The cellulases and their application in degrading agro-industrial waste

Las celulasas y su aplicación en la degradación de desechos agroindustriales

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ABSTRACT
A huge amount of lignocellulosic biomass is available which can be used to produce storable energy and basic material for the chemical industry. Its use is especially beneficial for a country's economy if it is waste material, which can be obtained at almost no cost and which presents an environmental burden. However, the polysaccharides present in biomass are difficult to degrade due to their heterogeneity and crystalline structure. This article addresses the enzymatic hydrolysis of cellulose by its natural degraders, the anaerobic bacteria. The difficulties of cellulose digestion are explained and the strategies used by the hydrolytic enzymes and enzyme systems, allowing for efficient degradation. The multitude of enzymes is uniform in having an identical chemical specificity, but differs in each component's action mode. Only by combining this with binding modules can efficient hydrolysis be performed. The variation of modular structures within a single enzyme family is an example of enzymatic activity's evolutionary diversification. A model for hydrolytically degrading natural cellulose is presented, but much more research has to be done to explain and describe the process on the molecular level, and to optimize an industrial enzymatic cellulose hydrolysis process.

Key words: cellulose hydrolysis, enzyme system, cellulosome

RESUMEN
Una cantidad de biomasa lignocelulósica está disponible y puede ser usada para producir energía almacenable, material básico de la industria química. Su uso es especialmente benéfico para un país, si esta biomasa hace parte de material de desecho que pude ser obtenido casi sin ningún costo y está presente en la carga ambiental. A pesar de esto, los polisacáridos presentes en ese tipo de biomasas son difíciles de degradar debido a su heterogeneidad y a su estructura cristalina. Este artículo está dirigido a la hidrólisis enzimática de la celulosa realizada por microorganismos que la degradan, las bacterias anaeróbicas. Se explican las dificultades para la digestión de la celulosa, así como las estrategias usadas por hidrolasas y complejos enzima ticos que permiten una degradación eficiente. La especificidad química de todas estas enzimas es idéntica, pero el modo de acción de cada uno de sus componentes es diferente. Sólo cuando se combinan con módulos de unión, se realiza una hidrólisis lenta pero eficiente. La variación de las estructuras modulares entre cada familia son un ejemplo de la diversificación evolutiva de la actividad enzimática. Se analiza un modelo para la hidrólisis de la celulosa en bruto (como está presente en la naturaleza); sin embargo, no se han realizado muchas investigaciones para explicar y describir el proceso a nivel molecular ni para optimizar la hidrólisis de la celulosa en el ámbito industrial.

Palabras clave: hidrólisis de celulosa, complejo enzimático, celulosoma

BIOPROCESSING OF LIGNOCELLULOSIC BIOMASS

Widespread thinking about the use of biomass is that, "You eat it or you burn it!" Not discussing eating (which can be a necessity and/or a pleasure), to suggest burning is a dramatic underestimation of the potential contained in biomass. We have recently learned that mankind gains a lot of surplus in usability by biotechnology

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-especially if it is biowaste, which is not used otherwise. The large-scale biological fermentation of lignocellulosic biomass could be a solution to many problems (Sheehan & Himmel, 1999).

What is biomass then? Biomass is primarily the product of CO_2 and light, and is produced by the photosynthetic apparatus in bacteria, algae and plants. 2,000 Gt (1 Gt = 10^9 t) of biomass, dead or living, has been calculated to be around on the surface of the continents, most of it in the tundra marches of the subarctic regions, much less in the grass-lands and the great jungles of the tropics. Biomass is a sink for CO_2 and keeps its level about constant in a natural cycle of production and degradation/mineralisation. Plant biomass, the greatest part of whole biomass, is composed of cellulose, hemicellulose, lignin, proteins and some other substances, and is called lignocellulosic biomass (LCB).

