

Realistic approach for full-scale bioethanol production from lignocellulose: a review

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Received 15 July 2008; revised 18 September 2008; accepted 22 September 2008

This paper reviews current status of bioethanol production including substrates, fermenting microorganisms and technology for a full-scale process development. Considering main drawbacks, several parameters (high substrate loadings, sugar recovery after pretreatment, tolerance to inhibitory compounds and xylose fermentation by yeast) must be optimized for a successful industrial process for bioethanol production from lignocellulose.

Keywords: Bioethanol, Lignocellulosic materials, Xylose

Introduction

Depletion of world's petroleum supply and greenhouse gas (GHG) effects have resulted in a growing interest in alternative fuels¹. For ethanol production, main feedstock among sugar crops is sugarcane as cane juice or molasses²⁻⁴. Among starchy biomass, most of bioethanol is produced from corn^{5,6} or wheat⁷⁻⁹, but also from cassava¹⁰, rye^{9,11}, barley⁸ or sorghum^{12,13}. These raw materials, also employed for animal or even human feeding, seem not to be sufficient for supplying increasing demand. In this context, bioethanol produced from lignocellulosic biomass (LB) is an interesting alternative since LB do not compete with food crops and are also less expensive than conventional agricultural feedstocks¹⁴. It is reported¹⁵ that total potential bioethanol production from LB is about 16 times higher than current ethanol production from sugars or starch biomass¹⁵. Also, bioethanol from LB can reduce net CO₂ emissions to almost zero¹⁶⁻¹⁸. Enzymatic hydrolysis of cellulose provides opportunities to improve technology being this ethanol competitive when compared to other liquid fuels on a large scale¹⁹.

This review offers present status of bioethanol production based on enzymatic hydrolysis (EH), with emphasis on different process configuration and

drawbacks that have to be overcome for a successful industrial process.

Lignocellulosic Biomass (LB): Composition and Feedstocks

LB is expected to be major feedstock in future bioenergy²⁰. Besides LB (**hardwoods**, softwoods or herbaceous biomass), other lignocellulosic sources such as municipal solid wastes (MSW)^{21,22} or waste paper²³ have also proven as raw material for bioethanol production. Fraction of some agricultural residues is difficult to quantify because it depends on weather, crop rotation, soil fertility, land slope and tillage practices¹⁵. For example, in case of wheat straw, each 1 kg of grain produced 1.1 kg of straw²⁴ and according to FAO²⁵, 680 million tonnes (MT) of wheat straw were produced in 2007 worldwide. Considering wheat straw used for animal feeding and for soil maintenance, about 60% of world production could be assigned to energetic purposes¹⁵, being one of the most abundant lignocellulosic sources.

