

Ethanol from Cellulosic Biomass with Emphasis of Wheat Straw Utilization. Analysis of Strategies for Process Development

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Received: October 08, 2015

Accepted: December 14, 2015

Published: December 22, 2015

Abstract: The "Green and Blue Technologies Strategies in HORIZON 2020" has increased the attention of scientific society on global utilization of renewable energy sources. Agricultural residues can be a valuable source of energy because of drastically growing human needs for food. The goal of this review is to show the current state of art on utilization of wheat straw as a substrate for ethanol production. The specifics of wheat straw composition and the chemical and thermodynamic properties of its components pre-determined the application of unit operations and engineering strategies for hydrolysis of the substrate and further its fermentation. Modeling of this two processes is crucially important for optimal overall process development and scale up. The authors gave much attention on main hydrolysis products as a glucose and xylose (C6 and C5 sugars, respectively) and on the specifics of their metabolism by ethanol producing microorganisms. The microbial physiology reacting on C6 and C5 sugars and mathematical approaches describing these phenomena are discussing, as well.

Keywords: Cellulosic biomass, Hydrolysis, Wheat straw, Ethanol, Modeling.

Current state of the art: Cellulose as renewable sources of energy from biomass. Sources of energy from biomass-cellulose

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Crude oil has been the major resource to meet the increased energy demand. Campbell and Laherrere [19] used several different techniques to estimate the current known crude oil reserves and the reserves as yet undiscovered and concluded that the decline in worldwide crude oil production will begin before 2010. They also predicted that annual global oil production would decline from the current 25 billion barrels to approximately 5 billion barrels in 2050. Because the eight great economies (except Brazil) and many other nations depend on oil, the consequences of

inadequate oil availability could be severe. Therefore, there is a great interest in exploring alternative energy sources. Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. Ethanol is widely used as a partial gasoline replacement worldwide. Fuel ethanol that is produced from corn has been used in gasohol or oxygenated fuels since the 1980s. These gasoline fuels contain up to 10% ethanol by volume. As a result, the U.S. transportation sector now consumes about 4540 million liters of ethanol annually, about 1% of the total consumption of gasoline. Recently, U.S. automobile manufacturers have announced plans to produce significant numbers of flexible-fueled vehicles that can use an ethanol blend – E85 (85% ethanol and 15% gasoline by volume) – alone or in combination with gasoline. Using ethanol-blended fuel for automobiles can significantly reduce petroleum use and exhaust greenhouse gas emission.

The Brazilian experience in the area is priceless and the research on ethanol production and use as a fuel can be considered as the most advanced. Moreover, using ethanol as a fuel has made Brazil independent from oil availability [100].

Ethanol is also a safer alternative to methyl tertiary butyl ether (MTBE), the most common additive to gasoline used to provide cleaner combustion. MTBE is a toxic chemical compound and has been found to contaminate groundwater. The U.S. Environmental Protection Agency recently announced the beginning of regulatory action to eliminate MTBE in gasoline [16]. However, the cost of ethanol as an energy source is relatively high compared to fossil fuels. A dramatic increase in ethanol production using the current corn starch-based technology (or other biomass) may not be practical for small countries because corn production for ethanol will compete for the limited agricultural land needed for food and feed production. An alternative potential source for low-cost ethanol production is to utilize lignocellulosic biomass (LCB) such as crop residues, grasses, sawdust, wood chips, and solid animal waste. Extensive research has been completed on conversion of (LCB) to ethanol in the last two decades [6, 21, 36, 38, 41-43, 59, 69, 70, 74, 79, 80, 91, 93, 96, 103, 108]. The conversion includes two processes: hydrolysis of cellulose in the (LCB) to fermentable reducing sugars, and fermentation of the sugars to ethanol. The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeasts or bacteria. The factors that have been identified to affect the hydrolysis of cellulose include porosity (accessible surface area) of the waste materials, cellulose fiber crystallinity, and lignin and hemicellulose content [2, 75]. The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis. The contents of cellulose, hemicellulose, and lignin in common agricultural residues are listed in Table 1. Removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity in pretreatment processes can significantly improve the hydrolysis [2, 75]. The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. Physical, physical-chemical, chemical, and biological processes have been used for pretreatment of (LCB) and will be summarized further.

Table 1. The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes

Lignocellulosic materials	Cellulose, (%)	Hemicellulose, (%)	Lignin, (%)
Hardwoods stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	n.a.	24-29
Swine waste	6.0	28	n.a.
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

*Source: Reshamwala et al., 1995 [87], n.a. – not available.

Chemical structure of lignocellulosic biomass-cellulose, hemicellulose, lignin

LCB has the potential to substantially reduce the ethanol production cost because it is less expensive than corn and available at large quantities. Cellulosic feedstock includes agricultural wastes (wheat straw, corn stover, rice straw, bagasse, grasses, etc.), forest residues, and other low-value biomass such as municipal wastes. LCB are mainly composed of cellulose, hemicellulose, lignin and other minor components such as ash and protein. Cellulose is a linear polymer of anhydro D-glucose units connected by β -1,4 glycosidic bonds. Native cellulose exists in the form of microfibrils, which are paracrystalline assemblies of several dozen (1 \rightarrow 4) β -D-glucan chains with hydrogen bonds connected to one another [20]. The cellulose micro-fibrils are embedded in a matrix of non-cellulosic polysaccharides, mainly hemicellulose and pectin substances.

