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Review: Continuous hydrolysis and fermentation for cellulosic ethanol production

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ABSTRACT

Ethanol made biologically from a variety of cellulosic biomass sources such as agricultural and forestry residues, grasses, and fast growing wood is widely recognized as a unique sustainable liquid transportation fuel with powerful economic, environmental, and strategic attributes, but production costs must be competitive for these benefits to be realized. Continuous hydrolysis and fermentation processes offer important potential advantages in reducing costs, but little has been done on continuous processing of cellulosic biomass to ethanol. As shown in this review, some continuous fermentations are now employed for commercial ethanol production from cane sugar and corn to take advantage of higher volumetric productivity, reduced labor costs, and reduced vessel down time for cleaning and filling. On the other hand, these systems are more susceptible to microbial contamination and require more sophisticated operations. Despite the latter challenges, continuous processes could be even more important to reducing the costs of overcoming the recalcitrance of cellulosic biomass, the primary obstacle to low cost fuels, through improving the effectiveness of utilizing expensive enzymes. In addition, continuous processing could be very beneficial in adapting fermentative organisms to the wide range of inhibitors generated during biomass pretreatment or its acid catalyzed hydrolysis. If sugar generation rates can be increased, the high cell densities in a continuous system could enable higher productivities and yields than in batch fermentations.

1. Introduction

According to the recent report of the Intergovernmental Panel on Climate Change (IPCC) warming of the world's climate system is unequivocal and is very likely due to the observed increases in anthropogenic greenhouse gas concentrations. Atmospheric concentrations of carbon dioxide (CO_2), the dominant greenhouse gas, have increased from a pre-industrial value of about 280 ppm to 379 ppm in 2005, primarily as a result of fossil fuel use (IPCC, 2007). Overall, petroleum is the source of about 170 quadrillion (10^{15}) BTUs or quads of energy of the total of more than 460 quads the world uses, far more than derived from coal, natural gas, hydroelectric power, nuclear energy, geothermal, or other sources. Over half of petroleum in this total is used for transportation, and demand is projected to grow rapidly as vehicle traffic increases throughout the world and even accelerates in Asia. Besides the negative global warming impact of fossil fuels, volatile oil prices and dependency on politically unstable oil exporting countries resulted in a significant increase in international interest in alternative fuels and led policy makers in the EU and the US to issue ambitious goals for substitution of alternative for conventional fuels (Galbe and Zacchi, 2002; Wyman, 2007).

Ethanol made biologically by fermentation from a variety of biomass sources is widely recognized as a unique transportation fuel with powerful economic, environmental and strategic attributes. First generation ethanol made from starch-rich materials such as corn and wheat or from sugar feedstock is a mature commodity product with a worldwide annual production of over 13 billion US gallons in 2007. However, these raw materials are insufficient to meet the increasing demand for fuels, and concerns have heightened recently that competition between the use of agricultural commodities for fuel production is driving up food costs. Furthermore, the use of food crops for fuel production may lead to environmentally detrimental indirect land use changes, e.g. the deforestation of tropical rainforest to gain more farmland. In addition, the reduction of greenhouse gases resulting from use of starch-based ethanol is not as high as desirable (Farrell et al., 2006; Hahn-Hägerdal et al., 2006). Alternatively, ethanol can be produced from lignocellulosic materials such as agricultural residues, wood, paper and yard waste in municipal solid waste, and dedicated energy crops, which constitute the most abundant renewable organic component in the biosphere (Claassen et al., 1999).

Regardless of the feedstock, the final ethanol selling prize must be competitive with that for gasoline, but gasoline benefits from over a century of learning curve improvements and largely paid for capital. Thus, profit margins in ethanol production processes are low, and returns on capital are uncertain due to the tremendous

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price swings in petroleum prices. In this context, costs must be kept as low as possible, and continuous fermentation of cellulosic biomass to ethanol can offer important advantages in terms of greater productivity and lower costs. Unfortunately, although process designs have been conceptualized based on continuous enzymatic hydrolysis and fermentations to take advantage of their low cost potential, limited studies have actually been reported from which to design or advance the technology. Thus, more information is sorely needed on this subject to guide the advancement of lower cost approaches to making ethanol and overcome the significant cost barriers to market entry.

In this paper, we will provide a short introduction to concepts and characteristics of continuous fermentations. Then a summary is presented of experiences and research activities with first generation industrial continuous ethanol fermentations as these provide the foundation for second generation cellulose-based processes. Following that, we review current knowledge of continuous fermentation of lignocellulosic material, including those based on chemical and enzymatic hydrolysis of cellulose to glucose.

2. Concept of continuous fermentations

In a true continuous fermentation system, substrate is constantly fed to the reaction vessel, and a corresponding flow of fermented product broth is discharged to keep the reactor volume constant. Furthermore, the balance between feed and discharge is maintained for long enough times to achieve steady state operation with no changes in the conditions within the reactor. Compared to a batch reaction, this mode of operation offers reduced vessel down time for cleaning and filling providing improved volumetric productivity that can translate into smaller reactor volumes and lower capital investments plus ease of control at steady state.

Two basic types of continuous reactors can be employed: the continuous stirred tank reactor (CSTR) or the plug flow reactor (PFR). In an ideally mixed CSTR, the composition in the reactor is homogenous and identical to that for the outgoing flow. In an ideal PFR, the reactants are pumped through a pipe or tube with a uniform velocity profile across the radius, and the reaction proceeds as the reagents travel through the PFR with diffusion assumed to be negligible in the axial direction. Consequently, PFR operations imply that inoculum has to be constantly fed to the reactor for fermentation processes. Cascading a large number of CSTRs in series will have similar performance to a PFR.

In a system with constant overall reaction stoichiometry that can be described by a single kinetic equation, performing the reaction in two or more bioreactors may lead to a higher product concentration, a higher degree of conversion, a higher volumetric productivity, or a combination of these factors compared to operation of a single CSTR. One approach to optimizing a continuous process is to determine the reactor configuration that gives the lowest residence time to achieve a certain degree of conversion. If the kinetics are known, a plot of the reciprocal rate against the dimensionless substrate concentration S/S_{feed} can be employed to estimate the reaction residence time and therefore the reaction volume (de Gooijer et al., 1996). For a CSTR, the area corresponding to a rectangle whose height equals the reciprocal of the rate at the desired conversion will equal the residence time for reaction to this conversion, whereas the residence time for a PFR will correspond to the area under the curve (see Fig. 1). If the desired conversion is higher than the minimum in the curve, a combination of reactors will require less reaction volume. Thus, for the situation depicted in Fig. 1, the combination of a CSTR followed by a PFR will be preferred if a conversion of 98% is targeted.

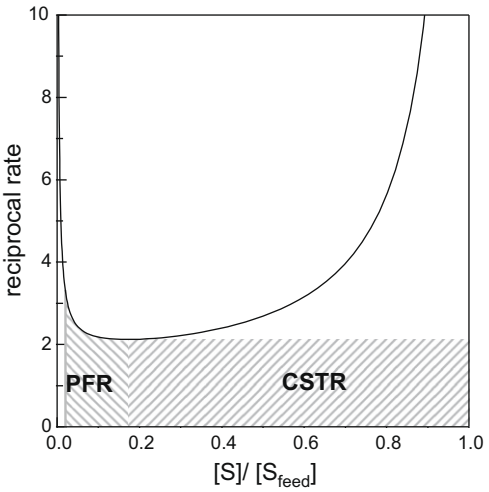

Fig. 1. Graphical design method to estimate reactor residence times and corresponding volumes. The reciprocal of reaction rate is plotted versus dimensionless substrate concentration S/S_{feed} on the basis of a literature kinetic model (de Gooijer et al., 1996; Lee et al., 1983).

An important performance criteria is the productivity of the fermentation system, i.e., the amount of product formed per unit of time and reactor volume, which depends on several factors including substrate concentration, cell concentration, and dilution rate. We used a simple model based on Monod growth kinetic that includes terms for product and cell inhibition (Lee et al., 1983) to illustrate the influence of some operational parameters. Generally, the productivity in a continuous fermentation system is higher than in a batch reactor. In the model example, a productivity of 3.57 g L^{-1} h^{-1} was calculated for a batch process inoculated with 1 g L^{-1} yeast cells. In a standard single stage CSTR without cell retention, where the biomass concentration would be fixed, a maximum productivity of 4.24 g L^{-1} h^{-1} was calculated for a dilution rate of 0.136 h^{-1}, however, the substrate conversion was only 83%. Generally it is desirable to achieve almost complete substrate conversion at the highest possible productivity to avoid loss of substrate or the need for recycle. In a two stage system with properly designed unequal reactor sizes (see Fig. 2a), the maximum possible overall productivity would be lower than in a single stage system, but the substrate conversion at identical productivities would be higher. In the two stage system, a maximum overall productivity of 4.16 g L^{-1} h^{-1} was calculated at a substrate conversion of 92% (Fig. 2b). If a substrate conversion of 99% were the goal, the productivity in a single stage CSTR would be 2.77 g L^{-1} h^{-1} but 3.94 g L^{-1} h^{-1} for a two stage system. Generally, a cascade of fermentors would be superior to a single vessel for autocatalytic reactions such as cell growth which are product-inhibited, but for situations with substrate inhibition, a single stage CSTR is often more favourable to remove as much reactant as possible (de Gooijer et al., 1996).