In this short review we will concentrate on the cellulose in LCB. Cellulose is by far the most abundant carbohydrate and polysaccharide on earth. About 40 Gt of cellulose are produced per year on land -not calculating the cellulose produced in the oceans. The cellulose is completely recycled by natural processes. This means that 40 Gt of cellulose are naturally degraded in the biosphere by enzymatic processes. Using cellulose for industrial bioprocessing does not thus influence the carbon cycle and is considered to be environmentally neutral, according to the Rio and Kyoto protocols.

Man traditionally uses biomass directly, for food, animal feed and construction purposes (e.g. wood), or indirectly by hydrolysis and chemical conversion to useful products like solvents (ethanol, butanol, acetone, etc.), organic acids (acetic, citric, lactic, succinic, etc.), aromatics (like phenol) and many other substances (Himmel *et al.,* 1999). As an example: about 10 million m^3 of ethanol are currently being produced from starch, mainly in the USA and in Brazil. This amount will be doubled in the USA by 2010, and cellulose will be the substrate. Most of this solvent is used in cars as a gasoline additive. The bright future perspectives were outlined by B. Cook and many others at the 1st World Conference on Biomass for Energy and Industry (2000).

The application of biotechnology in principle is a "low energy / low pollutant" way of technology;in addition, it is environmentally compatible: all substances - products, by products, waste are biodegradable. LCB is produced by forestry or agriculture. More advantages are (Van Wyck, 2001):

- Improvement of agriculture (diversification of production, increase in productivity, employment, enhanced rural economy)
- Improvement in the economy (independence from other sources, e.g. imports; employment etc.)
- Improvement in overall technology, innovation and investment; and
- Improvement in the environment (reduction of greenhouse gasses and landfill burden, improved water quality).

However, there are also disadvantages of an extended use of agricultural biomass for industrial bioprocesses:

- An inherent inefficiency of the products
- The use of fertile but marginal lands, which could be used for food production
- Reliability of supply (harvest season etc.); and
- High cost (labor intensive).

Is there really enough LCB available for industrial processes? The newest data collected in Germany for example are promising: most of the biomass is used for other purposes, but enough lignocellulosics are not used otherwise, like some of the straw (9.4 Mt), wood (55 Mt from forestry and 14 Mt from industry leftovers), and biowaste and green material (6.9 Mt from the food industry, gardening etc.), altogether representing more than 75 Mt (1 Mt = 10^9 metric tons) (Hartmann & Kaltschmitt, 2002). There is thus a realistic potential for a large scale industrial process, even in a country like Germany where most of the biowaste is used or recycled. There is an even broader basis for bioprocessing in more agriculturally-based countries.

The hydrolysis of lignocellulose in an enzymatic process can be highly efficient as demonstrated by the digestion of grass and straw in the rumen of cows,

where about 65% of the fiber material is hydrolyzed (mostly cellulose). In camelids, like guanacos, alpacas or llamas, this natural process works even more efficiently: up to 85% of the lignocellulosic material is digested in less than 12 hours. Interestingly, most biological processes in rumen and intestine have been well investigated, but fiber digestion has not, although the greatest part of a cow's energy is derived from fiber degradation, and thus from cellulose.

What is cellulose?

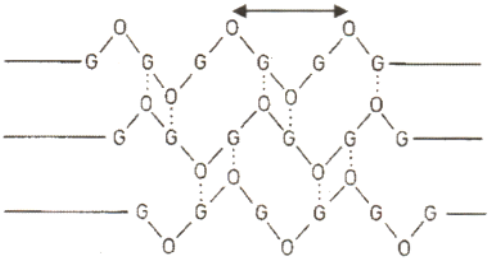

Figure 1: Structure of cellulose. Gluco-pyranose residues (G) are connected by 3-1,4-glucosidic linkages (-O-). The structural subunit ¡s cellobiose (-G-O-G-O-) The linear molecules are arranged in parallel in fixed into a crystalline structure by hydrogen inter- and intramolecular bonds.

Cellulose is a chemically simple but highly stable β-1,4-linked glucose residue homopolymer. It is linear and unbranched. Due to a 180° turn between neighboring glucose residues, the repeat unit is cellobiose (two glucose residues, figure 1). The cellulose molecules are synthesized in parallel strands which undergo self-assembly by strong binding forces, with inter- and intra-strand proton-bridges and stacking forces, and immediately form a crystal. Natural cellulose is thus crystalline and completely insoluble in water. These crystals, called microfibrils (3 nm thick), align in the secondary cell walls of plants to form larger fibers which are embedded in a matrix of hemicellulose and lignin, which in turn are extremely heterogeneous (figure 2) (Hayashi *et al.,* 1998).

The secondary cell walls of plants are therefore extremely recalcitrant substrates for enzymatic digestion: the hemicellulose-lignin complex has to be removed first by the cooperation of a large number of different enzymes to give the cellulases access to the crystalline cellulose. The substrate crystals are insoluble and tightly packed -they do not present single molecules which could be uptaken by the activity pockets of enzymes. A new type of chemistry has to be created to explain the action of enzymes on crystalline surfaces: the substrate is bigger than the enzyme and cannot be penetrated (figure 2). The kinetics of enzyme binding and hydrolysis are not yet understood, and Michaelis-Menten kinetics cannot be applied (Lynd *et al.,* 2002). The enzymes also have to cope with "frozen" (= highly ordered) layers of water molecules on the substrate's crystalline surface.

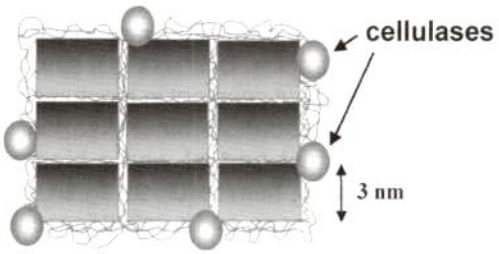

Figure 2: Structure of cellulose microfibrils. The basic crystalline units of cellulose (3 nm thick) are arranged in packages to cellulose fibrils. The microfibrils are embedded in hemicellulose and lignin. The size of single cellulase molecules is indicated for comparison.

However, the cellulose crystals are not perfect and contain amorphous regions, about every 30 nm, and also edges and ends, where enzymes could gain access to and "pull out" single molecules to fit them into their substrate pockets (figure 3). Degradation then proceeds progressively and reaches far into the crystalline regions. Binding modules also play an important role in this process (see below).

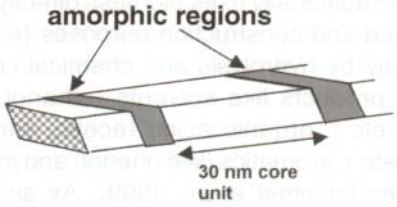

Figure 3: Substructure of cellulose microfibrils. Crystalline regions are interspersed by amorphic regions. The crystalline core is about 30 nm long.

Cellulose degrading bacteria

A number of bacteria thrive on cellulose in natural habitats, readily degrading it. Two types can basically be distinguished: 1). bacteria producing limited amounts of sugar from cellulose (most saccharolytic bacteria)

Table 1. Comparison between *Clostridium thermocellum* and *Clostridium stercorarium*

	C. thermocellum	*C. stercorarium*
Growth on:		
glucose	-	+
hemicellulose	-	+
Starch	-	+
cryst. cellulose	+	+
Cellulosomes	+	-
G + C (mol%)	38-40	39
Optimum temperature	55-69°C	65°C

and 2). bacteria effectively dissolving crystalline cellulose fibers ("true" cellulose degraders), a task only a few can fulfil (for a list of cellulolytic bacteria see: http://www.wzw.tum.de/mbiotec/cellmo.htm). A number of reviews on bacterial cellulose degradation have been published recently (Bayer *et al.,* 1998, 2000; Schwarz, 2001).