Hemicellulose is a complex, heterogeneous mixture of sugars and sugar derivatives that form a highly branched network [52]. The monomers that comprise hemicellulose are hexoses (glucose, galactose, and mannose) and pentoses (arabinose and xylose). Some monomers are acetylated. Hemicellulose can be classified into three groups, namely, xylans, mannans, and galactans based on the polymer backbone that is very often homopolymeric with β -1,4 linkage. Xylan is by far the most important component because of its large quantities in the biomass. It was reported that grasses contain 20-40% of arabinoxylans, while the principal hemicellulose in hardwood is glucomannan and methylglucuronoxylan [15].

Cellulosic materials also contain lignin, a three dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon-carbon bonds. When the plant matures and the cell growth ceases, the middle lamella (the cement between the primary walls of adjacent cells) and the secondary cell wall (inside of primary wall) have a large degree of

lignin. The lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together [37].

The structures of the lignocellulosic biomass, especially cellulose crystallinity, the heating of hemicellulose, and the lignin barrier, make it more recalcitrant to enzymatic hydrolysis compared to corn starch. Mechanical or chemical pretreatment is used to break down the hemicellulose and lignin structures in order to improve the substrate digestibility.

Cellulose

Cellulose has been said to be the most abundant organic polymer on the earth, with annual production of 4×10^{10} Mg [45]. It is found mainly in the secondary cell wall of plants, and is the major structural component of higher plants [88]. Celluloses from all sources have the same linear polysaccharides of D-glucopyranose units linked without branches. The straight chains of cellulose rotate 180° every other β -1 \rightarrow 4 glycosidic linkage, providing spatial sites to form intermolecular hydrogen bonds (Fig. 1). Parallel cellulose chains are associated by these hydrogen bonds and Van der Waals forces among molecules to produce three-dimensional microfibrils, in which a regular and repeating crystalline structure is interspersed by amorphous regions. The crystalline structure makes cellulose very water insoluble and impermeable to water, so that the highly associated microfibrils can act as an outside matrix to protect the inner environment of plant cells. This crystalline structure is one of the major limitations for cell wall hydrolysis. A simplified graphical illustration of the cross-linking of cellulose microfibrils and hemicellulose in the LCB can be found elsewhere [52].

The cellulose concentration of corn cobs has been reported in the range of 32.2-45.6% d.b. [39, 104] and 33.5-38.4% d.b. for corn stalks [66]. When β -1 \rightarrow 4 glycosidic linkages in this cellulose are broken down by enzymatic hydrolysis or moderate acids, glucan and glucose are released from the polysaccharide, which can be fermented into ethanol, lactic acid, or other chemicals.

Hemicellulose

Hemicelluloses are a heterogeneous group of polysaccharides including four basic types: D-xyloglucans, D-xylans, D-mannans, and D-galactans. In each type, two to six various monomers are aggregated through β -1 \rightarrow 4 and β -1 \rightarrow 3 linkages in main chains and α -1 \rightarrow 2, 3, and 6 linkages in branches. The monomer subunits can include D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucouronic acid. Hemicelluloses vary in subunits, compositions, polymer components, and concentrations from plant to plant and from one plant part to others.

The hemicellulose concentration of corn stover has been reported as 35% d.b. [104], with D-xylan as the major type [88]. The main chain structure of D-xylan is similar to that of cellulose except that the monomer is xylose, which lacks a primary alcohol group at the C-5 site. Moreover, glucopyranosyl uronic acid units are linked to the main chain at every four or five xylose residue through α -1 \rightarrow 2 linkage. The absence of the primary alcohol group reduces the chances of formation of intermolecular hydrogen bonds and microfibrils. Lacking intermolecular hydrogen bonds among the polysaccharide chains, xylan does not form a crystalline structure. Furthermore, uronic acids in the branches make xylan an acidic polysaccharide. Thus, xylan is much more water soluble than cellulose and reactive to chemical treatment. However, the heterogeneous monomers and linkages of hemicellulose spatially hinder enzyme attachment, which reduces the effectiveness of hemicellulose during hydrolysis. Utilization of hemicellulose monomers (xylose and arabinose) by genetically

modified ethanol producing microorganisms is a crucial step in development of cost-effective biomass-to-ethanol technologies.

Lignin

Lignin and associated phenolic acids, although present in relatively small concentrations, play an important role in cell wall degradation. Unlignified or slightly lignified plant tissues can be degraded much more easily than intensively lignified tissues [4]. The complicated composition and structure of lignin greatly limits the complete understanding of lignin synthesis and degradation. In terms of chemical composition, lignin is historically divided into core lignin and non-core lignin. Core lignin includes the highly-condensed polymers formed by dehydrogenative polymerization of the hydroxycinnamyl alcohols, p-coumaryl alcohols, coniferyl alcohols, and sinapyl alcohols. Non-core lignin includes esterified or etherified phenolic acids bound to core lignin or to non-cellulosic polysaccharides [78]. The chemical structure of lignin is also very complicated [90], as it is a three-dimensional cross-linked aromatic polymer made up from phenylpropane units. No single established structural scheme for lignin has been established thus far.

Lignin is mainly located in the middle lamella of the plant cell wall, cross-linked with hemicellulose directly or through phenolic acids. It provides vascular plants with strength and rigidity and helps the cell wall resist microbial attacks and enzymatic hydrolysis. Lignin concentration differs considerably from plant to plant. Corn stover contains 10.00-14.67% d.b. lignin [60, 62] while the lignin concentration of softwood stems range from 25-35% d.b. [104]. Different parts of corn stover have different levels of lignin with 10.9% in husks [65], 6.6% in cobs, 11.8-20.8 % in node [18]. Furthermore, the lignin content also changes over time, increasing with the maturity of plants during the harvest season [85]. Partial lignin chemical bond structures can be found in the work of Sarkanen [90].

The complete knowledge about chemical structure of cellulose and hemicellulose help to organize the complex research and development efforts to reach optimal and cost effective ethanol industrial schemes.