3. Continuous ethanol production from starch and sugar feedstocks

3.1. Industrial continuous ethanol production from sugar cane

Sugar cane is a tropical and subtropical crop that is the primary feedstock for ethanol production in Brazil, India, and Colombia. It contains mainly sucrose, a dimer of glucose and fructose, which is readily assimilated by *Saccharomyces cerevisiae* (Sanchez and Cardona, 2008). Both sugar cane juice and molasses normally contain sufficient minerals and nutrients for *S. cerevisiae* to ferment

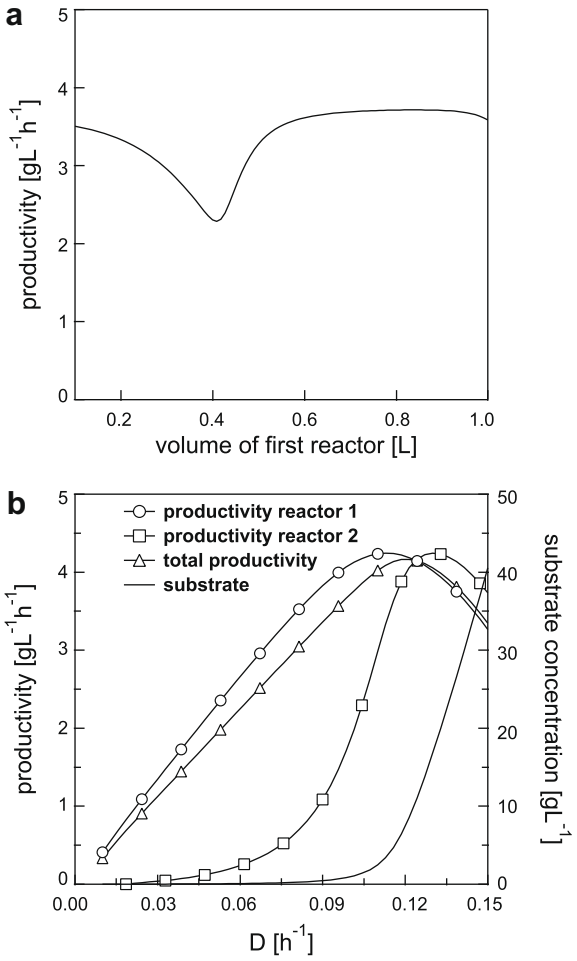

Fig. 2. Design considerations for a multistage continuous fermentation system as described by the kinetic model of Lee et al. (1983): (a) the volume of the first CSTR in a series of two CSTRs comprising a total volume of 1 L influences the productivity of the whole system. The dilution rate was set to 0.1 h^{-1}. (b) Influence of the dilution rate (based on the whole system) on productivity and steady state substrate concentration of a volume optimized system of two CSTRs in series. The first reactor has a volume of 0.83 L and the total volume of the system is 1 L.

them directly to ethanol (Wheals et al., 1999). In Brazil, 70–80% of the distilleries employ fed-batch processes (Melle-Boinot Process) for fermentors with outputs ranging from 400 to 2000 m^3 ethanol per day. Typically, high yeast cell concentrations of between 8% and 17% achieve fermentation times of only 6–10 h and final ethanol concentrations of up to 11% v/v, corresponding to an average ethanol yield of 91%. After each fermentation cycle, the yeast cells are separated, treated with dilute sulphuric acid to kill contaminating bacteria, and then recycled to start a new fermentation. This sequence can be repeated up to 200 times and minimizes carbon consumption for yeast growth while providing very high ethanol productivities (Dorfler and Amorim, 2007; Godoy et al., 2008; Wheals et al., 1999; Zanin et al., 2000). The first continuous versions of the Melle-Boinot process appeared in the 1970s, but several operational problems were detected, such as a high level of contamination, low productivity, low yields, and problems with solids flow. Today's continuous fermentation processes are optimized based on kinetic models to achieve high productivities (typically 10 mL L^{-1} h^{-1}), high process flexibility and stability, and low consumption of chemicals and are considered to be less expensive for ethanol production than batch processes (Zanin et al., 2000).

An important feature of state-of-the-art continuous processes is the use of multiples stages (typically four or five) of variable sizes. The sugar substrate is fed to the top of the first reactor together with the recycled yeast cream and leaves through the bottom, flowing then by gravity to the middle of the next stage. Each reactor typically uses an external plate-type heat exchanger for cooling of the fermentation broth, with the kinetic energy of the liquid leaving the heat exchanger outlet used to agitate the reactor. The yeast cells produced are separated from the "wine" by disk-bowl centrifuges, forming a yeast cream, which is then sent to acid treatment prior to being recycled back to the first reactor (Zanin et al., 2000).

Guerreiro et al. (1997) described an expert system for the design of such an industrial continuous fermentation plant which combines expert knowledge and industrial practices with kinetic modelling. As parameters are taken from industrial fermentations, differences between theoretical calculations and practical results are claimed to be minimal. In the example presented, an input medium containing 170–190 g L^{-1} sugar was fed at a rate of 143 m^3 h^{-1} to a four stage reactor train with volumes of 215, 274, 324, and 213 m^3 to maximize performance. In this case, the steady state concentrations in the first and last stages were 54 and 1 g L^{-1} of sugar, 42 and 66 g L^{-1} of ethanol, and 29 and 31 g L^{-1} of yeast biomass, respectively, and the process productivity was given as 7.7 g L^{-1} h^{-1}. Generally, the volumes of the tanks influence the productivity which varied from 6.1 to 7.9 g L^{-1} h^{-1}. Through optimization, it was possible to replace a previous fed-batch plant that consisted of 24 fermenters of 200 m^3 volume each (total 4800 m^3) producing 400 m^3 of 96% ethanol per day with a continuous plant with a total volume of 2500 m^3 producing about 440 m^3 of 96% ethanol per day (Guerreiro et al., 1997). However, larger continuous plants exist with capability to produce up to 600 m^3 ethanol per day (Zanin et al., 2000).

In some Brazilian distilleries, processes based on flocculent yeast strains are employed, with cell separation in settlers to avoid costly centrifuges. Yeast flocculation is a reversible, asexual, calcium dependent process of self-aggregation in which cells adhere to form flocs consisting of thousands of cells. Because of their macroscopic size and mass, the yeast flocs rapidly settle out of the fermenting medium, thus providing natural cell immobilization (Verbelen et al., 2006). Compared to the classical Melle-Boinot process, it is claimed that up to 1.5% higher fermentation efficiency is obtained, ethanol production costs are ca. \$7/m^3 lower, and consumption of chemicals such as antifoam is reduced (Zanin et al., 2000).

Despite such desirable attributes, there are also critical opinions about replacing batch fermentations with continuous processes. In one study of the advantages and disadvantages of continuous and batch fermentation processes for 62 distilleries over a time span of 9 years (1998–2007), batch processes with yeast recycle were shown to be less susceptible to bacterial contamination and the corresponding loss in productivity (Godoy et al., 2008). *Lactobacillus* contaminations, in particular, are regarded as the major factor that can reduce ethanol yield and also impair yeast centrifugation, and greater quantities of antibiotics are needed to address this issue for continuous processes. Also, slightly more sulphuric acid was consumed in continuous processes. Yet, continuous processes have the advantages of lower installation costs due to smaller fermentor volumes and less heat exchanger demands as well as lower costs due to greater automation (Godoy et al., 2008).

3.2. Continuous ethanol production from corn

Up to now, corn is the major feedstock for ethanol production in the US, which surpasses Brazil as the largest ethanol producer. Corn kernels contain about 70% by weight starch on a dry weight basis. Starch is a D-glucose polymer, consisting of about 30% amylose, a linear chain of α-1,4 linked glucose units with a helical

structure and 70% amylopectin, a highly branched polymer with additional α-1,6 glycolytic bonds. Ethanol from corn can be produced by either a dry grind (67% of the fuel ethanol) or wet mill (33%) process with recent growth in the industry mostly with dry grind plants due to their lower capital costs (Bothast and Schlicher, 2005).

In the wet mill process, the grain is separated into its four basic components of starch, germ, fiber, and protein to recover higher value co-products including corn oil, corn gluten meal, corn gluten feed, and germ. On the other hand, the dry grind process is much simpler in that the entire corn kernel is ground and mixed with water to form a mash.

