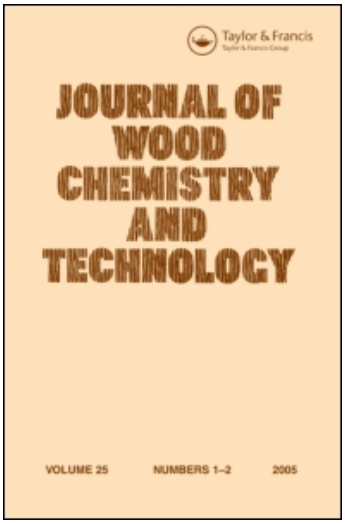

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ENZYMATIC CONVERSION OF LIGNOCELLULOSIC MATERIALS TO SUGARS

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ABSTRACT

Cellulase was produced by cultivating T. reesei QM 9414 and MCG 77 on spruce sulfite pulp. The solid enzyme prepared by acetone precipitation had 0.73 FPU/mg, 0.2 units ß-glucosidase/mg and 10 units xylanase/mg. The average molecular weight was 48000 dalton. Analytical and preparative (20 mg) separation of the cellulase by chromatofocusing revealed the presence of 18 different proteins. Identified were: two exocellulases (C_1-enzymes), five endocellulases (C_x-enzymes), one ß-glucosidase, one galactomannase, two xylanases and one ß-xylosidase.

The cellulase was used to hydrolyze cellulose (spruce sulfite pulp) and various lignocellulosic waste materials (newspaper, wheatstraw, cornstover, wood and reed grass a.o.). With 10, 5, 2.5, 1 and 0.5% cellulose slurries percentage of saccharification (24 hours) was 25, 42, 45, 53 and 69 %. Saccharification increased with increasing enzyme concentration up to a level of 0.60 FPU/ml and 0.36 units ß-glucosidase/ml. A further increase of either one of the enzymes did not improve hydrolysis. The various lignocellulosic materials were mechanically pretreated and yield of hexoses upon enzymatic hydrolysis (10 FPU/g, 50°C, pH 4.8, 48 - 75 hours) was on average 50 - 60% of the theory.

The sugars in raw materials and hydrolysates were determinated by capillary GC as alditol acetates or by HPLC. These methods were preferred since other determinations gave either too high (dinitrosalicylic acid procedure) or too low (glucose oxidase assay) values when employed with lignocellulosic hydrolyzates.

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INTRODUCTION

The conversion of lignocellulosic material to fermentable sugars requires three basic processes, i.e. enzyme (cellulase) production, pretreatment of the raw material and hydrolysis. If the glucose solution is fermented by yeast to ethanol, saccharification and fermentation can be carried out simultaneously as proposed in the "SSF process" developed by Emert (1). Many cellulolytic microorganisms (Clostridia, Actinomyces, Trichoderma, Penicillium, Sporotrichum, Fusarium, Aspergillus) are known to produce cellulose degrading enzymes and should therefore in principle be suitable for this process. Nearly all technically relevant studies in this field, however, have been performed with strains of Trichoderma reesei.

This fungus secretes large quantities of a complete cellulase system which is able to degrade crystalline cellulose. The isolation of the wild type of Trichoderma QM 6a and the consequent strain improvement by mutation and selection programs must be credited to the research team at the US Army Research and Development Center in Natick, USA, in particular to Dr. Reese. The selection program performed by this institution led to the well-known mutant T. reesei QM 9414 and to the highly productive strain T. reesei MCG 77 (2,3) which were also used in the fermentation experiments described in this paper (The T. reesei MCG 77 is now patented through US Patent 4.275.163 assigned to USA as represented by the Secretary of the Army; inventor: B. J. Gallo). The mutation program performed at the Rutgers University led to another highly productive strain now classified as T. reesei Rutgers C 30 (4).

The great interest in cellulose utilization and the anticipated significance of this main renewable resource for future technology is clearly indicated by the increasing number of publications. While in the period 1970 - 1975 about 110 papers related to enzymatic cellulose conversion and similar subjects were published each year, the number of publications rapidly increased after the first energy crisis reaching about 210 publications in 1980 (Fig. 1). Within a few years significant advances were made in

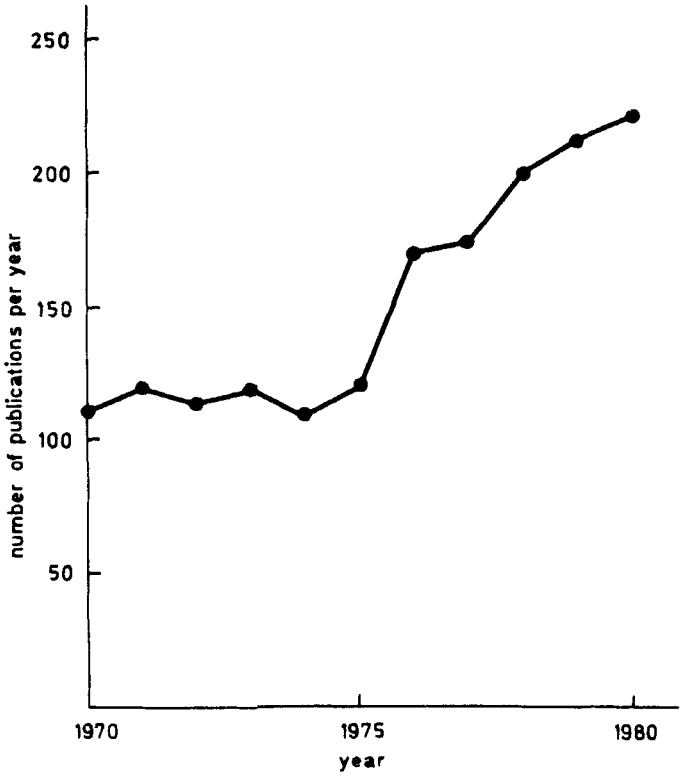

FIGURE 1: Number of publications related to enzymatic hydrolysis of lignocelluloses during the period 1970 - 1980.

this field and many review articles covering the various aspects of enzymatic cellulose utilization have already been published (5 - 9).

We report in this article on our own experience in enzyme production, enzyme characterization and enzymatic hydrolysis of various lignocellulosics available in this country as well as on sugar analysis in the hydrolyzates.

