

New improvements for lignocellulosic ethanol

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The use of lignocellulosic biomass for the production of biofuels will be unavoidable if liquid fossil fuels are to be replaced by renewable and sustainable alternatives. Ethanol accounts for the majority of biofuel use worldwide, and the prospect of its biological production from abundant lignocellulosic feedstocks is attractive. The recalcitrance of these raw materials still renders proposed processes complex and costly, but there are grounds for optimism. The application of new, engineered enzyme systems for cellulose hydrolysis, the construction of inhibitor-tolerant pentose-fermenting industrial yeast strains, combined with optimized process integration promise significant improvements. The opportunity to test these advances in pilot plants paves the way for large-scale units. This review summarizes recent progress in this field, including the validation at pilot scale, and the economic and environmental impacts of this production pathway.

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Introduction

Liquid transport fuels derived from renewable lignocellulosic resources offer unique and desirable features: a secure source of supply, limited conflict with land use for food and feed production, and lower fossil fuel inputs. The biological production of ethanol from forest and agricultural residues, or dedicated lignocellulosic crops, offers these benefits but its development is still hampered by economic and technical obstacles [1[•],2].

The ‘conventional’ process for producing ethanol from lignocellulosic biomass includes four main steps (Figure 1):

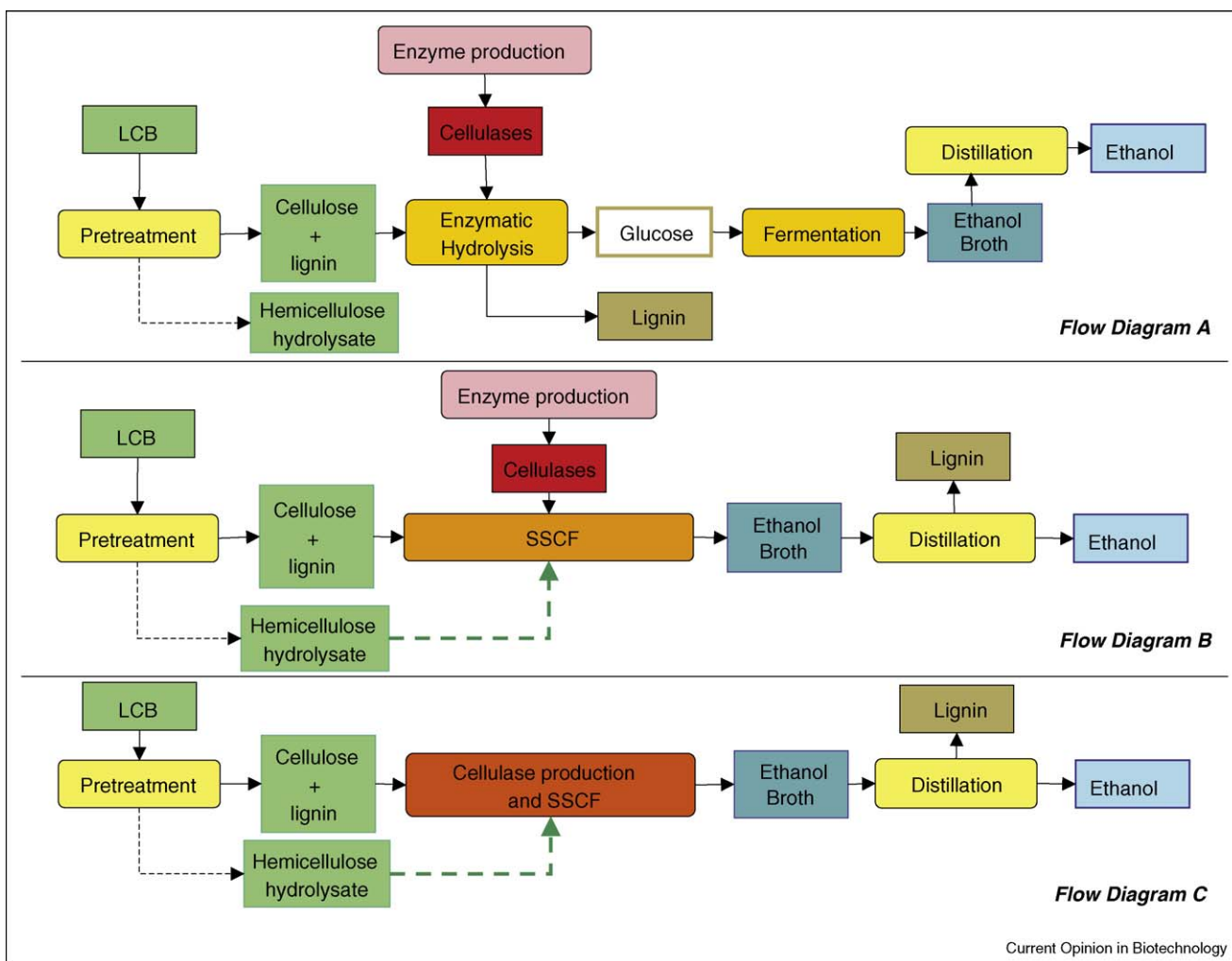
- (1) *Pretreatment*—breaking down the structure of the lignocellulosic matrix.
- (2) *Enzymatic hydrolysis*—depolymerizing cellulose to glucose by means of cellulolytic enzymes.
- (3) *Fermentation*—metabolizing the glucose to ethanol, generally by yeast strains.
- (4) *Distillation-rectification-dehydration*—separating and purifying the ethanol to meet fuel specifications.