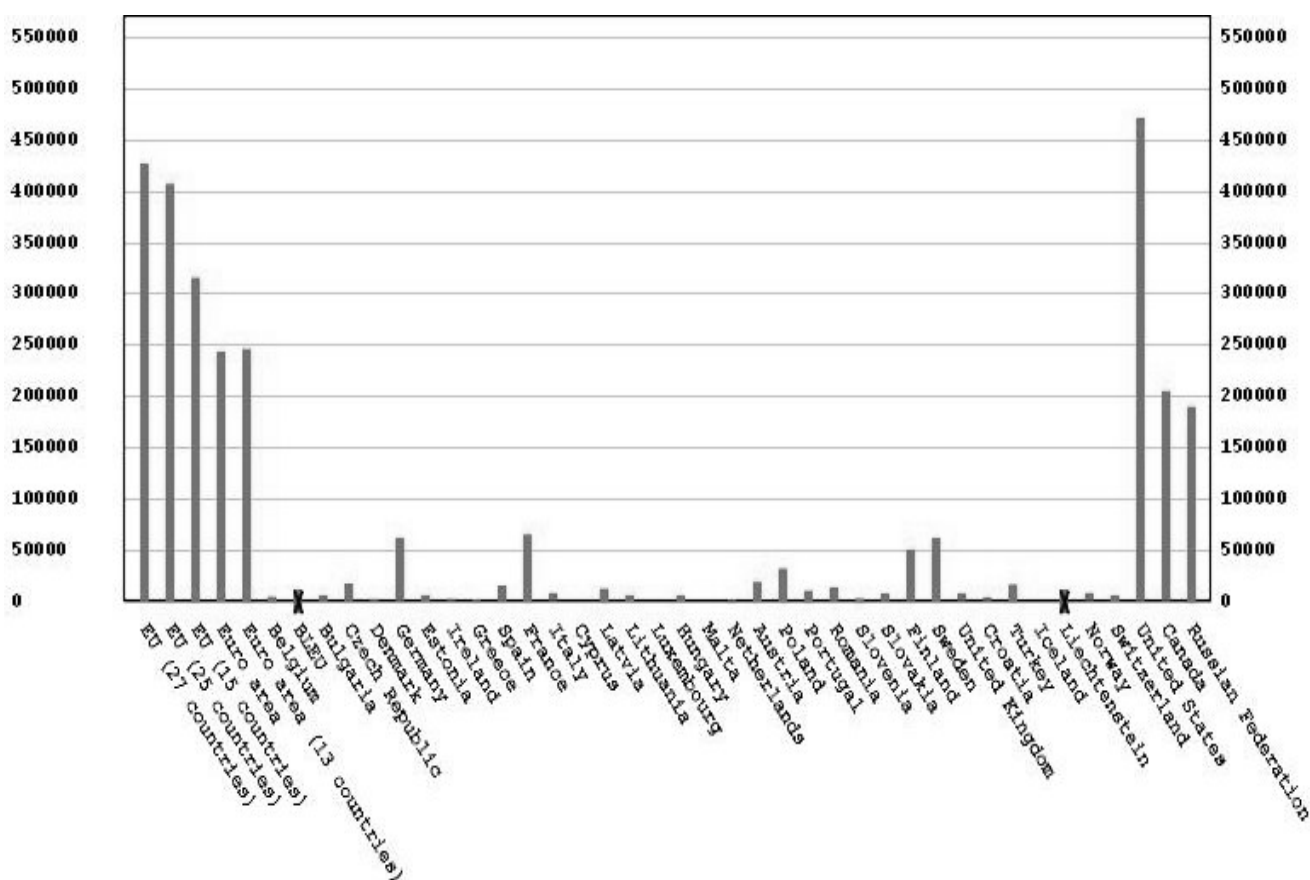
Wood residues production in European Union (EU) was about 534 millions m³ in 2006²⁵ (Fig. 1) and these LBs are potential raw material for ethanol production. In the specific case, olive trees generates world over an annual volume of lignocellulosic residues estimated at 3000 kg/ha²⁶. Apart from residual biomass, energy crops seem to be a promising future resource of biomass due to the ability of obtaining numerous harvests

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Table 1—Composition of different lignocellulosic materials (% dry wt)

Biomass	Glucan	Xylan	Mannan	Galactan	Arabinan	Lignin
Poplar ²⁸	43.5	15.5	2.5	2.3	1.5	26.2
Pine ²⁹	46.4	7.8	10.6	n.d	2.2	29.4
Spruce ³⁰	49.9	5.3	12.3	2.3	1.7	28.7
Salix ³¹	41.5	15.0	3.0	2.1	1.8	25.2
Birch ³²	38.2	18.5	1.2	n.d.	n.d.	22.8
Corn stover ³³	36.8	22.2	n.d.	2.9	5.5	23.1
Wheat straw ³⁴	30.2	18.7	n.d	0.8	2.8	17.0
Barley straw ³⁵	33.1	20.2	n.d.	0.9	3.8	16.1
Olive tree pruning ³⁶	25.0	9.82	0.7	1.4	2.1	18.8

n.d., no detected

Fig. 1. Wood residues production (1000 m³) in European Union

from a single planting. However, land availability and yield levels in energy crop production are very uncertain²⁷.

