

MICROBIAL CULTURES IN THE UTILIZATION OF CELLULOSIC MATERIALS

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ABSTRACT

This review elaborates on the most recent microbial development in saccharification of cellulose and cellulase formation. A particular highlight is a new genetic-immunochemical approach investigating the mechanism of adhesion of bacterial cellulase to cellulose during cellulose conversion. New developments and recent reviews in hemicellulose and lignin degradation are also covered.

KEYWORDS

Microorganisms, Renewable Substrate, Cellulose, Hemicellulose, Lignin, Cellulases, Xylanases, Hydrolysis, Saccharification.

INTRODUCTION

Plant material, one of nature's largest renewable biomass resources, will undoubtedly become an important energy and food resource, which can be supplied more readily than petroleum or natural gas in future. The decomposition or conversion of plant cellular material, however, is a very complex process and in nature it involves mixed populations of many

microorganisms, often in interacting and antagonistic relationships (12). The structural material cellulose derives both strength and resistance to degradation from its potential to crystallize and, in the case of wood, its encapsulation in lignin (20). Of the cellulose materials available, primarily as waste substances, the most important ones are rice and wheat straw, rice hull, bagasse and paper mill wastes. These native cellulosic materials consist of appreciable amounts of cellulose and hemicellulose, which yield on enzymatic hydrolysis a mixture mainly of glucose, cellobiose, xylose and cellodextrins (22). Although the majority of microbial development for cellulose degradation has been effected with fungi, which excrete the responsible enzyme complex, increasing literature becomes available on streptomycetes and on aerobic, facultative anaerobic and anaerobic bacteria (32, 72, 80, 85) depending upon the required product formation. The greatest challenge of the past decade is still with us, the exploration of economic enzymatic saccharification of cellulose (54).

The three main problems are: (1) the present need for a long cultivation time to obtain maximal saccharification or cellulase activity, (2) the repression by glucose and intermediate products of the cellulase synthesis and (3) the difficulties of cultivation at high concentration of cellulose in view of fluid dynamical constraints. The economics of cellulose utilization hinges therefore on three main operations, the pretreatment of cellulosics and hemicellulosics, cellulase and hemicellulase production, and the enzymatic hydrolysis of cellulose to glucose or hemicellulose to xylose, followed by specific conversions to useful products.

This review will deal with the latest microbial development for increasing the economy of these process operations. For completeness, the reader's attention is drawn to the review articles by Duong et al. (12), Sonnleitner (72) on thermophilic bacteria, and by Jeffries (36), McCracken and Gong (51), Schneider et al. (71), Kurtzman (43), Janson and Tsao (34), Volesky and Szczesny (79) and Janshekar and Fiechter (35) on lignin and xylose utilization.

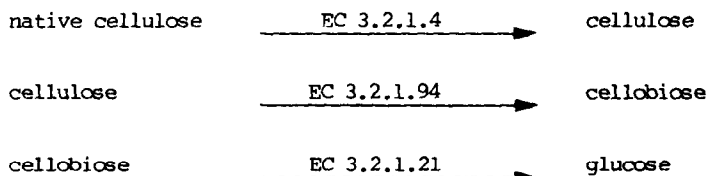
Over the past few years two main approaches have developed, whereby the first concerns known microorganisms and their improvement by mutation or genetic engineering with concomitant optimization of process development. The second approach concerns itself with the search for new

microorganisms, involving isolation, identification and selection with a specific goal such as the improvement of saccharification of the substrate, higher cellulase activities or specific product formations. Since pure cultures are inherently variable in their metabolic activities, the selection of stable and genetically uniform isolates producing maximal amounts of the desired product and a minimal number of unwanted metabolites had first priorities before further development of higher-producing strains commenced.