EXPERIMENTAL

Cultivation of T. Reesei

Cultures of T. reesei QM 9414 or MCG 77 were grown in a stirred 5 l fermenter with 2% bleached spruce sulfite pulp as carbon

source. The pulp was oven dried (110°C) and milled to a particle size of 0.25 mm. The mineral medium was a slightly modified Mandels' medium (2), pH control was performed with 3% ammonia and 3% sulfuric acid. After a fermentation time of 100 to 150 hours the mycelium was separated by coarse filtration followed by centrifugation. The clear enzyme solution was used for all further investigations.

Preparation of Solid Cellulase Powder

The clear enzyme solution was concentrated ten fold by ultrafiltration using an Amicon PM 10 membrane with an exclusion limit of 10.000 dalton. The protein was then precipitated by addition of two volumes of acetone at 4°C. After 12 hours at 4°C the protein precipitate was recovered by centrifugation at 3000 x g, transferred to a 1 l round bottom flask and dried under reduced pressure on a rotary evaporator at 20°C to remove most of the acetone. The residue was dissolved in water and freeze dried. The cellulase prepared in this way was a dry, white powder. In some experiments the cellulase protein was precipitated by addition of three volumes of isopropanol at 4°C. The subsequent work up procedure in this case was the same as used for the acetone precipitate.

Enzymatic Saccharification

Bleached spruce sulfite pulp was air dried and milled to 0.25 mm and suspended in 0.05 M citrate buffer pH 4.8 - 5.0 at the concentrations indicated in the text. Cellulase enzyme was added in the form of the clear solution harvested from the culture broth or as acetone dried powder. For most experiments the enzyme : substrate ratio was 10 FPU/g substrate. Saccharification was carried out at 50°C with or without shaking for periods between 24 to 50 hours. In most cases the incubation mixture contained 0.04% thimerosal as biocide to avoid growth of microorganisms. The sugars were analyzed by HPLC or GC. The bleached spruce sulfite pulp used in the hydrolysis experiments contained, as reported previ-

ously (13), 83.7% glucan, 7.2% mannan, 5.1% xylan and 0.8% benzene/ethanol extractibles. The theoretical glucose yield is therefore 93 g glucose per 100 g dry sulfite pulp. The % saccharification was calculated according to:

$$\% \text{ saccharification} = \frac{\text{mg glucose/ml + mg cellobiose/ml}}{\text{mg initial pulp/ml x 0.93}}$$

where glucose/ml, cellobiose/ml are the values obtained by HPLC. In experiments with lignocellulosic materials the % saccharification was related to the dry matter only.

Analytical Chromatofocusing

The cellulase powder isolated by isopropanol precipitation was dissolved in distilled water, desalted by a Sephadex G 25 column (25 x 2.6 cm) and freeze dried. 4 mg of the dry salt free material were then dissolved in 0.2 ml of 0.025 M histidine/HCl buffer pH 6.3 and separated by fast protein liquid chromatography (FPLC) on a Mono P HR5/20 column (Pharmacia) filled with Polybuffer exchanger PBE 94. The start buffer was 0.025 M histidine/HCl pH 6.3; the elution was performed by pumping 40 ml Polybuffer 74, pH 4.0 followed by 50 ml Polybuffer pH 3.0 through the FPLC column. The flow rate was kept at 1.0 ml/min, the upper pressure limit of the HPLC pump was set to 800 psi. The Polybuffer 74 (Pharmacia) used was diluted 1:9 with water and the pH was adjusted with HCl. The column effluent was monitored at 280nm to obtain the protein profile and fractionated, if desired, into fractions of individual peaks identified by the UV absorbance. The distribution curve of the specific enzyme activities was determined by performing the various enzyme assays with fractions or pooled fractions of the column effluent. These test were carried out without removing the Polybuffer and it was therefore carefully controlled that the Polybuffer did not change the pH of the enzyme test medium.

Preparative Chromatofucusing

20 - 30 mg of the Sephadex G 25 desalted protein were applied to the FPLC column and separated as described above for the analy-

tical runs, except that 70 ml Polybuffer pH 3.0 were used instead of 50 ml; the buffer was diluted 1:14. The protein distribution profile was measured by monitoring the column effluent at 280 nm. The effluent was collected in 2 ml fractions. Based on the UV absorbance peak fractions (see Fig.4) were pooled and freed from Polybuffer by precipitating the protein with 80% ammoniumsulfate. The protein precipitate was recovered by centrifugation, washed twice with 80% ammoniumsulfate, dissolved in water and desalted on a Sephadex G 25 column. The salt free solution was lyophilized to dryness. From 50 mg crude cellulase the yield of the major single protein (= exocellulase represented through peak K in Fig. 4) was 20 mg. This material was a white, dry powder.

Enzyme Assays

The *filter paper activity* was estimated principally as described by Mandels et al. (10). One milliliter 0.05 M citrate buffer pH 4.8 + 50 mg Whatman No. 1 filter paper + 0.5 ml enzyme sample solution were incubated 1 hour at 50°C. The reducing sugars were then estimated by the dinitrosalicylic acid (DNS) procedure (11). The incubation mixture (1.5 ml) was mixed with 3.0 ml DNS reagent, heated 5 min on a boiling water bath and the absorbance was measured in 1 cm cuvettes at 575 nm. The original enzyme was always used in such a dilution that the absorbance at 575 nm was between 0.5 and 1.0 corresponding to a saccharification of 0.5 - 1 %. The sugar content (mg glucose) was calculated from glucose standards subjected to the same procedure. FPU/ml parent enzyme solution = mg glucose x 0.185 x dilution factor. One FPU corresponds to the formation of 1 µmol glucose/min.

ß-Glucosidase activity was measured with p-nitrophenyl-ß-D-glucoside as substrate essentially as described by Herr et al. (12), except that pH was 4.8 and temperature was kept at 50°C. The absorbance of the liberated p-nitrophenol was measured at 405 nm and its concentration was calculated using an ε-value of 18500. One unit ß-glucosidase is the amount of enzyme which forms 1 µmole p-nitrophenol/min.

ß-Xylosidase activity was measured like ß-glucosidase, but with o-nitrophenyl-ß-D-xylanoside as substrate.

Xylanase activity was assayed by incubating 1 ml 1% xylan solution in 0.05 M citrate pH 4.8 + 0.5 ml enzyme sample solution at 50°C for 30 min. After adding 3 ml DNS reagent, the solution was heated for 5 min on a boiling water bath, cooled and the absorbance at 575 nm was measured against a blank without enzyme. The xylan stock solution was prepared from 1 g larchwood xylan (Sigma) +100 ml 0.05 M citrate buffer pH 4.8; the mixture was boiled for 5 min, cooled and mixed with 0.02% thimerosal. The xylose content was calculated based on the absorbance of xylose standards subjected to the same procedure. The enzyme solution was used in such a dilution that the sugar yield was about 1 mg xylose corresponding to a 10% saccharification. Units xylanase/ml parent enzyme solution = mg xylose formed x 0.444 x dilution factor. One unit is defined as the amount of enzyme producing 1 µmole xylose/min.

