-1}$)	8.4	6.5	9.5	9.0
$(q_p)_m$ ($gg^{-1}h^{-1}$)	3.1	3.0	4.5	3.8
Max. specific rates				
Xylose				
μ_m (h^{-1})	–	–	0.02	0.01
$(q_s)_m$ ($gg^{-1}h^{-1}$)	1.1	0.6	2.1	2.1
$(q_s)_m$ ($gg^{-1}h^{-1}$)	0.5	0.3	1.0	0.8
Residual xylose (48 h)	0	20	0	0
Overall yields				
$(Y_{x/s})$ (gg^{-1})	0.02	0.02	0.03	0.03
$(Y_{p/s})$ (gg^{-1})	0.46	0.46	0.48	0.46

μ_m: maximum specific growth rate (h^{-1})
$(q_s)_m$: maximum specific sugar uptake rate ($gg^{-1}h^{-1}$)
$(q_p)_m$: maximum specific ethanol production rate ($gg^{-1}h^{-1}$)
$(Y_{x/s})$: overall cell yield (based on total sugar utilized) (gg^{-1})
$(Y_{p/s})$: overall ethanol yield (based on total sugar utilized) (gg^{-1})

served over the lower dilution rate range of D = 0.04–0.08 h^{-1}, that both strain CP4 (pZB5) and a biomass hydrolysate adapted variant of 39676(pZB4L) exhibited values of m and "true biomass yield" that were significantly lower [35].

Results with a potentially high productivity cell recycle system using a membrane bioreactor are shown in Fig. 5 [29]. From Fig. 5(a), at sugar concentrations of 50 g L^{-1} glucose and 50 g L^{-1} xylose and D = 0.1 h^{-1}, an ethanol productivity of 5 g L^{-1} h^{-1} was achieved with an ethanol yield based on total sugars utilized ($Y_{p/s}$) = 0.50 g g^{-1}. No decline in specific ethanol productivity was evident up to 70 h, however as shown in Fig. 5(b), a decrease in total viable cells was observed after an initial steady state (40–50 h). This indicates that for effective longer term operation, high cell concentrations should be

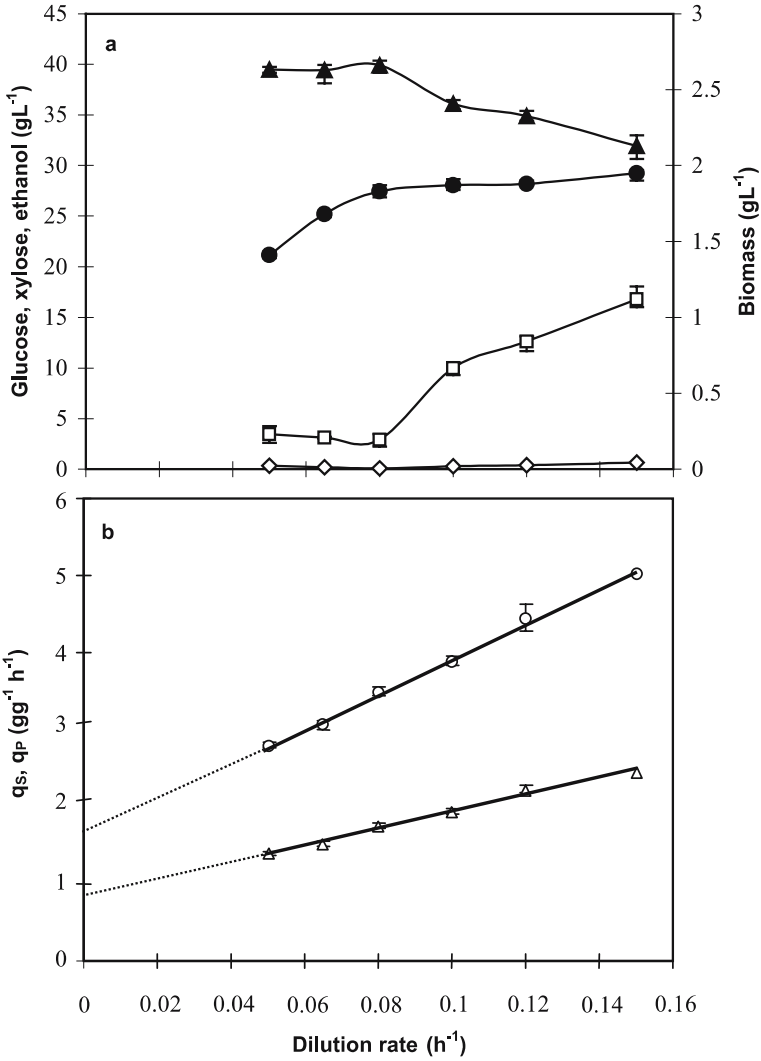

Fig. 4 (**a**) Kinetics of ethanol production by *Z. mobilis* ZM4 (pZB5) in continuous culture on medium containing 40 g L^{-1} glucose and 40 g L^{-1} xylose (T = 30 °C, pH = 5.0). Symbols: biomass •; glucose ◊; xylose □; ethanol ▲ (**b**) Effect of dilution rate on specific rates of total sugar uptake (q_s) and ethanol production (q_p). Estimation of maintenance energy (m) value at D = 0 by extrapolation. Symbols: q_s ○; q_p △

achieved by less stressful methods than membrane-based cell recycling (e.g., by use of flocculent cells and cell settling).

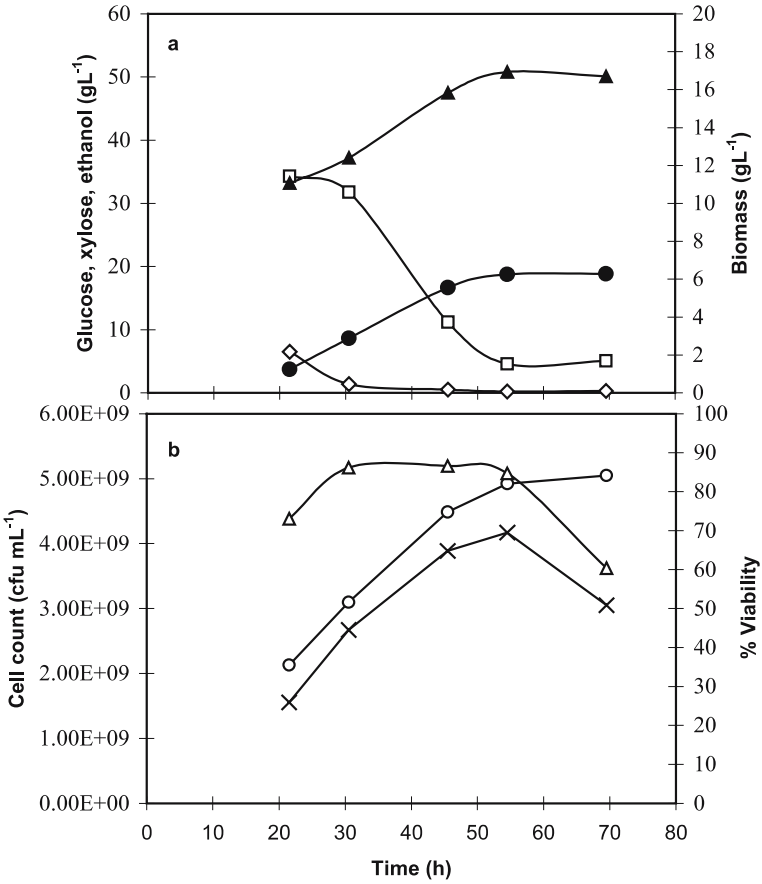

Fig. 5 **a** Time profile for *Z. mobilis* ZM4 (pZB5) for high productivity continuous system with total cell recycle using a membrane Filtron ultrasette and medium containing 50 g L^{-1} glucose and 50 g L^{-1} xylose ($D = 0.1\ h^{-1}$, $T = 30\ °C$, pH = 5.0). Symbols: • biomass; ◊ glucose; □ xylose; ▲ ethanol **b** Total and viable cell counts, and % viability, for continuous cell recycle system. Symbols: total cell count ○; viable cell count △; % viability *x*

2.5 Kinetic Model Development

On the basis of earlier kinetic modelling for the conversion of glucose to ethanol by wild-type *Z. mobilis* [59], a further model has been developed for the fermentation of glucose/xylose mixtures by ZM4 (pZB5) [60]. A two-substrate model was constructed based on Monod kinetics for substrate limitation, as well as functions for product (ethanol) inhibition and substrate inhibition at the higher glucose and xylose concentrations. The model simulation data for various glucose/xylose concentrations were compared with the

experimental results using a Microsoft Excel-based program and statistical analysis for error minimization. Using this approach, it was established that the model (with relevant values of the constants) provided good agreement with the experimental batch culture data for 25/25, 50/50 and 65/65 g L^{-1} glucose/xylose media. It should be noted that the model did not include any repression of xylose uptake by glucose as experimentally both glucose and xylose are taken up simultaneously even at the high initial glucose concentrations. However, this does not preclude the possibility that some glucose repression of xylose might be occurring. The results indicate that ethanol inhibition of xylose utilization is likely to be the more dominant factor in influencing its kinetics.

2.6 Effect of Inhibitors in Lignocellulosic Hydrolysates

A number of components in lignocellulosic hydrolysates can inhibit the growth and ethanol production of bacteria and yeasts, and acetic acid has been identified as a major potential inhibitor of *Z. mobilis* in such acid-produced hydrolysates [61–65]. Lawford and Rousseau [32] examined the role of glucose feeding as a means of improving fermentation performance in acetate-containing media. Another approach to solving this problem has been to use a hydrolysate-fed chemostat to produce adapted or mutant strains [33, 34]. Following chemical mutagenesis, Joachimsthal et al. [66] isolated a mutant strain, designated ZM4/Ac^R with a higher acetate resistance than the parent strain. This strain was then transformed by Jeon et al. [67] to the mutant recombinant ZM4/Ac^R (pZB5). Compared to ZM4 (pZB5), this strain showed enhanced kinetics in batch culture in the presence of 12 g L^{-1} sodium acetate (8.8 g L^{-1} acetic acid) at pH = 5.0 in batch culture on 40 g L^{-1} glucose, 40 g L^{-1} xylose medium. In continuous culture there was evidence of increased maintenance energy requirements/uncoupling of metabolism in the presence of acetate.

In more recent studies Saez-Miranda et al. [68] have determined ATP levels for growth on glucose/xylose media in the presence of different concentrations of acetic acid. From their results they have found that ATP production and accumulation rates are most sensitive to acetic acid at lower pH values—a result consistent with the earlier NMR studies by Kim et al. [57] which demonstrated increasing de-energization of the cells as the inhibitory effects of acetic acid increased. The greater toxicity of acetic acid at lower pH is related to its pK_a value as only unprotonated acid can be transported into the cells.

The effects of a range of inhibitory compounds at levels reported previously for a pre-treated hardwood hydrolysate [65] on specific rates of xylose utilization and ethanol production for ZM4 (pZB5) have been analyzed by Kim et al. [57]. From the results, sodium acetate was found to have the

greatest inhibitory effect at the concentration tested (10.9 g L^{-1} at pH = 6.0), followed by vanillin (0.04 g L^{-1}), syringaldehyde (0.13 g L^{-1}) hydroxymethylfurfural (0.9 g L^{-1}) and furfural (0.3 g L^{-1}). Vanillic acid (0.08 g L^{-1}) did not show any inhibitory effects at this experimental concentration. At the levels tested, these inhibitory compounds did not affect ethanol yields on xylose. Volumetric rates of xylose utilization and ethanol production were reduced by up to 20% by addition of the individual inhibitory components.

2.7 Application to Industrial Raw Materials

Several studies on ethanol production by wild-type strains of *Z. mobilis* on industrial starch-based raw materials have been reported. Bringer et al. [69] investigated an industrial-scale process and Poosaran et al. [70] evaluated a cassava-derived starch hydrolysate. In the latter case in a batch culture at controlled $T = 30\,^{\circ}C$ and pH = 5.0, fermentation using *Z. mobilis* ZM4 gave an ethanol yield of 95% theoretical, a productivity of 6 g L^{-1} h^{-1} and a final ethanol concentration of 114 g L^{-1}. Under the same conditions, a strain of *Saccharomyces uvarum* gave an ethanol yield of 90% theoretical, a productivity of 4 g L^{-1} h^{-1} and a final ethanol concentration of 106 g L^{-1} for a cassava starch suspension (23% glucose equivalent). A comparative batch and continuous culture study with starch hydrolysate using yeast and *Z. mobilis* 29191 has also been reported by Beavan et al. [71].

Extensive studies with various strains of *Z. mobilis* have been reported by using sugar cane syrup and molasses [72–76] and for sugar beet molasses [77, 78] with evidence of yield reductions on sucrose based media due to production of the fructose polymer levan as by-product [3, 6] and rate reductions due to high salt concentrations in the molasses. Improved productivities were reported following membrane desalting of high salt-containing sugar cane molasses [72].

Most recently, Davis et al. [79] studied the fermentation of a hydrolyzed waste starch stream from flour wet milling using both *Z. mobilis* ZM4 and an industrial ethanol-producing strain of *S. cerevisiae*. With glucose concentrations in the range 80–110 g L^{-1}, *Z. mobilis* ZM4 demonstrated superior fermentation characteristics. In a repeated batch process (five cycles), rapid concentration of the cells and increased productivities were achieved by cell settling between batches using the flocculent strain *Z. mobilis* ZM401 (ATCC 31822) as characterized by Skotnicki et al. [80]—see Fig. 6.

Similar flocculent mutants of wild-type *Z. mobilis* strains CP4 and ATCC 29191 have been isolated by Lawford et al. [16] and Fein et al. [81] using a specially designed chemostat. These strains were deposited with the ATCC as strains 35 000 and 35 001, respectively. The use of such flocculent cultures was demonstrated to increase volumetric productivity by as much as ten-fold [82]

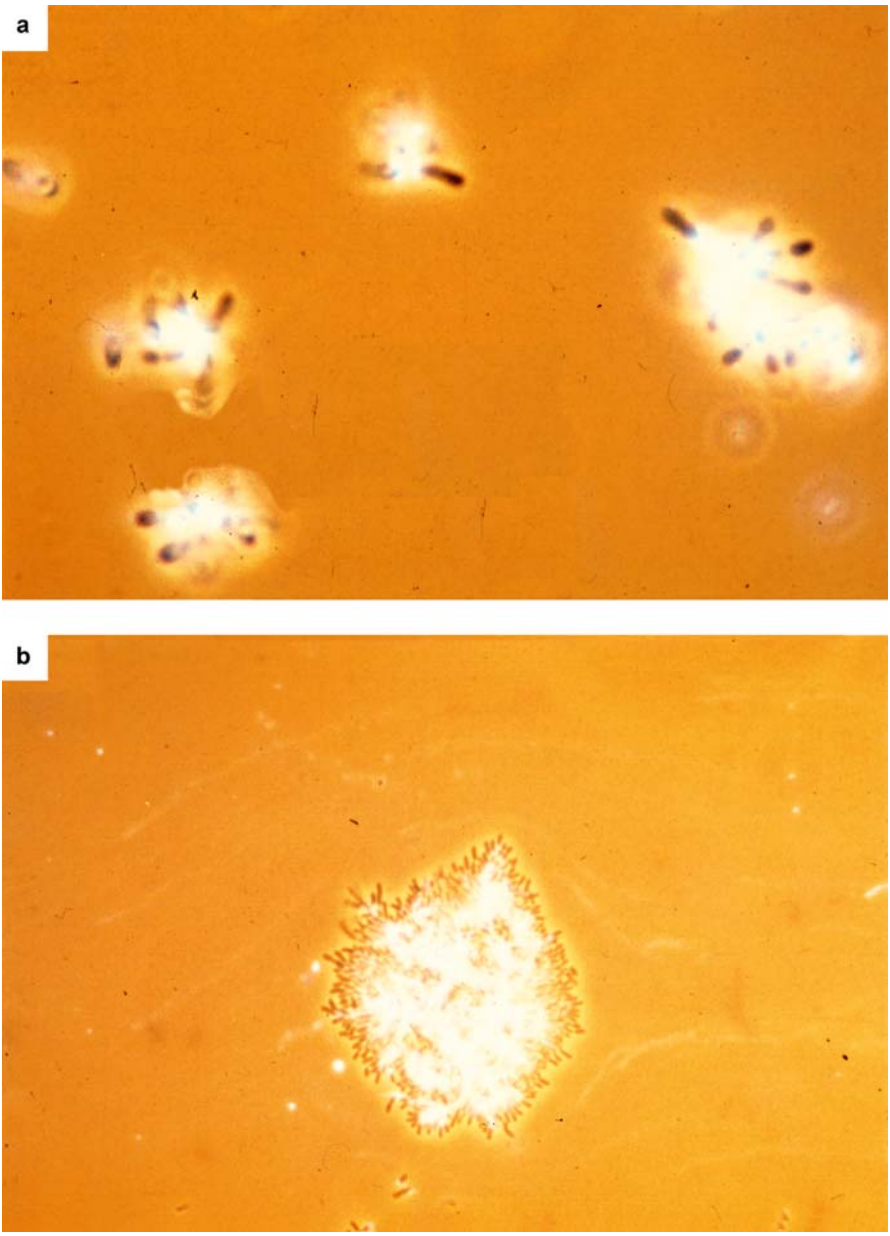

Fig. 6 **a** Photograph showing initial floc formation by a mutant strain of *Z. mobilis* ZM401. This is indicated by cell/cell attachment and fluorescence under UV light following addition of calcafluor which is known to bind to cellulose. **b** Photograph showing formation stable floc of ZM401 and its fluorescence following addition of calcafluor. Floc diameter is approx. 130 microns

and may have considerable potential in future large-scale processes for more stable fermentations.

Recombinant strains of *Z. mobilis* developed for xylose utilization have been evaluated on various agricultural residues including oat hull hydrolysate produced by the Iogen process [40]. Oat hull hydrolysate contains glucose, xylose and arabinose in a mass ratio of 8 : 3 : 0.5. Synthetic hydrolysate (6% w/v glucose; 3% w/v xylose; 0.75% w/v acetic acid) at initial pH 5.75 was mixed with either 2 ml L^{-1} corn steep liquor (CSL) or 1.2 g L^{-1} di-ammonium phosphate as N source and used for evaluation of ethanol production. From the results it was concluded that the highest productivity was achieved with *Z. mobilis* ZM4 (pZB5). In this and other studies, CSL was also found to be an effective nutrient source to replace yeast extract in the fermentation media for *Z. mobilis* [83–85].

Further studies were reported by Mohagheghi et al. [41] with an integrant strain (designated *Z. mobilis* Fig. 8b) derived from ZM4 (pZB5) using overlimed corn stover hydrolysate. The hydrolysate contained 16 g L^{-1} glucose, 69 g L^{-1} xylose and 11 g L^{-1} acetic acid at pH = 5.0. This medium was supplemented with 100 g L^{-1} glucose and diluted to various concentrations prior to fermentation. The authors found that up to 50 g L^{-1} ethanol was produced by the integrant strain with diluted 80% corn stover hydrolysate. Yields of 83–87% theoretical (based on sugars utilized) were reported.

One of the potential issues for large-scale *Z. mobilis* fermentations is whether or not contamination control is needed particularly in the presence of ethanol-tolerant strains of *Lactobacilli*. Such contamination constitutes a problem in many yeast-based processes and can reduce yields by an estimated 2–5%. However, its impact is reduced as pH decreases to 3.0–3.5 towards the end of batch fermentation (in the absence of pH control). "Acid washing" of the residual yeast at this pH or lower is often used to minimize contamination in yeast subsequently used in a repeated batch process. *Z. mobilis* is more sensitive to low pH than *S. cerevisiae* and contamination was identified as a problem by Bringer et al. [69] in their study on an industrial-scale process for conversion of starch to ethanol using *Z. mobilis* although Lawford and Rousseau [63] demonstrated that lactic acid in such circumstances is not likely to be inhibitory to *Z. mobilis*. Interestingly, although rarely observed in *Z. mobilis* fermentations due to the usual high metabolic flux rates in the ED pathway, conditions have been reported which can promote lactic acid synthesis in *Z. mobilis* [37, 85].

The issue of contamination control was addressed directly by Grote et al. [86] in which a continuous culture of *Z. mobilis* ZM4 was directly contaminated with a 10% (v/v) inoculum of *Lactobacillus* sp. isolated as an ethanol-tolerant contaminant from an industrial plant (Grain Processing Corporation, Muscatine, Iowa). It was found at $D = 0.1\ h^{-1}$ under conditions of glucose limitation, pH control at 5.0 and ethanol concentrations of 60–65 g L^{-1}, that the addition of the contaminant caused only a temporary

disturbance in the process. Steady state conditions with no evidence of sustained contamination were regained within five to six generations. These results suggest that contamination is not likely to be a significant problem once an active culture of *Z. mobilis* is established providing that the pH is maintained above 3.5–4.0. A similar conclusion was reached in a recent study [87] using an acid-tolerant strain of *Z. mobilis* under non-sterilized feed and operating conditions.

3 Genome Sequence of *Z. Mobilis*

As discussed earlier, the complete genome sequence of *Z. mobilis* ZM4 has been reported recently [12] following earlier related studies by Korean scientists [88–90]. It was found that the genome consists of 2 056 416 base pairs forming a circular chromosome with 1998 open reading frames (ORFs) and three ribosomal RNA transcription units. As reported by the authors, "the genome lacks recognizable genes for 6-phosphofructokinase, an essential enzyme in the Embden–Meyerhof–Parnas pathway, and for two enzymes in the tricarboxylic acid (TCA) pathway, the 2-oxoglutarate complex and malate dehydrogenase. Glucose can be metabolized therefore only by the Entner–Doudoroff pathway".

Comparison of whole genome microarray data for *Z. mobilis* ZM1 (ATCC10988) and ZM4 (ATCC 31821) revealed that the 54 ORFs present in ZM4 were absent for ZM1. Four of these ORFs that encode transport proteins or permeases, and two that encoded for specific enzymes—NAD(P)H:quinone oxidoreductase and an oxidoreductase related to short-chain alcohol dehydrogenases, were found to be highly expressed in *Z. mobilis* ZM4. The authors suggested that it is possible these genes relate to the higher specific rates of sugar uptake and ethanol production for ZM4 when compared to ZM1. They also reported that two genes encoding capsular carbohydrate synthesis enzymes were only actively expressed in ZM4 and may contribute to its relatively high resistance to increased osmotic pressure found in high sugar solutions (e.g. in 250–300 $g\,L^{-1}$ glucose media).

4 Applications for Higher Value Products

4.1 Metabolites and Related Products

The production of a range of byproducts from *Z. mobilis* is reviewed comprehensively by Johns et al. [6] and Panesar et al. [8] with the former authors

identifying potential commercial opportunities for the following products: fructose (using sucrose and a fructokinase negative mutant), sorbitol and gluconic acid, levan (a fructose polymer), fructo-oligosaccharides and various enzymes. As pointed out in a review by Scopes [91], *Z. mobilis* is a rich source (on an enzyme content per g cell basis) of many of the enzymes currently used in diagnostic analysis and research. Interestingly, in other studies by Park et al. [92], it was established that the activities of some of the key ED enzymes (e.g. glucokinase, G-6-phosphate dehydrogenase) were unaffected by the relatively high ethanol concentrations produced during fermentation, while the activity of an enzyme such as transketolase decreased appreciably above ethanol concentrations of 60 g L^{-1}. It has been estimated that for actively growing cells, as much as 30–50% of the cellular protein is comprised of ED enzymes [93]. However, the greatest difficulty for the commercial production of such enzymes is the low cell yield of *Z. mobilis* which is typically 0.02–0.03 g g^{-1} substrate sugar, compared to cell yields close to 0.5 g g^{-1} for many aerobically grown microorganisms.