Two examples for truly cellulolytic bacteria are the anaerobio, thermophilic bacteria *C. thermocellum* (CTH) and *C. stercorarium* (CST) which are abundant in soil containing rotting biomass and in compost (table 1). Both strains are closely related phylogenetically and belong to the same subclass of saccharolytic clostridia (Schwarz *et al.,* 1995). They have a slightly different G + C content in their DNA but a similar optimum temperature for growth (65°C). CST grows well on hemicellulose, starch and cellulose, whereas CTH is a true specialist for cellulose and cellodextrins and does not use any other substrate.

CTH has been the most efficient cellulose degrading microorganism isolated so far. It possesses a huge enzyme complex bound to the cell surface (the cellulosome) which is absent in CST (Lamed *et al.,* 1987). This complex contains a number of different enzymes responsible for effective cellulose hydrolysis, but also degrades hemicellulose and attaches the cell to the insoluble substrate. A list of the 25 cellulosomal genes cloned so far can be found in Schwarz (2001) and at http://www.wzw.tum.de/mbiotec/celoscomp.htm.

The enzyme activities in the culture supernatant do not differ dramatically between the two bacteria (table 2). Yet CST hydrolyses hemicellulose much faster. Consistently, CST also possesses more activities for the degradation of xylosides and arabinosides, constituents of hemicellulose. The higher cellobioside degradation by CTH is not a result of (3-glucosidase activity (as it might be in CST), but specific cellobiohydrolases (certain xylanases and exo-glucanases) and may suggest a high cellulose hydrolysis potential. However, cellulase activity in CTH culture supernatants is underestimated because most of the enzyme binds to the substrate, thus escaping an assay.

In contrast to CTH, CST hydrolyses crystalline cellulose with only two cellulases, CelZ and CelY, also called Avicelase I and Avicelase II (after the microcrystalline substrate Avicel which is used internationally for assaying "true" cellulases) (Bronnenmeier & Staudenbauer, 1988; Bronnenmeier *et al.,* 1990). No other cellulases could be identified, either by protein purification or by gene cloning. The two enzymes are not bound to a complex ("non-cellulosomal") and can be easily purified. CelZ is an endo-glucanase, introducing "nicks" (cuts) anywhere in a cellulose molecule. It produces cellodextrins of various length. CelY is an exo-glucanase with a predominantly processive mode, releasing cellobiose or cellotetraose, presumably by binding to the non-reducing end of a cellulose chain and threading the molecule through a tunnel around the active site pocket and cutting each second or fourth glycosidic bond. The endo-mode and

Table 2. Enzymatic activity in cell free culture fluid (grown on cellobiose)

Substrate	*C. thermocellum* (mU/ml)	*C. stercorarium* (mU/ml)
Microcrist. cellulose (Avicel)	2	2
Phosphoric acid swollen cellulose	13	10
Carboxymethyl cellulose (CMC)	140	120
1,3-1,4-ß-glucan (lichenan)	6,500	12,000
Arabino-xylan	3,000	20,000
*p*NP-ß-glucopyranoside	1.3	7.0
*p*NP-ß-cellobioside	12.0	1.7
*p*NP-ß-xylopyranoside	0.3	2.0
*p*NP-α-arabinofuranoside	1.3	21.0

1 mU = release of 1 nmol of reducing ends (glucose equivalents) or *p*-nitrophenol / min

the processive exo-mode enzymes work synergistically, i.e. the sum of the activities of the two enzymes would be smaller than the activity if both enzymes were combined (Riedel *et al.,* 1997). However, the activity of the recombinantly expressed enzymes seems to be much smaller than the activity in the culture, which might be an effect of cell-substrate interaction in an as yet unknown way.