Among main components²⁸⁻³⁶ (cellulose, hemicellulose and lignin) of LB (Table 1), cellulose, a

linear polymer of cellobiose, consist of two D-glucose, linked by β -1,4 bonds. In fact, there are several forms of cellulose with different degrees of polymerization and molecular weight^{37,38}. More ordered or crystalline cellulose is less soluble and less degradable³⁹.

Hemicelluloses are consisted of non-cellulosic polysaccharides (D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic) and related substances (hexuronic acid and its by-products). Hemicellulose differs from cellulose by composition of several sugar units, by presence of shorter chains and by a branching of main chain molecules³⁸, which made structure easier to hydrolyze than cellulose. Softwood hemicelluloses have mannose as majority constituent in main chain, and also present higher amount of galactose than hardwoods, which usually contain higher proportion of xylose units and acetyl groups^{38,40}. Herbaceous biomass has hemicellulosic components similar to hardwoods, but present lower amount of acetyl groups³⁸.

Lignin is linked to hemicelluloses and cellulose forming an impermeable barrier preventing enzymatic activity⁴¹. Lignin building units are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which are polymerized into an amorphous monocrystalline polymer⁴². These three aromatic alcohols give rise to guaiacyl units, syringyl units and p-hydrophenil units which proportion also differs among **hardwoods**, softwoods or herbaceous biomass^{38,43}. In LB, lignin is closely bound to cellulose and hemicellulose and this strong association affects enzymatic degradation. Thus, different chemical or physical delignification methods have been reported for obtaining higher sugar yields in hydrolysis step^{44,45} although addition of delignification step into bioprocess can reduce process cost-effectiveness⁴⁶.

Pretreatment

Owing to structural characteristics of LB, pretreatment is an essential step for obtaining potentially fermentable sugars in hydrolysis step⁴⁷. During pretreatment, fibers structure is altered and enzyme accessibility to cellulose is enhanced^{48,49}. Pretreatment that represents 33% of the total cost of process¹⁶ requires development of efficient pretreatment technologies for reducing ethanol production costs⁴⁶. Regarding applicability, several parameters (pentoses recovery, chip size required, concentration of toxic compounds formed during pretreatment and low energy demand) have been described as deciding factors in an effective pretreatment^{46,50,51}.

Different pretreatment technologies for pretreatment of LB include biological, chemical, physical and physico-chemical processes^{2,46}. Biological

pretreatments employ microorganisms (brown, white and soft-rot fungi), which degrade lignin and hemicellulose^{52,53}. Main problem in using biological methods is that fungi mainly attack cellulose and hydrolysis rate in most biological materials is very low⁵¹. Chemical pretreatments employ different chemical agents² (ozone, acids, alkalis, peroxide and organic solvents). Among all chemical pretreatments, dilute acid pretreatment has been successfully developed, improving significantly the subsequent process of EH^{54,55}. Alkaline pretreatment, ozonolysis, peroxide pretreatment and organosolv pretreatment are focused on lignin removal. Costs of pretreatments are so high that these methods are not competitive for large scale⁵¹. Physical pretreatments (chipping, grinding and milling) reduce cellulose crystallinity^{2,51} but require high energy and capital costs. Pyrolysis has also been used for pretreating LB, since biomass can be used as substrate for a fast pyrolysis for thermal conversion of cellulose and hemicellulose into fermentable sugars with good yields⁵⁶.

Steam explosion (SE) is most widely employed^{134,57-59} physico-chemical pretreatment for LB. **Liquid** hot water (LHW) and ammonia fiber explosion (AFEX) have been also reported as a good physico-chemical pretreatments for LB^{28,60}. LHW subjects biomass to hot water in liquid state at high pressure during a fixed period⁶⁰ and it presents elevated recovery rates for pentoses and generates low amount of inhibitors⁶¹. AFEX, where biomass is exposed to liquid ammonia and pressure for a time period and then pressure is quickly reduced, is suitable for herbaceous and agricultural residues^{62,63}, works moderately on hardwoods and unsuitable for softwoods⁶³.