The flowchart of biomass-to-ethanol conversion process

The structures of the lignocellulosic biomass, especially cellulose crystallinity, the sheathing of hemicellulose, and the lignin barrier, make it more recalcitrant to enzymatic hydrolysis compared to corn starch. The flowchart of biomass-to-ethanol conversion process is shown in Fig. 1.

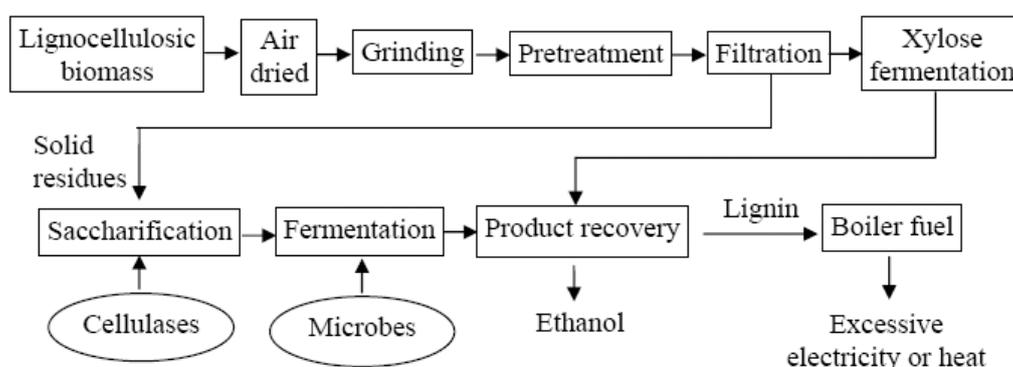


Fig. 1 Schematic overview of biomass-to-ethanol conversion process

The cellulose-based bioethanol production includes the following steps: cellulose pretreatment-chemical or enzymatic; cellulose saccharification; sugars fermentation and product recovery. Further, these steps will be discussed in details and some modeling approaches will be underlined.

Pretreatment

Physical or chemical pretreatment is used to break down the hemicellulose and lignin structures in order to improve the substrate digestibility and these process operations are crucial in the economic evaluation of the biomass-to ethanol technology. The most important engineering solutions in the area can be summarized as follows: Physical pretreatment such as – Mechanical comminuting [76]; Pyrolysis [61, 95] – Physical-chemical pretreatment such as – Steam explosion (autohydrolysis) [46, 75, 117]; Ammonia fiber explosion (AFEX) [50, 106, 110]; CO₂ explosion [25, 122] – Chemical pretreatment such as – Ozonolysis [109]; Acid hydrolysis [75, 111]; Alkaline hydrolysis [37, 75]; Oxidative delignification [12]; Organosolv process [107]; – Biological pretreatment [1].

Especially important is to analyze the structure, chemical composition and pretreatment methods of wheat straw [105] as a renewable source of energy in production of ethanol (see Fig. 2). Among the agricultural residues, wheat straw is the largest biomass feedstock in Europe and the second largest in the world after rice straw [62]. About 21% of the world's food depends on the wheat crop and its global production needs to be increased to satisfy the growing demand of human consumption [84]. Therefore, wheat straw is very challenging alternative in energy production and could serve as a great potential renewable source for production of ethanol in 21st century.

Hemicellulose hydrolysis and xylose utilization

Hemicellulose hydrolysis from biomass results in monomer pentose products such as xylose and arabinose. Utilization of these sugars for ethanol production is a crucial step and a key for development of modern cost-effective and competitive biomass-to-ethanol technology. Many efforts have been made to genetically modified ethanol producing yeasts of *Saccharomyces cerevisiae* to utilize simultaneously glucose and xylose avoiding glucose catabolic repression.

Xylose utilization strategies have been examined in *S. cerevisiae* [68]. Other researchers examined the xylose fermentation to ethanol using a recombinant strain of *Pachysolen tannophilus*. Several metabolism pathways of this strain have been explored, and one of which is illustrated below (Fig. 3). Xylose is first converted to xylulose, which is dissimilated via the pentose-phosphate pathway after phosphorylation. Overall ethanol yield of 0.39 kg/kg and a specific ethanol-production rate of 0.06 kg/kg/h were observed, which are comparable to those of glucose fermentation. Xylitol is produced as a by-product with an overall yield of 0.14 kg/kg. The development of several engineered microorganisms is advancing rapidly [29, 48, 120], and these are being considered for use in commercial processes.

