ΠΡΟΓΡΑΜΜΑ ΔΙΑ ΒΙΟΥ ΜΑΘΗΣΗΣ ΑΕΙ ΓΙΑ ΤΗΝ ΕΠΙΚΑΙΡΟΠΟΙΗΣΗ ΓΝΩΣΕΩΝ ΑΠΟΦΟΙΤΩΝ ΑΕΙ (ΠΕΓΑ)

«Οι σύγχρονες τεχνικές βιο-ανάλυσης στην υγεία, τη γεωργία, το περιβάλλον και τη διατροφή»
Barriers to the commercialization of lignocellulosic ethanol include the development of more robust biocatalysts, reduction of cellulase costs, and high capital cost associated with a complex process. Improvements have been made in all areas during the past two years. Oxidoreductases, transporters, and regulators have been identified that can increase the tolerance of biocatalysts to inhibitors formed during pretreatment. Biocatalysts are being developed that grow under conditions that are optimal for cellulase activity and others have been engineered to produce glycoside hydrolases. Ethanol yields resulting from most current process configurations are similar, approximately 0.21 g ethanol/g dry cellulosic feedstock. Potentially, this can be increased to at least 0.27 g ethanol/g biomass (83 gal/ton) using simpler processes.

This review highlights advances in the fermentative production of ethanol from lignocellulose during the past two years. Improvements are noted in the areas of pretreatment, biocatalysts, saccharification and liquefaction, and process simplification.

Advances in pretreatment

Pretreatments using dilute sulfuric acid require reactors made of exotic metals. Although all mineral acids have been explored to some extent, recent studies have proposed the use of phosphoric acid [4**,5]. As a weaker acid, phosphoric acid pretreatment produces lower levels of toxic side products than sulfuric acid pretreatment and can be used with a stainless steel reactor [4**]. Autohydrolysis produces the lowest levels of side products [2]. With autohydrolysis, hemicellulose components are solubilized as oligosaccharides that require further hydrolysis with enzymes or acid. Ethanologenic Escherichia coli strains have been adapted to phosphoric acid hydrolysates and can now ferment hemicellulose and cellulose derived sugars together in a single vessel, termed simultaneous saccharification and co-fermentation (SScF). The use of phosphoric acid could eliminate the need for separation of hydrolysates from pretreated fiber, detoxification, and reactors of exotic metals resulting in a simpler process quite analogous to that for corn ethanol (Figure 1). Using this process, ethanol yields of up to 0.27 g/g bagasse (dry weight) have been obtained (83 gal/ton) [6**].

The SPORL process (sulfite pretreatment to overcome recalcitrance of lignocellulose) and SO2 impregnation use sulfur compounds to disrupt the LCB structure [7**,8]. SPORL is better suited for biomass with high lignin content and SO2 impregnation for agricultural residues [8]. SPORL pretreatment was shown to increase sugar yields (from 57% to 88%) and reduce inhibitors by up to 65% compared to dilute sulfuric acid pretreatment of softwood [7**,9].

Ammonia-based AFEX pretreatment (ammonia fiber expansion) is very effective at increasing fiber digestion while producing lower levels of inhibitors than sulfuric
Lignocellulose to ethanol process configurations. The cellulose could be hydrolyzed alone before fermentation (separate hydrolysis and fermentation, SHF) or with the hemicellulose (separate hydrolysis and co-fermentation, SHcF) followed by fermentation of the resulting slurry. Cellulose hydrolysis could also occur simultaneously with fermentation in the presence (SScF) or absence (SSF) of hemicellulose. In the liquefaction followed by simultaneous saccharification and co-fermentation (L + SScF) process, there is a cellulose prehydrolysis step in the presence of hemicellulose hydrolysate followed by fermentation but the cellulases continue to hydrolyze the cellulose during fermentation. The consolidated bioprocessing process involves a biocatalyst that is capable of producing all the hydrolytic enzymes required for cellulose hydrolysis and is also capable of fermenting all the resulting sugars in the presence of hydrolysate inhibitors.

