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Cellulases and their application in the conversion of lignocellulose to fermentable sugars

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Several processes have been developed for the enzymic conversion of lignocellulose to fermentable sugars. Most of these processes have employed the cellulolytic enzyme system from *Trichoderma reesei*.

The action of a commercial cellulase preparation, Celluclast, from *Trichoderma reesei* on microcrystalline cellulose is compared with that of cellulolytic enzymes from other microorganisms. It is concluded that the *Trichoderma* system is not unique. More effective enzyme complexes can be produced from other microorganisms. In particular, systems from different *Aspergillus* species are shown to be more effective.

The enzymic conversion of lignocellulose to fermentable sugar has not yet been scaled up, undoubtedly for technical and economic reasons.

The cellulose substrate is very inaccessible, and furthermore the enzymic hydrolysis involves several consecutive reactions, each of which may be rate limiting.

Enzyme costs have a great impact on process economy. The conversion of cellulose into fermentable sugar requires approximately 100 times more enzyme protein than the corresponding hydrolysis of gelatinized starch. Therefore, with today's enzyme technology, the use of cellulose as a raw material is not competitive with processes based on starch.

Introduction

As a result of photosynthesis approximately 70 kg of lignocellulose is produced per person per day, and quite naturally this vast renewable resource has inspired numerous biotechnologists to propose processes for the conversion of cellulose into fermentable sugars that can be used either for the production of ethanol or single cells, thereby solving two serious problems facing mankind today, the energy and food shortages. During the last decades the development of many processes using enzymic conversion has been claimed, but none of these have been scaled up industrially, the reason undoubtedly being unforeseen economic and technical problems.

From an enzyme technological point of view, the hydrolysis of cellulose to glucose is an extremely difficult process because of the insolubility and the dense crystalline structure of the substrate. Further, the product glucose is formed through several consecutive reactions, each of which may be rate limiting.

Novo's Research Laboratories have been working with these problems for several years, concentrating their effort on the classical enzyme system produced by *Trichoderma reesei*. However, enzyme systems from other microorganisms have also been investigated. Microcrystalline cellulose, Avicel, was used as a model substrate because of its availability in a uniform standardized quality.

The problems that are essential for a nearly complete hydrolysis will be discussed in this paper. The conclusion is that an industrial process based on native crystalline cellulose is not economically feasible at present. A physical or chemical pretreatment of the substrate may increase the reaction rate, but has a negative impact on the process economy.

The structure of cellulose

Cellulose is an unbranched polymer containing glucose molecules linked by 1,4-β-glucosidic bonds. The glucose molecules are rotated through 180° in relation to each other, and the repeating unit in the glucan is therefore not glucose but cellobiose (see figure 1). All of the glucose molecules are in the chair conformation, and because of the β configuration all of the hydroxyl groups are in an equatorial position. This explains why cellulose has a layered structure where the single chains in a layer are bonded together by intermolecular hydrogen bonds. The forces holding the layers together are of van der Waals character.

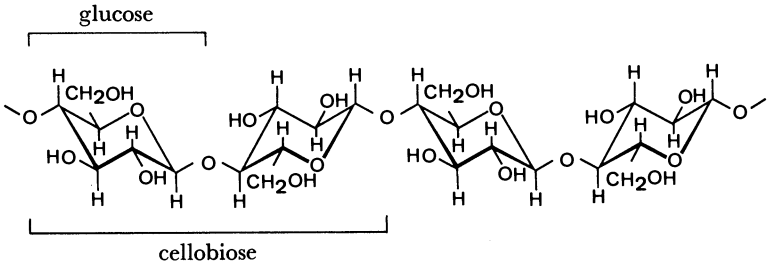

Figure 1. Conformation of the cellulose molecule.

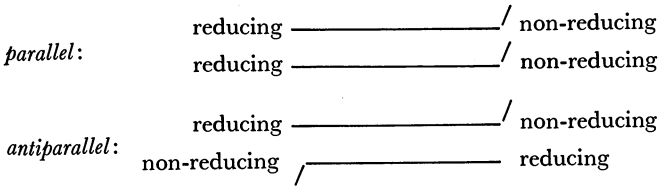

Figure 2. Conformation of the cellulose fibril.