4.2 Metabolic Engineering for Organic Acids and TCA Cycle Intermediates

There has recently been considerable interest in the redirection of metabolism in bacteria such as *E. coli* for the overproduction of specific metabolites and higher value products. At a commercial level, the large-scale production by Tate & Lyle/Dupont of 1,3-propandiol using a highly engineered strain of *E. coli* is indicative of an increasing trend towards such bio-based processes. The fast specific rates of sugar uptake by *Z. mobilis*, its highly efficient metabolism for a specific product (ethanol), and its relatively small genome size (facilitating genetic manipulation) may make it an ideal candidate for producing other metabolites via its genetic engineering.

As shown in Fig. 7, *Z. mobilis* has an incomplete TCA cycle and the potential exists via "knock out" mutation to redirect metabolism away from end-products such as lactate and ethanol, towards higher value products like succinic acid. As reported recently by Kim et al. [42], succinic acid overproducing *Z. mobilis* strains have been developed by disruption of the genes for pyruvate decarboxylase (*pdc*) and lactate dehydrogenase (*ldh*). Such strains can produce relatively high concentrations of succinic acid at yields of 1.73 mole/mole glucose (86% theoretical). The yield was reported to be more than 30% greater when compared to those of other succinic acid-producing bacteria such as *Actinobacillus succinogenes* and *Mannheimia succiniciproducens* (about 1.34 mole/mole glucose). These strains of *Z. mobilis* were also reported to exhibit higher overall rates of succinic acid production (1.62 g L^{-1} h^{-1}) under Na bicarbonate supplemented conditions compared to those of other succinic acid producing bacteria (1.35 g L^{-1} h^{-1}).

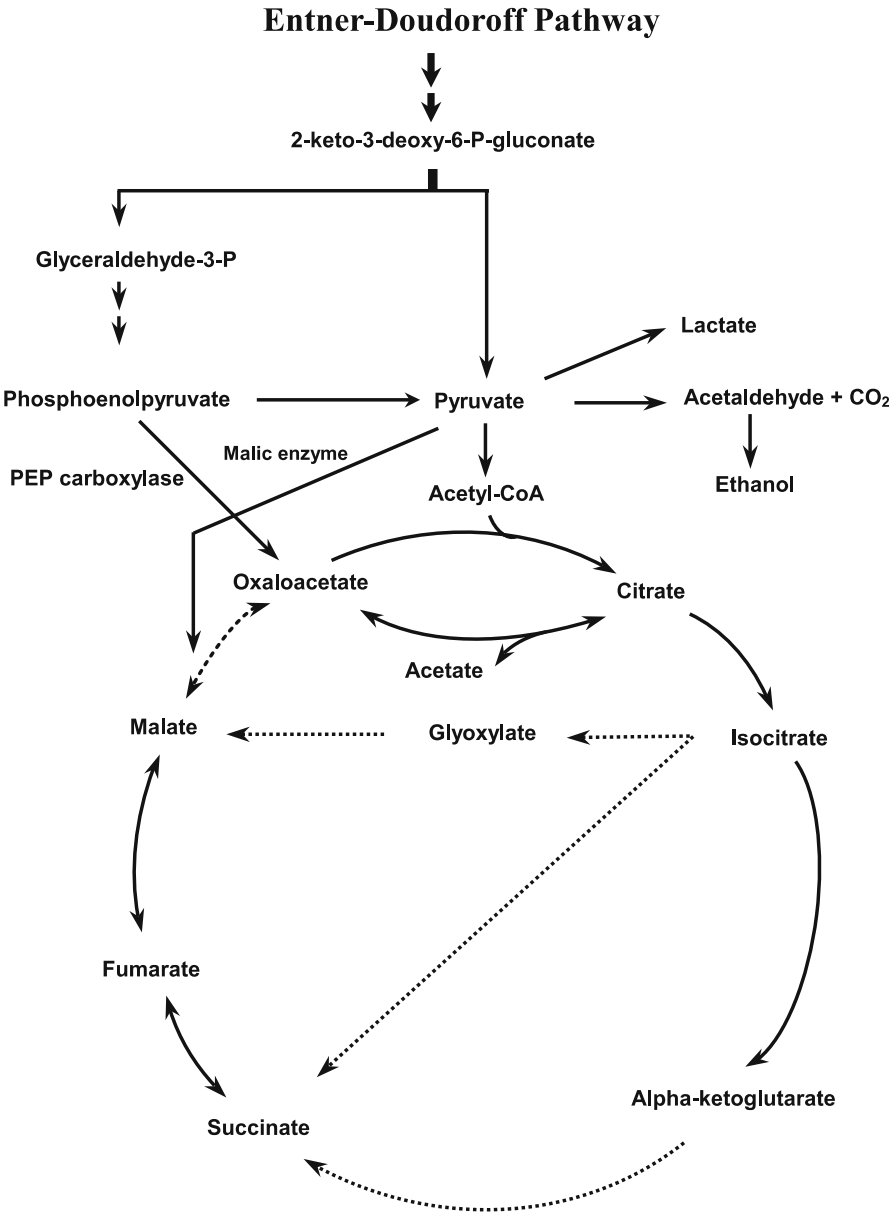

Fig. 7 Incomplete TCA cycle in *Z. mobilis* (after Seo et al. [12]) showing the potential for redirection of its rapid and efficient glucose metabolism to TCA intermediates by the use of "knockout mutants". Missing reactions are shown by *dotted lines*

4.3
Enzyme Based Biotransformations

4.3.1
Sorbitol/Gluconate Production

The production of sorbitol by *Z. mobilis* when grown on sucrose or a mixture of glucose and fructose has been reported earlier by several groups [94, 95]. In subsequent studies on the mechanism of sorbitol production, an enzyme complex was identified by Leigh et al. [96] which was capable of oxidizing glucose to gluconic acid concomitant with the reduction of fructose to sorbitol. This enzyme was described as a glucose-fructose oxidoreductase with a tightly coupled (non-dialyzable) co-factor identified as NADP [97]. The mechanism for sorbitol/gluconic acid production and the associated enzymes are shown in Fig. 8 with the pathway from gluconate to ethanol not being functional if cells of *Z. mobilis* are fully permeabilized. As shown in Fig. 8, the possibility exists also of producing a mixture of sorbitol and gluconolactone if gluconolactonase activity is deleted.

Kinetic studies have been reported for a 60% sugar solution (300 g L^{-1} glucose and 300 g L^{-1} fructose) using toluene-treated permeabilized cells of *Z. mobilis* in which a sorbitol concentration of 290 g L^{-1} and a gluconic acid concentration of 283 g L^{-1} were achieved after 15 h in a batch process [98]. A continuous process with immobilized cells was developed with only a small loss of enzyme activity (less than 5%) evident after 120 h. With a strongly basic anion exchange resin and a buffer system at pH = 9.0, good separation of sorbitol and gluconic acid was achieved. Subsequent studies using immo-

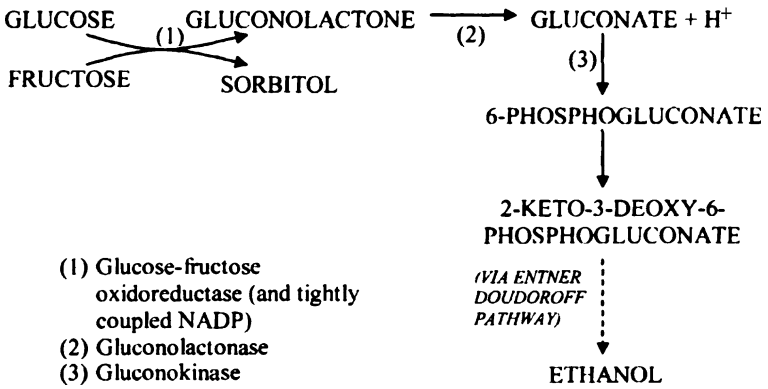

Fig. 8 Mechanism for rapid and efficient conversion of glucose to gluconic acid and fructose to sorbitol via action of glucose-fructose oxidoreductase (and tightly coupled NADP) and gluconolactonase in toluene treated cells of *Z. mobilis* ZM4

bilized (permeabilized) cells in a membrane bioreactor have confirmed the potential of this process for rapid and efficient product formation [99].

4.3.2 Pharmaceutical Intermediates and Fine Chemicals

R-phenylacetylcarbinol (PAC), an intermediate in the production of ephedrine and pseudoephedrine, is currently produced by the controlled addition of benzaldehyde to an actively growing culture of yeast (usually *Saccharomyces cerevisiae*). A decarboxylation/condensation biotransformation is effected by pyruvate decarboxylase (PDC) between pyruvate produced by the yeast and added benzaldehyde (see Fig. 9). Using this traditional process, 12–15 g L^{-1} PAC is usually produced in 10–12 h with a yield of 70% theoretical based on benzaldehyde [100].

Confirmation of PAC production from benzaldehyde and pyruvate using purified PDC from various sources, including *Z. mobilis*, *S. carlsbergenis*, *S. cerevisiae*, *S. fermentati* and *S. delbrueckii*, was demonstrated by several groups during the late 1980s to mid 1990s [101–105]. Bringer-Meyer et al. [106] isolated and characterized PDC obtained from *Z. mobilis*. By comparison with yeast PDC (*Saccharomyces* sp., *Candida* sp.), bacterial PDC (*Zymomonas* sp.) had a lower benzaldehyde affinity and was inhibited more strongly by benzaldehyde, even though its affinity for pyruvate was similar or higher than that of yeast PDC.

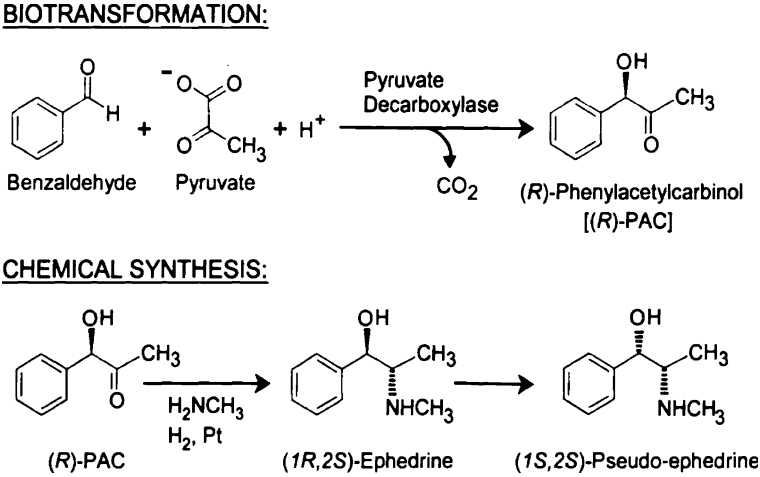

Fig. 9 Mechanism for production of pharmaceutical intermediate R-PAC from benzaldehyde and pyruvate via decarboxylation and condensation using an enzymatic process based on pyruvate decarboxylase present in fungi, yeasts and bacteria (including *Z. mobilis*)

However, interest in PDC from *Z. mobilis* continued due to its greater stability than yeast PDC at room temperature with an enzyme half-life in the absence of benzaldehyde of over 100 h [107, 108]. Unlike yeast PDC, it is also able to utilize the lower cost acetaldehyde as an alternative substrate to pyruvate for production of PAC [109]. Advances in site-directed mutagenesis techniques have facilitated the production of mutant PDC from *Z. mobilis* with greater carboligase activity and higher stability towards acetaldehyde [110]. This mutant enzyme, designated PDCW392M, resulted from replacement of the bulky tryptophan residue 392 with methionine. A continuous process with PDCW392M was used in a biotransformation process for conversion of acetaldehyde and benzaldehyde to PAC in an enzyme membrane reactor. A volumetric productivity (space-time yield) of 81 $g\,l^{-1}\,day^{-1}$ was reported with final PAC concentration of 22 mM and molar yields of 45% (initial substrates), based on 50 mM reaction mixture of both aldehydes [111, 112].

In further studies by Rosche et al. [113], a biphasic enzymatic biotransformation system for production of PAC from acetaldehyde and benzaldehyde with *Z. mobilis* PDCW392 was evaluated. Higher concentrations of benzaldehyde and PAC in the organic phase (octanol) provided protection for the aqueous phase PDC. As a result, a specific PAC production of 11 mg PAC U PDC^{-1} was achieved compared with 1.2 mg PAC U PDC^{-1} in the absence of an organic phase. A similar two-phase system has been developed subsequently for conversion of pyruvate and benzaldehyde to PAC using PDC from yeast (*C. utilis*) with higher concentrations and productivities being attained [114, 115].

A similar aqueous/organic two-phase system has been used also to screen a number of yeasts and bacteria for the enantio-specific reduction of the alpha, beta-unsaturated carbon bond in citral to produce citronellal [116]. In comparison to the bacteria tested, the eukaryotes showed at least 5-fold lower citral reductase activities. Bacterial strains were found to produce the (S)-enantiomer of citronellal preferentially with ee values > 99% for *Z. mobilis* and 75% for *Citronella freundii*. The possible use of a *Z. mobilis* biofilm bioreactor for production of other fine chemicals has been proposed also [117] as it has been demonstrated that increased tolerance to aromatic substrates such as benzaldehyde can occur with such a bioreactor.

5 Discussion and Conclusions

As outlined in the earlier reviews and summarized in Table 3, wild-type strains of *Z. mobilis* (and their mutants) can convert simple sugars to ethanol at faster rates and higher yields compared to yeasts. However, the ethanol industry has traditionally used yeasts, and despite the apparent advantages of *Z. mobilis*, there appears to be little incentive for change with sugar and

Table 3 Characteristics of *Z. mobilis* for production of fuel ethanol and higher value products

1. Considerably faster specific rates of sugar uptake and ethanol production (specific rates 2–3 times faster than yeasts).
2. Higher ethanol and lower biomass yields compared to yeasts due to different carbohydrate metabolism (Entner–Doudoroff vs. glycolytic pathway).
3. Higher reported productivities (120–200 $g\,L^{-1}h^{-1}$) in continuous processes with cell recycle (maximum reported values for yeasts are 30–40 $g\,L^{-1}\,h^{-1}$).
4. Simpler growth conditions. *Z. mobilis* grows anaerobically (not strict anaerobe) and does not require the controlled addition of oxygen to maintain cell viability at high ethanol concentrations.
5. Ethanol tolerance comparable if not better than yeasts. Ethanol concentrations of 85 $g\,L^{-1}$ (11% v/v) reported for continuous culture and up to 127 $g\,L^{-1}$ (16% v/v) in batch culture.
6. Laboratory scale studies with strains of *Z. mobilis* over many years in controlled fermentations (pH = 5.0, $T = 30\,°C$) have not revealed any significant contamination or bacteriophage infection problems.
7. The wide range of techniques developed for the genetic manipulation of bacteria (such as *Escherichia coli*) can be applied to developing recombinant strains of *Z. mobilis* and/or their metabolic engineering.
8. Integrant rec strains of *Z. mobilis* available for efficient ethanol production from glucose, xylose and arabinose. Ethanol concentrations above 60 $g\,L^{-1}$ in 48 h reported for medium containing 65 $g\,L^{-1}$ glucose, 65 $g\,L^{-1}$ xylose.
9. Sequencing of ZM4 genome now provides information for its metabolic engineering for additional higher value products (e.g., succinic acid).
10. Potential for use of its enzymes for fine chemical biotransformations.

starch-based raw materials. Some of the reasons lie in the concerns that *Z. mobilis* may be less robust than yeast and more susceptible to contamination in large-scale processes, as well as the lack of ethanol industry experience with large-scale bacterial fermentations. In addition, an established feed market exists for the high protein yeast by-product (as dried distiller's grains) and any new market for a high protein by-product from a Zymomonas process would need to be established. The key issues and alternative capabilities are summarized in Table 4.

The construction of recombinant strains of *Z. mobilis* able to use the additional C5 sugars xylose and arabinose have now opened up new opportunities as illustrated by the recently announced Dupont/Broin partnership to develop a Zymomonas-based process for conversion of corn stover to ethanol [43]. In an Integrated Corn Biorefinery (ICBR), this would be associated with conversion also of the corn starch to higher value products (e.g. to 1,3-propandiol using recombinant strains of *Escherichia coli*). Experience with large-scale recombinant bacterial fermentations could provide a future platform as well for an increased range of higher value products generated via the metabolic engineering of micro-organisms such as *Z. mobilis* which are capable of both rapid and highly efficient sugar metabolism.

Table 4 Possible reasons for non-commercialization of Zymomonas process for ethanol production from sugar and starch-based raw materials

	Issue	Comments
1.	Yeast is established ethanol producer for sugar and starch-based fermentations.	Established industry practice outweighs potential advantages of higher productivities and yields with Zymomonas-based process. No glycerol by-product with latter process may be potential advantage in minimizing 'fouling' problems during distillation.
2.	Yeast is more robust in terms of tolerance to inhibitors, salts and low pH conditions.	Both yeast and *Z. mobilis* influenced by inhibitors although *Z. mobilis* is more sensitive to high salt concentrations (e.g. in molasses) and low pH (less than 3.5). Inhibitor resistant strains of *Z. mobilis* have been isolated from lignocellulosic hydrolysates.
3.	Yeast has high protein by-product value in dried distiller's grain (DDG) for animal feed supplementation.	*Z. mobilis* is a GRAS organism and has higher crude protein content (65–70%) than yeast (50–55%). Niche nutritional/high protein feed market would need to be developed for *Z. mobilis* following animal feeding trials.
4.	Contamination control (e.g., with lactic acid bacteria) is easier to achieve with yeast using low pH conditions.	Laboratory experiments in continuous culture (pH = 5.0) have shown that *Z. mobilis* is very resistant to contamination once fermentation established (presumably due to much higher competitive rates of sugar uptake). High density cultures (e.g. use of flocs) would further minimize any contamination problems and facilitate high productivity fermentations. No evidence reported of any bacteriophage contamination with *Z. mobilis*. Evidence of high restriction enzyme activity in some strains.
5.	Lack of industry experience as well as regulatory issues with large-scale bacterial fermentations including those with recombinant bacteria.	Experience now being gained by industry with large-scale bacterial fermentations (e.g. production of 1,3-propandiol using rec *E. coli*). Similar regulatory issues would apply for use of recombinant yeasts with lignocellulosic hydrolysates.

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Coproduction of Bioethanol with Other Biofuels

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Abstract Large scale transformation of biomass to more versatile energy carriers has most commonly been focused on one product such as ethanol or methane. Due to the nature of the biomass and thermodynamic and biological constraints, this approach is not optimal if the energy content of the biomass is supposed to be exploited maximally. In natural ecosystems, biomass is degraded to numerous intermediary compounds, and we suggest that this principle is utilized in biorefinery concepts, which could provide different fuels with different end use possibilities. In this chapter we describe one of the first pilot-scale biorefineries for multiple fuel production and also discuss perspectives for further enhancement of biofuel yields from biomass. The major fuels produced in this refinery are ethanol, hydrogen, and methane.

We also discuss the applicability of our biorefinery concept as a bolt-on plant on conventional corn- or grain-based bioethanol plants, and suggest that petroleum-base refineries and biorefineries appropriately can be coupled during the transition period from a fossil fuel to a renewable fuel economy.

Keywords Biorefinery · Fuel cells · Hydrogen · Methane · Reforming

1 Introduction

Traditionally, the development of biological processes to transform biomass to more versatile energy carriers has focused on the production of one energy carrier, either hydrogen, methane, or ethanol. Among these products, only methane is released from the conversion of organic matter in nature; both hydrogen and ethanol are intermediates during anaerobic degradation and are further metabolized to methane in nature [1]. The production of these two energy carriers, therefore, demands a physical separation of individual processes in the anaerobic degradation chain, or the use of defined microbial cultures under controlled conditions. This can be carried out in a biorefinery, which is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass [2, 3]. The biorefinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum.

Instead of concentrating on the biological production of only one energy carrier, the simultaneous production of hydrogen, methane, and ethanol leaves the possibility to optimize the exploitation of the specific energy carriers to suit specific needs, corresponding to the current use of specific fossil fuels for specific purposes. Hydrogen can for instance be used in fuel cells for urban transportation. Ethanol can be used in fuel cells in rural areas, and methane can be used in fuel cells for local electricity and heat production in fuel cells or micro-turbines [4]. Although the fuel cell technology was developed initially for molecular hydrogen, this technology is in rapid progression, and fuel cell systems dealing with more complex compounds such as ethanol are currently being developed [5, 6].

Despite the obvious advantages of combining the production of different energy carriers, only a few concepts have been published. Common to the known concepts is a much better exploitation of the biomass by suiting specific microbiological processes to the conversion of different fractions of the substrates to different fuels. The different processes are thereby exploited in an additive sequential fermentation, transforming most of the energy available in the substrate to usable energy carriers. Furthermore, biorefineries might be considered as more environmentally friendly processes since process water and nutrients from the different processes can be recirculated, and waste production can be kept minimal [4].

By producing multiple products, a biorefinery can also take advantage of the differences in biomass components and intermediates and maximize the value derived from the biomass feedstock. A biorefinery might, for example, produce one or several low-volume, but high-value, chemical products and a low-value, but high-volume liquid transportation fuel, while generating electricity and process heat for its own use and perhaps enough for sale of electricity. The high-value products enhance profitability, the high-volume

fuel helps meet national energy needs, and the power production reduces costs and avoids greenhouse-gas emissions.

2 Hydrogen Production

Biological conversion of biomass to hydrogen either proceeds through photofermentation or dark fermentation. In dark fermentation the yield is only 10–20% of the potential hydrogen amount that theoretically can be derived from organic matter ([7] and Westermann P, Jørgensen B, Lange L, Ahring BK, Christensen CH (2007) Int J Hydrogen Energy (accepted for publication)). Typical hydrogen yields are from 0.52 mol H_2/mol hexose, when molasses was the substrate in a batch culture of *Enterobacter aerogenes* [8], to 2.3 mol when glucose was the substrate in continuous culture of *Clostridium butyricum* [9]. Besides the low hydrogen yield, a major problem of fermentative hydrogen production is hydrogen-consuming microorganisms such as methanogens and acetogenic bacteria. In these processes, hydrogen is inevitably converted into methane or acetate, respectively, unless the responsible microorganisms are excluded by sterilization of the biomass before fermentation and inoculation with specific hydrogen-producing microbes, or the process is carried out under conditions adverse to the hydrogen utilizers. A combination of biohydrogen production with fuel cell technology is, however, rather straightforward since the fuel cell technology is available [10]. An upgrading of produced gases might be necessary before they are introduced into the fuel cells [11].

As a stand-alone process, fermentative hydrogen production from biomass is currently not feasible due to the low yield attained.

