J. Microbiol. Biotechnol. (2008), **18**(7), 1257–1265

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

High-Solid Enzymatic Hydrolysis and Fermentation of Solka Floc into Ethanol

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Received: December 3, 2007 / Accepted: February 7, 2008

To lower the cost of ethanol distillation of fermentation broths, a high initial glucose concentration is desired. However, an increase in the substrate concentration typically reduces the ethanol yield because of insufficient mass and heat transfer. In addition, different operating temperatures are required to optimize the enzymatic hydrolysis (50°C) and fermentation (30°C). Thus, to overcome these incompatible temperatures, saccharification followed by fermentation (SFF) was employed with relatively high solid concentrations (10% to 20%) using a portion loading method. In this study, glucose and ethanol were produced from Solka Floc, which was first digested by enzymes at 50°C for 48 h, followed by fermentation. In this process, commercial enzymes were used in combination with a recombinant strain of *Zymomonas mobilis* (39679:pZB4L). The effects of the substrate concentration (10% to 20%, w/v) and reactor configuration were also investigated. In the first step, the enzyme reaction was achieved using 20 FPU/g cellulose at 50°C for 96 h. The fermentation was then performed at 30°C for 96 h. The enzymatic digestibility was 50.7%, 38.4%, and 29.4% after 96 h with a baffled Rushton impeller and initial solid concentration of 10%, 15%, and 20% (w/v), respectively, which was significantly higher than that obtained with a baffled marine impeller. The highest ethanol yield of 83.6%, 73.4%, and 21.8%, based on the theoretical amount of glucose, was obtained with a substrate concentration of 10%, 15%, and 20%, respectively, which also corresponded to 80.5%, 68.6%, and 19.1%, based on the theoretical amount of the cell biomass and soluble glucose present after 48 h of SFF.

Keywords: High-solid fermentation, enzymatic hydrolysis, saccharification followed by fermentation (SFF), Solka Floc

The demand for petroleum products continues to rise. In 2004, global oil consumption jumped 3.5%, or 2.8 million barrels per day [18], plus the U.S. Energy Information Administration has projected demand rising from the current 84 million barrels per day to 103 million barrels by 2015 [2]. Meanwhile, if China and India, where cars and factories are proliferating, consume oil at just one-half of current U.S. per-capita levels, global demand would jump 96%, according to Dr. Amos Nur (Stanford University).

Thus, with the increase of oil consumption, the production of bioethanol is looking ever more promising. The source of all biological fuels ultimately involves the photosynthetic fixation of CO_2 into biomass, which is performed by plants and algae to produce primarily lignocellulosics and starches. Moreover, biomass-based fuel technologies provide increasingly favorable alternatives, since these processes do not contribute to any net increase in atmospheric CO_2.

One of the most immediate and important applications of biological energy systems could be in the production of ethanol from biomass. In excess of 1.4 billion gallons per year of ethanol fuel is currently produced in the U.S. by fermentations, using corn starch as the substrate [3, 6]. However, this is a conventional technology that is only economically competitive as a result of large government subsidies to encourage corn production and its conversion into ethanol fuel. Although some process improvements are possible, no major breakthroughs are foreseen, and neither is the cost of corn starch likely to become more attractive, in light of the increasing food needs of the world's population. Energy and greenhouse gas emissions balances are also not particularly favorable for corn-to-ethanol systems, being at best only slightly positive, and then only if more expensive capital-intensive technology is used. Thus, lower-cost fermentation substrates and processes are required.

The polysaccharide fraction of lignocellulosic residues can be hydrolyzed using acids or enzymes as catalysts [16]. Cellulases catalyze the hydrolysis of cellulose, which is the major structural component of biomass, the most abundant organic material on earth [16]. The complete hydrolysis of cellulose yields the easily fermentable sugar, glucose, allowing biomass to be a potential renewable energy source [17]. As a result, there is strong interest in

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understanding the process of enzymatic cellulose degradation at a high initial solid concentration.

Typically, as much as 90% or more of the broth is water that must be removed during saccharification followed by fermentation (SFF). This separation is costly and also produces a large aqueous stream that must then be disposed of or recycled. Thus, a high initial cellulose concentration combined with a favorable conversion yield of cellulose into soluble sugars reduces the cost of water removal. When concentrated slurries are processed, the medium mixing/enzyme homogenization becomes difficult and often results in low bioconversion yields [9], which partly accounts for the lack of literature concerning fermentation of biomass suspensions at concentrations greater than 10% [13, 15]. For high-solid saccharification and fermentation, the reaction rate and bioreactor configuration are of critical importance to the economic feasibility of a larger scale industrial process, since this unit operation requires the longest residence time relative to the other major biomass conversion reactions of enzyme hydrolysis and fermentation. These longer residence times during saccharification translate into higher operating and capital costs per unit of product output.

However, this approach appears to be the simplest and most economically viable way to attain suitable ethanol concentrations in broths for distillation. On the whole, several process parameters must be optimized: substrate concentration, enzyme-to-substrate ratio, dosage of the active components (β-glucosidase-to-glucanase ratio) in the enzymatic mixture, bacteria concentration, and reactor conditions. Lastly, one of the most important factors affecting the overall economics is a compatible temperature between the enzymatic hydrolysis and the fermentation process for high slurries during saccharification followed by fermentation (SFF).

Accordingly, the present study investigated the optimal conditions for the enzymatic hydrolysis and fermentation of concentrated Solka Floc to produce the highest glucose and ethanol yields when using the SFF process in a 3-l bioreactor. The influence of the dry matter concentration and bioreactor configuration on the yield of glucose and ethanol was also investigated.

Materials and Methods

Materials

Solka Floc (Fiber Sale & Development Corporation, Urbana, OH, U.S.A.), a delignified spruce pulp, was the biomass used as the raw material in this study. The composition of the material was analyzed according to NREL Standard Procedures 001, 002, and 005. The glucan content was 88%.

Enzymes and Microorganism

Commercially produced Spezyme CP and Novozyme 188 were used for the enzymatic hydrolysis. The cellulose enzyme Spezyme CP, secreted by *Trichoderma longibrachiatum*, formerly *Trichoderma reesei*, was from Genencor International, Inc. The enzyme had an activity of 82 GCU/g, as provided by the manufacturer, and 55 FPU/ml, as determined by NREL Standard Procedure 006 [7, 20]. The Novozyme 188 purchased from Sigma was used for cellulose hydrolysis with a volume ratio of 4 FPU Celluclast/CBU Novozyme to alleviate any end-product inhibition by cellobiose.