Physical pretreatments (pyrolysis, grinding, chipping, etc.), chemical methods as ozonolysis and other as fungus pretreatment are unsuitable for commercial scale because of high cost⁵¹. However, SE, LHW, dilute acid, and AFEX pretreatments, have been reported as cost-effective pretreatments⁴⁶. SE has been extensively studied for pretreating LB and its feasibility for being employed at industrial scale have been shown with different raw materials⁶⁴⁻⁶⁶.

Steam Explosion (SE) Pretreatment

SE, compared to other pretreatments, offers potential for lower capital investment, significantly lower environmental impact, more potential for energy

efficiency, less hazardous process chemicals and conditions and complete sugar recovery⁵⁸. SE combines mechanical forces and chemical effect due to hydrolysis (autohydrolysis) of acetyl groups in hemicellulose. Most important factors affecting effectiveness of SE are particle size, temperature and residence time and the combined effect of both temperature and time is described by severity factor (R_o), which is optimal for maximum sugar yield between 3.0–4.5⁶⁷. Autohydrolysis takes place when high temperatures promote formation of acetic acid from acetyl groups. In combination with sudden depressurization, hemicelluloses are partially hydrolyzed and solubilized. Lignin is redistributed and to some extent removed from material⁶⁸. Although higher temperatures result in an increased removal of hemicelluloses from solid fraction and an enhanced cellulose digestibility, higher temperatures promote higher sugar degradation.

SE is reported a very suitable pretreatment method for ethanol production from poplar⁶⁹, eucalyptus⁷⁰, olive residues^{71,72}, herbaceous residues as corn stover^{73,74}, wheat straw³⁴ and sugarcane bagasse⁷⁵. SE has successfully performed with hardwoods and agricultural residues or herbaceous biomass but it is not very effective for softwoods due to low acetyl groups in hemicellulosic portion. SE offers advantages of using high chip size, unnecessary addition of acid catalyst (except for softwoods), high sugar recovery, good hydrolysis yields in EH and its feasible industrial scale development. Energy use for obtaining small chip size before pretreatment can make up one third of power requirements of entire process⁷⁶. Although avoiding acid catalysts is stated as an advantage, addition of an acid catalyst increases cellulose digestibility, improves hemicelluloses hydrolysis and decreases production of degradation compounds^{51,77}. Utilization of relatively high particle sizes as well as no acid addition would be desirable to optimize effectiveness on the process^{76,78}. Regarding higher hydrolysis yield in the following EH step, studies demonstrated that SE pretreatment improves EH of biomass^{35,78,79}.

For maximizing sugar recoveries, some studies have suggested two-step pretreatment^{80,81}. In the first step, steam is performed using low temperature to solubilize hemicellulosic fraction, and cellulose fraction is subjected to a second SE pretreatment step at a temperature higher than 210°C. It offers some additional advantages⁸² (higher ethanol yields, better use of raw material and lower enzyme dosages during EH). An

economic evaluation is needed to determine effectiveness of an additional SE⁸³. Its main drawbacks are partial hemicellulose degradation and generation of some toxic compounds derived from sugar degradation during pretreatment that could affect following hydrolysis and fermentation steps^{69,84}. Toxic compounds generated and their amounts depend on raw material and harshness of pretreatment. Major inhibitors are furan derivatives, weak acids and phenolic compounds. Main furan derivatives are furfural and 5-hydroxymethyl furfural (5-HMF) derived from pentoses and hexoses degradation, respectively; both are reported inhibitors by lag phase prolongation during batch fermentation⁸⁵.

Weak acids generated during pretreatment are mostly acetic acid, formed from acetic groups present in hemicellulosic fraction, and formic and levulinic acids derived from further degradation of furfural and 5-HMF. Phenolic compounds, generated due to lignin breakdown, vary widely between different raw materials. As the presence of toxic compounds is a significant obstacle for development of large scale ethanol production from lignocellulose⁸⁶, beside detoxification, several approaches such as genetic modification, evolutionary engineering or adaptive strategies are appearing as promising alternatives to obtain more tolerant yeasts⁸⁷.