3 Methane Production

In open anaerobic ecosystems where biomass is not sterilized, the degradation carried out by ubiquitous microorganisms normally follows a rather well-defined pathway as shown in Fig. 1. If no inorganic electron acceptors such as sulfate or nitrate are present, methane is the inevitable terminal biofuel product since all intermediates from the fermentative bacteria can be degraded to methane, carbon dioxide, and water. The natural end products of the fermentative bacteria in such open systems are short-chained volatile fatty acids, hydrogen, and carbon dioxide. Alcohols are only formed in small amounts. Approximately 90% of the energy of the converted biomass is conserved in the end products, and only 10% is used by the fermentative bacteria [1]. In the terminal formation of methane from the fermentation

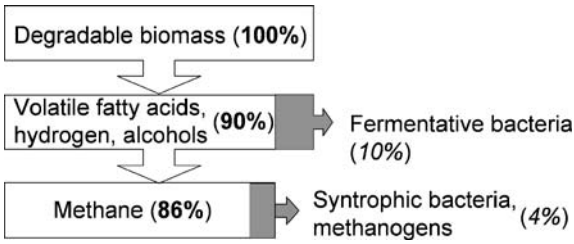

Fig. 1 Degradation pathway and available energy to participating microorganisms and in intermediates and end products during anaerobic degradation of organic matter. The percentages refer to residual energy in substrate and fermentation products (*in bold*), and to the energy used by the microorganisms (*in italics*)

products, the biomass carbon is sequestered completely to the most oxidized (CO_2) and the most reduced (CH_4) states. Only 4% of the original biomass energy is utilized by the terminal link, leaving 86% of the original energy content in the formed methane (Fig. 1), which constitutes the sound energetic rationale for the extensive exploitation of the biogas process. The obligate biology leading to methane formation has an intrinsic stability governed by thermodynamics, which ensures that methanogenesis proceeds within a wide spectrum of physical and chemical conditions. In most methanogenic fermentations the methane yield lies close to the theoretical maximum of 3 mol of methane per mol of glucose, calculated from the Buswell equation [12, 13]:

$$C_aH_bO_c + (a - b/4 - c/2)H_2O \rightarrow$$
$$(a/2 - b/8 + c/4)CO_2 + (a/2 + b/8 - c/4)CH_4$$

4 Production of Biofuels Using the Maxifuel Concept

We have combined biological production of ethanol, hydrogen, and methane in the Maxifuel concept (Fig. 2). The concept is designed to address the major barriers for bioethanol production from lignocellulosic materials. The overall process outline has been defined to yield the maximum amount of biofuels per unit of raw material and to increase the process benefit by utilization of the residues for further energy conversion and by-product refining. The main product is bioethanol for use as transportation fuel and emphasis has been on optimizing ethanol production. The supply and efficient conversion of the raw material is the major economic burden of bioethanol production and full and optimized use of the raw material is a key to success. Production of other biofuels such as methane and hydrogen, and other valuable by-products such as

a solid fuel from the parts of the biomass not suitable for ethanol production, adds full value to the overall process. The concept exploits an environmentally friendly way of producing bioethanol where recirculation and reuse of all streams produced in the process are integrated into the process. This is in contrast to most other bioethanol process schemes where water has to be added continuously and toxic waste water is left after the process. The basic ideas of producing biogas along with bioethanol and then to recycle the process water, or part of the process water, within the process are patented [14]. A combination of these innovative ideas along with the best available technologies has ensured an economic feasibility with a competitive advantage over other concepts. The development of the optimized process of bioethanol production from lignocellulosic biomass can be further integrated into a conventional bioethanol production where corn or grain fibers will be a residue of low value. Conversion of this fraction into ethanol can increase the productivity by up to 20% along with an improvement of the protein feed produced in the process (Fig. 3).

The Maxifuel concept is patented and consists of the following process steps (Fig. 2):

- Pretreatment
- Hydrolysis
- Fermentation of C6 sugars
- Separation
- Fermentation of C5 sugars
- Anaerobic digestion of process water and recirculation

All fermentable carbohydrates in the raw material are converted into ethanol and hydrogen, while much of the unused fraction such as residues from the

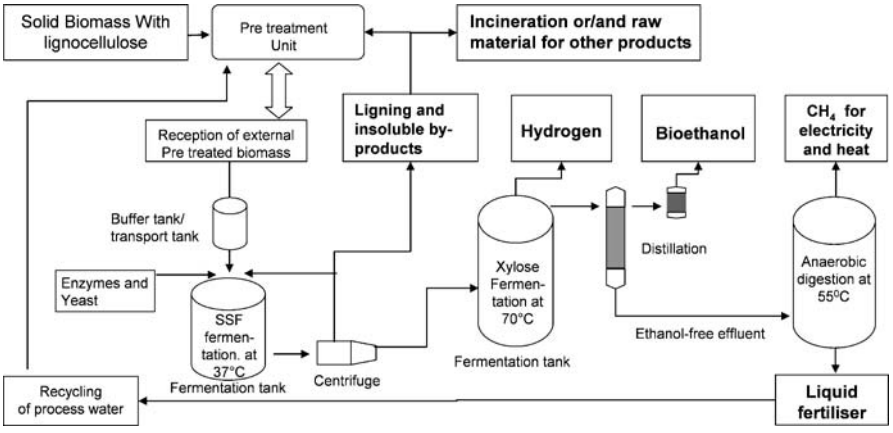

Fig. 2 Flow sheet of the Maxifuel concept. All major processes and process streams from solid lignocellulosic biomass to ethanol, hydrogen, and methane are shown

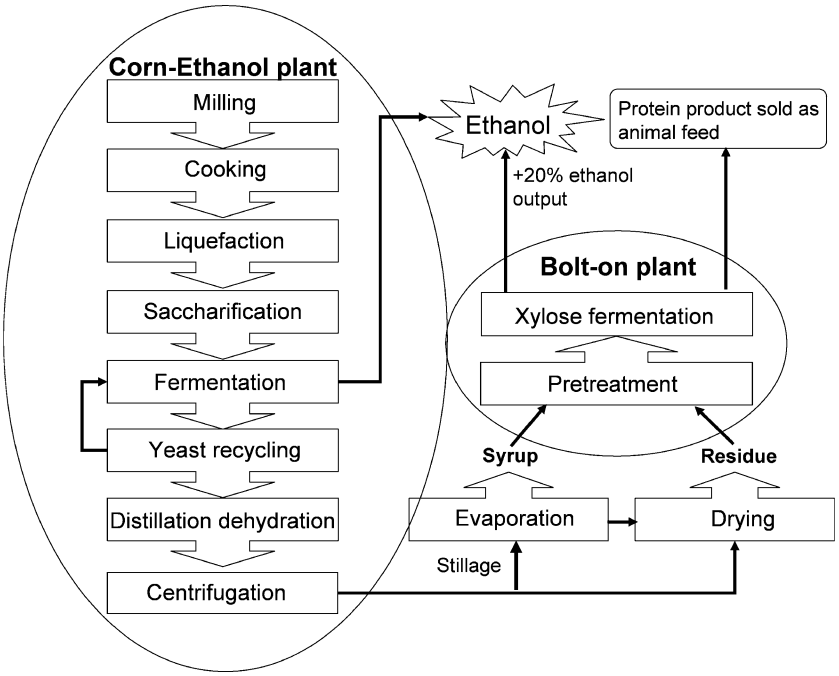

Fig. 3 Increasing the ethanol yield from a conventional corn-to-ethanol plant by bolting on a pretreatment and xylose fermenting unit. The ethanol output is increased by 20%

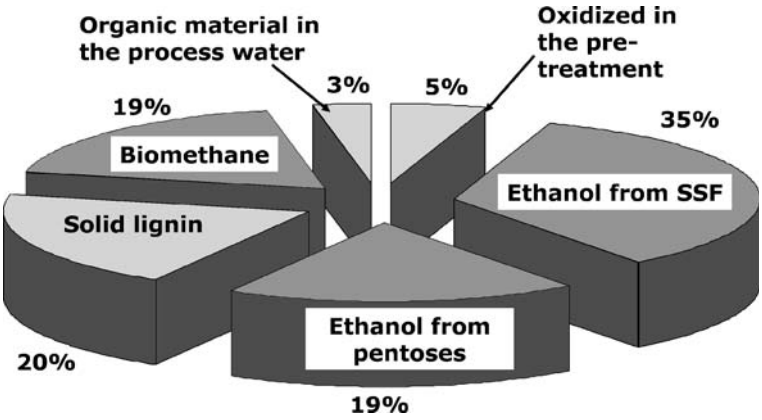

Fig. 4 Mass balance based on COD of different products from the Maxifuel processes. The percentage values represent the relative contribution to the total COD

pretreatment and cells are converted into methane. Up to 19% of the input material will be separated out as a solid, which can be used for combustion (Fig. 4). If desired, this fraction can be increased and, if unwanted, this frac-

tion can be recirculated to the pretreatment unit and used together with fresh raw material.

4.1 Pretreatment

Ethanol production from lignocellulosic biomass has to include a pretreatment more intensive than those used in processing sugar and starch-rich biomass in order to release the sugar compounds contained in the biomass. Agricultural residues like wheat straw or other types of biomass derived from plant material contain lignin, which is constructed to resist microbial attack and to add strength to the plant. Pretreatments are used to open the biomass by degrading the lignocellulosic structure and by partially hydrolysing the substrate. Current pretreatment methods, however, contribute to 30–40% of the total costs of bioethanol production from lignocellulosic biomass. The National Renewable Energy Laboratories (NREL) estimates that in an Nth generation plant (mature technology), feedstock handling and pretreatment would account for approximately 20% of the total ethanol production costs [15].

Several pretreatment methods have been developed [16] (see also Zacchi, this volume). However, in all methods the biomass concentrations need to be higher than 20% dry weight to ensure a suitable ethanol concentration for the subsequent distillation process. A new patented pretreatment process, Wet-ox explosion (WE), has been developed in our laboratory combining steam-explosion and wet oxidation using small amounts of oxygen [17]. The optimal combination of process parameters such as temperature (170–200 °C), pressure (12–30 bar), amount of oxygen addition, and residence time (2–15 min) has been tested. Depending on the biomass material used, the method will yield variable sugar yields but overall the results show that the method will be efficient and cost-effective for opening of most major biomass materials such as straw, corn stover, bagasse, and woody materials. Table 1 shows the apparent advantages and disadvantages of this pretreatment method.

Table 1 Advantages and disadvantages of Wet-Ox-Explosion

Advantages	Disadvantages
Fast and efficient	Requires water supply
No emission products	Advanced technology
Low heat consumption	No standard equipment
No detoxification	Only tested on pilot scale
Easily convertible substrates	
No waste products	

4.2 Hydrolysis

The hydrolysate released from the pretreatment is typically treated with enzymes in order to break down cellulose and hemicellulose into hexoses and pentoses that are then further fermented to ethanol. Enzyme costs are, however, generally high, so that the search for new enzymes with high efficiency that can be produced at low costs is the key to overcome the bottleneck of this process step [18] (see also Viikari, this volume). Another possible way to reduce treatment costs is to implement recycle loops in order to feed backwashed enzymes into the vessel of enzymatic hydrolysis [19, 20]. Enzymes could further be produced at the plant using a stream of the pretreated material in an on-site enzyme production.

The hydrolysis step is optimized by performing the enzyme treatment together with yeast fermentation of glucose (simultaneous saccharification and fermentation, SSF). The temperature optimum of the enzymes is, however, often higher than the optimum for yeast. This can depress the advantages of SSF compared to separation of the two processes.

4.3 Separation

Lignin is separated out after glucose fermentation in the Maxifuel concept. Using a filter-type separator, it is possible to obtain the high dry weight lignin necessary to avoid simultaneous removal of xylose and ethanol still present in the liquid phase after initial hydrolysis and fermentation.

4.4 Fermentation

Biomass or agricultural residues consist of the polymers cellulose, hemicellulose, pectin, protein, and lignin. Of the carbohydrate monomers, xylose is second-most abundant after glucose in most plant cell walls [21]. Because the raw material cost is > 50% of the overall cost of the ethanol process, fermentation of xylose is needed to improve the yield and lower the production cost of ethanol since many biomasses and agricultural wastes contain xylose, in the order of 10–40% of the total carbohydrate mass. Fermentation of both xylose and glucose is therefore crucial to reduce the costs of ethanol production from lignocellulosic raw materials.

The baker's yeast *Saccharomyces cerevisiae* is a desired process organism for fuel ethanol production due to its extensive use in current large-scale industrial ethanol production processes. Also, the excellent ethanol productivity and tolerance towards ethanol and the inhibitors found in biomass

hydrolysates are important reasons for using this organism, even though its natural xylose utilization capability is poor [22].

In the Maxifuel concept, a pentose and hexose fermenting thermophilic microorganism *Thermoanaerobacter* BG1 is used to ferment the residual sugars in the hydrolysate left after yeast fermentation [23]. Similar to the industrial yeast strains, the thermophilic microorganism is able to grow under the harsh conditions provided by the hydrolysate whilst fermenting sugars efficiently. This genetically modified strain has been shown to produce 38.7 g/L or 5.4% v/v of ethanol in a continuous system running directly with non-detoxified lignocellulosic hydrolysate material. The yield from the process is 0.40 g/g total influent sugar or 78% of the theoretical possible value, and productivity is 0.85 g/L/h. The strain is tolerant to 7% of ethanol and higher dry weight in the pretreatment could be used for reaching this concentration.

Furthermore, it grows in temperatures of up to 75 °C, which eases the distillation of ethanol from the reactor. Operation of the fermentation process at thermophilic conditions counteracts contamination by other bacteria, which is generally a problem for mesophilic yeast fermentation. During the residual sugar fermentation, between 0.5 and 1.1 mol of hydrogen/mol of substrate is produced. This is in the same magnitude as hydrogen yields from dedicated dark fermentation of complex substrates such as sugar beet extract (1.0–1.7 mol hydrogen/mol substrate) [24] and molasses (0.52–1.58 mol hydrogen/mol substrate) [8]. BG1 and all its mutants are covered by different patent applications.

To optimize the feasibility of the bioethanol production process the thermophilic fermentation is conducted in an immobilized reactor system. The immobilization of the fermenting organism in an up-flow reactor brings an array of important traits to the fermentation process like increased ethanol tolerance, high substrate conversions, and decreased sensitivity towards process imbalances.

4.5 Waste Water Treatment

The effluent from bioethanol production still contains a large amount of organics that are not composed of carbohydrates. Anaerobic digestion (AD) has been used for a long time to treat organic waste streams with a high concentration of organic matter. The benefits of anaerobic treatment are stabilization of the waste stream, the high reduction of organic matter, and the production of methane, which can be used as energy source [25]. This gives an overall positive energy balance of the waste treatment process compared to aerobic waste treatment. The income from the methane produced after bioethanol production constitutes a value corresponding to a lowering of the ethanol production price by 34%.

The effluent from the fermentation step of bioethanol production contains low-molecular weight lignin degradation products primarily generated during the physical-chemical pretreatment. These aromatic compounds are generally difficult to degrade under anaerobic conditions. Furthermore, a repeated reuse of the process water has the potential to cause a build up of these fermentation inhibitors. It is therefore important to achieve an anaerobic purification technology that is able to remove these compounds from the process water. Experiments in our laboratory have shown that all problematic organic components can be removed in the anaerobic step. The low hydraulic retention times and the removal of organics are of great importance, looking at the overall process feasibility [26].

The Maxifuel concept has been implemented at pilot scale at the Technical University of Denmark, DTU (Fig. 5) and the concept is planned to go into demonstration phase in 2008.

Fig. 5 The pilot plant at DTU. **a** Inlet. **b** Fermentation tanks (2700 L each). **c** Fermenters and holding tanks. **d** Distillation tank

The plant is dimensioned to convert 150 kg dry biomass/day, and consists of 17 tanks (fermentation, reactors, and holding tanks). The ethanol fermentation takes place in two 2700 L fermenters. The plant includes all processes from straw to ethanol, and was brought into operation in the autumn of 2006.

4.6 Bio/Catalytic Refineries

A further development of biorefineries is the use of hybrid techniques combining biological conversion with catalytic downstream processing (Westermann P, Jørgensen B, Lange L, Ahring BK, Christensen CH (2007) Int J Hydrogen Energy, accepted for publication). For instance, highly efficient autothermal reformers capable of converting 1 mol ethanol to 5 mol hydrogen have recently been demonstrated [27]. Since 2 mol of ethanol can be achieved for each sugar molecule, the hydrogen yield of this two-step process is 83% of the theoretical maximum, compared to the 10–20% achieved by direct hydrogen fermentation. Hydrogen produced in the thermophilic ethanol fermentation process described above would add to this yield, approaching the theoretical maximum yield of 12 mol hydrogen/mol monosaccharide.

Hydrogen has been suggested as a future energy carrier to succeed the fossil fuel era [28]. The introduction of downstream catalytic conversion of biofuels leaves the possibility of combining a less complex fuel handling technology (ethanol instead of hydrogen) for transportation purposes with all the benefits of the fuel cell technology. In the transition period before a hydrogen-based energy economy has been realized, a gradual change to the use of renewable energy can be facilitated by the use of catalytically converted biofuels in existing internal combustion engines. Although ethanol in even high ethanol:gasoline mixtures can be used for ground transportation with few modifications of the engines, biogasoline produced by catalytic conversion of methane and bioethanol will have potential use as a high energy alternative for aviation and air transport. If these transportation means are sustained in the future, the availability of safe liquid fuels with high energy content storable under ambient conditions is a prerequisite.

4.7 Integrating Conventional and Bio/Catalytic Refineries

Despite the high interest and rapid development of biomass-based fuels, it is not anticipated that oil-based fuels will be completely replaced by renewable fuels in a foreseeable future of 50 years [29]. Conventional refineries converting crude oil to fuels, starting chemicals, and other products, therefore, will operate decades ahead. In the biorefinery literature it has been a common practice to compare conventional petroleum-based refineries with biorefiner-

ies [30, 31], but to our knowledge a combination of the two refinery types has not previously been suggested. Integrating conventional and bio/catalytic refineries in the transition period from petroleum-based to biomass-based refineries might lead to several potential synergies with respect to processes, chemicals, and logistics.

Several process streams of intermediates, wastes, and heat from a conventional refinery might be utilized in a biorefinery (Fig. 6). Cooling water and some effluent water streams can be used as process water in the biorefinery. A conventional refinery has big volumes of low temperature energy, which could be exchanged and used as process energy in the biorefinery.

Products from the biorefinery can be used as input for various conventional refinery processes. As discussed elsewhere in this book, ethanol is mainly used as a blending component in gasoline products. Integrating the two refineries will improve the logistics of this mixed fuel production.

Hydrogen produced from fermentation processes of the biorefinery can for instance be used in the traditional hydrogenation processes of a conventional refinery. Methane produced in the biorefinery can be used as fuel gas, but also as a raw material for further catalytic reforming, producing more hydrogen. It could also be used for production of H_2/CO (synthetic gas), which is a feed gas for gas-to-liquids or methanol production. Introducing catalytic steps between the two refineries might further enhance the beneficial coupling since the hydrocarbon output from catalytic conversion of methane and ethanol might serve as a substrate for further refining and modification in the conventional refinery process streams.

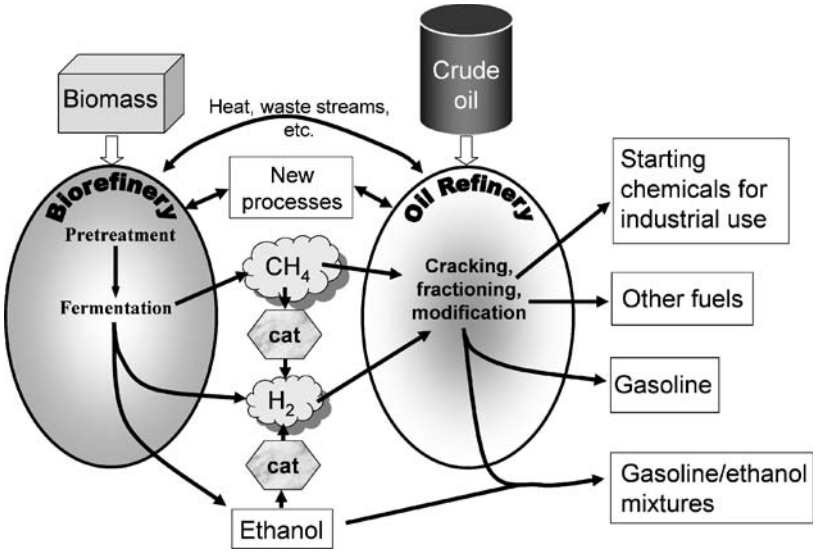

Fig. 6 Combination of bio/catalytic refinery and petroleum-based refinery. *cat* indicates chemical catalytic conversion

5 Conclusion

In this chapter we have shown the potential of producing more than bioethanol out of biomass raw material. While carbohydrates will be the precursor for ethanol production, the rest of the biomass can be used for production of other fuels. By this integration the net energy production will increase and the CO_2 reduction will be higher than in biorefineries without the integration. Furthermore, reuse of water and nutrient will allow for a more sustainable process with much lower environmental impact on the ecosystem.

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Process Engineering Economics of Bioethanol Production

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Abstract This work presents a review of studies on the process economics of ethanol production from lignocellulosic materials published since 1996. Our objective was to identify the most costly process steps and the impact of various parameters on the final production cost, e.g. plant capacity, raw material cost, and overall product yield, as well as process configuration. The variation in estimated ethanol production cost is considerable, ranging from about 0.13 to 0.81 US$ per liter ethanol. This can be explained to a large extent by actual process differences and variations in the assumptions underlying the techno-economic evaluations. The most important parameters for the economic outcome are the feedstock cost, which varied between 30 and 90 US$ per metric ton in the papers studied, and the plant capacity, which influences the capital cost. To reduce the ethanol production cost it is necessary to reach high ethanol yields, as well as a high ethanol concentration during fermentation, to be able to decrease the energy required for distillation and other downstream process steps. Improved pretreatment methods, enhanced enzymatic hydrolysis with cheaper and more effective enzymes, as well as improved fermentation systems present major research challenges if we are to make lignocellulose-based ethanol production competitive with sugar- and starch-based ethanol. Process integration, either internally or externally with other types of plants, e.g. heat and power plants, also offers a way of reducing the final ethanol production cost.

Keywords Bioethanol production · Biomass · Flowsheeting · Process economics

1 Introduction

There is no single process design offering the most cost-efficient way to produce ethanol from biomass. Many factors that affect the desired product have to be taken into consideration. Regarding ethanol production, some of the most important parameters are the capital cost of the plant, the type and cost of raw material, the utilization efficiency of the materials involved in the process and the energy demand. The design of the plant, as well as its individual process steps, must be based on accurate and reliable data. These comprise both physical and chemical data and cost estimation data. It is naturally best to use data gathered from the same or a similar type of plant as the intended one. Most of the data required are available, or can be adapted and used for a new plant design. This is not the situation when lignocellulosic materials are considered as feedstock for ethanol production.

Ethanol has traditionally been produced from sugar cane and sugar beet juice [1] or from various starch-containing materials, e.g. corn or wheat [2–4]. Figure 1 shows a simplified flowsheet of an ethanol production process based on starch-containing materials. Liquefaction of the starch fraction is accomplished by adding hydrolytic enzymes (α-amylases) at temperatures of around 90 °C. After the liquefaction step the starch molecules are further hydrolyzed by the addition of glucoamylases. This produces sugars, which are readily fermented by yeast, e.g. *Saccharomyces cerevisiae*, to ethanol. The main co-product is usually animal feed, consisting of the remaining fraction of the raw material, mainly proteins and fiber, which is sometimes referred to as DDGS—distillers dried grains with solubles [5]. There is considerable experience in starch-based ethanol production, and the technology can be considered mature. The design and cost estimates of new plants are, therefore, rather accurate.

The availability of agricultural land for non-food crops and the limited market for animal feed places a limit on the amount of ethanol that can be produced from starch-based materials in a cost competitive way [6]. Ethanol production from lignocellulosic raw materials, on the other hand, reduces the potential conflict between land use for food (and feed) production and energy feedstock production. The raw material is less expensive than conventional agricultural feedstock and can be produced with lower inputs of fertilizers, pesticides, and energy. Lignocellulosic materials contain about 50–60% carbohydrates in the form of cellulose (made up of glucose) and hemicellulose (consisting of various pentose and hexose sugars), which may be fermented to ethanol, and 20–35% lignin. The latter is the main co-product, which could be used for the production of heat and electricity or, in the longer perspective, for the production of specialty chemicals. There is thus no co-product limitation on the use of lignocellulosic materials for ethanol production. The only limitation is the availability of the raw material and, of course, the production

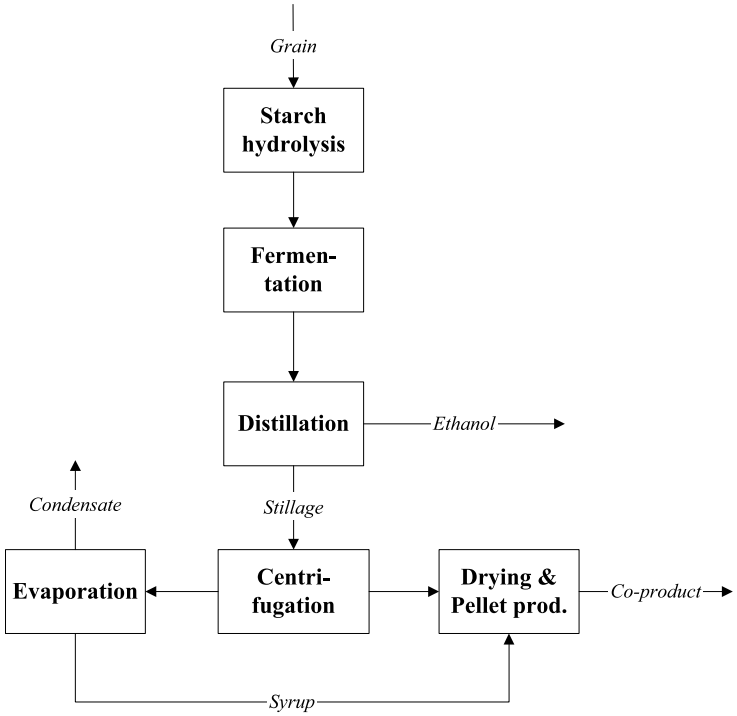

Fig. 1 Schematic flowsheet for the production of ethanol from starch-containing materials

cost. During recent years, there has been a considerable increase in interest in research on and the development of the conversion to ethanol of lignocellulosic materials, such as agricultural and forest residues, as well as dedicated energy crops.

However, in contrast to starch-containing materials, cellulose-containing raw materials, such as forest residues and straw, have not yet been commercialized in the ethanol industry. The reasons for this are several. For instance, there are physical barriers such as:

- the complex structure of lignocellulosic materials, making them recalcitrant to hydrolysis;
- the presence of various hexose and pentose sugars in hemicellulose, making fermentation more difficult; and thirdly,
- the presence of various compounds that inhibit the fermenting organism. These compounds either originate from the raw material itself, e.g. extractives, or are formed during the early process steps, e.g. degradation products of sugars and lignin. This makes it difficult to reach high ethanol concentrations during fermentation, which in turn results in a high energy demand and thus high production cost.

There is a big risk involved in being the first to invest in commercialization of a lignocellulose to ethanol plant and this is the main reason why there is no full-size plant in operation today.

Interest in lignocellulose-based ethanol production has recently brought about action on high political levels. For example, in the USA, the Energy Policy Act of 2005 requires blending of 7.5 billion gallons ($\approx$ 28.4 million m^3) of alternative fuels by 2012 [7] and recently, in his State of the Union Address (Jan 31, 2006), the US President announced the goal of replacing more than 75% of imported oil with alternative fuels by the year 2025 [8]. The major part of this alternative fuel will probably consist of ethanol, and to be able to meet these demands this will have to be largely produced from lignocellulosic materials. In Europe the European Commission plans to progressively replace 20% of conventional fossil fuels with alternative fuels in the transport sector by 2020, with an intermediate goal of 5.75% in 2010 [9]. Bioethanol is also expected to be one of the main means of achieving this goal.

Experience in the production of ethanol from lignocellulosic materials is limited, at least using modern technology. Full-scale plants have only been run occasionally during times of war. Examples are the Bergius process (concentrated HCl) operated in Germany during World War II, and the Scholler process (dilute H_2SO_4), which was used in the former Soviet Union, Japan and Brazil [10]. Thus, design and cost estimation for lignocellulosic-based processes cannot be based on reliable operational experience, but data gathered on lab scale, or at best on pilot scale, must be used. It is true that some of the process steps are of the same type as in a starch-based process, but there are several major differences. For example, the by-products from the various processes are not the same. Some of these are considered valuable co-products, which will contribute to the profit from the process, while others are waste materials that must be dealt with in wastewater treatment plants, or disposed of by other means.

During the past 20 years or so, a great deal of effort has been devoted to research on various areas, such as the pretreatment of raw material, enzymatic hydrolysis of cellulose, including the production of more cost-effective enzymes, and the development of new microorganisms and fermentation techniques to ferment all the sugars available in lignocellulosic materials. An enormous amount of data has been generated (see the work by Galbe, Vikarii, Cherry, Hahn Hägerdal, and Ingram, all in this volume), which today forms the basis for techno-economic calculations. However, although the results may be accurate, there is still a huge scale-up problem involved in going from batch pretreatment reactors on the liter scale, to continuous reactors of several cubic meters, and from 1- to 100-liter fermentors to vessels with a volume of 1000 cubic meters or more. Issues such as material corrosion, rapid heat evolution, excessive foaming, and precipitation of solids and incrustation, which may not even be considered on the lab scale may become serious problems in a full-scale process.

Pilot-scale trials have been run in several places during the past decade. The National Renewable Energy Laboratory (NREL) (Golden, Colorado, USA) has constructed a pilot fermentation facility to test bioprocessing technologies for the production of ethanol and other fuels or chemicals from cellulosic biomass [11]. The Process Development Unit (PDU) of the Bioethanol Pilot Plant was set up to investigate biomass fuel and chemical production processes from start to finish on a scale of about 900 kg day^{-1} of dry feedstock. The plant is, however, not a fully integrated unit that can run continuously.

A 1000 kg day^{-1} plant, using spruce as the raw material, has been in operation in Örnsköldsvik in Sweden since the middle of 2004 [12]. Abengoa Bioenergy Corp. has constructed a pilot plant in York, Nebraska, USA [13] and is now constructing a demonstration scale plant in Salamanca, Spain, with an annual production capacity of 5000 m^3 ethanol. This will be brought into operation at the beginning of 2007 [14]. This demonstration plant, which will be co-located with a 195 000 m^3 y^{-1} starch-based plant, will utilize the straw from wheat and thus contribute to the overall production capacity. Furthermore, Iogen Corp. is operating a pre-commercial demonstration facility, located in Ottawa, Canada, where ethanol is made from agricultural residues [15]. The plant is able to handle up to 40 metric tons of feedstock daily, consisting of wheat, oat, and barley straw, and is designed to produce up to 3 million liters of ethanol annually.

Data from these types of plants will increase the reliability of cost estimates significantly. They can also be used to identify process problems associated with continuous processing, such as the accumulation of toxic substances in various process streams, and fouling of heat exchanger surfaces. However, in most cases this will be proprietary information not available in the scientific literature.

Two process concepts have been investigated more than others regarding ethanol production from lignocellulosic materials. The main difference between the two is the way in which the cellulose chain is broken apart; either dilute sulfuric acid or cellulolytic enzymes are used to hydrolyze the cellulose molecules. Figure 2 shows the main features of a dilute acid hydrolysis process. The raw material is treated with 0.1–3% (w/w) H_2SO_4 at temperatures normally ranging from 160 to 200 °C. It may be advantageous to perform dilute-acid hydrolysis in two steps since the hemicellulose fraction is more easily degraded than is the cellulose fraction. A disadvantage of the dilute acid process is the somewhat low ethanol yield and the necessity of using expensive construction materials that are resistant to corrosion by acid at high temperatures. The acid must also be neutralized, which leads to the formation of large amounts of gypsum, $CaSO_4$, or other compounds that have to be disposed of.

An alternative to acid hydrolysis is enzymatic hydrolysis (Fig. 3). Cellulolytic enzymes are produced by microorganisms and have the ability to cleave off short sugar units from the cellulose chain, as described in detail

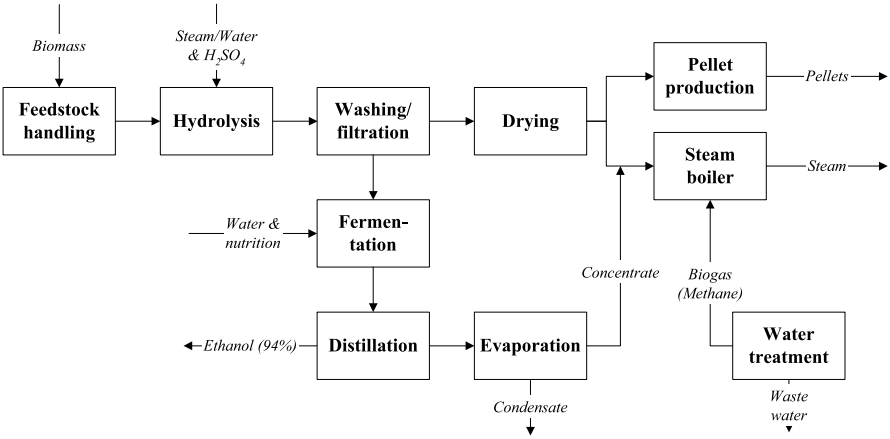

Fig. 2 Schematic flowsheet for the production of ethanol using dilute acid hydrolysis of cellulose-containing materials

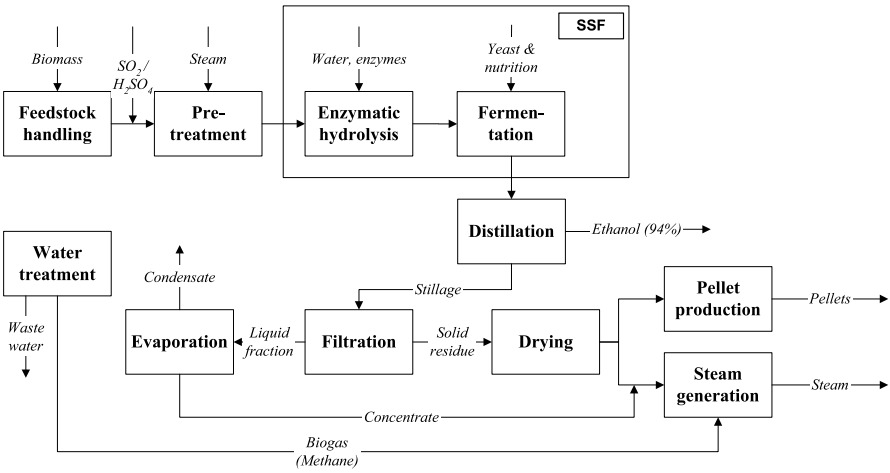

Fig. 3 Schematic flowsheet for the production of ethanol using acid-catalyzed steam pretreatment followed by enzymatic hydrolysis/SSF of cellulose-containing materials

by Vikarii 2007 and Merino 2007 (this volume). The enzymatic process is operated at much milder conditions than the dilute acid process, which is of great importance for several reasons. The yield can be expected to be higher, the construction materials will be less costly and the formation of toxic by-products will also be reduced. However, the enzyme action suffers from being slow if the raw material is not pretreated prior to enzymatic hydrolysis. Pretreatment can be performed in a number a ways. Depending on the type of raw material (hardwood, softwood or agricultural residue) a certain pre-

treatment method can be more or less successful. Pretreatment is described in more detail by Galbe 2007 (this volume). Fermentation can be performed either in a separate fermentor tank, a process configuration normally referred to as separate hydrolysis and fermentation (SHF), or simultaneously with the hydrolysis of the cellulose chains, so-called simultaneous saccharification and fermentation (SSF). If the pentose sugars are also fermented, the process is sometimes referred to as simultaneous saccharification and co-fermentation (SSCF). The downstream processing section is similar for the dilute acid hydrolysis and the enzymatic processes, or at least includes the same process steps (Figs. 2 and 3).

Simulation of processes with the aid of flowsheeting programs is an invaluable tool in studying how changes in process design affect the overall performance of a plant. Plants operating 24/7 cannot be experimented on, since the profit loss may be considerable if an ill-planned test causes standstill for a day or two. By performing "experiments" on a plant using computers the outcome of a design change can be evaluated beforehand, which will make a change in the process less risky.

This work will focus on the process economic aspects of ethanol production from lignocellulosic materials and provide targets for where process improvements should be investigated. The enzymatic process will be considered in detail, as most research over the years has been concentrated on this type of process. However, as mentioned earlier, the process suffers from the fact that process data from large production plants are very scarce. Nevertheless, the data gathered so far on lab and bench scales can be used as input data in flowsheeting programs for comparison of various process alternatives and to help identify bottlenecks in a process. A summary of various published reports and papers will be made. Unfortunately, this is an area that has clearly been neglected by many researchers, since the number of publications is small.

2 Flowsheeting

Flowsheeting programs, e.g. Aspen Plus, HYSYS and ChemCad, may be used to perform rigorous material and energy balance calculations, with the use of detailed equipment models, to determine the flow rates, composition and energy flow for all streams in the process. Because of their flexibility, the programs have many advantages when comparing different process configurations or scenarios in terms of overall efficiency, minimum energy demand or lower production cost. Also, they serve as a powerful tool when performing sensitivity analyses, due to the ease of changing a certain parameter. All flowsheeting programs are based on a modular approach where each module is a mathematical model of a unit operation. The fundamental equations needed

to accurately describe standard process equipment, such as columns (distillation, absorption, etc.), heat exchangers, pumps, reactors and splitters, are normally available as part of the program. The actual simulation is performed by arranging different unit operation modules into a complete flowsheet that represents the process to be simulated.

Construction of a process model in a flowsheeting program can be summarized in the following three steps.

- *Flowsheet definition*: The flowsheet defines the process configuration. It shows all streams entering the system as well as all unit operations and their interconnecting streams. The flowsheet also indicates all product streams that will be determined by the simulation program.
- *Chemical components:* The user must specify all the chemical components to be used in the system. All necessary physical and thermodynamic properties must be defined for each component. Normally, a database containing these properties for a large number of chemical compounds is included in the flowsheeting software. In general, the size of this database, which varies greatly between different simulators, determines the cost of the flowsheeting program. If data for some compounds are missing the user has to define them.
- *Operating conditions:* For every unit operation the user has to specify the operating conditions, such as temperature, pressure, heat duties, etc. In addition, all input streams have to be completely defined. Enough information has to be provided to result in a single steady-state solution based on material and energy balances coupled with phase equilibrium equations.

2.1 Simulation of Ethanol Production from Lignocellulosic Materials

Process simulations cannot replace experiments, but constitute a useful tool in the planning and evaluation of experiments. Furthermore, they highlight factors that are sometimes neglected in experimental studies, for example, the amounts of chemicals needed in the process (catalyst in pretreatment, acid/base for pH adjustment, nutrients and not least enzymes and yeast), which constitute a significant contribution to the production cost. The overall demand of steam, process water and cooling water are other important factors.

Optimization of ethanol production from lignocellulosic feedstock requires a model that includes all the major process steps, since changing the conditions in one process step is likely to affect other parts of the process. Although no full-scale plant based on enzymatic hydrolysis has yet been built, most of the process steps (e.g. distillation, evaporation, drying and incineration) are considered to be technically mature, i.e. their operational performance is well

known. Of course, the application of these unit operations in a lignocellulose-to-ethanol plant still requires to be verified on pilot scale before a full-scale plant can be constructed. However, the ethanol process includes other process steps, which are associated with greater uncertainties regarding design and performance on full scale. This is definitely true for the pretreatment step, irrespective of the pretreatment method chosen, or how it is configured (Galbe, in this volume). It also applies to enzymatic hydrolysis or SSF at high solids concentrations, as well as solid-liquid separation of the stillage.

The modeling of a lignocellulose-to-ethanol process poses a number of unique challenges. In contrast to well-defined systems, such as pure ethanol-water systems, it involves not only vapor and liquid phases but also a solid phase, including atypical compounds like cellulose, lignin and yeast. Therefore, when simulating such a process it is necessary to use a flowsheeting program that is able to handle solid components. In most techno-economic evaluations of the lignocellulose-to-ethanol process that have been performed during the past 10 years, Aspen Plus from Aspen Technologies has been used [16–20]. In Aspen Plus, a separate solid stream is used that does not interact with the liquid phase and never ends up in the vapor phase. There is thus no need to estimate vapor phase data such as heat of vaporization or vapor pressure for components treated as solids (lignin, glucan, yeast, etc.).

3 Process Economics

The number of studies on economic aspects of ethanol production from biomass is rather limited. This depends to a large extent on the fact that the ethanol production from biomass has not yet been demonstrated on commercial scale. The ethanol production cost varies between the studies performed from about 0.13 to 0.81 US$ per liter ethanol, see Table 1. During 2006 the selling price of bioethanol, produced from starch or sugar-based materials, has fluctuated around 0.65 US$ per liter of ethanol with a peak at 1.12 US$/liter [21]. The future selling price will be dependent on demand and availability, which is influenced by political decisions, such as the EU directive mentioned before. Also tax exemptions, e.g. exemption of CO_2 tax and energy tax adopted in Sweden, and protective duty, as that applied in the EU, will impact the pricing for customers.

The large differences in ethanol production costs in Table 1 can be explained by variations in the process design and in the assumptions underlying the techno-economic evaluations. Process variations are due to different conversion technologies, e.g. an enzymatic process with SHF, SSF or SSCF or the use of various types of raw materials. Thus, for meaningful comparison the actual differences have to be identified. The discrepancies that arise due to various assumptions, in many cases, overshadow the actual differences.

Table 1 Some results from various techno-economic evaluations in order of increasing raw material capacity. Costs have been converted from SEK to USD using a conversion factor of 7.0 SEK/USD. However, costs have not been updated by index

Type	Capacity (tons of dry raw material/year)	Capital cost (million US$)	Capacity/ raw material (US$ton^{-1})	Prod. cost (US$L^{-1})	Refs.
Enz-SHF	196 000 (S)	169	862	0.76	[38]
Enz-SSF	196 000 (S)	130	663	0.69	[38]
Enz-SSF	268 000 (H)	64	239	0.34	[25]
Dilute acid	263 000 (H)	67	255	0.36	[25]
Dilute acid	300 000 (S)	186	620	0.53	[37]
Enz-SSF	620 000 (H)	395	475	0.51	[36]
Enz-SSF	658 000 (H)	150	228	0.31	[23]
Enz-SSF	658 000 (H)	150	228	0.34	[24]
Enz-SSF	700 000 (H)	234	334	0.38	[16]
Enz-SHF	700 000 (CS)	197	281	0.28	[26]
Enz-SHF[a]	700 000 (A)	260	371	0.34	[27]
Enz-SSF	700 000 (CS)	186	266	0.26	[30]
Enz-SSCF	1 550 000 (H)	465	300	0.31	[36]
Enz-SSCF	2 738 000 (H)	268	99	0.13	[23]
Enz-CBP	3 110 000 (H)	820	263	0.20	[36]

S = Softwood, H = Hardwood, CS = Corn Stover, Enz = enzymatic A = agricultural residue

[a] Based on Iogen technology

Typical examples are the raw material cost (even if the same raw material is used), plant capacity and investment parameters, e.g. pay-off time and interest on capital. Also, the country in which the proposed plant is assumed to be located is of importance. One of the main influences on the production cost originates from the assumed annual capacities of the ethanol plants, which varied from 196 000 to 3 110 000 tons of raw material. This has a considerable influence on the total production cost (Fig. 4). Another difference is found in the overall ethanol yields assumed, e.g. if pentoses are converted to ethanol or not. Also, changes in process configurations, or a change in the equipment included in the ethanol production process also influences the overall cost, e.g. whether utilities such as process steam and electricity are included. Therefore, care must be taken when comparing ethanol production costs from different studies. However, this does not mean that the economic studies are without value. They give important information about which parts of the process are most costly, and where bottlenecks, which need to be addressed by further research, can be expected.

Most economic studies performed on the enzymatic bioethanol process during the past ten years have been for a configuration using some kind of

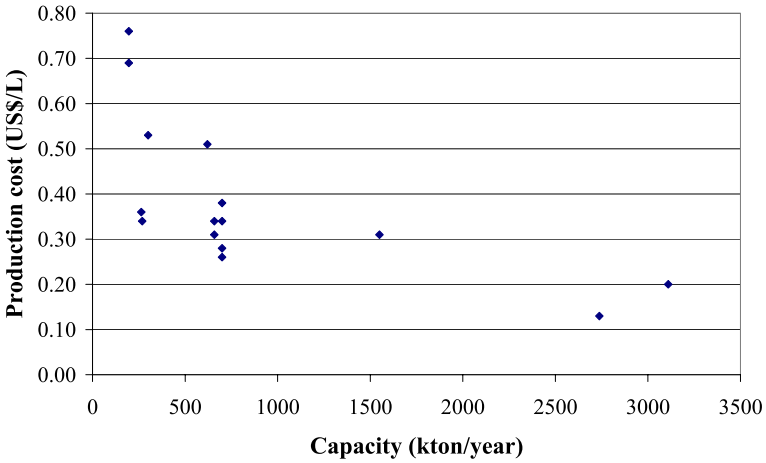

Fig. 4 Production cost vs. yearly raw material capacity in dry tons

steam pretreatment employing an acid catalyst. NREL has for many years been conducting detailed techno-economic evaluations of ethanol production from lignocellulosic materials. In the 1999 report [16] hardwood (poplar) was considered as the raw material and the proposed annual capacity was 700 000 dry metric tons. The following process configuration was assumed. The raw material is pretreated with dilute sulfuric acid at 190 °C for 10 minutes. The liquid hydrolyzate is detoxified by ion exchange and overlimed, after which an SSF step is employed. Some of the slurry following pretreatment is used for enzyme production. In the SSF step the remaining cellulose is converted to glucose and both hexoses and pentoses are considered fermentable. The ethanol is removed from the mash through stripping and the stillage is dewatered by means of centrifugation. The solids, together with the concentrated liquid from the evaporation step, are transferred to a boiler for steam and electricity production. The estimated ethanol production cost was 0.38 US$ L^{-1}. Especially the database of physical properties [22], but also several of the unit operation models from this study, has been used by many other investigators.

Lynd et al. [23] evaluated a process based on dilute acid hydrolysis, pentose fermentation and SSF using hardwood as the raw material, assuming the following procedure. Distillation bottoms are centrifuged and the solid residue, together with methane and sludge from the anaerobic digester, is sent to the boiler where process steam and electricity are generated. The plant capacity was assumed to be 658 000 dry tons per year. The ethanol production cost was estimated to be 0.31 US$ L^{-1}. A techno-economic evaluation based on the same process concept and the same raw material as the above, with an annual capacity of 640 000 tons, was conducted by Stone et al. [24]. This resulted in an estimated production cost of 0.34 US$ L^{-1}. In the study presented

by Lynd et al. [23] a more advanced scenario was also evaluated where future improvements in conversion technology were included. These include, but are not limited to, a higher overall ethanol yield, the use of a microorganism capable of not only fermenting sugars to ethanol but also of hydrolyzing the cellulose (direct microbial conversion), and shorter residence times in the process steps. With these improvements, together with an increased capacity (2 738 000 dry tons per year) the projected ethanol production cost was 0.13 US$ L^{-1}.

A comparative study of an SSF-based process and a process using dilute acid hydrolysis was performed by So and Brown [25]. The SSF process used the same conversion technology as the process evaluated by Lynd et al. [23], while the dilute sulfuric acid step was assumed to be carried out at 180 °C with an acid concentration of 5 g L^{-1}. Estimated production costs at a plant capacity of 25 million gallons of ethanol per year (equivalent to around 260 000 tons of dry raw material per year) were 0.34 US$ L^{-1} for the SSF-based plant and 0.36 US$ L^{-1} for the process employing a dilute sulfuric acid process.

In the NREL report of 2002 [26] the raw material was changed to corn stover. Several changes were also made to the model from 1999. Instead of running SSF, an SHF configuration (including pentose fermentation) was employed, where the saccharification was carried out separately prior to fermentation. The reason for this was to be able to carry out saccharification at a higher temperature than in the fermentation step. The enzyme production step was also removed and it was assumed that the enzymes had to be purchased from an enzyme-producing company at an estimated cost of 0.10 US$ per gallon of ethanol. This represents a projected future cost rather than the present cost. Changes were also made to the separation of solids from the stillage stream. Instead of centrifuges, as suggested in the 1999 report, a horizontal belt filter (of the type manufactured by Pneumapress Filter Corporation, CA, USA) was employed and the concentration of water insoluble solids (WIS) in the filter cake was assumed to be about 50%. The overall ethanol yield increased significantly compared to the 1999 report (from 73.6% to 85.5% concerning cellulose, while 85% yield was assumed from all hemicellulose sugars). The estimated production cost was reduced to 0.28 US$ L^{-1}.

According to a techno-economic evaluation of ethanol production from biomass performed by "SRI Consulting's Process Economic Program" (PEP) [27], the capital investment required for a plant producing ethanol from 2000 metric tons of straw per day would be around 260 million US$. The plant is assumed to produce 190 million liters of ethanol per year, which gives an investment cost of around 1.37 US$ per liter ethanol and 370 US$ per ton raw material. This is about 2.5 times higher than the investment cost of a corn-based ethanol plant with the same feed capacity. On the basis of the ethanol produced, the ratio would increase to above 3 as the yield of ethanol per ton raw material is higher for corn than for lignocellulosic materials.

The highest contribution to the capital cost, 45% of the total, was equipment for the production of heat and electricity for the process and for sale to the grid, wastewater treatment and other utilities. This is not a direct cost of the ethanol production equipment, and in our opinion is often underestimated in most studies on ethanol production cost. Another cost that differs widely between studies is the cost of raw material. This depends on both differences in the type of raw material (agricultural residues, forest residues or energy crops) and on the location of the raw material. According to the Road Map for Agricultural Biomass Feedstock Supply in the US presented by the DOE [28] the goal is to reach a feedstock cost of 30 US$ per dry ton. On the basis of this figure the net raw material cost, i.e. after by-product credit, in the PEP study would be about 0.07 US$ per liter of ethanol, which corresponds to 20% of the total production cost of 0.34 US$ L^{-1}. This production cost is, however, without any profit. The cost of biomass in Sweden, and other European countries is much higher, exceeding 90 US$ per metric ton of dry matter [29], which results in a net raw material cost of about 60 US$ per metric ton. This would have increased the total production cost in the PEP study to about 0.44 US$ L^{-1}. However, according to the PEP study, the main reason for the higher investment cost for biomass-produced ethanol is due to the cost of conditioning and pretreating the biomass to make the cellulose accessible to enzymatic hydrolysis, which was estimated to represent 27% of the total fixed capital.

Eggeman et al. [30] investigated the pretreatment cost in ethanol production from corn stover for five different pretreatment methods: dilute acid, hot water, ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP) and lime. The pretreatment design was based on experimental data from various research groups [31] and was implemented in the Aspen Plus model for a full-scale bioethanol plant previously developed by NREL [26]. The model was based on a corn stover feed rate of 2000 dry metric tons per day. The process configuration was based on pretreatment, SSF, ethanol recovery and internal production of heat and electricity from the syrup and solid residue from the process. The process configuration was identical for all processes except for the pretreatment step. The dilute acid pretreatment process resulted in the lowest ethanol production cost, 0.26 US$ L^{-1} for the base case alternative where oligomers released in the pretreatment and hydrolysis steps were not considered for ethanol production. The production cost includes depreciation, but no income tax or return on capital, to make it comparable to the other costs presented in this review. The total investment cost was estimated to be 185.8 million US$, of which the pretreatment step constituted 25 million US$, i.e. 13.5% of the total. The largest investment cost was for steam and power, 41.8 million US$, which represents 22.5% of the total investment cost.

Two-step pretreatment has been suggested to improve the overall sugar yield in several studies [32–34]. The first step is performed at low severity

to release hemicellulose sugars, which are then removed, followed by the second step at more severe conditions to make the cellulose more accessible to enzymatic attack. Wingren et al. [35] compared the ethanol production cost of two-step steam pretreatment of SO_2-impregnated spruce with that for one-step pretreatment, based on experimental data for pretreatment at optimal conditions. The production plants considered were designed for a yearly capacity of 200 000 tons raw material, and the only difference between the two plants was the pretreatment step. The process was based on SSF of the pretreated material. The two-step process resulted in a higher ethanol yield (see Table 2) and a lower requirement for enzymes. However, due to the higher energy demand and higher capital cost the estimated ethanol production cost was the same, 0.55 US$ L^{-1}. In the most optimistic scenario, where the material from the first step was dewatered to 50% dry matter (DM) without reducing the pressure, and the overall ethanol yield was assumed to be the highest achieved in the experimental work, 77%, the production cost decreased by 5.6% to 0.52 US$ L^{-1}. This shows the potential of the two-step pretreatment process, which, however, remains to be verified in pilot trials.

Hamelinck et al. [36] investigated the effect of expected future improvements in the conversion of biomass to ethanol, with poplar as a model raw material. The paper contains detailed information on the technical and economic data used in the analysis. Three scenarios were investigated: short-term (5 y), middle-term (10–15 y) and long-term (> 20 y). Improvements were mainly expected to be in enhanced pretreatment and bioconversion steps, changing from SSF to SSCF of hexose and pentose sugars and finally Consolidated BioProcessing (CBP), resulting in higher ethanol yield and reduced capital costs. This is based on improvements of both enzyme efficiency and fermentation microorganisms. For CBP a completely new microorgan-

Table 2 Comparison of ethanol production cost for one- and two-step steam pretreatment of softwood. Ethanol yield, temperature and concentration of water insoluble material (WIS) in the filtration and washing of the material between the two pretreatment steps are also given. Data from Wingren et al. [35]

Pretreatment	Ethanol yield[a] (% of theor.)	Filtration and washing		Prod. cost (US$ L^{-1})
		Temp (°C)	WIS %	
1-step	71.8	–	–	0.55
2-step I	74.6	60	30	0.55
2-step II	74.6	20	30	0.57
2-step III	74.6	180	50	0.53
2-step IV[b]	77.0	180	50	0.52

[a] Based on the hexose content in the raw material;
[b] No washing between the pretreatment steps

ism, not yet known, was assumed. The production capacity is also assumed to increase, from a biomass input (in metric tons per year) of 620 000 to 1 550 000 and 3 110 000 for the short, medium and long term, respectively. Also, the cost of the raw material was assumed to fall from about 58 US$ per metric ton for the short term to 48 and 39 US$ for the middle and long term.

The total investment cost for the short-term scenario was about 395 million US$, i.e. 475 US$ per ton raw material yearly capacity. The ethanol production cost was determined to be 0.51 US$ L^{-1}. Forty-five percent was due to capital cost and about 35% to the net raw material cost, i.e. after credit for co-produced electricity. For the middle-term scenario the total investment cost increased to around 465 million US$, corresponding to 300 US$ per ton raw material yearly capacity. The ethanol production cost decreased to 0.31 US$ L^{-1}, of which about 44% arose from capital cost and about 31% due to net raw material cost. For the long-term scenario the total investment cost increased to around 820 million US$, corresponding to 263 US$ per ton raw material yearly capacity. The ethanol production cost decreased to 0.20 US$ L^{-1}, of which about 50% was due to capital cost and about 42% to the net raw material cost. The long-term ethanol production cost was still considerably higher than that predicted by Lynd et al. [23] who estimated the future ethanol production cost for a plant based on CBP to be between 0.1 and 0.16 US$ L^{-1}. The main reasons for their lower cost are a higher conversion yield and lower capital costs. It must be pointed out that these are scenarios based on future projected improvements, which are very uncertain.

The number of studies on softwood is more limited. Fransson et al. [27] studied the potential for a two-stage dilute acid process using softwood as raw material. The plant is assumed to be co-located with a heat and power plant from which steam is purchased and co-products (solid residue) are sold. The first hydrolysis step is assumed to be run in co-current fashion, whereas the second reactor is working in counter-current mode to maximize sugar yield and reduce sugar degradation. A sulfuric-acid concentration of 5 g/L is used in both steps. The sugar stream is detoxified in an overliming step and then fermented to ethanol. The dilute ethanol is concentrated in a distillation step and the stillage is then evaporated. The plant is designed to process around 300 000 tons of raw material annually. The estimated ethanol production cost was 0.53 US$ L^{-1}.

In another study by Wingren et al. [38], the production of fuel ethanol from spruce using the enzymatic process was investigated. The softwood was steam pretreated after impregnation with SO_2. Two configurations, one based on SSF and the other on SHF, were evaluated and compared. The process conditions selected were based mainly on laboratory data and the processes were simulated using Aspen Plus, while the capital costs were estimated using Icarus Process Evaluator. The ethanol production cost was estimated to be 0.69 and 0.76 US$ L^{-1} for the SSF and SHF cases, respectively, based on a raw material cost of 63 US$ per dry ton. The main reason for SSF being less ex-

pensive was due to the capital cost being lower and the overall ethanol yield higher. Improvements in the SSF process, by running SSF at 8% WIS rather than at 5%, and by recycling of process streams, were shown to result in a decrease in production cost to 0.51 US$ L^{-1}.

3.1 Effect of Various Parameters on the Energy Demand and Production Cost

Process simulation of ethanol production from spruce using a process concept based on SO_2-catalyzed steam pretreatment followed by SSF, as shown in Fig. 3 ([20], Wingren et al. 2007 (submitted)), has been used to illustrate the effect of various process parameters on the energy demand and on the ethanol production cost. The general conclusions are, however, also valid for most of the process configurations described in Table 1. The model input was based on experimental data obtained from a process development unit. SSF was performed at 10% WIS with 2 g L^{-1} yeast. In the model, the overall ethanol yield was 296 liters per metric dry ton, corresponding to 69.4% of the theoretical based on the hexosan content in the raw material. Pentose fermentation was not included. Regarding production cost data, the proposed ethanol plant is assumed to be located in Sweden, with a capacity of 200 000 dry tons of raw material annually.

The ethanol yield affects both the raw material and capital costs and is the single most important parameter in reducing the cost of ethanol production, as was already stated in 1988 [39]. High energy efficiency is also of great importance for the process to be economically feasible. In most techno-economic evaluations, live steam for the process is generated in a steam boiler by burning part of the solid residue. From the excess solids it is possible to generate heat and electricity or pellets that can be sold to improve the process economics. Thus, the energy demand of the process affects the amount of solid residue that may add to the income as a solid fuel co-product and, therefore, it is very important for the process to be energy-efficient.

The heat duty of the process depends to a large extent on the process configuration. For the process alternative described above, the heat duty of the energy-demanding process steps is shown in Fig. 5. The white bars represent the primary steam demand while the gray bars represent the amount of secondary steam that is generated in each process step. The overall process heat duty, i.e. the total energy demand in the form of boiler-generated steam, is the sum of the black bars. Distillation (including preheating of the SSF broth) and evaporation account for the major part of the process energy demand. The contributions from pretreatment and drying, with the latter assumed to work as a steam dryer, are comparatively small, due to the generation of secondary steam in these process steps.

The energy demand of the distillation step, in which the ethanol in the mash from fermentation is concentrated, is highly dependent on the ethanol

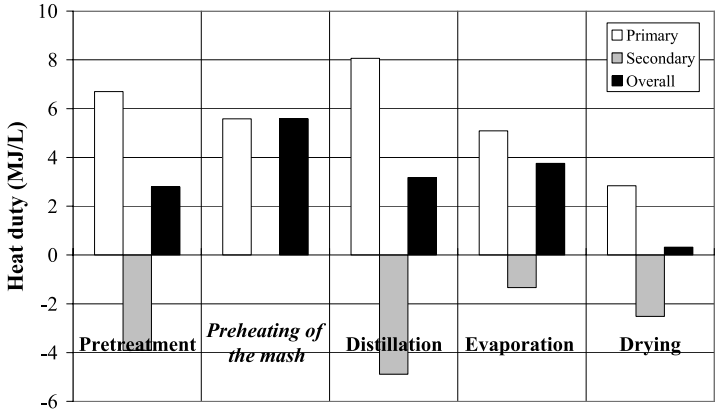

Fig. 5 Heat duty of the energy-demanding process steps in the proposed ethanol production process. The *white bars* represent the primary steam demand while the *gray bars* represent the amount of secondary steam that is generated. The *black bars* are the difference between the primary steam demand and the generated secondary steam. The sum of the *black bars* is equal to the overall process heat duty

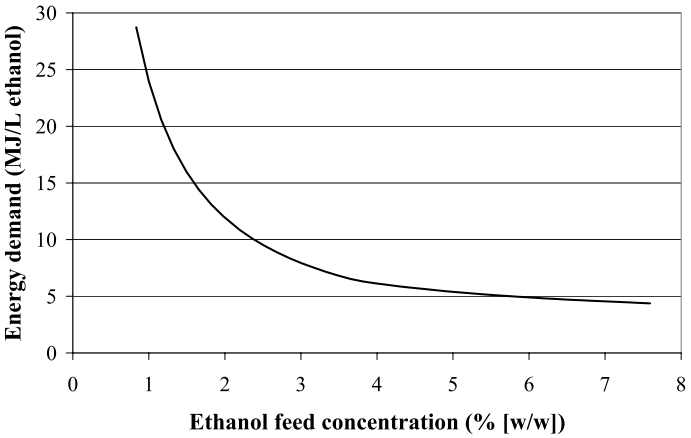

Fig. 6 Energy demand in the distillation step, where ethanol is concentrated to 94 wt %, as a function of the ethanol feed concentration. The step was assumed to consist of two stripper columns (25 trays each) and a rectification column (35 trays) heat integrated by operating at different pressures. The inlet feed temperature was increased from 80 °C to the boiling temperature before entering each stripper column

feed concentration, as shown in Fig. 6. The distillation step normally consists of a stripper column, in which the ethanol is separated from all solid and non-volatile compounds, and a rectification column, in which the ethanol is concentrated close to the azeotropic point. The implementation of heat integration, for instance by using the overhead vapor from the stripper as the heat

source in the reboiler of the rectification column, significantly reduces the energy demand. Nevertheless, it is of great importance to obtain a high ethanol concentration in the distillation feed. In a starch-based process the ethanol concentration in the stream entering the distillation step is normally above 8% (w/w). In a lignocellulose-based process, however, the aim has been to reach at least 4–5% (w/w) ethanol. In addition, a high ethanol concentration results in a high concentration of non-volatile compounds, which also leads to a decrease in energy demand in the evaporation step.

Recirculation of process streams is one way of reducing the overall energy demand, which results in a decrease in overall production cost, as shown by Wingren et al. [38]. Recirculation of part of the stream after distillation back to the fermentation step would result in an increased concentration of non-volatiles and thus a reduction in the energy demand in the evaporation step. Recirculation of part of the stream before distillation would also result in an increase in the ethanol concentration and thus a reduction in the energy demand in both the distillation and evaporation steps. This is true for both the SSF and SHF configurations. However, in the same study it was shown that it is even more beneficial to increase the substrate concentration in the SSF step. This would affect not only the costs related to distillation and evaporation, but also the cost of SSF. On the basis of this fact, one of the main objectives of several experimental studies performed during recent years has been to increase the substrate concentration in SSF [40–43]. This results in reduced water consumption, which greatly reduces the energy demand for distillation and evaporation, provided that the ethanol yield is maintained at a high level. In Fig. 7, the process heat duty (in MJ L^{-1}) and the overall production cost

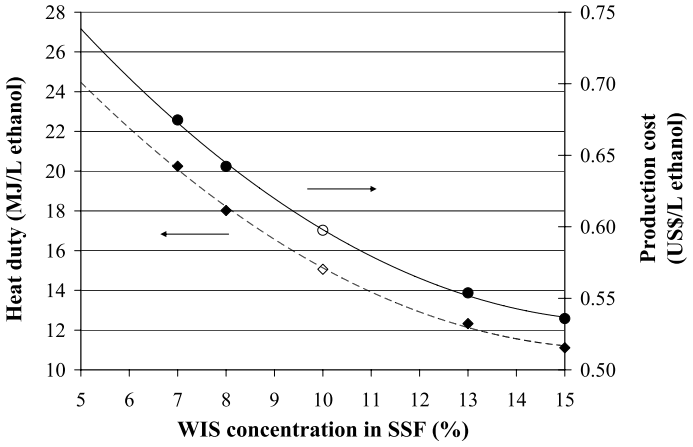

Fig. 7 Overall process heat duty (––) and ethanol production cost (—) as a function of the WIS concentration in SSF for the proposed ethanol production process. The ethanol yield was maintained the same as the base case (*open symbols*) while varying the WIS concentration

(in US$ L^{-1}) are presented as functions of the WIS concentration in SSF. The ethanol yield and the amount of yeast (NB: not the yeast concentration), were the same as in the 10-% WIS case when varying the WIS concentration. The reduction in production cost is due to an increase in co-product credit and a reduction in the fixed capital cost.

Process simulations clearly demonstrate the potential reductions in production cost and energy demand that can be obtained by running SSF at higher substrate concentrations. However, given the large number of compounds involved, and due to the fact that they may act synergistically, it is impossible to predict the impact of increased concentrations on the performance of the yeast and enzymes using process models. Effects on parameters such as productivity (yield, residence time), yeast and enzyme dosages have to be determined experimentally, preferably on pilot scale.

Savings in energy demand can also be accomplished by changes in the process design. Evaporation is the traditional, but energy-demanding, way to concentrate the water-soluble, non-volatile components in the stillage stream. To reduce the energy requirements for evaporation, multiple evaporation effects are used. This has a significant effect on the overall process heat duty, as shown in Fig. 8. (In the simulation results presented in Figs. 6 and 7, evaporation was carried out with five effects.) The energy savings have, of course, to be weighed against the increase in capital cost. Also shown in Fig. 8 is a case where the use of mechanical vapor recompression (MVR) has been implemented in the evaporation unit. In a traditional multiple-effect evaporator system, a large proportion of the energy supplied ends up as latent heat in the vapor phase leaving the last effect in the evaporator. This vapor is

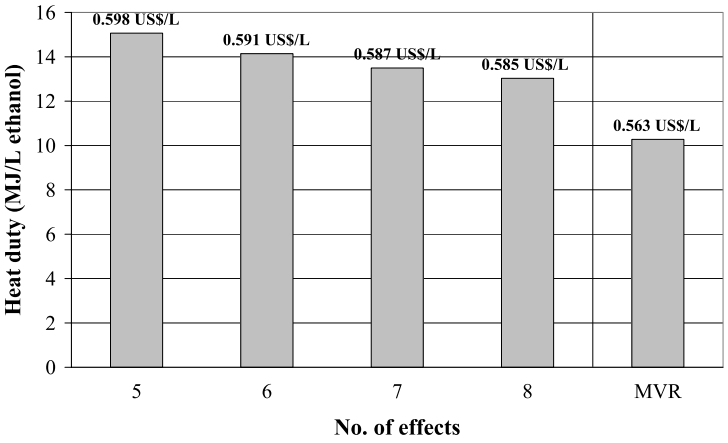

Fig. 8 Overall process heat duty in the proposed ethanol production process as a function of number of effects (5–8) in evaporation (MVR: mechanical vapor recompression). The overall production cost for each case is presented above each *bar*

normally condensed using cooling water. Another option is to compress the vapor, thereby raising the temperature to a level at which the latent heat can be utilized. The vapor can then be used as a heating medium to replace most of the primary steam. When compression is carried out by aid of a mechanical compressor the process is referred to as MVR. An electrical motor or a steam turbine provides power to the compressor. The overall process heat duty was reduced from 15.1 (base case configuration) to 10.3 MJ L^{-1} when MVR was applied to the evaporation step (Fig. 8), while the overall electric power requirement was estimated to increase from 2.2 (base case configuration) to 2.8 MJ L^{-1} (data not shown).

It has also been proposed that the entire evaporation step be replaced by an anaerobic digestion step, in which most of the organic material (unfermented sugars, acids, yeast, etc) is converted to biogas mainly consisting of methane and carbon dioxide. This was estimated to reduce the production cost by about 7%. The performance of such a system is dependent on a number of parameters such as the composition of the feed, residence time, temperature, etc. A crucial question is also how to handle the sludge from the anaerobic digestion. Further investigation is required since very limited data regarding the performance of this kind of system have been published.

3.2
Lignocellulose versus Starch—a Comparison

Production of ethanol from starch-based crops such as wheat and corn is a well-known technology. Such processes have been optimized over a long time and are reaching a level of maturity where further cost reductions, based on improvements in conversion technology, are becoming more difficult. In contrast, processes using lignocellulosic raw materials are still under development and significant reductions in ethanol production cost can be expected.

With some modifications it is possible to make a basic evaluation of a starch-based process (Fig. 1) and compare it with a process based on lignocellulosic material (Fig. 3). This was done by Wingren [17]. The purpose of the evaluation was not to determine the absolute ethanol production cost, but to compare the processes using the same fundamental cost basis and the same assumptions in the investment analysis. A comparison of this kind provides valuable information on the major differences between a commercial process and a process under development.

Both plants were designed for an annual ethanol production of 55 000 m^3, which is a rather small plant. This value is on a pure ethanol basis, although the actual distillate was assumed to be 94% (w/w), i.e. no dehydration step was included as this would have been the same in both cases. Also, no offsites, e.g. production of heat and electricity, were included, only the pure ethanol production facility. In the evaluation no credit was given for carbon dioxide. The cost of the enzymes in a starch-based plant is lower than in a lig-

nocellulosic plant. In the study it was assumed to be 0.014 US$ L^{-1} ethanol, which is slightly higher than the cost reported for the enzymes in a corn-based plant located in the USA [18].