Adapted from [2].

Advances in biocatalyst
The need for more robust biocatalysts is one of the weakest links in the LCB to ethanol process. These biocatalysts need to be resistant to inhibitors formed during lignocellulose pretreatments, co-utilize a variety of sugars at high yields, secrete cellulase enzymes, and remain active under conditions that are near optimal for cellulase function (pH 5, 50 °C). Much of the complexity in lignocellulosic ethanol processes stems from the need for toxin mitigation (solid liquid separation after pretreatment; sugar cleanup) before fermentation. Additional complexity comes from the requirement for external sources of cellulase enzymes. Developing biocatalysts that ferment under conditions that are near optimal for fungal cellulase activity can reduce the requirement for external enzymes. Engineering the fermenting biocatalyst to produce some or all of the cellulase enzymes provides a complementary route to further reduce enzyme cost. The development of a fermentation-based lignocellulose to ethanol industry depends on research advances to minimize these biological impediments.

Developing tolerance to hydrolysate inhibitors
Furans from sugar dehydration, acetate, and soluble products from lignin are the primary inhibitors in hemicellulose hydrolysates from dilute acid pretreatment [13]. Of these, furans appear to be particularly important and have been the focus of many recent papers. Biocatalysts have been developed with increased resistance to furfural [14**,15,16**,17,18,19**], 5-hydroxymethylfurfural [15,19**,20], acetate [21], and to unfractionated dilute acid hydrolysate [6**,15,19**,22**,23**].
Furans can be reduced to less toxic furan alcohols by most organisms using native enzymes. Expression arrays have noted many changes among oxidoreductases in response to furans with yeast [14,15,24,25*], Zymomonas mobilis [19*,21*], and ethanologenic E. coli [16*,17,18,20]. In some cases, regulators have been identified [18,19*]. Several of the furfural reductase enzymes in E. coli have sufficiently low \( K_m \) values for NADPH that growth is inhibited until furan metabolism has been completed. Silencing of these low \( K_m \) enzymes (less than 20\% of total furfural reductase activity) was beneficial for furfural tolerance in E. coli [17]. Other higher \( K_m \) enzymes (NADPH), NADH-dependent enzymes [15], and transhydrogenase enzymes have been shown to confer partial resistance to furfural [16*,18,20].

Reduced sulfur compounds have long been used to improve the fermentation of dilute sulfuric acid hydrolysates of wood although the mode of action remains unknown [23*,26*,27]. *Saccharomyces cerevisiae* fermentation of acid hydrolysates (sugarcane bagasse and spruce wood) was improved by the direct addition of reduced sulfur compounds to slurries containing complex media, termed 'in situ detoxification' [26*]. The use of sodium metabisulfite and sodium hydrosulfite was also shown to improve the fermentation of slurries containing phosphoric acid pretreated sugarcane bagasse (L + SSeF) using E. coli in mineral salt medium [23*]. Surprisingly, addition of metabisulfite did not decrease the toxicity of furfural or acetate when each was tested alone. It is possible that metabisulfite neutralizes the toxicity of soluble products from lignin.

**Improving hexose and pentose sugar co-utilization**

The co-utilization of hexose and pentose (xylose and arabinose) sugars remains a challenge for biocatalysts, especially in the presence of hydrolysate inhibitors. Derivatives of *S. cerevisiae* have been previously engineered to ferment xylose and these continue to be improved by additional genetic changes in xylose metabolism [28*,29,30]. Although ethanologenic *E. coli* have the native ability to metabolize all sugars from LCB, xylose utilization lags behind glucose and was also improved by further genetic changes [31]. Inhibitors present in acid hydrolysates retarded xylose metabolism during *E. coli* fermentation even with this genetic change. The lag in xylose metabolism was substantially relieved by injection of small amounts of air during fermentation, termed microaeration [6*,32].

