Brazilian potential for biomass ethanol: Challenge of using hexose and pentose co-fermenting yeast strains

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This paper reviews Brazilian scenario and efforts for deployment of technology to produce bioethanol vis-à-vis recent international advances in the area, including possible use of hexose and pentose co-fermenting yeast strains.

Keywords: Biomass ethanol, Brazilian ethanol production, Ethanologenic yeasts, Hexose-pentose co-fermentation

Introduction

Brazil produces annually 5 x 10⁹ gallons of ethanol from sugarcane grown on 5 million ha. Sugarcane is processed in 365 sugar/ethanol producing units, and it is forecasted that 86 new distilleries will be built before 2015. Sugarcane juice can go either directly to fermentation vats for ethanol production, or be used to produce sugar (crystal sucrose, and its residue molasses), depending on which product is priced more favorably. As a consequence, 75 million tonnes of bagasse are produced annually. In each sugar and/or ethanol mill, approx. 85% of bagasse is burned for the production of steam (heat) and power/electricity generation.

Bioethanol from Sugarcane

Brazil has been a front-runner to substitute liquid fuel gasoline by renewable ethanol1-5. State of São Paulo is biggest sugarcane producer (68% of total sugarcane plantations in Brazil). Out of 365 sugar/ethanol producing units in Brazil, 240 produce sugar and ethanol, 109 produce only ethanol and 16 produce only sugar. Brazil will produce around 27 billion litre of ethanol in 2008, and plans to build 41 new distilleries before 2010, and 45 more until 20156. Land use for energy crops as compared to food crops has been opted for renewable fuels with use of biomass polysaccharide part for the production of fuel ethanol7-9. A simple mass balance (Fig. 1) for harvested sugarcane indicates that lignocellulosic-derived fuel ethanol could add up to > 50% bonus over total amount of ethanol currently produced from sugarcane, considered that the amount of sugars found in sugarcane biomass could be recovered and fermented at ~90% efficiency4,10-12.

Structure of lignocellulosic biomass (LB) is represented by physicochemical interaction of cellulose, with hemicellulose and lignin13-15. Cellulose is a highly ordered polymer of cellobiose whose length may present 10,000 glycosyl units in cellulose chain that form fibrils. Hemicelluloses are shorter (degree of polymerization within 100-200), highly branched heteropolymers of xylose, arabinose, mannose, galactose and glucose as well as uronic acids. Depending on predominant sugar type, hemicelluloses are referred to as mannans, xylans or galactans. Pentose and hexose sugars, linked through 1,3,
1,6 and 1,4 glycosidic bonds and often acetylated, form a loose and very hydrophilic structure that acts as a glue between cellulose and lignin.

Parallel to the implementation of Brazilian Proálcool Program, in 1970s, several research institutions and companies have carried out research for the production of bioethanol, using primarily and municipal cellulosic solid wastes. The first pilot-scale facility was built in 1981 at Fundação de Tecnologia Industrial (FTI, Lorena, SP) to run using the Scholler-Madison process based on concentrated sulphuric acid to hydrolyze Eucalyptus paniculata to produce 500 l ethanol per day.

Brazilian company Dedini, in Piracicaba (SP), began in 1987 the development of a biomass-to-ethanol production technology (called Dedini Hidrolise Rapida, DHR), in partnership with Copersucar (presently Centro de Tecnologia Canavieira) and State of São Paulo Research Supporting Foundation (FAPESP). DHR technology for ethanol production is an organosolv hydrolysis single-stage process that employs very diluted H$_2$SO$_4$ for hydrolysis of cellulose and hemicellulose to sugars, and an organic solvent for lignin extraction. DHR’s unique feature is reduced hydrolysis reaction time (few min) in a continuous high-throughput process, with quick cooling of hydrolysate. However, all projects that studied acid hydrolysis observed low sugar yields, formation of inhibitors for subsequent ethanol fermentation step, and corrosion of equipments. Besides, acidic pH of sugar hydrolysate requires a neutralization step prior to fermentation.