Galactomannase activity was measured like xylanase activity, except that locust bean gum (galactomannan) was used as substrate for the enzyme.

Endocellulase activity (C_x activity, carboxymethylcellulase) was assayed by measuring the reducing sugars liberated from CMC. 1.0 ml carboxymethylcellulose in 0.05 M citrate buffer pH 4.8 + 0.5 ml enzyme solution were incubated at 50°C for 1 hour. After adding 3 ml DNS reagent the solution was heated at 100°C for 5 min. From the absorbance at 575 nm the amount of reducing sugars was calculated as glucose equivalents. Glucose standards were used for the calculation. Endocellulase units/ml parent enzyme solution = mg glucose x 0.185 x dilution factor. One endocellulase unit is the amount of enzyme necessary to produce 1 µmole reducing sugar/min.

Exocellulase activity (C_1 activity, cellobiohydrolase) was measured by following the formation of cellobiose from Walseth cellulose. 2.3 ml of an approx. 5% suspension of phosphoric acid swollen cellulose in 0.05 M citrate pH 4.8 + 0.3 ml enzyme solution were incubated at 50°C for five hours. The sugar mixture was

then analyzed by HPLC on a Bio Rad HPX 87 P column. Enzymes which produced as major product cellobiose with only trace amounts of glucose or cellodextrines were defined as exocellulases. No attempt was made to express the activity of these enzymes in terms of international units.

Sugar Analysis

The individual sugars (cellobiose, glucose, xylose, arabinose) present in the enzyme hydrolyzates from cellulose or lignocellulosics were commonly determinated by HPLC (13) using the Bio Rad HPX 87P column and water at 80°C as eluant. The column effluent was monitored with a refractive index detector. For analysis of solutions containing a complex mixture of sugars in great varying concentrations capillary GC was preferred to HPLC. The sugars were converted to the trimethylsilyl derivatives (14) and separated on a 50 m SE 30 fused silica column as described (13). In some cases the sugars were also converted into the alditol acetates (15) and separated on a 50 m CP Sil 88 glass column. The temperature program was 150 - 200°C with 10°/min, 200 - 240°C with 5°/min and 25 min at 240°C; FID detector 250°C; injection was done with a programmed temperature injection (PTV) system) set at 50°C for 9 sec and then heated ballistically to 250°C. For the GC analysis of the alditol acetates the parent sugar solution was spiked with ribose as internal standard.

To determinate the sugars of the various lignocellulosic raw materials the dilute sulfuric acid hydrolysis described by Wilke et al. (16) was used. A portion of the hydrolyzate was neutralized with $CaCO_3$ and the sugars were separated by capillary GC either as trimethylsilyl derivatives or as alditol acetates.

Other Analysis

Protein was determinated with the Biuret method calibrated with bovine serum albumine.

Polyacrylamide electrophoresis was carried out on flat gels with tris/borate buffer pH 8.9: SDS electrophoresis was done in a

similar way except that 0.1% sodium dodecylsufate was included in the buffer. The protein samples were boiled for 5 min in buffer containing 1% SDS and 1% mercaptoethanol.

Average molecular weight, molecular diameter and effective hydrodynamic volume of the cellulase complex were obtained from light scattering measurements.

RESULTS

Enzyme Production

The cellulase enzyme complex was produced by cultivating T. reesei QM 9414 or MCG 77 on bleached spruce sulfite pulp in a 5 l fermenter. A typical fermentation profile is shown in Fig. 2. The enzyme titer in the culture broth was followed by measuring the filter paper activity (FPU/ml) and the ß-glucosidase activity. The enzyme titers increased rapidly when most of the cellulose substrate had been consumed. The final titer was dependent on the initial cellulose concentration. With 1%, 2.7% and 6% cellulose pulp, QM 9414 produced 1.4, 2.3 and 3.5 FPU/ml. Similar results were found for MCG 77. By variation of the pH and temperature profile during the fermentation and changes in the medium composition the enzyme production could be improved. With the strain MCG 77 the best solution so far produced had 5.2 FPU/ml and 2.80 ß-glucosidase units per ml and a protein concentration of 8.0 mg/ml. The physical structure of the cellulose material which is used as substrate for enzyme production seems to be of great importance for the cellulase yield. For example, fresh pulp (wet from the pulp mill, never dried) was not as good as substrate as oven dried (110°C) pulp. This may depend on the fact that drying leads to a hornification of the cellulose fibers, which renders them less accessible for the fungus. This reduced accessibility appears to induce the microorganism to a higher secretion of the cellulose degrading enzymes. Untreated wheat straw, cornstover or newspaper were poor substrates for enzyme production, whereas cardboard was nearly as good as sulfite pulp.

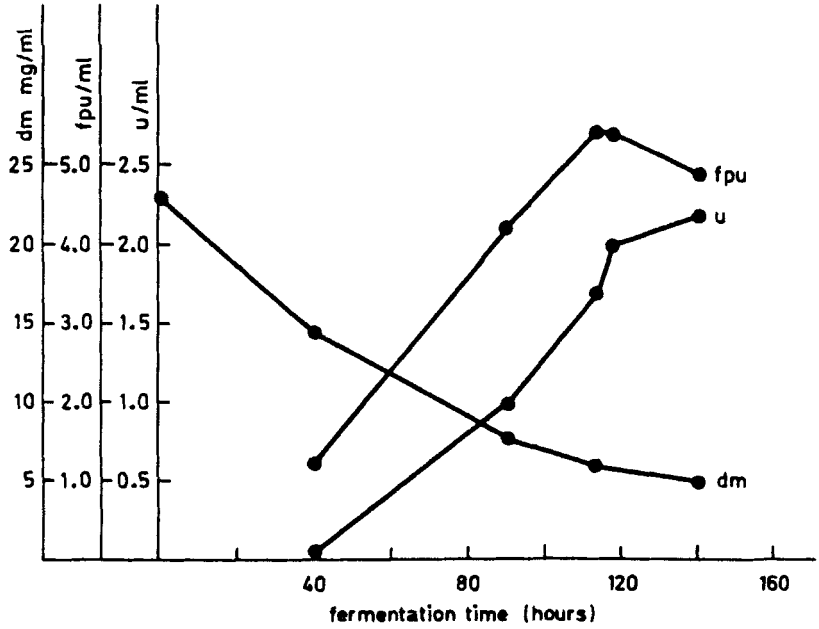

FIGURE 2: Typical fermentation profile for T. reesei MCG 77 on 2% spruce sulfite pulp in a 5 l fermenter. fpu= filter paper units, u = ß-glucosidase, dm = dry matter (cellulose + mycelium).

Enzyme Characterization

For biochemical and physicochemical studies the culture broth was separated from the mycelium by coarse filtration and centrifugation. The protein could be quantitatively precipitated with acetone or ispropanol in the cold and recovered as dry, powdered material by lyophilization. Some biochemical data on the enzyme prepared in this way are summarized in Tab. 1. A comparison of our preparation with 11 other products commercially available shows a remarkable variation of enzyme activities in different products. Differences in experimental results obtained by different laboratories may therefore rely to some extent on the fact that the different cellulase products show a great variation in their composition.

TABLE 1

Product Parameters of the Solid Cellulase Enzyme Prepared by Acetone Presipitation from Cultures of T. Reesei MCG 77 and Comparison with 11 Other Commercially Available Cellulase Preparations.

	T.r. enzyme	other enzymes (range)
solubility in water (mg/ml)	40	5 - 50
colour	white	white - brown
protein content (%)	96	18 - 43
filter paper units (FPU/mg)	0.73	0.02 - 0.24
ß-glucosidase (U/mg)	0.16	0.01 - 0.67
xylanase (U/mg)	9.8	0.1 - 20
CMC activity (U/mg)	4.9	not estimated
average molecular weight	48.000	"
average molecular diameter (Å)	65.8	"
effective hydrodynamic volume (cm^3)	1.49×10^{-19}	"

Further experiments aimed at an analytical and preparative separation by chromatofocusing of the enzyme complex into its individual components. Fig. 3 demonstrates the result obtained by an analytical chromatofocusing experiment. The protein profile (absorption at 280 nm) shows at least 18 different protein peaks. The major peak, eluted at about 70 min, represents about 60% of the total protein material in our cellulase preparation. For identifying the distribution of the various specific enzyme activities the column effluent was fractionated and each fraction was assayed for exocellulase (C_1-enzyme, cellobiohydrolase), endocellulase (C_x-enzyme, carboxymethylcellulase), ß-glucosidase, xylanase, galactomannase and ß-xylosidase. As indicated in Fig. 3, nine of the protein peaks could be definitely ascribed to specific enzyme activities. The enzymes identified are:

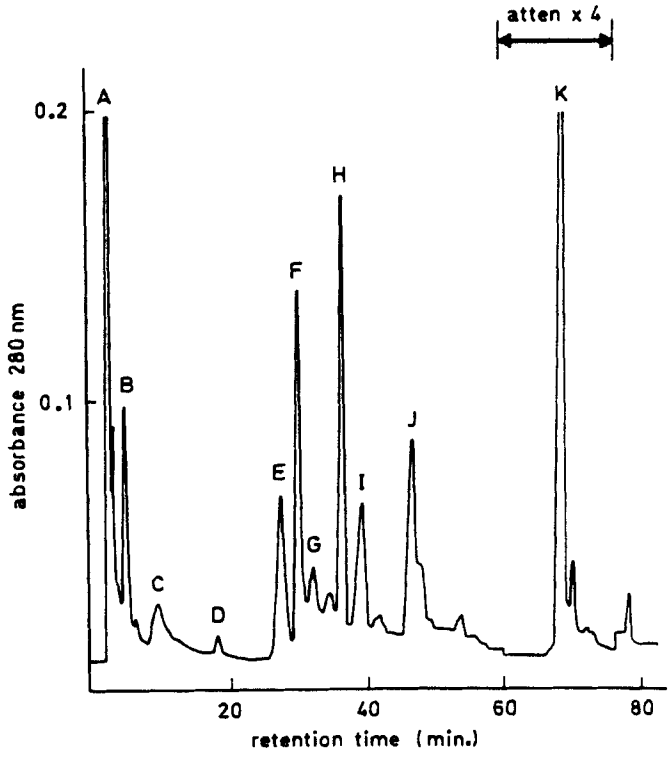

FIGURE 3: Analytical separation of the cellulase enzyme complex from T. reesei MCG 77 by chromatofocusing. A = ß-glucosidase, ß-xylosidase, xylanase, B = endocellulase (Cx), C = galactomannase, D = endocellulase (Cx), E = unknown, F = exocellulase (C_1), G = endocellulase (Cx), H = xylanase, I = endocellulase (Cx), J = endocellulase (Cx), K = exocellulase (C_1).

a) Two exocellulases (C_1-enzyme, cellobiohydrolase, E.C. 3.2.1.91), represented by peaks F and K. The exocellulase material represented by peak K comprises 60 - 70% of the total protein, as mentioned earlier. Both exocellulases were found to degrade cellulose (Avicel) swollen in phosphoric acid nearly exclusively to cellobiose, as shown by HPLC analysis of the sugar solution produced by incubation of the enzyme. The two exocellulases showed practically no activity towards carboxymethylcellulose.

b) Five endocellulases (C_x-enzymes, glucan-glucanohydrolases E.C. 3.2.1.4) represented by peaks B, D, G, I, J. These enzymes show a high activity towards carboxymethylcellulose, as measured by the reducing sugar analysis method.

c) At least one ß-glucosidase present in peak A (cellobiase, E.C. 3.2.1.21). No attempt was hitherto made to further fractionate, by using other pH profiles of the Polybuffer, the components present in peak A.

d) Several hemicellulose degrading enzymes like one galactomannase (peak C), two xylananses (xylan-xylanohydrolase, E.C. 3.2.1.8) represented by peak A and H, and one ß-xylosidase also present in the peak A material.

The analytical chromatofocusing separation was found to be well reproducible. This technique is therefore an extremely useful tool for the comparison of different enzyme preparations with different cellulose degrading activities or for the rapid analysis of cellulases produced under different fermentation conditions.