**Advances in saccharification**

**Reducing the cost of enzyme production**

The cost of cellulase enzymes remains a major concern for the commercialization of LCB ethanol processes. Cost estimation software (e.g. Aspen Plus and Aspen Icarus Process Evaluator) has been used to compare the minimum ethanol selling prices of processes involving the purchase of commercial cellulase and on-site cellulase production [33]. International enzyme companies such as Novozymes and Genencor have formed partnerships with Poet LLC and DuPont Danisco Cellulosic Ethanol LLC, respectively, to commercialize lignocellulosic ethanol (The New York Times: URL: http://www.nytimes.com/cwire/2010/02/16/ 16climatewire-economics-improve-for-first-commercial-cellul-93478.html?scp=1&sq=cellulosic%26ethanol%20 plants&st=cse). Both have reported that enzymes will cost approximately $0.50/gallon of ethanol. This represents an 80\% price reduction during the last two years.

Efforts continue to reduce the cellulase requirement. Novel cellulases have been isolated from a variety of organisms using improved screening methods [34]. Current research has focused on developing improved cellulase enzyme cocktails, development of biocatalysts with fermentations that match the optimal conditions for cellulase activity, and novel cellulases [34,35*]. *Trichoderma reesei* is currently the primary industrial organism used for the production of cellulase enzymes. Sequencing of the *T. reesei* genome will facilitate further improvements in enzyme production [36].

**Improving cellulase performance**

Compounds have been identified that increase cellulase effectiveness and enzyme usage [37,38*,39]. These include surfactants (Tween 80, cetylpyridinium chloride, and cetyl trimethylammonium bromide) and divalent metals (calcium and magnesium). Up to 35\% improvement in saccharification was reported. All are proposed to act by reducing the nonproductive binding of cellulases to lignin. *Bacillus coagulans* is a thermotolerant biocatalyst capable of growth at temperatures and pH (55 °C, pH 5.0) that are optimal for fungal cellulases. Using this organism, the cellulase was reduced to 5 FPU/g cellulose during lactate production [35*,40]. Similar benefits would be expected for ethanol production after further metabolic engineering, and for other biocatalysts that can function under these conditions.

**Toward consolidated bioprocessing**

Consolidated bioprocessing without the need for externally supplied enzymes remains a goal for many scientists [41*,42*,43*,44,45]. Expression of endoglucanase I and II genes from *T. reesei* QM6a allowed the resulting strain to ferment phosphoric acid swollen cellulose (amorphous) when beta-glucosidase was supplied [41*]. Tsai et al. and Wen et al. reported the development of recombinant *S. cerevisiae* strains capable of displaying functional mini-cellulosomes on their surface exhibiting enzyme synergy and producing 3.5 g/L and 1.8 g/L ethanol, respectively, using phosphoric acid swollen cellulose [42*,43*]. Previous studies have demonstrated up to 11 g/L ethanol production from phosphoric acid swollen cellulose using *Klebsiella oxytoca* strain SZ21 expressing endoglucanase.
genes from *Erwinia chrysanthemi* (*celY, celZ*) [44]. Synergies between purified cellulases and xylanases from the thermostophilic bacterium *Thermobifida fusca* displayed on ‘designer cellulosomes’ were found to possess higher activity on wheat straw than the corresponding free enzymes [45].