Structure of the cellulosome

Cellulose hydrolysis by CTH is much more complex. However, it has been intensively investigated and the structure of the cellulosome has been quite well documented. A cell-wall binding protein (Olp - outer layer protein) grasps a structural protein by protein-protein interaction (CipA - cell integrating protein, called "scaffoldin"), which has nine binding sites for different catalytic and at least one non-catalytic components (figure 4). To date, the genes for 11 endo-glucanase, 4 exo-glucanase, 5 xylanase, 1 chitinase, 2 mannanase, 1 β-1,3-1,4-glucanase and 1 non-catalytic components have been detected. The genomic sequence which is expected to be published later this year will surely disclose even more genes.

The scaffoldin and some catalytic components are joined to carbohydrate binding modules (CBMs) which bind the protein complex tightly to the substrate. The CTH cellulosomes form complexes of up to 6 MDa big (poly-cellulosomes). The cloned components have been biochemically characterized, many have been crystallized and their 3D-structure has been solved. All components are modular proteins composed of more than one independently folding protein module.

Why so many components?

Two reasons for the great number of different components in the cellulosome can be thought of. The first is easy to explain: the matrix covering the cellulose crystals has to be degraded; this material, mainly hemicellulose, is heterogeneous and a number of different hemicellulases has to act on them -hence the 5 xylanases etc., in the cellulosome. A sixth xylanase, not integrated in the cellulosome, can be detected. The great number of hemicellulases is surprising because CTH cannot make use of the sugars produced by these enzymes -hence the absence of glycosidases; the polymer is dissolved but the soluble oligosaccharides are not split down to monosaccharides useful for the hosts' metabolism. The mannanase, chitinase and B-glucanase present in the cellulosome may fulfill the same purpose.

The other reason is less well understood: all cellulases exhibit the same specificity for β-1,4-glycosidic linkages; this makes them difficult to describe and to distinguish with classical biochemical methods. But cellulose is topologically heterogeneous -different cellulases may be specifically working either on the flat surfaces or the edges of the crystals, for example. In addition, the local fixation of the substrate to a large crystal restricts free movement of the enzymes and a number of enzymes with different topological specificity may work side-by-side. Furthermore, due to the crystallinity of the substrate,

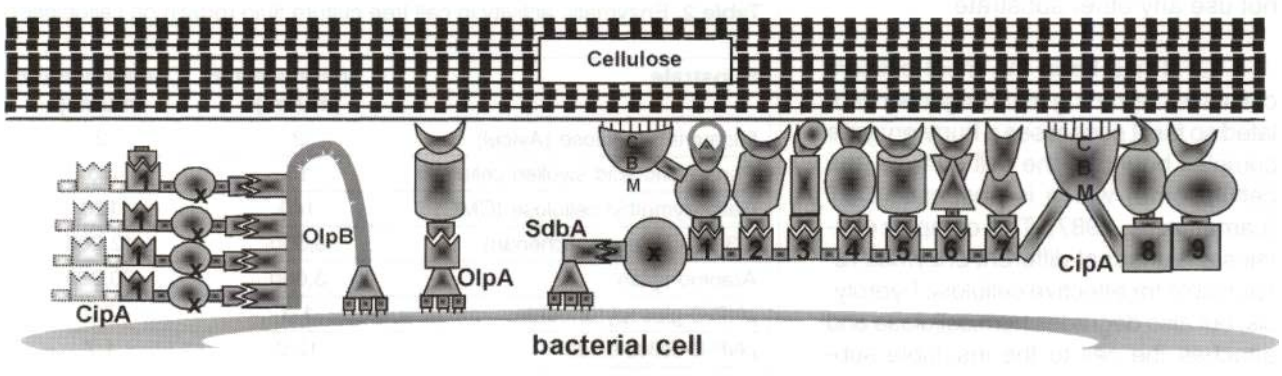

Figure 4: The cellulosome between bacterial cell and cellulose crystal. Cell wall binding proteins (OlpB, SdbA) attach the cellulosome to the bacterial surface. They are connected to the scaffolding protein CipA, which holds specifically the catalytic cellulosome components with its binding sites 1-9. CipA as well as some catalytic components have cellulose binding modules (CBM), which bind tightly to the crystal surface. SdbA holds one and OlpB four scaffoldins.

the endo-glucanases have to open the molecules and exo-glucanases work from the newly formed ends ¡n two directions, namely from the reducing or the non-reducing end.