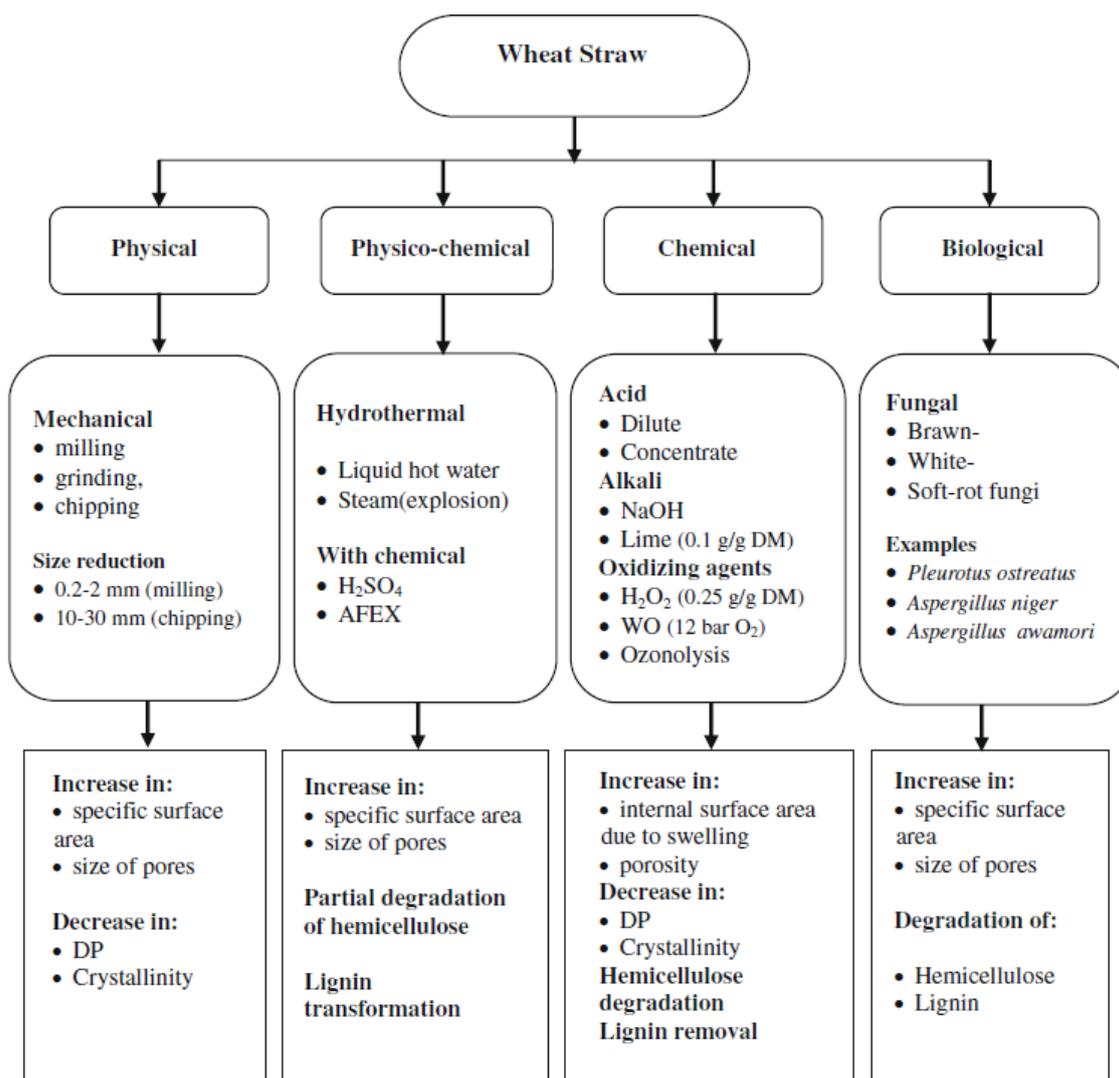


Fig. 2 The most common pretreatment methods used on wheat straw and their possible effects (DP – degree of polymerization; WO – wet oxidation) [105]

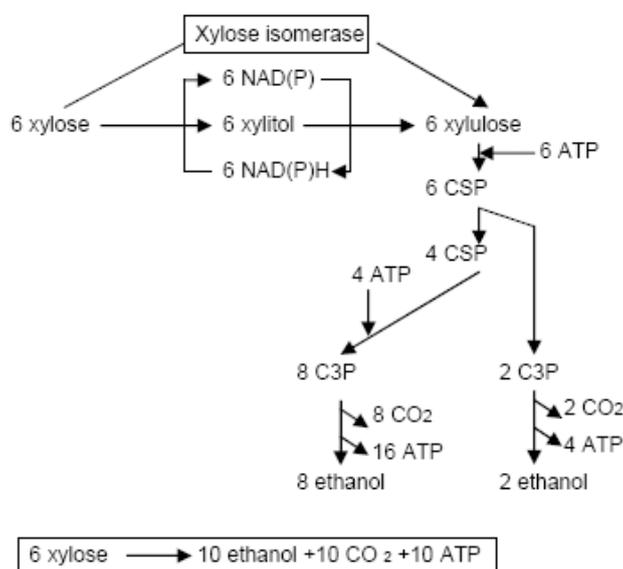


Fig. 3 Metabolic pathway for alcoholic fermentation of xylose with a calculated redox balance

A very useful review about the subject has been published recently [104] underlying the main problems and achievements in genetic engineering constructing species utilizing xylose for ethanol production.

Hydrolysis of cellulose modeling

Analysis of cellulose hydrolysis is especially important, because obtained information about the process could be incorporated into models and can be used to predict the behavior of multi-component cellulase enzyme systems. Comparison of such predictions to experimental measurements is the most systematic and rigorous means available by which to test whether understanding of cellulase components and their interactions is sufficient to explain a given observation [121]. In addition, once a quantitative model is validated, it can be used to rapidly formulate new hypotheses of significance in both fundamental and applied contexts. The review paper gives detailed analysis of models applied to describe the hydrolysis of cellulose by cellulases. Many models were tested based on different hypothesis, but the review tried to systematize the knowledge in the field. As well, beneficiary will be to have in mind a comprehensive review of enzymatic cellulose hydrolysis kinetic models by [37].