The recombinant *Zymomonas mobilis* strain ATCC 39679, carrying the plasmid pZB4L (designated as *Zm* 39679:pZB4L), was provided from M. Zhang (NREL, Golden, CO, U.S.A.). Stock cultures were stored in glycerol at −70°C.

Preculture and Inoculation Procedures

A 2.0-ml aliquot of a glycerol-preserved culture was removed from cold storage (freezer) and transferred to 200 ml of a complex medium (RM), containing about 2% (w/v) glucose and 2% (w/v) xylose supplemented with tetracycline (20 mg/l) in 500-ml Erlenmeyer flasks, and grown overnight at 30°C.

The batch fermentations were inoculated by transferring around 10% (v/v) of the overnight flask culture directly into the medium in a stirred-tank bioreactor (BioFlo 3000; New Brunswick Scientific Co. Inc., Edison, NJ, U.S.A.). For the fermentations, the initial cell density was monitored spectrophotometrically to give an OD_{600} in the range of 0.4 to 0.5, corresponding to growth in the mid-exponential phase.

Saccharification Followed by Fermentation

To maximize the glucose and ethanol concentrations, substrate concentrations were employed from 10% to 20% (200–400 g) on a dry basis, corresponding to cellulose concentrations of 8% to 17% (176–352 g). In previous studies of traditional batch enzyme reactions and fermentation of a high substrate concentration (>10%), no visible liquid phase was observed, owing to complete absorption of the liquid by the biomass. Moreover, no sugar or ethanol products were reported for tests between 10% and 20%. Thus, to overcome this problem, Solka Floc was added to the reactions in three portions, during both the enzyme reaction and fermentation up to a final substrate concentration of 20%. The portions were added to the reaction during the initial 4 h of the reaction. A 10% inoculum, prepared based on the volume of the total working volume (2 l), was then transferred into the reactor after enzymatic hydrolysis for 48 h. The enzyme loading was 20 FPU per gram of cellulose, supplemented by β-glucosidase to prevent any product inhibition by cellobiose. The SFF experiments were operated for 96 h, initially at 50°C and finally at 30°C. Fig. 1 presents the strategy used for the high solid loading.

Whereas the substrate and nutrient media were autoclaved (120°C for 20 min), the enzyme solutions were not sterile. The Solka Floc slurry, diluted to different dry weights of solid material (10%, 13%, 15%, and 20%), was used as the substrate.

Impellers and Baffle

The mixing-tank fermentation using a Rushton impeller consisted of an ellipsoidal cylindrical tank with four equally spaced wall-mounted baffles, extending from the vessel bottom to the free surface, and stirred by a centrally located six-blade Rushton turbine impeller.

The bioreactor was tested with a 4.6-cm-diameter Rushton and 1.0-cm-diameter marine impeller for glucose conversion, with and without wall baffles to give four bioreactor configurations: Rushton, baffled Rushton, marine, and baffled marine. The Rushton impeller had six blades, where each blade was 1.0 cm (height) by 1.5 cm (width) by 0.05 cm (thickness). The marine impeller had three inclined

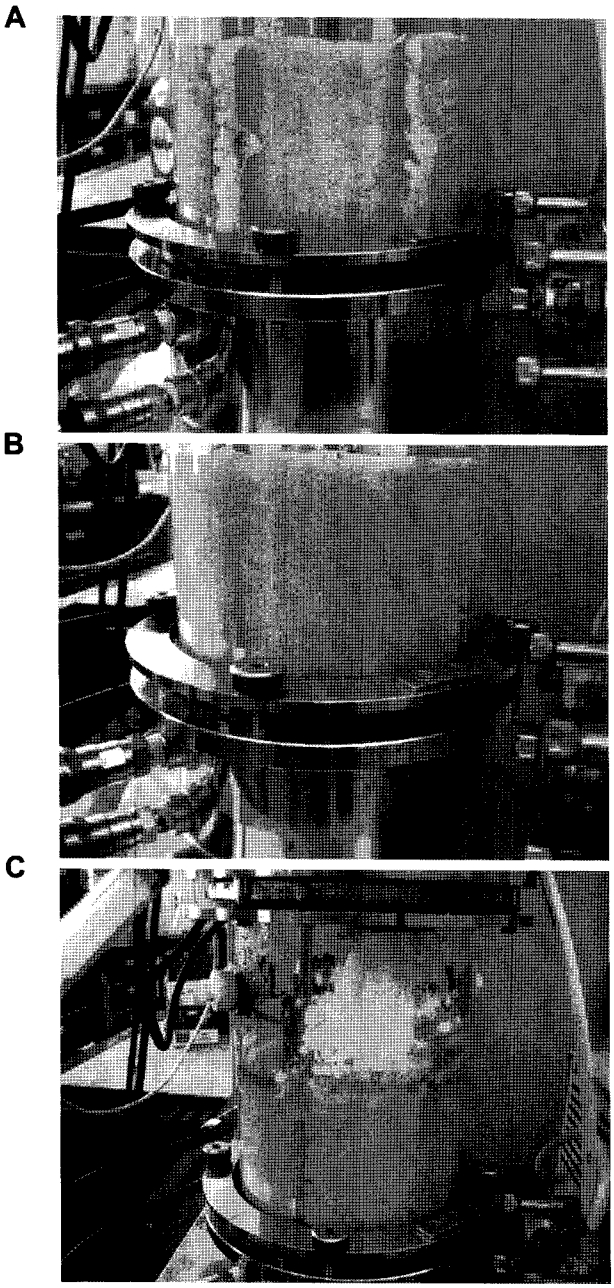

Fig. 1. High-solid loading strategy for enzyme hydrolysis and fermentation: **A**. 5% (w/v) suspension (t=0); **B**. 5% (w/v) suspension (t=0 to 4 h); **C**. Reloaded 5% (w/v) suspension (t=4 h).

curved blades of standard configurations. Each of the four wall baffles was 17. 5 cm (height) by 1.5 cm (width) by 0.05 (thickness).