There are cellulases with completely different primary and secondary protein-structure and folding types: a/B8-barrel, B-sheet sandwich, etc. They have been categorized into different glycosyl hydrolase families (GHF) (Coutinho and Henrissat, 1999). But even within one GHF different hydrolytic specificities or modes of action are found, e.g. GHF5 and GHF9 cellulases contain both endo- and exo-glucanases, whereas GHF8 and GHF48 cellulases show only endo- or exo-mode, respectively.

The enzyme activity of a given catalytic module may be modulated by the addition of various non-catalytic modules, as shown in figure 5. The different combinations within GHF9 are a good example for the playground of evolution and have been discussed extensively by Bayer *et al.* (2000). It is of interest to note that all cellulosomal systems identified so far have contained only one type of GHF48 cellulase, whereas many different GHF5 and GHF9 enzymes are present. GHF8 enzymes are not found ¡n all cellulosomes.

While CTH is very well hydrolyzing cellulose and produces a highly complex cellulase system to perform this difficult task, it is a mystery how the CST cellulase system works with only two components,

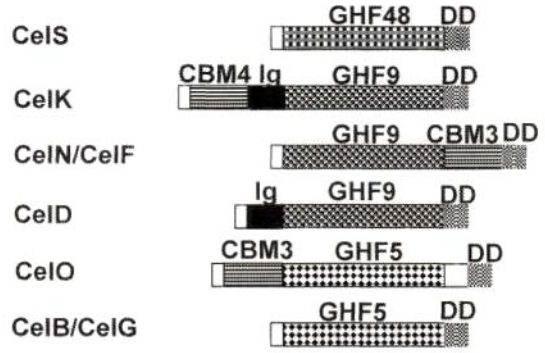

Figure 5: Diversity of *Clostridium thermocellum* cellulases. Catalytic modules of glycosyl hydrolase families GHF5, GHF9 and GHF48 connected to carbohydrate binding modules (CBM), immune-globulin like modules (Ig) and dockerin domains (DD) for binding to the cellulosomal scaffoldin. The enzymes have acquired different non-catalytic modules, which modify their action mode.

CelZ (GHF9) and CelY (GHF48), and still allows its host to grow well on pure crystalline cellulose.

Carbohydrate binding modules

Cellulases could not work on crystalline cellulose without the aid of binding modules. A wide range of CBMs have been identified: 28 families, from 75 to 150 amino acid residues. They usually bind the substrate with an arrangement of hydrophobic tryptophane residues. They are either in the loose vicinity of the catalytic module, connected by a flexible arm, or closely attached to it. The CBMs may bind to flat crystal surfaces or bind single cellulose molecules (Carrard *et al.,* 2000). Some CBMs are tightly attached to the catalytic module and seem to have lost their binding capacity -they rather perform a stabilizing function, and removal by genetic engineering may produce a drop in temperature stability by up to 20°C. Another CBM is connected to the scaffoldin of the cellulosome and obviously holds the huge complex on the surface of the substrate. Its binding is very tight with unmeasurable off rates and it is not clear if sliding along the substrate is possible. Still other CBMs embrace a cellulose molecule and feed it into the activity pocket of an endo-glucanase, thus modulating an enzyme's mode of activity, making ¡t a "processive endo-glucanase" (Irwin *et al.,* 1998).

The major role of the CBMs might be to hold the catalytic module in the close vicinity of the substrate, thus increasing the local substrate concentration. The loosening of cellulose molecules on the crystal surface by binding strongly to it could also be important, or the breaking up of "frozen" water layers on top of the crystals, thus enabling the catalytic module access to the substrate. The last two processes could also be positively enhanced by increasing the reaction temperature, giving thermophilic systems an advantage. One cannot think of a "true cellulase" without thinking about such binding modules.

APPLICATION OF CELLULASES - A FUTURE PERSPECTIVE

From a scientists' point of view, cellulases are fascinating and very complex nano-molecular machines. Their knowledgeable application in the hydrolysis of biomass would allow the production of a great amount of fermentation substrates (sugars) from a renewable source. A lot of research still needs to

be done. But it is not a dream for the far-distant future: it is actually being addressed by a multi-million dollar US program (DOE/NREL) which undertakes to improve the hydrolysis rate and production efficiency of a multi-component cellulase system by genetic engineering and molecular modeling. Wooden waste from forestry will be used in the near future to produce sugar which in turn will be fermented to ethanol fuel by a conventional, large-scale, yeast-based process (Sheehan and Himmel, 1999). The production of 10 Mt ethanol from wood is projected by the year 2010, leaving the presently used starch-based process far behind. Moreover, direct fermentation of biomass to solvents by bacteria seems to be economically feasible and efficient and could eventually be established without the addition of external enzymes, if cellulolytic microorganisms were to be used (Gapes, 2000; Zaldivar *et al.,* 2001). Conventional process technology can be applied, except that innovative and progressive use of designer-cellulases and of an environmentally beneficial substrate will make the process much more economical. However, its realisation demands the combined effort of biochemistry, biophysics, molecular biology, bioinformatics and engineering -and of a public opinion concerned about the disappearance of natural resources and a future for our children.

It could be inspiring to reread the statement made by, T. K. Ghose, one of the pioneers of cellulase research, about bacterial cellulolysis in 1969, "Microorganisms have no difficulty digesting cellulose. They accomplish it readily and effectively. Why is it then that we cannot utilize their systems to develop a practical conversion of cellulose to sugar? The answer is rather simple: we can -if we pour into this problem the effort it rightly deserves."

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Endo-glucanase	produces new ends and falls off
Processive endo-glucanase	produces new ends and remains on the substrate, cutting oligo-saccharides off the non-reducing end
Exo-glucanase from non-reducing end	splits cellobiose residues off the non-reducing end (cellobiohydrolase)
Exo-glucanase from reducing end	splits cellobiose residues off the reducing end (cellobiohydrolase)

Future perspectives

1) **Development of industrial processes** (like in the USA: DOE with Iogen and Novo):

   recombinant enzyme system for hydrolysis of wood
   - Optimisation of catalytic activity
   - Optimisation of binding capacity
   - Optimisation of synergism

   Financial break-even with chemical hydrolysis

2) **Screening for bacteria** capable of direct conversion of LCB

3) With the availability of **genomic sequences**:
   - Increasing interest in complete enzyme systems: **cellulosome**

   (*C. thermocellum, Ruminococcus albus, Butyrivibrio fibrisolvens*)

REFERENCES

Bayer, E. A., Morag, E., Lamed, R., Yaron, S., and Shoham, Y. 1998. Cellulosome structure: four-pronged attack using biochemistry, molecular biology, crystallography and bioinformatics. In M. Claeyssens, W. Nerinckx, and K. Piens (eds.), Carbohydratases from *Trichoderma reesei* and other microorganisms. The Royal Society of Chemistry, London. pp. 39-65.

Bayer, E. A., Shoham, Y, and Lamed, R. 2000. Cellulose-decomposing prokaryotes and their enzyme systems. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (eds.), *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community,* 3rd edition. Springer-Verlag, New York.

Bronnenmeier, K., and Staudenbauer, W. L. 1988. Resolution of *Clostridium stercorarium* cellulase by fast protein liquid chromatography (FPLC). *Appl. Microbiol. Biotechnol.* 27: 432-436.