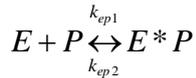
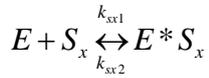
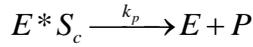
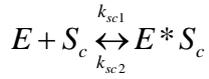
Example: Over the years several different mechanisms have been proposed for the actual conversion of cellulose to glucose. The initial concept was put forward by Reese et al. [86], and is known as the (C_1 - C_x) concept. They reported that the C_1 component “activates” cellulose chains and C_x enzymes carry out depolymerisation:



The following assumptions were made to simplify the mathematical representation:

- The cellulase system (E) of endo-gluconase, cellobiohydrolase, and glycosidase is considered as having a single combined catalytic function in the hydrolysis of cellulose to produce reducing sugars represented as a single product (P). No attempt was made to distinguish the differing functions of different cellulose enzyme components as they are difficult to quantify and require incorporating excessive kinetic parameters in the model.
- The complex structure of the cellulosic material is simplified into a hydrolysable region composed of exposed cellulose microfibrils (S_c) and a non-hydrolysable inert region (S_x).
- Cellulase absorbed at the active cellulose and inert binding sites form $E*S_c$ and $E*S_x$ complexes.
- Instead of using a total mass concentration, substrate concentration is based on surface concentration of active cellulose which is accessible to enzymes for adsorption and subsequent catalysis. This allows the consideration of the effect of substrate particle size and cellulose quality of the substrate particles.
- New cellulose and inert substrate emerge from the inner region of substrate solids after hydrolytic dissolution of the first layer of cellulose fibrils. The reaction interface moves towards the inside of the substrate solids structure. The quality of the reaction interface gradually decreases as the surface concentration of inert substrate increases and the accessibility of the reaction interface to enzyme molecules becomes more restricted due to increased internal diffusion resistance.
- The reducing sugars inhibit the enzyme in a reversible and competitive manner, forming complex EP.
- Enzyme deactivation by factors other than product inhibition is related to shear field residence time.

Based on these assumptions, the heterogeneous enzymatic cellulose hydrolysis can be represented by the following reaction scheme:



In this representation, k_{sc1} and k_{sc2} are the primary rate constants for the reversible formation of active E^*S_c intermediate, k_{sx1} and k_{sx2} are the primary rate constants for the reversible formation of non-productive E^*S_x complex, k_p is the rate constant of product formation, and k_{ep1} and k_{ep2} are the forward and reverse reaction rate constants for the formation of the enzyme-product complex.

According to the proposed reaction scheme in a closed system, the dynamic changes of concentrations and the reaction intermediates can be expressed by first-order differential equations:

$$\frac{dC_{E^*S_c}}{dt} = k_{sc1} C_E C_{S_c} - k_{sc2} C_{E^*S_c} - k_p C_{E^*S_c} \quad (1)$$

$$\frac{dC_{E^*S_x}}{dt} = k_{sx1} C_E C_{S_x} - k_{sx2} C_{E^*S_x} \quad (2)$$

$$\frac{dC_{E^*P}}{dt} = k_{ep1} C_E C_P - k_{ep2} C_{E^*P} \quad (3)$$

$$\frac{dC_P}{dt} = k_p C_{E^*S_c} \quad (4)$$

$$\frac{dC_{S_c}}{dt} = k_{sc2} C_{E^*S_c} - k_{sc1} C_E C_{S_c} + \sigma(1 - \varphi_{S_x}) \frac{dC_P}{dt} \quad (5)$$

$$\frac{dC_{S_x}}{dt} = k_{sx2} C_{E^*S_x} - k_{sx1} C_E C_{S_x} + \sigma\varphi_{S_x} \frac{dC_P}{dt} \quad (6)$$

$$C_E = C_{E0} - C_{E^*S_c} - C_{E^*S_x} - C_{E^*P} \quad (7)$$

$$\varphi_{S_x} = \frac{C_{S_x}}{C_{S_x} + C_{S_c}} = \frac{C_{S_x}}{C_{S_0}} \quad (8)$$

where C_E , C_{S_c} , C_{S_x} , and C_P represent concentrations of free enzymes, digestible cellulose, inert and product, respectively; $C_{E^*S_c}$, $C_{E^*S_x}$, and C_{E^*P} represent the intermediates reaction complexes; φ_{S_x} – corresponds to a non-reacting cellulose fraction; the third term of the right-hand side of Eq. (5) and Eq. (6): the rate of emergence of new cellulose and inert, respectively, at the reaction interface; σ – accessibility coefficient of newly exposed substrate.

The system can be solved for the following initial conditions:

$$C_{E^*S_c}(0) = 0, C_{E^*S_x}(0) = 0, C_{E^*P}(0) = 0, C_P(0) = 0, \quad (9)$$

$$C_{S_c}(0) = C_{S_{c0}}, C_{S_x}(0) = C_{S_{x0}} \quad (10)$$

The simulation results and dynamic profiles of S_c , S_x and P are shown in Fig. 4.

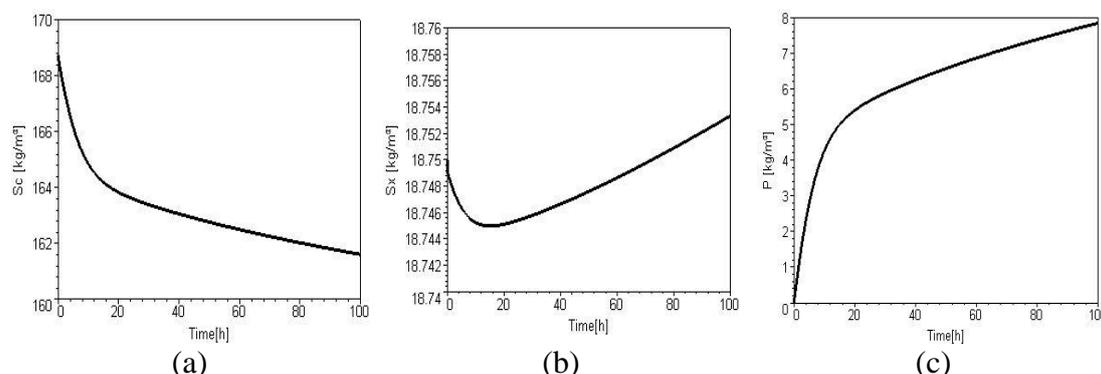


Fig. 4 Simulations of Gan et al. [40] model with $S_0 = 190$, $\varphi_{S_x} = 0.1$:

- (a) hydrolysable cellulose concentration; (b) non-hydrolysable cellulose concentration; (c) glucose concentration.