RESULTS

Enzymatic Hydrolysis Below 10 Percent (w/v)

Fig. 2 shows the cellulose conversion to ethanol for the 2-l batch hydrolysis during the initial 6-h enzyme reaction

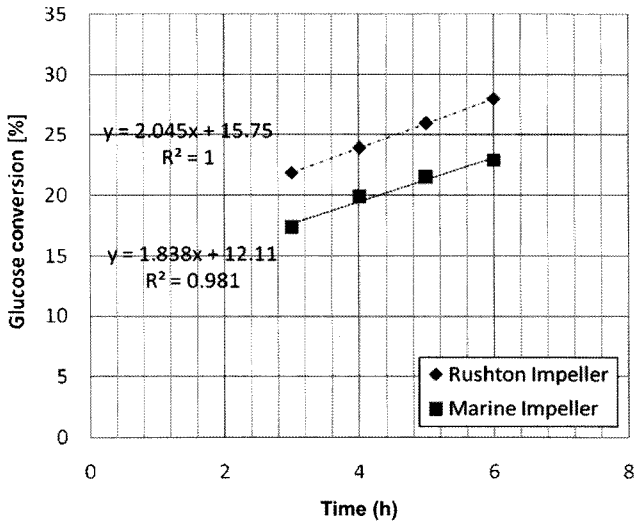

Fig. 2. Glucose conversion rate during initial 6-h enzymatic hydrolysis of 5% (w/v) Solka Floc.
Enzymatic hydrolysis conditions: 20 FPU/g cellulose, pH 4.8 to 5.0, 50°C, 120 rpm.

with the other enzyme parameters (T and enzyme loading) held constant. The experiments were conducted using 5% initial insoluble solid by weight and 20 FPU/g cellulose, plus a 20 CBU/g cellulose addition of Novozyme to reduce any cellobiose inhibition. The plot shows that the conversion rates exhibited a linear behavior at the beginning of the reaction, allowing the cellulosic material to be sufficiently liquidized within 4 h.

Many researchers have already shown that the initial rate of hydrolysis is much higher than the subsequent rate [21], where possible explanations include the selective initial hydrolysis of amorphous cellulose [4, 11], decreases in specific enzyme adsorption or subsequent inability of bound cellulases to reach new catalytic sites [5, 10], and steric preferences [19, 21]. In the current study, the specific rate at which the majority of the cellulose was utilized remained approximately constant until a critical value was reached at approximately 80% cellulose conversion. However, to improve the weak mass and heat transfer, Solka Floc was added to the reaction in three portions over four hours during the enzymatic hydrolysis up to a final substrate concentration of 20%.

The extent of digestion (enzymatic digestibility) as a function of time for lower solid concentrations is shown in Fig. 3A, where the digestibility was 79%, 68%, and 63% for solid concentrations of 1%, 3%, and 5%, respectively. The conversion yields were generally higher at the lower substrate concentrations (~5%, w/v), due to lower mass transfer limitations within the reaction medium. Fig. 3B shows the glucose conversion during the enzymatic hydrolysis as a function of the impeller type. On average, the baffled Rushton bioreactor generated a 63% conversion of cellulose

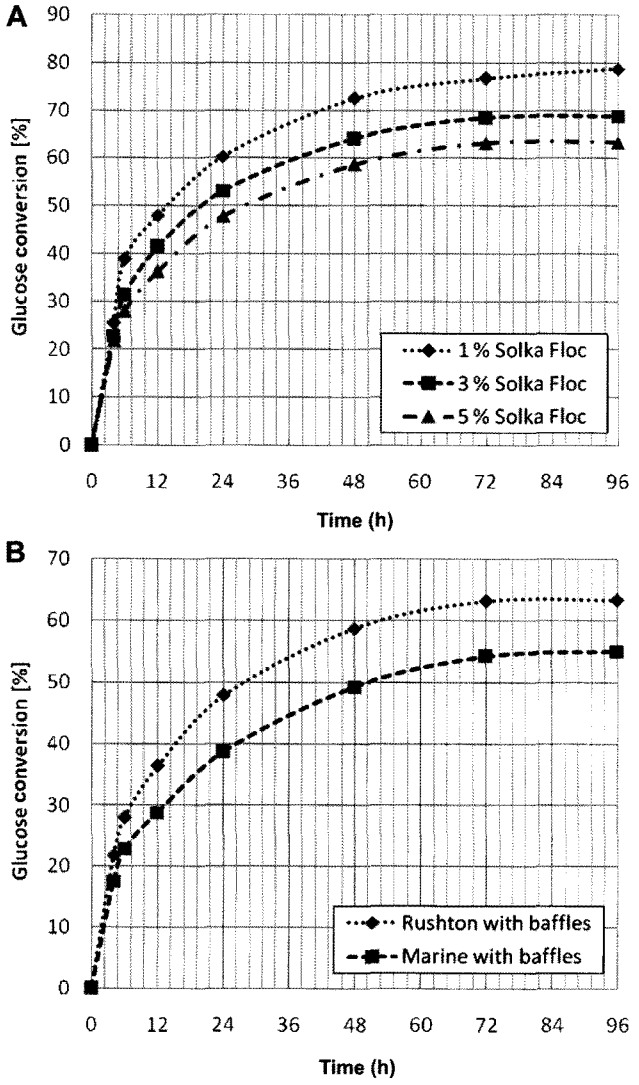

Fig. 3. Enzymatic hydrolysis of Solka Floc at lower percent solid concentrations (**A**) and 5% (w/v) with different impeller types (**B**) as a function of time for constant cellulase activity. Enzymatic hydrolysis conditions: 96 h, 20 FPU/g cellulose, pH 4.8–5.0, 50°C, 120 rpm.

with a 5% solid concentration, representing 5% more glucose than that generated by the baffled marine bioreactor.

Stirring Power in Three-Liter Fully Baffled Tanks

The generally accepted measurements of the stirring power in fully baffled tanks containing non-Newtonian fluids with various impellers were made by Rushton *et al.* [14]. Fig. 4 shows the power consumption for the baffled 3-l bioreactor with 5% and 15% Solka Floc suspensions based on a working volume of 2 l. Fig. 4A shows that the measurements were made with a 0.046 m diameter Rushton impeller, with and without wall baffles. The power consumption increased when increasing the rotational speed, yet was unaffected by the presence or absence of wall baffles up to 400 rpm. However, a significant difference in the power consumption was seen beginning at 600 rpm. Fig. 4B shows the results obtained with Rushton and marine impellers, respectively. With a 5% solid suspension, the power consumption was significantly lower when using the marine impeller compared with the Rushton impeller. Figs. 4C and 4D are analogous to Figs. 4A and 4B, respectively, and show the power consumption with a relatively high solid concentration (15%, w/v) and otherwise unchanged operating conditions. The diagrams reflect the difference in the power consumption between the impellers in this type of baffled mixing tank. Essentially, the power consumption exhibited the same trend, as seen in Figs. 4A and 4B. However, at an agitation speed of 900 rpm in the Rushton bioreactor, the power consumption with the 15% solid concentration was much higher than that with the 5% solid concentration, implying that the fluid flow and power consumption increased with an increasing viscosity. Meanwhile, a 1,000 rpm agitation speed required power inputs of 30 and 3.6 W for the baffled Rushton turbine and baffled marine configuration, respectively. Fig. 4C shows the power consumption for the Rushton impeller with and without baffles, where the power required for the baffled configuration was 10 W higher with a 15% solid concentration.

Therefore, these findings demonstrate the similarity of the hydrodynamic effects caused by the viscosity and baffles, which both added resistance to the flow. The effect was the same: more mechanical power needed to be introduced to the system to maintain fluid motion.

Effect of Bioreactor Configuration on Bioconversion

The bioreactor was tested with a 4.6-cm-diameter Rushton and 1.0-cm diameter marine impeller for glucose conversion, with and without wall baffles, to give four bioreactor configurations: Rushton, baffled Rushton, marine, and baffled marine. The Rushton impeller had six blades, where each blade was 1.0 cm (height) by 1.5 cm (width) by 0.05 cm (thickness), whereas the marine impeller had three inclined curved blades of standard configurations. Each of the four wall baffles was 17. 5 cm (height) by 1.5 cm (width) by 0.05 cm (thickness). The substrate used for the hydrolysis studies was shown to be primarily composed of cellulose, since glucose constituted the majority among the sugars in the substrate (88%). Fig. 5A illustrates the effect of the baffles on the glucose conversion with the Rushton turbine and 5% and 10% solids. The baffles became more important for glucose conversion as the solid concentration increased. Fig. 5 shows the enzymatic digestibility (g released glucose/g initial cellulose), as recommended by the NREL Standard Procedure 009, for the various reactor configurations with a high solid concentration. All the portion methods were performed starting with a 5% solid concentration. The baffled Rushton bioreactor produced 10% more glucose than the baffled marine bioreactor (Fig. 5).

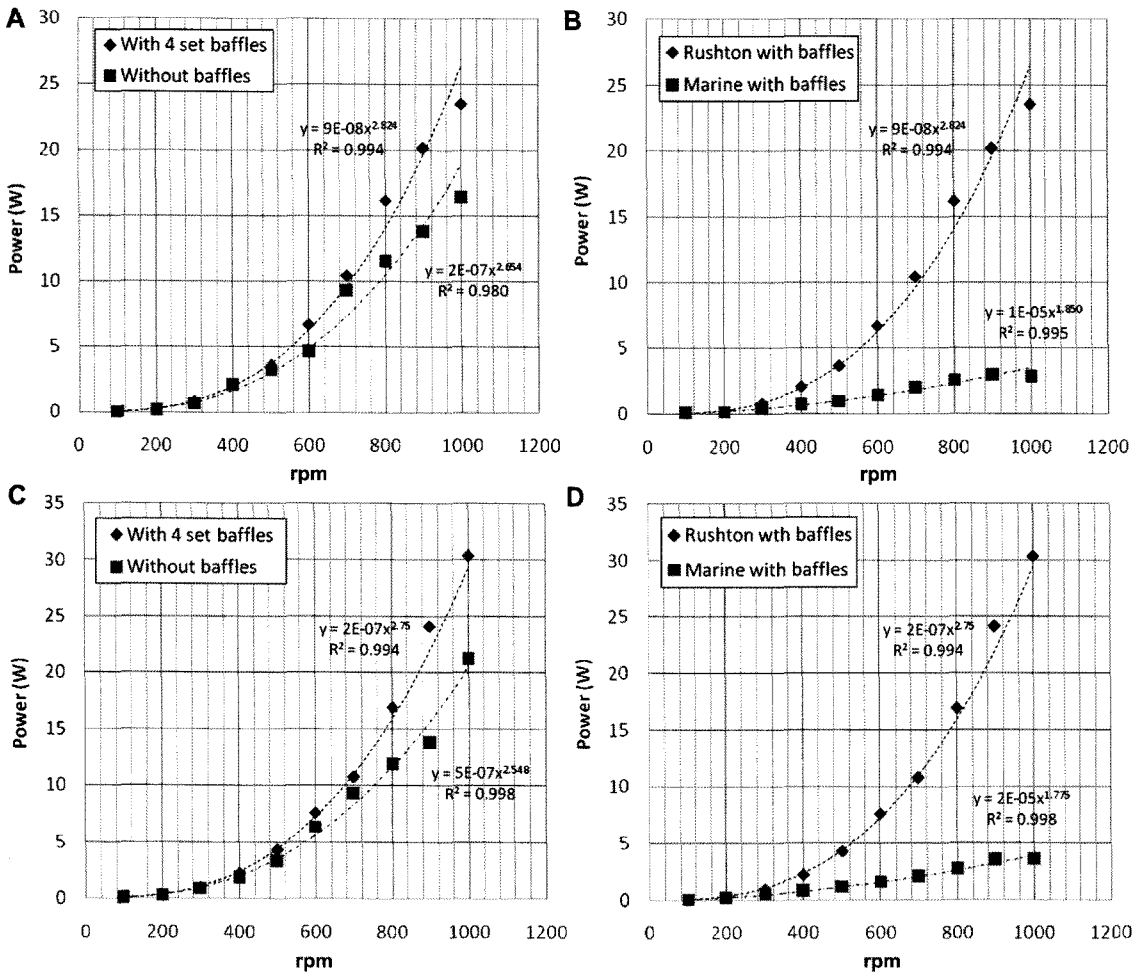

Fig. 4. Power consumption in two-liter bioreactor with 5% (**A**, **B**) and 15% (**C**, **D**) (w/v) Solka Floc suspensions and various bioreactor configurations as a function of RPM.

Enzymatic hydrolysis conditions: 20 FPU/g cellulose, pH 4.8–5.0, 50°C. **A**, **C**. Rushton impeller with and without baffles. **B**, **D**. Baffled with Rushton or marine impeller. All data points given are average yields for duplicate determinations.