No other separation technique gives a comparable complete separation of the complex protein pattern of the cellulase complex. In addition to that, the separation needs only 90 min, which has to be compared with a several day operation when the cellulase complex is separated, for example, by a combination of molecular sieve and ion exchange chromatography (17). Electrophoretic methods, which are less time consuming and commonly give excellent separations, are difficult to quantify and have the main disadvantage that the various protein bands can hardly be related to specific enzyme activities. The analysis of cellulase proteins by HPLC separation on a DEAE-glycophase anion exchange column (18) would be a rapid procedure, however its efficiency is much lower than that achieved by the focusing method.

Moreover, FPLC chromatofocusing allowed fractionation of the cellulase complex into its individual enzyme proteins on a preparative scale. For preparative separation the FPLC column was loaded with approx. 20 mg of crude cellulase and eluted with Polybuffer as described in the experimental part. The column effluent

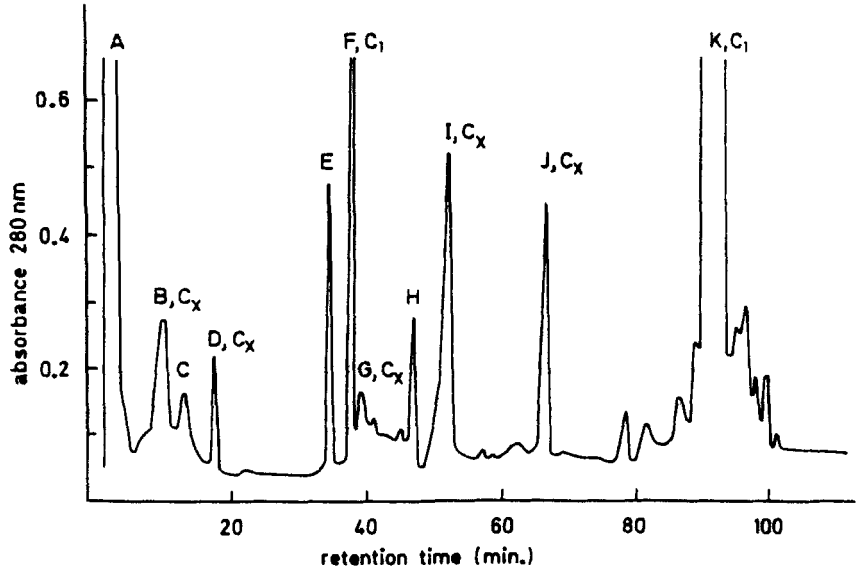

FIGURE 4: Preparative separation of the cellulase enzyme complex from T. reesei by chromatofocusing.
Peak identification as in Fig. 3, Cx = endocellulases, C_1 = exocellulases.

was fractionated into the individual protein peaks detected by the UV monitor. As can be seen from the protein profile in Fig. 4 an excellent separation comparable to that obtained in analytical runs was achieved. It is therefore possible to isolate in one single step most of the individual proteins contained in the cellulase complex.

We first directed our efforts on the major protein component, i.e. the exocellulase of peak K. The column fraction containing this protein was treated with 80% ammonium sulfate to precipitate the enzyme and desalted by Sephadex G 25 column chromatography and then freeze dried. In two preparative runs 50 mg crude cellulase protein was separated and 20 mg of pure exocellulase were obtained.

The purity of the isolated enzyme was ascertained by polyacrylamide electrophoresis and by SDS electrophoresis. The SDS electrophoresis showed only one single band indicating homogeneity of

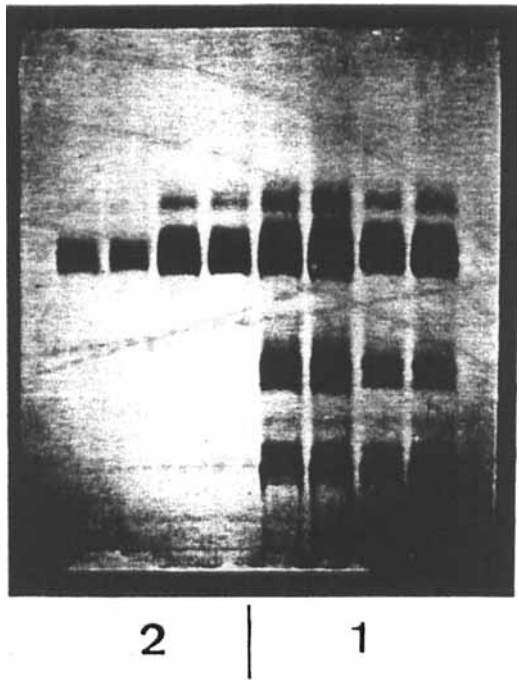

FIGURE 5: Polyacrylamide gel electrophoresis of the total cellulase (1) and of the exocellulase of peak K (2) isolated by preparative chromatofocusing.

the protein with respect to molecular weight. Based on a calibration of the SDS electrophoresis with reference proteins the molecular weight of the isolated exocellulase was estimated to be approx. 58500 $\pm$ 1500 dalton. This value is in good agreement with data reported by others for the major exocellulase of the T. reesei enzyme complex (19). The isolated exocellulase showed only one major band in the polyacrylamide electrophoresis (Fig. 5). A minor contamination which appears as a weak shadow near the major band on the stained gel could only be detected, if large amounts of the exocellulase were applied to the gel. The isolated exocellulase can therefore be considered as a homogeneous protein suitable for the further biochemical and physicochemical characterization. Investigations in this direction are now under way.

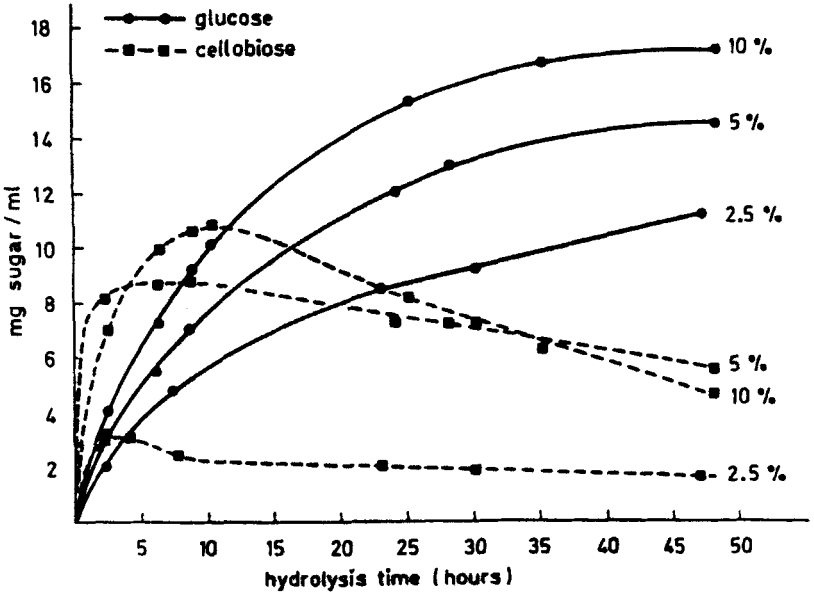

FIGURE 6: Kinetics of formation of cellobiose and glucose during enzymatic hydrolysis of 2.5, 5 and 10% spruce sulfite pulp; 1 mg enzyme/ml, pH 4.8, 50°C.
solid line: glucose; dashed line: cellobiose

Saccharification of Cellulosic and Lignocellulosic Materials

Hydrolysis experiments were done with cellulose fibers as well as with a great variety of different indigenous lignocellulosic raw materials. Fig. 6 shows as example a kinetic experiment in which the formation of soluble sugars (mainly glucose and cellobiose) from a 2.5%, 5% and 10% cellulose pulp was followed over a time period of 50 hours. The rate of sugar formation depended on the cellulose concentration and duration of hydrolysis. During the initial phase of hydrolysis sugars were produced at a maximum rate of about 5 - 6g/l, hour from the 5 or 10% cellulose suspension. It is likely that during this period the more accessible amorphous parts of the cellulose fibers are attacked by the cellulase. With increasing hydrolysis time the saccharification rate rapidly decreased, although a remarkable proportion of the cellulose substrate would still be available for the enzyme.

The reason for this behaviour may be manyfold. Factors such as crystallinity of the cellulose, the surface area, pore diameter and degree of swelling have been recognized as important parameters which influence the extent of saccharification (20). Furthermore enzyme composition, enzyme inactivation, irreversible adsorption to the cellulose, product inhibition and inhibition by the added biocide thimerosal must be considered as possible reasons for the rapid retardation of the hydrolysis rate and for the incomplete hydrolysis of 5 - 10% cellulose even at long exposure to the enzyme. The proportion of cellulose which could be hydrolyzed within 24 hours in our experiments depended strongly on the initial slurry density as shown in Tab. 2.

A 0.5% slurry was saccharified by 1 mg/ml enzyme to 70%, while a 10% slurry gave only 25.2% saccharification in 24 hours. After 50 hours the 0.5 % slurry was hydrolyzed to 85 - 90%.

The cellulose used in our experiments could therefore in principle be saccharified nearly completely, if its concentration was low enough. This strongly suggests that inaccessibility of the fibers for the enzyme is likely not the only reason for the incomplete hydrolysis of 5 to 10% cellulose slurries. For such high slurry densities the inhibitory action of accumulated cellobiose on the exo- and endocellulases as well as the inhibition of ß-glucosidase by the high concentration of accumulated glucose will probably be of equal importance. It is evident from Fig. 6 that cellobiose rapidly increases during the first hours of incubation reaching a level of nearly 10 mg/ml with the 5 or 10% cellulose slurries. During this initial phase, the rate of cellobiose formation is considerably higher than the rate of glucose formation. The kinetic experiment clearly shows that the low rate of cellobiose hydrolysis by ß-glucosidase represents a bottleneck which leads to the accumulation of cellobiose. Cellobiose in turn inhibits the exo- and endocellulases. The insufficient activity of ß-glucosidase is also evident from the fact that cellobiose concentration decreases very slowly on prolonged hydrolysis (Fig.6), although no noteworthy amounts of cellobiose are newly generated by cellulose degradation.

TABLE 2

Effect of Cellulose Concentration on the % Saccharification by Cellulase.
Sulfite pulp was incubated 24 hours at 50°C, pH 4.8, under shaking with cellulase (0.73 FPU/mg, 0.16 units ß-glucosidase/mg).

sulfite pulp mg/ml	cellulase mg/ml	after 24 h incubation		
		cellobiose mg/ml	glucose mg/ml	saccharification %
100	1	8.1	15.4	25.2
50	1	7.3	12.1	41.7
50	2	3.5	15.3	40.4
25	1	2.0	8.5	45.0
10	1	0.4	4.5	52.7
5	1	0.2	3.0	68.8
5	2	0.1	3.5	77.4

It is known that T. reesei cellulase supplemented by ß-glucosidase from other sources (Aspergillus niger, Aspergillus phoenicii) gives higher rates and degree of cellulose saccharification. In our opinion it is economically more realistic for a technical process to improve the ß-glucosidase production by the Trichoderma strain than to use ß-glucosidase from other microbial sources. It was found that ß-glucosidase production by Trichoderma can be greatly enhanced by variation of the fermentation parameters (data on that will be reported in a following paper). For enzymes produced under different conditions the cellulase activity ranged from 1.1 to 5.2 FPU/ml and the ß-glucosidase activity from 0.31 to 2.96 units/ml (Tab.3). Enzyme solutions containing only 0.3 to 0.5 ß-glucosidase units/ml were unable to degrade cellulose to a significant extent when used in a dilution of 3 ml enzyme + 7 ml buffer, while those with 1 or more U/ml gave 35 - 40% saccharification (Tab.3).

TABLE 3

Comparison of the Effectiveness of Different Enzyme Preparations from T. Reesei MCG 77 for Hydrolysis of Cellulose.
Conditions: 7 ml 0.05 M citrate buffer pH 4.8 + 1g air dried spruce sulfite pulp + 3 ml enzyme solution with the indicated activities + 0.04% thimerosal were incubated without shaking at 50°C for 24 hours.