Different Hydrolysis and Fermentation Configurations

Main options when using EH are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). An important advantage of SSF comparing to SHF is reduction of end-product inhibition during hydrolysis step resulting in higher yields, shorter residence time and lower requirements of enzyme loading^{67,88}.

Ethanol production cost using SSF is lower than using SHF since capital cost in SSF processes is reduced due to higher overall yield and employment of one single vessel⁸⁹. In an industrial SSF process, higher substrate concentrations to achieve higher final ethanol concentration have to be used and lower enzyme and yeast loading are crucial. Operating hydrolysis at high initial substrate loading faces problems such as end-product inhibition and high viscosity, which make medium more difficult to handle. Wingren *et al*⁸⁹ checked that running SSF with 8% of WIS instead of 5% would result in a 19% decrease in production cost.

After pretreatment, concentration of water insoluble solids in pretreated material is high and water is needed to dilute feedstock to fixed substrate loading. Use of fresh water is slightly feasible because large effluents are created and capital cost would be increased (lower ethanol concentrations)⁸⁹. Since energy consumption depends on dilution level, when initial loading of substrate increases, production costs are reduced significantly. By recycling stream after distillation back to SSF process, overall ethanol production cost would be reduced (14%)⁸⁹.

Among some other bottlenecks, each step can be conducted at its optima conditions in SHF whereas a compromise have to be reached in SSF processes^{67,90}. Whereas EH has an optimum temperature around 50°C, most fermenting microorganisms have an optimum temperature between 30-37°C⁶⁷. In this case, employment of thermo tolerant yeasts such as *Kluyveromyces marxianus* appears as an attractive alternative⁹¹. Several other advantages when using thermo tolerant yeast for ethanol production are energy savings through a reduction in cooling cost, higher saccharification yields, significant decreased risk of contamination and the possibility of continuous ethanol removal^{4,92}.

Biomass composition in hemicellulosic or cellulosic sugars also strongly influences SSF or fermentation performance. Since most of pentoses are present in liquid fraction after SE pretreatment, fermentation of liquid fraction separately from solid fraction could be shown as an option. Higher ethanol concentrations could be obtained from SSF experiments with whole pretreated material when comparing to SSF with solid fraction and fermentation of prehydrolysate separately⁸⁸. Using whole slurry, operational costs could be reduced because filtration step after pretreatment is avoided and amount of wastewater as well as freshwater requirements are diminished⁹³. If both hexose and pentose are present in SSF broth and pentose-fermenting yeast is used, the process is called simultaneous saccharification and co-fermentation (SSCF) (Fig. 2). When whole slurry has to be employed, inhibitory compounds released during pretreatment step are present in broth⁹⁴. Hence, it is important to employ a robust strain capable of growing or fermenting with high yield in presence of inhibitors. Future overall performance will depend strongly on development of more efficient microorganisms for fermentation of both pentoses and hexoses for a successful SSCF process (Fig. 2).

Pentose-Fermenting Microorganisms for Ethanol Production

Only enteric bacteria and some yeast are able to ferment pentoses but with low yields. Natural xylose fermenting yeast (*Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus*) are not tolerant to high ethanol concentrations, require microaerophilic conditions and are very sensitive to inhibitors and pH changes⁹⁵. With introduction of ethanol genes in enteric bacteria, hard efforts are carried out to incorporate pentose conversion pathways in natural ethanol producers such as *Saccharomyces cerevisiae* or ethanologenic bacterium *Zymomonas mobilis*.