In pure native cellulose, e.g. cotton fibres, the chains lie in parallel so that the reducing ends are adjacent to each other, but by chemical treatment rearrangement can take place, allowing a reducing end of one chain to be adjacent to a non-reducing end of another chain, a so-called antiparallel configuration (see figure 2).

The 1,4-β-glucan chain does not exist as such in Nature, the smallest unit being the elementary fibril with a diameter of approximately 3 nm. The elementary fibrils are bonded into microfibrils with a diameter of 10–40 nm, and these are again bonded into macrofibrils visible in a light microscope. Further, the structure of the microfibrils and macrofibrils is strengthened by a matrix of hemicelluloses and lignin.

In native cellulose most of the fibrils are in a crystalline state. The exact degree of crystallinity is difficult to estimate; it may vary with the source and the treatment of the material. Several models have been proposed to explain the occurrence of amorphous parts in otherwise totally crystalline cellulose. The basis of most of these models is the view that the glucan chains are folded.

It is quite obvious that the very dense structure of cellulose and the presence of the hemicellulose and lignin matrixes make diffusion of cellulolytic enzymes into the fibrils extremely difficult. The hydrophobic character of the 1,4-β-glucan chains may also contribute to the very low reaction rate observed. With regard to diffusion resistance it should be mentioned that the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

distance between parallel layers of the glucan chains is of the same order of magnitude as the average diameter of most cellulolytic enzymes.

Granular starch (showing partial crystallinity) is composed of glucan chains similar to those of cellulose, but with the difference that the glucose molecules are linked by 1,4-α and 1,6-α-glucosidic bonds. It is strongly hydrophilic and should therefore be easier to hydrolyse enzymically. However, experiments have shown that under reaction conditions acceptable on an industrial scale it is impossible to achieve a degree of hydrolysis of granular starch greater than 30–40 %. By carrying out a simultaneous alcohol fermentation, thereby constantly removing the glucose formed, one can obtain 100 % hydrolysis.

A starch hydrolysis process, where the energy-demanding process steps of gelatinization and liquefaction could be avoided, would be very attractive to the industry, but so far no such process has been transferred to the large scale because of the very high enzyme dosages required. This comparison gives rise to rather pessimistic thoughts about the feasibility of enzymic hydrolysis of cellulose without prior physical and/or chemical treatment.

The enzyme complex from *Trichoderma reesei*

In the last decade many workers have investigated the composition and the technical application of the cellulolytic enzyme complex produced by *Trichoderma reesei*. At Novo, Schülein *et al.* (1980) have characterized a crude preparation from this strain, Celluclast, by immunochemical methods. More than 20 antigenic components could be identified by crossed immunoelectrophoresis (figure 3). The enzymically active components were separated by ion-exchange chromatography on DEAE-Sephadex A-50 (see figure 4), and the pooled fractions analysed for enzymic activity. They were also characterized by immunoelectrophoresis with the use of polyspecific as well as more specific antisera.

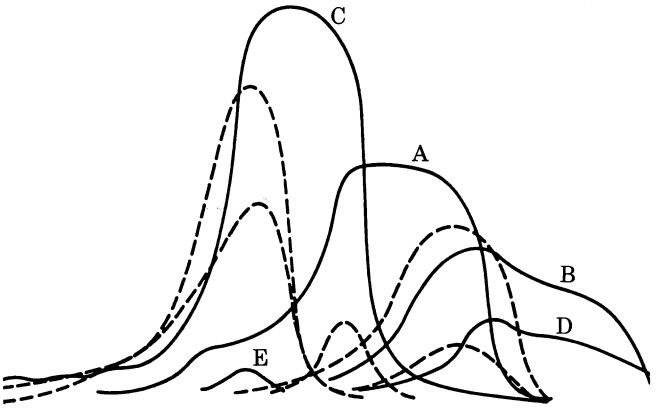

Figure 3. Crossed immunoelectrophoresis of Celluclast. First dimension: 90 min, 10 V cm^{-1} in agarose (10 g l^{-1}) in veronal buffer, pH 8.6, ionic strength 0.02; anode to the left; antibodies: 0.5 ml rabbit anti-Celluclast serum plus 11 ml agarose. Second dimension: 20 h, 1 V cm^{-1}, 15 °C, anode at the top; staining Coomassie brilliant blue R; the letters indicate the major antigenic components.