Cellulase Preparation			Sugar Yield, mg/ml			
Enzyme No,	Activity/ml FPU	ß-Gluc.	Cellobiose	Xylose	Glucose	Total Sugar
B 1	3.3	0.68	5.9	1.6	14.3	21.9
D 1	1.4	0.54	1.7	0.7	5.6	8.0
D 2	1.1	0.38	0.7	0	2.4	3.1
D 3	1.1	0.31	0.6	0	2.0	2.6
E 1	2.1	1.25	7.4	2.7	29.6	39.7
E 2	5.2	2.90	6.4	2.6	30.5	39.4
E 3	3.3	2.97	6.0	2.4	26.6	35.1
E 4	1.9	2.40	6.3	2.6	23.6	32.4
E 5	3.3	2.96	5.5	2.4	18.3	26.2
Mixture	3.3	1.10	7.8	3.2	24.0	35.0

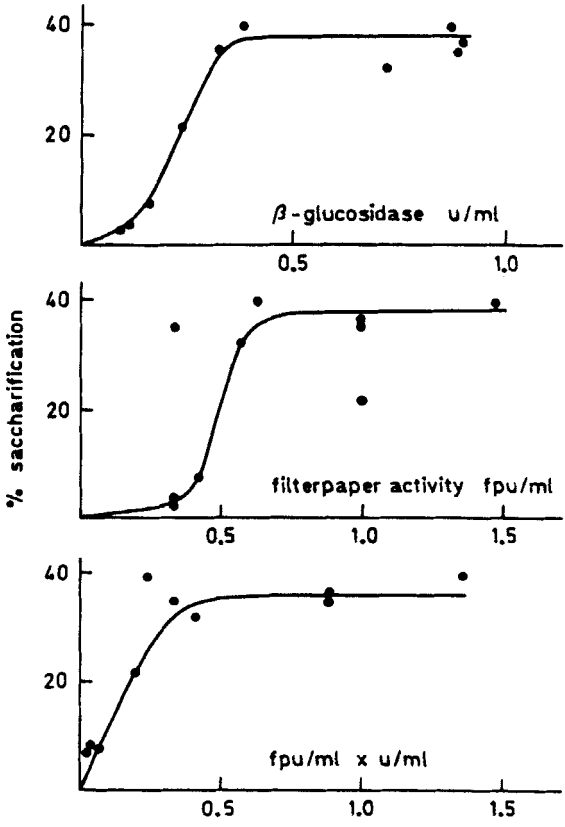

FIGURE 7: Relationship between enzyme activity and % saccharification.
The data are from Tab.3, the enzyme activities are units/ml incubation medium.

A plot of % saccharification against FPU/ml or ß-glucosidase units/ml (Fig. 7) shows that saccharification increases with increasing enzyme activity up to a level of about 40%, which was reached, when the incubation medium contained 0.60 FPU/ml and 0.36 ß-glucosidase units/ml. A further increase of either one of the enzyme activities brought no significant enhancement of saccharification. The data points fit even better, if % saccharification is related to the term ß-glucosidase activity x filter paper activity.

TABLE 4

Yields of Hexoses and Pentoses from Various Lignocellulosic Materials.
The raw materials were pretreated mechanically and saccharified in 10 to 15% slurries with 10 FPU/g dry matter at 50°C for 48 h. Liters ethanol were calculated assuming a conversion rate of 0.45 kg EtOH/kg hexose.

	kg/Ton Dry Matter		Hexoses % of Th.	Liters EtOH per Ton
	Hexoses	Pentoses		
newspaper	338	22	56	192
card board	440	10	70	250
MSW	310	160	-	176
wheat straw	241	103	50	137
corn stover	256	38	60	146
reed grass	290	273	66	166
sugar beet residue	334	91	-	190
bark, spruce	196	19	-	112
groundwood, spruce	215	41	47	122
poplar wood	340	80	66	193
pulp mill sludge	260	110	-	148

The finding that the sugar yield does not linearily increase with the enzyme concentration has important technical implications since it clearly shows that for 10% spruce sulfite cellulose pulp 40% saccharification within 24 hours is the upper limit, which cannot be exceeded by increasing the enzyme concentration (i.e. FPU/ml or ß-glucosidase/ml). A further improvement of saccharification is therefore only possible by means of a suitable pretreatment of the cellulosic material and/or by a process design which eliminates the product inhibition. Results of saccharification experiments with various lignocellulosic materials, most of them pretreated by mechanical means, are compiled in Tab. 4.

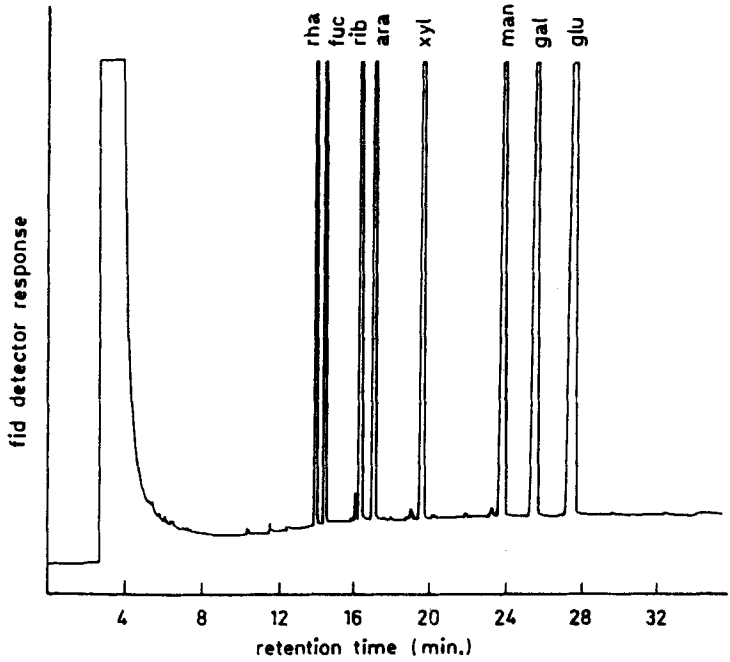

FIGURE 8: Capillary GC of alditol acetates from reference sugars. rha = rhamnose, fuc = fucose, rib = ribose (internal standard), ara = arabinose, xyl = xylose, man = mannose, gal = galactose, glu = glucose.

Sugar Analysis

Depending on the purpose and the aim of the analysis, various methods were used for the estimation of the sugars contained in the raw materials and hydrolyzates. For raw material analysis the milled product was extracted with benzene/ethanol and then subjected to a dilute sulfuric acid hydrolysis according to Wilke et al. (16). The monomeric sugars were determinated by capillary GC as trimethylsilyl derivatives (13) or alditol acetates (15). The latter procedure proved to be particularly useful for the analysis of complex mixtures containing very different amounts of individual sugars. Fig. 8 shows a separation of reference alditol acetates, which can be expected in biomass hydrolyzates, and Fig. 9 shows the capillary GC of a wheat straw sulfuric acid hydrolyzate.