Engineered Bacteria

Some ethanologenic bacteria (*Escherichia coli*, *Klebsiella oxytoca* and *Z. mobilis*) have shown promising alternatives for industrial exploitation. *E. coli* was the first successful bacterium genetically modified for ethanol production⁹⁶. It can grow on a wide range of carbon sources, can sustain high anaerobic and aerobic glycolytic fluxes and presents quite good ethanol tolerance⁹⁷. Wild-type *E. coli* shows low ethanol yield because it converts sugar most efficiently to organic acids (acetic or lactic acid) instead of ethanol. Due to that, several approaches have been performed with the aim of redirecting fluxes to ethanol. Most successful approach has been transformation of *E. coli* with a plasmid containing genes from *Z. mobilis* encoding for PDC (pyruvate decarboxylase) and ADH (alcohol dehydrogenase)^{96,98} in an artificial operon (PET). In recombinant strain *E. coli* KO11, both enzymes are overexpressed to high level^{99,100}. Strain KO11 has been evaluated at laboratory scale for producing ethanol from many types of LB including barley hull¹⁰¹, water energy crops¹⁰², orange peel¹⁰³, corn cobs¹⁰⁴ or even waste house wood¹⁰⁵. *E. coli* LY01, derived from KO11, showed higher tolerance to inhibitors present in lignocellulosic hydrolysates¹⁰⁶. Major disadvantage in using *E. coli* is public acceptance because of the existence of some pathogenic strains as well as neutral pH. Due to that is not widely employed in SSF approaches because its optimum pH (6.5) is not compatible with optimum pH for cellulolytic enzymes (4.8)⁹⁷.

Apart from *E. coli*, xylose is employed by engineered bacterium *Z. mobilis*. Glucose can easily cross cell membrane by facilitated diffusion and can efficiently convert it into ethanol by an overactive PDC-ADH system¹. Wild strain can not produce ethanol from

Table 2.— Engineered xylose fermenting *S. cerevisiae* employed for SSCF processes with different raw materials

Strain	Xylose pathway	Medium
<i>S. cerevisiae</i> TMB3001 ¹¹⁷⁻¹²⁰	XR-XDH-XK	Sugar cane baggasse
<i>S. cerevisiae</i> TMB3400 ^{115,117,121,122}	XR-XDH-XK	Corn stover, wheat straw, barley straw, spruce hydrolysates
<i>S. cerevisiae</i> TMB3066 ¹¹⁵	XI-XK	Undetoxified spruce hydrolysates
<i>S. cerevisiae</i> F12 ^{88,123}	XR-XDH-XK	Wheat straw
<i>S. cerevisiae</i> H158 ¹²⁴	XR-XDH-XK	Birch hydrolysates
<i>S. cerevisiae</i> CEN.PK ¹²⁴	XR-XDH-XK	Birch hydrolysates

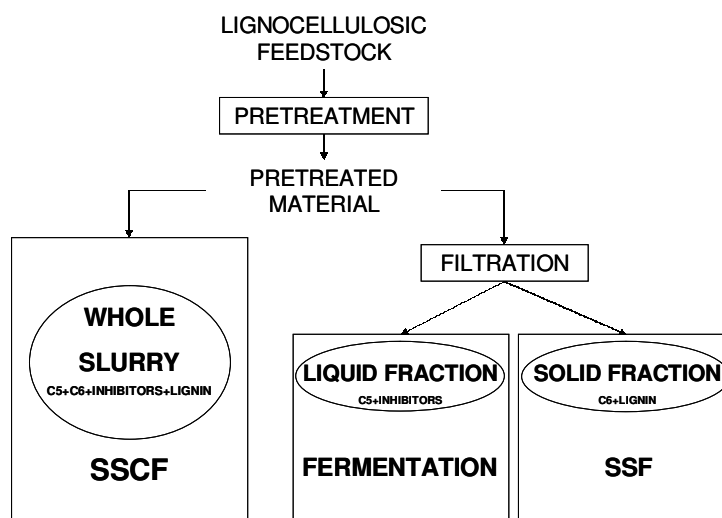


Fig. 2.— Different process configurations for an industrial bioethanol production process from biomass

xylose but it has been successfully engineered by introducing a xylose-metabolizing pathway from *E. coli*¹⁰⁷. It presents high ethanol yields selectivity and specific productivity as well as low pH and high ethanol tolerance¹. *Z. mobilis* has been mainly used in processes for ethanol production from starch¹⁰⁸ or sugar biomass³ but newly strain AX101 can use xylose after glucose consumption in lignocellulosic hydrolysates but showing high sensitivity to inhibitors present in the broth¹⁰⁹.