**Process simplification**

Reducing process complexity remains a major challenge for the commercialization of LCB to ethanol. Current research is focused on eliminating the need for detoxification of hydrolysates, developing robust biocatalysts capable of fermenting pentose and hexasugar sugars simultaneously, reducing water usage, increasing ethanol yield and titer, and decreasing cellulase usage. Considerable progress has been made during the past two years by developing robust biocatalysts capable of fermenting pentose and hexasugar sugars simultaneously. Further progress is needed to increase ethanol titers and to decrease water and cellulase usage. Collaborative research projects have focused on comparing pretreatment options for specific biomass types (e.g. corn stover or poplar wood [1,46**]). Various process configurations are shown in Figure 2. These decrease in complexity from separate hydrolysis and fermentation (SHF) to consolidated bioprocessing (CBP). The SHF process involves separation of the cellulose-rich solid from the hemicellulose hydrolysate and separate fermentation trains. L + SScF and SScF processes combine C-6 and C-5 sugar fermentations in a single vessel [6**,22**,23**]. The consolidation of bioprocessing steps is hindered by the fibrous nature of suspensions at loadings of 10–20% solids [47,48]. Models have been described relating viscosity, solubilized sugars, time, and enzyme loadings for slurries of sugarcane bagasse [4**,48,49]. On the basis of these studies, a partial saccharification step using a CSTR (one to six hours residence) was proposed [4**]. This liquefaction step can produce slurries containing 10–15% solids (solids plus solubles) that can be readily pumped and mixed.

**SScF of lignocellulosic biomass**

Pretreatment processes typically require solid–liquid separations and neutralization of hydrolysate toxins before fermentation. With the development of hydrolysate resistant biocatalysts such as *E. coli* MM160 [22**,23**] and *S. cerevisiae* 424A [43**,44,45,46**,47], comparable yields could be obtained with less process complexity. The development of robust biocatalysts allowed the fiber and liquid from pretreatment to be fermented without separation [6**,22**,23**]. The resulting process is analogous to the mature corn dry milling ethanol process (Figure 1) that combines all components in a single vessel after an initial liquefaction step (L + SScF process). Ethanol yields for LCB processes have continued to improve during the past two years (Table 1). Despite differences in process complexity, similar ethanol yields were obtained by most researchers, approximately 0.21 g/g (63 gal/ton). Higher yields are obtained when purified cellulose was used (e.g. paper sludge [55**]) or starch combined with lignocellulose (e.g. corn silage and whole corn plant [53]). The use of SPORL pretreatment is making similar progress toward process simplifications (e.g. L + SScF) although part of the hydrolysate was removed before fermentation [56]. AFEX treated corn stover supplemented with corn steep liquor was fermented after an initial 96 h prehydrolysis (cellulases and hemicellulases added) to produce 40 g/L ethanol.
(0.22 g ethanol/g corn stover [51**]) and dilute acid pretreated sugarcane bagasse was fermented to high ethanol yields (0.27 g ethanol/g bagasse) when air was added to the headspace during a L + SSF process [6**].

**Dual uses of process residues, chemicals, and water**

Beneficial products must be derived from all materials entering LCB to ethanol processes. Vinasse, stillage from sugarcane ethanol processes, has been used for many years as a fertilizer for biomass crops [57]. Pretreatment processes with phosphoric acid offer a similar opportunity by producing an ammonium phosphate fertilizer that includes magnesium sulfate and trace metals [4**,22**,49]. A phosphoric acid LCB-ethanol process can be viewed as a temporary stop for water and fertilize en route to farms for new crop growth, sharing the cost of these materials. Lignin-rich residues can be used as boiler fuel or converted to higher value products [58,59]. Lignin could also be formed into inert blocks as an effective means for carbon sequestration.

**Conclusions**

The challenge of producing 36 billion gallons of ethanol by the year 2022 is being met with an expansion of research in the biofuel arena. Major improvements have been made as researchers learn more about the genetic basis of resistance to inhibitors in acid hydrolysates and pentose utilization. Pretreatment processes have been optimized to minimize inhibitor formation and to improve enzymatic hydrolysis of cellulose. The cost of cellulase enzymes remains a concern. Approaches have been proposed to minimize external enzyme usage by
producing enzymes in the biocatalyst and by providing conditions that increase the effectiveness of cellulases.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