The adapted values of primary rate constants in simulations are presented in Table 2.

Table 2. Adapted values of primary rate constants

Rate constant	Value	Reference
k_{sc1}	0.20	Ryu et al., 1982 [89]
k_{sc2}	0.05	Howell and Mangat, 1978 [53]
k_{sx1}	0.02	Ryu et al., 1982 [89]
k_{sx2}	0.002	Ryu et al., 1982 [89]
k_p	9.05	Howell and Mangat, 1978 [53]
k_{ep1}	0.1	Mangat, 1977 [73]
k_{ep2}	0.03	n.a.

Analyzing the structure of cellulose and hemicelluloses and their content in used raw materials for ethanol production several conclusions can be made:

1. The pretreatment processes can be carefully chosen based on the specificity of lignocellulosic materials (LCM).
2. In case of wheat straw, the hydrolysis process should be chosen to minimize toxic compounds for ethanol producing bacteria.
3. In case of wheat straw, present of high C5 sugars required engineering solutions of two steps fermentation process or utilization of genetically modified strains capable to utilized C6 and C5 sugars simultaneously. Construction of simultaneous saccharification and fermentation (SSF) of LCM should take into account this key phenomenon.
4. Modeling the hydrolysis process of wheat straw should take into account the key state parameters of the process for the given pretreatment method.

- Further, the model can be incorporated into the complex model of SSF of wheat straw to ethanol.

Enzymes and genetically modified microorganisms involved in cellulose hydrolysis-last achievements in the field

Based on the cellulose structure a generalized step-by-step scheme of cellulose hydrolysis can be completed as follows (see Fig. 5).

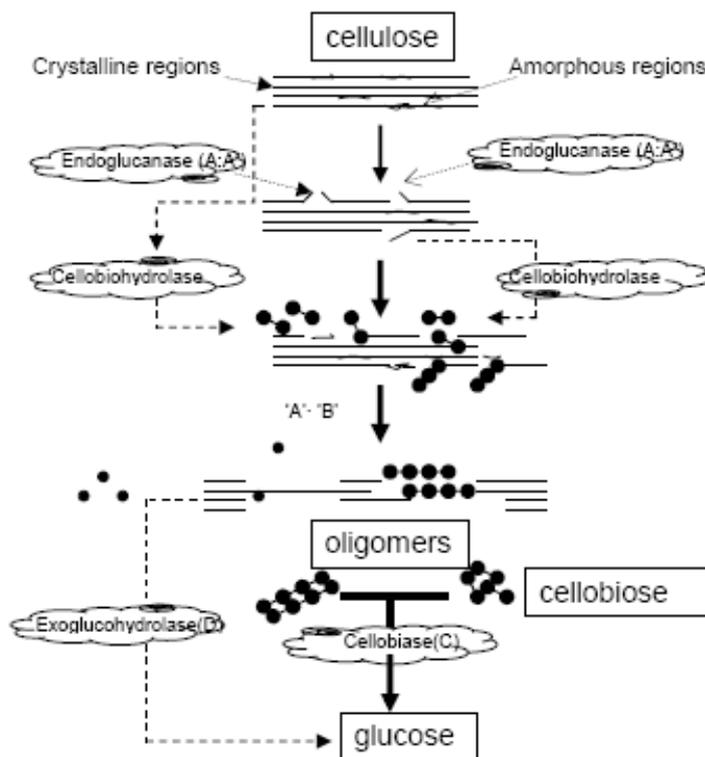


Fig. 5 Generalized scheme for cellulolysis

Enzymatic hydrolysis of cellulose is carried out by cellulases which are highly specific [10]. The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50 °C) and does not have a corrosion problem [34]. Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases [11]. *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteriodes cellulosolvens* produce cellulases with high specific activity, they do not produce high enzyme titres [34]. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi [34].

Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium*

[34, 37, 101]. Of all these fungal genera, *Trichoderma* has been most extensively studied for cellulase production [101].

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process (see Fig. 5): (1) endoglucanase (EG, endo- 1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-b-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) beta-glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose [23]. In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, b-xylosidase, galactomannanase and glucomannanase [34]. During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol.

Fermentative microorganisms

One of the major technical obstacles to commercialization of a process for converting lignocellulose to ethanol is strain development. Traditional microorganisms used for ethanol fermentation (e.g., *S. cerevisiae* and *Z. mobilis*) do not metabolize pentoses. Consequently, considerable effort has been expended over the past 20 years searching for pentose-fermenting organisms, especially those able to ferment xylose, the dominant hemicellulose sugar in many hydrolyzates [35]. A number of bacteria, yeasts, and fungi are able to ferment xylose (Fig. 3). Bacteria generally accomplish conversion of xylose to xylulose in one step with a xylose isomerase, while yeasts use a xylose reductase to reduce xylose to xylitol and a xylitol dehydrogenase to convert xylitol to xylulose [99]. While many bacteria can ferment xylose and a large variety of other carbohydrates, these microorganisms produce a mixture of fermentative products. Therefore, selectivity for ethanol and yields obtained using these bacteria are much lower than those obtained with yeast, fungi, or *Z. mobilis*.