Around the world there are numerous R&D projects seeking to overcome the remaining obstacles to commercialization. Some of the projects, principally those in USA, include pilot and demonstration facilities. The key obstacles being tackled are: pretreatment selection and optimization; decreasing the cost of enzymatic hydrolysis; maximizing the conversion of sugars (including pentoses) to ethanol; process scale-up and integration to minimize energy and water demand; characterization and valuation of the lignin co-product; and lastly, the use of representative and reliable data for cost estimation, and the determination of environmental and socio-economic impacts. Besides seeking to improve the conventional process, which utilizes *Trichoderma reesei* cellulolytic enzymes and *Saccharomyces cerevisiae* yeast strains, alternative and novel schemes are also being investigated, for example, the use of thermophilic enzymes [3^{••}], recombinant ethanol-producing strains [4,5] and consolidated bioprocessing [6]. This review focuses on recent advances in the four-step process, underlined by efforts performed within the framework of a European research project: the NILE (New Improvements for Lignocellulosic Ethanol) project. It should be noted, however, that major breakthroughs here could also benefit these other production pathways.

Pretreatment of lignocellulosic materials

Pretreatment of lignocellulosic biomass aims at rendering cellulose accessible to the action of hydrolytic enzymes by altering the lignocellulosic cell wall [7,8]. Pretreatment effects include: an increase of the accessible surface area, cellulose decrystallization, partial cellulose depolymerization, hemicellulose and/or lignin solubilization, and the modification of the lignin structure. Many pretreatment technologies have been proposed generally on the basis of combined physical and chemical actions. These include, steam explosion, liquid

Figure 1



Flowsheet of production of bioethanol from lignocellulosic biomass. Flow diagram (A), 'conventional' process. Flow diagram (B), simultaneous saccharification and co-fermentation process (SSCF). Flow diagram (C), consolidated bioprocessing (CBP). LCB, lignocellulosic biomass. Dotted lines represent optional process configuration (depends on pretreatment used).

hot water (LHW), ammonia fiber expansion (AFEX), acid cooking, lime, Organosolv extraction, and so on. The main constraints are minimizing sugar degradation and the formation of inhibitors (furanic and phenolic compounds), and limiting the consumption of chemicals, energy and water, and the production of wastes. In addition to steam explosion and dilute acid cooking that are already used at pilot scale (methods used in the NILE project), AFEX appears to be a promising technology owing to recent progress made [9], for example, in new process designs [7,10]. Recycling of chemicals is also crucial for other novel methods based on cellulose dissolution [11^{*}–13^{*}]. Selecting an optimal technology remains difficult because the performance is dependent on the biomass used. A comparative study concluded that methods like steam explosion, lime pretreatment, the LHW process, and ammonia-based pretreatments gave

