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Enhancement of Enzymatic Cellulose Hydrolysis Using a Novel Type of Bioreactor with Intensive Stirring Induced by Electromagnetic Field

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ABSTRACT

The use of the intensive mass transfer reactor (IMTR) for enzymatic saccharification of cellulose, where the reaction mixture is intensively stirred by ferromagnetic particles (FMP), enhances the process rate and productivity drastically. The most significant enhancement of the process was observed when microcrystalline cellulose was used as a substrate. A concentration of sugars up to 5% was obtained after 1 h of cellulose hydrolysis using a cellulase activity level of 2 filter paper units (FPU)/mL (20 FPU/g substrate). In the hydrolysis of two types of industrial cellulosic wastes, the enhancement effects were less pronounced. Parameters related to the IMTR design, such as the shape, dimensions, and mass of FMP, as well as the magnetic field strength, strongly affected the process of hydrolysis. Among various kinds of FMP tested, the most efficient were found to be cylindrical particles (0.25 × 4 mm). In general, the hydrolysis rate enhanced when the magnetic field strength increased from 26,000 to 64,000 A/m. An optimal FMP loading existed at each level of the field strength. Hydrolyzates obtained in the IMTR under the action of *Trichoderma reesei* and *Penicillium verruculosum* cellulases contained glucose and

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cellobiose as soluble products, cellobiose being predominant (> 50%). Only when a high level of extra β-glucosidase was added to the IMTR (10 CBU/mL), did glucose made up more than 90% of the products. Owing to extreme shear conditions in the IMTR, significant enzyme inactivation took place.

Index Entries: Cellulase; cellulose hydrolysis; electromagnetic field; Intensive Mass Transfer Reactor.

INTRODUCTION

One of the pressing problems in the development of lignocellulose bioconversion processes is a choice of bioreactor for enzymatic saccharification of cellulose. An ordinary batch-stirred reactor has been used for cellulose hydrolysis in a large-scale process of lignocellulose bioconversion to acetone-butanol (*1*). Some other approaches described in the literature include continuous or semicontinuous ultrafiltration membrane reactors (*2–5*), column plug-flow (packed-bed) reactors (*6–13*), attrition or ball mill reactors (*14–19*). The use of these types of reactors may help to solve various problems, such as the partial elimination of product inhibition, enzyme recovery, enhancement of the hydrolysis rate, and productivity.

The intensive mass transfer reactor (IMTR) is a novel type of bioreactor where the energy of an electromagnetic field of special configuration is transformed into kinetic energy of ferromagnetic particles (FMP) added to the reactor (*20*). Rapidly moving FMP provide very intensive agitation of the reaction mixture. Mass-transfer processes proceed very efficiently in such reactors. This is particularly important in a heterogeneous reaction system like cellulose-cellulases. The concentration of sugars of 5–6% has been achieved in the IMTR after 1–2 h of enzymatic hydrolysis of microcrystalline cellulose with a productivity of 30–50 g/L·h (*20*). For a shorter process time, the productivity has been even higher. In other types of bioreactors used for cellulose saccharification, the hydrolysis time is much longer, and the productivity is considerably lower (*8,12*). In an attrition bioreactor, which claims to be one of the most efficient hydrolysis apparatus, only 4.4% concentration of sugars has been obtained after 6 h hydrolysis of Avicel with a much lower productivity (7.3 g/L·h) (*17*).

In this article, the effects of various factors on the efficiency of enzymatic cellulose hydrolysis in the IMTR are discussed.

MATERIALS AND METHODS

Enzymes

Industrial cellulase preparation from *Trichoderma reesei* (Privolzhsk Biochemical Plant, Russia) and freeze-dried laboratory preparation from

Penicillium verruculosum (Leipzig Institute of Biotechnology, Germany) (*21*) was used for cellulose hydrolysis. The *T. reesei* enzyme had cellulase activity of 147 filter paper units (FPU) (pH 4.8, 50°C) (*22*), and β-glucosidase (cellobiase) activity of 1.2 CBU/g (pH 4.5, 40°C, 2 m*M* cellobiose as a substrate) (*23*). The respective activities of *P. verruculosum* preparation were 240 FPU/g and 80 CBU/g. Partially purified β-glucosidase from *Aspergillus* sp. (NPO Biotechnika, Russia) had an activity of 2000 CBU/g.