Because yeasts produce superior ethanol yields, efforts were directed toward discovering natural xylose fermenting yeasts in the late 1970s. We were the first to discover yeast (*Pachysolen tannophilus*) that fermented xylose well and filed a patent application April 28, 1981 [14]. Subsequently, *Pichia stipitis* and *Candida shehatae* were discovered, and all three yeasts received considerable attention [26, 27, 33, 47, 57, 58, 72, 81, 94, 98]. The ethanol yields of D-xylose fermentations by these yeasts are lower than those for hexose fermentations, with the major difference between hexose and pentose metabolism being that all pentoses have to be shuttled through the pentose phosphate pathway [97]. Other problems include low ethanol productivity, sensitivity to low concentrations of inhibitors commonly found in hydrolysates (i.e., acetate), an inability to grow without oxygen, and a relatively low ethanol tolerance. Also, some of these yeast strains will, if the oxygen tension is below their fermentation optimum, produce significant amounts of xylitol. If the oxygen tension is too high, all these yeasts will metabolize ethanol. A variety of efforts have been applied to improve their fermentation performance. Novel bioreactor designs with cell recycle have been used to improve ethanol productivity, and adaptation techniques have been applied to increase tolerance to inhibitors. In addition, these yeasts do not have the ability to ferment arabinose, a major component (11%) of corn fiber. We have conducted an extensive yeast screening (117 strains) to identify those capable of fermenting arabinose [28]. Four yeast species (*Candida aungiensis*, *Candida succiphila*, *Ambrosiozyma monospora*, and *Candida sp.* (YB-2248)) were determined to ferment arabinose, but unfortunately, maximum ethanol

production was 4.1 g/L. Consequently, there is significant impetus to continue to develop microorganisms that ferment D-glucose, D-xylose, and L-arabinose to ethanol.

Much of the more recent work to improve fermentation of multiple substrates has been in the development of genetically engineered microorganisms. Two fundamental molecular approaches have evolved for the development of superior ethanogenic microorganisms that can ferment pentose and hexose sugars. The first approach is to genetically engineer the ability to use multiple substrates in microorganisms (such as *S. cerevisiae* and *Z. mobilis*) that normally make ethanol. The second approach is to genetically engineer enhanced ethanol production in microorganisms (such as *Escherichia coli*, *Klebsiella oxytoca*, and *Erwinia* species) that naturally use multiple substrates.

The first approach is illustrated by the research at the National Renewable Energy Laboratory. Zhang et al. [120] have produced a functional xylose metabolic pathway through the introduction of genes encoding xylose isomerase, xylulokinase, transaldolase, and transketolase. This pentose metabolism pathway converts xylose to central intermediates of the Enter-Doudoroff pathway and enables *Zymomonas* to ferment xylose to ethanol.

The xylose-utilizing recombinant (*Zymomonas mobilis* CP4 (pZB5)) grew on xylose (25 g/L) as the sole carbon source at a specific growth rate of 0.057 1/h and produced ethanol at 86% of the theoretical yield based on xylose consumed. The strain fermented a mixture of xylose and glucose (25 g/L each) to ethanol at 95% of theoretical yield within 30 h. Xylose was utilized much more slowly than glucose. Subsequently, Zhang et al. [120] developed L-arabinose fermenting strain. Five genes encoding L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate-4-epimerase, transaldolase, and transketolase were cloned from *E. coli* and introduced into *Z. mobilis* under the control of constitutive promoters. The resultant strain (ATCC 39676 (pZB186)) grew on medium containing arabinose 25 g/L as the sole carbon source, producing ethanol at 98% of the theoretical yield based on arabinose consumed. However, a significant amount of arabinose remained. In the presence of glucose and arabinose (both 25 g/L), the overall ethanol yield (based on sugars supplied) was 84% of theoretical, due to incomplete utilization of arabinose. Arabinose was used at a much slower rate than glucose, and only after glucose was nearly depleted.

More recently, Chou et al. [22] and Zhang et al. [119] reported the construction of a single strain of *Z. mobilis* capable of fermenting both xylose and arabinose. The strain contains seven plasmid-borne genes encoding xylose- and arabinose-metabolizing and pentose phosphate pathway enzymes. A mixture of glucose (30 g/L), xylose (30 g/L), and arabinose (20 g/L) was fermented to ethanol at an overall yield of 82-84% of the theoretical (based on sugar supplied). The consumption of arabinose was slow compared to that of glucose and xylose, accounting for the long fermentation time (80-100 h at 30 °C).

Advances in the genetics of engineered yeasts for pentose fermentation, particularly *S. cerevisiae*, are most attractive to the corn processing industry because of their familiarity and experience with yeast fermentations and the potential robustness of the organisms. Stevis and Ho [102], Gong et al. [44], Sarthy et al. [92], Amore et al. [3], Moes et al. [77], and Walfridsson et al. [112] have introduced bacterial xylose isomerase genes into *S. cerevisiae*, which does not normally metabolize xylose. This approach for producing a *Saccharomyces* capable of converting xylose to ethanol has met with limited success because of the following possibilities [35]: differences in internal pH between bacteria and yeasts; incorrect folding of the enzyme; and unsuitable post-translational modifications. Whatever the cause, overall

ethanol yields have been low, and further developments will be required for this approach to reach commercial reality.

Most recently, Ho and Tsao [49] and Ho et al. [48] constructed recombinant *Saccharomyces* strains containing plasmid-borne xylose reductase and xylitol dehydrogenase genes from *P. stipitis* and extra copies of the *S. cerevisiae* xylulokinase gene. Strain 1400 (pLNH32) was able to ferment xylose (80 g/L) as the sole carbon source to produce ethanol (27 g/L) (66% of theoretical yield based on sugar supplied). From a synthetic carbohydrate mixture containing glucose (31 g/L), xylose (15 g/L), arabinose (10 g/L), and galactose (2 g/L), the recombinant strain produced ethanol (22 g/L) in 24 h, an overall yield of 90% (based on sugar supplied, arabinose excluded). As expected, arabinose was not fermented to ethanol, although a portion was converted to arabitol. Additional genetic efforts will be necessary for yeast to ferment arabinose produced in the hydrolysis of corn fiber.