The isolated starch from wet-milling and the mash from the dry grind process are treated identically to produce ethanol. First, a thermostable alpha-amylase, which breaks down the starch polymer to soluble dextrins by hydrolyzing α 1–4 bonds, is added. The mixture is heated to over 100 °C to liquefy the mash over a holding time of at least 30 min. Then, glucoamylase is added, which converts liquefied starch to glucose at an optimal temperature of 65 °C. In the final fermentation step, which is performed either coupled (simultaneous saccharification and fermentation, SSF) or subsequent to glucoamylase treatment (separate hydrolysis and fermentation, SHF), the mash is cooled to 32 °C, and yeast is added as well as ammonium sulphate or urea as a nitrogen source. Alternatively, proteases are added to break down corn protein to free amino acids for use as a nitrogen source. Fermentation is completed in 48–72 h to a final ethanol concentration of 10–12% v/v and higher. Over the course of fermentation, the pH drops to 4.0 or lower, which helps to prevent bacterial contamination. Many plants use simultaneous saccharification and fermentation, because it lowers the risk of contamination, lowers the initial osmotic stress on the yeast, and is generally more energy-efficient (Bothast and Schlicher, 2005).

According to a United States Department of Agriculture (USDA) survey in 2002, 27% of the dry grind distilleries in the US employ continuous fermentation processes which are more common in large plants producing more than 400 m^3 ethanol per day (Shapouri and Gallagher, 2005). To the best of our knowledge, no performance data for industrial continuous corn ethanol fermentations are published. However, Bai et al. (2008) described in their review a commercial plant employing a self-flocculating yeast with a production capacity of 680 m^3 per day which started operation in 2005 in China. In this system, six fermentors with volumes of 1000 m^3 each were arranged in a cascade, and corn meal hydrolyzate, with a sugar concentration of 200–220 g L^{-1}, was fed to the fermentation system at a dilution rate of 0.05 h^{-1}. The final ethanol concentration was reported to be 11–12% v/v. Yeast flocs were retained within the fermentor by baffles to effectively immobilize them, and the yeast concentration within the fermentors was maintained at 40–60 g DCW L^{-1}.

4. Continuous production of second generation ethanol from lignocellulosic materials

Although composition of lignocellulosic materials varies in different plants, the three main components are cellulose (36–61%), hemicellulose (13–39%), and lignin (6–29%) (Olsson and Hahn-Hägerdal, 1996). Cellulose is a D-glucose polymer, where the subunits are linearly linked by β-1,4 glycosidic bonds and exists in crystalline and amorphous forms. Hemicellulose is composed of linear and branched heteropolymers of pentoses (i.e., xylose and arabinose) and hexoses (i.e., mannose, glucose, and galactose). Lignin is a polymer that can consist of three different phenylpropane units (*p*-coumaryl, coniferyl and sinapyl alcohol) that bind the plant together. In order to release fermentable sugar monomers, cellulose and hemicellulose are hydrolyzed chemically, enzymatically, or by their combination (Gray et al., 2006; Hendriks and Zeeman, 2009; Wyman et al., 2004).

Lignocellulosics can be hydrolyzed chemically by addition of acids, with sulphuric acid most often preferred based on price and toxicity, and acid hydrolysis can be divided in two categories: concentrated acid hydrolysis and dilute acid hydrolysis. Concentrated acid processes operate at low temperatures, e.g., 40 °C, and give high sugar yields, e.g., 90% of theoretical glucose yield. However, acid consumption is high, a lot of energy is consumed for acid recovery and recycle, the equipment can suffer from corrosion, and reaction times of 2–6 h are required. The dilute acid process is characterized by a low acid consumption and very short reaction times at high temperatures. Hemicellulose is generally much more susceptible to acid hydrolysis than cellulose, and yields of more than 85% can be obtained at relatively mild conditions, with only a small part of the cellulose converted to glucose. More severe conditions required to achieve high glucose yields from cellulose, however, lead to degradation of hemicellulose sugars, resulting in low yields and unwanted side-products that are also strong fermentation inhibitors. Potential inhibitors that can be formed or released from hemicellulose, cellulose, and lignin during such thermochemical routes include furfural, 5-hydroxymethylfurfural (HMF), levulinic acid, acetic acid, formic acid, uronic acid, 4-hydroxybenzoic acid, vanillic acid, vanillin, phenol, cinnamaldehyde, and formaldehyde. To reduce degradation of monosaccharides at high temperature, dilute acid hydrolysis is typically carried out in two stages, with hemicellulose solubilized in the first under relatively mild conditions and the residual solids hydrolyzed in the second under the more severe conditions needed to breakdown cellulose. With this procedure, hemicellulose derived sugar yields are in the range of 90%, while glucose yields are only about 40–60% at realistic residence times. However, it has been reported that alternative reactor configurations to classic batch reactors, such as a shrinking-bed reactor give glucose yields of up to 90% (Taherzadeh and Karimi, 2007).

Cellulase enzymes from the fungus *Trichoderma reesei* can hydrolyze biomass to sugars at near ambient temperatures, resulting in little degradation. However, because sugar yields from raw biomass are very low, the biomass is subjected to a pretreatment step. Numerous pretreatment methods have been developed including pretreatment with steam, liquid hot water, dilute acid, lime, ammonia, and wet oxidation and are discussed in more detail elsewhere (Hendriks and Zeeman, 2009; Mosier et al., 2005; Wyman et al., 2005, 2009). Effective pretreatments are thought to enhance enzymatic digestibility of biomass due to several effects: disruption of the lignocellulosic structure by loosening the hemicellulose lignin entanglement, hemicellulose hydrolysis, lignin solubilisation and disruption, decrystallization of cellulose, and increased accessible surface area (Lynd et al., 2002; Zhang and Lynd, 2004, 2006). During many pretreatments, fermentation inhibitors such as acetic acid, lignin breakdown products, and furfural are released. After pretreatment, several process configurations are possible, as recently reviewed in detail (Cardona and Sanchez, 2007). In the separate hydrolysis and fermentation (SHF) approach, the liquid and solid phases are separated after pretreatment, and the solid phase may be subjected to additional washing steps. The solids, in case of dilute acid and steam pretreatment, contain most of the lignin and cellulose from the raw biomass, with the latter hydrolyzed to glucose by addition of cellulolytic enzymes that are comprised of endo- and exoglucanase and β-glucosidase activities, often supplemented with additional β-glucosidase derived from *Aspergillus niger*. The resulting hexose solution is then fermented to ethanol using conventional yeast or other suitable microorganisms. A suitable pentose fermenting strain can convert the liquid stream from pretreatment containing

solubilized hemicellulose to ethanol in a separate unit usually after a detoxification step such as overliming to reduce fermentation inhibitors and make the hydrolyzate fermentable. Hydrolysis and fermentation were initially separated to better match the pH and temperatures to those that are optimal for each step, with about 50 °C preferred for enzymatic hydrolysis and about 32 °C often best for fermentations. Alternatively, enzymes could be added to the whole pretreatment slurry without separation of the liquid from the solids, followed by fermentation of pentoses and hexoses to ethanol, a process which we call separate hydrolysis and co-fermentation (SHcF). This more integrated approach is economically very attractive, but the fermentation step is much more challenging than in SHF and complicates hydrolyzate conditioning to remove inhibitors due to the presence of the solids (Cardona and Sanchez, 2007).

Because cellulases are inhibited by their hydrolysis products cellobiose and glucose, a favoured processing mode is to combine hydrolysis and the fermentation, a process termed simultaneous saccharification and fermentation (SSF), thereby keeping sugar concentrations low (Gauss et al., 1976; Spindler et al., 1987; Takagi et al., 1977; Wright et al., 1987; Wyman et al., 1986). Although initial applications subjected only the washed solids fraction from pretreatment to SSF with the liquid pentose stream processed separately, these two steps can be combined in what is termed simultaneous saccharification and co-fermentation (SScF) (Wooley et al., 1999). Despite the need to reduce the temperature for SSF from the optimal levels for enzymes to accommodate fermentative organisms available to date, SSF was shown to achieve higher rates, yields, and concentrations than SHF by overcoming the major effects of end-product inhibition (Spindler et al., 1987; Wright et al., 1987). In addition, SSF, and even more so SScF, reduces fermentation equipment demands, and the presence of ethanol impedes invasion by unwanted organisms. Thus, until enzymes are found that can overcome end-product inhibition, SSF or SScF are likely to be preferred in terms of productivity, yields, and ethanol concentrations. In the following sections, results for continuous fermentations with hydrolzates from acid and enzymatic hydrolysis and for SSF applications are summarized.

4.1. Fermentation of hexoses in enzymatic hydrolyzates

Fermentation of hexose sugars derived from enzymatic hydrolysis of washed pretreated lignocellulosic material generally does not pose special difficulties (see Table 1), as the inhibitor concentration should be very low. However, compared to starch and sugarcane fermentations, the sugar concentration after hydrolysis are often low with values approaching typically not more than 70 g L^{-1} due to challenges in feeding solids concentrations higher than about 10% by weight to the fermentors and end-product inhibition of cellulase enzymes by the sugars released. Thus, a concentration step, e.g., vacuum evaporation, might be needed to achieve higher concentrations, with additional extra costs possibly counterbalanced by savings in the final distillation step (Maiorella et al., 1984).