Substrates

Microcrystalline cellulose (Chemapol, Czechoslovakia) with a mean particle size 23 μm (*24*), lignocellulosic industrial residue after the production of furfural from mixed wood ("cellolignin") with 45% cellulose content (*25*), and short fiber (3–5 mm) cellulose wastes from the pulp and paper industry ("skop") were used as substrates in the enzymatic hydrolysis. Cellolignin is a dark-brown material with a wide particle size distribution (10–500 μm) that cannot be precisely estimated, since the particles form crumbly aggregates of different sizes.

Hydrolysis Conditions

The principles of the IMTR operation and its main design features have been described previously (*20*). Two vessels, constructed of stainless steel (130 mL) and glass (20 mL), were used as working cells in the IMTR. The cells were filled to the top with a suspension of cellulosic substrate in order to minimize enzyme inactivation at an air–liquid interface under shear conditions (*26,27*). The mass of FMP added to the reactor varied from 0.03–0.75 g/mL of working cell volume. The oscillation frequency of the electromagnetic field was 50 Hz. Unless otherwise stated, the magnetic field strength was 64,000 A/m. For a comparison, hydrolysis experiments were also carried out in glass cells (50-mL volume) equipped with a thermostat jacket and agitated on a shaker (250 rpm). In all experiments (in the IMTR and controls), the substrates were hydrolyzed at 50°C, and the reaction was carried out in 0.1*M* acetate buffer, pH 4.5.

Analysis of the Hydrolysis Products

The products of hydrolysis were analyzed by HPLC with a Knauer HPLC system (Germany) using a Silasorb-NH_2 column (4.6 mm × 25 cm) and acetonitrile–water (70:30) as the mobile phase. A differential refractometer was used as detector.

In all cases, the products of hydrolysis were glucose and cellobiose. Unless otherwise stated, the concentration of sugars indicated represents the combined amounts of glucose and cellobiose.

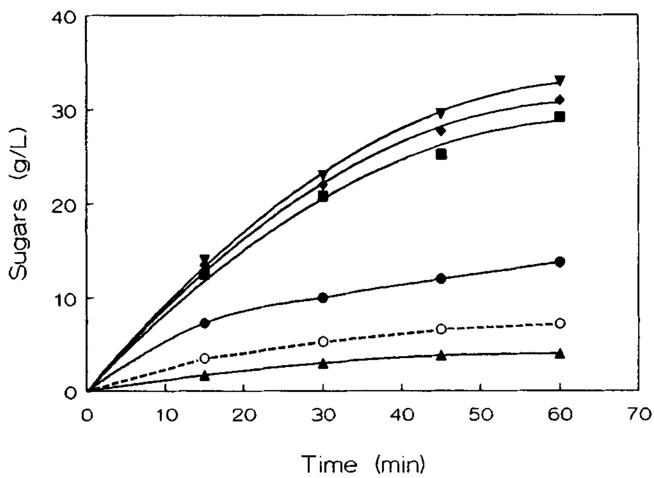

Fig. 1. Effect of FMP shape and dimensions on the hydrolysis of microcrystalline cellulose (100 g/L) by *T. reesei* cellulase in the IMTR (enzyme activity 2 FPU/mL, FMP loading 100 g/L). Cylindrical particles: ●, 2 × 10 mm; ■, 0.36 × 4 mm; ◆, 0.5 × 6 mm; ▼, 0.25 × 4 mm; ▲, circular particles (5-mm diameter); ○, control (hydrolysis in a shake flask).

RESULTS

Effect of FMP Parameters and Strength of the Magnetic Field on the Hydrolysis Efficiency

The main parameters related to the IMTR design that affect hydrolysis efficiency are the shape, dimensions, and mass of the FMP and the magnetic field strength.

Figure 1 shows the effect of FMP shape and dimensions on the kinetics of cellulose hydrolysis in the IMTR. The most pronounced effect of enhancement of the hydrolysis efficiency was observed in the case of cylindrical particles (0.25 × 4 mm), where the initial reaction rate in the IMTR was 4.6 times higher than the hydrolysis rate in the control experiment; the increase in a product yield after 60 min of the hydrolysis was 4.2 times. In the case of circular particles (5-mm diameter), the effect of hydrolysis enhancement was not observed, and the reaction rate in the IMTR was even lower than in the control.

In the subsequent experiments, cylindrical FMP having the optimal dimensions (0.25 × 4 mm) were used.