Applying the second molecular approach, Dr. Lonnie Ingram and colleagues [8, 17, 31, 32, 54-56, 83, 115] have developed a series of recombinant *E. coli* and *K. oxytoca* strains for the efficient fermentation of pentose and hexose sugars to ethanol. The alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdhC*) genes from *Z. mobilis* were cloned under the control of a single promoter to form the *pet* (production of ethanol) operon [55]. The *pet* operon was introduced into the chromosome of these bacteria, and expression of the *pet* genes resulted in high production of ethanol with good selectivity [83, 115]. Excellent conversion of sugars (glucose, xylose, and arabinose) to ethanol has been reported for model fermentation broths [9, 13] and for hemicellulose hydrolyzates [7, 67].

Bioconversion of cellulose – comparison between two-step biomass-to-ethanol process and SSF of cellulose to ethanol

Compared to the two-stage hydrolysis-fermentation process, SSF has the following advantages: (1) increase of hydrolysis rate by conversion of sugars that inhibit the cellulase activity; (2) lower enzyme requirement; (3) higher product yields; (4) lower requirements for sterile conditions since glucose is removed immediately and ethanol is produced; (5) shorter process time; and (6) less reactor volume because a single reactor is used. However, ethanol may also exhibit inhibition to the cellulase activity in the SSF process. Wu and Lee [118] found that cellulase lost 9%, 36% and 64% of its original activity at ethanol concentrations of 9, 35 and 60 g/L, respectively, at 38 °C during SSF process. The disadvantages which need to be considered for SSF include: (1) incompatible temperature of hydrolysis and fermentation; (2) ethanol tolerance of microbes; and (3) inhibition of enzymes by ethanol.

A new model for SSF of starch to ethanol was developed by Kroumov et al. [64]. A very detailed analysis of processes on molecular and population levels was performed. The approaches in system analysis, modeling and parameters identification can be successfully for SSF of materials giving glucose and other sugars for ethanol production.

Research and development future prospects

The U.S. fuel ethanol industry produced more than 6.2 billion liters of ethanol in 2000, and is planned to produce about 14.4 billion gallons in 2014 and to 15 billion gallons in 2015 most of which was produced from corn [5, 71, 73]. However, an increase of ethanol production from corn will compete for the limited land against corn based food and feed production. On the other hand, there is a huge amount of low-value or waste LCB that are currently burned or wasted. Utilization of LCB can replace the equivalent of 40% of the gasoline in the U.S. market [113]. Using LCB such as agricultural residues, grasses, forestry wastes and other

low-cost biomass can significantly reduce the cost of raw materials (compared to corn) for ethanol production. A reduction of the cost of ethanol production can be achieved by reducing the cost of either the raw materials or the cellulase enzymes. It was predicted that the use of genetically engineered raw materials with higher carbohydrate content combined with the improvement of conversion technology could reduce the cost of ethanol by \$0.11 per liter over the next ten years or to reduce costs for enzymes from \$1 per gallon up to \$0.1 dollars [82, 116]. Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of LCB. Genetic techniques have been used to clone the cellulase coding sequences into bacteria, yeasts, fungi and plants to create new cellulase production systems with possible improvement of enzyme production and activity. Wood et al. [114] reported the expression of recombinant endoglucanase genes from *Erwinia chrysanthemi* P86021 in *E. coli* KO11 and the recombinant system produced 3,200 IU endoglucanase/l fermentation broth (IU, international unit, defined as a micromole of reducing sugars released per minute using carboxymethyl cellulose as substrate). The thermostable endoglucanase E1 from *Acidothermus cellulolyticus* was expressed in *Arabidopsis thaliana* leaves [123], potato [24], and tobacco [51]. Using genetically engineered microorganisms that can convert xylose and/or pentose to ethanol can greatly improve ethanol production efficiency and reduce the cost of the production. The constructed operons encoding xylose assimilation and pentose phosphate pathway enzymes were transformed into the bacterium *Z. mobilis* for the effective fermentation of xylose to produce ethanol [120]. The recombinant strain of *E. coli* with the genes from *Z. mobilis* for the conversion of pyruvate into ethanol has been reported in 2000 by Dien et al. [29]. The recombinant plasmids with xylose reductase and xylitol dehydrogenase genes from *P. stipitis* and xylulokinase gene from *S. cerevisiae* have been transformed into *Saccharomyce* spp. for the co-fermentation of glucose and xylose [49]. Although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations. These challenges include maintaining a stable performance of the genetically engineered yeasts in commercial scale fermentation operations [30], developing more efficient pretreatment technologies for LCB, and integrating the optimal components into economic ethanol production systems.

Kroumov [63] has developed a model describing effects of catabolic repression mechanism when two substrates were involved in ethanol production process. The model clearly showed that simultaneous, sequential and separate utilization of the substrates was possible depending on the ratio of sugars' concentrations. Similar approach can be used to describe SSF of wheat straw and consequent utilization of pentose and hexose to ethanol.

Conclusions

The main conclusion of the above analysis of ethanol production process on LCB and especially on wheat straw as renewable source of biomass is that simultaneous utilization of C5 and C6 sugars to ethanol remains the key problem. Especially, beneficiary will be application of models taking into account catabolic repression mechanism which is involved when these two substrates are present in the cultural broth.

Acknowledgements

The authors are grateful to the National Science Fund of Bulgaria which sponsored this work under the grand NSF-E02/16, 2014-2016.

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