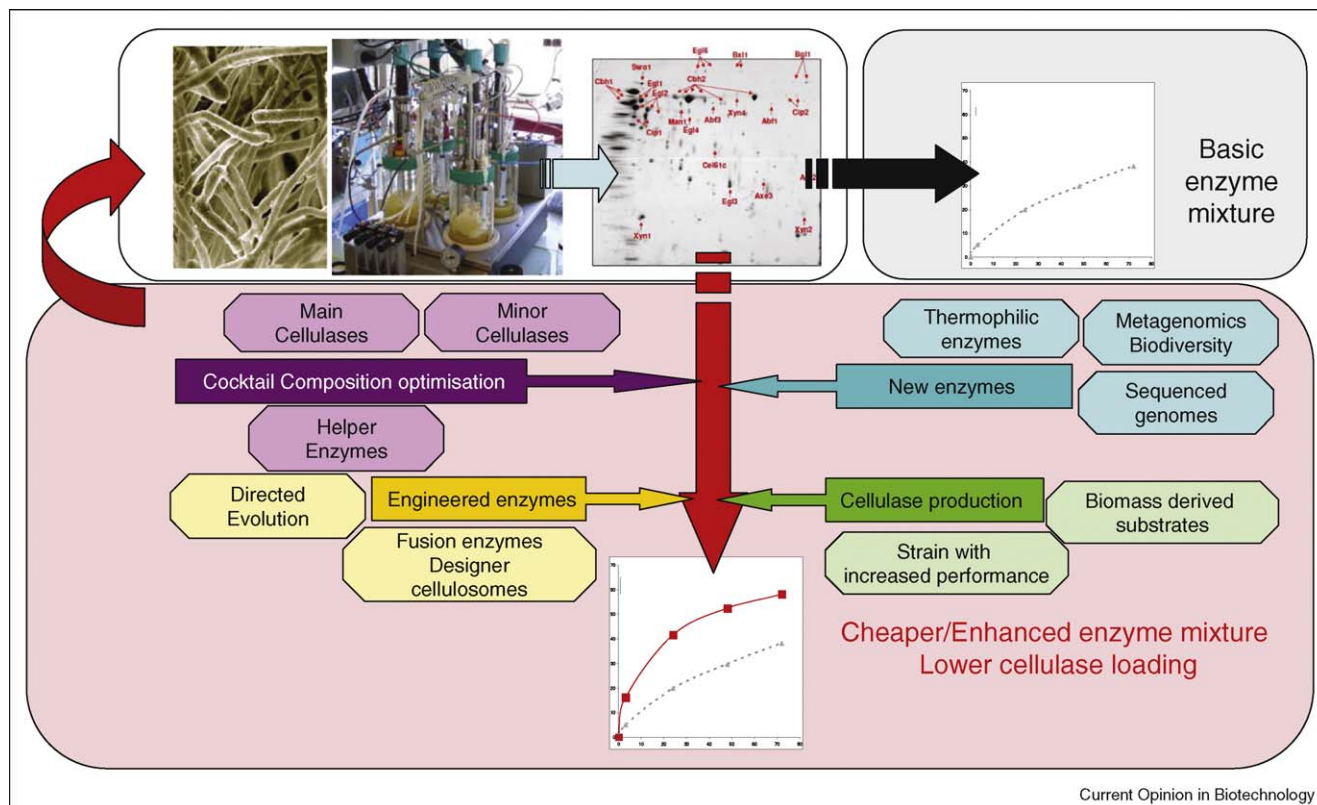
minor differences in projected economic performance [14].

Enzymatic hydrolysis

Overcoming the recalcitrance of lignocellulosic biomass

The goal of enzymatic hydrolysis is to depolymerize the polysaccharides in the water insoluble solid fraction that remains after pretreatment. After most pretreatments, the bulk of these remaining polysaccharides are cellulose. Three classes of enzymes act synergistically to hydrolyse cellulose: endo- β -1,4-glucanases (EG, EC 3.1.2.4) attack the endogenous part of cellulose chain, cellobiohydrolases (CBH, EC 3.2.1.91) attack the ends of the polymer, releasing cellobiose that is ultimately cleaved into two glucose molecules by β -glucosidases (BG, EC 3.2.1.21) [15]. In addition, accessory or 'helper' enzymes including hemicellulases [16] and ligninases [17] may also play a

Figure 2



Most promising biotechnological research areas for enhancement of the lignocellulose hydrolysis step, from basic cellulase mixtures (above, black arrow), to enhanced cellulase mixtures (red arrow). The key is either to lower cost of the enzymes or to produce more efficient enzymes. Most of these changes (enzyme addition, modification, or enhancement of the fungal strain) have to be integrated into the *T. reesei* genome (red arrow on the left).

role in hydrolysis by clearing access to cellulose for the main enzymes. The high costs of enzyme production and the excessive enzymatic dosages necessary to hydrolyse pretreated biomass are often considered to be the major bottleneck on the path to a commercial lignocellulosic ethanol industry [1]. Several ways are considered for improvement (Figure 2).

Improving cellulase production

The main industrial source of cellulases and hemicellulases is the mesophilic soft-rot fungus *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) owing to the high protein secretion capacity of mutant strains obtained by random mutagenesis [18]. Further enhancement of these strains will require precise metabolic engineering taking into account genetic expression in process conditions [19]. Attempts to produce the enzymes using cheaper carbon sources, for example, those originating from cellulosic biomass, are also being made [20].

Much work has been undertaken on the induction mechanisms of cellulases by the industrial inducer lactose in *T. reesei*, which involves an alternative D-galactose metabolism pathway [21]. Besides, three main

transcription factors are known to be involved in cellulase induction: ACE1, ACE2 and the major inducer XYR1, the latter regulating both hydrolytic enzymes and the lactose inducer pathway [22,23]. With the recent release of its complete genome sequence [24**], *T. reesei* has entered the post-genomic era, and the availability of genome-wide tools will permit systems biology approaches, developing a much needed knowledge of existing mutants and leading to new high-performance strains [25*].

Improving enzymatic cocktail efficiency

The two cellobiohydrolases (CBH I and CBH II) and the two endoglucanases (EG1 and EG2), in a rough proportion of 60:20:10:10, can make up to 90% of the enzyme cocktail secreted by *T. reesei*, while β -glucosidase typically makes up less than 1% [15,26]. Hydrolysis conditions within the ethanol process are far from the natural environmental conditions where *T. reesei* may be found. It can therefore be hypothesized that proportions of enzymes in the native cocktail are not optimal under these process conditions. A first, straightforward, way to improve lignocellulose hydrolysis is thus to refine basic components of the cocktail [27].

Recent genetic and biochemical technologies have improved our knowledge of the full set of *T. reesei* enzymes. Three more endoglucanases were reported to be secreted, together with 12 hemicellulases [28]. Complete analysis of the 'secretome' of an industrial strain revealed 22 secreted enzymes [26]. Complete genome sequencing [24**] has identified a far higher number of genes encoding enzymes (200 glycoside hydrolases as defined by the CAZy database [29**]). It is possible that overexpression of some of these genes may lead to significant enhancement of enzymatic hydrolysis. Still, *T. reesei* genome remains surprisingly poor in terms of number and diversity of enzymes likely to be involved in biomass degradation by comparison with other fungi [24**]. Thus, expressing enzymes from GH families absent from *T. reesei*, would seem another promising option. The ever increasing number of sequenced genomes is a potent source of enzymes [30]. Metagenomics is also a promising field, as recently demonstrated for a termite hindgut microbial community [31**].

A good example of such a successful approach is the cloning and expression of thermostable cellulases that has revealed that some of these enzymes have interesting properties for lignocellulose degradation [3**] and that some could replace *T. reesei* enzymes. Finally, the study of bacterial cellulosomes shows that one way to increase synergies between cellulolytic enzymes is to physically link them, either by direct genetic fusion [32] or by constructing 'designer cellulosomes' [33**].

Ethanolic fermentation of lignocellulose

Ethanolic fermentation of lignocellulose hydrolysates requires that the organism ferments both the hexose

sugars glucose, mannose, and galactose, and the pentose sugars, xylose and arabinose in the presence of inhibitory compounds including weak acids, furaldehydes and phenolics. Baker's yeast *Saccharomyces cerevisiae*, which has been the preferred organism for fermentative ethanol production throughout recorded human history is also tolerant toward lignocellulose derived metabolic inhibitors [34,35**]. However, *S. cerevisiae* lacks the ability to utilize the pentose sugars xylose and arabinose.