Considering detrimental aspects of acid hydrolysis, studies focused on enzyme-based route that involves biomass pretreatment because LB are structured for strength and resistance to biological, physical and chemical attack. Pretreatments (steam explosion, hydrothermolysis or using catalytic amounts of acid), render raw cellulose digestible by cellulases. Sugarcane bagasse was pretreated via steam explosion using a 1.6 l reactor. These studies paved way for development of sugarcane bagasse steam explosion process used to date in Brazil for the production of cattle feed.

Presently, “Bioethanol Project: Bioethanol Production through Enzymatic Hydrolysis of Sugarcane Biomass”, initiated in 2006, is supported by Brazilian Ministry of Science and Technology through its Research and Projects Financing agency (FINEP), and is carried out by a network of more than 20 institutions, including universities and research institutes. This project aims to develop a viable technology for conversion of sugarcane bagasse and straw into fuel ethanol, minimizing expansion of sugarcane fields. It is expected that Brazilian sugar mills will be gradually converted into biorefineries, able to eventually process all fractions of sugarcane. This

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![Fig. 1—Bioethanol production from sugarcane [current technology of sugarcane processing and fuel ethanol production from sucrose (black arrows) is illustrated at the right side of the figure, while the required technological achievements for bioethanol production from sugarcane bagasse and straw (biomass pre-treatment, hydrolysis and pentose fermentation, gray arrows) are shown on the right side of the figure.](image-url)
technology will use all already existing facilities in sugar and alcohol plants including juice/sugars syrup treatment system, fermentation & distillation, cogeneration, wastewater & residues treatment and recycling, instrumentation and automatic control systems, management, and commercialisation. Biomass sugar syrups will be blended with molasses or with sugarcane juice prior to fermentation carried out by *S. cerevisiae* yeast strains. As a result, lower biomass ethanol production cost is expected compared with plants dedicated to produce biomass ethanol solely. For enzymatic hydrolysis of pretreated biomass, the project is developing enzyme blends in situ for sugarcane bagasse.

To produce ethanol from LB economically, it is essential to have a biocatalyst able to ferment hexose and pentose sugars under adverse conditions of an industrial environment. Yeast *S. cerevisiae*, applied successfully in industrial production of ethanol from sugarcane juice and molasses in Brazil, is able to ferment hexoses rapidly and efficiently, and exhibits high ethanol tolerance, GRAS status, and tolerance to low pH. However, *S. cerevisiae* for use in a second generation process (lignocellulose-to-fuel ethanol process) cannot metabolize pentoses, although development of *S. cerevisiae* pentose-utilizing strains could be integrated into existing ethanol plants. Engineering industrial strains that would be able to co-ferment efficiently all sugars in lignocellulose hydrolysates to ethanol is, however, still a challenge for molecular biologist and engineers worldwide.

**Pentose Fermentation**

In 1922, Willaman and co-workers showed that *Fusarium lini* could ferment xylose into ethanol. Since then, many bacteria have been found to metabolize and ferment hexose and pentose sugars, but all produced an unwanted mixture of fermentation products. Although, homo-ethanol pathway is found in *Z. mobilis*, substrate range this organism can metabolize (glucose, fructose and sucrose) is very limited. Many yeasts able to ferment xylose were also found, including several *Candida*, *Pichia*, *Hansenula*, *Debaromyces*, *Schwanniomyces*, etc. If xylose was converted to xylulose, several more yeast could ferment it directly to ethanol, including *S. cerevisiae*. Thus, although many yeasts are able to ferment xylose, large-scale utilization is hampered by their low tolerance to ethanol and hydrolysate inhibitors, requirement for microaerophilic conditions, and an inability to ferment xylose at low pH.

Best yeast strains known for fermenting xylose are *P. stipitis*, *P. tannophilus* and *C. shehatae*. Yeast *P. stipitis* is capable of converting xylose and all major lignocellulosic sugars to ethanol (yield, 0.3-0.44 g/g of substrate). Although this is suitable for some waste streams, commercial fermentation for fuel ethanol requires higher performance.