5. The authors show a decrease in side reactions during dilute acid pretreatment when phosphoric acid is used instead of sulfuric acid. Sugar yields higher than 80% were obtained for total sugars when using 0.5 FPU/g WIS.
7. The authors were able to improve sugar utilization by injecting small amounts of air into the headspace, resulting in increased ethanol yields. The process was successfully scaled up to 80 L with yields of up to 0.27 g ethanol/g bagasse.
9. Successful SHC process of softwood using an inhibitor tolerant strain S. cerevisiae VS. A yield of 270 L/tonne wood was obtained using 15 FPU cellulase/g wood.
Using an engineered S. cerevisiae strain, the authors were able to ferment corn stover without detoxification or added nutrients to obtain a yield of 238 L/tonne corn stover.
17. The authors identified ADH1 and an uncharacterized open reading frame, YKL071W, as genes that conferred furfural resistance to S. cerevisiae.
20. The authors proposed a model of furfural inhibition in which growth is limited by the assimilation of sulfur into amino acids due to the depletion of NADPH during furfural reduction.
This study reports the involvement of the global regulator Hqf in the resistance of inhibitors. For the purpose of this study, the authors created a plasmid for gene expression and mutant complementation in Z. mobilis.
The authors used a combination of microarray-based CGS and next-generation 454 pyrosequencing to identify genes related to acetate tolerance in Z. mobilis.
Successful L + SScf of sugarcane bagasse without detoxification. A yield of 260 L ethanol per tonne bagasse was obtained using 10 FPU/g bagasse and a solids loading of 14%.
The authors tested the effect of various reduced sulfur compounds on the performance of an ethanologenic E. coli strain during the fermentation of phosphoric acid pretreated sugarcane bagasse. Addition of sodium metabisulfite at the start of fermentation resulted in increased ethanol yields.
29. Ma M, Liu ZL: Comparative transcriptome profiling analyses during the lag phase uncover YAP1, PDR1, PDR3, RPN4, and HSFF1 as key regulatory genes in genomic adaptation to the


28. Bettiga M, Bengtsson O, Hahn-Hagerdal B, Gorwa-Grauslund MF: Arabinoxylose and xylose fermentation by recombinant Saccharomyces cerevisiae expressing a fungal pentose utilization pathway. Microb Cell Fact 2009, 8:40. The authors express a fungal pathway for pentose utilization, resulting in a decrease in α-arabinose yield when compared to previously engineered strains with α-arabinose reductase activity co-fermenting α-arabinose and α-xylose.


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43. Tsai SL, Oh J, Singh S, Chen R, Chen W: Functional assembly of minicellulosomes on the Saccharomyces cerevisiae cell surface for cellulose hydrolysis and ethanol production. Appl Environ Microbiol 2009, 75:6087-6093. The assembled minicellulosomes maintained the synergistic effect for cellulose hydrolysis and produced ethanol directly from phosphoric-acid-swollen cellulose with a 95% conversion of the carbohydrate consumed.

44. West F, Sun J, Zhao H: Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Appl Environ Microbiol 2010, 76:1251-1260. The recombinant strain was able to create cell-associated multifunctional minicellulosomes and produce ethanol directly from phosphoric-acid-swollen cellulose.


51. Lau MW, Dale BE: Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST). Proc Natl Acad Sci U S A 2009, 106:1368-1373. This article describes a SHF process in which there is no need to add nutrients or detoxify the hydrolysate. AFEX treated corn stover was used to produce 0.18 g ethanol/g dry corn stover (40 g/L ethanol) using S. cerevisiae 424A (LNH-ST).


The authors report the production of over 40 g/L ethanol using waste paper sludge as feedstock. Two recombinant strains, Z. mobilis 8b and S. cerevisiae RWB222, were compared.


The authors used a fed-batch SSF strategy to obtain 20% more ethanol using steam exploded wheat straw and K. marxianus CECT10875. They obtained 0.18 g ethanol/g dry wheat straw from a final solids loading of 14%.


The authors compared a batch and fed-batch SSF process as well as enzyme feeding strategy using sulfite pretreated spruce and S. cerevisiae. They found no definite relationship between ethanol production and enzyme feeding strategy but found that the fed-batch mode provided the best mixing.