For glucose and ethanol production, the mixing efficiency in the bioreactor is affected by various parameters, including baffles, the impeller speed, impeller type, clearance, tank geometry, solubility of the substance, and eccentricity of the impeller [12]. A vortex is generated owing to the centrifugal force acting on the rotating suspension. However, if the vortex reaches the impeller, severe air entrainment occurs. The depth and shape of the vortex depend on the impeller and vessel dimensions, rotational speed, and presence of baffles.

Fig. 5B show the results of the cellulose digestibility with and without baffles at a relatively high solid concentration (13% and 15%, w/v). As expected, the baffled configurations gave a higher glucose conversion than the unbaffled configurations with 13% and 15% solid concentrations. In the baffled tanks, a better concentration distribution was achieved throughout the tank, thereby improving the mixing efficiency and yielding a high glucose conversion with high solid substrates. Meanwhile, in the unbaffled vessel with the impeller rotating in the center, the centrifugal force acting on the fluid raised the fluid level at the wall and lowered the level at the shaft.

Effect of Rotational Speed on Glucose Yield

One important parameter in the current bioreactor design was the rotational speed of the impeller. Filamentous fungal fermentations in a stirred tank bioreactor usually experience a high apparent viscosity and non-Newtonian broth behavior that can lead to the use of a high agitation speed to provide adequate mixing and oxygen transfer to improve the cell and ethanol production rate. However, mycelial damage with a high power input and rigorous agitation can limit the acceptable range for the agitation speed. This damage probably results from the higher shear rates present at the impeller tip, and a high rate of cell damage can lower growth and product formation [1, 8].

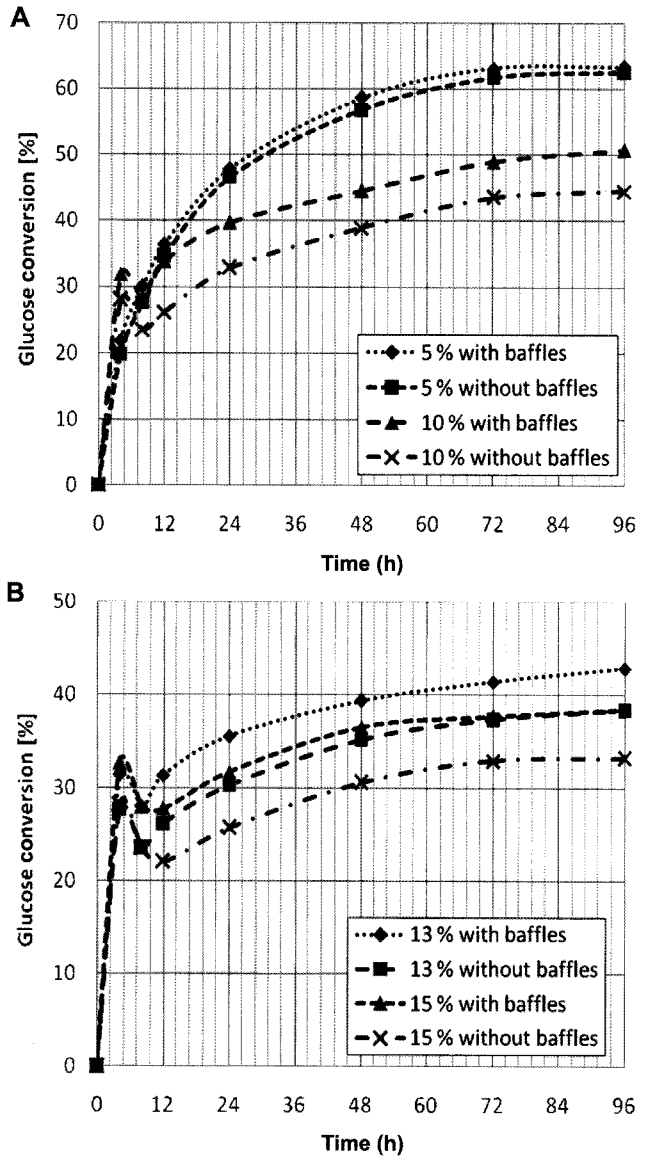

Fig. 5. Effect of baffles on glucose conversion of 5% and 15% solid concentrations of Solka Floc with baffled and unbaffled Rushton bioreactors as a function of time for constant cellulase activity.

Enzymatic hydrolysis conditions: 96 h, 20 FPU/g cellulose, pH 4.8 to 5.0, 50°C, and 120 rpm. The substrate was added to the reactions in portions during the enzymatic hydrolysis, up to a final substrate concentration of 10%, 13%, and 15%.

Similarly, for enzyme suspensions in a stirred-tank bioreactor, the rotational speed of the fibrous matrix influences the glucose conversion by cellulase. Thus, since a rotational speed higher than 200 rpm was anticipated to damage the enzyme and/or *Zymomonas mobilis*, owing to the high shear rate around the impeller, the effect of the rotational speed was determined at 60, 120, and 180 rpm with solid concentrations of 10%, 13%, and 15%. Fig. 6 shows the glucose conversion by the enzymatic hydrolysis at different rotational speeds and various solid concentrations. Although the rotational speeds tested in this study did not greatly affect the glucose yield between 120 rpm and 180 rpm, an increased glucose productivity was observed at the high rotational speeds (Figs. 6A, 6B, and 6C). Meanwhile, the low rotational speed (60 rpm) did not appear to be sufficient to produce contact between the substrate and the enzyme, resulting in conversion yields that were 5% to 10% lower than those obtained at 120 and 180 rpm. There was no significant conversion difference at the various rotational speeds with the relatively low solid concentration, probably due to the substantial decrease in the viscosity of the reaction mixture and better interaction between the enzymes and the remaining substrates. In addition, Fig. 6 shows the rotational speed profiles, where the mixing speeds of 120 and 180 rpm showed no significant difference as regards their effect on the glucose conversion after 96 h. Therefore, in this study, a rotational speed setting of 120 rpm with a lower power input was selected for the ethanol fermentation, suggesting that a threshold value for the rotational speed (mass transfer related) has to be achieved for efficient glucose conversion.

High-Solid Saccharification by Portion Loading

To improve the process economics of the lignocellulosic biomass to ethanol process, a bioreactor system was developed for the enzymatic saccharification of high solid concentrations. The saccharification was performed in a three-liter bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ, U.S.A.) with a baffled Rushton turbine and baffled marine propeller. As discussed in the Materials and Methods section, substrates were added to the reactions in portions during the enzymatic hydrolysis, up to a final substrate concentration of 20 % (w/v).