The realization, however, that the majority of polymer degrading microorganisms do not themselves form the desired products together with the observations that polymer hydrolysis rates can be significantly different from the monomer uptake and metabolic utilization rates, leads to a third developmental line. This third approach puts its emphasis on the search for and development of microbial strains which produce high amounts of all the hydrolytic enzymes responsible for the degradation of the polymer to monomers and then using the latter for separate production processes using specifically known microbial cultures. The problems encountered in this latest approach are the accumulation of the monomer, which in turn represses polymer hydrolysis, and the control of fermentation processes with mixed culture populations.

SACCHARIFICATION OF CELLULOSE

Despite the research efforts over the past decade on the elaboration of the mechanisms of enzymatic hydrolysis of cellulose (15, 45, 63), problems relating to the elucidation of the cellulase complex, the required ratio of the individual component enzymes involved and their individual and collective mode of action are still far from being solved (48). It has been generally accepted that the enzymatic degradation of cellulose into glucose is carried out by at least three enzymes:



The first enzyme, originally designated as C_1 , represents the 1,4- β -D-glucan 4-glucanohydrolase, often abbreviated as avicelase or endoglucanase. The second enzyme, originally designated as C_x , represents the exo-1,4- β -D-glucan cellobiohydrolase, often abbreviated as CMCase (carboxymethylcellulase) or exoglucanase and the third enzyme involves β -glucosidase, often referred to as cellobiase in the past. The existence of these three enzymes in the cellulase complex was confirmed with High Performance Liquid Chromatography. Using a DEAE on silica (10 mm diam) column with ammonium acetate buffer containing 5 mM sodium azide at 12°C (17), the corresponding proteins were separated and characterized from the supernatant of Trichoderma reesei cultures.

There is no doubt that the intense investigations of cellulose hydrolysis using Trichoderma reesei has led to a considerable understanding of the saccharification process. The observation that crude culture filtrates from T. reesei ITCC-1433 (30) were able to produce 4% glucose from a 10% cellulose suspension and an enzyme concentrate led to an accumulation of 8% glucose causing a glucose-cellobiose ratio of 75:1, which subsequently led to an inhibition of cellobiose hydrolysis and the formation of cellobiose and oligosaccharides, demonstrated the problems facing saccharification of cellulose. Removal of glucose with an ultra-filtration device resulted in a 90% saccharification in 48 hours. This severe effect of glucose on the glucosidase leading to cellobiose accumulation brings the saccharification process to a halt. Cellobiose accumulation should be avoided; this can be done by glucose removal or, in the case of low glucosidase activity, by the addition of an immobilized glucosidase (77). The glucosidase used was obtained from Aspergillus phoenicius and immobilization was by sorption on controlled pore alumina with about 90% activity retention. During an on-stream reaction period of 500 hours, only 10% of the original activity was lost using cellobiose as the substrate. This combination of culture filtrate and immobilized glucosidase was successfully used for the saccharification of Solka Floc, corn stover and exploded woods.

A very similar saccharification was carried out with 5% pretreated barley straw, wheat straw, corn stover, corn stalks and alfalfa stalks. The addition of 15 IU of Trichoderma E 58 cellulase per g dry weight of residue resulted in a 46%, 42%, 38%, and 31% hydrolysis, respectively (56).

The search for microorganisms that hydrolyze cellulose and either do not use glucose or convert the glucose to protein (SCP) or fuel, such as ethanol, butanol or methane has continued. A mesophilic anaerobe, Acetovibrio cellulolyticus (38) and two anaerobes belonging to the family of Bacteroidaceae, strain BAS and strain SPB-25, were isolated. A comparative study (27) revealed that the mesophilic anaerobes produce fairly large amounts of sugar; 50 gl^{-1} cellulose gave 12.9 - 14.5 gl^{-1} glucose, a 50% conversion efficiency in 21 days. One of these cultures, Acetovibrio cellulolyticus has been used as a co-culture in the fermentation of cellulose to methane. In this fermentation system (47), using a triculture of A. cellulolyticus, Desulfovibrio sp. and Methanosarcina barkeri methanogenesis was found to be the rate-limiting step. A pH control at 6.8 gave high cellulose hydrolysis rates with low methane yields, whereas no pH control resulted in a better methane production with a slower cellulose hydrolysis. Acetovibrio cellulolyticus was also adapted to the conversion of cellulose to ethanol, acetate, H_2 and CO_2 (4). Using strain CD 2 (ATCC 33288) and pH control at 6.8, ethanol production was favoured over acetate. Ethanol formation could be increased under low redox potential conditions. Changes in physical and chemical parameters in particular redox potential, appear to be of great significance for cellulose conversion with A. cellulolyticus.