Over the past 20 years, metabolic engineering has been used to construct bacterial and yeast strains with advantageous traits for lignocellulosic ethanol production. Efforts are concentrated on three most promising microbial platforms (*S. cerevisiae*, *Z. mobilis*, and *E. coli*). Since *S. cerevisiae* has been used for mature industrial technologies of ethanol production from cane and grain sugars, several strategies that have been used to engineer this yeast towards pentose fermentation (Fig. 2) include insertion of bacterial and yeast/fungal pentose-utilization genes, selection or overexpression of the own *S. cerevisiae* xylose utilization pathway, improvement of xylulose consumption through overexpression of xylulokinase and pentose-phosphate pathway enzymes, engineering redox cofactor regeneration to reduce by-product formation, in particular xylitol, and application of random mutagenesis and evolutionary engineering to select for improved traits.

Unfavorable kinetic properties of enzymes with an imperfect match of cofactor, and an inadequate pentose phosphate pathway, apparently limit ability of recombinant yeast to ferment xylose. Despite development of recombinant strains with improved xylose fermentation performance, high products yields and increased product concentrations are key targets that have yet to be achieved. Recent transcriptome and proteome analysis have indicated that xylose is not perceived as a fermentable sugar by *S. cerevisiae* probably because signaling pathways that ensure efficient utilization of hexoses do not recognize pentoses xylose or arabinose. Thus, genetic modifications that could have greatest impact on economic feasibility of these fermentations should include amplification and deregulation of rate-limiting reactions.

Sugar uptake from medium is major rate limiting step for fermentation of even naturally fermentable sugars (glucose, fructose, maltose, maltotriose) by *S. cerevisiae*. In general, overexpression of cognate sugar transporters, which can mediate facilitated diffusion or active transport of sugar across plasma membrane.
(Fig. 3), improves significantly fermentation performance of cells\textsuperscript{56-60}. Xylose and arabinose uptake across plasma membrane is also rate-limiting for pentose fermentation by \textit{S. cerevisiae}, specially because this yeast transports xylose and arabinose with very low affinity, compared with uptake of other fermentable sugars\textsuperscript{34,36,47,70,71}. All yeast species that use xylose and/or arabinose have both low- and high-affinity pentose transport activities, covering a wide range of sugar concentrations extending to at least 2-3 orders of magnitude. Affinities for xylose are always lower than those for glucose. While most strains present both high-affinity arabinose/xylose-H\textsuperscript{+} symport and low-affinity facilitated transport of sugars\textsuperscript{72,73} (Fig. 3) in \textit{S. cerevisiae}, these pentoses are transported solely by facilitated transport mediated by hexose permeases. Expression of any of the major \textit{HXT} transporters (\textit{HXT1}-\textit{HXT7} and \textit{GAL2}) allows growth of an \textit{hxt}-null strain on xylose\textsuperscript{74,75}. Of these transporters, mid-to-high-affinity glucose permeases (\textit{HXT4}, \textit{HXT5}, \textit{HXT7} and \textit{GAL2}) were more effective arabinose and xylose transporters\textsuperscript{36,71,74,75}, which is in good agreement with analysis of mutated and evolved \textit{S. cerevisiae} cells showing improved pentose fermentation due to an increased expression of \textit{HXT5}, \textit{HXT7} and \textit{GAL2} permeases\textsuperscript{36,51-54,76,77,78,79,80}. Although genome of this yeast indicates presence of a large family of sugar transporter genes\textsuperscript{77}, only three sugar permeases characterized in \textit{P. stipitis} are high affinity glucose permeases that transport xylose with significantly lower affinity\textsuperscript{80}. This study also revealed first example of sugar permeases whose expression is regulated by oxygen availability, which may be also present in other yeasts and fungi\textsuperscript{72,79}. Although pentose transport activities are described in several other yeasts, only other known xylose/glucose permease genes were isolated from \textit{C. intermedia} through functional expression in an \textit{hxt}-null \textit{S. cerevisiae} strain\textsuperscript{80}. While there are still many
limitations towards functional expression of heterologous active pentose permeases in \textit{S. cerevisiae} \cite{71,81}, overexpression of a xylose/glucose facilitated diffusion transporter from \textit{P. stipitis} is reported to significantly enhance xylose fermentation by a \textit{S. cerevisiae} strain engineered to metabolize this sugar \cite{82}.