The raw material flow is higher in the lignocellulosic process, 200 000, compared to 126 500, dry metric tons y^{-1} for the starch-based plant, due to the lower overall ethanol yield and the somewhat lower amount of fermentable sugars in the raw material. The overall energy demand in the lignocellulosic process was estimated to be 16 MJ L^{-1} ethanol compared to 10 MJ L^{-1} for the wheat-based process. The fixed capital investment was estimated to be 99 and 53 million US$ for the lignocellulosic and the starch-based processes, respectively. A breakdown of costs is presented in Table 3. The estimated ethanol production cost was 0.60 and 0.58 US$ L^{-1} for the lignocellulosic and starch-based processes, respectively. Major differences were found in the cost of raw material, enzymes, capital, steam as well as income from the co-products. It should be noted that, although significantly higher than in the starch-based process, the enzyme cost in the lignocellulosic process was based on a projected future cost. In the starch-based process the cost of the raw material constitutes as much as 65% of the total production cost. This is typically the case for well-established, mature processes. Thus, the economics of a starch-based process is very dependent on the cost of feedstock.

The lignocellulosic process is more dependent on the income from the co-products. However, the potential price of the syrup is uncertain since its fuel properties are unknown. At 12.9 US$ MWh^{-1} the income from this co-product was estimated to be 0.03 US$ L^{-1}. In a scenario where the co-product instead has to be disposed of and cannot be utilized as a fuel, the ethanol production cost for the lignocellulosic process would be 0.63 US$ L^{-1}. The

Table 3 Breakdown of costs for the starch- and lignocellulosic-based processes in US$ L^{-1}, as evaluated by Wingren [17]

	Starch	Lignocellulosics
Raw material	0.380	0.200
Chemicals	0.019	0.041
Enzymes	0.014	0.091
Co-products	– 0.100	– 0.147
Syrup	n.a.	– 0.030
Steam	0.076	0.130
Other utilities	0.017	0.031
Maintenance & insurance	0.029	0.054
Labor	0.033	0.033
Capital	0.107	0.194
Total	**0.575**	**0.597**

n.a.: not applicable

income from the pellets in the lignocellulosic plant reduces the ethanol production cost by 0.15 US$ L^{-1} at 20 US$ MWh^{-1}. As in the case of the syrup, the true price of this co-product will be dependent on its fuel properties.

The results of this comparative study led to some important conclusions regarding potential cost reductions in the lignocellulosic process, compared with the starch-based process. The overall ethanol yield in the lignocellulosic process evaluated is 68% of the theoretical based on the available glucan and mannan in the raw material, a figure that can probably be increased. In addition, a pentose- and galactose-fermenting organism could increase the ethanol production per unit raw material without increasing the capital cost. This is especially important if the raw material is rich in pentoses, e.g. as in straw or hardwood. A reduction in enzyme loading would also be rewarding provided that the ethanol yield could be maintained. Figure 9 shows a breakdown of capital costs together with energy costs for the two processes. The largest difference in costs is seen in the conversion steps and in the evaporation step. The pretreatment step in the lignocellulosic process represents around 0.093 US$ L^{-1} ethanol. This cost is attributed to both a high energy demand and to the high cost of the reactor system. This shows the need to improve pretreatment and/or enzymatic hydrolysis so that less severe pretreatment is required. The higher cost of the SSF step compared with the fermentation step in the starch-based process is due to the longer residence

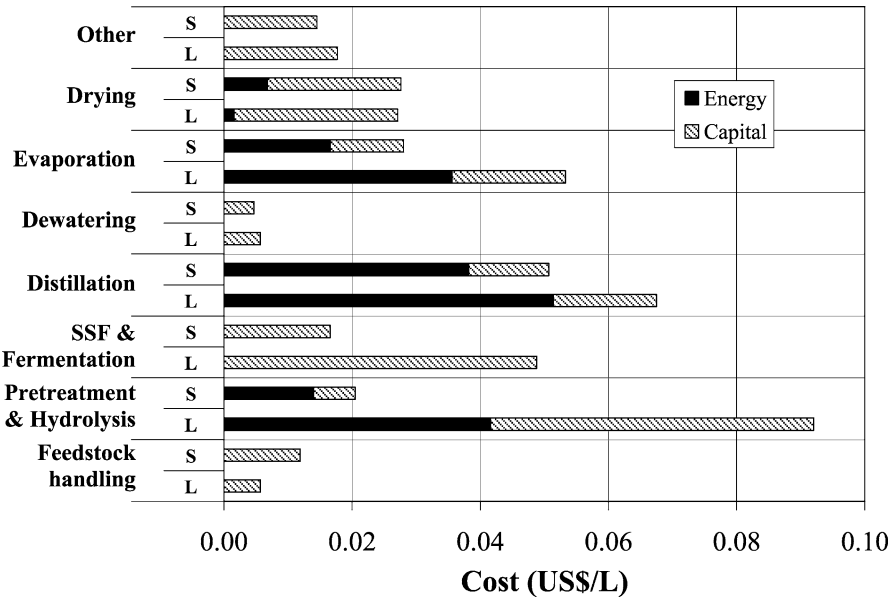

Fig. 9 Breakdown of energy (steam) and capital costs for a starch-based (S) and a lignocellulosic-based (L) process, according to Wingren [17]

time and the lower substrate concentration in the lignocellulosic process. An increase in substrate load and productivity in the lignocellulosic process would reduce this difference. The difference in cost between the starch-based process and the lignocellulosic process in the downstream processing steps (evaporation and distillation) would also be reduced if the ethanol concentration in the SSF step could be increased.

3.3 Co-location with other Plants

One approach to reducing the production cost is integration of ethanol production with another suitable plant, e.g. a combined heat and power plant, a starch-based ethanol plant or a pulp and paper mill. Significant reduction of the production cost was obtained in a study on co-production of ethanol and electricity from softwood, based on conditions in California, USA [44]. One of the benefits is that the syrup or lignin residue can be used for steam production without prior drying. Another option is to integrate cellulosic ethanol production with starch-based ethanol production to utilize the whole agricultural crop. This will increase the production capacity drastically, and it may also help to boost the ethanol concentration resulting from the lignocellulosic process, if the ethanol-containing streams can be distilled in the same distillation units. This will have a beneficial effect on the energy demands in the distillation and evaporation steps. It might be a disadvantage if the residue cannot be used for animal feed (DDGS). However, it will still have a fuel value, which will help to improve the economics of the overall process. The biorefinery concept is also an interesting option. Using chemical and biological transformations, the raw material is processed to produce ethanol and, e.g., modified lignin, specialty chemicals and maybe anaerobic biogas, adding value to the main product. In this case the income from other products improves the overall process economics [45, 46].

4 Conclusions

Flowsheeting, combined with estimates of the production cost, is a valuable tool for the comparison of process alternatives and to determine bottlenecks that require further improvement. It is, however, difficult to compare production costs from different studies due to the many assumptions made in the simulations, such as ethanol yield, productivity and concentration, as no commercial-scale plants are in existence. Also, differences in capacity and cost of raw material, as well as currency exchange rates, add to the uncertainty. This is clearly illustrated by the large variation in the estimated ethanol production cost, from 0.13 to 0.81 US$ L^{-1} ethanol.

The most important parameters for the economic outcome are the feedstock cost, which varied between 30 and 90 US$ per metric ton, and the plant capacity, which influences the capital cost. It is thus very important to reach a high overall ethanol yield as this is directly related to feedstock and capital costs for a given production capacity.

One of the major research challenges is to improve the hydrolysis of carbohydrates through more efficient and less expensive pretreatment methods, but also by enhanced enzymatic hydrolysis with superior enzymes at a reduced enzyme production cost. The latter is one of the most uncertain costs in most economic analyses.

It is also important to achieve a high ethanol concentration in the fermentation or SSF steps to reduce the energy demand. This requires new technology for enzymatic hydrolysis (or SSF) at high solids concentrations and the development of robust fermenting organisms that are more tolerant to inhibitors. They also have to be able to ferment all sugars in the raw material in concentrated hydrolyzates, while maintaining high ethanol productivity and a high ethanol concentration.

Finally, process integration within the process and with other types of industrial processes, e.g. a combined heat and power plant or a starch-based ethanol plant, will reduce the production cost further. Regarding the immediate future, we believe that these integrated plant concepts will be used in the first successful industrial-scale production of lignocellulosic fuel ethanol.

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Policy Options to Support Biofuel Production

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Abstract Biofuels for use in the transportation sector have been produced on a significant scale since the 1970s, using a variety of technologies. The biofuels widely available today are predominantly sugar- and starch-based bioethanol, and oilseed- and waste oil-based biodiesel, although new technologies under development may allow the use of lignocellulosic feedstocks. Measures to promote the use of biofuels include renewable fuel mandates, tax incentives, and direct funding for capital projects or fleet upgrades. This paper provides a review of the policies behind the successful establishment of the biofuel industry in countries around the world. The impact of direct funding programs and excise tax exemptions are examined using the United States as a case study. It is found that the success of five major bioethanol producing states (Illinois, Iowa, Nebraska, South Dakota, and Minnesota) is closely related to the presence of funding designed to support the industry in its start-up phase, while tax exemptions on bioethanol use do not influence the development of production capacity. The study concludes that successful policy interventions can take many forms, but that success is equally dependent upon external factors, which include biomass availability, an active industry, and competitive energy prices.

Keywords Biofuels · Direct funding · Excise tax exemptions · Policy · Renewable fuel mandates

1 Introduction

Biofuels derived from sustainable biological sources, including agricultural crops, waste vegetable oils, and woody biomass, is advocated by many including MacLean et al. [1] and McMillan [2] as a potential substitute for petroleum-derived fuels such as gasoline and diesel. The use of biofuels is generally associated with lower greenhouse gas emissions and improved energy balance compared to petroleum-based fuels [3], which makes them an attractive option for combating climate change and meeting national or international targets of environmental performance. As the biofuel industry is based on agricultural (or potentially forest) biomass, development of the industry will lead to a diversified rural economy and increased employment, which can support domestic development goals [4–6]. The industry has long been promoted as a means to substitute renewable, sustainable biomass for fossil reserves of oil, which may in turn increase the security of energy supplies and reduce dependence upon foreign oil [7]. These attributes make biofuel an attractive option for policymakers, offering solutions to a number of domestic challenges. At the same time, policy is needed in order to increase the competitiveness of bio-based fuels, which are generally more expensive to produce than petroleum-based counterparts [8].

Policy options to support biofuel production may take a number of forms. Some options are "top-down" in form, as they are enacted on a national or regional basis and impact all producers and consumers. One such option is the national target, in which policymakers make a public declaration of their intention to meet a certain level of production (often expressed as a percentage of overall production) in domestic transportation fuel supply. Top-down policy places the emphasis upon governments, which are then responsible for creating an environment supportive towards industrial expansion. The national target should not be confused with a renewable fuels standard (or obligation), which sets legal standards for the minimum levels at which biofuels must be blended into transportation fuels. A renewable fuel standard places the emphasis upon industry, who must then meet the renewable fuel standards with their products in order to be eligible for sale. One commonly observed policy option is exemption of biofuels from national excise taxation schemes, which has the effect of reducing producer costs and thus increasing potential profits. This type of incentive can be identified as a subsidy to industry, although lower prices can be and are passed to consumers in competitive markets.

Other policy options act in a "bottom-up" fashion, impacting only particular industrial or consumer participants in the biofuel marketplace. One such option is direct government funding of capital projects to increase capacity or upgrade distribution networks. Normally, these types of policies are enacted in a competitive fashion, wherein various industrial producers can

compete for projects, which are then carried out in conjunction with government. Another bottom-up type of policy is targeted at increasing biofuel use in government or corporate vehicle fleets.

In some countries, multiple policies covering the range of options described above have been enacted to support biofuel development (e.g. [9–11]). The presence of multiple policies within these jurisdictions means that determining the effectiveness of individual policies is quite difficult. In this paper, implementation of biofuels in several countries is examined. The ability of two measures to promote domestic biofuel production is compared. The first measure considered is exemptions on fuel excise taxes; the second is funding designed to support projects, infrastructure, or capacity development for bioethanol production. The industry is then evaluated on its ability to successfully promote broad policy goals of employment, environmental performance, and fuel security. A number of recommendations for the formulation of future policies are proposed.

2 Biofuel Production

Today, the most commonly used biofuels are bioethanol, generated from sugar- and starch-based processes, and biodiesel, generated from animal fats or vegetable oils. As of 2005, worldwide production capacity for bioethanol fuel was about 45 million L year^{-1} [12]. Global capacity for biodiesel is much lower at about 4 million L [13–16], although certain countries (notably Germany) are investing in expanded capacity for this fuel [14]. The installed capacity for both fuels is rising dramatically in the face of high oil prices; biodiesel production has risen by an average of 50% annually between 2000 and 2005, while about 15% annual growth has been observed in bioethanol production over the same period. While biodiesel is increasing in importance, it is clear that bioethanol will remain the dominant biofuel for some years to come.

The simplest way to generate bioethanol is to use yeast to ferment hexose sugars such as glucose, which can be obtained directly from agricultural crops such as sugarcane or sugar beet. In Brazil, the sugar-based industry currently has the capacity to produce almost half of the world's bioethanol supply, or about 17 billion L year^{-1} [12]. Another source of the sugars required for fermentation is starch, produced in corn, wheat, and other cereal crops. Starch must be broken down through acid or enzymatic hydrolysis in order to release glucose, which can then be fermented to bioethanol [6]. Both sugar and starch-based processes are employed in Europe, with France (629 million L) and Spain (520 million L) currently leading production [12]. In North America, corn (or maize) is currently the dominant biomass source for the bioethanol industry, due in part to the high proportion of starch

found in its kernels and its high yield per hectare in comparison to other cereal crops. Corn, like sugarcane or switchgrass, is a C4 plant, which can utilize an extra carbon molecule in the photosynthetic process as compared to wheat or trees, which are C3 plants. Warmer growing conditions found across the USA favor C4 plants, while cooler regions (including the Canadian prairie) are well-suited to C3 plant production. Comparatively, C4 plants have relatively high water efficiency, while C3 plants have the ability to increase photosynthetic activity in the presence of elevated CO_2 levels. Thus, growing conditions in any given year will determine optimal bioethanol feedstocks for specific regions [17].

The USA has a bioethanol production capacity of over 18 billion L [18], while Canada's bioethanol production capacity is currently about 245 million L but expected to grow to more than 1 bill L by 2008 [15]. Various other countries around the world have increased bioethanol production significantly since the mid-1990s. The dominant emerging bioethanol producers include China, which is home to Jilin Fuel Alcohol, the world's largest corn-based bioethanol plant with a current capacity in excess of 350 million L year^{-1}. The development of biofuel capacity over the past quarter century may be seen in Fig. 1.

As bioethanol is the most dominant biofuel found today, it is useful to look at the policies that supported development of this fuel in different jurisdictions around the world, and to evaluate the impact that different policies may have on creating increases in production capacity.

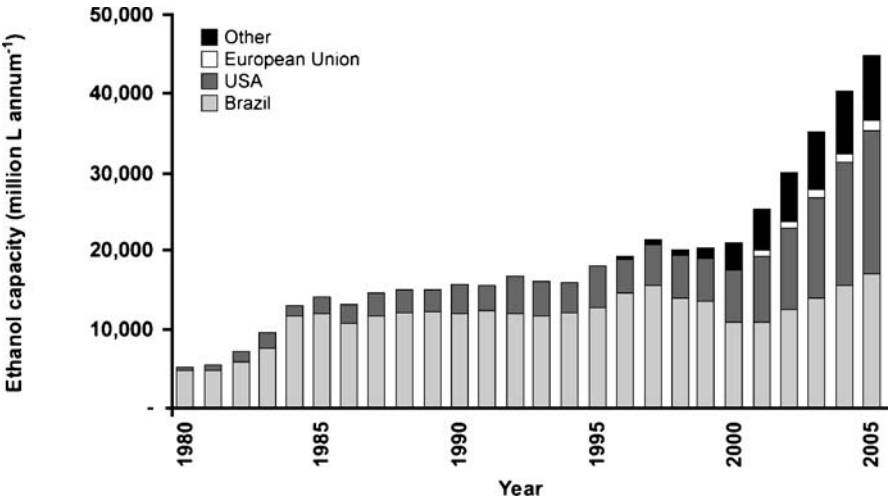

Fig. 1 Global bioethanol production capacity identifying major producers from 1980–2005 [12]

2.1 Brazil

The oldest example of widespread biofuel development is found in Brazil, which produces bioethanol from sugar- or starch-based material in the form of sugarcane and sugarcane residues. Because of Brazil's optimal climate, two seasons of sugarcane growth can be achieved, adding greatly to the potential production of both sugar and bioethanol products. In response to the first oil crisis of the 1970s, Brazil invested heavily in fuel alcohol primarily as a means of increasing fuel security and saving foreign currency on petroleum purchases. The original policy choice was to create direct funding sources to create biofuel capacity. In 1975, a diversification program for the sugar industry called Proálcool was created with large public and private investments supported by the World Bank, allowing expansion of the sugarcane plantation area and construction of alcohol distilleries, either autonomous or attached to existing sugar plants [19].

The second group of policies introduced in Brazil provided a subsidy for bioethanol use. Two related financing schemes were organized to guarantee fuel sale price; the FUPA program guaranteed US $ 0.12 L^{-1} for E22 (a blend of 22% ethanol in gasoline), while the FUP program provided US $ 0.15 L^{-1} for E100 (or pure, anhydrous ethanol) fuel. By 1996/97, the total subsidy delivered via these programs reached about US $ 2 billion $year^{-1}$ [19].

The presence of a renewable fuel standard and of strong subsidies to E100 production, combined with the second oil shock of the early 1980s, resulted in the successful adaptation of engines to E100 fuel use. By 1984, E100 vehicles accounted for 94.4% of domestic automobile manufacturers' production, and in 1988 participation in the E100 program reached 63% of total vehicle use in the country [20]. The upward trend ended, however, when high global sugar prices led to a crash in availability of fuel alcohol, resulting in a consumer shift away from E100 vehicles.

From 1989 to 1996, the sugar export market was very strong, and thus the cost of sugar to the bioethanol industry soared and fuel bioethanol shortages resulted. In response, the Brazilian government made a failed attempt to restrict sugar exports, and then announced that the fuel market would be deregulated as of 1997. While deregulation began with E100 fuels, subsidies for blended fuels remained in place for an additional period, which had the effect of increasing overall alcohol production at the time. When price controls on E22 were removed in 1999, however, the prices for bioethanol collapsed [19].

Faced with an excess of bioethanol and collapsed prices at home, major producer groups joined together to form Brasil Alcool SA in March 1999, and made the decision to export excess bioethanol at any price. Later that year, a mechanism to create a monopoly on fuel bioethanol named Bolsa Brasileira de Alcool Ltda was created by the founders of Brasil Alcool. This

monopoly drove a dramatic increase in bioethanol export prices for a period after its inception, with prices doubling within a year [20]. Since 1999, the total production of bioethanol in Brazil has risen; this trend has been driven by the expansion of export markets for bioethanol, rising world prices for oil, and an increase in domestic oil supply. The Brazilian industry today follows a simple biorefinery model, where the production of a combination of products, including refined sugar, bioethanol, and energy from the combustion of sugarcane residues (bagasse) improves both economic and environmental performance. Brazil controls more than 75% of the world's export market, with primary exports going to the USA, Europe, Korea, and Japan; Brazil's estimated total exports will be approximately 3.1 billion L in 2006 [12]. Many countries that lack significant biomass resources, such as Japan, have made Brazilian bioethanol a part of their renewable fuel strategies.

Brazil's domestic market still utilizes the single largest portion of fuel bioethanol capacity in the country. The presence of a Renewable Fuel Standard means that all Brazilian gasoline has a legal alcohol content requirement that has ranged between 20% and 25% (currently 23%, as of 20 November 2006) [21]. Most vehicles are being run on E20 or E22, but sales of flex-fuel vehicles capable of operating on E85 blends are strong. Brazil has developed a unique distribution infrastructure for this fuel, with a network of more that 25 000 gas stations with E20 pumps.

Today, Brazil remains a dominant bioethanol producer and the single largest exporter of this fuel, with shipments expected to hit a record 3 billion L in the 2006–07 harvest. Rising demand for bioethanol – in part caused by policies in other countries – has created an impetus for new product capacity. Recently, it was reported that UNICA plans to open 77 new bioethanol plants by 2013, adding to the existing 248 plants. When complete, this will raise the country's production capacity to about 35.7 billion L [21].

2.2 United States

The second series of data illustrated in Fig. 1 shows that development of the bioethanol industry in the USA began in the 1980s. The drivers for the industry were in part the rapid surges in global oil prices experienced in the 1970s and 1980s, which led to rising prices of fuel. There was also the presence of a strong agricultural lobby which was (and is) interested in creating additional revenue streams for farmers. The US bioethanol industry uses corn, and to a lesser extent wheat, as a feedstock for wet- and dry-milling processes. A number of different policy options have been employed to help build the industry. Both federal and state governments have offered the industry direct funding in the form of public–private partnerships and research funds, as well as tax incentives and state-level renewable fuel mandates, i.e., legislated amounts of renewable fuels contained in fuel sales within the state, defined

by blending level or by renewable fuels [22, 23]. A more focused discussion of state-level funding and tax incentives, and the effectiveness of these options, may be found in Sects. 3 and 4, respectively.

In the USA, most bioethanol production capacity is concentrated in the Midwest, where corn is found in abundance, and where state and federal government incentives have combined to make an attractive environment for investment in the infrastructure required for bioethanol production. Over half of US production capacity is found in just three states, each of which have supplied significant capital resources to the bioethanol industry. The US states with the highest bioethanol capacities include Illinois (annual bioethanol production capacity, 5.1 billion L), Iowa (3.7 billion L), South Dakota (2.2 billion L), Minnesota (1.9 billion L), and Nebraska (1.8 billion L) [18]. These states are notable in that they have provided direct funding incentives in addition to federal funding, as discussed in Sect. 3.

The total financial commitment that the USA has made to biofuels dwarfs the investment that other countries have made. By 2006, total cumulative US funding through national or state programs applicable to bioethanol has exceeded US $ 2.5 billion [23]. The largest amount of funding has been offered by the federal government. Annual program spending by all government agencies, primarily the US Department of Agriculture and the US Department of Energy, on alternative fuels exceeded US $ 253 million in 1998 and has risen since to more than US $ 300 million [18, 24]. This has resulted in improving the technology that is utilized by the industry, and has broadened the potential number of coproducts that can be generated from the bioethanol production process. The remainder of federal funds supports a number of incentive programs, including the Alcohol Fuel Credit (a corporate tax credit designated for industry producing bioethanol), deductions for both clean-fuel vehicles and refueling properties, and the Renewable Energy Systems and Energy Efficiency Improvements Program. The latter program is designed to aid in the construction of new facilities, and will cover up to 25% of construction costs. Maximum grants for a single project under this program are US $ 500 000, and the fund generally pays out between US $ 3–5 million in any given year [22, 23]. Finally, it should be pointed out that significant funding in the USA has been directed towards developing cost-effective coproducts from the biofuel production process, allowing the creation of "biorefineries" with improved economic and environmental performance. Pilot facilities are already operating under some of these funding programs [23].