Clostridium thermocellum is another organism under development for the conversion of cellulose to ethanol. This thermophilic anaerobe (72, 84) requires 60°C and pH 7.0 for a 52% (w/w) conversion of raw bagasse, mild alkali-treated bagasse and solka floc, and a 79% conversion of mild alkali- and steam treated bagasse (42). However, the end products, ethanol and acetic acid, have a severe effect on the viability of the culture. It is therefore necessary to remove the products from the system - technically easier given the higher culture temperature - avoiding the accumulation of more than 8 - 14 gl^{-1} ethanol and acetic acid. Clostridium thermocellum has also been under investigation as coculture. In conjunction with Methanobacterium thermoautotrophicum, methane instead of ethanol was obtained (84), whereas higher ethanol yields were obtained in the presence of Zymomonas mobilis (70).

Clostridium thermocellum, in contrast to most fungi, adheres to the cellulose molecule for its hydrolytic process. At least four additional protein components were considered to be associated with the cellulase

complex (6), which may be responsible for the adhesion of the organism to the insoluble cellulose molecule. With the help of cellular antigens (1), a cellulose binding factor was discovered, isolated and characterized (46). The results demonstrate that the cellular binding factor comprises a discrete, multisubunit complex or group of closely related complexes, which exhibit separate antigenic and multiple cellulase activities in addition to the property of cellulose binding. It is thought therefore that the complex may be structured in such a way to ensure (a) effective delivery to the substrate of all enzymes, and (b) to facilitate the transfer of the intermediates to their corresponding enzymes for further hydrolysis. It appears that the cellulase subunits are arranged within the cellulose-binding-factor complex in a defined supramolecular fashion designed for highly efficient cellulose hydrolysis. Such an arrangement would undoubtedly help in the latter relationship between cellulose hydrolysis and glucose transport and utilization.

Another suggestion to overcome low glucosidase activities in cellulolytic organisms is the use of yeasts as coculture for further utilization of the glucose and cellobiose. It must be realized, however, that in the case of yeasts, glucose is preferentially utilized in mixtures of glucose and cellobiose (25), which would result in the sequential diauxic utilization of the two substrates. The yeast Candida wickerhamii is one of those yeasts, which are able to ferment both glucose and cellobiose to ethanol (25, 39, 40), in which the ability to ferment cellobiose is dependent upon the initial glucose concentration (41).

The problems of glucose and cellobiose inhibition of the corresponding enzymes of the cellulase complex lead to an intensive search for microbial strains and conditions, which produce the most efficient cellulase complex in a short time with high activities, and a closer investigation into the functions of the individual enzymes and their differences amongst microorganisms. They have emphasized the need for investigators to measure and report "cellulase activity" with particular care.

CELLULASES

In maintaining a pH control between 4 and 4, glucosidase activity can be stabilized (29), since about 75% of its activity is otherwise lost in the case of T. reesei ITCC-1433. Under these conditions it is claimed to be

equivalent to the mutant strains QM 9414 and QM 9123. Significant increases in total cellulase activity can also be obtained in fermenters by using higher cellulose concentrations. Sternberg and Doval (73) found that an increase to 8% cellulose does not require a change in salt concentration provided NH_4OH is used as a nitrogen source and pH control. If one adds a change of cultivation system, continuous culture increases the productivity by 15 - 20% through recycling operations (21) and fed-batch cultivation by as much as 33% (23). The advantage of the latter system is, of course, that it allows slow addition of cellulose at high concentrations. The use of gamma-irradiated bagasse as carbon substrate only increased Avicelase and CMCase activities in T. reesei QM 9414 (49). A change from liquid state to solid state fermentation of wheat straw by T. reesei QM 9414 (8) increased cellulase yields by 72% from 160-250 IU to 250-430 IU per gram of cellulose.