The investigations confirm the results obtained by others, namely that the enzymes in the complex can be divided into three groups (Petterson *et al.* 1979): endoglucanases, exoglucanases or cellobiohydrolases, and cellobiases or β-glucosidases. Two endoglucanases, two cellobiohydrolases and one glucosidase were isolated and characterized with regard to their activity towards

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

the different substrates (table 1). The molecular masses were in the range 45–65 kDa, and the isoelectric points between 4 and 7.

Later investigations have indicated that one of the endoglucanases (enzyme A) might be an exoglucosidase that splits off glucose from the non-reducing end of the glucan. The approximate composition of the protein in Celluclast is: endoglucanase A or exoglucosidase, 10 %; endoglucanase B, 10 %; cellobiohydrolase C, 55 %; cellobiohydrolase D, 10 %; cellobiase E, 1 %; enzymically inactive protein, 15 %. Cellobiohydrolase C is therefore the main component, and

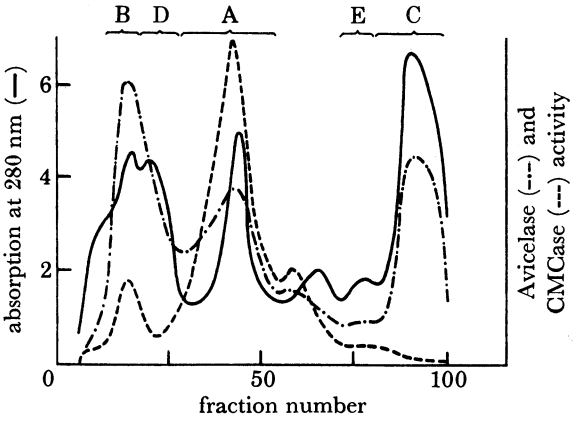

FIGURE 4. Ion-exchange chromatography of Celluclast on DEAE-Sephadex A 50. Conditions: 0.05 M Tris buffer, pH 7.0, with a 0–0.2 M NaCl gradient; the fractions were analysed for cellulolytic activity by using Avicel and carboxymethylcellulose (CMC) as substrates (Avicelase and CMCase activity).

TABLE 1. CELLULOLYTIC ACTIVITY OF DIFFERENT ENZYME FRACTIONS ISOLATED BY ION-EXCHANGE CHROMATOGRAPHY OF CELLUCLAST

enzyme	type of enzyme	CMCase activity units mg^{-1} protein	Avicelase activity units mg^{-1} protein	CMCase/ Avicelase
A	endoglucanase	103	1.0	103
B	endoglucanase	16	1.6	10
C	exoglucanase	3	0.8	4
D	exoglucanase	15	1.7	9
E	β-glucosidase	8	0.4	20

it is observed that the complex contains only minute amounts of cellobiase, which is characteristic for preparations produced from *Trichoderma reesei*. To obtain full conversion to glucose it is therefore necessary to add cellobiase from another microorganism, e.g. from *Aspergillus niger*.

Celluclast has been tried out in numerous hydrolysis experiments with Avicel as a substrate, and a characteristic experiment at optimal pH and temperature conditions, pH 5 and 50 °C, over 24 h with 20 % substrate, is shown in figure 5. Celluclast alone, even at high concentrations, can only hydrolyse 10 % of the cellulose, but if cellobiase is added approximately 25 % hydrolysis can be obtained. This indicates that cellobiose has a product-inhibiting effect that can be compensated for by the addition of cellobiase so that only small amounts of the disaccharide are present in the reaction mixture. At lower substrate concentrations higher degrees of hydrolysis can be obtained; however, from a practical point of view this is less interesting. In experiments with acid- and base-treated straw, even lower degrees of hydrolysis were

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

observed with a Celluclast–cellobiase mixture. Thus on the basis of our experiments we consider it doubtful whether the enzyme complex from *Trichoderma* combined with cellobiase can be used for the complete hydrolysis of native crystalline cellulose.