Most recent policy developments in the USA stem from the Energy Policy Act of 2005, H.R. 6, which was signed into law by President G.W. Bush on 8 August 2005 [25]. This act created a nationwide renewable fuels standard (RFS) that will raise the use of biofuels (mostly bioethanol and biodiesel) to 28.4 billion L $year^{-1}$ by 2012, which is effectively 5% of the total fuel sales. The Act also introduced credits for the purchase or lease of flex-fuel vehicles by taxpayers, although these credits diminish as the sales of flex-fuel vehicles

progress by manufacturer through the fiscal year [25]. The 2005 Energy Policy Act has had some unintended consequences as related to biofuels, however. Section 701 of the Act requires flex-fuel vehicles in the US federal fleet to operate on alternative fuels 100% of the time. By Executive Order 13149, federal flex-fuel vehicles were previously required to operate on alternative fuels the majority of the time (i.e., 51% or more) [26]. Thus, Section 701 has effectively doubled E85 use by the federal fleet, and the increased demand has raised prices and decreased the practical availability of E85 fuels. The long-term impact of this policy on the market has yet to be seen.

The recently-announced "20/20" vision for biofuels (introduced as a Senate Bill on 29 July 2005) defines a future biofuel production goal for the USA as 20 billion gal (approximately 75.7 billion L) by 2020 [27]. As the US starch-based bioethanol capacity is already quite high, it is unlikely that continued growth could achieve this goal. Accordingly, in his State of the Union Address for 2006, the President outlined the Advanced Energy Initiative, which seeks to reduce US dependence on imported oil by accelerating the development of new, renewable alternatives to gasoline and diesel fuels [28]. These alternatives include bioethanol and other future biofuels derived from cellulosic biomass. Cellulosic biomass is an attractive energy feedstock because it is an abundant, domestic, renewable source that can be converted to liquid transportation fuels including bioethanol, which can be used readily by current-generation vehicles and distributed through the existing transportation-fuel infrastructure. To determine feedstock availability for cellulosic bioethanol processes, the US Department of Agriculture commissioned a report that explored the technical feasibility of a billion-tonne annual supply. This report found that approximately 1.24 billion t of dry cellulosic biomass can be sustainably produced each year, with about 910 million t coming from agriculture and an additional 330 million t from the forest sector [29]. Using the efficiency of conversion technologies observed in the literature to date [6], this would translate to between 110 and 250 million L $year^{-1}$, compared to current US gasoline use of approximately 500 million L $year^{-1}$.

US production of biofuels is significant, but today only comprises about 2.6% of liquid fuel consumption. In order to become a more significant component of the transportation fuel sector, biofuel production must grow tremendously, which will require access to cellulosic biomass. The Advanced Energy Initiative includes the Biorefinery Initiative, which sets a goal of making cellulosic bioethanol cost-competitive by 2012 and which provides significant funding to achieve this goal (US $ 91 million in 2006, US $ 150 million in 2007) [30]. Biorefining pilot facilities are already operating with starch-based feedstocks, and these processes have the potential to be applied to cellulose-based biofuel production facilities, which will contribute to the economic viability of these operations. If these measures are successful, cellulosic bioethanol production could easily become the dominant biofuel within the USA.

2.3 European Union

For the member states of the European Union, the primary policy tool behind the development of a bioethanol industry is the Directive on the promotion of the use of biofuels for transport (Directive 2003/30/EC) [31]. The motivations behind this Directive include improving the security of energy supply, and reducing the environmental impact of the transportation sector [32]. The Directive mandates an increasing share of biofuels from 2% of total fuel supply in 2005 to 5.75% of total fuel supply in 2010 (based on energy content) in order to meet these priorities. Due to relatively slow growth in the industry, it is currently anticipated that renewable fuels will occupy about 4.8% of the market by 2010, which is significantly less than the existing policy target.

The overriding priorities of the European Commission will impact the behavior of each member nation in setting national policies relating to biofuels. It can be expected that, while economic factors are not the political priority of the EU, the member nations will have a strong interest in utilizing the proposed Directive to meet national goals of employment and economic diversification. From an economic standpoint, it is anticipated that a biofuel contribution of 1% of the total EU fossil consumption will create between 45 000 and 75 000 new jobs [32].

At the time of writing, many member states have passed the biofuels Directive into national law, including Belgium [33], the Czech Republic [34], France [35], Germany [36], Greece [37], Latvia [38], Lithuania [39], and Sweden [9]. Some countries have announced indicative targets that are below that of the Biofuels Directive, including Malta (target value for 2005 of 0.3%) [40], Hungary (0.4–0.6%) [41], Poland (0.5%) [42], Spain (0.55–0.65%) [43], and Cyprus (1%) [44]. Each of these countries still plan to achieve national targets of 5.75% for the end of 2010. Slovenia follows a slightly different set of targets, ranging from 1.2% in 2006 to at least 5% in 2010 [45]. The Netherlands has set a target percentage of 2% biofuels for 2006, which will be followed in 2007 by requiring suppliers to ensure that these blends are achieved [46]. The UK has announced a Renewable Transport Fuel Obligation, which will place a legal requirement on transport fuel providers to ensure that a specific percentage of their fuel sales is renewable, ranging from 2.5% in 2008/09 to 5% in 2010/11 [47].

In implementing the biofuels Directive, some countries have set slightly more aggressive targets, including Austria (revised Fuels Ordinance, 4 November 2004: BGBI. II, No 417/2004), which mandates that all petrol and diesel marketers blend at least 2.5% biofuels on an energy content basis in all fuels sold within the country [48]. Sweden has set their national target of at least 3% biofuels after 2005, and has mandated that renewable fuels be made available at petrol stations, starting with the largest stations ($> 3000\ m^3\ year^{-1}$) in 2006, and progressing to smaller stations ($> 1000\ m^3\ year^{-1}$) by 2009 [9]. Swe-

den also has a very aggressive long-term target of 40–50% reduction of fossil fuel use, which should engender significant increases in biofuel use over the next 13 years [49].

Another important piece of legislation is the Directive restructuring the community framework for the taxation of energy products and electricity (Directive 2003/96/EC), which allows excise-tax exemptions for biofuels produced or blended within European countries [50]. This legislation is very important within European nations due to the high level of excise tax that is currently levied on petroleum and diesel in these countries, particularly when compared to North America. Within these countries, a reduction of even a few percent can mean cents per liter, which translates into significant cost savings. For instance, in Austria, a 10% reduction in excise taxes on biodiesel reduces the cost by US $ 0.028 L^{-1} [51]. This sum is almost equivalent to the federal excise taxes paid for diesel fuel in Canada. A similar percentage reduction in the US federal excise tax for diesel would result in a selling price of US $ 0.058 L^{-1} and a savings of only US $ 0.006 L^{-1} [32, 52].

Before the release of the second Biofuel Directive, European governments did not always utilize excise tax exemptions to the same extent as their Canadian and US counterparts. This was because national controls over excise tax rates were complicated by the rules of the European Economic Community (EEC). A Directive issued by the EEC on 16 October 1992 was intended to harmonize the structures of excise duties among all member nations [52, 53]. When France decided to create an aid scheme for biofuels that would exempt these fuels from national excise taxes, objections were raised and an appeal to the Commission of the European Communities was made by BP Chemicals [54]. Ultimately, however, the Commission decided to validate the French decision, allowing an exemption amounting to US $ 0.06 L^{-1} to be extended through 31 December 2003 [13, 55, 56]. This move created the precedent within the EU to allow excise tax exemptions for biofuels, freeing a powerful policy tool for decision-makers within the nations of the Union. The second Directive Regarding Tax Relief Applied to Biofuels (2003/96/EC) was issued in 2003, permitting other countries to make the decision to grant excise tax exemptions as biofuel production becomes more widespread within Europe.

Today, most EC member states, including Austria, Belgium, Cyprus, Denmark, Estonia, France, Germany, Hungary, Italy, Latvia, Lithuania, Luxembourg, Malta, Poland, Slovakia, Slovenia, Spain, Sweden and the UK have introduced exemptions at various levels up to 100%, using the precepts laid down in Directive 2003/93/EC. These exemptions are summarized in Table 1.

In implementing tax exemptions, Germany was careful to include a measure that allowed for adjustments to be made in the case of overcompensation. Perceived overcompensation has recently been observed in regards to vegetable-oil based fuels, and accordingly, the German government has introduced an Energy Tax Act, which from 1 August 2006 places a tax on these fuels [36].

Table 1 Excise tax rates and exemptions for gasoline, diesel, and renewable fuels in North America and Europe, in US cents L^{-1} [31, 50, 55]

Country	Leaded gas	Unleaded gas	E10	Diesel	Biodiesel
Canada[a]	9.5	8.6	7.8	3.7	n/a
Mexico[b]	n/a	66.6%	78.9%	43.5%	n/a
United States[c]	4.9	4.9	3.5	6.4	n/a
Austria	59.8	50.8	50.8	35.3	31.9
Belgium	68.8	61.5	61.5	36.2	36.2
Czech Republic	36.8	36.8	36.8	27.7	27.7
Denmark	65.3	54.6	54.6	37.6	37.6
Finland	79.4	69.9	69.9	40.6	40.6
France	n/a	73.1	65.8	48.5	48.5
Germany	80.4	73.9	72.9	51.1	51.1
Greece	43.2	37.1	37.1	31.4	37.1
Hungary	48.4	44.7	44.7	36.2	36.2
Ireland	57.3	46.7	46.7	27.7	27.7
Italy	69.4	69.4	69.4	37.6	37.6
Luxembourg	52.9	46.4	46.4	40.6	40.6
Netherlands	80.7	72.3	71.7	36.2	36.2
Norway	92.4	95.6	95.6	64.1	95.6
Poland	46.7	41.9	41.9	n/a	41.9
Portugal	68.4	41.7	41.7	30.6	30.6
Russia[b]	30%	30%	25%	30%	25%
Spain	50.5	46.3	41.7	33.1	33.1
Sweden	74.5	64.9	64.9	42.4	42.4
Switzerland	n/a	56.8	56.8	59.0	56.8
United Kingdom	105.3	94.0	94.0	94.0	94.0

Currency exchange rates (December 2006): US$ 1.0000 = € 0.7567 = CDN$ 1.1545

[a]Canadian Federal excise tax rate is shown. Provincial rates are variable, ranging from US 4.5 ¢ L^{-1} (Yukon Territory) to US 12.1 ¢ L^{-1} (Newfoundland and Labrador). Provincial excise tax exemptions range from US 0.7 ¢L^{-1} (Alberta) to US 1.8 ¢ L^{-1} (Manitoba)

[b] Mexican and Russian rates are *ad valorem* and vary on a monthly basis, depending on world petroleum prices

[c] US Federal excise tax rate is shown. State rates are variable, ranging from US 2.0 ¢ L^{-1} (Georgia) to US 7.7 ¢ L^{-1} (Rhode Island). State excise tax exemptions range from US 0.1 ¢ L^{-1} (Florida) to US 0.7 ¢ L^{-1} (Idaho)

Italy also incorporated measures to adjust in the case of overcompensation; that country currently provides tax exemptions for an annual quota of 200 000 t of biodiesel for the period 2005–2010, as well as reduced excise duties on bioethanol and related bio-derived additives [57]. Several countries have experimented with pilot excise tax exemptions on a project-by-project basis, including Finland [58], Ireland [59], and the Netherlands [46]. Latest reports indicate that Greece [37] is also considering tax exemptions for biofuels.

As of late 2005, only one country exceeded the goals set out in the Directive. German biofuel use (primarily biodiesel) accounted for 3.75% of total fuel consumption in 2005 [36]. Swedish biofuel use (primarily bioethanol) accounted for 2.2% of the total in the same year [9], which came closest to achieving the goal; however, since most cars in Sweden are now running at E5 bioethanol blends, the country has encountered a constraint in the form of the EU Directive on Fuel Quality, which limits renewable fuel blends to 5%. Other countries, including the UK, have identified this Directive as a barrier to achieving the goals of the Directive on Biofuel Use [47]. In France, about 1.2% of fuel sales consisted of renewable fuels in 2005, mostly in the form of bio-ETBE or bioethanol [35]. In Austria, biodiesel production had reached almost 100 million L, which is approximately 1.1% of national fuel consumption [13, 48]. Spain used significant amounts of both bioethanol (1.49% of total petrol) and biodiesel (0.10% of total diesel) [43].

Most EU members had not yet reached their biofuel use goals under the biofuel Directive in 2005, although the situation is changing rapidly as new capacity comes on-line. Lithuania's use of biofuels has grown, rising to 0.72% in 2005 [39]. Both Italy and Malta report increasing biofuel production characterized by significant amounts of biodiesel, achieving about 0.57% and 0.52% biofuel use, respectively, in 2005 [40, 57]. Other growing biofuel producers include Poland (0.48%) [42] and Latvia (0.33%) [38]. Countries with less than 0.2% biofuel use in 2005 include Greece (0.18%) [37], the UK (0.18%) [47], and Finland (0.1%) [58]. In the case of Finland, it should be noted that a new biodiesel plant designed to be online in 2007 will produce about 200 million L annually, raising this percentage significantly.

Countries reporting less than 0.1% biofuel use in 2005 include Hungary (0.07%) [41], the Czech Republic (0.046%) [34], and Luxembourg (0.021%) [60]. Countries with no appreciable biofuel use include Cyprus [44], Ireland [59], the Netherlands [46] and Slovakia [61]. In Denmark, a limited number of Statoil stations began selling 5% bioethanol blends in 2005, but total sales are as yet unknown and unlikely to meet 2% of total transportation fuel sales. Biodiesel is produced in Denmark but exported, primarily for use in Germany [10]. Estonia has some biofuel production, but this volume is completely exported to other EC member countries [62].

Direct funding mechanisms have been implemented in a number of EU member states. In Belgium, the Federal Public Service of Finance has issued a call for tenders to market increasing amounts of biofuels, beginning in November 2006 for biodiesel and in October 2007 for bioethanol. Some research funding has also been made available [33]. In Cyprus, legislation to comply with the biofuel Directive includes a grant scheme for energy conservation and renewable energy utilization. Under this legislation, four applications for biodiesel plants have been submitted [44]. In the Czech Republic, state aid for biodiesel production has been introduced at a level of about US $ 39 million (CZK 821 million) [34]. In Estonia, about US $ 5000 (EEK

57 600) was granted as support to draw up business plans for the production of liquid biofuel in 2005 [62]. Ireland announced a renewable energy grant aid package in 2005 which provides up to US $ 86 million (€ 65 million) annually to a range of projects, including biofuel initiatives [59]. Latvia provided about US $ 680 000 (LVL 358 980) for bioethanol and US $ 380 000 (LVL 201 770) for biodiesel production [38]. Lithuanian producers of biofuels may claim refunds on every tonne of feedstock used in biofuel production [39]. Poland has provided approximately US $ 550 000 (PLN 1 601 700) in funding to research projects related to biofuels, and an additional US $ 95 000 (PLN 271 500) towards two production start-up projects [42]. Sweden has provided an investment of approximately US $ 120 million (SEK 815 million year^{-1}) for energy research, which includes research into transportation fuels [9]. The UK has created grant programmes to help upgrade infrastructure and to provide direct support for the development of a biofuels industry [47].

Some countries have also implemented other measures to promote biofuels. The Czech Republic has introduced policies that provide resources to support biomass production for non-transport energy purposes [34], and Estonia has set aside resources to support the expansion of energy crops [62], as has Slovenia [45]. Ireland has established a number of initiatives, including tax exemptions for corporate fleets and for flex-fuel vehicle sales [59]. The UK created a fleet biofuel mandate for its Government Car and Dispatch Agency in 2005, which specifies 5% biodiesel use in the fleet [47]. Sweden has created a number of progressive measures, including a provision that state-owned vehicles be environmentally sound (which includes power by biofuels), and introduction of a congestion charge for Stockholm to which biofuel-powered vehicles are exempt [9]. Sweden has also released a report entitled "Making Sweden an oil-free society", which has among its goals the reduction of petrol and diesel in transportation fuels of 40–50% by 2020 [49].

2.4 Other Biofuel Producing Nations

Other major biofuel producers include China, which has grown its bioethanol production sector rapidly since 2000 to become the third-largest single bioethanol producer after the USA. Total capacity from four plants in 2005 was about 1.3 billion L, but continued high prices for international oil has led the National Development and Reform Commission to announce that biofuel production will increase dramatically, providing China with the ability to replace about 2 million t of crude oil by 2010, and 10 million t by 2020 [63]. The Commission also announced that China would begin shifting to non-grain feedstocks, including sweet sorghum, for bioethanol production [63]. Jilin Fuel Alcohol remains the world's largest corn-based bioethanol plant with a current capacity in excess of 350 million L year^{-1} [64]. The biofuel industry in China has been subsidized, mostly in terms of funds to construct biofuel

plants. Some Chinese provinces have announced biofuel mandates, although the national government has not yet made any decision about legislating biofuel use [63].

A country poised to be a major biofuel producer is Canada, which currently produces about 250 million L annually [15]. Much of the funding being made available to fund research and development in biofuels in Canada has depended upon the federal government's environment strategy. This strategy has evolved significantly with the ascension of a Conservative minority federal government in 2005, who made a campaign promise to introduce a 5% biofuels mandate. An agreement with provincial governments on the 5% mandate was reached in May, 2006, which will see this mandate take full effect by 2010 [65]. Recently, the federal government announced the proposed Clean Air Act, which was tabled on 19 October 2006 [66]. Unfortunately, the proposed Act does nothing to codify the government's biofuels target, and does not provide concrete policy incentives for additional biofuel use. To help spur some biofuel development, Agriculture and Agri-Food Canada is providing CAD $ 10 million (approximately US $ 8.7 million) in the fiscal year 2006/2007 through the Biofuels Opportunities for Producers Initiative (BOPI). The objective of the Initiative is to help agricultural producers develop business plans for new biofuels projects [67].

Previous governments have provided more substantial support to biofuels, including a cumulative investment of CAD $ 2.7 billion (US $ 2.34 billion) into the implementation of the former Climate Change Plan for Canada [68], which included incentives for the development and use of environmentally-friendly technologies including bioethanol. The federal Canadian government provided direct funding for the industry through the Ethanol Expansion Program, which in 2004 and 2005 provided a total of CAD $ 118 million (US $ 102 million) in direct funding for 11 projects, six of which are currently in active development [69]. The federal government provides an excise tax exemption for biofuels, as do the provinces of Manitoba, Ontario, and Alberta [70]. Most recently, the Alberta government has announced a commitment of CAD $ 239 million (US $ 207 million) to expand the province's bioenergy sector by encouraging products including biofuel development [71]. Other nations with biofuel-friendly policies include Australia, where a bioethanol production subsidy is in place that replaces excise tax exemptions at a rate of approximately US $ 0.21 L^{-1} produced. Capital subsidies have been provided for two bioethanol production plants [64]. In Thailand, excise taxes are waived for bioethanol. In Latin America, production schemes in Peru and Columbia have been linked to urban renewable fuel standards in Columbia [64]. In a move designed to utilize surplus production, the sugar industry in India has successfully lobbied the government for state-level E5 fuel mandates, which were passed in September 2002 and which apply to nine states and four territories. In order to support these mandates, an excise tax exemption was granted and bioethanol prices have been

fixed by a Tariff Commission [72]. Production from other nations will become more important as capacity comes on-line and the international market for bioethanol continues to develop.

3 Direct Funding Programs in the USA

As seen in the review of major biofuel producers, a common policy instrument used to support the industry is direct government program funding, in the form of contracts, loans, grants, or fiscal guarantees. It is difficult to evaluate the effectiveness of direct funding by comparing different countries, where synergistic policies (such as renewable fuel mandates, excise tax exemptions, etc.) or simply more favorable market conditions may play a role in determining capacity. However, within a single country it may be easier to see the impact of direct funding on the establishment of biofuel capacity. The bioethanol industry in the USA has been chosen for an analysis of the effectiveness of direct funding towards establishing biofuel production capacity. For the purpose of this study, direct funds are considered to be funds earmarked for all aspects of research, development and demonstration, including all biofuel production as well as biomass production for general energy purposes. When different funding sources were considered, the only real criteria applied to warrant their inclusion in this study were (1) that the funds be applicable to research, development, and demonstration (RD&D) projects for bioethanol, including construction or modification of production facilities, and (2) that bioethanol is accounted as an eligible product. Funding sources that recognized bioethanol as a co-product of material or bioenergy generation were also included. Estimates of the cumulative, total funding available to support the bioethanol industry are shown in Fig. 2. Canada is included in this graphic for comparison's sake.

In Fig. 2, direct funds available in each state are indicated by the shading on the map, from blue (base levels of cumulative funding provided by the federal government as of 2005) to light or dark red (additional state funding, depending upon the cumulative amount of funds available as of 2005). Existing bioethanol production capacity for 2005 is indicated by the yellow circles, logarithmically sized according to the scale indicated. Additional bioethanol production capacity expected to be online as of 2007 is indicated by the dark orange circles, again plotted logarithmically. The graph indicates that bioethanol production is likely to be found where funding is available for infrastructure development, biomass procurement, and plant operation. Each of the major bioethanol-producing states has followed a different approach in creating these incentives. Each approach represents a successful strategy for attracting the industry and expanding bioethanol production capacity.

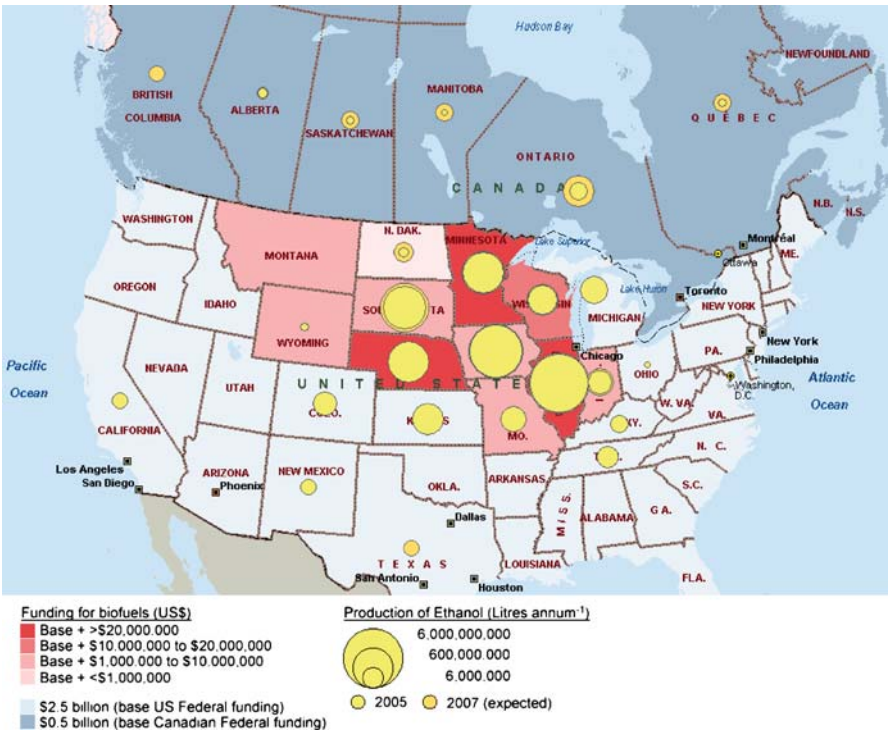

Fig. 2 Geographic distribution of North American federal and state/provincial-level funding programs for renewable fuels (cumulative to 2005), existing bioethanol production capacity (2005), and projected bioethanol production capacity (2007) [15, 17, 21, 22, 69]

In Illinois, the primary incentive offered to bioethanol producers is the Illinois Renewable Fuels Development Program, which offers up to US $ 5.5 million per facility in grants for the construction or retrofitting of renewable fuels plants, provided that they are a minimum of 114 million L in capacity and that the total grant award does not exceed 10% of total construction costs, or US $ 0.026 L^{-1} of additional biofuels capacity created [73]. Both bioethanol and biodiesel production facilities are currently the primary recipient of these funds. In addition, the Renewable Energy Resources Program offers funding at various levels to promote the development and adoption of renewable energy within the state. With two new plants under construction in 2006, the total funding available to the bioethanol industry is estimated at approximately US $ 30.15 million [23]. Currently, Illinois has five operating facilities with a capacity of 5.1 billion L $year^{-1}$, while two new facilities are under construction [18].