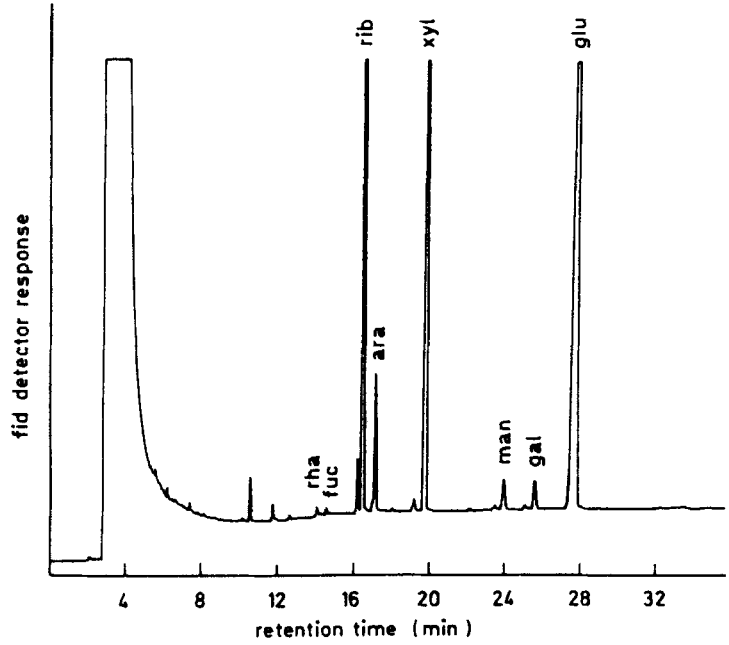

FIGURE 9: Capillary GC of the sugars in wheat straw. Wheat straw was hydrolyzed with dilute sulfuric acid (16) and the sugars were converted to the alditol acetates. Peak identification as in Fig. 8.

For routine analysis in enzyme hydrolyzates and for estimation of cellobiose or cellodextrins the best method in our hand is HPLC separation on a HPX 87P column with water as eluant and refractive index detection. Fig. 10 gives as example the HPLC analysis of a newspaper hydrolyzate. Cellodextrines, cellobiose, hexoses and pentoses were well separated. The quantitative evaluation of the chromatograms was possible by both peak height and peak area measurements. Calibration curves established with reference mixtures showed linearity over a wide concentration range from 0.5 mg/ml to 50 mg/ml.

On the other hand colorimetric determination of total reducing sugars by the dinitrosalicylic acid method (11) or the measurement of glucose by the enzymatic glucose oxidase assay (21) -

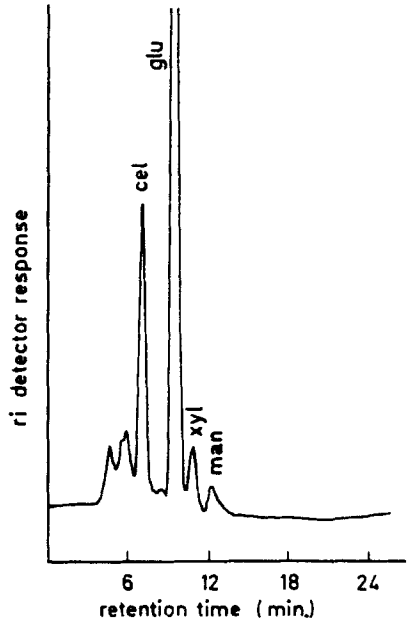

FIGURE 10: HPLC separation on a HPX 87P column of the sugars contained in an newspaper enzyme hydrolyzate.
cel = cellobiose, glu = glucose, xyl = xylose, man = mannose.

TABLE 5

Determination of Sugars in Various Enzyme Hydrolyzates by the Dinitrosalicylic Acid Method (DNS), GC Separation of the TMS Derivatives and Glucose Oxidase Assay (GOD).

Hydrolyzate	Total Sugars, mg/ml		Glucose, mg/ml	
	DNS	GC	GOD	GC
spruce bark	39.1	25.9	18.9	21.4
wheat straw	52.4	35.5	18.0	22.5
newspaper	40.8	32.8	26.4	26.8
sugar beet residue	32.5	21.8	8.5	15.3
paper mill sludge	62.2	48.0	29.7	33.8

methods frequently used for studying enzymatic hydrolysis - are only of limited value since many substances other than sugars can interfere. The values in Tab. 5 show that, compared to the sugar concentration determinated by GC and/or HPLC, the dinitrosalicylic procedure gives generally values which are too high, while on the other hand the glucose oxidase assay gives values which are often too low.

CONCLUSIONS

The work on enzymatic conversion of lignocellulosic materials to sugars has been greatly stimulated in the recent years by the anticipation that this process could be developed to an alternative technology based on renewable resources. Considerable progress has been made within a few years in improving enzyme production, and it can be anticipated that an inexpensive cellulase enzyme can be produced in the near future. Both, the work performed in our laboratory as well as at many other places clearly show that most process parameters like enzyme production, raw material pretreatment, hydrolysis and sugar recovery are well reproducible and controllable in the lab scale. Lignocellulosics from very different sources (Tab. 4) can be saccharified on an average to 50 - 60% of the theory. The sugar yield can certainly be improved, if the raw materials can be properly pretreated. Further research is urgently needed to develop inexpensive pretreatment procedures which improve the rate of saccharification. The use of chemicals like alkali, acids, cadoxene, sulfur dioxide, hypochlorite a.o. is most likely not a promising approach since it would lead into new problems of waste disposal and pollution control. It seems more realistic to put more efforts in designing mills which can effectively reduce particle size and increase surface area at low energy costs.

The scaling up from the lab or pilot scale to factory proportions is an engineering and biotechnology problem which will probably be successfully solved, if the impetus is strong enough. The production of sugars from lignocellulosics has to compete with other

well-established sugar sources (grain, sugar cane, sugar beet, potato) and therefore the process economy depends strongly on the costs of these products and, if the sugar is converted to ethanol, on fuel prices. Nevertheless we are cautiously optimistic that the utilization of renewable cellulosic materials for production of valuable basic chemicals will be an important aspect in fulfilling future demand in a resource limited world.

The biochemical characterization of the cellulase enzyme is in a very early stage, when compared with other enzymes which were apparently more attractive to biochemists. To understand the synergistic action of the various cellulolytic enzymes and the mechanistic details of cellulose hydrolysis it is essential to isolate, purify and define each of the individual enzymes involved in cellulose hydrolysis. Advances have been made, as described in this paper, in the fractionation of the cellulase complex into separate, individual enzymes by chromatofocusing. This rapid procedure for characterizing a cellulase preparation should enable us to eludicate the cellulase composition optimally for the enzymatic hydrolysis process.

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We dedicate this paper to Prof. Dr. E. Ziegler at the occasion of his 70th birthday.

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