**Current Fuel Ethanol Technology in Brazil: Challenges and Opportunities for Bioethanol**

Industrial production of fuel ethanol in Brazil uses raw sugarcane juice and/or molasses as substrate. Sugarcane juice contains significant amounts of yeast and bacteria, depending on different factors mainly related to cane variety and age, time lag between cane harvesting and processing, etc. \cite{83,86}. While bacterial infections can be controlled by antibiotics and acid treatment of the yeast cells, yeast contaminations can have a significant impact in fermentation performance of distillery. In wine fermentations, \textit{D. bruxellensis}, major contaminant in industrial fuel ethanol plants in Brazil, can cause decreased productivity as well as other operational problems \cite{85,86}. Thus, to ensure high productivities and efficiency in industrial process, currently fuel ethanol plants (> 80%) work with an adaptation of \textit{Melle-Boinot} process \cite{87-89}. This process is a fed-batch fermentation that uses high cell densities (~10 g yeast/l) allowing very short residence times for fermentation to be completed (<8-10 h), and thus up to 3 fermentation runs can be performed per day, contributing to overall process success. Normally, final sugar concentration employed is also high (~180 g/l), with high conversion yields (up to 92%) due to high cell density process that limits cell growth. Crucial to the performance of this process is constant recycling of yeast slurry into fermentors, after treatment with \text{H}_2\text{SO}_4, a procedure that ensures less bacterial and wild yeast contaminations. Consequently, yeast fitness for industrial process includes high tolerance to fermentative stresses (high temperatures, ethanol and osmotic pressure, low pH, production interruptions, etc.), and yeast should also dominate fermentation tanks during whole crop season. Indeed, analysis of yeast population dynamics during fuel ethanol production in Brazil has revealed existence of selected yeast strains that tend to dominate a given industrial process \cite{85}. Actually, some yeast strains (PE-2, CA-1) are commercially available as starters for fuel ethanol industry (www.fermentec.com/en/index.html), showing high alcohol productivity and increased resistance to stressful
conditions found in this industrial process\textsuperscript{5,87}, again contributing significantly to overall process economical success.

Regarding introduction of sugarcane biomass hydrolysates into actual fuel ethanol production setup, one major concern is that even with the best performing \textit{S. cerevisiae} yeast strains\textsuperscript{22,82}, rates of pentose uptake by cells (<0.25 g sugar/g yeast/h) are far slower than rate of fermentable sugar uptake by industrial yeast strains (2-4 g sugar/g yeast/h). This would mean either longer fermentation cycles (affecting the overall process productivity), or presence and persistence for longer periods of pentose sugars in fermentor would favor contaminant bacteria and wild yeasts that use these sugars more efficiently\textsuperscript{91}, probably affecting whole fermentation process. Thus, even if pentose fraction in sugarcane represents only ~14\% of total sugar available for fermentation from this energy crop (Fig. 1), all these sugars need to be used by \textit{S. cerevisiae} cells present in fermentor in order to not increase the risk of contaminations in fuel ethanol process.

Since transport systems for hexoses present in this yeast also transport pentoses with lower affinity, xylose and arabinose fermentation can only take place after hexoses have been significantly depleted from medium\textsuperscript{92}. An interesting alternative for Brazilian sugarcane fuel industry, which uses sucrose-rich broths (Fig. 1), could be to use \textit{S. cerevisiae} yeast strains that would not hydrolyze outside the cells the sucrose present in cane juice, but sugar would be transported directly into cytoplasm and hydrolyzed intracellularly. Efforts towards this goal are already in development in Brazil\textsuperscript{93,94}. Finally, selected industrial yeast strains mentioned above should be seriously considered for metabolic engineering of pentose fermentation pathways (Fig. 2) and other desired phenotypic characteristic required for bioethanol production from sugarcane biomass\textsuperscript{22}, and indeed recent data indicates feasibility of such strains for genetic transformation\textsuperscript{45}, paving the way towards implementing bioethanol production processes in Brazil.

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