In one study, sugar cane bagasse was delignified by autoclaving in 1% NaOH for 1 h, and the solids were washed several times prior to hydrolysis by *T. reesei* cellulases. In a single stage continuous fermentation of *S. cerevisiae* with a 16% glucose feed, several dilution rates were tested to find a maximum ethanol productivity of 4.1 g L^{-1} h^{-1} at a dilution rate of 0.13 h^{-1}. At this point, steady state concentrations of 90 g L^{-1} glucose, 31 g L^{-1} ethanol, and 3.8 g L^{-1} biomass were measured. To avoid washout of large amounts of unfermented glucose, a continuous single stage cell recycle fermentation system was set up, and the maximal productivity reached 18.3 g L^{-1} h^{-1} at a dilution rate of 0.3 h^{-1} with a steady state glucose concentration of 22 g L^{-1} (Ghose and Tyagi, 1979).

Lee et al. (2000) employed an enzymatic hydrolyzate derived from washed steam exploded oak chips (3 min at 215 °C). To reduce fermentation inhibitors, the hydrolyzate was sterilized for 120 min at 60 °C, rather than at a typical temperature of 121 °C. Continuous cultures were performed in a reactor equipped with an internal membrane filtration module to retain cells inside the reactor. At a dilution rate of 0.22 h^{-1} and a feed glucose concentration of 180 g L^{-1}, 77 g L^{-1} ethanol was produced, corresponding to a productivity of 16.9 g L^{-1} h^{-1} and a yield of 0.43 g g^{-1}. In a batch fermentation in a similar medium containing 170 g L^{-1} glucose, only 57 g L^{-1} ethanol was produced in 210 h, with 35 g L^{-1} glucose not utilized, leading to a very low productivity of 0.3 g L^{-1} h^{-1}. No problems were experienced with bacterial contamination despite the low sterilization temperature.

When the solid–liquid separation and solids washing steps are omitted and the whole slurry is enzymatically hydrolyzed, fermentations are much more difficult, as exemplified by the work of Palmqvist et al. (1998) who employed a hydrolyzate of spruce pretreated by steam explosion for 5 min at 215 °C after sulphur dioxide impregnation. Two different batches of hydrolyzate were used, containing 25–50 g L^{-1} glucose and approximately 10 g L^{-1} mannose, that were both supplemented with mineral media. *S. cerevisiae* ATCC 96581, a strain isolated from a spent sulphite liquor (SSL) fermentation plant running since 1940 and showing a 7-fold higher maximum growth rate on SSL than bakers yeast, was employed for the study. At a pH of 4.6 in a batch system, cells metabolized

Table 1
Fermentation of hexoses in enzymatic hydrolyzates.

Medium	Sugar concentration (g L^{-1})	Reactor type	Dilution rate (h^{-1})	Ethanol (g L^{-1})	cell dry weight (g L^{-1})	Ethanol yield (g g^{-1})	Ethanol productivity (g L^{-1} h^{-1})	References
Sugar cane bagasse pretreated with NaOH; washed solids enzymatically hydrolyzed, concentration by vacuum evaporation; addition of "cheap nitrogen source", $CaCl_2$, $MgSO_4$	Reducing sugars: 160	Single stage CSTR	0.13	31	3.8	0.19	4.1	Ghose and Tyagi (1979)
		Single stage CSTR with cell recycle	0.3	58	30	0.36	18.3	
Steam exploded oak chips, washed solids enzymatically hydrolyzed, concentrated by vacuum evaporation, sterilization for 120 min at 60 °C	Glucose: 180	Single stage CSTR with cell retention by membrane module	0.22	77	n.d.	0.43	16.9	Lee et al. (2000)
	Glucose: 170	Batch	–	57		0.34	0.3 (fermentation time of 210 h)	
Steam exploded spruce, whole slurry enzymatically hydrolyzed, addition of complete mineral medium salts	Glucose: 25–50, mannose: 10	Single stage CSTR	0.05	20	0.9	0.32	0.5	Palmqvist et al. (1998)
			0.1	Washout				
		Single stage CSTR with cell recycle	0.1	23	Maximum 26	0.51	2.3	

glucose without growing and produced only a minor amount of ethanol. When the pH was raised to 5.0, the cells started to grow, and ethanol productivity increased. At the point that glucose was completely consumed, the system was switched to continuous operation at a dilution rate of 0.05 h^{-1} while raising the pH further to 5.5. At steady state, 20 g L^{-1} ethanol was measured, corresponding to a yield of 0.32 g g^{-1} and a productivity of 0.5 g L^{-1} h^{-1} at a yeast concentration of 0.9 g L^{-1}. Raising the dilution rate further to 0.1 h^{-1} resulted in washout of the cells.

In a second continuous fermentation run with a dilution rate of 0.1 h^{-1}, the pH was adjusted to 5.7, and cells were recycled by filtration to increase productivity. Cells were removed at a low rate to maintain a constant cell density of approximately 6 g L^{-1} between 115 and 200 h, but when removal was stopped, the cell density reached a maximum value of 26 g L^{-1} after 290 h of fermentation. Glucose, which was fed at a concentration of 36 g L^{-1} during this run, was depleted over most of the time, and ethanol concentrations reached values as high as 23 g L^{-1}, corresponding to a mass yield of 0.51 g g^{-1}. The volumetric productivity of 2.3 g L^{-1} h^{-1} was 4.6 times higher than for the case without cell recycle. Low growth rates were blamed on the presence of inhibitors such as HMF and furfural that are known to induce a lag phase in cell growth and ethanol production while they are being reduced by the yeast. As in a continuous mode, the inhibitors were constantly fed with the result that the resulting low growth rate limited the maximum possible dilution rate. The strong effect of the fermentation pH was explained by the presence of weak acids (e.g., acetic, formic, levulinic, *p*-hydroxybenzoic, vanillic, and syringic acid), which are growth-inhibiting in their undissociated form (Palmqvist et al., 1998).

4.2. Fermentation of hexoses in acid hydrolyzates

Acid hydrolysis of lignocellulosic materials releases several kinds of fermentation inhibitors, as described above. Chemical detoxification methods such as overliming, ion exchange adsorption, addition of activated carbon, solvent extraction, or steam stripping, can improve the fermentability of an acid hydrolyzate, but these steps are costly and can also result in considerable sugar loss (Olsson and Hahn-Hägerdal, 1996). However, these inhibitors, especially HMF and furfural, can alternatively also be converted *in situ* to non-toxic forms by yeast under appropriate process conditions. Because continuous cultivations keep such inhibitors at lower concentrations than seen initially for batch operations, we would expect continuous processes to be better able to cope with these compounds and avoid an extended lag phase and possibly loss of cell viability. *In situ* detoxification is even more effective at higher cell densities, which can be achieved by different cell retention techniques in a continuous fermentation setup.

The fermentability of two stage dilute acid hydrolyzates was studied by several authors (Table 2). For example, in the first step, 30% w/w wood chips, water, and 5 g L^{-1} H_2SO_4 were heated by steam injection and kept at 12 bar for 7–10 min. After rapid decompression, the liquid phase was separated, and the solids heated again to a pressure of 21 bar and held there for 7 min. Brandberg et al. (2005) did not detoxify the hydrolyzate and employed the yeast strain *S. cerevisiae* ATCC 96581 originally isolated from spent sulphite liquor which proved to be relatively tolerant to the inhibiting environment of dilute acid wood hydrolyzate. Continuous experiments were performed with and without cell recycling by a cross-flow filter unit, and the effects of nutrient addition were investigated. Recirculation of 90% of the cells in the outflow allowed a specific growth rate of 10% of the dilution rate and still avoided washout. Furthermore, the use of cell recirculation limited the losses of carbon in the form of cell biomass, as the cell biomass yield was reduced by 50%. However, in continuous fermentations with 90% cell recycle by cross-flow filtration, unsupplemented hydrolyzate could still not be fermented at dilution rates of 0.1 and 0.06 h^{-1}. However, with supplementation of mineral media ingredients, 99% of the sugars were converted at a cell concentration of 6 g L^{-1} leading to an ethanol concentration of 17 g L^{-1} and a productivity of 1.6 g L^{-1} h^{-1}. Microaerobic conditions resulted in higher biomass growth and gave more stable and robust fermentations. Glycerol production, by which cells reoxidize NADH and maintain the redox balance, was generally low using acid hydrolyzate, apparently due to the fact that *S. cerevisiae* can use reduction of furfural as an alternative redox sink.