In Iowa, a number of innovative programs are in place. The Iowa Renewable Fuel Fund's Financial Assistance Program offers a combination of forgivable and traditional low-interest loans for projects involving biomass

and alternative fuel technologies, while the Alternative Fuel Loan Program offers zero-percent interest loans for up to half the cost of biomass or alternative fuels related fuel production projects, up to a maximum of US $ 250 000 per facility [74]. Approximately 20% of the money awarded under this program is in the form of forgivable loans, while the remaining 80% are low-interest loans. A number of other incentives, including the Ethanol Infrastructure Cost-Share Program, provide incentives for installation or conversion of E85 refueling stations [75].

In Minnesota, the chief incentive is the Ethanol Production Incentive. Originally, this incentive provided direct payments to producers at a rate of approximately US $ 0.052 US L^{-1} bioethanol, although the passage of bill SF 905 (2003) has reduced this amount to US $ 0.034 L^{-1} from 2004–2007. In 2007, the original incentive will be restored and producers may be reimbursed for lost incentive if funds are available. The total fund available is US $ 37 million, although there is a cap of US $ 3 million per producer, which essentially means that producers of more than 15 million L $year^{-1}$ are ineligible for extra incentive [76]. Perhaps due to this restriction in funding, the program has resulted in the establishment of 15 individual facilities by 2006 with a total production capacity of 1.9 billion L $year^{-1}$ [18]. The Ethanol Production Incentive expires June 30, 2010 [77]. Ethanol infrastructure grants are also available to help upgrade service stations for dispensation of E85 fuels [23]. Minnesota has also enacted legislation for a bioethanol blend mandate, currently enforcing a 10% bioethanol blend for consumers (to increase to 20% bioethanol in 2013) [77].

In South Dakota, the Ethanol Production Incentive is designed as a direct payment of US $ 0.052 L^{-1}, with a maximum of US $ 1 million annually or US $ 10 million in total to any single facility. Unlike the incentives described for Minnesota, Illinois, or Nebraska, this particular program is targeted specifically at bioethanol from cereal grains and expires this year [22]. While this level of support is lower than in many other states, South Dakota also has an excise tax exemption on bioethanol which provides additional financial incentive for production. Currently, South Dakota has 11 operating facilities, with four additional plants under construction and a total production capacity of 2.2 billion L $year^{-1}$ [18].

In Nebraska, the main program is the Ethanol Production Incentive, which offers a tax credit of US $ 0.048 L^{-1} bioethanol for up to 60 million L of annual production per facility, or 473 million L in total production over the course of a 96-month consecutive period [78]. This credit, which will expire in 2012, is limited to a total of US $ 22.5 million. As a tax credit, these funds can be considered to be defrayed costs in direct support of the industry [23]. Nebraska currently has a production capacity of 1.8 billion L annually in ten facilities, with three new installations currently under construction [18].

As these examples demonstrate, a range of policy tools have been deployed in areas with significant bioethanol production capacity. The tools of pro-

duction incentives, tax exemptions, direct loans, and cost-share schemes are shown to be effective in attracting capacity to individual jurisdictions, and the tools are shown to be flexible in achieving different results. The Minnesota example, in particular, shows the potential impacts of small changes to policy. By limiting the capacity to which the incentive applied, the state government was able to spur the creation of many individual facilities, which will in turn have a direct impact on jobs and the local economy. It is important to remember, however, that each of these strategies build upon the US federal government's strong commitment to research and development. Without that commitment, the rapidly improving technology that makes these facilities possible would not exist. However, it is interesting to note the differences that small amounts of local funding might have on productivity.

In Fig. 3, the relation between state funding for biofuels is compared to actual bioethanol production capacity, using the funding data and bioethanol production capacities for 2003 and 2005. The two years of data are differentiated by the shaded and white circles. In 2003, a strong correlation was found between state-level funding and bioethanol production capacity ($r^2 = 0.85$). This indicates that direct funding likely played a role in attracting new bioethanol capacity, and thus it could be concluded that this is an effective policy tool. By 2005, the changes in production capacity and direct funding levels in many states has reduced this correlation significantly ($r^2 = 0.64$). It may be postulated that a shift is taking place, in which the amount of funding available to capital projects has become less important in relation to some other factor, such as feedstock availability or mar-

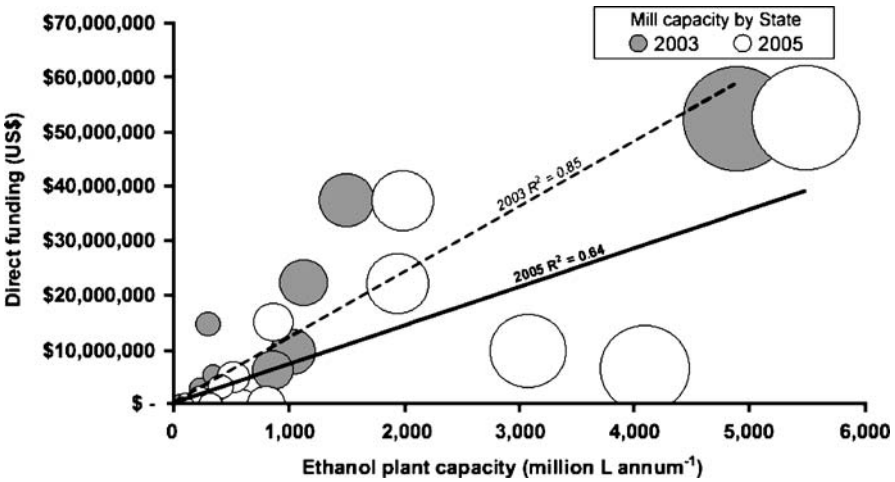

Fig. 3 Sum of federal and state/provincial-level funding programs for renewable fuels vs. cumulative state/provincial bioethanol production capacities, 2003 and 2005 [15, 17, 21, 22, 69]

ket influences. Indeed, follow-up analyses using corn production data [79, 80] indicate that in the same period, the relation between bioethanol production capacity and corn harvest figures on a state level show the opposite trend. In 2003, the correlation between the two was fairly weak ($r^2 = 0.58$), while in 2005, this correlation had grown stronger ($r^2 = 0.83$). In 2003, availability of corn seemed to be less important than direct funding for bioethanol facilities. It may be postulated that the rapid growth in bioethanol capacity seen to 2005, coupled with strong prices for bioethanol, has made feedstock availability more important than funding for construction purposes.

4
Excise Tax Exemptions in the USA

Another common policy instrument to promote biofuel use and consumption is exemption from excise taxes or mineral spirits taxes. Excise taxes are commonly used in the transportation sector and are designed to fill the gap between property and income taxes. These types of taxes can be imposed on the sale or use of certain articles, including fuels, and on certain transactions and occupations. In many cases, these taxes are not itemized in sales receipts and cannot be easily detected, and thus result in a hidden cost to the consumer [81, 82]. As shown previously in Table 1, excise tax rates for the countries under consideration range considerably.

In North America, excise taxes have been used as a tool to support renewable biofuels for some time. The federal governments of both Canada and the USA offer an exemption on bioethanol, which results in a slightly reduced tax rate for E10 blends. In addition, some state and provincial governments also offer exemptions. The largest North American exemption on excise taxes is currently offered in Manitoba, although that status is dependent upon the value of Canadian and American currency.

In Fig. 4, the excise tax exemptions are shown for the USA, and are related to bioethanol production capacity. The federal and state exemptions are illustrated by the shading on the map, with blue indicating the base federal exemption, and shades of red from light to dark indicating increasingly higher state-level exemptions. Bioethanol production capacity in 2005 is indicated by the size of the yellow circles, increasing on a logarithmic scale as shown in the legend. Expected additional bioethanol capacity for 2007 is shown by the dark orange circles.

In the USA, Idaho offers the largest combined exemption on E10 fuels at US $ 0.021 L^{-1}, but has no active production of bioethanol. Of the largest bioethanol-producing states, South Dakota and Iowa are the only two producing states that offer an additional exemption on state excise taxes. It may be inferred that excise tax exemptions provide a benefit for producers, but are

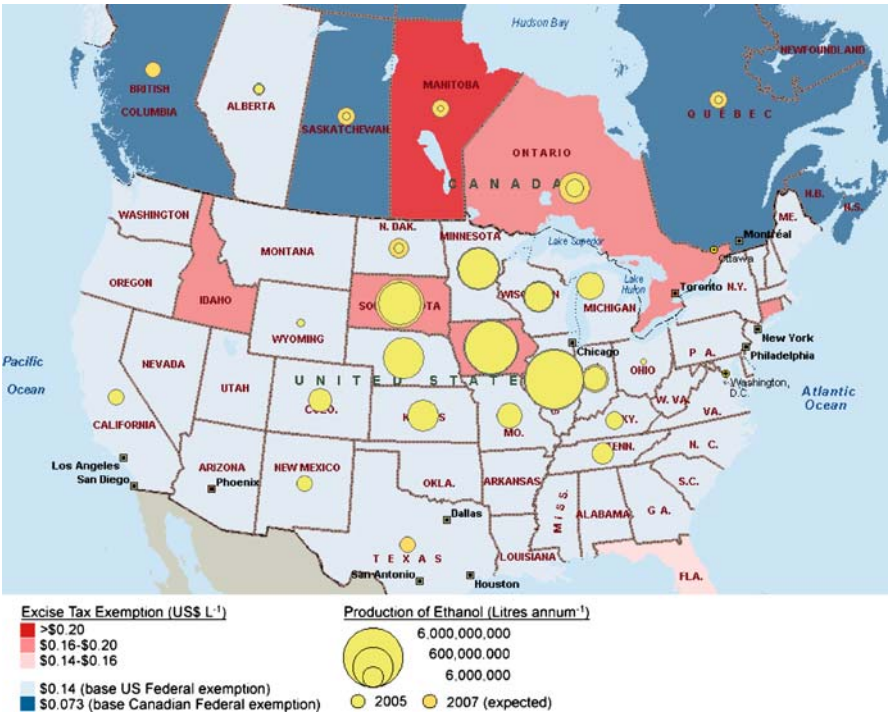

Fig. 4 Geographic distribution of North American federal and state/provincial-level excise tax exemptions (2005), existing bioethanol production capacity (2005), and projected bioethanol production capacity (2007) [15, 17, 21, 22, 69, 81]

not the deciding factor in determining where to install capacity for production.

Similarly, exemptions on excise taxes cannot be simply related to bioethanol production in Canada or Europe. In Canada, Manitoba offers combined exemptions that are higher than any offered in the USA. Combined federal and provincial excise tax exemptions on E10 reach as high as US $ 0.0256 L^{-1} in Manitoba, as compared to US $ 0.0181 L^{-1} in Ontario. At the current time, however, Ontario continues to lead Canada in the amount of bioethanol produced, while Manitoba currently lags behind jurisdictions such as Saskatchewan (which has individual incentives) and Quebec (where exemptions are limited to the federal level). In Europe, high excise taxes mean that exemptions for bioethanol (and other biofuels) are very significant and orders of magnitude larger than those found in North America. France offers the largest incentive in the form of tax exemptions, but has focused production of biofuels on ETBE, while Spain produces a significant amount of bioethanol under a significantly lower excise exemption regime, as indicated in Table 1 [35, 43].

In Fig. 5, the level of excise tax exemptions are plotted against bioethanol production capacity and the correlation between the two is examined, using bioethanol production capacities for 2003 and 2005. The two years of data are differentiated by the shaded and white circles. In 2003, no correlation was found between state-level excise tax exemptions and bioethanol production capacity ($r^2 = 0.01$). This may be evidence that the federal level exemption, which applies to all states, is a sufficient incentive for producers, and that additional incentives are not required to spur development of bioethanol capacity. It could thus be concluded that this is a less effective policy tool for state-level planners. By 2005, the changes in production capacity has slightly changed this correlation, but not to any significant extent ($r^2 = 0.04$). It may be postulated that excise tax exemptions have far less influence over the development of bioethanol capacity than does the amount of funding available to capital projects, feedstock availability, or other market influences. Follow-up analyses compared excise tax exemptions to estimates of bioethanol consumption [83, 84] and indicate that in the same period, no correlation ($r^2 < 0.01$) could be found between the use of bioethanol in gasoline blends and state-level excise tax exemptions. This indicates that excise tax exemptions do not serve as a particularly effective tool in enforcing the use of renewable fuels, and that there is no clear cause-and-effect relationship between the level of these exemptions and the establishment of the industry within individual jurisdictions. While excise tax exemptions are undoubtedly an important economic component of a bioethanol producer's business plan,

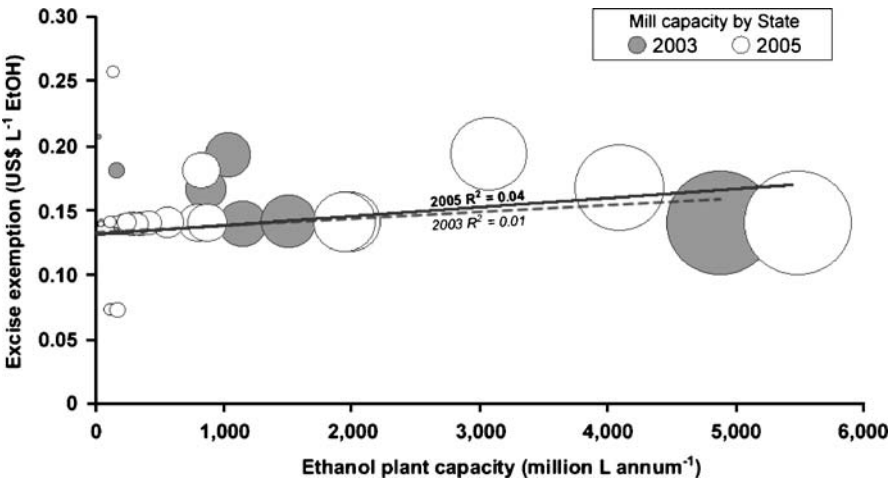

Fig. 5 Sum of federal and state/provincial-level excise tax exemptions for bioethanol vs. cumulative state/provincial bioethanol production capacities, 2003 and 2005 [15, 17, 21, 22, 69, 81]

they would seem to have less effectiveness as a policy tool to create biofuel capacity or increase its consumption.

5 Political Goals and Bioethanol-Related Policy

The ability of biofuels to contribute positively to the environmental and economic performance of a country, and to improve energy security in the long term, makes the nascent industry a tool that policymakers can employ to meet national priorities in these areas. A review of the priorities that governments are pursuing when designing biofuel-related policy illustrates some issues that the emerging bioethanol industry might consider. These issues may have particular relevance to the commercialization of the lignocellulosic-based component of the industry.

In the USA, the primary political drivers that support research and development into bioethanol for fuel are related to the economy and to energy security. Two agencies have become the primary implementing bodies for US policies related to bioethanol. The Department of Agriculture (USDA) has a mandate to increase rural employment, diversify agricultural economies, and stimulate rural development by harnessing crops and crop residues and identifying new uses for this material. The Department of Energy (DOE) has a mandate to diversify the energy supply, expand the availability of renewable energy sources, and develop new technologies to exploit renewables in all forms.

From an economic perspective, bioethanol policy in the USA has been highly successful. Since 1976, bioethanol production capacity has grown significantly. Almost a decade ago, the US industry passed 5 billion L in annual production and was credited with the creation of an estimated 200 000 new jobs and US $ 500 million in annual tax receipts [4]. Today, there are 94 bioethanol plants in the USA, producing about 18.5 billion L year^{-1}, with an additional 16 plants and 2.5 billion L of capacity under construction [13]. Urbanchuk [85] estimated that expansion to this level would require US $ 5.3 billion investment in new facilities and would increase demand for crops by 1.6 billion bushels per year. In that report, the author anticipates that a bioethanol industry of this size could reduce the US trade deficit by US $ 34 billion year^{-1}, create 214 000 new jobs within the USA, and generate US $ 51.7 billion in new US household income. It should be noted that the success of the US industry is in part due to the presence of import tariffs on bioethanol (duty of 2.5% market value, plus US $ 0.143 L^{-1}) [23]. While some regions (notably the Caribbean) may export duty-free bioethanol within a quota, the maximum amount of duty-free bioethanol entering the USA is currently 7% per year. This means that it is not cost-effective to import large supplies of bioethanol from other producers, such as Brazil.

From a security perspective, bioethanol policy has been less successful. American demand for petroleum continues to outpace domestic supply, resulting in growing petroleum imports, anticipated to be nearly 70% by 2020 [18]. Only about 3% of US energy requirements are supplied by biomass [56], and only about 2.6% of American total transportation fuel consumption is derived from biofuels [18]. Five individual US states (South Dakota, Nebraska, Minnesota, Iowa, and Illinois) now produce enough bioethanol to provide an E10 option to their entire local population. From the perspective of energy security, the USA could benefit from continued expansion of the bioethanol industry and increased utilization of the industry's potential.

Globally, Germany has the best capacity to substitute biofuels for fossil-based fuels, with current capacity of about 3.75% total demand, followed by the USA (2.6%), Sweden (2.2%), France (1.2%), Austria (1.1%), and Spain (0.44%) [9, 35, 36, 43, 48, 60].

The issue of climate change has become a major, global concern, but the sectors most closely linked to bioethanol production – including energy producers, farmers, and foresters – will feel the impact of this issue more closely. Climate change is the driver behind many new policies that influence the actions taken by these sectors. Perhaps the best-known of these is the Kyoto Protocol, which has been ratified by Russia, by the members of the EU, and by Canada in North America. The Clean Skies Initiative in the USA is another example of these policies. Because the use of bioethanol has the potential to significantly reduce net greenhouse gas emissions compared to petroleum products, an expansion of bioethanol production may become a significant part of national climate change strategies. It must be noted, however, that significant amounts of bioethanol must be substituted for petroleum products in order for these reductions to make a significant impact on total greenhouse gas emissions.

6 Conclusions

Successful policy options to support biofuel production may take a number of forms, including targets and mandates, exemption of biofuels from national excise taxation schemes, direct government funding of capital projects to increase capacity or upgrade distribution networks, or consumption mandates for government or corporate vehicle fleets. As discussed in this review, these policies can be differentiated by their relative emphasis on government, industry, or consumer actions. In most biofuel-producing countries examined here, a number of policies have been enacted in order to develop industrial capacity and encourage consumption. It is very difficult to measure the indi-

vidual success of these policies because of the synergistic effects that multiple policies may have.

In the USA, an analysis of state-level excise tax exemptions shows no correlation with bioethanol industry capacity, which suggests that these exemptions are not a crucial factor in the creation of industrial facilities. Direct funding and support was found to play a much more positive role in the creation of production capacity. It was noted that strong funding for establishment of facilities, including all aspects of research, development, and deployment, was present in each of the states where significant bioethanol production was present. In a comparison of production capacity between 2003 and 2005, it was observed that the correlation between direct funding opportunities and bioethanol production capacity has dropped somewhat. This indicates that other factors, including feedstock supply, the presence or absence of interested industrial players, and other market forces play a significant role in the establishment of the industry.

In advising governments on the creation of bioethanol-friendly policy, the US experience offers some valuable lessons to consider. The US goals behind policies supporting the bioethanol industry are dominated by (1) economic and social issues, and (2) security-based concerns. Of these priorities, the bioethanol industry has been more successful in meeting social criteria such as rural employment. The starch-based segment of the bioethanol industry has enjoyed particular success in the USA, particularly in Minnesota, Illinois, and Iowa. In the past, these jurisdictions have utilized a number of schemes, including direct payments, grants, corporate tax breaks, and excise tax exemptions, as incentives to lure the industry and build bioethanol capacity.

The ability of the industry to increase energy security in the USA, on the other hand, has been limited by the relatively small capacity of their production facilities at the current time. This should serve as a cautionary measure for governments in both Canada and the EU, who have invested biofuel-related policy with more emphasis on the environment and on energy security than they have upon social or economic concerns. Improved energy security through biofuel production can only be achieved when enough capacity is brought on-line. Thus, security-related policy geared to the short-term cannot succeed to any great extent. Policymakers must realize that, in the immediate future, the goals of most successful policies will be related to the economy, and perhaps to the environment. The implication here is that security-related policy, such as mandated renewable fuel use, is likely to take the form of long-term programs that have very little immediate reward.

One important finding was that a balance between research funding and funding for the creation of facilities might be more conducive to supporting the industry. It was noted that the USA has devoted a significant amount of funds to research as well as to supporting facility creation. A commitment to advancing the technology and improving efficiencies may serve to

increase the industry's comfort level in committing resources to this sector. The US example may have important lessons for other countries, where an effective balance between research and commercialization has not been reached. For instance, the total French commitment to biofuels in 2002 was just under US $ 200 million, of which about US $ 180 million is devoted to investment subsidies for biofuels, and a further US $ 11 million was put towards wood energy programs. Only about US $ 9 million was earmarked for research and development into renewables, including biofuels research [32]. Although the incentives that the French government offer are dramatic, the research focus of this country has been in other areas, notably nuclear power. This may in part explain the relatively low level of bioethanol production in France, which is currently at about 140 million L year^{-1} (or 629 million L when bio-ETBE production is considered) [35]. In Spain, the total investment is much lower at approximately US $ 30 million per year, but over half of this amount (US $ 17 million) is available for research and development into various renewables, while the other half may be used for commercial facilities or demonstration plants [32]. Perhaps because of this, Spanish production of bioethanol is at about 521 million L year^{-1} [43]. The balance between research and production incentives that is present in both Spain and the USA, and the resultant human capital, may in part account for the success that these nations have had in nurturing the bioethanol industry.

The experiences gained in developing bioethanol capacity, using both sugar- and starch-based processes, contain many lessons for other biofuels, including biodiesel and the lignocellulose-based bioethanol industry. These fuels can be seen as a response to a variety of domestic issues, including the need to diversify local economies, increased concerns over environmental damage associated with fossil fuel use, and a growing security rationale for a shift to domestic fuel sources. The emerging industry, including the lignocellulosic-based sector, may in turn find opportunities for strategic linkages and partnerships that capitalize upon these political issues.

Our findings indicate that successful policy interventions can take many forms, but that success measured as biofuel production capacity is equally dependent upon external factors, which include feedstock availability, an active industry, and competitive energy prices. It is important that policies be crafted that reflect "realistic" use scenarios for bioethanol and other biofuels over future time-frames.

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