K. oxytoca has been also transformed with PET operon diverting carbon flow to ethanol production (strain P2). It can grow on sugars including hexoses and pentoses as well as cellobiose and cellotriose¹¹⁰. This latter trait makes strain attractive for processes for ethanol production from LB presenting chance of adding

lower amount of extracellular β -glucosidase for cellobiose hydrolysis¹¹¹. *K. oxytoca* has been employed successfully on corn fiber¹¹² or sugarbeet pulp¹¹³. However, bacterial processes for ethanol production are not still commercial.

Engineered Yeast

S. cerevisiae shows high ethanol productivity, high tolerance to ethanol and tolerance to inhibitory compounds present in hydrolysate of LB¹¹⁴. However, wild type *S. cerevisiae* has limitation being unable to ferment pentoses and hard efforts have been made to design a suitable engineered *S. cerevisiae*. Main strategies have been the construction of recombinant strains by introduction of genes *XYL1* and *XYL2* encoding for xylose reductase (XR) and xylitol dehydrogenase

(XDH) respectively or by introduction of gene encoding for xylose isomerase (XI) due to its ability to ferment xylulose to ethanol^{116,118} (Table 2). Former strategy also need an overexpression of endogenous xylulokinase (XK) for efficient xylose metabolism¹¹⁵. Another hurdle to overcome when using xylose fermenting *S. cerevisiae* is that xylose uptake competes with glucose uptake, because they are sharing membrane transporters^{125,126}. *S. cerevisiae* takes up xylose by both low and high-affinity glucose transport systems, however, xylose uptake through these transporters is significantly less efficient compared to glucose¹²⁷. Therefore, various metabolic engineering efforts involving recombinant *S. cerevisiae* have led to improvements in the initial rate of xylose consumption¹²⁶, being improvement of xylose transport in *S. cerevisiae* a great challenge to optimize xylose metabolic pathway.

P. stipitis grows rapidly without ethanol production under aerobic conditions and it ferments xylose and glucose under oxygen-limited conditions. Control aeration is difficult and it would be preferable to use a fermentation microorganism that is able to grow and ferment xylose anaerobically. Due to that, expression of *S. cerevisiae* *URA1* in *P. stipitis* has been concluded as a suitable approach. *URA1* gene encodes for dihydroorotate dehydrogenase that employs fumarate as an alternative electron acceptor, which enables *P. stipitis* to grow in anaerobic conditions¹²⁸. Further improvement of *P. stipitis* have been also achieved by modification of its respiratory chain by deleting *CYC1* gene coding for cytochrome *c*¹²⁹.

Conclusions

Pretreatment operation itself must be low in cost and avoid high consumption of expensive chemicals, high energy demands, and feedstock degradation. In this context, SE pretreatment at commercial scale proved sustainability for pretreating LB in an industrial process for ethanol production. High substrate loadings, sugar recovery after pretreatment, tolerance to inhibitory compounds and xylose fermentation by yeast must be optimized for a successful industrial process for bioethanol production from lignocellulose. Moreover, lower enzyme and yeast loading are crucial for an economically viable process. To counter presence of toxic compounds in fermentation broth, genetic modification, evolutionary engineering or adaptative strategies are promising alternatives to obtain more tolerant yeasts. When using whole pretreated material, operational costs

could be reduced because filtration step after pretreatment is avoided and amount of wastewater as well as freshwater requirements are diminished. For a successful SSCF process, future overall performance will depend strongly on development of more efficient microorganisms for fermentation of pentoses and hexoses.

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