In a later work, because wheat hydrolyzate is available in large amounts at a relatively low price, the authors tested it as a cheap alternative to the expensive ingredients of a complete defined mineral media including trace metals and vitamins (Brandberg et al., 2007). When dilute acid spruce hydrolyzate was supplemented with 10% wheat hydrolyzate, only a minimal level of biological activity could be sustained in a continuous cultivation at $D = 0.1$ h^{-1}. However, when 7.5 g L^{-1} $(NH_4)_2SO_4$ and 20 μg L^{-1} biotin were added along with wheat hydrolyzate, steady state fermentation of the dilute acid hydrolyzate was achieved with a hexose conversion of 76%, a cell concentration of 1.9 g L^{-1}, and an ethanol productivity of 1.7 g L^{-1} h^{-1}. To improve the productivity, three different types of cell retention were evaluated: cross-flow filtration with 75% recirculation, sedimentation in a settler equally sized as the working volume of the reactor, and immobilization in calcium alginate. When 75% of the cells were retained by filtration at a fermentation dilution rate of 0.1 h^{-1}, the cell concentration in the reactor tripled compared to the classic chemostat, hexose conversion increased to 94%, and the ethanol production rate was 2.3 g L^{-1} h^{-1}. Comparable results were achieved with cell recirculation by a settler and immobilization. When the dilution rate was increased to 0.2 h^{-1}, neither filtration nor sedimentation could prevent washout of the cells. On the other hand, culture immobilization increased the ethanol productivity to 3.5 g L^{-1} h^{-1}, although hexose conversion dropped. Ethanol was still produced at a rate of 4.5 g L^{-1} h^{-1} for a dilution rate of 0.3 h^{-1}, but hexose consumption decreased even more (Brandberg et al., 2007).

A similar dilute acid hydrolyzate supplemented with defined mineral media was fermented by yeast strain *S. cerevisiae* CBS 8066 immobilized in Ca-alginate beads at dilution rates of 0.3, 0.5, and 0.6 h^{-1}. Glucose consumption was found to drop with increasing dilution rate from 86% to 79% and mannose consumption from 72% to 55%. Ethanol yields based on consumed sugars varied between 0.45 and 0.47 g g^{-1}. In contrast, fermentation of the hydrolyzate with free cells experienced washout at a dilution rate of only 0.2 h^{-1}. However, when the experiments were repeated with a second batch of hydrolyzate, none of the described fermentations were successful without detoxification of the hydrolyzate by overliming (Taherzadeh et al., 2001).

In a follow up work by the same authors with immobilized yeast cells, different reactor configurations were examined, and experiments were performed with a defined glucose based mineral media in detoxified hydrolyzate. In a single stage CSTR operated at dilution rates between 0.22 and 0.86 h^{-1}, conversion of an initial glucose concentration of 20 g L^{-1} in the control mineral media decreased from 100% to 77%, but productivities increased from 2.1 to 6.6 g L^{-1} h^{-1}. If an equal sized fluidized bed bioreactor (FBBR) was connected to the CSTR, glucose conversion was higher than 99% even for the highest dilution rate calculated based on the first reactor only. In contrast, a single FBBR gave a higher glucose conversion of 92% at a dilution rate of 0.86 h^{-1} and a higher ethanol productivity of 7.4 g L^{-1} h^{-1}. When hydrolyzate containing initially 28.4 g L^{-1} sugars was employed as carbon and energy source, conversions dropped from 98% to 54% as dilution rate increased from 0.22 and 0.86 h^{-1} in a single stage CSTR, and ethanol productivities

Table 2
Fermentation of hexoses in acid hydrolyzates.

Medium	Sugar concentration ($g\,L^{-1}$)	Reactor type	Dilution rate (h^{-1})	Ethanol ($g\,L^{-1}$)	Cell dry weight ($g\,L^{-1}$)	Hexose conversion (%)	Ethanol productivity ($g\,L^{-1}\,h^{-1}$]	References
Two stage dilute acid hydrolyzate of spruce	30–40	Single stage CSTR with 90% cell recirculation	0.06–0.1	Washout				Brandberg et al. (2005)
Two stage dilute acid hydrolyzate of spruce, supplemented with full mineral medium salts	30–40		0.1	17	6	99	1.6	
Two stage dilute acid hydrolyzate of spruce, addition of wheat hydrolyzate, $(NH_4)_2SO_4$, biotin	Glucose: 34, mannose: 4	Single stage CSTR	0.1	n.d.	1.9	76	1.7	Brandberg et al. (2007)
		Single stage CSTR with either 75% cell retention by filtration, or cell sedimentation, or cell immobilisation in alginate beads			5.7	94	2.3	
		Single stage CSTR with cell immobilization in alginate beads	0.2		n.d.	n.d.	3.45	
Two stage dilute acid hydrolyzate of wood, addition of full mineral medium ingredients	Glucose: 10.2, mannose:19.8	Single stage CSTR with cell immobilization in alginate beads	0.3	n.d.	n.d.	86 (glucose), 72 (mannose)	n.d.	Taherzadeh et al. (2001)
			0.6			79 (glucose), 55 (mannose)		
Mineral medium	Glucose: 20	Single stage CSTR with cell immobilization in alginate beads	0.22	n.d.	n.d.	100	2.1	Purwadi and Taherzadeh (2008)
			0.86			77	6.6	
		Fluidized bed bioreactor (FBBR)	0.86			92	7.4	
		Equally sized CSTR and FBBR in series	0.86 (based on first reactor only)			99		
Two stage dilute acid hydrolyzate of wood, detoxidfied by overliming, addition of full mineral medium ingredients	Glucose: 28.4	Single stage CSTR with cell immobilization in alginate beads	0.22			98	2.7	
			0.86			54	6	
		CSTR and FBBR in series	0.86			87	5.5	
Second stage dilute acid hydrolyzate of wood, addition of full mineral medium ingredients	Glucose: 19.3; mannose 6.6	Single stage CSTR with free cells	0.1	n.d.	n.d.	90 (hexoses)	0.86	Talebnia and Taherzadeh (2006)
		Single stage CSTR with encapsulated cells	0.1			95 (glucose), 98 (mannose)	1.14	
			0.5			71 (glucose), 79 mannose	4.2	
Two stage dilute acid hydrolyzate	Glucose: 28, mannose: 6, galactose: 2	Single stage CSTR with flocculating yeast	0.52	n.d.	23–30	96	n.d.	Purwadi et al. (2007)
Concentrated sulphuric acid hydrolyzed coniferous wood, supplemented with corn steep liquor, KH_2PO_4, $MgSO_4$, $CaCl_2$	Glucose: 106, mannose 11	Single stage tower type reactor with flocculating yeast	0.3	ca. 60	n.d.	82	20	Tang et al. (2006)
		Two tower type reactors in series	0.2	56 (1), 63 (2)		100	12.6	

were in the range of 2.7–6 $g\,L^{-1}\,h^{-1}$. The addition of a FBBR increased sugar conversion to 86.6% at the highest dilution rate of 0.86 h^{-1}. However, productivities were slightly lower in the two stage system, with values between 1.4 and 5.5 $g\,L^{-1}\,h^{-1}$. This reactor segregation gained about 11.6% in hexose assimilation if hydrolyzate was fermented but only 1.2% in synthetic media (Purwadi and Taherzadeh, 2008).

Alternatively, yeast cells can also be encapsulated rather than traditionally entrapped in an alginate matrix, which has the advantages that resistance to diffusion of nutrients as well as cell leakage is lower and higher cell concentrations are possible. Talebnia and Taherzadeh (2006) encapsulated *S. cerevisiae* CBS 8066 cells and second stage hydrolyzate, obtained as previously described, was used for the experiments and supplemented with mineral media ingredients. In continuous hydrolyzate fermentations at dilution rates of 0.1, 0.2, 0.3, 0.4, and 0.5 h^{-1}, and glucose conversion dropped from 95% to 71% and mannose conversion from 98% to 79% over this range. Ethanol productivity increased with increasing dilution rate from 1.1 to 4.2 $g\,L^{-1}\,h^{-1}$, and cell viability was high under all conditions with values between 77% and 90%. In contrast, if the hydrolyzate was continuously fermented by free yeast cells at a dilution rate of 0.1 h^{-1}, 90% of the hexoses were converted, the ethanol yield was low at 0.34 $g\,g^{-1}$, and cell viability was only 25%.

To date, almost no industrial processes employ encapsulated or immobilized cells due to worries about long term stability and the additional costs for immobilization (Sanchez and Cardona, 2008; Verbelen et al., 2006). In addition, there are significant challenges in applying immobilized cells at the scales typical of ethanol production processes, and they will become even more challenging to apply when using hydrolyzates containing lignin and other solids. However, the natural form of immobilization – cell flocculation – has greater potential to be employed on an industrial scale and is already used for cane sugar fermentations. Thus, Purwadi et al. (2007) investigated the fermentability of the dilute acid hydrolyzate described above with a flocculating yeast strain of *S. cerevisiae*

isolated from an ethanol plant. One stage continuous cultivations of unsupplemented hydrolyzate with the flocculating yeast were carried out successfully at dilution rates of 0.13, 0.24, 0.38, and 0.52 h^{-1} with cell concentrations in the reactor between 23 and 30 g L^{-1} achieved by separation from the effluent by a settler. Even at the highest dilution rate, 96% of the sugars were assimilated, and ethanol yields based on consumed sugars decreased with increasing dilution rate from 0.46 to 0.42 g g^{-1}. HMF and furfural were almost completely removed. Cell growth rate was controlled by limiting the nitrogen source in the medium, so that ethanol was produced over 37 retention times at a constant cell density. If nitrogen was supplemented, cell density quickly reached concentrations of more than 50 g L^{-1}. Continuous cultivation was also carried out using two bioreactors connected in series, where cells were recycled from the last reactor to the first with dilution rates of 0.24, 0.38 and 0.52 h^{-1} based on the total volume of the reaction system. The results were similar to those for single stage experiments with respect to sugar assimilation, ethanol yields, and cell concentrations.