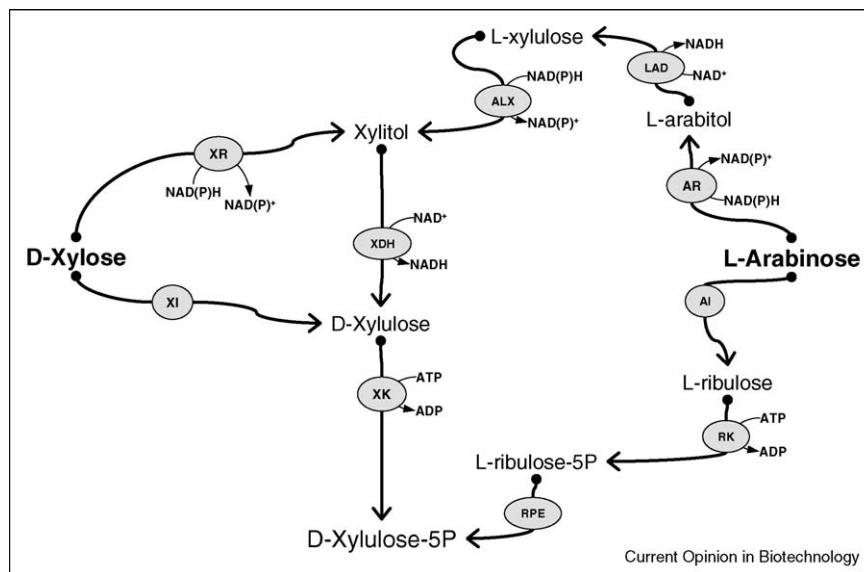
Pentose fermentation

In natural xylose and arabinose utilizing microorganisms, bacteria use an isomerase pathway to channel the pentose sugars to the central metabolism, whereas yeast and filamentous fungi mainly use a reductase/dehydrogenase pathway (Figure 3). Both pathways have been introduced in *S. cerevisiae* (reviewed in [35**,36**]).

High ethanol yields have been obtained when the xylose isomerase pathway was introduced in *S. cerevisiae* and transformants were subsequently exposed to evolutionary engineering protocols [37]. Similarly, the arabinose isomerase pathways have been introduced [38]. However, isomerase pathways have so far only been expressed from multi-copy plasmids, which lack the stability required for industrial applications [39].

Yeast and fungal reductase/dehydrogenase include enzyme reactions using different redox co-factors (Figure 3), which may result in carbon-wasting by-product formation (reviewed in [35**,36**]). Nevertheless the two best-performing industrial xylose-fermenting *S. cerevisiae* strains are based on reductase/dehydrogenase

Figure 3



Pentose metabolism. AI: arabinose isomerase; ALX: L-xylulose reductase; AR: arabinose reductase; LAD: arabitol dehydrogenase; RK: ribulokinase; RPE: ribulose 5-phosphate epimerase; XDH: xylitol dehydrogenase; XI: xylose isomerase; XK: xylulokinase; XR: xylose reductase (designed by Maurizio Bettiga).

pathways [34]. However, when a fungal arabinose pathway was expressed in *S. cerevisiae*, ethanol formation was limited [40].

The pentose redox metabolism has been a subject of numerous engineering approaches (reviewed in [35••,36••]). Most recently, targeted protein engineering resulted in significantly increased ethanol yield and productivity (O Bengtsson *et al.*, unpublished). Furthermore, aldehydes and ketones present in non-detoxified hydrolysate significantly reduce by-product formation [41].

Despite enzyme cross-affinities [42] co-utilization of xylose and arabinose was significantly better in a xylose reductase/xylytol dehydrogenase expressing strain than in a corresponding isogenic isomerase strain [43]. For co-utilization of pentose sugars by isomerase strains a dedicated evolutionary engineering protocol was required [44•].

Inhibitor tolerance

S. cerevisiae strains display great variation in inhibitor tolerance [45]. Whereas the biochemistry of lignocellulose inhibitors has been individually investigated [46••] the molecular basis for yeast tolerance toward lignocellulose hydrolysates remains to be elucidated. Furaldehydes are reduced to the less inhibitory alcohols by the yeast itself (JRM Almeida *et al.*, unpublished) and have been used to evolutionary engineer yeast for improved fermentation of lignocellulose hydrolysates [47].