Improvements in Trichoderma reesei strains also contributed significantly to increased cellulase formation (81, 87). Whereas the mutant QM 9414 exhibited 7.5 IU ml^{-1} (filter paper activity) and a productivity of 54 IU $\text{l}^{-1}\text{h}^{-1}$ (82), the mutant D 1-6 produced 11 IU ml^{-1} or 57 IU $\text{l}^{-1}\text{h}^{-1}$ (24) and the mutant RUT c-30, 12.01 IU $\text{ml}^{-1}\text{h}^{-1}$ (83) on pilot plant scale.

Although studies with Trichoderma reesei are the most advanced, the relatively low glucosidase activity in this strain led to further intensive investigations of cellulases from other microorganisms. A comparison of the extracellular activities of Clostridium thermocellum and Trichoderma reesei (60) revealed significant differences in the component enzymatic activities, mode of action, and stability to temperature and chemical effectors. It appears that in Cl. thermocellum the extracellular activities are highly coordinated with the intracellular saccharide transport activities (cf previous section). Cl. thermocellum also had a distinctly different ratio of the various enzymes in the cellular complex to each other compared with T. reesei. A great number of other fungi have recently received investigation, such as Aspergillus fumigatus (74), Pleurotus sajor-caju (52), Aspergillus wentii Pt 2804 (61), Pichia etchellsii (33), Dekkera intermedia (26), Myrothecium verrocaria (64), Fusarium moniliforme (62), Pryicularia oryzae (31), Sporotrichum thermophile (57), Scytalidium lignicola (10), Humicola sp. (3), Pestalotiopsis versicolor (67), Aspergillus terreus (19), Aspergillus foetidus (68) and Penicillium funiculosum (11).

In the case of Penicillium funiculosum, a quantitative elution and separation from glucose of the cellulase complex was obtained after hydrolysis of alkali-treated bagasse, alkali-treated cellulose powder and steam-treated bagasse (65) using polyacrylate gel (78). The recovery of all three major components was quantitative with a 10-fold concentration (11). DEAE Sephadex studies revealed that the cellulase complex comprises a cello-biohydrolase, three or more endo-(1 4) β -D-glucanases and two or three glucosidases (86). In isolation, these enzymes had very little capacity to solubilize cotton cellulose. If one recombines these enzymes in their original proportion, the actual activity was recovered quantitatively. A mutant UV-49 was obtained (37), which gave higher (50%) cellulase activity compared to the wild strain. The highest activities were 5.5 IU ml⁻¹ or 52 IU l⁻¹h⁻¹ (filter paper activity). In combination with a hypersecretive mutant Cu-1 in a medium favouring pH maintenance above 4.5 and supplemented with 3% rice bran, extracellular glucosidase activities between 30 and 36 IU ml⁻¹ were observed (44).

Desai and co-workers (10) used growth condition optimization with Scytalidium lignicola CD-48 for increases in cellular production, which resulted in 28 times higher glucosidase activity compared with T. reesei QM 9414. Since the other two enzymes are also present in significant amounts, this fungus appears to have great industrial potential. Using DEAE Sephadex, two glucosidase isozymes were found, both of which revealed strong inhibition by glucose.

Two glucosidases were also found in Humicola sp. (3), a thermophilic cellulolytic fungus. One of these glucosidases was an extracellular enzyme with substantially higher activities compared to T. reesei, whereas the second glucosidase was an extracellular enzyme.

A cellulase system rich in glucosidase activity was also reported from Pestalotiopsis versicolor (67) with Avicelase, OMCase and glucosidase activities of 0.25, 0.42 and 2.8 IU ml⁻¹, respectively. It was of particular interest to see that during cultivation, the pH of the medium increased from 5.0 to 6.5 in the initial stages, which is in contrast to all the other reports from cellulolytic microorganisms.

Pyricularia oryzae (31) and Sporotrichum thermophile (57) contain at least two different glucosidases. Both isozymes are concomitantly induced by

cellobiose or cellulose. Their different roles are thought to be connected with glucose regulation or the regulation of glucose levels inside the cell.

Mesophilic Streptomyces strains (32) and thermophilic Actinomycetes strains (50) exhibit significant cellulase activity. In Thermomonospora sp., the glucosidase is cell associated, whereas the avicelase and carboxymethylcellulase are to be found in the culture filtrate (28). Treating the wild strain Thermo-monospora sp. YX by a combined method of UV irradiation, 8-methoxysporalen and nitrosoguanidine, a mutant strain N-35 was obtained (58) which produced twice as much enzyme activity as measured by the filter paper activity method. The excellent thermostability of the Thermomonospora cellulases at 55 - 60°C, compared to below 45°C of T. reesei (16), has obvious potential industrial advantages. Grown on cellulose mineral salts medium multiple forms of endo- β -1,4-glucanase were obtained with different kinetic properties. The enzyme pattern changes with culture age. In Clostridium thermocellum, a thermophilic bacterium, only one cell-associated glucosidase was found and characterized (2). The presence of several groups of endoglucanase isozymes (68), differing in affinity for cellulose by three orders of magnitude, was demonstrated in cellulase complexes of various fungal origin (T. reesei, Aspergillus terreus, Asp. foetidus). A cellulase active on crystalline cellulose was purified from Cellulomonas uda (59) with an estimated molecular weight of 66,000 daltons, a pH optimum between 5.5 and 6.5 and a temperature optimum of 45 to 50 C. The purified enzyme produced cellobiose.

UTILIZATION OF HEMICELLULOSE

Apart from cellulose, plant materials contain significant amounts of hemicellulose (20-30%) and lignin (15-30%). The major hemicellulosic constituents of lignocellulosic wastes are the hetero-1,4- β -D-xylans and the hetero-1,4- β -D-mannans. As the xylans are structurally of mixed constitution, total hydrolysis occurs by synergistic actions of the endo-1,4- β -D-xylanases and various exo-glycosidases, which lead to the end products D-xylose, L-arabinose and D-glucuronic acid. The degree of hydrolysis is influenced by the amount of β -xylosidase present in the hemicellulolytic enzyme preparation (9), which indicates that the heteroxylan hydrolysis could face similar problems as the cellulose

hydrolysis if one considers the equivalent roles of xylosidase and glucosidase in the processes. A xylanase isolated and purified from Sporotrichum thermophile (55) exhibited a molecular weight of 25,000 which is significantly smaller than the corresponding cellulose degrading enzymes. In Bacillus subtilis, Roncero (69) revealed the existence of two genes controlling xylan utilization. The data indicate that these two genes code for two xylan-degrading enzymes, an extracellular beta-xylanase and a cell-associated beta-xylosidase. The existence of two enzymes agrees with reports from Aspergillus niger (18) and Cryptococcus albidus (7).

The most important development over the past few years has, however, occurred in the investigation of xylose utilization (36, 43, 51, 71), the conversion of pentoses to 2,3-butanediol (34) and to acetone and butanol (79). The extensive reviews cited in the introduction indicate that microorganisms growing on xylose can choose between two principal degradation pathways depending upon the presence or absence of three enzymes: D-xylose isomerase (EC 5.3.1.5), NADPH-linked D-xylose reductase (EC 1.1.1.21) and NAD-linked xylitol dehydrogenase (EC 1.1.1.9). These enzymes allow D-xylose to be either first directly isomerized to D-xylulose, or to be reduced to xylitol first with a subsequent oxidation to D-xylulose. It appears that the isomerase pathway is preferred by bacteria, the oxido-reductase pathway by yeasts and some fungi may have both. This has been confirmed (76) using Fusarium oxysporum, Candida utilis and Lactobacillus brevis. The biggest problem encountered in xylose fermentation is the conversion to xylitol or xylulose, as xylitol is inhibiting the D-xylose reductase. If the yeasts use predominantly the oxidoreductive pathway, pentose fermentation by yeast must be aerobic or at least partially aerobic. Using Candida sp. XF 217, Baillargeon and co-workers (5) found that optimal ethanol production rates are obtained at an oxygen uptake rate of $9 - 12 \text{ mmol l}^{-1} \text{ h}^{-1}$. The highest ethanol yields were obtained with 26 g l^{-1} in 40 hours using Candida shehatae CSIR-Y 492 (13). Xylitol formation was inversely related to the degree of aeration. With xylulose as substrate, Saccharomyces uvarum showed a 76% conversion efficiency (75). Although the fungus Pachysolen tannophilus produces ethanol as major end product from pentoses, mutants have been obtained (53) which predominantly produce acetic acid.

Another aspect of lignocellulosic degradation is the formation of fungal biomass or single cell protein (19,66), which may be of considerable importance in some countries for animal feed.

For lignin degradation, the reader is referred to the excellent review by Janshekar and Fiechter (35).

CONCLUSIONS

The effective utilization and conversion of plant materials, cellulosic and lignocellulosic wastes is still far from being solved. However, some important progress has been made in aspects of cellulose and hemicellulose conversion studies. In the case of cellulose as substrate, it appears that it is not the specific surface area but rather the crystallinity of cellulose which shows a highly linear relationship to the rate of hydrolysis. This means that it is the fine structural order of cellulose which may dictate the hydrolysis rate. Structural effects, cellulase adsorption, product inhibition and cellulase deactivation must also be considered in order to increase the conversion efficiency.

Amongst the most elegant research over the past two years is that of Bayer and his co-workers (6, 46), who applied a combination of genetic and immunochemical methods to investigate the mechanism of the strong adhesion of the cellulases to cellulose. From a working hypothesis that adhesion of Cl. thermocellum to cellulose may be mediated by cellulases associated with the bacterial cell wall, they isolated a mutant AD₂, which was less adherent. Immunochemical comparisons between the parent strain and the mutant revealed a deficiency in a single major surface antigenic component, which they called CSF or cellulose-binding factor. The isolation of this factor revealed a particulate, multisubunit entity of complicated quaternary structure with a molecular size of approximately 18 nm. Treatment with sodium dodecyl sulfate resulted in the resolution of 14 polypeptide bands, which, according to immunoprecipitation, all belong to the same complex. Of the 14 components, only one unit (MW 210,00) was found to be antigenically active and at least 8 of the remaining components exhibit cellulase activity. Further research should reveal the final mechanism of cellulose degradation by those microorganisms which adhere to the cellulose molecule.

For other microorganisms, which excrete the cellulase complex, the picture is still very obscure. The finding of multiple endoglucanases may have some relationship with the different structures in the cellulose molecular, and the variable numbers of glucosidases may be related to the regulation and transport of glucose. It is evident, however, that a relatively high glucosidase activity is very desirable for an efficient and rapid cellulose hydrolysis. The use of co-cultures for the speedy removal of glucose shows great promise, but we still lack knowledge and experience in the handling of mixed populations. One-step conversions by thermophilic microorganisms and anaerobic microorganisms, on the other hand, may have certain advantages, as the adhesion complex appears to ensure a good relationship between cellulose hydrolysis and glucose uptake and thus a high conversion efficiency. The next few years may see major breakthroughs in this area of research.

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