A thermotolerant flocculating yeast strain was also investigated for the fermentation of a sugar stream produced by hydrolyzing coniferous wood with concentrated sulphuric acid to obtain a solution containing 106 g L^{-1}glucose, 11 g L^{-1} mannose, 3 g L^{-1}galactose, and 12 g L^{-1} xylose. The pH of the acid hydrolyzate was adjusted to 3.0 by adding $Ca(OH)_2$ and holding overnight before adding further media components. Media optimization experiments showed that yeast extract and peptone could be replaced by 1% corn steep liquor supplemented with 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.01% $CaCl_2 \cdot 2H_2O$. Acid hydrolyzate fermented at temperatures below 35 °C with a dilution rate of 0.3 h^{-1} resulted in an ethanol productivity of 20 g L^{-1} h^{-1} and a yield based on total sugars in the feed of 82% and compounds in the hydrolyzate did not significantly inhibit the fermentations. In a scale up experiment, two 4.5 L tower reactors were connected in series to ferment acid hydrolyzate. Growth of contaminating bacteria was repressed at pH 4 but not 4.5 for operation at 35 °C with a dilution rate of 0.2 h^{-1} without adding $K_2S_2O_5$. However, at this condition, washout occurred at a dilution rate of 0.3 h^{-1}. At a dilution rate of 0.2 h^{-1}, ethanol concentrations of 56 and 63 g L^{-1} were measured in reactors 1 and 2, respectively, corresponding to an overall productivity of 12.6 g L^{-1} h^{-1}. All hexose sugars were converted completely in reactor 2. The system was run for 30 days (Tang et al., 2006).

4.3. Co-fermentation of pentoses and hexoses in solution

To be economical, all sugars, both pentoses and hexoses, must be converted to ethanol or other useful products. However, *S. cerevisiae* does not ferment pentoses, and other strains have to be employed for fermentation of mixtures containing arabinose and xylose (see Table 3). Generally, natural pentose fermenting organisms are less inhibited by hemicellulose hydrolyzate than recombinant gram negative xylose fermenting strains such as *E. coli*, *K. oxytoca* or *Zymomonas mobilis* (Georgieva and Ahring, 2007). For example, although *Pichia stipitis* yeast can naturally ferment pentose sugars to ethanol, hexose sugars are used preferentially, and pentose uptake is competitively inhibited by hexoses. Thus, pentose fermentation is only possible at very low glucose concentrations. In addition, microaerophilic conditions are required, which are difficult to maintain in large scale systems, and even then yields are low. Because a series of reactors has the advantage over a single CSTR in that optimal conditions for each vessel can be adjusted separately, Grootjen et al. (1991) applied a three reactor system with *P. stipitis* in which they adjusted the residence time such that glucose was depleted in the last reactor and optimal pentose conversion took place. A synthetic medium containing 10 g L^{-1} xylose and 40 g L^{-1} glucose was employed to demonstrate this approach, and at a dilution rate of 0.027 h^{-1} with controlled aeration of the first two reactors, almost complete mixed sugar conversion was possible with the ethanol productivity being 0.51 g L^{-1} h^{-1}. However, if the dilution rate was doubled, only 20% of the xylose could be converted (Table 3).

Genetically engineered *Z. mobilis* strains were also investigated for their co-fermentation potential. Lawford et al. (1998) used a single stage CSTR to first characterize the continuous co-fermentation abilities of the strain with a synthetic medium containing 8 g L^{-1} glucose and 40 g L^{-1} xylose. With dilution rates between 0.04 and 0.1 h^{-1}, approximately constant ethanol yields of 20–22 g L^{-1} were achieved, and xylose conversion remained high with values between 83% and 93%. Furthermore, long term adaptation experiments were performed employing a detoxified dilute acid pretreatment hydrolyzate of poplar sawdust, starting with an initial hydrolyzate concentration of 10%. The co-fermentation abilities could be preserved, while the concentration of hydrolyzate was raised over a time period of 50 days to 35%, a level which was stated to be substantially inhibiting in batch fermentation thereby showing that long term continuous fermentation is a valuable tool to adapt the microorganism to toxic conditions. Krishnan et al. (2000) employed an immobilized recombinant *Z. mobilis* strain to co-ferment xylose and glucose. In batch experiments it was shown that fermentation results were comparable for concentrated acid rice straw hydrolyzate and a synthetic control medium. Continuous experiments in a fluidized bed reactor were performed with the control medium at dilution rates between 0.24 and 0.5 h^{-1} and excellent volumetric ethanol productivities of 6.5–15.3 g L^{-1} h^{-1} were achieved. However, xylose conversion varied from 10% to 90%.

Georgieva and Ahring (2007) investigated the ability of the thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 that was genetically modified to be lactate dehydrogenase deficient to ferment xylose in undetoxified hydrolyzate. The National Renewable Energy Laboratory (NREL) pretreated corn stover in dilute sulphuric acid at a solids concentration of 30% to produce hydrolyzate (the liquid phase) containing 16 g L^{-1} glucose and 68 g L^{-1} xylose. For the fermentations, the hydrolyzate was diluted 1:2 to 1:12, corresponding to total solids contents (TS) of 2.5–15%, and supplemented with yeast extract, minerals, trace metals, vitamins, and Na_2S. The strain was immobilized on a granulated carrier material and employed in a continuous fluidized bed reactor system operated at 70 °C and pH 7.0 with a residence time of 2 days for all but the highest TS concentration, for which the residence time was increased to 3 days. After start-up of the reactor with synthetic media, the hydrolyzate concentration was increased stepwise after reaching steady state to allow adaptation of the microorganism to inhibitors in the feed. Independent of the substrate concentration, ethanol yields based on sugars consumed were in the range of 0.39–0.42 g g^{-1} (76–82% of the theoretical possible), and the maximum ethanol concentration was 10.4 g L^{-1}. Xylose consumption ranged from 89% to 98% at 2.5–10% TS but dropped to 67% for 15% TS. Overall sugar conversion to ethanol was between 70% and 77% at 2.5–10% TS and 52% at 15% TS. The reactor was operated for 135 days without any bacterial contamination.

An identical fermentation system was used to convert wet exploded wheat straw hydrolyzate to ethanol. A 200 g L^{-1} wheat straw suspension was heated to 170 °C, and hydrogen peroxide was added to reach a final oxygen concentration of 3% per g of dry matter content. Pretreatment was stopped as soon as all hydrogen peroxide had reacted with the biomass, and the resulting suspension was diluted with water to a concentration equivalent to dry matter concentrations of 3–12%. Then, the pH was adjusted to 5.0 with sodium hydroxide, and the mixtures were autoclaved, which increased inhibitor concentrations further. Following enzy-

Table 3
Continuous co-fermentation of pentoses and hexoses in solution.

Medium	Sugar concentration ($g\ L^{-1}$)	Reactor type	Dilution rate (h^{-1})	Strain	Ethanol ($g\ L^{-1}$)	Sugar conversion (%)	Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	References
Growth medium with yeast extract and mineral salts	Glucose: 40, xylose: 10	Three CSTRs in series	0.027	*Pichia stipitis*	18.8	Almost complete	0.51	Grootjen et al. (1991)
Hydrolyzate of dilute sulphuric acid pretreatment of corn stover at a solids concentration of 30%, diluted 1:2 to 1:12, supplemented with yeast extract, minerals, vitamins and Na_2S	Glucose: 4.4, xylose: 21.1	Fluidized bed reactor with immobilized cells	0.02	*Thermoanaerobacter* BG1L1	9.1	Glucose: 91, xylose 89	0.18	Georgieva and Ahring (2007)
Enzymatic hydrolyzate of whole wet exploded wheat straw slurry, supplemented with yeast extract, minerals, vitamins and Na_2S	Glucose: 22.2, xylose: 11.2	Fluidized bed reactor with immobilized cells	0.02	*Thermoanaerobacter* BG1L1	11.6	Glucose: 93, xylose 76	0.23	Georgieva et al. (2008)
Dilute sulphuric acid pretreated spruce hydrolyzate, supplemented with yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$, $MgSO_4$, vitamins, and trace metal solution	Glucose: 13.5, mannose: 20.5, xylose: 7.9	Single stage CSTR CSTR with cell retention	0.1 0.2	*Mucor indicus*	Washout 17	 Hexoses:87–99, xylose 26	 3.3	Karimi et al. (2008)
Synthetic medium with yeast extract, KCl and K_2HPO_4	Glucose: 49.9, xylose: 12.9	Fluidized bed reactor with immobilized cells	0.24	*Zymomonas mobilis* CP4(pZB5)	26.9	Glucose: 99.8, xylose: 91.5	6.5	Krishnan et al. (2000)
	Glucose: 68.8, xylose: 23.1		0.25		34.5	Glucose: 99, xylose: 36.4	8.6	
35% hydrolyzate of dilute sulphuric acid pretreatment, detoxified by overliming, supplemented with corn steep liquor	Glucose: 8, xylose: 40	Single stage CSTR	0.04	*Zymomonas mobilis* 39676:pZB4L	ca. 20	Xylose: 92.5	0.8	Lawford et al. (1998)