During batch system saccharification in a bench-scale fermentor, the solid concentration is one of the most important variables affecting the rate and extent of conversion. Fig. 7 shows the effect of the Solka Floc solid loading on the rate and extent of glucose conversion when increasing the solid level with two different reactor configurations and a constant enzyme loading. From the data shown in Fig. 7, for a 20 FPU/g cellulose loading, increasing the solid loading up to 20% (w/v) significantly decreased the rate of hydrolysis and conversion of glucose. The most likely reasons for this decrease in the hydrolysis rate and glucose conversion were a combination of cellobiose and glucose inhibition of the enzyme system from the correspondingly higher sugar levels reached when using a higher solid loading and mass transfer limitations. Specifically, in the case of mass transfer, a relatively weak axial flow was found near the center bottom of the tank and below the baffle from a CFD simulation (data not shown).

Fig. 7A shows that for all the cases presented, glucose concentrations of more than 41 g/l were achieved during

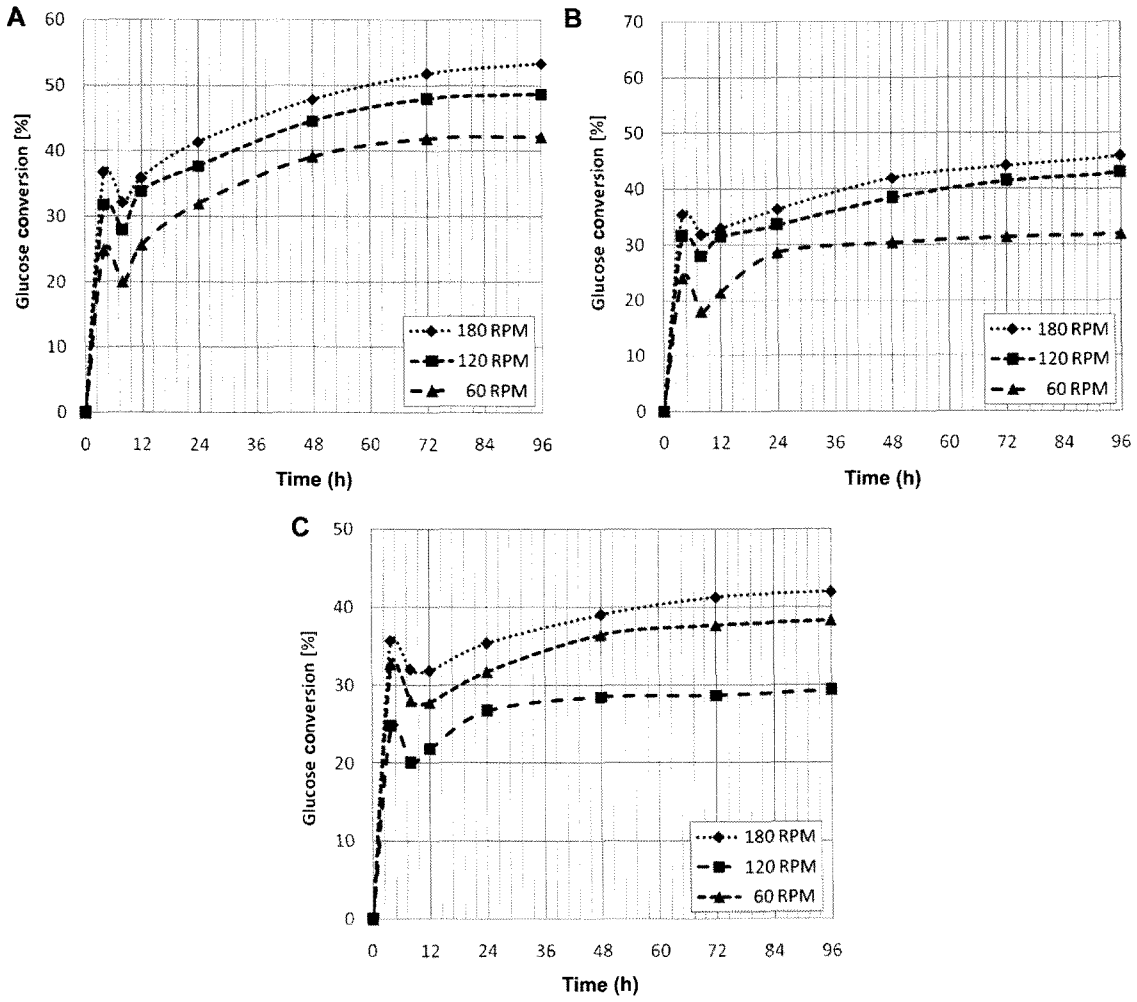

Fig. 6. Effect of RPM on enzymatic hydrolysis of 10% (**A**), 13% (**B**), and 15% (**C**) solid concentrations of Solka Floc in the baffled Rushton bioreactor as a function of time for constant cellulase activity.
Enzymatic hydrolysis conditions: 96 h, 20 FPU/g cellulose, pH 4.8 to 5.0, 50°C. Substrates were added to the reactions in portions during the enzymatic hydrolysis up to a final substrate concentration of 10%, 13%, and 15%.

the liquid phase, whereas more than 50 g/l of glucose was achieved in the case of the 20% solid loading after 48 h. However, the higher solid loadings required a significantly longer residence time to achieve these high liquid-phase sugar levels. As shown in a previous work [18], the effect of glucose on β-glucosidase activity is an important inhibition concern, as this can become the ultimate rate-limiting step when very high levels of glucose are accumulated. The glucose digestibility was lower with the 20% solid concentration than with the 10% solid concentration, indicating that the mixing limitations for these levels of solids had become a significant factor in addition to glucose inhibition. On average, the baffled Rushton bioreactor generated a higher glucose conversion than the baffled marine configuration, by as much as 15% (Figs. 7A and 7B).

However, most importantly, this work demonstrated that a cellulose conversion of greater than 20% could be achieved with an initial insoluble solid level as high as 20% when using a portion loading method. Without the portion loading, it was impossible to maintain a continuous liquid phase in the bioreactor with a solid concentration of 20%, plus the shear stress was 10 times higher than when portion loading the 20% solid concentration (data not shown), implying that the flowability depended on the presence of a continuous liquid phase.