Industrial strains fermenting non-detoxified hydrolysate are a source of genes encoding detoxifying enzymes. An NADPH using alcohol dehydrogenase 6 (ADH6) [48] and a mutated NADPH-dependent and NADH-dependent ADH1 [49] were identified in a strain isolated from a spent sulfite liquor fermentation plant [50]. The enzymes reduce furfural to its corresponding alcohol and confer inhibitor tolerance to laboratory strains of *S. cerevisiae* [51].

SSF of lignocellulose

In simultaneous saccharification and fermentation (SSF) [52••] low glucose concentrations are maintained in the reactor and this stimulates xylose fermentation without inhibiting xylose transport [53]. Thus, improved xylose fermentation has been demonstrated in SSF [54•] and a recent report on fermentation of low xylose concentrations with a recombinant *S. cerevisiae* expressing a heterologous glucose/xylose facilitator from *Candida intermedia* [55] suggests that SSF of lignocellulose can be further improved.

Process integration

Increasing production capacity to commercial scale can only be done with confidence when a process is shown to be robust at an intermediate, pilot scale. An ideal pilot plant needs to be fully integrated, able to evaluate the

complete system (e.g. enzymes and yeasts) while having sufficient flexibility to investigate alternative process configurations and test options for better heat integration and the recycling of process streams. Recently there has been a significant effort for building pilot facilities, but the mass and energy balances obtained are rarely published and recent literature on process integration is poor [56].

The pilot plant evaluated within the NILE project is able to handle 2 tons of dry biomass per day, a capacity enabling representative data on mass and energy balances to be gathered while maintaining flexibility. The enzymatic hydrolysis process could be scaled up from laboratory scale (1–10 L) to process development scale (100 L), and finally to pilot scale (10 000 L). The first pilot scale trials were based on the concept of separate hydrolysis and fermentation (SHF). Later trials incorporated SSF, and measured enzymatic decomposition rates of up to 80 percent of the cellulose. Further trials with both SHF and SSF will soon be performed in the pilot plant to validate new enzymes and yeast strains obtained in the project.

The decision to employ SHF or SSF has an important impact on the final production cost. In the SHF process both yeast and enzymes can work at their optimal temperature, but an accumulation of end products can reduce the efficiency of hydrolysis. In the SSF process, end product inhibition can be avoided but the reaction conditions are a compromise. Models based on laboratory data have confirmed the SSF process to be the most efficient option for both softwood [57] and wheat straw [58]. Nevertheless, it is still too early to ratify the SSF process concept at pilot plant scale.

The properties of the lignin (and other solid) residues from the pilot plant trials indicate that this material is suitable for heat and power generation, but because it has a higher calorific value than softwood granules, higher capacity heat exchangers may be required. There is also potentially a higher risk of NO_x and SO₂ formation during combustion owing to relatively high nitrogen and sulfur content in the residue [59].

From a process scale-up perspective, it is clear that the challenges lie not only in finding the most efficient organisms for the conversion of cellulose to ethanol but also in making intelligent use of the entire feedstock and exploiting all the opportunities for process integration. Process solutions that can decrease energy demand and increase energy outputs have in models demonstrated significantly better process economy [60•,61,62]. For example, in a basic scenario with softwood, the minimum selling price of ethanol shows variations from 0.38 to 0.47 €/L depending on for which energy purpose(s) the residue is utilized. In particular, the local demand for district heating will influence the price of ethanol [60•].

Table 1

Cost estimates for lignocellulosic ethanol production. Estimates are normalized for currency, year, and units.

Reference	Conversion process ^a	Capacity ^b (tonnes dry biomass year ⁻¹)	Ethanol production cost (₂₀₀₅ \$ L ⁻¹)
Von Sivers and Zacchi [69]	Enz./dilute acid/conc. acid	100 000 (S)	0.76/0.81/0.79
Lynd [65]	Enz. (SSF)	592 000 (H)	0.4
NREL: Wooley <i>et al.</i> [66]	Enz. (SSF)	700 000 (H)/(CS)	0.47/0.34
Aden <i>et al.</i> [74] Ruth and Jechura [67]			
Wingren [58]	Enz.(SHF)/Enz.(SSF)	196 000 (S)	0.8/0.68–0.64
Sassner <i>et al.</i> [68]	Enz.(SSF)	200 000 (H)/(CS)/(S)	0.71/0.71/0.57

^a Process classified according to principal hydrolysis step. Enz. = enzymatic hydrolysis; SSF = simultaneous saccharification and fermentation; SHF = separate hydrolysis and fermentation.