matic hydrolysis of the slurry, the remaining solids were removed, and the final fermentation media was made up as described above. No detoxification or washing steps were included. The glucose concentration in the wet exploded wheat straw hydrolyzate (WEH) varied between 9 and 27 $g\ L^{-1}$ and the xylose concentration between 3 and 14 $g\ L^{-1}$. With increasing WEH concentration, the ethanol concentration in the effluent increased from 4.6 to 14.4 $g\ L^{-1}$, corresponding to ethanol yields based on consumed sugars in the range of 0.39–0.42 $g\ g^{-1}$ (76–83% of theoretical). Provided the residence time was increased from 2 to 3 days for the highest WEH concentration, glucose utilization was higher than 90% for all tested conditions, whereas xylose conversion was lower at 72–80%. The total experiment lasted 143 days, and no contamination of the reactor occurred (Georgieva et al., 2008).

The dimorphic filamentous fungus *Mucor indicus* (formerly *M. rouxii*) is a promising alternative to *S. cerevisiae* as it is capable of xylose fermentation, is safe for humans, and produces ethanol from hexoses with comparable yields and productivities. In this case, forestry residues mainly from spruce were treated at a solids concentration of 33% with 0.5 $g\ L^{-1}$ sulphuric acid for 10 min at 15 bar to produce a liquid containing 44.5 $g\ L^{-1}$ sugars (20.5 $g\ L^{-1}$ mannose, 13.5 $g\ L^{-1}$ glucose, 7.9 $g\ L^{-1}$ xylose and 3.2 $g\ L^{-1}$ galactose). This solution was then supplemented with yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$, $MgSO_4$, antifoam, vitamin solution, and trace metal solution for continuous cultivations in a standard stirred tank reactor with synthetic glucose media as well as hydrolyzate at 32 °C and pH 5.5. With the pure glucose medium, conversion was more than 98%, an ethanol yield of 0.41 $g\ g^{-1}$ was reached at dilution rates of 0.1 and 0.2 h^{-1}, and washout occurred at a dilution rate of 0.3 h^{-1}. When hydrolyzate was employed, the fermentations failed, and cells were washed out at a dilution rate as low as 0.1 h^{-1}. Thus, a new bioreactor was constructed containing a stainless steel net with 0.5 × 0.5 mm square holes placed at the top where fluid left the reactor. During continuous cultivation, filamentous biomass quickly covered the screen, allowing permeation of liquid while partly retaining cells to prevent washout. With this configuration, continuous fermentations of hydrolyzate were possible up to a dilution rate of 0.3 h^{-1}. The best results were achieved at a dilution rate of 0.2 h^{-1}, with 87–99% hexose conversion, only 26% xylose conversion, and an ethanol concentration of 17 $g\ L^{-1}$, corresponding to a yield of 0.45 g/g and a productivity of 3.3 $g\ L^{-1}\ h^{-1}$ (Karimi et al., 2008).

4.4. Continuous simultaneous saccharification and fermentation

4.4.1. Experimental data

SSF is one of the most promising process configurations to convert pretreated lignocellulosic biomass to ethanol as it reduces end-product inhibition of cellulase by sugars. However, only limited information has been published about continuous SSF, presumably due to the inherent experimental challenges of homogenous delivery of solid substrate, the extended run times needed, and the complexities of continuous experimental systems. On the other hand, batch systems are hampered by mixing problems at high solid substrate loadings, which can be avoided by operation of a CSTR with high conversion of insolubles to ethanol. Furthermore, in a batch reactor, the high amounts of β-glucosidase added are only required at the beginning of the reaction when cellobiose production is highest, while we hypothesize that β-glucosidase loading can be reduced in a continuous system because cellobiose production slows with conversion. For consolidated bioprocessing by strains such as *Clostridium thermocellum*, the higher cell densities possible in a CSTR allows higher cellulase production levels.

In a pioneering study, South et al. (1993) presented experimental results for continuous conversion of pretreated hardwood flour to ethanol for two different systems: SSF that utilized the D5A strain of *S. cerevisiae* in combination with cellulase enzymes and direct microbial conversion (DMC) with the cellulose fermenting strain *C. thermocellum*. Hardwood flour was pretreated for 10 s at 220 °C with 1% H_2SO_4, with typical particle sizes of about 0.05 mm. A single stage CSTR was set up with a working volume of 1.25 L, and solid substrate was fed to the reactor from a 20 L feed reservoir by a progressing cavity pump that intermittently added biomass to achieve the target flow rate. The SSF system was run with cellulose loadings from 5 to 60 $g\ L^{-1}$ at cellulase loadings

ranging from 7 to 25 FPU g^{-1} cellulose but seemed relatively insensitive to variations in enzyme loadings and substrate feed concentrations. With a cellulose feed of 61 g L^{-1}, an enzyme loading of 12 FPU g^{-1}, and a dilution rate of 0.02 h^{-1}, an ethanol concentration of 21 g L^{-1} was reached, corresponding to a conversion of 60% and a productivity of 0.41 g L^{-1} h^{-1} (Table 4). Generally, conversion in a CSTR was found to be 8–13% lower than in batch reactors at residence or reaction times between 1 and 3 days. At comparable substrate concentrations (4–5 g L^{-1}) and residence times (12– 14 h), substrate conversion in the *C. thermocellum* system was 77%, significantly higher than the 31% achieved in the SSF system.

In a later paper, a semi-continuous laboratory scale fermentation system was applied to convert paper sludge to ethanol in an SSF process with the goal of reaching a final ethanol concentration of more than 4 wt.% to realize cost- and energy-effective distillation. Mixing of unreacted sludge proved to be almost impossible at the solids concentrations necessary to reach the targeted ethanol concentration in a batch system, making it necessary to employ a fed-batch or continuous process to reduce the viscosity and assure reasonable mixing. A solid feeding device was developed, for which a motor driven plunger advanced a plug of paper sludge into the reactor at 12 h feeding intervals. The system was operated with a residence time of 4 days (corresponding to a dilution rate of 0.01 h^{-1}) with a cellulase loading of 15–20 FPU g^{-1} cellulose, and *S. cervisiae* D5A was used to ferment the sugars to ethanol. After difficulties with the first generation system, a retrofitted design enabled stable operation for more than one month. In one run, cellulose fed at a concentration of 82 g L^{-1} (corresponding to a solid concentration of about 12%) achieved an average conversion of 92% and 42 g L^{-1} ethanol with an enzyme loading of 20 FPU g^{-1}. Based on the reported data, the productivity was calculated to be 0.42 g L^{-1} h^{-1}. However, at a higher cellulose loading of 120 g L^{-1} and lower cellulase concentration of 15 FPU g^{-1}, only 74% conversion was reached, corresponding to an ethanol concentration of 50 g L^{-1} (Fan et al., 2003).

The National Renewal Energy Laboratory (NREL) conceptualized two base case scenarios for large scale cellulosic ethanol production based on continuous SSF operation in reactor trains consisting of 5–6 vessels (Aden et al., 2002; Wooley et al., 1999). Furthermore, a bioethanol pilot plant, equipped with capabilities for feedstock handling, continuous dilute acid pretreatment, yeast inoculum production, and SSF with commercially available cellulase, was put into operation in 1995 (Schell et al., 2004). The plant was designed to process 900 kg dry biomass per day, and initial demonstration runs were performed using corn fiber, a corn wet-milling by-product, as the substrate. After pretreatment, the material was neutralized and sent directly to the first 9000 L fermentor of a cascade of three equally sized vessels, with the mean residence time in each fermentor being 36 h to give an overall dilution rate of 0.009 h^{-1}. Although substrate concentration, enzyme loading, and general performance were not specified, an ethanol concentration of approximately 40 g L^{-1} was measured early in the runs before bacterial contamination with *Lactobacilli* was reported to be a problem. After a run time of 400 h, lactic acid concentration increased to nearly 25 g L^{-1}, but liquid culturing techniques detected contamination after only 168 h. Concomitantly to the increase in lactic acid concentration, the concentration of arabinose, a sugar which cannot be fermented by the yeast employed, decreased from 25 g L^{-1} to 2 g L^{-1}, mirroring the lactic acid curve. Addition of 10 ppm penicillin every 12 h combated this problem temporarily but was overcome after 100 h. However, three doses of 10 ppm virginiamycin over 36 h decreased lactic acid concentrations to pre-contamination levels. Adding ethanol to the fermentor at the end of the run to increase its concentration to 50 g L^{-1} failed as a strategy to reverse contamination. The authors concluded that microorganisms that consume arabinose or any other biomass-derived sugar not utilized by the primary fermentative microorganism pose a significant challenge to the desired yeast mono culture. Furthermore, high levels of contaminant compete with the yeast for glucose, especially as the by-products such as lactic and acetic acid are inhibitory to the fermenting strain. Thus, they concluded that a microorganism that can ferment all sugars in the feed stream is vital to avoiding invasion by unwanted microbes. Also, separate saccharification and fermentation might be advantageous because of the ability to operate saccharification under conditions not favourable to contaminant growth and the much shorter fermentation residence times with sugars than possible with cellulose hydrolysis. Alternatively, a microorganism with outstanding tolerance to the fermentation inhibitors produced during pretreatment could be employed (Schell et al., 2007).