Enzyme complexes from other microorganisms

For many years attempts have been made to produce cellulolytic enzyme systems from other microorganisms than *Trichoderma* species, the aim being to find systems giving a more complete hydrolysis. In Nature, lignocellulose is degraded over a time span of 1–10 years; there are three main types of degradation, i.e. white rot, brown rot and soft rot. White and brown rot are mainly caused by basidiomycetes while soft rot, which is a much slower process, is mainly caused by ascomycetes. Certain bacteria also produce cellulases.

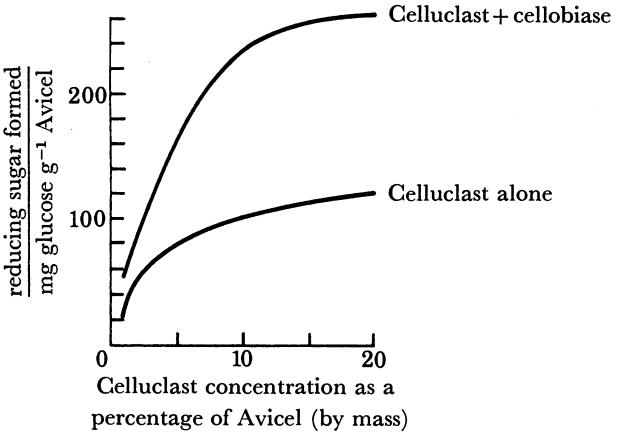

Figure 5. Hydrolysis of Avicel with Celluclast and Celluclast + cellobiase at different concentrations. The reaction conditions were: substrate concentration, 20% (by mass) Avicel; pH 5; temperature 50 °C; time of hydrolysis, 24 h. In the experiment with Celluclast + cellobiase the concentration of cellobiase was 20% (by mass) of the Celluclast concentration.

In a screening programme performed at Novo, strains from 27 different species were investigated for cellulase production: 18 of these produced large enough amounts of enzyme for further investigations to be carried out. Of the 18 strains, 14 were ascomycetes, 2 basidiomycetes, and 2 aerobic bacteria.

The most active preparations were investigated for their ability to hydrolyse Avicel at 50 °C and pH 5 for 24 h (see table 2). It appears that the preparations from *Aspergillus* sp. A and sp. B are by far the most effective, which may indicate that they are not product-inhibited to the same extent as the other preparations. It is interesting to note that a combination of the *Aspergillus* sp. A enzyme and Celluclast in only half the concentration of the first enzyme system gives the same degree of hydrolysis. Here we see an example of synergism between two enzyme systems.

By increasing the time of hydrolysis to 48 h under the same reaction conditions (see table 3), it is possible to obtain approximately 50% hydrolysis.

It can be concluded that it is possible to find microorganisms producing more effective enzyme systems than that of *Trichoderma reesei*. However, it is still doubtful whether one can find enzymes giving more than 90% hydrolysis, which must be the goal if an industrial production of fermentable sugars from native cellulose is to be possible.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 2. HYDROLYSIS OF AVICEL FOR 24 h WITH DIFFERENT CELLULOLYTIC ENZYMES

(Reaction conditions: 50 °C pH 5, 20% (by mass) substrate concentration.)

enzyme	strain	enzyme dosage (percentage of Avicel by mass)	hydrolysis (%)
KRF 68	*Aspergillus* sp. A	20	35
KRF 83	*Aspergillus* sp. B	20	34
SP 227	*Humicola insolens*	20	20
SP 129	*Myceliophtora thermophile*	20	20
KRF 55	*Sporotrichum pulverulentum*	20	17
KRF 71	*Irpex lacteus*	20	21
SP 116	*Trichoderma harzianum*	20	15
Celluclast	*T. reesei*	20	12
Celluclast + cellobiase	*T. reesei* + *Aspergillus niger*	20 + 4	26
KRF 68 + Celluclast	*Asp.* sp. A + *Trichoderma reesei*	5 + 5	34

TABLE 3. HYDROLYSIS OF AVICEL FOR 48 h WITH CELLUCLAST, CELLUCLAST + CELLOBIASE OR KRF 68 AT 50 °C, pH 5 AND 20% (BY MASS) AVICEL SUBSTRATE CONCENTRATION

enzyme	enzyme dosage (percentage of Avicel by mass)	hydrolysis (%)
Celluclast	20	20
Celluclast + cellobiase	20 + 4	36
KRF 68	20	50
KRF 68 + Celluclast	10 + 10	48
KRF 68 + Celluclast	5 + 5	41