Effect of Substrate Concentration on Ethanol Yield

In several previous studies, the solid content in the conventional fermentation of lignocellulosic biomass is limited to about 10%, resulting in a maximum ethanol concentration of 4% (v/v). However, if higher solid levels could be fermented, this might produce higher ethanol concentrations and reduce the downstream costs. SFF baffled Rushton bioreactors were operated in a three-liter fermentor after enzymatic hydrolysis (for 48 h) using a maximum of 20% dry matter (DM), corresponding to a

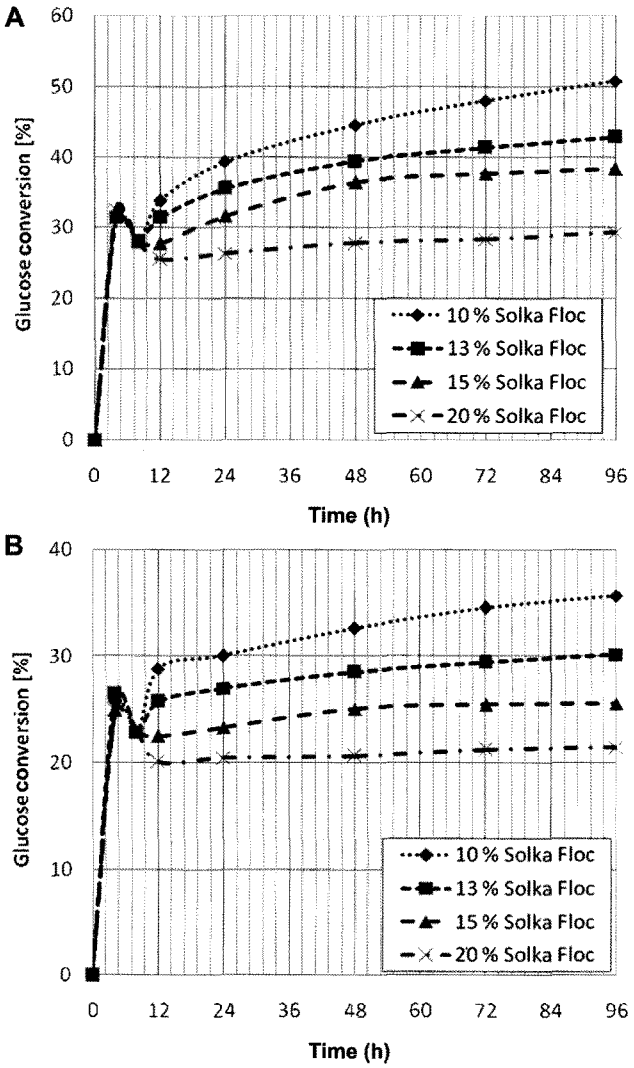

Fig. 7. Enzymatic hydrolysis with the baffled Rushton turbine (**A**) and marine propeller (**B**) as a function of time for constant cellulase activity.

Enzymatic hydrolysis conditions: 96 h, 20 FPU/g cellulose, pH 4.8 to 5.0, and 120 rpm. The substrate was added to the reactions in portions during the enzymatic hydrolysis, up to a final substrate concentration of 20%.

Table 1. Ethanol yield and conversion (%) by *Z. mobilis* after 48 h.

Substrate concentration	10%	15%	20%
Initial glucose after enzyme reaction (g/l)	42.6	55.5	58.4
Final ethanol concentration after 48 h (g/l)	18.2	19.7	6.3
Conversion of consumed glucose into ethanol (%)	83.6	73.4	21.8
Theoretical ethanol yield (%)	80.5	68.6	19.1
Total fermentation time based on portion method (h)	106	110	114

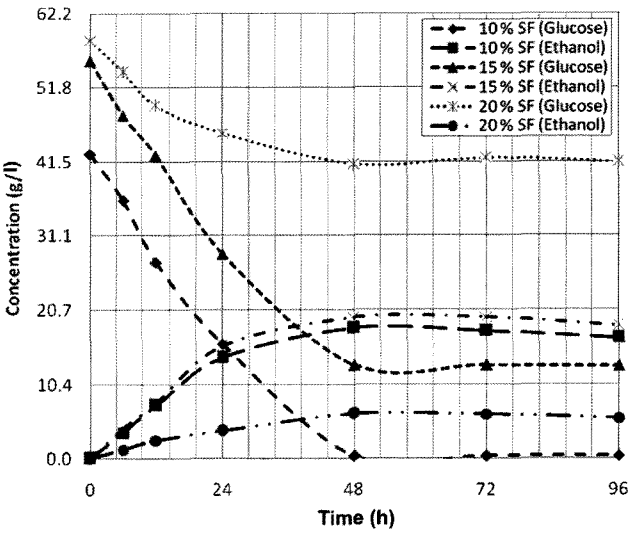

Fig. 8. Time course of substrate utilization and ethanol production by *Zymomonas mobilis* at solid concentrations of 10%, 15%, and 20% with the Rushton impeller as a function of time for constant cellulase activity.

Enzymatic hydrolysis conditions: 48 h, 20 FPU/g cellulose, pH 4.8 to 5.0, 50°C. Cell transfer after 48 h, Initial OD_{600} 0.52. Fermentation conditions: 96 h, *Zymomonas mobilis* (39679:pZB4L), pH 5.0, 30°C. The substrate was added to the reactions in portions during the enzymatic hydrolysis, up to a final substrate concentration of 20%.

cellulose concentration of 17%. As previously mentioned, to avoid poor mass and heat transfer, the substrate was added to the reaction in three portions during the enzymatic hydrolysis, up to a final substrate concentration of 20%. The portions were added to the hydrolysis at 4-h intervals (Fig. 2), as after 4 h, the rate of enzymatic hydrolysis became dramatically high, meaning that the previous 5% substrate had been sufficiently liquidized to allow another 5% substrate to be loaded (Fig. 1).

The effect of the substrate concentration on the ethanol yields after 96 h of SFF using Solka Floc is shown in Table 1 and Fig. 8. Fig. 8 shows the time course of the substrate utilization and ethanol production by *Zymomonas mobilis* at 10%, 15%, and 20% solid concentrations with a Rushton impeller as a function of time for a constant cellulase activity. When increasing the DM content to 20%, the ethanol yield was dramatically reduced compared with that with 10% and 15% solid concentrations, which was probably due to insufficient mass transfer caused by the different viscosity and flow patterns for those concentrations.