4.4.2. Kinetic modelling

Kinetic modelling is a useful tool to gain a deeper insight into a reaction system if performance of a process can be predicted under various conditions, but although many models have been developed to describe enzymatic hydrolysis of cellulose or SSF in batch systems, only few have been applied to simulate continuous processes. South et al. (1995) sought to develop a kinetic model of continuous SSF to predict cellulose conversion over a wide range of conditions, such as cellulose loading, enzyme loading, and reaction time but with minimal complexity. In this case, cellulose was assumed to be hydrolyzed to cellobiose which further broke down to glucose by the action of β-glucosidase, with no direct conversion of cellulose into glucose considered. Cellulase adsorption onto cellulose and lignin was described according

Table 4
Continuous SSF.

Substrate	Cellulose concentration (g L^{-1})	Reactor type	Dilution rate (h^{-1})	Enzyme loading (FPU g^{-1} cellulose	Ethanol (g L^{-1})	Cellulose conversion (%)	Ethanol productivity (g L^{-1} h^{-1})	References
Solids of dilute sulphuric acid pretreated hardwood flour	61	Single stage CSTR	0.02	12	21	60	0.42	South et al. (1993)
Paper sludge	82	CSTR with 12 h feed intervals	0.01	20	42	92	0.42	Fan et al. (2003)
	120			15	50	74	0.5	
Whole slurry of dilute acid pretreated corn fiber	n.s.	3 CSTRs of 9000 L each in series	0.009	n.s.	40 (last reactor)	n.s.	0.36	Schell et al. (2007)
Wastepaper	50	2 equally sized CSTRs in series	0.01875	10	8 and 12	43	0.225	Philippidis and Hatzis (1997)

to the Langmuir model, and the cellulose hydrolysis rate was taken to be proportional to the concentration of the enzyme–substrate complex divided by the specific capacity of the substrate for cellulase. Inhibition by cellobiose and ethanol was also integrated into the model, and the conversion of cellobiose was modelled to follow Michaelis–Menten kinetics with inhibition by the glucose released. Cell growth followed Monod kinetics and was taken to be inhibited by ethanol. Furthermore, the decreasing reactivity of cellulose with conversion was described by a cellulose hydrolysis rate equation of the form $k(x) = k^{*}(1-x)^{n} + c$ in which k is the hydrolysis rate constant, x is the fractional cellulose conversion, n is an exponent of the declining substrate reactivity, and c is the conversion independent component in the rate function. To describe continuous SSF, a particle population model was used to account for the effect of the distribution in particle exit age on the change in particle reactivity with conversion. Comparison of the population model to a soluble substrate model assuming a uniform mean residence time with experimental data showed that the chosen approach was necessary to describe the heterogeneous reaction accurately. Overall the model predicted the experimentally determined conversions in a CSTR quite well with a root mean squared difference of 5.2%.

The model was then extended to describe potentially more optimal reactor configurations than a single stage CSTR, such as a cascade of fermentors or a system that partially retained solids to increase their residence time relative to liquid residence time. The resulting predictions revealed that conversion in a single stage CSTR was lower than in a batch reactor but that a cascade of 5 CSTRs could attain an equal conversion of 95% after 5 days. Retaining the solids for a mean residence time 1.5 times longer than the hydraulic residence time reduced the time to reach 90% conversion from 2.5 days in a batch reactor to 1.2 days for the five stage cascade, a 47% reduction in overall reactor volume compared to a batch system. Modelling the influence of ethanol concentration showed that inhibition of cellulase was more important than inhibition of the fermenting organism for the SSF system, because the growth rate is far in excess of the hydrolysis rate (South and Lynd, 1994).

Philippidis and Hatzis (1997) developed a kinetic model for simultaneous saccharification and fermentation based on experimental data of a two stage continuous SSF of wastepaper containing 50% cellulose and 20% lignin. The solid loading in their experiments was 100 g L^{-1}, and the dilution rate was set to 0.01875 h^{-1}, corresponding to a total mean residence time of 53 h in the two reactor system with 8 L working volumes for each. Applying an enzyme loading of 10 FPU g^{-1} cellulose supplemented with 20 CBU of β-glucosidase resulted in steady state ethanol concentrations of approximately 8 g L^{-1} in the first stage and 12 g L^{-1} in the second stage, giving an overall yield of 43% and a productivity of 0.225 g L^{-1} h^{-1}. The model assumed that cellulose was simultaneously converted either directly to glucose or first to cellobiose which was then converted by β-glucosidase to glucose. Glucose was then catabolized to ethanol, cells, and CO_2. The rate equations for cellulose decomposition included enzyme inhibition by glucose, cellobiose, and ethanol as well as an exponential decay term of the form $e^{-\lambda \cdot time}$ to account for the time-dependent decline in the hydrolysis rate due to the loss of effective surface area of cellulose. The specific rate constants employed a Michaelis–Menten dependence on the cellulase concentration, cellobiose hydrolysis was assumed to be inhibited by only glucose, and loss of enzyme activity due to adsorption on lignin was accounted for in the specific rate constant. The model fit the continuous data well for both steady state operation and even for the transition from batch startup, providing more confidence in the model and its usefulness for their theoretical parameter study. They then applied the model to plot ethanol yield as a function of dilution rate to obtain a bell shaped curve with a distinct maximum. Thus, a given ethanol yield could be reached at two dilution rates, with the higher one more attractive in that a much higher productivity was realized. Adding ethanol to avoid bacterial contamination was then shown to decrease the ethanol yield from 46% if no ethanol was recycled to 31% if 80 g L^{-1} of ethanol was fed to the reaction. Then, the effect of doubling the specific cellulose hydrolysis rate constants that could result from better pretreatment methods was predicted to increase ethanol yields from 46% to 59% (a 27% increase), while 5 and 10-fold improvements enhanced yields to 70% and 73%. Thus, saturation was projected to be reached quickly, with the result that other kinetic parameters must be improved to obtain higher ethanol yields. One of the critical parameters was the specific rate loss factor λ in cellulose reactivity, with a reduction from 0.02 in the base case to 0.005 boosting ethanol yield to 65% at the base case dilution rate. Microbial parameters of maximal growth rate and ethanol resistance had a small effect on performance, supporting the notion that enzymatic hydrolysis is the rate limiting step for SSF.

5. Summary and conclusions

Continuous fermentations generally show higher productivities than batch processes and exhibit reduced vessel down time for cleaning and filling, thereby enabling a smaller plant size at an equal annual capacity. For conversion of lignocellulosic biomass to ethanol, continuous fermentations provide several additional advantages. First, costly conditioning of inhibitory compounds released into acid hydrolyzates can be omitted or at least reduced due to the *in situ* detoxification abilities of yeast, especially at high cell densities achievable in cell retention systems such as immobilization and self-flocculation. By properly adjusting the residence time, the viscosity of the solids can be reduced substantially, allowing continuous enzymatic hydrolysis systems to handle higher effective solids concentrations than could be mixed initially in a batch operation. Furthermore, we hypothesize that β-glucosidase loading can be reduced in a continuous system as the burst of cellobiose production is avoided at steady state. However, experience with continuous enzymatic hydrolysis of cellulosic biomass is extremely limited, and several important research topics require attention: (1) continuous SSF of unwashed substrates, (2) continuous fermentation of mixed pentose and hexose sugars, (3) optimal design of cascade systems for continuous SSF, (4) techniques to avoid and overcome bacterial contamination, (5) the effect of operating parameters on reactor stability and washout of organisms, and (6) tradeoffs in yields for continuous vs. batch operations at lower, more economically attractive enzyme loadings. To ensure applicability to commercial systems, conditions at the laboratory scale should be chosen that are compatible with an industrial cost effective process, and it should be kept in mind that such factors as media additives, fermentation pH, sterilization temperature, and cell density strongly influence performance.

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