^b S = softwood; CS = corn stover; H = hardwood.

Economics and environmental impacts

The markets for biofuels in North America and the EU are almost entirely dependent on policy mandates and fiscal incentives, predicated on the contribution of ethanol to greenhouse gas saving, security of supply, and employment policy objectives [63]. For example, the latest EU policy (10% biofuels by 2020) makes access to subsidized markets contingent on a minimum 35% GHG saving (increasing to 50% from 2017). Currently, it is uncertain which competing technology pathways will become dominant, but nonetheless, it is clear that for a technology to be commercially viable and future-proof it must be cost competitive and deliver the environmental benefits demanded.

Cellulosic ethanol's commercial viability depends upon: (i) feedstock cost and digestibility; (ii) conversion cost and efficiency; (iii) product revenues. R&D is improving the conversion process, but the issues of feedstock availability and revenue stability remain uncertain and subject to political risk. Feedstock costs are primarily determined by existing markets and tend to be geographically constrained. Digestibility requirements may also limit practicable supply. Supply-chain design will therefore play a crucial role in determining which process concepts are successful [64••]. Expectations of feedstock availability are reflected in estimates of production cost. Cost is an important metric for comparing alternative process designs, but caution is required when comparing different estimates. For example, US studies generally forecast a lower cost of ethanol (0.34–0.47₂₀₀₅\$ L⁻¹) [65–67] than EU studies (0.57–0.8₂₀₀₅\$ L⁻¹) [58,68,69] (Table 1). Partly, this is due to the US studies assuming greater feedstock availability and larger conversion plant.

Forecasting GHG savings is problematic for two reasons: (i) the definition of system boundaries and the allocation of co-product impacts are highly subjective; (ii) GHG emissions embodied in feedstocks depend upon the production location, method, and consequential impacts. Influential meta-studies aiming to bring greater consistency, transparency, and coherence to the life cycle

assessment (LCA) of biofuels have drawn the system boundary around an individual production plant and its supply-chain [70••,71]. The studies differ somewhat in approach, but agree upon a general conclusion: cellulosic ethanol results in greater carbon savings (75–150 g CO₂e km⁻¹) than wheat (15–110 g CO₂e km⁻¹) or maize (40–60 g CO₂e km⁻¹), but not necessarily as great as from Brazilian sugarcane (125–175 g CO₂e km⁻¹). Two subsequent studies contest this conclusion, asserting that the system boundaries should be expanded to include consequential impacts (direct and indirect land-use change) [72,73••,74]. The science for determining these impacts is in its infancy. Following Intergovernmental Panel on Climate Change 'tier 1' guidance the direct impacts from waste products (e.g. straw) and managed forestry (e.g. softwood in northern Europe), are nil. If the carrying capacity of existing managed forestry and agricultural residues is not exceeded, land use change could, therefore, reasonably be ignored. Replacing 10% of European transport fuel, however, requires a radical response. Consequently, land use change and indirect impacts will remain firmly on the political and scientific agenda. Maximizing GHG savings will require efficient supply-chain design and a better understanding of the spatial and temporal factors that affect overall performance.

Conclusions

Advances in the cost-effective conversion of lignocellulosic biomass are often difficult to assess accurately because of the lack of integrated testing, for example, lab and pilot scale trials, and the lack of appropriate tools, for example, process, cost, and environmental impact models. Integrated projects such as NILE are required because of the high level of interdependence between process steps and the necessity to give a global standpoint on the whole chain. All results generated in projects involving pilot plant trials will contribute to the process design developed for an industrial development. A good example is the industrial development unit (IDU) planned by SEKAB (URL: <http://www.sekab.com/default.asp?id=2028&refid=2038>). This IDU will have a

production capacity of 6000 m³ ethanol per year, which is 40 times higher than the existing pilot plant. It is planned to be in operation by 2010–2011 and by 2014 SEKAB expects to have developed the technology to a commercial level thanks to the incremental improvements obtained on lignocellulosic ethanol.

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