The effect of FMP loading at various magnetic field strengths on the yield of sugars after 60 min of hydrolysis is shown in Fig. 2. It is apparent that increased magnetic field strength generally results in a higher efficiency of the process. An optimal FMP loading existed at each level of the field strength. Below the optimum value, the hydrolysis enhanced with increased mass of added particles owing to intensification of stirring.

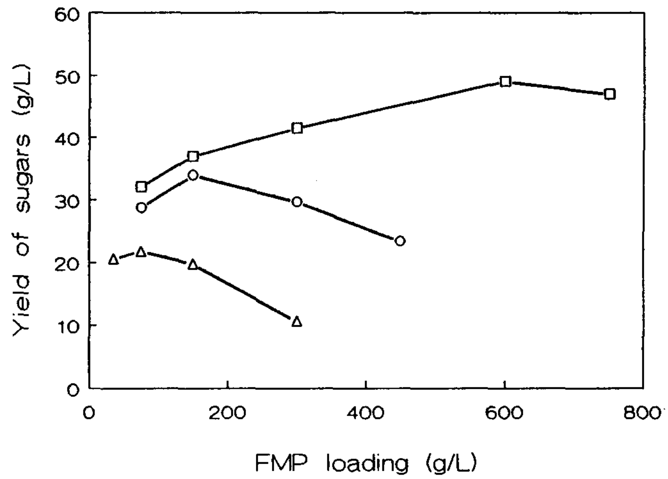

Fig. 2. Effect of FMP loading on the yield of sugars after 1 h of microcrystalline cellulose hydrolysis (100 g/L) by *T. reesei* cellulase (2 FPU/mL) in the IMTR. Magnetic field strength: Δ, 26,000 A/m; ○, 38,000 A/m; □, 64,000 A/m.

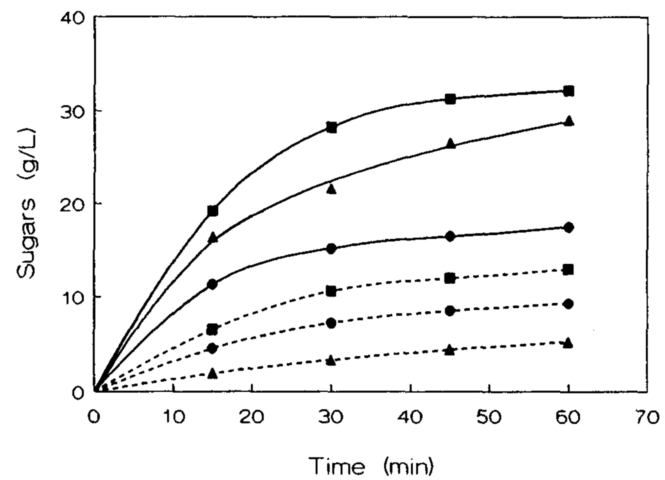

Fig. 3. Hydrolysis of different substrates by *T. reesei* cellulase in the IMTR (—) and shake flasks (---). Conditions: substrate concentration 75 g/L, enzyme activity 1 FPU/mL, FMP loading 150 g/L. ●, cellolignin; ■, skop; ▲, microcrystalline cellulose.

When loading was higher than optimal, the yield of sugars declined, because the regime of agitation became unstable and some attachment of FMP to the wall of working cell was observed.

Hydrolysis of Different Substrates by Different Cellulase Preparations in the IMTR

When different cellulosic substrates (other than microcrystalline cellulose) were hydrolyzed by *T. reesei* cellulase, the enhancement of the hydrolysis rate in the IMTR was also observed (Fig. 3), though the effect

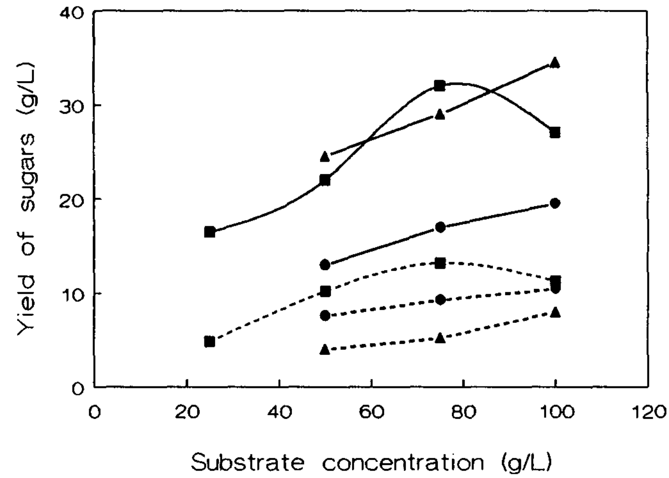

Fig. 4. Effect of substrate concentration on the yield of sugars after 1 h of cellulose hydrolysis by *T. reesei* cellulase (enzyme activity 1 FPU/mL, FMP loading 150 g/L). ●, cellolignin; ■, skop; ▲, microcrystalline cellulose. —, IMTR; ---, shake flask.

of enhancement was less pronounced. For skop and cellolignin, the initial rate of product formation increased 2.9 and 2.6 times, respectively, compared with the process in shake flasks, and the product yield increased 2.5 and 2.0 times.

In the hydrolysis of cellulosic substrates by *P. verruculosum* cellulase, the effects of enhancement of the process rate in the IMTR were similar to those observed in the case of *T. reesei* enzyme (the data are not shown). For example, when microcrystalline cellulose was hydrolyzed, the reaction rate in the IMTR increased up to five times (depending on the conditions used) compared to the control experiments; in the case of cellolignin, the rate increased 2–2.7 times.

Effect of Substrate and Enzyme Concentrations

The effect of substrate concentration on the hydrolysis efficiency was different for different substrates. For microcrystalline cellulose and cellolignin, the initial rate of hydrolysis and the yield of sugars increased with increased substrate concentration from 50 to 100 g/L (Fig. 4). However, in the case of skop, an optimal concentration was 75 g/L. At a higher substrate concentration, the hydrolysis efficiency decreased in both the IMTR and shake flask. It is probable that the main reason was lower density of skop and relatively high viscosity of the reaction mixture hindering its efficient stirring at high concentration of the substrate.

The effect of enzyme concentration (activity) on the yield of sugars in the IMTR is shown in Fig. 5. The yield increased 1.8 times when the cellulase activity changed from 0.5 to 4 FPU/mL. Such nonlinear dependence is rather typical for enzymatic cellulose hydrolysis (*28*), and the IMTR did not show any unusual features.

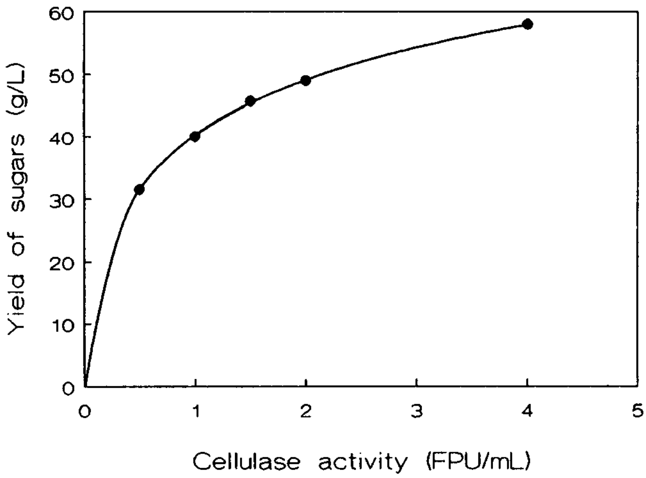

Fig. 5. Effect of enzyme concentration on the yield of sugars after 1 h of hydrolysis of microcrystalline cellulose (100 g/L) by *T. reesei* cellulase in the IMTR (FMP loading 600 g/L).

Product Composition in the IMTR and Effect of β-Glucosidase Activity

The soluble products of enzymatic cellulose hydrolysis were glucose and cellobiose in all cases. However, the ratio of these products in the IMTR and shake flask (control) was different.

In the control experiments, when different substrates were hydrolyzed by *T. reesei* cellulase, the weight content of glucose in the hydrolysis products after 60 min of the reaction varied from 23–33%. In the IMTR, at relatively low FMP loading (75 g/L), the glucose content was similar to that in the control; however, it considerably increased (from 31 to 48%) with increase in the amount of added FMP (Fig. 6). It should be noted that after a prolonged time of cellulose hydrolysis (24 h) by *T. reesei* cellulase in the shake flask, glucose content in the hydrolysis products was 70–80%.

In the case of *P. verruculosum* cellulase, the difference in the product composition for different reactors was more drastic. During hydrolysis of cellulosic substrates in shake flasks, glucose made up more than 80% of soluble products after 30–60 min of the reaction, whereas in the IMTR, glucose content was only 20–45%. After a prolonged time of cellulose hydrolysis in the shake flask (24 h), glucose was the only soluble product.

Addition of extra β-glucosidase may help to obtain the hydrolyzates containing glucose as a single product. When partially purified β-glucosidase from *Aspergillus* sp. was added to the cellulase solution in the IMTR, the glucose content increased. As can be seen from Fig. 7, only when a relatively high level of β-glucosidase was added (10 CBU/mL) to *P. verruculosum* cellulase was glucose a predominant product (>90%). A similar level of β-glucosidase activity was necessary in the case of *T. reesei* cellulase.

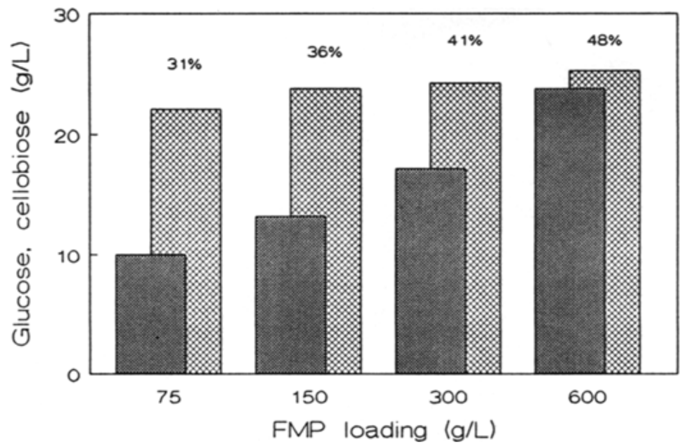

Fig. 6. Product composition in hydrolyzates of microcrystalline cellulose (100 g/L) after 1 h of hydrolysis by *T. reesei* cellulase in the IMTR at various FMP loadings (enzyme activity 2 FPU/mL). Left bars, glucose. Right bars, cellobiose. Figures over the bars show the relative percentage of glucose.

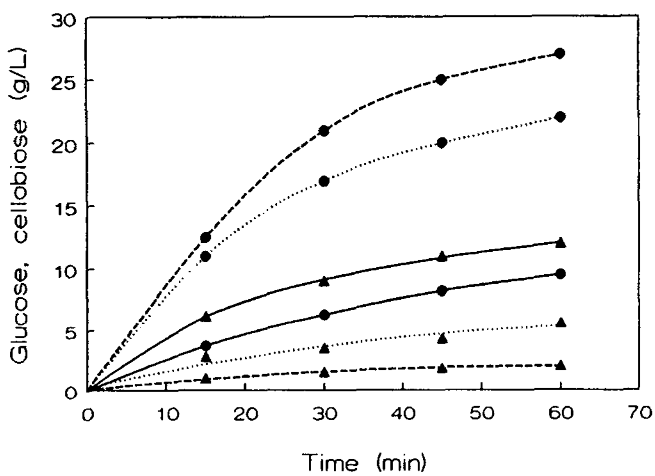

Fig. 7. Effect of β-glucosidase activity on product composition during hydrolysis of cellolignin (100 g/L) by *P. verruculosum* cellulase (2.4 FPU/mL) in the IMTR (FMP loading 100 g/L). ●, glucose; ▲, cellobiose. —, without extra β-glucosidase (inherent β-glucosidase activity 0.8 CBU/mL); ····, with 4 CBU/mL of extra added β-glucosidase; ---, with 10 CBU/mL of extra added β-glucosidase (from *Aspergillus* sp.).

Shear Inactivation of Cellulase and β-Glucosidase in the IMTR

It is well known that cellulase may lose activity at an air–liquid interface when the enzyme solution is intensively stirred (*26,27*).

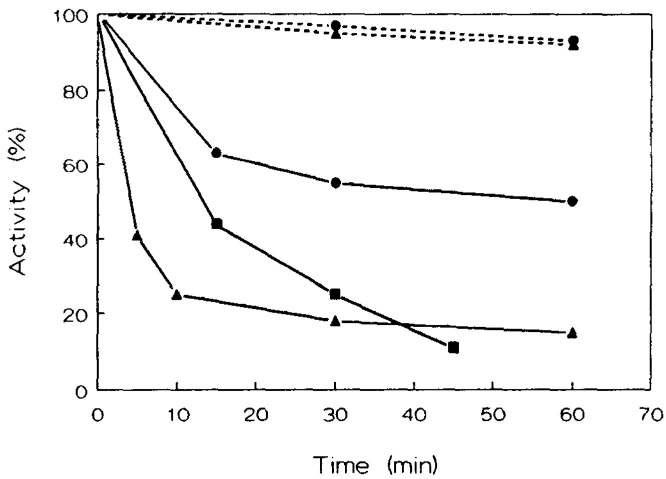

Fig. 8. *T. reesei* enzyme inactivation in the IMTR at FMP loading of 600 g/L (—) and shake flask (---). ●, cellulase (filter paper activity); ▲, β-glucosidase. ■, cellulase inactivation in the IMTR in a partially filled (1/2 vol) working cell.

Because of extreme shear conditions in the IMTR, significant enzyme inactivation took place. Even when a reaction vessel was filled with the enzyme solution to the top in order to eliminate an air-liquid interface and to minimize the enzyme inactivation (as in all hydrolysis experiments), almost 50% of cellulase activity and more than 80% of β-glucosidase activity were lost after 60 min of agitation of the solution in the absence of substrate, most of β-glucosidase activity being deactivated in the first 10 min (Fig. 8).

It was also interesting to study the enzyme inactivation in a partially filled working cell. As expected, when only half of the vessel volume was filled with the enzyme solution, the cellulase inactivation was more pronounced owing to an air-liquid interface in the system (Fig. 8).

Dramatic enzyme inactivation even in the absence of visible air-liquid interface may be explained by a formation of microbubbles under very intensive agitation of the enzyme solution. The microbubbles are the origin of "localized" air-liquid interface inside the solution. Some localized heating (induced by electromagnetic field or generated during the FMP collisions) seems to be another deactivating factor. Independent experiments without thermostat control showed that localized heating certainly takes place in the IMTR. In these experiments, depending on the magnetic field strength and FMP loading, the temperature of the enzyme solution increased by 2–8°C after 30 min of the agitation. Though in all hydrolysis experiments (also in that shown in Fig. 8) water circulating through a thermostat jacket around a working cell ensured the withdrawal of generated heat and the constancy of enzyme solution temperature (this was shown by measuring the temperature before and after hydrolysis), localized heating still could affect the enzyme stability.

DISCUSSION

The results presented above show that parameters related to the IMTR design strongly affect the process of cellulose hydrolysis. The most important parameters are the strength of the magnetic field and FMP geometry and loading. The hydrolysis efficiency changed dramatically on transition from circular to cylindrical FMP and, in general, on transition from larger to smaller particles (Fig. 1). An exception was the 0.5 × 6 mm FMP that showed higher efficiency than the 0.36 × 4 mm FMP. However, it should be noted that this fluctuation was also reproduced under the conditions of hydrolysis different from those shown in Fig. 1.

More studies need to be performed to find a theoretical background for the observed phenomena. Probably, a more systematic approach to FMP geometry and further optimization of FMP shape and dimensions will result in an even higher enhancement of the hydrolysis. Additional intensification of the process may be expected from a further increase in the magnetic field strength. Since the construction of the IMTR used in our experiments did not allow the creation of magnetic field strength higher than 64,000 A/m, the highest possible hydrolysis rates may still not have been achieved.

The effect of the hydrolysis enhancement was dependent on substrate properties. The most pronounced effect was observed in the case of microcrystalline cellulose that had the lower initial susceptibility to cellulases (Fig. 3). For this substrate, a considerable contribution into the hydrolysis intensification was brought about by an increase in cellulose reactivity (up to 2.5 times) owing to continuous mechanical treatment of the substrate by FMP in the course of hydrolysis (*20*). In the case of skop and cellolignin, having already more amorphous structure and higher initial susceptibility to hydrolysis (Fig. 3), such effect of mechanical treatment seems to be less significant.

The enhanced mobility of cellulase molecules along cellulose surface is the most probable reason for the enzyme activation in the IMTR. A major part of the enzyme is known to be adsorbed on the surface of insoluble substrate inside its pores and capillaries (*6,10*). In an ordinary stirred bioreactor, the increase in the agitation speed basically results in the enhancement of mass-transfer processes in the outer solution (in relation to substrate particles). However, in the IMTR, owing to frequent compressions of cellulose particles during the collisions with FMP, the intensification of mass-transfer processes inside the pores and capillaries of cellulose also occurs. As a result, the enzyme molecules can more quickly migrate from one reaction site to another. The probable increase in the rate of soluble products' withdrawal from the pores to the outer solution should also positively affect the hydrolysis efficiency (glucose and cellobiose are known to be inhibitory for cellulases [*5,12,28*]).

Since shake flasks used in control experiments may not adequately represent any typical stirred-tank reactors, it would be interesting to compare the results of cellulose hydrolysis in the IMTR to those obtained in true types of reactors. Lee and Wolf (*17*), when hydrolyzing microcrystalline cellulose (80 g/L) in a regular stirred reactor (0.5 L) at the agitator speed of 200 rpm and cellulase loading of 1 FPU/mL, have obtained 10 g/L of sugars after 1 h of the hydrolysis. In an attrition bioreactor under the same conditions, they have obtained 16 g/L of sugars. In the IMTR under very similar conditions of hydrolysis (microcrystalline cellulose, 75 g/L, cellulase activity of 1 FPU/mL, 1 h, *see* Figs. 3 and 4), the product concentration was considerably higher (29 g/L), though this case does not show the highest possible hydrolysis rate in the IMTR (the FMP loading is below optimum). This comparison shows the IMTR being more efficient than other types of reactors.

The major factor negatively affecting the process of cellulose hydrolysis in the IMTR seems to be a considerable shear inactivation of enzymes that was especially pronounced in the case of β-glucosidase (Fig. 8). Lower stability of β-glucosidase under the shear conditions had a significant influence on a product composition.

It is interesting to note that, if during cellulose hydrolysis in shake flasks glucose content was higher in the case of *P. verruculosum* cellulase compared to *T. reesei* cellulase, in the IMTR, the situation was the opposite. This may be explained by the fact that different key components of the cellulase multienzyme system are responsible for glucose formation when the enzymes from *T. reesei* and *P. verruculosum* are considered. Independent experiments (not presented here) showed that the enhancement of glucose formation from the intermediate product, cellobiose, catalyzed by β-glucosidases (from different fungi) does not take place in the IMTR. Moreover, the rate of cellobiose hydrolysis decreased in the IMTR because of β-glucosidase inactivation under the shear conditions in the reactor. Therefore, in the case of the *T. reesei* cellulase system having low specific β-glucosidase activity, the pathway of glucose formation directly from cellulose (or from high molecular fragments of its destruction) may become more important (*28,29*), since the relative contribution of this pathway to the rate of soluble products' formation significantly increased with increase in the FMP loading (Fig. 6). For the *P. verruculosum* cellulase system, which had high specific β-glucosidase activity, the pathway of glucose formation from intermediate cellobiose owing to the action of β-glucosidase was predominant. Therefore, when *P. verruculosum* cellulase was used in the IMTR, only enhancement of the rate of cellobiose formation from cellulose owing to activation of cellobiose-producing enzymes could be observed.

High cellobiose content in the IMTR may be a serious problem. In a regular stirred reactor, where the hydrolysis time is usually one or several days, the β-glucosidase level of 1 CBU/mL is sufficient to provide high glucose content in the hydrolyzates (*25*). In the IMTR, however, β-glucosidase limits glucose formation. Even if this enzyme could survive under the shear conditions, such limitation would likely be observed, since the hydrolysis time in the IMTR is much shorter. Only when a relatively large amount of extra β-glucosidase is added to the IMTR (10 CBU/mL), glucose becomes a predominant product (Fig. 7).

The use of immobilized β-glucosidase may be an alternative. However, since it is hardly possible that any of the commonly used immobilization matrices can withstand high shear forces in the IMTR, an additional treatment of hydrolyzates with high cellobiose content in a separate immobilized enzyme reactor would be more reasonable.

The most serious drawback that may hinder a practical application of the IMTR is its high power consumption. In the experiments described above, the power consumption varied in the range of 0.76–1.9 kW. For example, a typical power consumption of an attrition bioreactor (0.5-L working volume) was 20–50 W (*16*); for a regular stirred reactor, the value is even lower. However, it should be noted that the high power consumption of the IMTR can be partially compensated for by the dramatically reduced time of hydrolysis process.

More studies must be performed in order to minimize the power consumption and to find optimum conditions where the highest yield of sugars can be obtained with the lowest process costs. Even if a significant process cost reduction is impossible, the results obtained in the IMTR show the potential of enzymatic cellulose hydrolysis, and they may serve as a benchmark for designing highly efficient reactors for cellulose saccharification.

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