Bronnenmeier, K., Ebenbichler, C., and Staudenbauer, W. L. 1990. Separation of the cellulolytic and xylanolytic enzymes of *Clostridium stercorarium. J. Chromatogr.* 521: 301-310.

Carrard, G., Koivula, A., Sóderlund, H., & Béguin, P. 2000. Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. *Proc. Nat. Acad.* Se/., USA. 97: 10342-10347.

Cook, B. 2001. Perspectives on biomass industry futures. Kyritsis *et al.* (eds.), Proceedings of the 1st World Conference on Biomass for Energy and Industry, Sevilla, Spain. P. xli-xliii. James & James (Science Publishers) Ltd.

Coutinho, P. M. and Henrissat, B. 1999. Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/-cazy/CAZY/index.html

Gapes, J. R. 2000. The economics of acetone-butanol fermentation: theoretical and market considerations. *J. Molec. Microbiol. Biotech.* 2(1).

Ghose, T. K. 1969. Anonymous. "Mini-Manhattan project" for cellulases, Brochure #NREL/MK-336-5676. National Renewable Energy Laboratory; Golden, CO. 1993. (Cited from Sheehan and Himmel, 1999).

Hartmann, H., and Kaltschmitt, M. 2002. Biomasse ais erneuerbarer Energietráger. A Feasibility study supported by the Ministry of Food, Agriculture and Forestry of the Federal Government of Germany and the Fachagentur Nachwachsende Rohstoffe.

Hayashi, N., Sugiyama, J., Okano, T., and Ishihara, M. 1998. The enzymatic susceptibility of cellulose microf ibrils of the algal-bacterial type and the cotton-ramie type. *Carbohydr. Res.* 305: 261-269.

Himmel, M. E., Ruth, M. F., and Wyman, C. E. 1999. Cellulase for commodity producís from cellulosic biomass. *Curr. Opin. Biotechnol.* 10:358-364.

Irwin, D., Shin, D.-H., Zhang, S., Barr, B. K., Sakon, J., Karplus, P.A., and Wilson, D. B. 1998. Roles of the catalytic domain and two cellulose binding domains of *Thermomonospora fusca* E4 in cellulose hydrolysis. *J. Bacteriol.* 180:1709-1714.

Lamed, R., Naimark, J., Morgenstern, E., and Bayer, E. A. 1987. Specialized cell surface structures in cellulolytic bacteria. *J. Bacteriol.* 169:3792-3800.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., and Pretorius, I. S. 2002. Microbial cellulose utilization: fundamentáis and biotechnology. *Microbiol. Molec.* e/o/. *Rev.* 66: 506-577.

Riedel, K., Ritter, J., and Bronnenmeier, K. 1997. Synergistic interaction of the *Clostridium stercorarium* cellulases Avicelase I (CelZ) and Avicelase II (CelY) in the degradation of microcrystalline cellulose. *FEMS Microbiol. Let.* 147: 239-243.

Schwarz, W. H., Bronnenmeier, K., Landmann, B., Wanner, G., Staudenbauer, W. L., Kurose, N., and Takayama, T. 1995. Molecular characterization of four strains of the cellulolytic thermophile *Clostridium stercorarium. Biosci. Biotech. Biochem.* 59: 1661-1665.

Schwarz, W. H. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Appl. Microbiol. Biotechnol.* 56:634-649.

Sheehan, J., and Himmel, M. 1999. Enzymes, energy, and the environment: a strategic perspective on the U.S. Department of energy's research and development activities for bioethanol. *Biotechnol. Prog.* 15: 817-827.

Van Wyck, J. P. H. 2001. Biotechnology and the utilization of biowaste as a resource for bioproduct development. *Trends in Biotechnol.* 19:172-177.

Zaldivar, J., Nielsen, J., and Olsson, L. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl. Microbiol. Biotechnol.* 56:17-34.