With the relatively high substrate concentration, the enzymes were unable to liquefy the cellulose fibrous material, resulting in a low enzyme reaction rate and low ethanol yield of only 21%. The maximum ethanol yield of 83% was achieved with a 10% solid concentration after 48 h. However, the ethanol yield was also favorable at 73% when using a 15% solid concentration.

DISCUSSION

This study demonstrated a number of important novel conclusions related to high-solid saccharification following fermentation systems. First, critical to the design and optimization of agitated bioreactor processes is understanding and assessing the effect of the reactor configuration on glucose and ethanol production. In this investigation, high-solid enzymatic saccharification was performed using a 2-l working volume and various bioreactor configurations. At 120 rpm, the reactors with Rushton impellers achieved much higher concentrations of glucose than the reactors with marine impellers. Moreover, the results of the enzymatic hydrolysis indicated that wall baffles significantly increased the digestibility in the Rushton bioreactor, yet not in the marine bioreactor. Therefore, a baffled Rushton bioreactor is recommended for a high-solid bioconversion process.

Second, it was demonstrated that sugar inhibition of the enzymatic saccharification rate was not as compelling a concern as had previously been suspected, and that remarkably high concentrations of glucose were achievable from high-solid enzymatic reactions. Meanwhile, other parameters, including the bioreactor configuration and temperature, were identified as important for high-solid enzymatic saccharification. Therefore, the remarkably improved rate and high product concentrations may greatly improve the economics of enzymatic saccharification.

Most importantly, this study also demonstrated a glucose concentration and ethanol conversion greater than 50 g/l and 20%, respectively, with a 20% solid concentration, likely due to the adoption of an optimal substrate loading strategy.

Thus, the portion loading method provides a feasible method for fermenting cellulosic material, while avoiding the mass transfer limitations with a higher solid loading. In traditional batch fermentation, there is no visual continuous liquid phase with a 20% solid concentration in the bioreactor, likely due to the complete absorption of the liquid by the biomass before the reaction between the microorganism and substrate.

An additional observation from this study was the importance of keeping the fermentation anaerobic, even though an increased cellulosic biomass concentration is desirable for the SFF process, because when oxygen was introduced to the reaction, the production of acetate and glycerine increased, thereby decreasing the purity of the desired ethanol product.

REFERENCES

1. Amanullah, A., L. H. Christensen, K. Hansen, A. W. Nienow, and C. R. Thomas. 2002. Dependence of morphology on agitation intensity in fed-batch cultures of *Aspergillus oryzae* and its implications for recombinant protein production. *Biotechnol. Bioeng.* **77:** 815–826.
2. Beyond Petroleum. 2005. *Statistical Review of World Energy.* <http://www.bp.com>.
3. *Bioethanol: Fueling Sustainable Transportation.* <http://www1.eere.energy.gov/biomass/pdfs/bioethanol_fueling_sustainable_transportation.pdf>.
4. Davis, M., J. O. Baker, T. Rignall, and M. E. Himmel. 2002. Changes in cellulose morphology of pretreated yellow poplar during enzymatic hydrolysis. NREL Report No. PO-510-32125.
5. Eriksson, T., J. Karlsson, and F. Tjerneld. 2002. A model explaining declining rate in hydrolysis of lignocellulosic substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei. Appl. Biochem. Biotechnol.* **101:** 41–59.
6. Kang, H. J. and K. Ishikawa. 2007. Analysis of active center in hyperthermophilic cellulase from *Pyrococcus horikoshii. J. Microbiol. Biotechnol.* **17:** 1249–1253.
7. Kusuma, K., G. H. Chon, J. S. Lee, J. Kongkiattikajorn, K. Ratanakhanokchai, K. L. Kyu, *et al.* 2006. Hydrolysis of agricultural residues and kraft pulps by xylanolytic enzymes from alkaliphilic *Bacillus* sp. strain BK. *J. Microbiol. Biotechnol.* **16:** 1255–1261.
8. Li, Z. J., V. Shukla, K. Wenger, A. Fordyce, A. G. Pedersen, and M. Marten. 2002. Estimation of hyphal tensile strength in production-scale *Aspergillus oryzae* fungal fermentations. *Biotechnol. Bioeng.* **77:** 601–613.
9. Lübbert, A. and B. S. Jørgensen. 2001. Bioreactor performance: A more scientific approach for practice. *J. Biotechnol.* **85:** 187–212.
10. Lynd, L., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **6:** 506–577.
11. Mansfield, S. D., C. Mooney, and J. N. Saddler. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnol. Prog.* **15:** 804–816.
12. Oldshue, J. Y. 1983. *Fluid Mixing Technology,* pp. 1–23. McGraw-Hill, New York.
13. Philippidis, G. P. and C. Hatzis. 1997. Biochemical engineering analysis of critical process factors in the biomass-to-ethanol technology. *Biotechnol. Prog.* **13:** 222–231.
14. Rushton, J. H., E. W. Costich, and H. J. Everett. 1950. Power characteristics of mixing impellers. *Chem. Eng. Prog.* **9:** Part I: 395–450, Part II: 467–476.
15. Shin, D. G., A. Yoo, S. W. Kim, and D. R. Yang. 2006. Cybernetic modeling of simultaneous saccharification and fermentation for ethanol production from steam-exploded wood with *Brettanomyces custersii. J. Microbiol. Biotechnol.* **16:** 1355–1361.
16. Um, B. H. 2002. Effect of pretreatment reagent and hydrogen peroxide on enzymatic hydrolysis of oak in percolation process. *Appl. Biochem. Biotechnol.* **91/93:** 81–94.
17. Um, B. H., M. N. Karim, and L. L. Henk. 2003. Effect of sulfuric and phosphoric acid pretreatments on enzymatic hydrolysis of corn stover. *Appl. Biochem. Biotechnol.* **105/108:** 115–152.
18. USA Today, June 11, 2006. Debate brews: Has oil production peaked?
19. Väljamäe, P., V. Sild, G. Pettersson, and G. Johansson. 1998. The initial kinetics of hydrolysis by cellobiohydrolases I and II is consistent with a cellulose surface-erosion model. *Eur. J. Biochem.* **253:** 469–475.
20. Yin, L. and R. J. Chróst. 2006. Enzymatic activities in petroleum wastewater purification system by an activated sludge process. *J. Microbiol. Biotechnol.* **16:** 200–204.
21. Zhang, Y. H. P. and L. Lynd. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnol. Bioeng.* **88:** 797–824.