REACTION MECHANISMS

Many models for the degradation of native cellulose have been proposed, for example by Wood (1975), Fan *et al.* (1980) and Klyosov (1980). Our results from experiments with enzyme systems from *Trichoderma* and other microorganisms fit well with this last model, which is illustrated in figure 6. The breakdown of cellulose to glucose can follow several reaction paths. Different microorganisms produce different cellulolytic enzymes with different substrate specificities. Further, the catalytic activity of enzymes hitherto investigated is to a varying degree subject to substrate and product inhibition. Three reaction paths leading to glucose are possible: direct with exoglucosidase; combined action of endoglucanase, cellobiohydrolase and cellobiase; first endoglucanase, then exoglucosidase.

As an example can be taken a preparation with only little cellobiase. Cellobiose will be accumulated in the reaction mixture, and we have a reaction pattern as shown in figure 7. If the rate-limiting enzyme – in this case cellobiase – is added, the reaction will be as shown in figure 8, and now the other enzymes, endoglucanases and cellobiohydrolases, are the rate-limiting factors.

The Klyosov model also explains the synergistic effect observed when enzyme systems from different microorganisms are mixed. Even very closely related microorganisms such as *Trichoderma reesei* and *Trichoderma harzianum* produce enzyme systems showing synergism when mixed. If equal amounts of the two systems are mixed, more cellulose will be hydrolysed than when

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

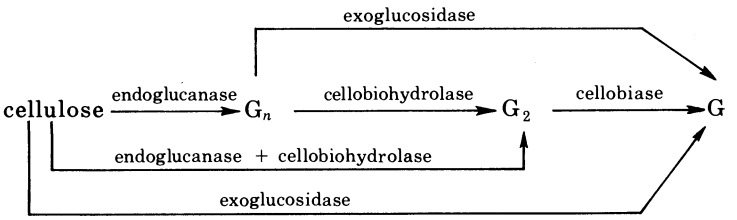

FIGURE 6. Model according to Klyosov for the breakdown of cellulose. G, glucose.

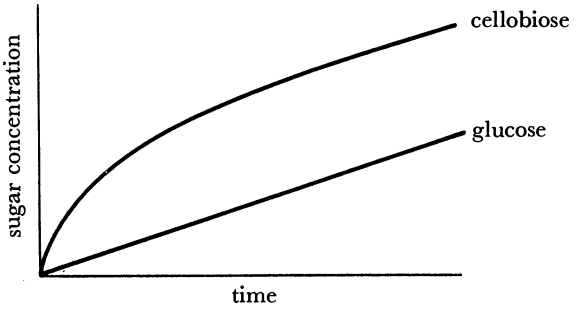

FIGURE 7. Hydrolysis of cellulose by a cellulolytic enzyme complex in which cellobiase is the limiting enzyme component.

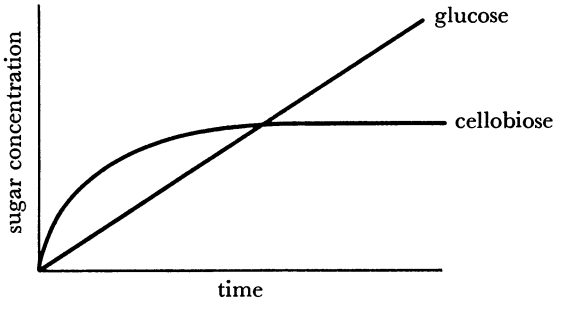

FIGURE 8. Hydrolysis of cellulose by a cellulolytic enzyme complex to which the limiting enzyme component cellobiase has been added.

applying double the amount of one of the enzyme systems. This indicates that several reaction paths exist, or that the different components are rate-limiting at different stages of the reaction.

PROCESS ECONOMY

The conclusion of our investigations is that by using high concentrations of the best enzymes developed, either alone or in combination, about 50 % degradation of native cellulose can be reached at 50 °C, pH 5, 20 % (by mass) substrate concentration, and a reaction time of 48 h. The degree of hydrolysis can be improved by constantly removing the glucose, either by using a membrane reactor or by a simultaneous ethanol fermentation. However, these solutions will hardly be practicable on the industrial scale, and thus it appears that an industrial process must include a pretreatment of the lignocellulose to make the cellulose more susceptible to enzymatic degradation, a point of view that is now shared by most biotechnologists. But it is still unknown whether this leads to an economic process.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

It is generally accepted that one of the decisive factors in the process economy is the enzyme cost. There is still room for reducing costs of enzyme production by mutations leading to hyper-producers, by optimization of fermentation and enzyme composition, and finally by screening for new and better strains. However, the starting point for optimization is very unfavourable: it can be calculated that to hydrolyse crystalline cellulose an amount of enzyme protein is required that is approximately 100 times that needed for the hydrolysis of starch. Assuming that

Table 4. Flow sheet for the Scholler–Tornesch process

process step	raw materials	products formed	heat consumption GJ
hydrolysis, 10 atm, 170 °C	wood d.m.: 1000 kg H_2SO_4: 130–450 kg water: 12000 l	sugars: 530 kg (4% solution (by mass))	5.9
↓ neutralization	$CaCO_3$: 133–460 kg	gypsum, CO_2	—
↓ three-stage evaporation	—	sugars: 530 kg (20% solution (by mass))	8.4
↓ fermentation and distillation	—	ethanol: 240 l	1.3

Table 5. Economics of the Scholler–Tornesch process

(Basis of calculation: (1) 1 ton of coal yields *ca.* 33 GJ (U.S. $ 6.1 GJ^{-1}); (2) coal-to-steam conversion efficiency is 76%; (3) cost of steam is U.S. $ 8.0 GJ^{-1}; (4) cellulose yields 17 MJ kg^{-1}; (5) costs of hydrolysis equipment are not included.)

	quantity	unit price U.S. $	cost U.S. $	distribution (%)
wood dry matter†	1000 kg ≈ 10.64 GJ	6.1 GJ^{-1}	64.9	29–26
sulphuric acid	130–450 kg	0.08 kg^{-1}	10.4–36.0	5–14
calcium carbonate	133–460 kg	0.019 kg^{-1}	2.5–8.7	1–3
heat of hydrolysis	5.86 GJ	8.0 GJ^{-1}	47.0	21–19
heat of evaporation	8.37 GJ	8.0 GJ^{-1}	67.2	31–26
fermentation and distillation			28.5	13–11
	costs per 240 l of ethanol:		220–254	
	cost per litre of ethanol:		0.92–1.05	

† Cost calculated on the basis of the combustion energy of cellulose. Value of lignin and hemicellulose not included.

the production costs for enzyme protein are the same for cellulases and amylases, a tremendous development effort is necessary to make enzymic cellulose hydrolysis economically feasible.

The present market price for fuel alcohol in the U.S. is approximately U.S. $ 0.46–0.48 per litre, and the process economy when using cellulose as a raw material instead of starch will not be competitive with the actual stage of enzyme technology.

It is characteristic that the only processes for the production of ethanol on the basis of lignocellulose on an industrial scale were developed in Germany before World War II. They were based on a war economy and would not have been contemplated under normal economic conditions.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

One of the best-known processes is the Scholler–Tornesch process, for which a flow sheet is shown in table 4. We have tried to calculate the process economics without including investments for an acid hydrolysis plant, but as can be seen from table 5 the variable costs will, even under these favourable conditions, be about U.S. $ 0.9–1.03 per litre of ethanol. In a future process including chemical pretreatment as well as enzymic hydrolysis there will be no room at all for enzyme costs.

Conclusion

The use of lignocellulose for the production of ethanol or other chemical feedstocks is one of the most difficult tasks encountered in the history of biotechnology. Cellulose has a very dense structure and is shielded by hemicelluloses and lignin, so it is very difficult to degrade enzymically. The enzyme systems to be used must contain at least three enzyme components in adequate amounts, which makes production optimization extremely difficult. It is therefore doubtful whether the enzyme production costs can be reduced by one or two orders of magnitude. Consequently it may be necessary to subject the lignocellulose to a pretreatment before the enzymic hydrolysis, thus increasing the reaction rate to such a degree that the dosage of enzymes can be drastically reduced. Against this background the outlook for enzymic cellulose hydrolysis is rather bleak, and only a dramatic breakthrough can change this picture.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES