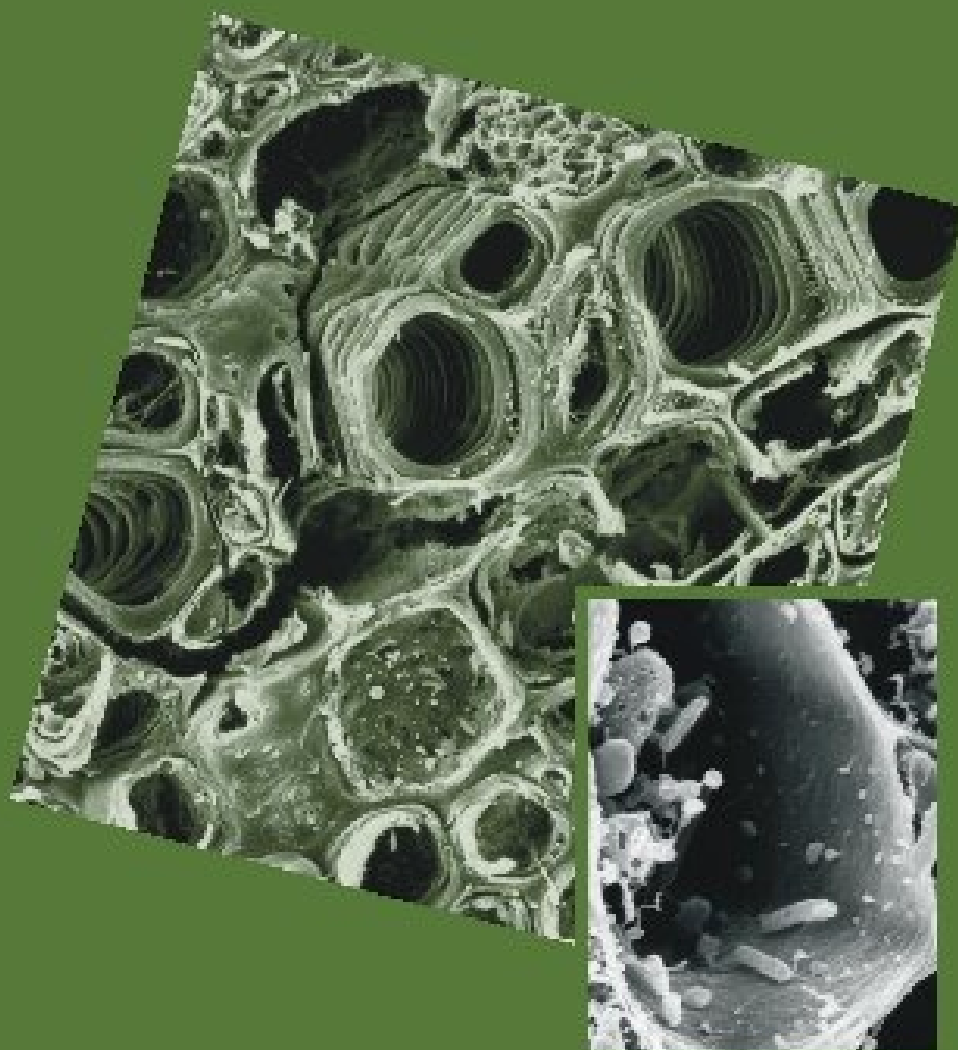# Utilization of Lignocellulosic Substrates by Solvent-Producing Clostridia

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des namiddags te half twee in de Aula

A mi familia

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Table of contents

Chapter 1

General Introduction

Mankind has used microorganisms in a variety of domestic applications, even without knowing it, for thousands of years. The oldest processes developed were involved in the production of food. Classical examples of microbial fermentations are the production of beer, wine and dairy products. Although archeological findings suggest that during the Neolithic period wine, beer and cheese were already made, the first detailed descriptions date from the Egyptians (around 3000 B. C.), who recorded grape harvest, brewing and cheese making on stone tablets and tomb walls. Different species of yeasts are used for brewing and wine making, and lactic acid bacteria are centrally involved in the second step of wine making and in the fermentation of milk. Nowadays, the mentioned processes still rely on the microorganisms and constitute an important part of the world's food industry.

The discovery of microbes by Antoni van Leeuwenhoek in the second half of the 17th century and the developments in microbiology initiated by Pasteur and others from the 19th century onwards favored the improvement of classical microbiological processes and stimulated the development of new ones. In the last 50 years many large-scale production processes using microorganisms have been developed. Some examples are the production of antibiotics by fungi, the production of amino acids by bacteria or the large-scale production of industrial enzymes (such as cellulases, amylases or proteases) by microbial cultures. The environmental problems caused by the use of petroleum-derived fuels and chemicals and the limited nature of the fossil oil reserves have stimulated a search for alternative, more environmentally friendly processes for the production of feedstocks for industry and for energy carriers. Renewable resources, described as those that can be used without depletion, include solar and wind energy, hydro power and biomass. Fermentation processes have a good potential for significantly contributing to a more sustainable world because a number of microorganisms utilize biomass for the production of interesting chemicals and energy carriers (11), e.g. pure hydrogen gas is produced by thermophilic bacteria belonging to the *Thermotoga* genus; ethanol is produced by several yeast strains; and the solvents acetone, butanol and ethanol are produced by several bacterial species belonging to the genus *Clostridium*.

The genus *Clostridium*

The production of the solvents acetone, butanol, isopropanol or ethanol by microbial fermentation was first described in the late 19th century. Large-scale industrial fermentation processes for the production of acetone, butanol and butanol (the ABE fermentation) were developed during the first half of the 20th century, becoming the second largest industrial fermentation (after ethanol production by yeasts). To date, this is the only fermentation process at industrial scale using clostridial strains. A large number of strains able to produce neutral solvents

(acetone, butanol, ethanol, isopropanol) from different carbohydrate substrates have been described and used in patent applications over the years (32). Initially, solvent-producing strains were classified mainly as *C. acetobutylicum* or *C. beijerinckii*, but important physiological and genetic differences observed between strains belonging to the same group made it necessary to make a clear classification of the existing strains. Detailed DNA similarity studies and 16S rDNA sequence comparisons between strains belonging to different culture collections showed that the existing strains can be classified into four distinct groups (29, 30, 37, 38) that are all members of the cluster I of the clostridia: *C. acetobutylicum* (type strain ATCC 824); *C. beijerinckii* (type strain NCIMB 9362); *C. saccharoacetobutylicum* (type strain NCP 262), and *C. saccharoperbutylacetonicum* (type strain N1-4). All solventogenic clostridia are mesophilic and contain DNA with low GC-content.

The genus *Clostridium*, formed by Gram-positive, anaerobic, rod-shaped and endospore-forming bacteria, constitutes a phylogenetically very diverse genus. The heterogeneity of this group is indicated by both the remarkable range of GC-content of the DNAs of different species, varying from a minimum of 24 mol% for *C. pasteurianum* to a maximum of 55 mol% for *C. barkeri*, and the wide range of optimal growth temperatures, from 34-37 °C for *C. beijerinckii* up to 75-78°C for *C. thermohydrosulfuricum*. However, members of *Clostridium* can still be considered descendants of a common ancestor that emerged early in the evolution of the Gram-positive bacteria. A phylogenetic tree containing low GC-content Gram-positive bacterial species that include the solvent-producing strains is shown in Fig. 1.1.

According to the latest release of the taxonomic outline of the Bergey's Manual of Systematic Bacteriology, the genus *Clostridium* comprises 144 validly described species (22). The phylogenetic relationships between Clostridial species have been revised by Stackebrandt and co-workers (72, 73). Based on 16S rDNA sequence analysis, the known species have been grouped into 19 clusters.

Among the clostridia there are both pathogenic (to human and animals) and non-pathogenic species. The pathogenic species are generally proteolytic and without exception all species produce toxins, generally enzymes, that are responsible for their toxicity. *C. botulinum* and *C. tetani* produce the most powerful toxins known so far, that selectively block neurotransmission in the peripheral and central nervous systems, resulting in botulism and tetanus, respectively. Other toxins produced by clostridia have other mode of action, such as degrading tissue (in case of *C. perfringens*, causing gas gangrene) or causing diarrhea (in case of *C. difficile*, causing pseudomembranous colitis). The molecular biology of pathogenesis by clostridia has been reviewed in a comprehensive overview (64).
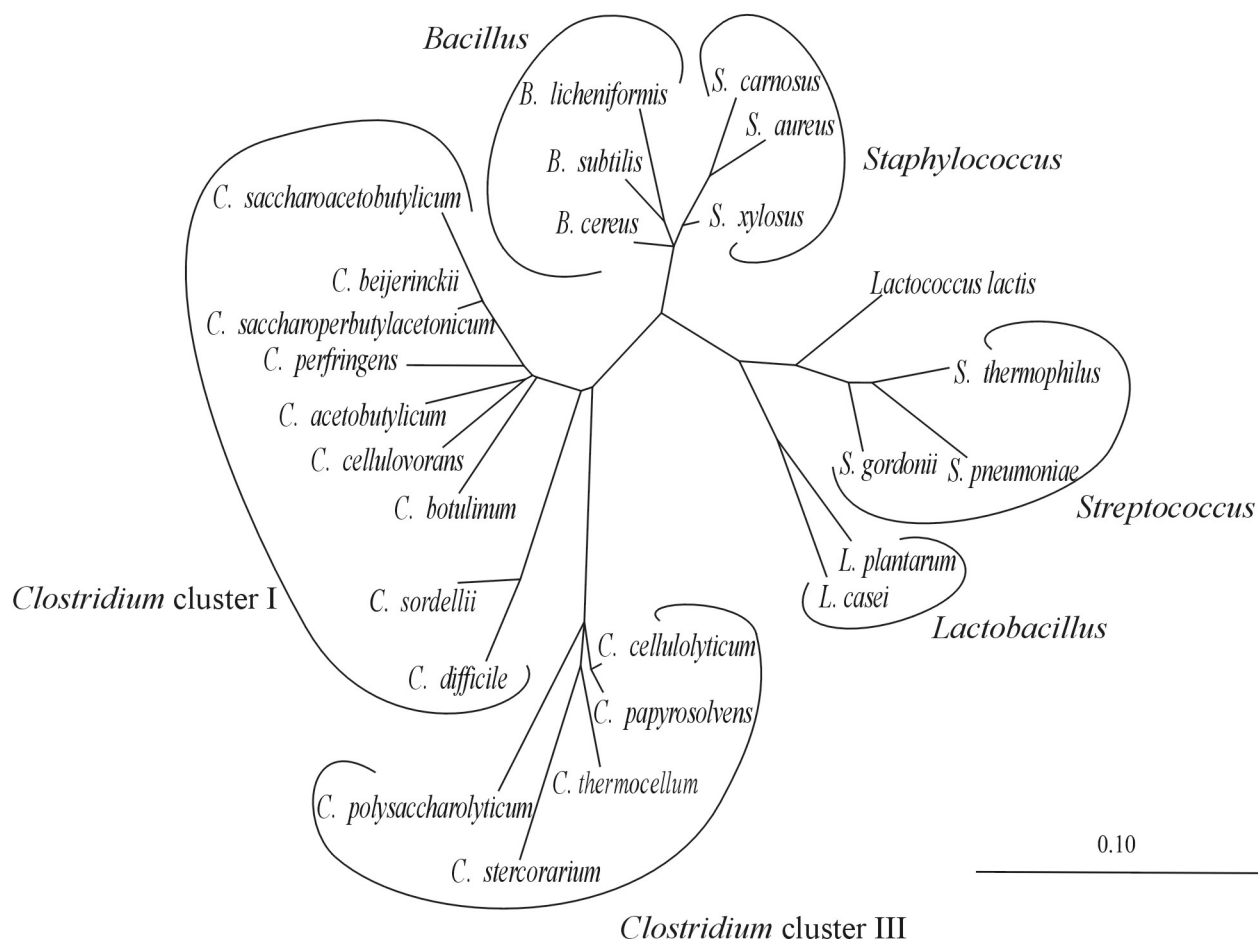
Figure 1.1 Phylogenetic tree based on 16s rDNA sequences of low GC-content Gram-positive bacteria. Alignment and phylogenetic analysis were performed with the software ARB (74). The reference bar indicates 10 nucleotide exchanges per 100 nucleotides.

Several non-pathogenic thermophilic clostridial species are considered to have potential for the production of ethanol or organic acids, mainly due to their capacity to ferment cellulose or pentose sugars. Members of the cellulolytic species *C. thermocellum* are able to ferment cellulose directly to ethanol, but their slow growth rate and limited tolerance to ethanol (approximately 3% (v/v)) makes their industrial application unattractive. Other non-cellulolytic strains able to produce ethanol are *C. thermosaccharolyticum* and *C. thermohydrosulfuricum*, but their industrial application is hampered by the same problems as with *C. thermocellum* (8). Several studies have shown that thermophilic clostridial strains are able to produce acetic acid (e.g. *C. thermoaceticum* (9)), butyric acid (e.g. *C. thermobutyricum* (7)) or lactic acid (e.g. *C. thermolacticum* (78)) as end products at high yields during growth on glucose or xylose, but more research is needed before the industrial production of these compounds by fermentation can be established.

The cluster III of the clostridia contains all cellulolytic clostridial species described so far with the exception of *C. cellulovorans*, which belongs to cluster I. Most of the cellulolytic clostridial species produce a large extracellular multiprotein complex specialized in cellulose degradation called cellulosome that is typical for anaerobic cellulolytic organisms (68). Cellulosomes are important for the hydrolysis of plant biomass, since they are able to degrade not only cellulose, but also other components in plant cell walls (51). In contrast to the cellulosome-producers, the thermophilic species *C. stercorarium* produces a very simple cellulase system, composed of two single enzymes (6).

History of the acetone-butanol-ethanol (ABE) fermentation

The competition of the petrochemical industry and an increase in the price of the substrates provoked a decline in the ABE fermentation, that was definitively abandoned at a commercial level in the early 1980s by the closure of the last fermentation plant in South Africa by National Chemical Products.

Acetone is currently produced by the cumene hydroperoxide process or by catalytic dehydrogenation of isopropanol. This product is widely used as a solvent (in printing inks, adhesives, etc) or as a precursor of other products such as acrylic plastics (54). Butanol (1-butanol) is synthesized by reduction of butyraldehyde or from ethylene oxide. Butanol is used primarily as a chemical intermediate in the production of butyl acrylate and methacrylate and in the production of glycol ethers and butyl acetate and as a solvent for fats, waxes, varnish, etc (54). Iso-propanol (2-propanol) is generally manufactured from propene, either by an indirect or a catalytic hydratation process, and it is mainly used in antifreeze solutions, and as a solvent in products such as oils, ink and cosmetics (54).

The development, rise and decline of the industrial ABE fermentation are well-documented and have been summarized from different perspectives by several authors (18, 34, 71). A very complete overview covering the history of the process, and the current state-of-the-art has been published recently (31). In this section a short summary of the history of the ABE fermentation is given.

Louis Pasteur described for the first time the production of butanol by microbial fermentation (56). A number of studies on butanol production by anaerobic bacteria followed, and in 1905 Schardinger first reported the production of acetone by fermentation of glucose (3, 66). The fermentation process at industrial scale was first developed at the early twentieth century aimed at the production of butanol, used as starting component for synthetic rubber. In 1913 Strange and Graham Ltd built the first production plant at Rainham (UK).

The outbreak of World War I in 1914 had a big impact on this process as acetone was needed in large amounts for the production of smokeless powder (cordite). The fermentation process was further developed and improved by Chaim Weizmann and several plants were built in the United Kingdom. As a result of the German blockade, the supplies of carbohydrate substrates became very difficult, and from 1916 on, new plants were built in different parts of the British Empire, USA and Canada, where suitable substrates were easily available.

During the 1920s the demand for butanol grew considerably due to its properties as good solvent for the nitrocellulose lacquers used in the automobile industry. New strains were isolated and the fermentation technique was improved in order to expand the substrate range and the solvent yields obtained. At the beginning the process developed mainly in the USA and the UK, but after 1936 plants were built in other countries such as Japan, India and Australia.

During World War II the process was aimed again at the production of acetone for military use. By 1945, two-thirds of the butanol and one-tenth of the acetone in the USA were still produced by fermentation. However, as already mentioned, the increase in the price of the substrates and the development of the petrochemical industry made the industrial ABE fermentation no longer economically viable in the years to follow.

Limitations of the conventional process

The increasing oil prices, starting from the crisis of 1973, and environmental policies to reduce CO_2 emissions have stimulated research into the development of technology to produce chemicals and fuels from renewable resources, including the ABE fermentation (11). Before the ABE fermentation can be established as an economically viable alternative process for the production of solvents, there are a number of specific bottlenecks that have to be improved. These bottlenecks, which played an important role in the decline of the process, include (31):

A) Low final solvent concentrations, resulting in low productivity, due to solvent toxicity. The solvents produced, especially butanol, are toxic to the bacteria at relatively low concentrations (between 1-2 % (v/v) for butanol). Low product concentrations in the fermentation broth results in high recovery costs.

B) The substrate costs influence significantly the economics of the process (accounting for up to 60% of total costs (16)). Acetone and butanol are relatively cheap bulk chemicals, with a price marginally higher than the substrates used for producing them. Suitable cheap substrates have to be found in order to achieve a competitive process.

C) The traditional fermentation process is highly sensitive to contamination and in the past bacteriophage infections caused major operation problems (33). The need to sterilize the

fermentation substrate and the operating plant adds a significant economic burden to the process.

During the last three decades considerable research has been carried out by many groups distributed all over the world in order to restore an economically viable ABE fermentation. As a result, important developments in the knowledge of the metabolism, physiology and genetics of the solvent-producing strains (23, 79) have been made, including the sequencing of the genome of the type strain *C. acetobutylicum* ATCC 824 (53) and the development of interesting mutants with enhanced solvent production (28, 55). Significant advances have been made in the fermentation technology as well, in which continuous fermentation processes and (in-situ) recovery methods alternative to the traditional destillation (i. e. gas-stripping, pervaporation) have been developed (44, 57-59).

Biochemistry of the process and metabolic engineering

In batch culture, solvent-producing clostridia produce hydrogen, carbon dioxide, acetate and butyrate during the initial growth phase (acidogenic phase), which results in a decrease of the pH of the culture medium. As the culture enters the stationary growth phase, the metabolism of the cells switches to the production of solvents (solventogenic phase). During the second growth phase acids are partially re-assimilated simultaneously with the consumption of carbohydrates, resulting in an increase of pH of the medium.

The biochemical pathways used for the conversion of carbohydrates into hydrogen, carbon dioxide, fatty acids, and solvents by solvent-producing clostridial species have been extensively studied and characterized (34). Hexose sugars are metabolized via the Embden-Meyerhof pathway (Fig. 1.2). One mol of hexose to is converted to 2 moles of pyruvate, with the net production of 2 moles of ATP and 2 moles NADH. The utilization of pentoses takes place via de pentose phosphate pathway (Warburg-Dickens pathway), yielding 5 mol of ATP and 5 mol of NADH and 2 mol of fructose-6-phosphate and 1 mole of glyceraldehyde-3-phosphate (which both enter the glycolytic pathway) per 3 mol of pentoses (35). The pyruvate resulting from the gylcolysis is cleaved by pyruvate ferredoxin oxidoreductase in the presence of Coenzyme A to yield CO_2, acetyl-CoA and reduced ferredoxin. Acetyl-CoA is the central intermediate in the branched fermentation pathways leading to both acid and solvent production. (Fig 1.2)

Despite numerous physiological studies, it is still not completely understood how the metabolic switch from acid to solvent production is regulated at the molecular level (25). Initiation of solvent formation requires low pH, threshold concentrations of acetic and butyric acid, and a suitable growth-limiting factor such as phosphate or sulfate (1, 65). Solvent formation appears to be

associated with the availability of ATP and NAD(P)H (49) and can be controlled, in continuous culture, by varying the glucose concentration (26). Increasing the reducing power in the cell by inhibiting hydrogenase or by creating a redox imbalance can also enhance solvent formation. The reported role of a DNA-binding protein, Spo0A, on the expression of genes that are involved in solvent production and sporulation in *C. beijerinckii* (63), suggests that these two phenomena may be related.

Most of the genes that encode enzymes involved in primary metabolism have been characterized, and there is extensive knowledge on the regulation of the expression of genes involved in acid and solvent production and their function in the metabolic pathways (23, 79). The best-studied strain at the genetic level is *C. acetobutylicum* ATCC 824. In this strain the genes involved in solvent production are located in a megaplasmid of 210 kb (pSOL1), whose loss produces strains unable to make solvents (12). The whole genome of *C. acetobutylicum* ATCC 824, including the megaplasmid, has been completely sequenced (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html) (53). The presence of a similar megaplasmid in other strains has not been reported.

Techniques like mutagenesis (5) and transformation have been developed for solvent-producing strains (80, 81), making possible the engineering of recombinant strains with altered product formation. Integration-vectors have been developed for *C. beijerinckii* and *C. acetobutylicum* and used to specifically disrupt genes (24, 25). In *C. acetobutylicum* ATCC 824 inactivation of the *aad* gene (coding for acetoacetate decarboxylase; see Fig. 1.2) eliminates acetone formation and reduces butanol production by 85%. On the other hand, inactivation of the *buk* gene (coding for butyrate kinase; see Fig. 1.2) reduces butyrate production but increases butanol production by 15% (25). Complementation of the *aad* mutant with a functional *aad* gene restored butanol production but not acetone production and complementation of the *buk* mutant with a functional *buk* gene restored the production of butyrate in acidogenic cultures (24). In addition, a mutant strain with an inactivated *solR* gene, located in the megaplasmid and encoding a putative regulator, produced higher amounts of solvents compared to the wild type (27). Antisense mRNA techniques have also been used successfully for the study of regulation of product formation (14).
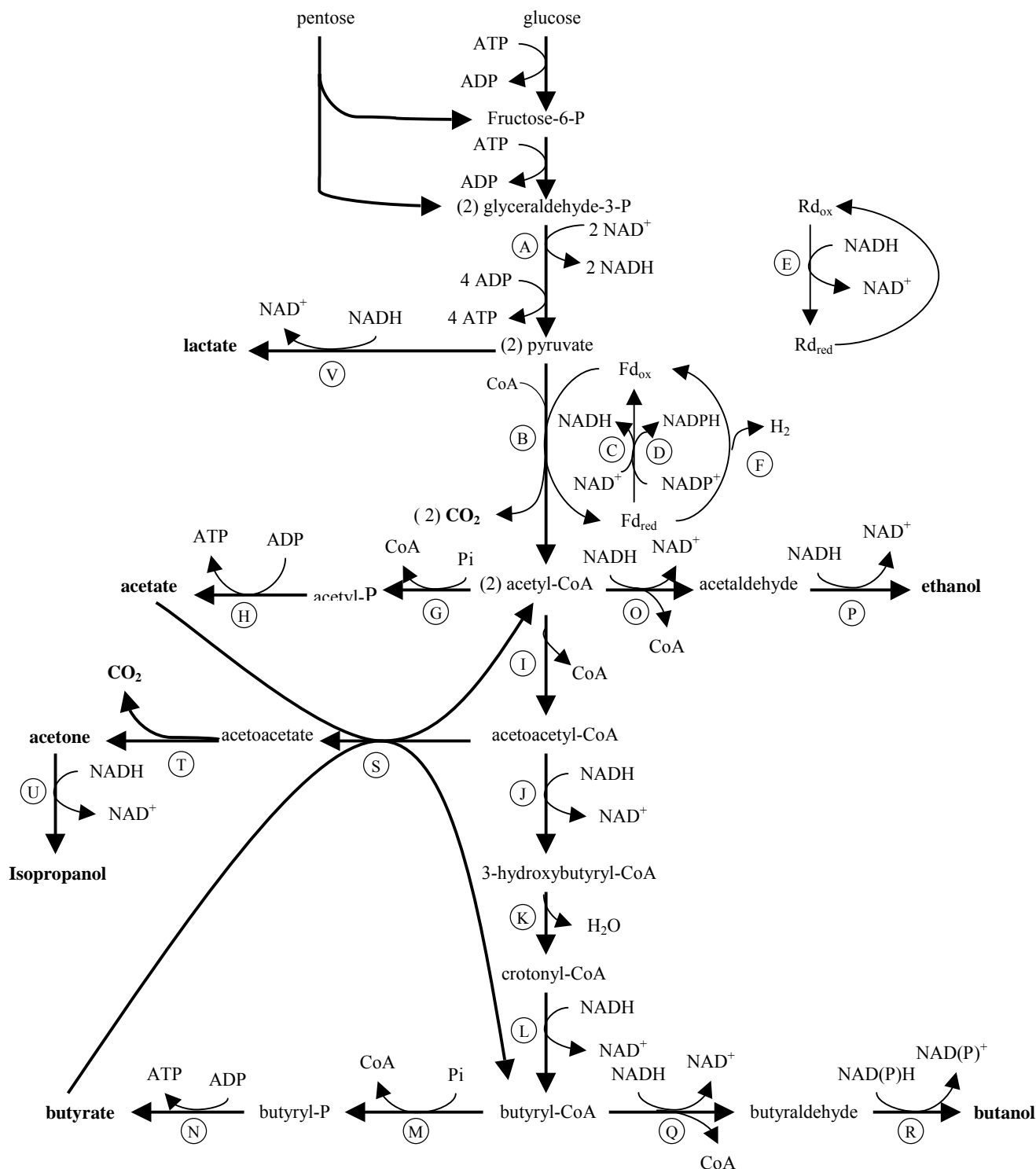
Figure 1.2 Biochemical pathways in *C. acetobutylicum* and *C. beijerinckii* (17, 34). Enzymes are indicated by letters as follows:A, glyceraldehyde-3-phosphate dehydrogenase; B, pyruvate-ferredoxin oxidoreductase; C, NADH-ferredoxin oxidoreductase; D, NADPH-ferredoxin oxidoreductase; E, NADH-rubredoxin oxidoreductase; F, hydrogenase; G, phosphotransacetylase; H, acetate kinase; I, thiolase; J, 3-hydroxybutyryl-CoA dehydrogenase; K, crotonase; L, butyryl-CoA dehydrogenase; M, phosphate butytransferase (phosphotransbutyrylase); N, butyrate kinase; O, acetaldehyde dehydrogenase; P, ethanol dehydrogenase; Q, butyraldehyde dehydrogenase; R, butanol dehydrogenase; S, acetoacetyl-CoA:acetate/butyrate:CoA transferase; T, acetoacetate decarboxylase; U, isopropanol dehydrogenase; V, lactate dehydrogenase. Abbreviations: CoA, coenzyme A; P, phospate; Rd, rubredoxin; Fd, ferredoxin.

A chemically-induced mutant of the strain *C. beijerinckii* NCIMB 8052, strain BA101, that produces significantly increased amounts of butanol (55) constitutes one of the most interesting strains developed so far. Strain BA101 has been used in a number of (economic) studies in order to develop an industrial ABE process using media based on corn starch or corn steep liquor combined with novel techniques for butanol recovery (57, 58, 61, 62).

Substrates for the ABE fermentation

As already mentioned, the costs of the substrates needed for the fermentation are a key factor in the economics of the ABE fermentation. In the past, commercial fermentations used starch (corn, wheat, etc) or sugar (molasses) substrates, but at present their market price is too high to be considered as viable feedstock. The ability of saccharolytic clostridia to utilize a wide range of carbohydrate substrates, including mono- and disaccharides as glucose, xylose or cellobiose and polymers such as xylan or starch (50), has stimulated a search for alternative cheaper substrates. A number of cellulosic and non-cellulosic substrates have been tested as possible alternatives with different solvent-producing strains.

Table 1.1 Several of the substrates tested as alternative for the acetone-butanol-ethanol fermentation

Substrates	Main carbon components	Organism	References
Non-cellulosic			
Apple pomace	fructose, glucose, sucrose	*C. beijerinckii*	(77)
Jerusalem artichokes	polyfructans	*C. beijerinckii*	(47)
Whey	lactose	*C. acetobutylicum,*	(46)
		C. beijerinckii	(67)
Low-grade potatoes	glucose	*C. beijerinckii*	(52)
Soy molasses	dextrose, sucrose, fructose	*C. beijerinckii*	(60)
Lignocellulosic			
Wood hydrolysate	glucose, mannose	*C. acetobutylicum*	(45)
Peat	glucose, xylose	*C. beijerinckii*	(20)
Palm oil effluent	oil, glucose, xylose	*C. aurantibutyricum*	(69)
Domestic organic waste	glucose, xylose	*C. acetobutylicum*	(42)

Lignocellulosic substrates, defined as those derived from plant material and composed mainly of lignin and carbohydrate polymers (cellulose and hemicellulose), and in particular agricultural wastes, are considered the substrates with the greatest potential for the ABE fermentation due to their wide availability, low price and sugar composition. Many studies have shown that acid and/or enzymatic hydrolysates of lignocellulosic material from a variety of biomass

sources are potential feedstock for ABE fermentation (10, 34, 45). In most cases, the material has to be pretreated in order to make it more accessible to chemical or enzymatic hydrolysis; the most common pretreatment is steam-explosion (19, 45), but extrusion has been also used (10). Yu and co-workers (82) investigated the utilization of cellulose and hemicellulose in acid-hydrolyzed, steam exploded wood. The production of 9 g/l of butanol, with nearly theoretical product yields (0.26 g of butanol per g of sugar consumed), indicates that the bioconversion of wood cellulose and hemicellulose carbohydrates is feasible.

Another approach to enable the utilization of biomass is the application of simultaneous saccharification and fermentation systems. These can be classified into two groups:

i) Co-culture systems. The use of mixed cultures of solventogenic clostridia with true cellulolytic organisms has the advantage of eliminating the pre-hydrolysis step. The direct conversion of cellulose to solvents by co-cultures of *C. acetobutylicum* with *C. cellulolyticum* or *C. thermocellum* has been shown. In both cases, the production of solvents was very poor, possibly due to the fact that the concentrations of sugars and butyric acid in the medium were too low to induce the solventogenic phase. To increase the level of butyric acid in the medium, co-cultures of *C. acetobutylium* and *C. beijerinckii* with butyric acid producing strains of *C. butyricum* and *C. pasteurianum* have been tested, but the amount of solvents produced was not higher than in the mono-cultures (34).

ii) Addition of cellulases to the culture medium. Supplementing the culture medium with cellulases is also an alternative to pre-hydrolysis of the substrate. Certain strains of *C. acetobutylicum and C. beijerinckii* are able to grow on and produce solvents from crystalline cellulose when a cellulase preparation from the fungus *Trichoderma reesei* is present in the medium (chapters 3 and 7 of this thesis). Fermentation by *C. acetobutylicum* of alkali pretreated wheat straw in a medium supplemented with cellulase from *T. reesei* resulted in 17.3 g solvents/l and solvent yields of 18.3% with respect to pretreated wheat straw after 36 hours of fermentation (70). These results may represent an economic improvement with respect to separate hydrolysis and fermentation of these substrates.

Cellulose utilization by anaerobic bacteria: The cellulosome

Cellulose is the most abundant component of plant biomass and constitutes a major component of plant cell walls. In most cases the cellulose fibers are embedded in a matrix formed by other structural polymers, mainly hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30 % of plant dry weight, respectively, depending on the material (43). The microbial degradation of cellulose is carried out by several fungal and bacterial species, including several clostridial species.

Due to its physical properties, cellulose is a highly recalcitrant substrate for enzymatic degradation. Cellulose molecules are composed of chains of β-1,4 linked glucose units. The chains are insoluble and form parallel bundles that are very stable due to interchain hydrogen bonds and Van der Waals interactions between the pyranose rings (2). In nature, however, cellulose fibers do not present a pure crystalline structure, but contain regions with variable degrees of crystallinity, from purely crystalline to purely amorphous (48).
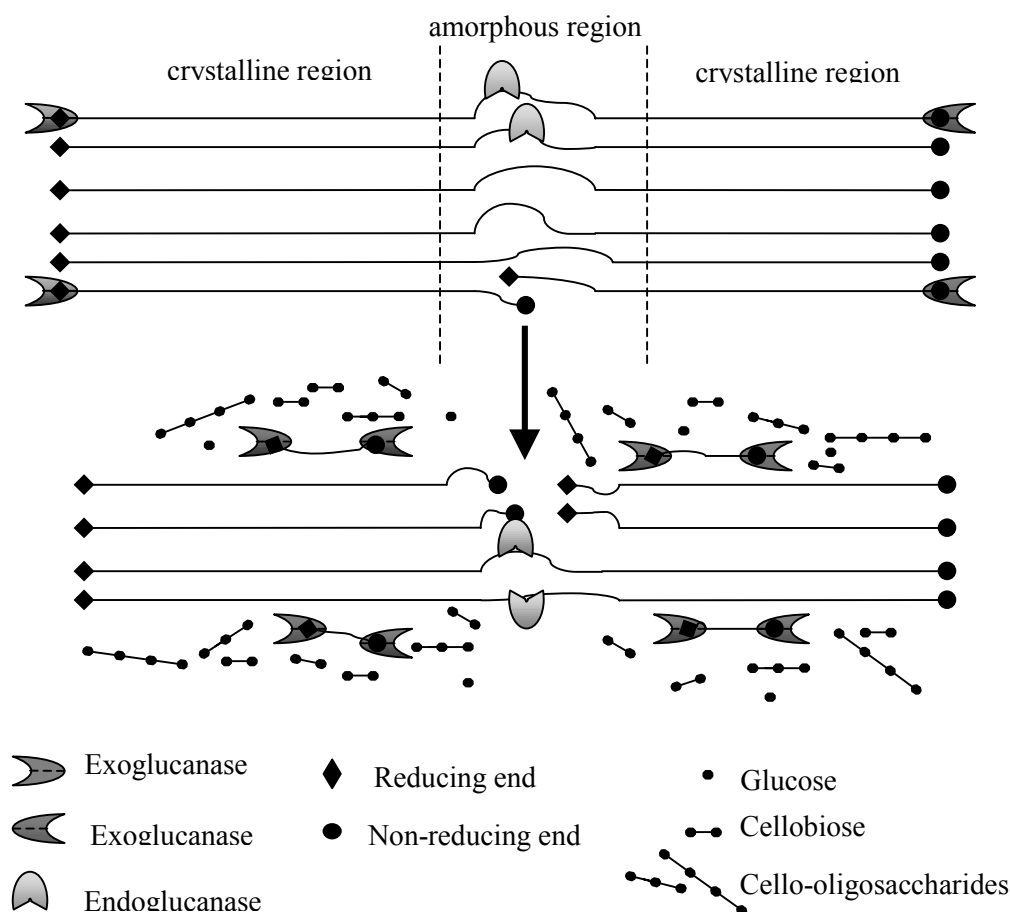
Figure 1.3 Schematic representation of the hydrolysis of cellulose by free cellulases, based on (43).

The microbial degradation of cellulose is carried out by a number of different enzymes that cleave the β-1,4- glycosidic bonds between the β-D-glucose units. Based on structural features, these enzymes have been divided into 14 different families of glycoside hydrolases (13). According to their mode of action, cellulases are subdivided in endo- and exoglucanases (Fig. 1.3). Endoglucanases (E.C. 3.2.1.4) randomly cleave the cellulose chains at exposed positions in amorphous regions and create new ends, while exoglucanases (also called cellobiohydrolases) (E.C. 3.2.1.91) degrade the polymeric chain either from the reducing or the non-reducing end, producing cellobiose as main product (Fig. 1.3). These two types of enzymes can be distinguished by their substrate specificity. Endoglucanases show high activity on soluble cellulose derivatives, such as

carboxy-methylcellulose (CMC) and very low (or not at all) on microcrystalline cellulose, while exoglucanases show relatively high activity on microcrystalline cellulose. During an efficient degradation of cellulose, both types of enzymes act synergistically. All cellulolytic organisms known so far produce at least one, but mostly several glucanases belonging to each type.

Various anaerobic organisms, including several fungal and bacterial species, degrade cellulose by producing an extracellular multi-protein complex called the cellulosome, which has been extensively studied (2, 15, 68). In an active cellulosome, glycoside hydrolases bind to a large non-enzymatic scaffolding protein called Cellulose-binding protein (Cbp) or Cellulose integrating protein (Cip) that binds to the cellulose polymer and to the cell. Cellulosomes have been proposed to provide a number of advantages to its host, such as positioning hydrolytic enzymes that act synergistically in their optimal orientation with respect to the substrate, or the minimalisation of product diffusion, since cellulosomes are attached both to the cell surface and to the substrate (Fig 1.4).

Figure 1.4 Schematic model of the *C. cellulovorans* cellulosome after Doi and Tamaru (15). The cellulose-binding protein is shown in black and the enzymes in gray. The numbers represent the family number in the corresponding classification of the carbohydrate active enzymes (13). The drawing is not on scale. Abbreviations; CBM, carbohydrate-binding module; SLH, surface layer homology; GH, glycoside hydrolase; PL pectate lyase.

Although cellulosomes from different organisms present significant variations, there are conserved characteristics between them. Cellulosomal subunits bind to the scaffolding protein by means of duplicated domains called dockerins (on average, 22 amino acids each) often located at

the C-terminal end of the protein. The dockerins interact with hydrophobic cohesin domains (of, on average, 140 amino acids each) in the Cbp. In addition to the cohesins, the scaffolding protein contains a cellulose-binding domain and hydrophilic domains with homology to surface layer proteins (SLH domains) that have been proposed to be involved in adhesion to the cell surface (15). The majority of the cellulosomal enzymatic subunits contain catalytic domains belonging to different families of the glycoside hydrolase classification, although enzymes with catalytic domains with other activities can be found, such as a cellulosomal pectate lyase from family 9 in the *C. cellulovorans* cellulosome (15). The presence of enzymes with different activities and substrate specificities in the cellulosome enables this complex to degrade cellulose as well as other polymers present in plant-cell walls, such as xylan or pectin (21, 39). In *C. cellulovorans* it has been observed that the growth substrate affects the enzymatic activity and composition of the cellulosomes produced (75). During growth on pectin, a structural polysaccharide composed of galacturonic acid present in cell walls of higher plants, cellulosomes are produced with higher cell-wall degrading activity as compared to those produced during growth on glucose, cellobiose, xylan or locus bean gum (51).

In bacteria, the genes encoding cellulases are either randomly distributed (e.g. *C. thermocellum*) or clustered in the chromosome (e.g. *C. cellulovorans*, *C. cellulolyticum*, *C. josui* (4, 36, 76)). The cellulosome gene cluster from *C. cellulovorans* is approximately 22 kb in size and contains nine genes coding for cellulosomal subunits, in which the first gene codes for the Cbp and the second gene for an exoglucanase belonging to glycoside the hydrolase family 48 (76). The organization of the gene clusters found in *C. cellulolyticum* and *C. josui* is very similar to the one in *C. cellulovorans*, suggesting the presence of a common ancestor (43).

Some of the solvent-producing clostridial strains are able to produce extracellular cellulolytic and xylanolytic activity (40, 41). However, none of the known strains is able to utilize cellulose for growth (50). Unexpectedly, in the chromosome of the strain *C. acetobutylicum* ATCC 824 genes encoding for putative cellulosomal components are present (53). Ten of these genes are forming a gene cluster that highly resembles the clusters found in *C. cellulovorans*, *C. cellulolyticum* or *C. josui*. The study of the genes encoding for putative cellulosomal components and their regulation in *C. acetobutylicum* ATCC 824 represents a first step towards enabling this strain to degrade cellulose and other plant polymers and the establishment of a process for the direct conversion of lignocellulosic substrates into acetone, butanol and ethanol.

Scope and outline of this thesis

The work presented in this thesis was initiated in relation to an ABE fermentation research program financed by the Commission of the European Union that focused on investigating the potential of converting cheap substrates into solvents. Within this program, the Agrotechnological Research Institute in Wageningen (ATO) carried out studies on the utilization of lignocellulosic hydrolysates for the ABE fermentation. As an attempt to develop strains able to convert biomass directly into solvents, and thus removing the expensive pre-hydrolysis step, research into substrate utilization and genetic improvement of the cellulolytic properties of well-known solvent-producing strains has been carried out both at ATO and at the Laboratory of Microbiology of the University of Wageningen, and the results are presented in this thesis.

Chapters 2 and 3 of this thesis concentrate on the characterization and fermentation of domestic organic waste (DOW), one of the interesting substrates for ABE production. In **Chapter 2** the utilization of saccharides in the substrate, both raw and pre-hydrolyzed by cellulases, by *C. acetobutylicum* ATCC 824 is studied. The results obtained showed that this strain was not able to utilize the polymeric sugars present in the DOW unless this substrate was subjected to pre-hydrolysis. Because lignocellulosic materials are insoluble, heterogeneous and often produce viscous suspensions, there is a preference to work with lignocellulosic hydrolysates as substrates, in which the majority of the polymers have been degraded and the sugars are soluble in a clear solution. **Chapter 3** shows that in concentrated hydrolysate from DOW there are toxic compounds that that can be specifically removed, making possible its fermentation by solvent-producing clostridia.

In **Chapter 4**, the construction of solventogenic strains with improved substrate utilization compared to the parent strain is described. Two different fungal genes encoding cellulases were cloned into *C. beijerinckii* resulting in transformants with enhanced cellulase and lichenase activities.

As already mentioned, one of the unexpected findings in the genome sequence of *C. acetobutylicum* ATCC 824 was the presence of several genes encoding potentially cellulosomal components in its chromosome. Since this strain does not show real cellulolytic properties, it was of interest to study whether the putative cellulosomal genes were expressed and if they were indeed coding for functional proteins. The results from these studies are shown in **Chapters 5, 6 and 7**.

Chapter 5 describes the cloning of *cbpA* gene from *C. cellulovorans*, encoding Cellulose-binding protein A, into *C. acetobutylicum* and the characterization of the transformants obtained. Two of the genes, *celG* and *celF*, encoding putative cellulosomal glycoside hydrolases from families 9 and 48, respectively, of the glycoside hydrolases classification, present in *C.*

acetobutylicum have been cloned into *E. coli*. In **Chapter 6**, the characterization of the *E. coli*-produced glycoside hydrolase from family 9 is shown. In addition, (hemi)cellulolytic extracellular activities produced by *C. acetobutylicum* grown on different carbon sources have been studied and the results are shown in **Chapters 6 and 7**.

In **Chapter 8** an overview of the presented results is given.

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Chapter 2

Utilization of Saccharides in Extruded Domestic Organic Waste by *Clostridium acetobutylicum* ATCC 824 for Production of Acetone, Butanol and Ethanol

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Abstract

Domestic organic waste (DOW) collected in The Netherlands was analyzed and used as substrate for acetone, butanol and ethanol (ABE) production. Two different samples of DOW, referred to as fresh DOW and dried DOW, were treated by extrusion in order to expand the polymer fibers present and to obtain a homogeneous mixture. The extruded material was analyzed with respect to solvent and hot water extractives, uronic acids, lignin, sugars and ash. The total sugar content in the polymeric fractions of the materials varied from 27.7% to 39.3% (w/w), in which glucose represented the 18.4 and 25.1 % of the materials, for fresh and dried DOW, respectively. The extruded fresh DOW was used as substrate for the ABE fermentation by the solventogenic strain *Clostridium acetobutylicum* ATCC 824. This strain was grown on a suspension of 10% (w/v) DOW in demineralised water without further nutrient supplement. This strain produced 4 g ABE/100g extruded DOW. When *C. acetobutylicum* ATCC 824 was grown on a suspension of 10% (w/v) DOW hydrolyzed by a combination of commercial cellulases and β-glucosidases, the yield of solvents increased to 7.5 g ABE/100g extruded DOW. The utilization of sugar polymers in both hydrolyzed and non-hydrolyzed DOW was determined, showing that the bacteria only had consumed a small proportion of the polymers. These results indicate that growth and ABE production on DOW is mainly supported by soluble saccharides in the medium.

Introduction

The production of acetone, butanol and ethanol (ABE) by solventogenic Clostridia is a well-known process. During the first part of this century it was, after the production of ethanol, the second largest biotechnological process in the world. Following World War II the ABE fermentation process was no longer economically viable as a result of the development of the petrochemical industry, which made the production of solvents from petroleum derivatives cheaper (6, 13). During the last 20 years there has been a revival of interest in the ABE fermentation, since renewable resources have become possible alternative substrates for the production of chemicals and fuels, instead of oil-derived feedstocks (5). At present, one of the major bottlenecks hampering its economic viability is the cost of substrates, accounting for up to 60% of the total production costs.

Since solventogenic Clostridia are able to utilize a wide range of carbohydrate substrates (18), considerable research into the use of substrates cheaper than molasses (the traditional substrate

for ABE production) has been done. From the compounds that have been investigated as possible substrates for ABE fermentation, waste products from the dairy industry (i.e. whey) and agricultural wastes seem to be the most interesting ones (4, 6, 13). The agricultural wastes are mainly composed of lignocellulose, which is considered to have great potential as a substrate for fermentation, provided that its cellulosic and hemicellulosic fractions can be de-polymerized and utilized efficiently. Although many solventogenic clostridia can utilize all sugars present in wood cellulose and hemicellulose hydrolysates and can degrade polymers such as starch or xylan, they are not able to grow on cellulose (16, 18). For the use of lignocellulosic substrates, several strategies have been studied, such as the use of hydrolysates, co-cultures with true cellulolytic organisms, or addition of cellulases to the fermentation medium (13). For the production of hydrolysates, the lignocellulosic material is first subjected to a pre-treatment, such as steam-explosion or extrusion, in order to expand the polymer fibers and facilitate their hydrolysis. The hydrolysis can be done chemically by acid treatment, or enzymatically. Both types of hydrolysates have been tested as media for fermentation (4, 17). In this study, the application of extruded domestic organic waste (DOW) as substrate for ABE fermentation is described.

Materials and methods

Microorganisms

Clostridium acetobutylicum ATCC 824 was kindly provided by Dr. P. Soucaille (Insa, Toulouse, France). Stock cultures were maintained as spore suspensions in sterile 10% (v/v) glycerol at -20°C. Spore suspensions were heat-shocked for 10 min at 70-80°C in a water bath prior to inoculation. For the production of pre-cultures, vegetative cells were grown in a semi-synthetic medium described previously (19) for 24 h at 37 °C. All experiments were performed anaerobically, in a Coy anaerobic chamber (Coy Laboratory Products, USA) under an atmosphere of 20% CO_2, 4% H_2, 76% N_2, unless indicated otherwise.

Substrates, pre-treatment and fermentation

Two different samples of DOW were used in this study. The first, referred to as fresh DOW, was collected in The Netherlands during the summer season and was stored at 4°C for 15 days. The second, referred to as dried DOW, was kindly provided by the composting company VAM (The Netherlands) after collection during the spring season. This sample was washed twice with water and dried up to 85% (w/w) dry matter content. Both samples were treated in a Clextral BC45 extruder (Clextral, Firminy, France) under the following conditions: screw speed 100 rpm;

production rate 12.8 kg/h for DOW and 9.9 kg/h for VAM-DOW, temperature 120˚C, reversed screw configuration RSE-25H10/RSE-25H6. Extruded samples were freeze-dried, milled to a size particle of 0.5 mm in a Retsch mill and stored at room temperature until further use.

Freeze-dried DOW was suspended in demineralised water at 10% (w/v) in serum flasks, closed with a rubber top and metallic cap. The solutions were flushed with N_2 to remove O_2 and then sterilized at 121˚C for 15 min. Inoculation was done with 2% (v/v) overnight pre-cultures. A device composed of a needle, a 0.22 μm sterile filter and a bicycle tyre valve were used to prevent the development of pressure in the flasks. Cultures were incubated at 37˚C without agitation.

For measuring growth, 100 μl of culture were taken and diluted ten-fold in sterile holding buffer (1mM $MgSO_4$, 25 mM $KHPO_4$, pH 7). The number of total cells was determined in a Neubauer counting chamber.

Enzymes

The commercially available enzyme preparations Celluclast 1.5L (cellulases) and Novozym N188 (β-glucosidase) were kind gifts from Novo Nordisk (Bagsvaerd, Denmark). The ratio of these enzyme preparations used for hydrolysis was 1:0.2 (Celluclast 1.5L: Novozym N188), as advised by the manufacturer. Concentrations of enzyme preparations are given as grams of enzyme preparation per 100 g of substrate. Enzyme preparations were filter-sterilized prior to addition to the substrate. The hydrolysis mixtures were incubated at 45˚C in a shaking water bath.

Analytical methods

The composition of DOW was determined as follows. First, samples were extracted according to the Technical Association of the Pulp and Paper Industry method T 264 om-88 (22). For this purpose, 1 g of freeze-dried sample was extracted in a Soxtec System HT6 (Tenacor, Höganäs, Sweden) with, successively, ethanol/toluene (2:1) and ethanol. Thereafter, the samples were extracted with hot water for 1 h at 100˚C. Extractive free samples were dried at 60˚C for 24 h and hydrolyzed with 12 M H_2SO_4 at 30˚C for 1 h followed by 1 M H_2SO_4 at 100˚C for 3 h. In the hydrolyzate, after reduction and acetylation, neutral sugars were determined by gas liquid chromatography in a Hewlett Packard model 5890A series II gas chromatograph (Hewlett-Packard, Avondale, USA) equipped with a CP-SIL 88 WCOT fused silica column (Chrompack, Middelburg, The Netherlands) and a flame ionisation detector. Helium was the carrier gas. Injector and detector temperatures were 300°C, and column temperature was 220˚C. Analysis of the chromatograms was carried out using ChemStation software (Hewlett-Packard, Avondale, USA). Acid-soluble lignin was determined spectrophotometrically at 205 nm following method T UM 250 (23). Uronic acids

were measured using the method of Blumenkrantz and Asboe Hansen (2). Acid-insoluble lignin was determined gravimetrically. Ashes were determined by combustion of the samples at 575 ˚C as described in method T 211 om-93 (21).

Sugars, saccharides, solvents, acids and furfural compounds were determined in clear supernatants of centrifuged culture samples. Glucose was determined enzymatically using the Biotrol Glucose Enzymatique Color kit (Merck, Darmstadt, Germany). Other mono- or disaccharides were determined by high-pressure liquid chromatography (HPLC). After passing the samples through Sep-Pak C18 cartridges (Waters, USA) previously equilibrated dropwise with 2 ml of methanol and 4 ml of milliQ water, sugars were separated on a CHO-682 column (InterAction, USA) at 85˚C. MilliQ water was used as eluent at a flow rate of 0.4 ml/min. Solvents, acids and furfural compounds were analysed by HPLC as described previously (11). As internal standards, propionic acid was used in solvents and acids analyses, while phenoxyacetic acid was used in furfural and 5-hydroxymethyl-2-furaldehyde (HMF) determination. Furfural compounds were extracted from the samples by passing 2 ml of sample through a Sep-Pak C18 cartridge (Waters, Milford, USA) previously equilibrated as described above and eluted from the cartridge with 2 ml methanol. Separation was carried out using a Shodex KC-311 column (Shodex, Japan) at 80˚C with 3 mM H_2SO_4 as eluent at a flow rate of 1 ml/min. A refractive index (RI) detector (Waters 410, Millipore, Milford, USA) and an UV absorbance detector (model VWM2141, Pharmacia, Uppsala, Sweden) were used in series. Concentrations of all mentioned metabolites were determined from the RI chromatograms, except furfural and HMF, which were determined from the UV chromatograms at 280 nm.

Scanning electron microscopy analysis

Samples of cultures were filtered using sintered glass filters. A small portion of the material on the filter was analysed by cryo-scanning electron microscopy (cryo-SEM), using a Philips 515 microscope equipped with an Oxford cryo-unit.

Results

Extrusion and hydrolysis of DOW

After storing the freshly collected DOW for 15 days at 4˚C, no signs of deterioration by microbial growth were observed. The collected material appeared to be very heterogeneous, with a high content of vegetables and fruits. The dried DOW, on the other hand, contained mainly garden

waste. After storage for 12 months in a closed container, this dried DOW did not show microbial degradation either, probably due to its low moisture content (15%; w/w). The composition of these samples was determined after extrusion and freeze-drying and is shown in Table 2.1.

Table 2.1 Composition (w/w % in dry matter) of fresh domestic organic waste (DOW) and dried DOW. Values are means $\pm$ SD ($n = 3$)

Fraction	Fresh DOW	Dried DOW
Solvent extractives	19.1 $\pm$ 0.8	5.9 $\pm$ 0.9
Hot water extractives	24.4 $\pm$ 2.5	7.2 $\pm$ 0.6
Acid Soluble Lignin	1.5 $\pm$ 0.05	1.2 $\pm$ 0.03
Acid Insoluble Lignin	1.5 $\pm$ 0.25	10.5 $\pm$ 0.8
Uronic acids	8.0 $\pm$ 0.41	4.9 $\pm$ 1.6
Sugars		
Glucose	18.4 $\pm$ 0.6	25.1 $\pm$ 1.7
Xylose	4.0 $\pm$ 0.2	8.4 $\pm$ 0.7
Arabinose	1.5 $\pm$ 0.03	2.3 $\pm$ 0.03
Mannose	2.0 $\pm$ 0.3	1.6 $\pm$ 0.1
Galactose	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1
Rhamnose	0.2 $\pm$ 0.01	0.3 $\pm$ 0.01
Ashes	9.1 $\pm$ 0.2	15.9 $\pm$ 1.1

The fresh DOW showed approximately three times more solvent and hot water extractives than the dried DOW. These extractives represent the solvent and water-soluble compounds present in the samples (proteins, free sugars, etc) and, in the case of the dried DOW, these were partially removed by the washing treatment applied. Also, the dried DOW had a higher content of acid-insoluble lignin, ashes and sugars.

In order to determine optimal conditions for the enzymatic hydrolysis of the DOW, different concentrations and combinations of cellulases and β-glucosidases were tested for the generation of glucose. The increase in glucose concentration in fresh DOW suspension during hydrolysis is shown in Fig. 2.1. When only Celluclast 1.5L was used, the highest concentration of glucose obtained was 5.2 g/l after 72 h of hydrolysis; and this did not increase by increasing the concentration of Celluclast 1.5L from 3% to 4% (w/w). However, an increase in glucose concentration was observed in the soluble fraction of the DOW suspension. It rose to 18 g/l using a combination of Celluclast 1.5L at 3% (w/w) and Novozyme N188 at 0.6% (w/w). The addition of β-glucosidases (Novozyme N188) results in hydrolysis of cellobiose to glucose and has been shown to

prevent the inhibition of cellulases, making the hydrolysis of the substrate significantly more efficient (3, 12).
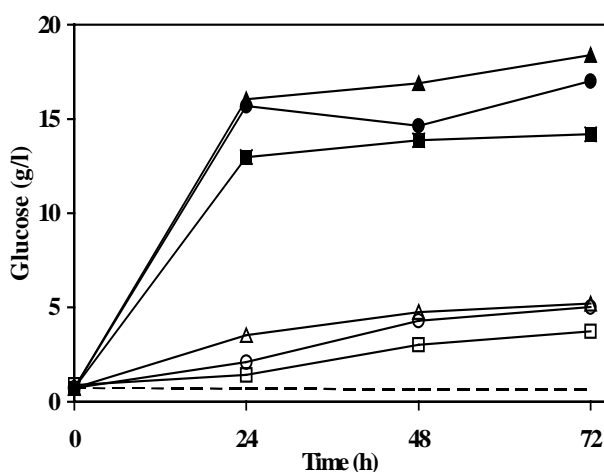
Figure 2.1 Hydrolysis of sterile 10% (w/v) fresh domestic organic waste (DOW) by different concentrations of Celluclast 1.5L (C) and Novozyme N188 (N). --- Blank, □ C 1%, ■ C 1%+ N 0.2 %, ○ C 3%, ● C 3% + N 0.6%, △ C 4%, ▲ C 4% + N 0.8 %. Concentrations given as % g preparation/g substrate. Values are averages of duplicate determinations

With the dried DOW, similar results were obtained, with maximum concentrations of glucose after 72 h of hydrolysis of 5.5 g/l when only Celluclast 1.5L at 3% (w/w) was added, and 9.6 g/l when Celluclast 1.5L at 3%(w/w) and Novozyme N188 at 0.6%(w/w) were added.

For further experiments, concentrations of 3% and 0.6% (w/w) of Celluclast 1.5L and Novozyme N188, respectively, were used. Only fresh DOW was used as substrate for fermentation, since the concentration of glucose obtained during hydrolysis was higher than that obtained from dried DOW.

Fermentation of DOW by *C. acetobutylicum* ATCC 824

Since the DOW suspensions were sterilised by autoclaving prior to fermentation, its effect on the composition of the samples was analysed. For sterilisation of the samples, extruded fresh DOW was resuspended at 10% (w/v) in demineralised water and this suspension was autoclaved at 121˚C for 15 min. The resulting solution was then freeze-dried, homogenized and used for analysis. The differences in composition between sterile and non-sterile samples were minimal (results not shown). During treatment of lignocellulosic materials at high temperatures and low pH, furfural compounds can be formed as a result of the degradation of sugars (15). Since furfural compounds at certain levels could inhibit bacterial growth, their formation should be avoided when the material is intended to be used for fermentation (15, 20). The furfural and HMF present in the soluble fraction

of 10% (w/v) DOW suspensions were measured before and after sterilisation. No furfural or HMF was detected before sterilisation and only 3.5 mg of HMF/l were found in sterile medium.

C. acetobutylicum ATCC 824 was grown on 10% (w/v) fresh DOW and 10% (w/v) hydrolyzed fresh DOW in demineralised water without further addition of nutrients. These media contained insoluble material and thus it was impossible to determine cell growth by measuring the optical density of the cultures. Therefore, growth was followed by total cell counting using phase contrast microscopy. The growth curves obtained on both media were very similar and show a short lag phase followed by an exponential growth phase, resulting in a cell concentration of approximately 10^9 total cells/ml (Fig. 2.2). To determine whether bacterial cells would be attached to the substrate particles, filtrated samples of fermentation cultures were analysed by cryo-SEM. In the cryo-SEM-photographs, no substrate-attached cells were observed (results not shown), which is in agreement with the previously observed lack of attachment of *C. acetobutylicum* ATCC 824 to cellulose (9).
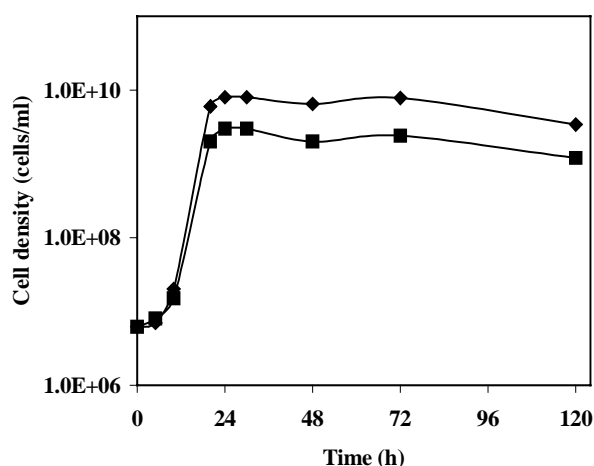
Figure 2.2 Growth curve of *Clostridium acetobutylicum* ATCC 824 in 10% (w/v) DOW and 10% (w/v) hydrolyzed DOW in demineralised water. ■ Total cells DOW, ◆ total cells hydrolyzed DOW. Values are average of duplicate determinations

The acids and solvents produced and the glucose consumed during fermentation of 10% (w/v) fresh DOW and 10% (w/v) hydrolyzed fresh DOW in demineralised water were determined (Fig. 2.3). During exponential growth of the bacteria, acetic and butyric acids were produced; and during the stationary growth phase, solvent production (ABE) and partial re-assimilation of the acids were observed. After 120 h of fermentation, 3 g of butanol/l were produced from fresh DOW and no acetone or ethanol were found in the culture medium; and butyric acid was the main product

(3.5 g/l). In contrast, when hydrolyzed fresh DOW was the substrate, after 120 h of fermentation the main product was butanol, with a concentration of approximately 4.2 g/l; and 1.5 g of acetone/l and 0.4 g of ethanol/l were found. In the fermentation of hydrolyzed fresh DOW, almost no change in the concentrations of acids and solvents in the medium after 48 h of fermentation were observed, while during fermentation of fresh DOW there was a continuous increase in product formation.
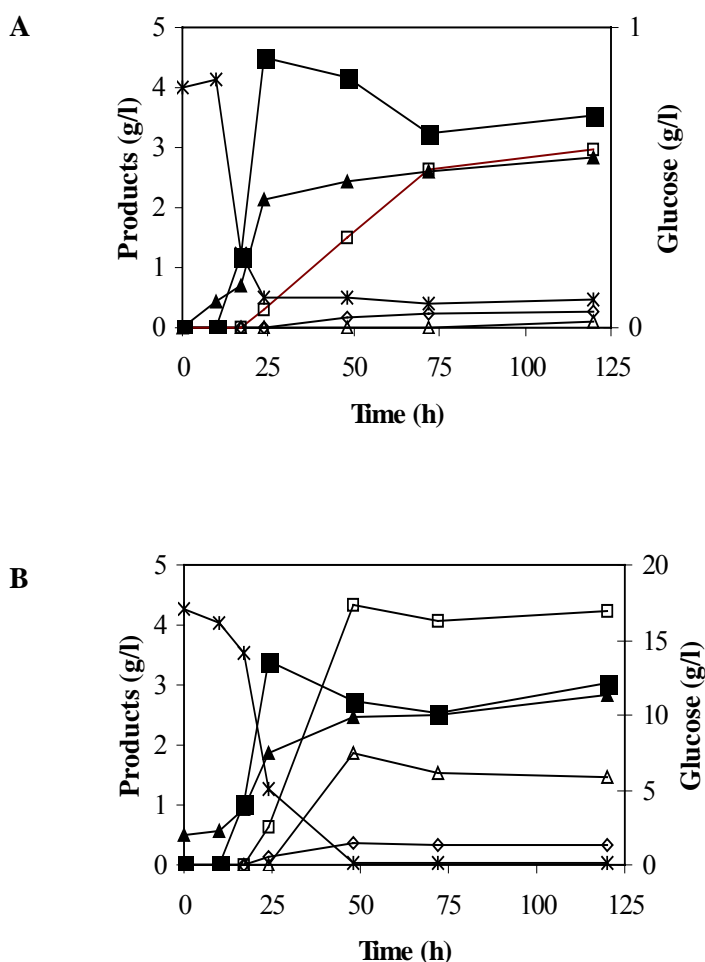
Figure 2.3 Acids and solvents production by *C. acetobutylicum* ATCC 824 on **A** 10% fresh DOW and **B** 10% hydrolyzed fresh DOW. □ Butanol, ■ butyric acid, Δ acetone, ▲ acetic acid, ◊ ethanol, * glucose. Values are averages of duplicate experiments

Saccharide utilization from DOW by *C. acetobutylicum* ATCC 824

Table 2.2 shows the mono- and disaccharides in the soluble fraction of sterile 10% (w/v) fresh DOW and 10% (w/v) hydrolyzed fresh DOW. The concentration of these saccharides in the soluble fraction of the media during fermentation was followed and, after 4 days, all of them except for arabinose, had been consumed. A small amount of unidentified material was detected in the RI chromatograms during HPLC saccharide analysis of the soluble fraction of 10% (w/v) fresh DOW

suspension. This material largely disappears during fermentation; and hence it could represent other mono-, di- or oligosaccharides that were utilized by the bacteria during growth. This material did not appear in samples from 10% (w/v) hydrolyzed DOW, indicating that it may correspond to oligosaccharides that are hydrolyzed by the cellulolytic enzymes.

Table 2.2 Mono- and disaccharides present in the soluble fractions of 10% (w/v) fresh DOW and 10% (w/v) hydrolyzed fresh DOW in demineralised water. Values (g/l) are the average of at least three different measurements ± SD. *ND* Not detected

	10% DOW	10% hydrolyzed DOW
Glucose	1.2 ± 0.2	18.1 ± 1.8
Fructose	2.5 ± 0.3	2.9 ± 0.3
Galactose	ND	0.2 ± 0.04
Arabinose	0.1 ± 0.004	0.3 ± 0.06
Cellobiose	0.6 ± 0.1	ND
Maltose	0.2 ± 0.1	ND

The insoluble fractions of fresh DOW and hydrolyzed fresh DOW remaining after fermentation were analysed in order to determine whether the (hemi)cellulosic polymers had been utilized by the bacteria during growth. All polymeric sugars present in the DOW were only partially utilized. After fermentation of the hydrolyzed fresh DOW, 73 % (w/w) of the initial amount of polymeric glucose was consumed, while after fermentation of fresh DOW, the polymeric glucose consumed was 12 % (w/w) of the initial amount. From these data we calculated that during enzymatic hydrolysis of the DOW about 60% of the total polymeric glucose was hydrolyzed to soluble glucose.

Table 2.3. Composition (in g/l) of the polymeric fraction of the media 10% (w/v) fresh DOW and 10% (w/v) hydrolyzed fresh DOW after fermentation by *Clostridium acetobutylicum* ATCC 824. Values are means of triplicate samples ± SD

	10 %DOW t = 0 h	10% DOW t = 96 h	10% hydrolyzed DOW t = 96 h
Glucose	16.8 ± 0.7	14.7 ± 0.2	4.6 ± 0.3
Xylose	4.8 ± 0.2	4 ± 0.3	2.3 ± 0.02
Arabinose	1.3 ± 0.1	0.5 ± 0.01	0.3 ± 0.02
Mannose	2 ± 0.3	1.8 ± 0.2	0.7 ± 0.02
Galactose	1.4 ± 0.1	1 ± 0.1	0.5 ± 0.02
Rhamnose	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.02

The soluble fraction of 10% (w/v) fresh DOW contained 1.2 g of glucose/l, while in the soluble fraction of 10% hydrolyzed fresh DOW the concentration of glucose was 18.1 g/l (Table 2.2). In the hydrolyzed DOW solution, some of the glucose in the soluble fraction could be derived from the hydrolysis of soluble disaccharides like maltose or cellobiose, amounting to 0.8 g/l (Table 2.2). Table 2.3 shows that the amount of glucose consumed from the polymeric fraction of hydrolyzed DOW is only 12.2 g/l. After correction of the total amount of soluble glucose in hydrolyzed DOW (18.1 g/l) with the amount of glucose coming from disaccharides, the polymeric fraction of the DOW and the soluble glucose already present before hydrolysis, the residual glucose is 3.8 g/l. This glucose could be a result of hydrolysis by the cellulases of soluble oligosaccharides present in the DOW suspension. These oligosaccharides may correspond to the observed unidentified material that appears in the RI chromatograms from samples of the soluble fraction of 10% (w/v) DOW. Since these peaks disappear during fermentation, it seems that *C. acetobutylicum* ATCC 824 utilizes these oligosaccharides during growth.

Discussion

The composition of DOW varies with the season. In spring and autumn the percentage of garden waste (rich in woods) in the DOW is higher than in winter or summer (8). In this study, DOW collected in two different seasons and subjected to different pre-treatments was analyzed. It is very likely that the washing of the dried DOW prior to extrusion affected the amount of extractives in the sample (Table 2.1). Therefore, the percentages in the composition of both samples cannot be compared directly. Even if the extractives are not taken into account, the percentages of glucose and

lignin in the polymeric fractions of the fresh DOW and the dried DOW were very different (61%
and 49% for glucose, respectively, and 10% and 23% for lignin, respectively). This indicates that
when the DOW had a higher content of food and fruits wastes (fresh DOW), the polymeric fraction
showed a higher content in glucan and a much lower content in lignin than when the DOW was
richer in garden waste (dried DOW).

As the extruded DOW was intended to be used as substrate for fermentation, the temperature
during extrusion was kept at 120˚C to prevent degradation of sugars and formation of furfural
compounds. In the case of the medium used in this study, the concentration of HMF was lower than
that found in hydrolysates made from similar, steam exploded lignocellulosic material (Dr J.
Ballesteros, CIEMAT, Madrid, Spain, personal communication).

After enzymatic hydrolysis followed by fermentation of the DOW, 27% of the glucose in the
polymeric fraction was still present (Table 2.3). The incomplete enzymatic hydrolysis of cellulose
in lignocellulosic materials could be due to a number of factors, including the presence of inhibitors
for the enzymes (7), product inhibition phenomena, inaccessibility due to a high degree of
crystallinity in the cellulose, or the presence of other components, such as hemicellulose or lignin
(12).

In the soluble fraction of 10% (w/v) fresh DOW in demineralised water, there were mono-
and disaccharides (Table 2.2) and, most probably, oligosaccharides as well. These substrates were
enough to support growth up to 10^9 total cells/ml, but not enough to support the shift from the
acidogenic to solventogenic phase; and thus butyric acid was the main product formed during
fermentation (Fig. 2.3). Butanol was the main solvent produced and no acetone was found after 120
h of fermentation. In the case of hydrolyzed DOW, the solvent production was higher, and after 48
h of fermentation the solvent production was complete. The total amount of solvents (ABE)
produced from hydrolyzed fresh DOW after 48 h of fermentation is 4.3 times higher than that from
fresh DOW during the same period of fermentation: 6.8g ABE/100 g extruded fresh DOW and 1.6 g
ABE/100 g extruded fresh DOW produced from fresh DOW. The amount of solvents produced per
gram of soluble substrate consumed after 120 h of fermentation, calculated taking into account the
sugars in Table 2.2 plus 2.8 g of oligosaccharides/l in the case of fresh DOW, is very similar in both
DOW and hydrolyzed DOW. However, in the case of hydrolyzed fresh DOW, the fermentation was
complete after 48 h and the concentration of solvents in the medium was higher than that from fresh
DOW, making this fermentation economically more efficient. As observed before (10, 13), these
data show that the level of sugars in the medium is a very important factor for triggering the shift
from the acidogenic to solventogenic phase in ABE fermentation and thus the success of ABE
fermentation.

DOW represents an interesting substrate for ABE fermentation due to its low price and a sugar composition that can sustain growth and solvent production by Clostridia if an efficient hydrolysis is performed. The inability of solventogenic Clostridia to utilize cellulose makes the hydrolysis of lignocellulosic substrates a necessary step prior to fermentation. However, the hydrolysis, either chemical or enzymatic, results in an increase in the costs of the process. Some solventogenic clostridial strains, including *C. acetobutylicum* ATCC 824, show endoglucanase and β-glucosidase activities (1, 14, 16). These activities are involved in cellulose utilization, but the exo-glucanase activity necessary for cellulose degradation seems to be lacking. The advances in our knowledge of the genetics and physiology of solventogenic Clostridia, which include the sequencing of the genome of the strain *C. acetobutylicum* ATCC 824 by Genome Therapeutics (Genome Therapeutics, 1999), would allow the construction of strains with a wider substrate range (i.e. able to degrade cellulose) and thus make ABE fermentation an economically viable process again.

Acknowledgements

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Chapter 3

Acetone, Butanol and Ethanol Production from Domestic Organic Waste by Solventogenic Clostridia

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Abstract

Domestic organic waste (DOW) was washed and dried to 85 % dryness by VAM (The Netherlands). This material contained 25.1 g glucose, 8.4 g xylose and 5.8 g other monosaccharides / 100 g dry matter. Using Mansonite steam explosion and enzymatic hydrolysis, a hydrolysate containing 15.4 g glucose, 2.2 g xylose and 0.8 g other monosaccharides per l was made. *Clostridium acetobutylicum* DSM 1731 produced 1.5 g/l ABE and *C. beijerinckii* B-592 0.9 g/l ABE and Clostridium LMD 84.48 1.9 g/l IBE, respectively, from this hydrolysate without further supplementation. Incubation with 2 fold concentrated hydrolysate completely impaired ABE production. After removal of unspecific inhibiting components, the yield of ABE production by *Clostridium acetobutylicum* DSM 1731 increased about 3 fold as compared to the non-treated hydrolysate. From 4 fold concentrated, partially purified, hydrolysate containing 34.2 g glucose/l, ABE production was 9.3 g/l after 120 h as compared to 3.2 g ABE/l from non-concentrated hydrolysate which contained 12.0 g glucose/l after elution over the same column. The concentration of butyric acid in the fermented hydrolysates was 2.2 and 0.4 g/l, respectively. This reasonably low amount of butyric acid showed that the fermentation had proceeded quite well.

Introduction

The anaerobic production of acetone, butanol and ethanol (ABE) by solventogenic clostridia has once been the second largest biotechnological industry in the world (7), initially for production of synthetic rubber precursors but later on for production of solvents in the lacquer industry. As a result of the development of the petrochemical industry and the increase in prices of the agricultural produce, especially molasses, used as substrate for the fermentation, the fermentative ABE production became no longer economically viable and came to a halt in the 1960's (1).

However, interest in the ABE fermentation has resurfaced because of the new global support for the exploitation of biomass as a sustainable source of energy.

At present, two major bottlenecks still hamper sound economics of the ABE fermentation:

a) the cost of the substrates contributing to over 60% of the overall costs and

b) the toxic nature of the products prohibiting high concentrations in the fermentation broth.

In the past, ABE fermentation was done employing easily fermentable carbohydrates in mashes derived from maize, grains, beets or potatoes. Because of the saccharolytic property of the solventogenic clostridia these starchy substrates could be converted to ABE without prior

pretreatments (9). In the present study, an attempt has been made to utilize the abundant supply of cellulose and hemicellulose in domestic organic waste (DOW). As the exoglucanase enzyme, required for cellulase activity, is not present in solventogenic clostridia (in contrast to endoglucanase activity) and as cellulose and hemicellulose are rather inaccessible because of being part of complex structures, this feedstock must be pre-treated and hydrolyzed in order to liberate fermentable monosaccharides. This paper describes the results obtained with fermentation of hydrolysate from DOW, produced using steam explosion and enzymatic hydrolysis.

Materials and methods

Microorganisms

Clostridium acetobutylicum DSM 1731 was obtained from the German culture collection, DSM (Deutsche Sammlung von Mikro-organismen, Braunsweig, Germany), *Clostridium* LMD 84.48 from the Dutch culture collection CBS (Centraal bureau voor Schimmelculturen, Delft, The Netherlands) and *Clostridium beijerinckii* B-592 was kindly supplied by Dr. Gapes (TU Wien, Austria). For standard procedures the strains were grown at 30 ºC (DSM 1731 and B-592) or 37 ºC (LMD 84.48) without agitation in medium described by Gapes (5). For maintenance of the cultures, spores were collected in distilled water from colonies grown on solid medium according to Gapes (5), supplemented with 2 g/l agar. Spores were stored at -20 °C until further use. Spore germination was started by heat shocking 1 ml spore suspension in 5 ml distilled water, 2.5 min at 80 °C. For growth of precultures, i.e. cultures with most cells in the exponential growth phase, inoculation was done with spore suspensions to a final concentration of 1% (^{v}/v). Inoculation of cultures for experiments was always done at 1% (v/v) from precultures in the exponential growth phase unless stated otherwise. Culture media were made anaerobic by sparging with N_2 prior to sterilization. After inoculation, valves equipped with sterile 0.2 µm filters were mounted on the flasks in order to prevent building up of pressure. All cultures were done in duplicate.

Pretreatment and hydrolysis

Hydrolysate from domestic organic waste (DOW; kindly supplied by VAM, The Netherlands) was prepared by Ms. M. Ballesteros (CIEMAT, Madrid) by applying Mansonite steam explosion at 200 °C for 6 min at DOW which had been washed and subsequently dried to 85 % dryness prior to steam explosion. The liquid and solid fractions were collected from the cyclone and subsequently filtered. The liquid residue was 2 fold concentrated using a flash evaporator and recombined with the solid residue in order to restore the initial mean solid/liquid ratio of DOW.

Enzymatic hydrolysis was carried out with cellulolytic enzymes using a solid/liquid ratio of 1 to 10 (30 FPU (Filter Paper Units)/g substrate at 50 °C for 72 hrs). The enzymes used were Celluclast 1.5L for the breakdown of cellulose into glucose and cellobiose, and Novozym 188 at a loading of 25 IU/g substrate for the conversion of cellobiose into glucose, both enzymes kindly donated by Novo Nordisk (Bagsvaerd, Denmark). Enzyme suspensions were sterilized by passing through a 0.2 μm filter prior to addition to the medium. The solid residue, which is not fermentable, was removed by filtration and after adjusting the pH of the filtrate 4.8 – 5 in order to prevent contamination, the filtrate was frozen until further use. For concentrating the amount of saccharides in the hydrolysate, this was lyophilized and subsequently re-suspended in a reduced amount of demineralised water.

Analytical methods

For preparing homogenous samples, the DOW was passed through a BC 45 Clextral extruder with the following screw configuration: RSE –25H10/ RSE –25H6. Extrusion was done at 120 ºC with steam. The mean residence time was 1 min. The homogenized samples were lyophilized and stored at room temperature until further analysis.

The analysis of the lyophilised DOW was started after milling and passing through a sieve of 0.5 mm. Solvent and hot water extractives were determined in the DOW using, in succession, ethanol/toluene (2:1) and 95% ethanol, and hot water during 1 h, respectively, (TAPPI (Technical Association of the Pulp and Paper Industry) method T 264 om-88). Chemical hydrolysis of the extractive free material was done in 12 M H_2SO_4 at 30 °C during 1 h, followed by 1 M H_2SO_4 at 100 °C during 3 h. After centrifugation and filtration, soluble lignin and sugars were determined in the supernatant. The lignin was determined spectrophotometrically at 205 nm. The sugars were derivatised using acetic acid and measured using GLC (CP-SIL 88 WCOT; He as carrier gas) equipped with FID. Acid-insoluble lignin and ash in the pellet were determined gravimetrically. By drying this pellet overnight at 105 °C, the amounts of lignin and ash were determined and by heating to 525 °C overnight the amount of ash only was determined (TAPPI method T211 om-93). Subtraction then gave the amount of insoluble lignin. Dry matter content, also needed for enabling comparisons of efficiencies of conversions, was determined by drying overnight at 105 °C.

During fermentation, glucose concentrations were measured either enzymatically using the GOD-PAP kit from Merck (Darmstadt, Germany) or, together with other sugars, by HPLC (CHO-682 carbohydrate column, eluted with distilled water). Concentrations of acetone, butanol and ethanol and organic acids were measured by HPLC (Shodex Ionpak KC-811 column, eluted with 3 mM H_2SO_4). Detection was done with a RI or UV detector (butyric acid at 210 nm).

Results and discussion

Composition of domestic organic waste (DOW) and hydrolysate

The composition of the DOW collected in the spring in the Netherlands and used in this study is given in Table 3.1. Since in this experiment the DOW had been washed for removal of sand, and dried to 85% dryness, soluble components like free mono- and oligosaccharides may have been lost from the samples which were passed through the extruder. However, the presence of glucose is prevalent, reflecting the large contribution of starch and (ligno)cellulosic compounds to this DOW. The amount of potential fermentable sugars in the studied DOW seems reasonable, whereas the amount of the lignin fraction is fairly low, indicating an interesting applicability for fermentations. As the distribution of the constituents is comparable to some other agrowastes (6) this feedstock may serve as a model for studying the utilization of waste, with low lignocellulose content, for ABE production.

Table 3.1 Composition of domestic organic waste (DOW) in g per 100 g dry matter. Values are means ± SD (n=3).

Fraction	DOW (g/ 100 g dry matter)
Solvent extractives	5.9 ± 0.9
Hot water extractives	7.2 ± 0.6
Acid soluble lignin	1.2 ± 0.03
Acid insoluble lignin	10.5 ± 0.8
Uronic acids in polymeric fraction:	4.9 ± 1.6
Sugars in polymeric fraction:	
Glucose	25.1 ± 1.7
Xylose	8.4 ± 0.7
Arabinose	2.3 ± 0.03
Mannose	1.6 ± 0.1
Galactose	1.6 ± 0.1
Rhamnose	0.3 ± 0.01
Total sugars	39.3
Ashes	15.9 ± 1.1

In The Netherlands, DOW is mainly composed of leftovers from fruits and vegetables, and garden waste like weeds and cuttings, and shows a rather constant supply during the year with some increase in the spring and autumn. The composition of the DOW will, to some extent, be affected by the season, since in spring and autumn the contribution from the garden waste is relatively large. The availability of DOW is estimated at 1000 Kton/year (2). Presently, DOW is largely aerobically digested to make compost. The significant amount of polymeric saccharides in the DOW, assumedly cellulose and hemicellulose which yield the fermentable sugars shown in Table 3.1, amounting to 39% of the dry weight, invite also other conversions which may generate valuable compounds or energy. However, for most of such applications, the mobilization of the saccharides from the biomass is a prerequisite.

Table 3.2 Composition of hydrolysate, prepared and supplied by CIEMAT (Spain) from domestic organic waste (DOW) provided by VAM. Acknowledgement: The analyses were done by CIEMAT.

Component	Concentration (g/l) in hydrolysate
Glucose	15.4
Xylose	2.2
Galactose	0.2
Arabinose	0.2
Mannose	0.4
Furfural	0.03
Hydroxymethylfurfural	0.0093

As shown in Table 3.2, the application of steam explosion followed by enzymatic hydrolysis, have mobilized almost 50% of the saccharides from the polymers. The high ratio of glucose to other sugars, especially xylose that as pentose may confer special demands on the fermentation, is of importance. Even though a simultaneous utilization of glucose and xylose has been observed in other experiments (results not shown), a high amount of glucose may inhibit xylose consumption in ABE fermentation (3, 4). This phenomenon, being dependent on the culture conditions and strain differences has not been the subject of this investigation but is certainly of interest when dealing with such complex substrates. Except for furfural and hydroxymethylfurfural, the hydrolysate has not yet been tested for the presence of other known inhibitory substances, as are phenolic compounds like ferulic acid, vanillin etc. As previous studies have shown (8), an inhibitory effect of 50% by furfurals occurs at concentrations exceeding 2 g/l, the low concentration of furfurals in our hydrolysate seems not significant, although there may be strain differences. Below

this concentration, the inhibitory effect of concentrated hydrolysate on ABE production is described, indicating that other strong inhibitors are indeed present in the hydrolysate.

At present, the combination of steam explosion and enzymatic hydrolysis for pretreament of DOW needs further development since, on the one hand, the efficiency, albeit promising, is only 50% and since, on the other hand, the concentration of the saccharides in the hydrolysate, until now never exceeding 30 g/l, is low, thus yielding large waste streams. Furthermore, as suggested previously, the cost of the enzymatic hydrolysis is a severe economic burden, even when the enzyme cocktail is being produced on site (8). Therefore, further improvement needs to be achieved by a reduction of enzyme consumption, e.g. by recycling or by coupling hydrolysis and fermentation.

Fermentation of hydrolysate by *Clostridium sp.*

For concentrating the amount of fermentable substrate, the hydrolysate was lyophilised and re-suspended in a smaller volume of demineralised water and adjusted to pH 7.0. The effect of lyophilization was checked by monitoring solvent production on non-treated and lyophilized hydrolysate and by recording HPLC profiles (organic acids column, Shodex Ionpack KC-811, with RI detection). There were no differences detected in the chromatograms. The hydrolysate was sterilized in an autoclave and used without removal of the precipitate. After sterilization the pH had dropped to 5.7. This pH drop was always observed when sterilizing DOW and was tentatively attributed to thermal decomposition to acidic compounds. The method of filter sterilization offered no alternative because filters became clogged within 2 min of usage.

Solvent production from hydrolysate and concentrated hydrolysate was tested using Clostridium LMD 84.48, *C. acetobutylicum* DSM 1731 and *C. beijerinckii* B-592 without further supplementation. The results are shown in Table 3.3. As always observed, strain LMD 84.48 was an IBE producer, reducing acetone to i-propanol, indicating that this strain should be classified as a *beijerinckii* strain. Besides i-propanol production, strain LMD 84.48 can also be distinguished because of its great sensitivity with respect to butanol concentration which is evident from the large amount of residual substrate in the culture grown on 6% glucose (Table 3.3). The poor performance of LMD 84.48 on hydrolysate may be explained by the presence of unspecific inhibitors in the hydrolysate, which is supported by the lack of IBE production in concentrated hydrolysate (Table 3.3). On the other hand, the low initial pH plus the low buffering capacity of the hydrolysate may also have negatively affected IBE production by LMD 84.48 since this strain is, in contrast to other solventogenic clostridia, very sensitive towards pH decreases to below 5.

Table 3.3 Production of acetone or isopropanol, butanol and ethanol (ABE or IBE) and butyric acid by *Clostridium* LMD 84.48, *Clostridium acetobutylicum* DSM 1731 and *Clostridium beijerinckii* B-592 from domestic organic waste. For concentration of the hydrolysate, lyophilization with subsequent dissolving in a 4 fold reduced volume of demineralised water was used. The initial pH was 5.7.

Strain	LMD 84.48				DSM 1731				NRRL B-592			
	Glu	IBE	Butyric acid	pH	Glu	ABE	Butyric acid	pH	Glu	ABE	Butyric acid	pH
Medium, [glu] at t=0	*Concentration in g/l at the end of the fermentation (180 h)*											
Hydrolysate [glu] = 16 g/l	8.7	1.9	1.3	4.8	2.0	1.5	2.1	4.4	5.7	0.9	1.8	4.4
Lyophilised hydrolysate (4:1)in water [glu] = 48 g/l	46.8	0.2	0	5.4	46.4	0.3	0	5.5	44.5	0	1.1	4.9
6% glucose-Gapes [glu] = 69 g/l	39.0	10.4	0.6	5.3	0	20.1	0	4.7	0.4	20.1	0.4	5.0

Solvent production from the hydrolysate by the ABE producing strains, DSM 1731 and B-592, was also quite poor even though substrate consumption was higher as compared to the LMD 84.48 strain (Table 3.3). As previously no growth inhibition has been observed with these strains at pH's as low as 4.5, the contribution of the initial pH to the poor performance seems unlikely. In all cultures, and especially in the culture of B-592, the production of butyric acid was extensive, showing that the bacteria were inhibited in the shift from acidogenesis to solventogenesis. In general, the triggering event for this switch is still obscure although one of the thriving factors may be the ample supply of substrate, necessitating the detoxification of the poisonous organic acids that precede the formation of solvents (7). This phenomenon, as well as the presence of unspecific inhibitors, illustrates the difficulty of fermenting saccharides in a hydrolysate.

In previous experiments (results not shown) the need for the addition of yeast extract to hydrolysate medium had already been tested. This supplementation was without positive effect. In the experiments presented below (Table 3.4), it will be shown that absence of nutrients or low buffering capacity of the medium with hydrolysate only, inflict no inhibition, at least as far as the DSM 1731 strain is concerned.

The inhibition by unspecific components in the hydrolysate was further investigated by adding concentrated hydrolysate to cultures of LMD 84.48, DSM 1731 and NRRL B592 which had been grown on 6% glucose-Gapes medium for 50 h and were producing butanol (Figs. 3.1 and 3.2). The final concentration of the hydrolysate in the medium was 4 fold, 2 fold and equal to the initial, non-concentrated, hydrolysate. For a blank the same amount of demineralised water was added to

the cultures. In all cases, ABE production came to a halt after the addition of hydrolysate to a final concentration of 4 fold the initial, just as observed in Table 3.3.

The results with lower final concentrations of hydrolysate are shown in Figs 3.1B,C and 3.2B,C for LMD 84.48 and DSM 1731, respectively. As in all fermentations ethanol production was very low, never exceeding 0.5 g/l, this metabolite is not shown in the figures. The data for B-592 were similar to DSM 1731.

The production of (I)ABE was completely impaired in all cultures with 2 fold the initial concentration of hydrolysate. The addition of lyophilized hydrolysate to a final concentration equal to the non-concentrated hydrolysate had no effect on IBE production by LMD 84.48 as the IBE production did not increase and as the additionally supplied glucose seemed to remain untouched (Fig. 3.1B). This is probably due to the sensitivity of this strain to butanol. In previous studies, growth of LMD 84.48 halted after the addition of butanol to a final concentration of 8 g/l, despite otherwise favorable growth conditions such as no butyric acid and pH 6. Therefore, it is assumed that the achieved butanol concentration in this culture may have provoked the end of the fermentation. However, as also discussed above, the additional effect of inhibitors on growth inhibition of this strain, can not be excluded. The pH was 5.2 in the blank as well as in the culture to which the hydrolysate had been added. In the cultures with a final hydrolysate concentration of 2 fold the non-concentrated, the pH had dropped to 4.7 from 6.4 (Fig. 3.1C). Here, the involvement of low pH and/or inhibitors seems clear. However, it remains to be established which of these parameters effects the greatest contribution to the inhibition of ABE production by LMD 84.48.

The cultures of the NRRL B592 and DSM 1731 strains to which concentrated hydrolysate had been added to make a final concentration equal to the non-concentrated hydrolysate (Fig. 3.2) showed a better performance as compared to LMD 84.48 and as compared to non-concentrated hydrolysate (Table 3.3). In the cultures of Fig. 3.2 C/D, all glucose was consumed, including the additional amount from the hydrolysate, yielding extra revenue in the fermentation due to the sugars from the hydrolysate. Furthermore, the additional glucose from the hydrolysate was converted to ABE with a greater efficiency than after growth on non-concentrated hydrolysate only. In the cultures, the utilization of the added hydrolysate yielded an additional production of 5 g ABE/l, whereas production of ABE from non-concentrated hydrolysate (Table 3.3) was only 1.5 g ABE/l, partially due to the production of butyric acid. Apparently, the 'mixed' substrate system has a beneficial effect with respect to substrate conversion and prevents the production of butyric acid. In the cultures with a final hydrolysate concentration of 2 fold the initial (Fig. 3.2E/F), ABE production was impaired in a similar way as observed in the cultures of the LMD 84.48 strain (Fig. 3.1E/F), possibly due to either a drop in pH or increase in inhibitor concentration.
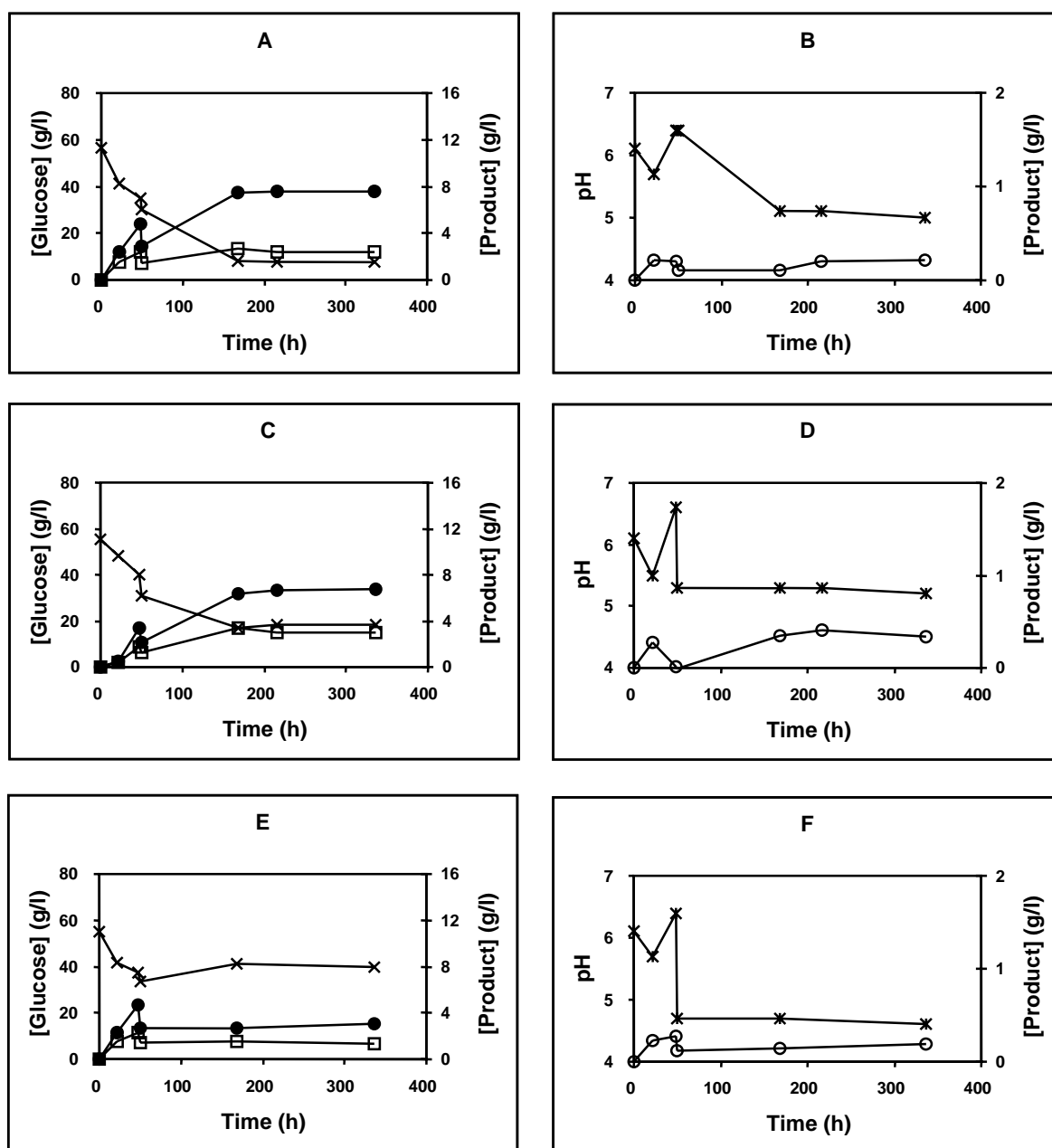
Figure 3.1 Production of i-propanol and butanol by *C.* **LMD 84.48** on glucose and hydrolysate of domestic organic waste. A/B: synthetic medium with 6% (w/v) glucose; C/D: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make the initial hydrolysate concentration at t = 50 h and E/F: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make the 2 fold the hydrolysate concentration at t = 50 h. Controls were done with demineralised water at t = 50 h, which showed no effect. In g/l: X glucose; • butanol; ○ butyric acid; □ i-propanol; * pH

The observed phenomena designate an important finding for the application of hydrolysates for solvent production: the drawback of the low fermentable sugar concentration in hydrolysates may be circumvented by offering 'mixed' substrates to the bacteria. These mixtures may be composed out of glucose, just sufficient to trigger the switch to solventogenesis, supplemented by hydrolysates of cheap lignocellulosic wastes for increased revenue. Another approach might be the removal of inhibiting

components in order to allow the utilization of concentrated hydrolysates. This approach will be discussed below.
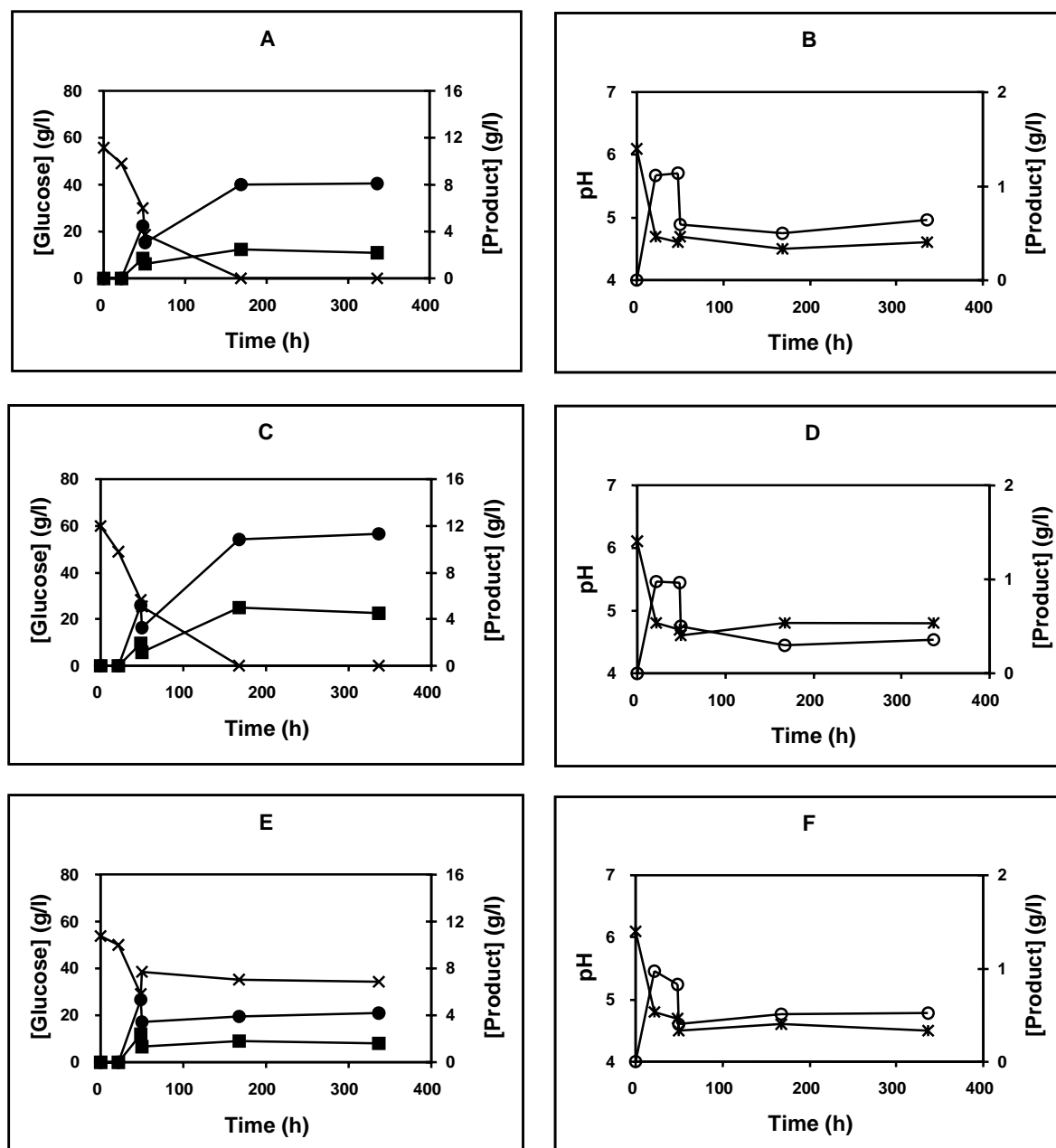
Figure 3.2 Production of acetone and butanol by *C. acetobutylicum* **DSM 1731** on glucose and hydrolysate of domestic organic waste. A/B: synthetic medium with 6% (w/v) glucose; C/D: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make the initial hydrolysate concentration at t = 50 h and E/F: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make the 2 fold the hydrolysate concentration at t = 50 h. Controls were done with demineralised water at t = 50 h, which showed no effect. In g/l: X glucose; ● butanol; ○ butyric acid; ■ acetone; * pH

Fermentation of partially purified hydrolysate by *C. acetobutylicum* DSM 1731

In order to remove unspecific inhibitors, the hydrolysate was first 10 fold concentrated. The precipitate was removed and the concentrated hydrolysate was eluted over a Dowex 1-X8 anionic

exchange column, equilibrated with 200 mM Na-acetate buffer, pH 4.8. The fractions containing glucose were pooled and diluted to make 4, 2 and 1 fold concentrated hydrolysate. After adjusting the pH to 6.0, the filter-sterilized hydrolysates were inoculated with a 5% inoculum. Because of an expected long lag phase, glucose consumption and ABE production measurements were started after 120 h of incubation. The results are shown in Table 3.4.

Table 3.4 Fermentation of partially purified, concentrated hydrolysate from domestic organic waste by *Clostridium acetobutylicum* DSM 1731. Purification was done by anion exchange. Media were prepared from dilutions of this purified hydrolysate in demineralised water to make 1, 2 or 4 fold the initial concentration. The initial pH was adjusted to 6.0.

	Fermentation time in h								
	Glucose g/l			ABE g/l			Butyric acid g/l		
	0	120 h	168 h	0	120 h	168 h	0	120 h	168 h
Medium									
Hydrolysate 1x concentrated	12.0	0	0	0	3.2	4.5	0	0.4	2.0
Hydrolysate 2x concentrated	21.0	0.2	0	0	6.2	6.7	0	1.4	1.6
Hydrolysate 4x concentrated	34.2	1.6	0.1	0	9.3	10.9	0	2.2	1.8
6% glucose-Gapes	59.9	3.7	1.7	0	17.3	17.9	0	0.7	0.8

As can be seen in Table 3.4, the removal of inhibiting components from the hydrolysate was quite successful, judged from glucose concentration and ABE production. It was surprising to note that in the medium with the lowest concentration of hydrolysate, in which glucose concentration amounted up to a mere 12 g/L , DSM 1731 showed no sign of remaining acidogenic in contrast to the culture described in Table 3.3 where this strain produced 1.5 and 2.1 g/l of ABE and butyric acid, respectively. Apparently, the triggering event of the switch to solventogenesis can be governed by other constituents in the medium besides the ample supply of glucose.

In 4 fold concentrated hydrolysate, glucose consumption was virtually complete and production of ABE occurred without any problems. Even though there was some production of butyric acid, the yield of ABE per g of glucose was 38%, which is probably a biased estimate because of the presence of other saccharides in the hydrolysate. If a proportional loss during purification and concentration is assumed, together with a complete utilization of the other saccharides, the yield on hydrolysate amounts to an average of 28 g ABE per 100 g monosaccharides in the hydrolysate, which is in line with the yield of 30% observed on glucose in the synthetic medium (Table 3.4). As shown in Table 3.1, the content of saccharides in the polymeric fraction of DOW was 39 g/100 g dry matter. The yield of 28% for ABE from hydrolysate

suggests that, on the basis of an average dry matter content of 17% in fresh DOW, there is a potential renewable resource for producing 19 g ABE per kg of fresh weight, provided that all saccharides are made available as fermentable feedstock.

Conclusion

The major drawback of the utilization of DOW for the production of ABE is the low content of fermentable feedstock in the raw material which results, even after successful pre-treatment and hydrolysis, in low substrate concentrations and thus in diluted product streams. There are two strategies optional: either to increase the concentration with a penalty due to the increase of inhibitors, or to use the DOW as a co-substrate, still necessitating the utilization of more expensive feedstock. A techno-economical analysis is required to evaluate the potential viability of either approach.

Acknowledgements

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Chapter 4

Clostridium beijerinckii **Expressing** *Neocallimastix patriciarum*

Glycoside Hydrolases Show Enhanced Lichenan Utilization and

Solvent Production

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Hans Mooibroek and Willem M. de Vos

Abstract

Growth and the production of acetone, butanol, and ethanol by *Clostridium beijerinckii* NCIMB 8052 on several polysaccharides and sugars were analyzed. On crystalline cellulose, growth and solvent production were observed only when a mixture of fungal cellulases was added to the medium. On lichenan growth and solvent production occurred, but this polymer was only partially utilized. To increase utilization of these polymers and subsequent solvent production, the genes for two new glycoside hydrolases, *celA* and *celD* from the fungus *Neo-callimastix patriciaru*m, were cloned separately into *C. beijerincki*i. To do this, a secretion vector based on the pMTL500E shuttle vector and containing the promoter and signal sequence coding region of the *Clostridium saccharobutylicum* NCP262 *eglA* gene was constructed and fused either to the *celA* gene or the *celD* gene. Stable *C. beijerinckii* transformants were obtained with the resulting plasmids, pWUR3 *(cel*A) and pWUR4 *(cel*D). The recombinant strains showed clear halos on agar plates containing carboxymethyl cellulose upon staining with Congo red. In addition, their culture supernatants had significant endoglucanase activities (123 U/mg of protein for transformants harboring *celA* and 78 U/mg of protein for transformants harboring *cel*D). Although *C. beijerinckii* harboring either *celA* or *celD* was not able to grow, separately or in mixed culture, on carboxymethyl cellulose or microcrystalline cellulose, both transformants showed a significant increase in solvent production during growth on lichenan and more extensive degradation of this polymer than that exhibited by the wild-type strain.

Introduction

The anaerobic conversion of carbohydrates into acetone, butanol, and ethanol, which is known as ABE fermentation, by several strains of *Clostridium* spp. was first described more than half a century ago. Interest in industrial applications of this process was lost following the development of the petrochemical industry, and currently ABE fermentation plants are operated only in the People's Republic of China (8). Recently, however, there has been increased interest in the production of chemicals and energy by using renewable resources as starting materials. Biomass is a widely available substrate, and utilization of biomass for production of energy carriers is considered an environmentally friendly process (3). For ABE fermentation the most interesting substrates appear to be agricultural or domestic organic wastes due to their low price, wide availability, and appropriate sugar compositions (3, 15). These materials contain mainly two major

sugar polymers: cellulose, an insoluble, linear, unbranched homopolysaccharide consisting of glucose units linked by β-1,4 glycosidic bonds; and hemicellulose, which consists of noncellulosic polysaccharides, including xylans, mannans, and glucans. The cellulose fibrils are partially arranged in a crystalline structure, integrated with hemicellulose, and embedded in lignin (a complex polyphenolic compound). Therefore, in contrast to other substrates used in the past, such as starch or molasses, lignocellulosic substrates are very recalcitrant to degradation, and an expensive hydrolysis step is often necessary prior to fermentation.

Many solventogenic clostridia can utilize a wide range of carbohydrates, including mono- and disaccharides (such as glucose, cellobiose, fructose, maltose, or xylose), that are generated during degradation of plant polysaccharides. However, none of the known solventogenic species is able to utilize cellulose, although some of these species do show some cellulase activity (14). With respect to other polysaccharides, most of the solvent-producing clostridia can efficiently ferment starch, and *Clostridium acetobutylicum* ATCC 824 utilizes xylan, although not very efficiently (18). During the last decade, important advances in our knowledge concerning the physiology and genetics of solventogenic clostridia have been made. Genetic tools for transformation have been developed for a number of strains, including *Clostridium beijerinckii* NCIMB 8052 (24). Transformation of this strain with suitable genes coding for active extracellular hydrolytic enzymes would increase its substrate utilization range and, eventually, enable it to degrade cellulose and hemicellulose more efficiently. Cloning and expression of the *Clostridium cellulovorans engB* gene in *C. acetobutylicum* have been described. However, the recombinant strain exhibited increased extracellular endoglucanase activity but was not able to grow on cellulose as a sole carbon source (13). The *engB* gene from *C. cellulovorans* codes for a glycoside hydrolase that belongs to family 5 of glycoside hydrolases and exhibits endoglucanase and xylanase activities but not hydrolytic activity on crystalline cellulose (9).

To provide an alternative approach, we directed our attention to cloning glycoside hydrolase genes from eukaryotic microorganisms and focused on the genes from the anaerobic rumen fungus *Neocallimastix patriciarum*, which grows on crystalline cellulose as a sole carbon source and exhibits high cellulolytic and hemicellulolytic activities. Another reason why we chose this fungus was its low DNA G+C content and its codon usage, which is compatible with that of *C. beijerinckii* (31). Several cDNAs coding for cellulases from this fungus have been cloned and functionally expressed in *Escherichia coli* (7, 30, 31). These cDNAs include the *celA* gene coding for cellobiohydrolase (EC 3.2.1.91), an exoenzyme that releases cellobiose as a main product from crystalline cellulose (7). *N. patriciarum* CelA belongs to family 6 of the glycoside hydrolases (5, 6), and its production is induced during fungal growth in the presence of cellulose. Another well-

characterized gene is the *N. patriciarum celD* gene, which encodes an endoglucanase (EC 3.2.1.4) belonging to family 5 of the glycoside hydrolases (6, 7). CelD is constitutively produced by the fungus and exhibits activity towards carboxymethyl cellulose (CMC) and, to a lesser extent, towards Avicel (microcrystalline cellulose) and amorphous cellulose (30). It is generally assumed that enzymes with complementary activities, such as exoglucanases (cellobiohydrolases) and endoglucanases, act synergistically during degradation of crystalline cellulose.

In this study we describe the growth of and solvent production by *C. beijerinckii* NCIMB 8052 on different glucose polymers including lichenan, CMC, and crystalline cellulose. In media with crystalline cellulose, growth and solvent production were observed only when a mixture of fungal cellulases was added to the medium. Subsequently, we cloned the *N. patriciarum celA* and *celD* genes individually into *C. beijerinckii* NCIMB 8052 using a secretion vector. Extracellular production of the fungal enzymes by *C. beijerinckii* transformants was observed, and substrate utilization by transformants was also studied in different combinations.

Materials and methods

Bacterial strains, media, and growth conditions.

C. beijerinckii NCIMB 8052 was kindly supplied by M. Young (University of Wales, Aberystwyth, United Kingdom). *Clostridium saccharobutylicum* NCP262 was kindly provided by S. R. Reid (University of Capetown, Capetown, South Africa). Stock cultures were maintained as spore suspensions in sterile 10% (vol/vol) glycerol at -20°C. Spore suspensions were heat-shocked for 1 min at 100°C (for strain NCIMB 8052) or for 3 min at 75°C (for strain NCP262) in a water bath prior to inoculation. For production of precultures, vegetative cells were grown in a semisynthetic medium described previously (20) or in clostridial basal medium (CBM) (21) overnight at 37°C. For growth experiments the same media were supplemented with 1.5% (wt/vol) glucose (Merck, Darmstadt, Germany), 3% (wt/vol) cellobiose (Sigma), 6% (wt/vol) Avicel (Merck), 6% (wt/vol) Sigmacell (type 50; Sigma), 1 or 2% (wt/vol) CMC (low or high viscosity; Sigma), or 2% (wt/vol) lichenan (Sigma) as the carbon source, as indicated below. For simultaneous saccharification and fermentation experiments, media were supplemented with Cellulast 1.5L (Novozymes, Bagsvaerd, Denmark) at a concentration of 2% (weight of Celluclast 1.5L/weight of substrate), unless indicated otherwise, at the beginning of the fermentation (zero time). All experiments were performed anaerobically in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) under a 20% CO_2/4% H_2/76% N_2 atmosphere unless indicated otherwise.

For vector construction *E. coli* XL1 blue (Stratagene) was used. This strain was usually grown in Luria-Bertani broth as described previously (26). When necessary, media were supplemented with ampicillin (50 μg/ml), isopropyl-β-thioga-lactopyranoside (IPTG) (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyr-anoside (X-Gal) (40 μg/ml), or erythromycin (10 μg/ml).

Plasmids pNPCA (7) and pBSFD (30) containing the cDNA sequences of the *celA* and *celD* genes, respectively, were kindly supplied by G.-P. Xue (CSIRO Tropical Agriculture, St. Lucia, Australia). Plasmid pMTL500E (22), used as cloning vector for *C. beijerincki*i, was kindly supplied by M. Young.

Transformation procedures, DNA manipulation, and PCR.

All general DNA manipulations in *E. coli* were carried out essentially as described previously (25). Transformation of *C. beijerinckii* NCIMB 8052 was performed by electroporation as described previously (22).

Restriction endonucleases and modification enzymes were purchased from Roche Diagnostics, Eurogentec, or Qiagen.

DNA isolation from *E. coli* was performed with a Wizard Plus SV miniprep kit (Promega Inc.). Plasmid DNA was isolated from *C. beijerinckii* by the modified alkaline lysis method described previously (22). Genomic DNA from *C. saccharobutylicum* NCP262 was isolated by the method of Pospiech and Neumann (23).

The oligonucleotides used for mutagenesis or PCR were purchased from Eurogentec. The DNA fragments containing the desired mutations were cloned into pGEMT-Easy (Promega Inc.). The mutations were verified by sequencing with an automated laser fluorescent ALF DNA sequencer (Amersham Pharmacia Biotech), using fluorescently labeled M13 universal and reverse primers. The promoter plus signal peptide fragment from the *eglA* gene of *C. saccharobutylicum* NCP262 was obtained by PCR as a 0.37-kb *Xho*I/*Nco*I fragment. The primers used were based on the sequence of the *eglA* gene (32) and included forward primer 5'GC<u>CTCGAG</u>CAAACTGCTTCCCCTAATTCCC3' and reverse primer 5'G<u>CCATGG</u>TTGCAGCTTCAGCTTTATAA3'; *Xho*I and *Nco*I sites (underlined) were added at the 5' and 3'ends of the fragment, respectively. The *Nco*I site was added in such a way that it allowed the in-frame fusion of the genes to be cloned downstream. The *celA* gene was amplified from plasmid pNPCA by PCR performed with forward primer 5'GC<u>ACGCGT</u>GTGGTGGTGCCTGGGCTCAATG3' and reverse primer 5'GC<u>TCTAGA</u>TTAAAATGATGGTCTAGC3'; this resulted in introduction of *Ml*uI and *Xba*I sites (underlined) at the 5' and 3' ends of the gene, respectively. The *celA* fragment was cloned after the

promoter-signal sequence fragment in pMTL500E as an *Ml*uI/*Xb*aI fragment, resulting in pWUR3 (Fig. 4.1). The *celD* gene was amplified from plasmid pBFSD by PCR performed with forward primer 5'G<u>CCATGG</u>AAGCTATACGATTTCGAACC3' and reverse primer 5'GC<u>TCTAGA</u>TTAGTTGGTTCTTCTGG3'; this resulted in introduction of *Nc*oI and *Xb*aI sites (underlined) at the 5' and 3' ends of the gene, respectively. The 1.2-kb *celD* fragment obtained was cloned as a *Nc*oI/*Xb*aI fragment after the promoter-signal sequence in pMTL500E, resulting in pWUR4.

Analytical methods

The concentrations of solvents (acetone, butanol, and ethanol) and acids (acetic and butyric acids) were determined in clear supernatants of centrifuged culture samples by high-performance liquid chromatography as described previously by using propionic acid as an internal standard (11). Separation was carried out by using a Shodex KC-311 (Shodex, Tokyo, Japan) column at 80°C and 3 mM H_2SO_4 as the eluent at a flow rate of 1 ml/min. A refractive index detector (Waters 410; Millipore) and a UV absorbance detector (model VWM2141; Pharmacia) were used in series. The concentrations of most of the metabolites were determined from the refractive index chromatograms; the only exception was the concentration of butyric acid, which was determined from UV chromatograms at 210 nm.

Preparation of culture samples for enzyme assays

Cells were sedimented by centrifugation at 10,000 x *g* at 4°C for 15 min. The culture supernatant was collected and concentrated (approximately 30-fold) by ultrafiltration through a Millipore PM10 membrane at 4°C. Low-molecular-weight compounds and medium components were removed from the concentrated material by dilution with 4 volumes of ice-cold 20 mM sodium phosphate buffer (pH 6.0); this was followed by reconcentration by ultrafiltration. This process was repeated three times, and the resulting fractions were used for enzymatic activity determinations. *C. beijerinckii* cell extracts were prepared as follows. One milliliter of culture cells was harvested by centrifugation, resuspended in 0.1 ml of 50 mM Tris-HCl (pH 8.0) buffer, and sonicated with a Branson Sonifier. Cell debris was removed by centrifugation (10,000 x *g* for 10 min), and the resulting supernatant was used for enzymatic activity determinations.

Cellulase activity assays

E. coli and *C. beijerinckii* transformants grown on agar plates were screened for endoglucanase activity by the Congo red staining method of Teather and Wood (27). Some

modifications were made, as follows: CMC was added to the medium at a concentration of 0.1% (wt/vol), and carboxymethyl cellulase (CMCase) activity was detected after 2 to 4 days of incubation at 37°C by washing the cells out of the plates with sterile demineralized water and staining the CMC with Congo red.

Endoglucanase and exoglucanase activities were determined as described previously (15). The following substrates were used (final concentrations): 0.5% (wt/vol) CMC, 0.2% (wt/vol) lichenan (Sigma), 0.5% (wt/vol) laminarin (Sigma), and 0.5% (wt/vol) Avicel in 50 mM citrate buffer (pH 5.7). The substrates were incubated with the enzyme samples for 60 min at 39°C in a water bath. The reducing sugars formed were measured by the 3,5-dinitrosalicylic acid method (10). One unit of activity corresponded to the formation of 1 nmol of reducing sugar (D-glucose) per min. Protein concentrations in the samples were determined by the Bradford assay (Bio-Rad).

Results

Growth of *C. beijerinckii* NCIMB 8052 on (hemi)cellulosic substrates

To analyze the potential for acetone, butanol, and ethanol production on substrates other than the well-studied compounds starch and mono- and disaccharides (17), *C. beijerinckii* NCIMB 8052 was grown on various forms of cellulose and lichenan (Table 4.1). Because microcrystalline cellulose and lichenan were insoluble, growth on these substrates could not be quantified accurately, and growth was indicated as positive or negative. In media with microcrystalline cellulose (Avicel and Sigmacell) or soluble cellulose (CMC) there was virtually no growth. Very small amounts of solvents were produced, which resulted from utilization of residual glucose in the medium (concentration, less than 4 g/liter), probably originated from impurities in the substrates and from the inoculum (10% [vol/vol] from an overnight culture grown in the presence of 6% [wt/vol] glucose). In medium containing cellobiose as a carbon source, the growth and solvent production levels were similar to those in the same media containing glucose (Table 4.1). Growth and solvent production were also observed in medium containing lichenan, but a residue was left after fermentation, indicating that utilization of this polymer was incomplete.

In order to study the possibility of increasing solvent production from cellulose (Avicel, Sigmacell, or CMC) or lichenan, a commercial cellulase mixture, Celluclast 1.5L, was added to media along with these substrates at the same time as the inoculum. This enzyme mixture is obtained by fermentation from the fungus *Trichoderma reesei* and catalyzes the breakdown of cellulose into glucose, cellobiose, and higher glucose polymers since it has mainly cellulase activity and exhibits relatively low β-glucosidase activity (2, 4). When the cellulolytic enzymes were added

to medium containing CMC as the sole carbon source, no growth was observed. This did not change even when the initial amount of cellulases was increased five-fold. However, in media containing microcrystalline cellulose supplemented with the cellulolytic enzymes, significant growth and solvent production were observed. The growth of *C. beijerinckii* on microcrystalline cellulose supplemented with cellulases was slower than the growth on glucose, since the optimal temperature of Celluclast 1.5L is higher (60°C) than the incubation temperature of the cultures (37°C) and growth depended on hydrolysis of cellulose. *C. beijerinckii* is able to utilize cellobiose, and addition of a purified preparation of fungal β-glucosidases (Novozyme N188) to medium containing microcrystalline cellulose supplemented with Celluclast 1.5L did not significantly affect solvent production (data not shown). Finally, addition of cellulolytic enzymes did not significantly improve the production of solvents on lichenan, and a solid residue was still present in the medium after fermentation.

Table 4.1 Growth of and production of solvents (acetone and butanol) by *C. beijerinckii* on semisynthetic medium with different substrates in absence or presence of cellulolytic enzymes[a]

	No addition		+ Celluclast 1,5L	
	Growth	Solvents[a]	Growth[b]	Solvents
Substrate				
None	-	0.8-0.6	ND	ND
Glucose	+	11.1-11.2	ND	ND
Cellobiose	+	9.2	ND	ND
Lichenan	+	4.1-4.3	+	4.9
CMC	-	1.0-1.2	-	1.8-1.4
CMC[c]			-	1-1.1
Avicel	-	0.8-1.1	+	7.7-6.7
Sigmacell Type 5	-	0.7-0.3	+	5.2*

[a] Semisynthetic media (20) were inoculated (10% vol/vol) with an overnight culture grown on glucose, and the resulting cultures were grown for 96 h in the presence of 6% (wt/vol) glucose, 3% (wt/vol) cellobiose, 2% (wt/vol) lichenan, 2% (wt/vol) CMC, 6% (wt/vol) Sigmacell type 50, or 6% (wt/vol) Avicel. Celluclast 1.5L was added to the cultures at a concentration of 2% (wt/wt), unless indicated otherwise. Growth was monitored by measuring the optical density of a culture at 600 nm (+, optical density at 600 nm of more than 1.0; – , residual growth optical density at 600 nm of 0.01 or less). Normally, the acetone/butanol solvent ratio was 1:4; however, in the presence of lichenan the ratio was 1:3. All experiments were carried out in duplicate.
[b] ND, not determined.
[c] Celluclast 1.5L was added at a concentration of 10% (wt/wt) relative to the concentration of CMC.

These results indicate that *C. beijerinckii* NCIMB 8052 can utilize the products of degradation of crystalline cellulose by the *T. reesei* cellulases added (Celluclast 1.5L) for growth and solvent production. In addition, *C. beijerinckii* grew to some extent on lichenan, and solvent production on this polymer increased, although slightly, when the mixture of fungal cellulases was added to the medium. Therefore, we expected that sufficient production of appropriate glycoside hydrolase enzymes by *C. beijerinckii* should enable it to utilize cellulose or lichenan more efficiently without exogenous addition of enzymes.

Cloning and expression of the *N. patriciarum celA* and *celD* cDNAs using a clostridial secretion vector

To allow extracellular production of fungal glycoside hydrolases by *C. beijerincki*i, a clostridial secretion vector was designed. This vector was based on the 6.4-kb shuttle vector pMTL500E and the well-characterized promoter and signal peptide coding sequences of the 1,4-β-endoglucanase *(egl*A) gene from *C. saccharobutylicum* NCP262 (32).
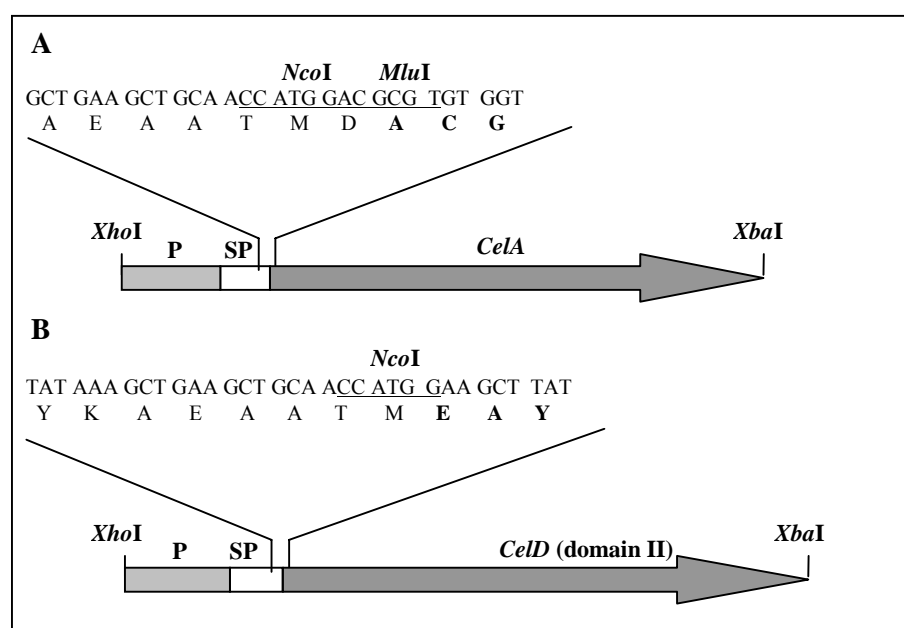
Figure 4.1 Schematic representation of the constructions in pMTL500E that generated pWUR3 (A) and pWUR4 (B). Amino acids in boldface type are amino acids of the CelA or CelD protein. The position of the *C. saccharobutylicum eglA* promoter (P) is indicated, as is the position of the signal sequence (SP). The fungal cDNA is indicated by the dark grey arrow. The restriction sites in the fusions are underlined.

In-frame fusions between the *eglA* signal peptide sequence and the *N. patriciarum celA* or *celD* coding sequence were created by adding adequate restriction sites at the 3' end of the *eglA*

sequence as well as at the 5' end of the *celA* or *celD* coding sequence (Fig. 4.1). Transformants of *E. coli* harboring the resulting 6.8-kb plasmid pWUR3 *(cel*A) or the 6.9-kb plasmid pWUR4 *(cel*D) were readily obtained and were found to have the expected configuration upon sequence analysis of the fusion sites (Fig. 4.1). Subsequently, *E. coli* transformants harboring either pWUR3 or pWUR4 were analyzed for production of endoglucanase activity. Both strains were found to produce clear halos around colonies grown in agar plates supplemented with CMC upon staining with Congo red (results not shown). This confirmed that the *N. patriciarum celA* and *celD* genes were functionally expressed in *E. coli* (6, 16).

The expression plasmids pWUR3 and pWUR4 were subsequently introduced by electroporation into *C. beijerincki*i. While transformants with pWUR4 were obtained at a frequency similar to that observed with cloning vector pMTL500E (~10^2 transformants/ μg of DNA), the transformation frequency of pWUR3 was more than 10-fold lower. However, restriction analysis showed that the size and architecture of the expression plasmids isolated from *C. beijerinckii* transformants were as expected in all of the transformant colonies tested.
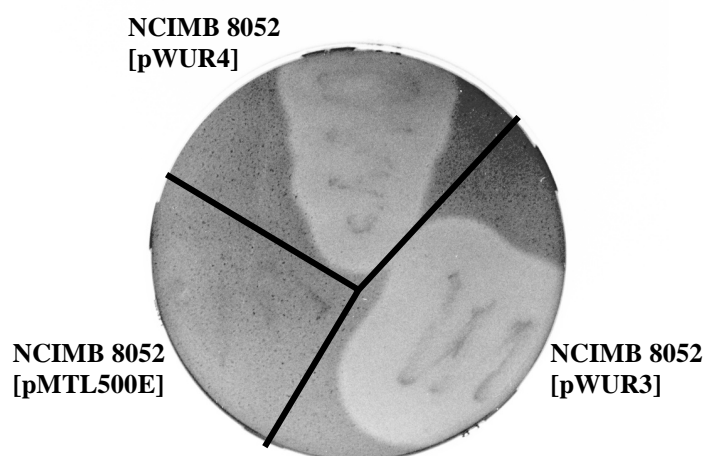
Figure 4.2 Endoglucanase activity plate assay with *C. beijerinckii* NCIMB 8052 harboring pMTL500E, pWUR3 (*celA*), or pWUR4 (*celD*). Cells were grown on agar plates supplemented with 0.2% (wt/vol) CMC for 48 h. CMC was stained with Congo red, and hydrolysis of CMC is indicated by clear halos around cells.

To prevent plasmid loss, erythromycin was added to the media at a concentration of 10 μg/ml when the transformants were cultivated. To detect production of the fungal glycoside hydrolase activity by the *C. beijerinckii* wild-type strain or the clones harboring pWUR3, pWUR4, or the cloning vector pMTL500E, colonies were grown on agar plates supplemented with 0.2%

(wt/vol) CMC. Subsequently, CMCase activity was determined by staining CMC with Congo red (27). Around the colonies of *C. beijerinckii* harboring pWUR3 or pWUR4 clear halos were visible, indicating that degradation of CMC occurred. These halos were significantly larger than the halos around colonies of the untransformed strain or the transformant harboring pMTL500E (Fig. 4.2).

Enzymatic degradation of different polymeric substrates was determined in both cell extracts and concentrated extracellular medium from early-stationary-phase cultures of wild-type and recombinant *C. beijerinckii* grown on CBM with cellobiose or glucose as the carbon source. We determined these activities after 24 h of growth because at this time all of the sugar had been utilized and because in previous studies the highest levels of endoglucanase activity in supernatants of solvent-producing clostridial cultures were found at the end of the exponential growth phase or the early stationary phase (1, 14). No significant differences in glycoside hydrolase activity were found in cultures grown on cellobiose and cultures grown on glucose (results not shown). In the wild-type strain or transformant harboring pMTL500E, no hydrolytic activity with CMC was detectable, but the transformants harboring pWUR3 *(cel*A) or pWUR4 *(cel*D) showed significant endoglucanase activity (Table 4.2). The CMCase activity was predominantly (around 70% of the total activity) located in the culture supernatant (data not shown). This indicates that the fungal cellulases were produced in an active form and efficiently secreted into the medium. The recombinant enzymes were not detectable on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, even after silver staining (results not shown).

Lichenase activity was found in all cultures of *C. beijerinckii*, independent of the presence of the plasmids that were constructed. However, higher levels of activity were found in the cultures of *C. beijerinckii* harboring the fungal cellulase genes. CelA has higher lichenase activity than CelD (7, 30), and in agreement with this, *C. beijerinckii* harboring pWUR3 showed the highest lichenase activity (Table 4.2).

The activities of the cultures producing the fungal enzymes were also determined with laminarin, a polymer composed of β-1,3-linked glucose residues. We tested this substrate because we expected that the lichenase activity found in wild-type and recombinant cultures could be due to hydrolysis of β-1,3 link-ages in lichenan by clostridial enzymes. We did not find laminarinase activity in cell extracts of *E. coli* harboring pWUR3 or pWUR4. There was not a significant difference in the laminarinase activities determined for the wild-type and recombinant cultures (Table 4.2).

Table 4.2 Glucanase specific activities in *C. beijerinckii* culture supernatants[a]

Substrate	Sp act (U/mg of protein)			
	Wild-type	pMTL500E	pWUR4	pWUR3
CMC	< 1	< 1	78±3	123±37
Lichenan	201±17	181±5	297±34	447±98
Laminarin	1,271±122	1,144±106	1,089±57	1,395±50

[a] Untransformed wild-type and plasmid-harboring cultures were tested for enzymatic activity on CMC, lichenan, and laminarin. One unit of activity corresponded to the release of 1 nmol of reducing sugars per min. Cultures were grown in duplicate, and every sample was assayed in duplicate. The values are means ± standard deviations.

Substrate utilization by *C. beijerinckii* producing fungal glycoside hydrolases

Cultures of untransformed *C. beijerinckii* or cultures harboring pMTL500E, pWUR3, or pWUR4 were grown in CBM containing glucose, cellobiose, lichenan, Avicel, or CMC as the sole carbon source. In media containing glucose or cellobiose as the carbon source the transformants grew as well as the wild type. None of the strains grew in media containing CMC or Avicel. Also, cocultures of *C. beijerinckii* harboring pWUR3 and *C. beijerinckii* harboring pWUR4 did not grow on these substrates. Cultures were also grown in CBM containing 1% (wt/vol) CMC (high or low viscosity) or 6% (wt/vol) Avicel supplemented with cellobiose or glucose at a concentration of 1 or 0.1% (wt/vol). CMC and Avicel were not utilized by any of the strains, even after extended incubation (14 days). As expected, the growth and solvent production of these cultures were comparable to those of cultures containing only the sugars as carbon sources (results not shown).

In media containing lichenan, cultures of *C. beijerinckii* harboring the *celA* or *celD* gene produced significantly more solvents than the untransformed wild type or the transformant harboring the pMTL500E vector produced (Fig. 4.3). *C. beijerinckii* harboring pWUR3 grown on lichenan produced an amount of solvents similar to the amount produced by the wild-type strain grown on glucose at the same concentration in CBM when a 1% (vol/vol) inoculum was used (4.9 and 5.4 g of solvents per liter, respectively). At the concentration used in the media, lichenan was partially insoluble, and after fermentation residual undegraded polymer remained in all of the cultures except those of *C. beijerinckii* harboring pWUR3 (results not shown). This indicates that there was extensive degradation of this polymer by the fungal cellulase CelA, which can be explained by the high lichenase activity found in cultures of *C. beijerinckii* expressing the *celA* gene (Table 4.2).

A

B
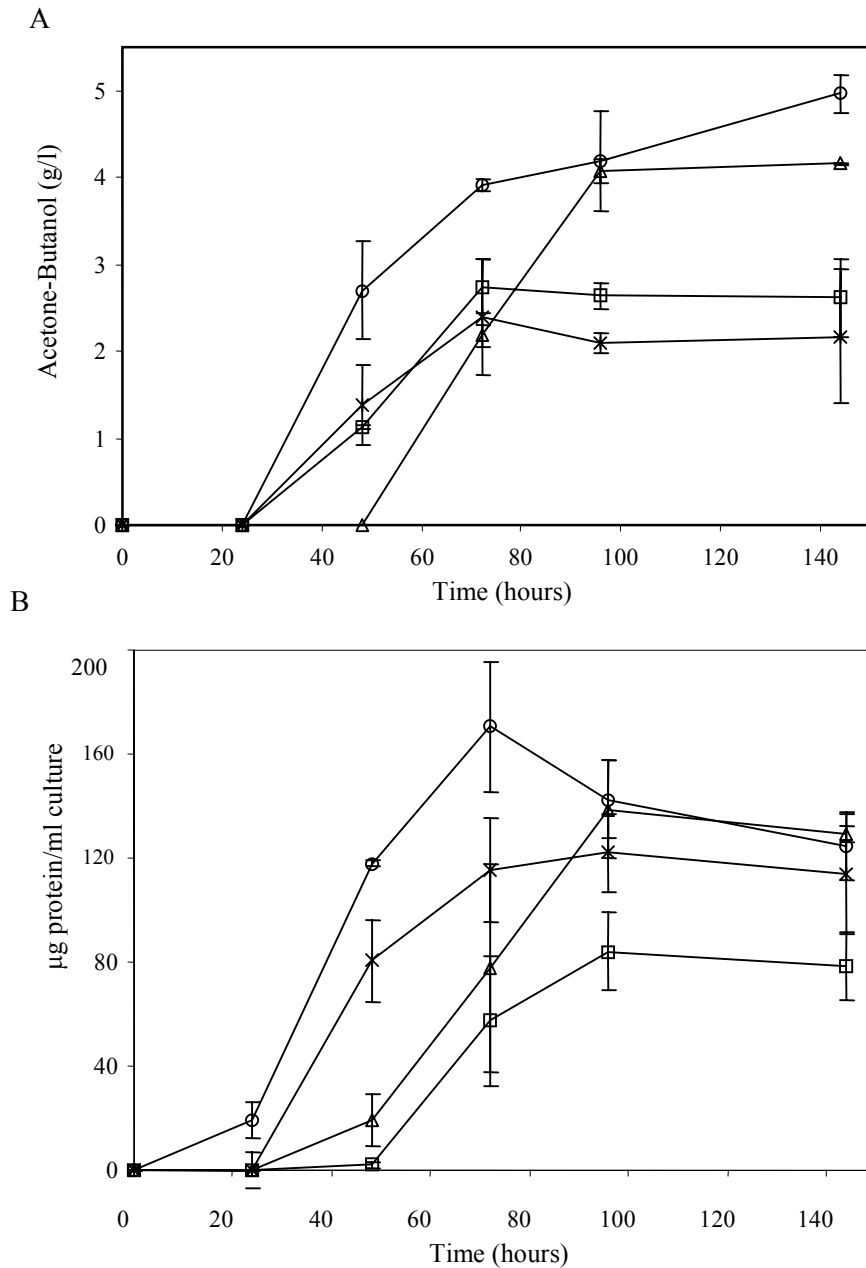
Figure 4.3 Production of solvents by and growth of *C. beijerinckii* NCIMB 8052 carrying no plasmid (X), pMTL500E (□), pWUR4 (Δ), or pWUR3 (○) on CBM containing 20 g of lichenan per liter as the sole carbon source. The 1% (vol/vol) inoculum was obtained from an overnight preculture grown in the presence of 1.5% (wt/vol) glucose. Because lichenan was insoluble and cultures were very turbid, growth was estimated by measuring the protein contents of cell extracts of culture samples. Solvents were produced at acetone/butanol ratios between 1:2 and 1:3. Averages based on two independent experiments are shown. The error bars indicate standard deviations based on at least triplicate samples.

Discussion

C. beijerinckii NCIMB 8052 belongs to the group of well-known solventogenic clostridia, and its physiology and substrate utilization range have been the subjects of a number of studies (17). In this study we found that saccharification of cellulose, growth, and solvent production take place simultaneously when the growth medium is supplemented with fungal cellulolytic enzymes (Table 4.1). Remarkably, growth on CMC (either high or low viscosity) was not observed even when the same cellulases were added to the medium at high concentrations (Table 4.1). Since the bacteria can grow in media containing CMC and supplemented with glucose or cellobiose, the presence of toxic impurities in this substrate can be ruled out as an explanation for this observation. It seems likely that the carboxymethyl groups that are present in 7 of the 10 glucose residues in CMC confer a negative charge that could inhibit utilization of the oligosaccharides generated by the bacteria.

Addition of fungal cellulases (Celluclast 1.5L) to a medium with microcrystalline cellulose as the sole carbon source enabled *C. beijerinckii* to grow on this substrate (Table 4.1). Celluclast 1.5L contains a mixture of different cellulases, including exo- and endoglucanases, which act synergistically in degradation of sugar polymers (4, 16).

For cloning of the *N. patriciarum* glycoside hydrolase genes we used the promoter and signal sequences of the *eglA* gene from *C. saccharobutylicum* NCP262 (32), which appeared to be adequate to control expression of a cellulase gene. This system has been used recently for cloning of another heterologous gene in *C. acetobutylicum* (28); the only difference was that the signal peptide sequence used here was one codon shorter. In this study we predicted a signal peptide with 38 amino acids (29) and showed that this is effective in directing the secretion of fungal cellulases. Although both CelD and CelA were successfully produced and excreted into the extracellular medium in an active form by C. *beijerinckii* NCIMB 8052 (Fig. 4.2 and Table 4.2), the transformant strains, alone or in combination, failed to utilize Avicel or Sigmacell as a carbon source under the conditions tested. While this finding could be attributed to a number of reasons, one of the possible explanations is that the levels of the fungal enzymes produced were too low to efficiently degrade cellulose. Moreover, the degradation of cellulose is a complex reaction in which many enzymes are involved, and it is likely that more than the two cloned fungal glycoside hydrolases is necessary for efficient hydrolysis of this substrate.

In contrast to the results obtained with other solventogenic clostridia (14), no CMCase activity was found in liquid cultures of *C. beijerinckii* NCIMB 8052 (Table 4.2) or in plate assays

(Fig. 4.2). The experiment was repeated with a new culture of strain NCIMB 8052 received from the National Collections of Industrial, Food and Marine Bacteria, and the same results were obtained (data not shown). Since CMC is a soluble derivative of cellulose that is considered a typical substrate for endoglucanases, we concluded that this strain does not produce 1,4-β-endoglucanase activity, which contrasts with the conclusions of previous reports (13, 19). *C. beijerinckii* NCIMB 8052 showed extracellular hydrolytic activity on lichenan (1,3-1,4-β-glucan) and laminarin (1,3-β-glucan), indicating that it produces 1,3-β-glucanases and lichenases. These enzymes, which have been placed in family 16 of the glycoside hydrolases, are widely distributed in bacteria (12). A number of lichenases (EC 3.2.1.73), 1,3-β-glucanases (EC 3.2.1.39), and 1,3(4)-β-glucanases (EC 3.2.1.6) have been characterized at the genetic level in several *Bacillus* spp. and *Clostridium thermocellu*m. In solventogenic clostridia these activities have not been studied in detail yet, and this is the first report of lichenase and laminarinase activities in *C. beijerinckii* NCIMB 8052. During growth of wild-type or transformant strains on lichenan, the acetone/ butanol ratio in the media varied between 1:2 and 1:3, in contrast to the ratio found during growth on glucose (1:4). The influence of substrate concentration on the solvent ratio has been described previously (26). Transformants harboring pWUR3 or pWUR4 showed increased extracellular lichenase activity as a result of expression of either the *celA* or *celD* gene. This increased activity resulted in increased solvent production when lichenan was the substrate (Fig. 4.3). Only the transformant carrying pWUR3 was able to utilize lichenan completely, which resulted in clear media after fermentation, whereas the turbidity did not disappear in cultures of the other strains. This observation is in agreement with the fact that in cell extracts of *E. coli*, fungal CelA exhibits approximately fivefold-higher lichenase activity than CelD (7, 30). Remarkably, the solvent production by the pWUR3-harboring transformant on lichenan was comparable to that of the wild-type strain on glucose.

This is the first example of cloning of cellulase genes from a eukaryotic organism into *C. beijerinckii*. The *N. patriciarum celA* and *celD* genes were functionally expressed in *C. beijerinckii* NCIMB 8052, and the resulting enzymes were exported to the medium. However, the recombinant strains individually or in cocultures did not grow on microcrystalline cellulose or CMC as a sole carbon source. It is likely that more proteins are needed for efficient degradation of cellulose to support growth. The *C. beijerinckii* strains producing the fungal enzymes showed increased utilization of lichenan, a polymer very similar to the mixed 1,3-1,4-β-glucans that are part of the cell walls of cereals such as barley, rice, and sorghum. In conclusion, we show that cloning of fungal genes into *Clostridium* strains can produce strains with a substrate utilization range different

from that of the wild-type strain. This may open possibilities for generating solventogenic strains that are able to grow on polymeric substrates suitable for economically viable ABE fermentation.

Acknowledgments

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Chapter 5

Heterologous Expression of a Cellulose-Binding Protein by

Clostridium acetobutylicum

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Abstract

The genome sequence of the anaerobic, Gram-positive, solvent-producing bacterium *C. acetobutylicum* ATCC 824 predicts the production of cellulosomes, extracellular multiproteic complexes that efficiently degrade microcrystalline cellulose. However, this strain is not able to utilize microcrystalline cellulose (Avicel) as sole carbon source unless the medium is supplemented with external cellulases. In the gene cluster containing most of the genes coding for cellulosomal components, there is a unique gene that codes for a large non-enzymatic scaffolding protein essential for cellulose degradation, the cellulose-binding protein. To restore cellulolytic and cellulose-binding capacity, *C. acetobutylicum* was transformed by electroporation with a plasmid containing the *cbpA* gene from *C. cellulovorans*. The transformants obtained did not show improved utilization of cellulose compared to the wild-type. Using antibodies against *E.coli*-produced CbpA, the protein produced by *C. acetobutylicum* could only be detected in the extracellular growth medium as a number of small protein fragments, and not as a single band corresponding to the complete protein. This indicates that CbpA was produced, but it was probably subjected to proteolysis.

Introduction

Some anaerobic microorganisms degrade cellulose by means of an extracellular enzyme complex, called the cellulosome, which contains a variety of cellulolytic enzymes attached to a non-enzymatic scaffolding component, generally termed as Cellulose binding protein (Cbp) or Cellulose integrating protein (Cip). The cellulosomes from several cellulolytic clostridial species have been extensively studied at the physiological and genetic levels. Although they are not identical, they show a number of common characteristics (1, 19). The Cbp plays an essential role on the cellulosome structure and function (20). This protein, of a high molecular weight compared to the other subunits, shows no detectable enzymatic activity and contains a cellulose-binding domain. All cellulosomal enzymatic subunits contain duplicated amino acid sequences (on average 2x 22 residues) often located at their C-terminal end called dockerins that bind to hydrophobic domains, called cohesins, in the Cbps. Cellulose-binding proteins from different organisms contain a different number of cohesin domains consisting of, on average, 140 residues each often located at their C-terminal end and repeated several times. Interaction between the cohesin and the dockerin domains is the means by which the cellulolytic enzymes are incorporated into the cellulosome.

Although the solvent-producing strain *Clostridium acetobutylicum* ATCC 824 produces some cellulases and hemicellulases during growth on carbohydrates, it is not able to utilize crystalline cellulose or to bind to it (4, 12). The genome of this bacterium has been recently sequenced, and, unexpectedly, several genes were identified that potentially encode cellulosomal subunits (14). These genes are organized similarly to those found in true cellulolytic clostridia such as *C. cellulovorans*, *C. cellulolyticum* and *C. josui*, and, with the exception of two genes, all genes are located in a cluster. The predicted cellulosomal proteins show high sequence similarity to the ones from corresponding proteins from the true cellulolytic clostridia.

In the known cellulosome gene clusters (including those in *C. acetobutylicum* and *C. cellulovorans*) the gene coding for the Cbp is the first in the cluster. The amino acid sequences and domain structures from the different clostridial Cbps known are well conserved. Since the reason for the lack of cellulose degradation by *C. acetobutylicum* is not known, the cloning into this organism of genes coding for cellulosome components could help to determine the factor(s) involved in the production of functional cellulosomes.

In this study, the *cbpA* gene encoding for the CbpA from *C. cellulovorans* has been cloned into *C. acetobutylicum* under the control of its own promoter. The CbpA protein was produced by *C. acetobutylicum* and was secreted to the extracellular medium. However, it was found to be unstable and transformants harboring the *cbpA* gene did not show improved cellulolytic properties compared to the parent strain.

Materials and Methods

Bacterial strains, media, and growth conditions.

C. acetobutylicum ATCC 824 was kindly supplied by Dr. P. Soucaille (INSA, Toulouse, France) and cultivated as described previously (7). Celluclast 1.5L was a gift from Novozymes (Bagsvaerd, Denmark) and was added at a concentration of 2% (weight Celluclast 1.5L/weight substrate), unless indicated otherwise, at the beginning of the fermentation (zero time)

C. cellulovorans DSM 3052 was obtained from the DSMZ culture collection and cultivated as described (22) with cellobiose (Sigma) or cellulose MN 300 (Macherey-Nagel, Düren, Germany) as carbon sources. For vector construction and protein production the *E. coli* strain XL1 blue (Stratagene, Amsterdam, The Netherlands) was used. This strain was grown in Luria-Bertani broth as described (17), supplemented, when appropriate, with ampicillin (50 μg/ml), isopropyl-β-thiogalactopyranoside (IPTG; 50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal; 40 μg/ml) or kanamycin (50 μg/ml).

Transformation procedures, DNA manipulation and PCR

All general DNA manipulations in *E. coli* were carried out essentially as described (17). Restriction endonucleases, modification enzymes or oligonucleotides for polymerase chain reaction (PCR) were purchased from Roche Diagnostics (Mijdrecht, The Netherlands), Eurogentec (Maastricht, The Netherlands) or Qiagen. DNA isolation from *E. coli* was performed with the Wizard Plus SV miniprep kit (Promega Inc, Leiden, The Netherlands). Genomic DNA from *C. acetobutylicum* ATCC 824 was isolated as described previously (15).

The plasmid pCB1 (21) containing the *cbpA* gene was kindly supplied by Dr. R. H. Doi (Davis University, California, USA). Primers based on the sequence of the *cbpA* gene from *C. cellulovorans* (NCBI Accession nr M73817) were used to confirm the presence of the gene in recombinant *E. coli* or *C. acetobutylicum*; the forward primer was 5'-GAATTTTACAACTCTAACAAATCAGC- 3' (BG1016) and the reverse primer was 5'-GCTAATGTACCATCTGCAAATACAGG- 3' (BG1017), which generate an internal PCR-fragment of 1,567 bp. The *cbpA* gene was cut out of the pCB1 plasmid by restriction with *EcoR*I and ligated into the unique *EcoR*I site of the *E.coli-C. acetobutylicum* shuttle vector pSYL2 (6), resulting in pWUR113. Plasmids were methylated using the methylating plasmid pAN1 (10) prior to transformation. *C. acetobutylicum* was transformed by electroporation as previously described (11). Plasmids pSYL2 and pAN1 were kind gifts from Dr. G. Bennett (Rice University, Houston, Texas, USA)

Analytical methods

Fermentation products were determined in the extracellular culture medium by high performance liquid chromatography (HPLC) as described previously (7).

Preparation of culture samples for Western blotting

Cells were sedimented by centrifugation at 10,000 x g at 4°C for 15 min. The culture supernatant was collected, filtered through a 0.22 µm filter and concentrated approximately 30-fold by ultrafiltration through a PM10 membrane (Millipore, Etten-Leur, The Netherlands) at 4°C. Low-molecular-weight compounds and medium components were removed from the concentrated material by dilution with 4 volumes of ice-cold 50 mM sodium citrate buffer (pH 5.7), followed by re-concentration by ultrafiltration. This process was repeated 3 times and the resulting fractions were used for Western blotting assays.

For the preparation of cell-free extracts of *C. acetobutylicum*, cells (1 ml) were harvested by centrifugation, resuspended in 0.1 ml 50 mM Tris-HCl (pH 8.0) buffer and sonicated using a Branson sonifier. Cell debris was removed by centrifugation (10,000 x g for 20 min.). The insoluble fraction was re-suspended in 0.1 ml of the same buffer.

Cellulose-bound proteins in *C. cellulovorans* cultures were harvested by centrifuging an aliquot of the culture and washing the pellet, consisting of cellulose, cellulose-bound proteins, and cells, briefly with a buffer containing 8 M urea. Proteins bound to the cellulose denatured and became soluble, whereas bacterial cells remained mostly intact. The protein-containing soluble fraction was obtained by centrifugation.

Immunological procedures

The anti-CbpA antibodies were kindly supplied by Dr. R. H. Doi (20). Western blot analyses were carried out with antiserum diluted 750 times according to a standard protocol (17)

Results

Growth of *C. acetobutylicum* on microcrystalline cellulose

Although it is not able to utilize microcrystalline cellulose, *C. acetobutylicum* ATCC 824 is able to grow on starch and other hemicellulosic polymers (xylan) and produce extracellular cellulolytic enzymes when grown on a range of substrates (Chapters 6 and 7) (12).

Table 5.1 Acids (acetic and butyric acid) and solvents (acetone, butanol and ethanol) produced by *C. acetobutylicum* ATCC 824 on 6 % (w/v) Avicel, 6% (w/v) glucose or 2% (w/v) CMC. Fermentations were terminated when growth was arrested at the time indicated (in brackets). The medium was supplemented, as indicated (+CL), with Celluclast 1.5L at 2% (weight Celluclast 1.5L/weight substrate)

Growth substrate	Products (g/l)				
	acetic acid	butyric acid	acetone	butanol	ethanol
Glucose (144 h)	1.6	0.9	3.4	10.9	0.8
Avicel (96 h)	0.5	0.6	0.1	0.4	0.1
Avicel + CL (216 h)	2.0	1.7	2.4	7.2	0.8
CMC + CL (72 h)	0.3	0.9	0.1	0.4	0

In contrast to the cellulolytic strain *C. cellulovorans*, *C. acetobutylicum* did not utilise CMC (a substrate typical for cellulases with endo-mode of action), not even when Celluclast 1.5L, a

commercial cellulase mixture from the fungus *Trichoderma reseei*, was added to the medium (Table 5.1). On microcrystalline cellulose (Avicel) the presence of Celluclast 1.5L in the medium was enough to enable the bacterium to grow and produce solvents. The fermentation of glucose occurred in a faster manner than that of Avicel (144 hours and 216 hours, respectively) indicating that the degradation of the Avicel into soluble sugars (mostly glucose and cellobiose) was a limiting step for the fermentation.
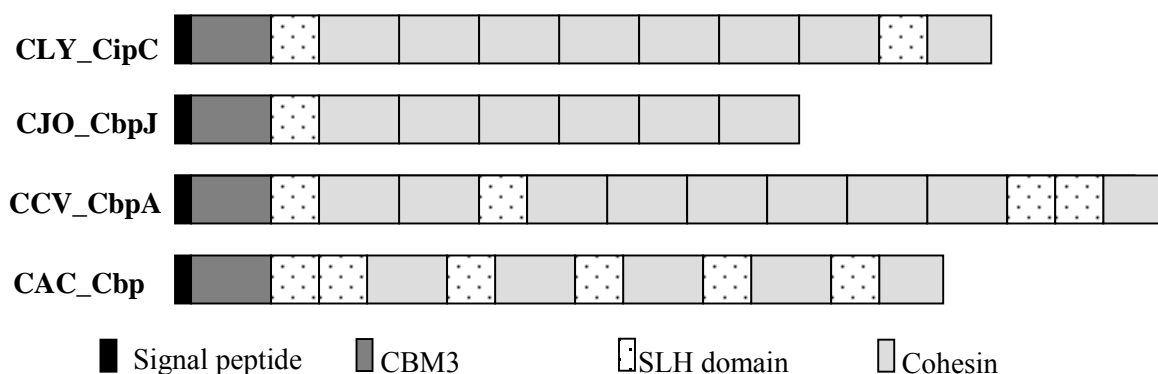

Figure 5.1 Comparison of the domain structures of the cellulosomal cellulose-binding proteins of *C. acetobutylicum* (CAC) Cellulose-binding protein, *C. cellulovorans* (CCV) Cellulose-binding protein A (CbpA), *C. cellulolyticum* (CLY) Cellulose-integrating protein C (CipC) and the *C. josui* (CJO) Cellulose-binding protein J (CbpJ). Abbreviations: CBM3, carbohydrate-binding module from family 3; SLH, surface layer homology.

Sequence analysis of the Cbp of *C. acetobutylicum*

The putative Cbp from *C. acetobutylicum*, encoded by the gene number CAC0910 (gene identification at the NCBI, gi:15023809) has an N-terminal signal peptide sequence of 27 amino acids typical for gram-positive bacteria. The mature Cbp consists of a carbohydrate-binding module from family 3 (2), followed by two hydrophilic modules (Surface layer homology domain, SLH) and five cohesin domains where there is a hydrophilic module (SLH) between each cohesin (Fig. 5.1). This domain architecture is unique compared to the ones found in other Cbps (Fig. 5.1). The CbpA from *C. cellulovorans* shows a 28 amino acids signal peptide sequence, a family 3 carbohydrate binding module, a hydrophilic domain followed by 9 cohesin domains where there is one hydrophilic domain between cohesins 2 and 3, and 2 more hydrophilic domains between cohesins 8 and 9 (3,19) (Fig. 5.1). The overall amino acid sequence of the *C. acetobutylicum* Cbp showed homology to other cellulosomal scaffolding proteins, including CbpA from *C. cellulovorans* (27% identity and 43% similarity), CipC from *C. cellulolyticum* (27% identity and 41% similarity) and CipA from *C. josui* (26% identity and 40% similarity), where the carbohydrate-

binding modules and cohesin domains show highly conserved regions (Fig. 5.2). The insert of the plasmid pCB1, used for heterologous expression, does not contain the complete *C. cellulovorans* *cbpA* gene, but a 5.4-kb fragment that lacks the last 135 bp at the 3' end (21). Interaction of smaller mini-cbpAs with cellulosomal components has been demonstrated before (3), and therefore we expected that this smaller CbpA would interact with cellulosomal components from *C. acetobutylicum*.

A-Cellulose-binding Module

```
CJO_CbpJ: TGVISVQFNNGSSPTSSSSIYARFKVTNTSGSPINLADLKLRYYFTQDENKQMTFWCDHA :  89
CLY_CipC: TGVVSVQFNNGSSPASSNSIYARFKVTNTSGSPINLADLKLRYYYTQDADKPLTFLGDHA :  89
CCV_CbpA: TSSMSVEFYNSNKSAQTNSITPIIKITNTSDSDLNINDVKVRYYYTSDGTQGQTFWCDHA :  89
CAC_Cbp : NSGVQIQFADTNTSTIMNTIAPKFKITNNTGAPLDITTLKLRYYFTADGTQDENFWCDHA :  93

CJO_CbpJ: GYLSGNNYMDVTSKVSGTF-NEVSPAVTNADHYLEVALSSDAGSLPAGGSIEIQTRFARN : 148
CLY_CipC: GYMSGSNYIDATSKVTGSF-KAVSPAVTNADHYLEVALNSDAGSLPAGGSIEIQTRFARN : 148
CCV_CbpA: GALLGNSYVDNTSKVTANFVKETASPTSTYDTYVEFGFASGRATLKKGQFITIQGRITKS : 149
CAC_Cbp : GMLNGYNYQTITSNVVGTF-VAMDNATATADHYLEISFSNGAGQLDAGSSLEVQCRVAKN : 152

CJO_CbpJ: DWSNFDQSNDWSYTSAGSY-MDWQKIAAFVGGTLVYGSTPNGDDNPTQDPKISPTSISAK : 207
CLY_CipC: DWSNFDQSNDWSYTAAGSY-MDWQKISAFVGGTLAYGSTPDGGNPPPQDPTINPTSISAK : 207
CCV_CbpA: DWSNYTQTNDYSFDASSSTPVVNPKVTGYIGGAKVLGTAP-GPDVPSS--IINPTSATFD : 206
CAC_Cbp : DWSNYDQSNDYSFTSNASDFTDWDKITGYVNGDLVFGNPP--VVDP----VITPTTATFD : 206
```

B-Cohesin domain

```
CJO_CbpJ: ALKVIVGTAEGNVGETVTVPVTFADVAKVNNVGTCNFYLAYDASLLDVVSVDAGPIVKNA : 490
CLY_CipC: ELKVAVGTANGKPGDTVTVPVTFADVVNVGNVGTCNFYLAYDASLLEVVSVDAGPIVKNA : 493
CCV_CbpA: RMQISVGTATVKAGEIAAVPVTLTSVPSTG-IATAEAQVSFDATLLEVASVTAGDIVLNP : 492
CAC_Cbp : TVKLSVGTVSGNPGDTVKVPVTISQVSTPV--GLICMDISYDASKFTVKDVLPNTDLVKD : 672

CJO_CbpJ: AVNFSSSASN---GTISFLFLDNTITDELITSDGVFANITFKLKNVSTKTTTPITFKDGG : 547
CLY_CipC: AVNFSSSASN---GTISFLFLDNTITDELITSDGVFANIKFKLKSVATKTTTPVTFKDGG : 550
CCV_CbpA: TVNFSYTVNG---NVIKLLFLDDTLGSQLISKDGVEVTINFKAKAVTSTVTTPVTVSGTP : 549
CAC_Cbp : TDNYSFIVNTSTPGKISITFTDPTLANYPISVDGILAYLDFIINSNATAGDSALTVDPAT : 732

CJO_CbpJ: AFGDGTMSKITTVIKTNGSVTIIPG : 572
CLY_CipC: AFGDGTMAKIATVTKTNGSVTIDPG : 575
CCV_CbpA: VFADGTLAEVQSKTAA-GSVTINIG : 573
CAC_Cbp : LIVADENDKDIKDAASNGKITVTGS : 757
```

Figure 5.2 Amino acid sequence alignment of the conserved carbohydrate-binding module (CBM) (A) and one of the cohesin domains (B) from the *C. acetobutylicum* (CAC) Cellulose-binding protein, *C. cellulovorans* (CCV) Cellulose-binding protein A (CbpA), *C. cellulolyticum* (CLY) Cellulose-integrating protein C (CipC) and the *C. josui* (CJO) Cellulose-binding protein J (CbpJ). The numbers indicate the aminoacid number including the signal peptide, shadows indicate conserved aminoacids.

Promoter analysis

The regulation of carbon metabolism in gram-positive bacteria is mediated by a transcriptional factor called catabolite control protein (CcpA) (13). The CcpA binds to specific *cis*-acting DNA sequences known as catabolite response element (CRE), repressing or activating

expression of the gene downstream. In the genome of *C. acetobutylicum* ATCC 824 a gene is present (CAC3037) that encodes a protein highly homologous to the *Bacillus subtilis* CcpA (5)(Genebank accesion number NP_390852) (42% identity and 63% similarity). The promoter regions of the *cbpA* gene from *C. cellulovorans* and the *cbp* gene from *C. acetobutylicum* have been screened in order to find putative catabolite repression elements (CRE) or XylR-binding regulatory sequences, involved in transcriptional regulation of pentose utilization genes (18). No putative CREs were detected using the consensus motif WTGNAANCGNWNNCW, in which W indicates T or A and N indicates any nucleotide (13) or XylR sequences using the consensus sequences TTGAAAGCGCTTTCAA or GWAAWCGNTNNCA (16).

Cloning of the *cbpA* gene from *C. cellulovorans* gene into *C. acetobutylicum*

The 6.5-kb *EcoR*I-fragment containing the *C. cellulovorans cbpA* gene and its promoter region was cloned into the *E. coli*-*C. acetobutylicum* shuttle vector pSYL2, generating pWUR113. The resulting construct, of a total size of approximately 15.2 kb, was methylated and electroporated into *C. acetobutylicum*. The transformation efficiency obtained with pWUR113 (28 transformants per μg of DNA) was approximately five times lower than that obtained with the empty vector pSYL2 (135 transformants per μg of DNA).

The recombinant plasmids could not be isolated from the transformants by using standard protocols. However, the presence of DNA derived from the pWUR113 construct into the transformants could be confirmed by PCR analysis on their total DNA using primers BG1016 and BG1017, specific for an internal fragment of the *cbpA* gene. Only in samples containing DNA from the *C. acetobutylicum* transformants harboring pWUR113 a PCR product of approximately 1.5 kb was observed (data not shown).

To determine if pWUR113 was integrated into the chromosome of *C. acetobutylicum*, total DNA isolated from the wild-type or the transformants was restricted with *BamH*I, that cuts once in pSYL2 or pWUR113, or with *Bcl*I, that cuts once inside the *cbpA* gene sequence and does not cut pSYL2. Southern blotting of the digested or undigested DNA samples followed by hybridization with labelled probes specific for the pSYL2 vector or the internal fragment of the *cbpA* gene generated by PCR using primers BG1016 and BG1017 were carried out. The restriction enzyme *Bcl*I cuts once in the internal *cbpA* fragment used as a probe and if pWUR113 was integrated into the chromosome two bands would be expected in the Southern blot corresponding to *Bcl*I-restricted total DNA from the transformant harbouring pWUR113. Alternatively, if no integration occurred, a single band would be expected. In Figure 5.3 (lane B 10) only a single band corresponding to *Bcl*I-restricted pWUR113 is observed, indicating that integration did not take place. Also, no cross-

reaction was observed between the labelled *cbpA* fragment and DNA from *C. acetobutylicum* wild-type, reflecting the lack of homology at nucleotide level between the *cbpA* gene from *C. cellulovorans* and the *cbp* gene of *C. acetobutylicum*.
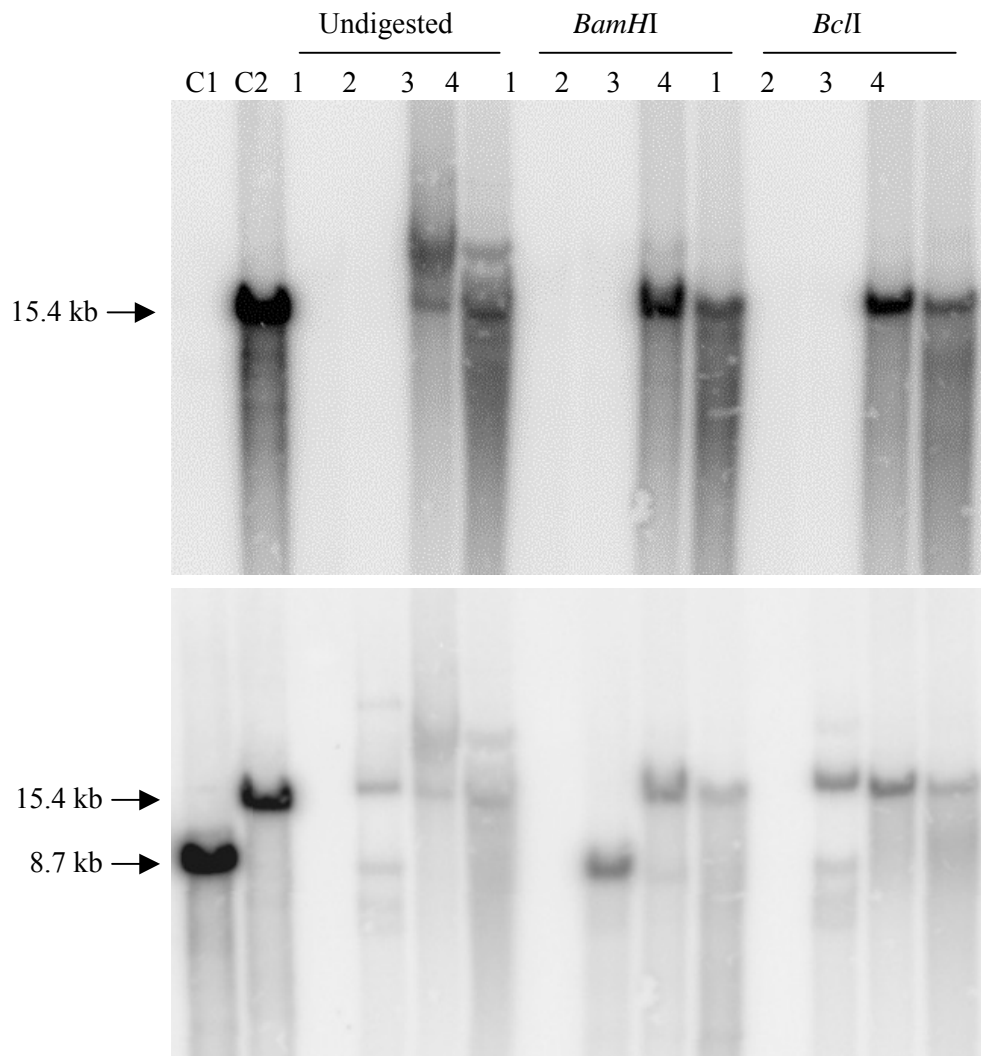
Figure 5.3 Southern blot analysis of genomic DNA (2 μg/slot) from wild-type *C. acetobutylicum* ATCC 824 (lane 1) and transformants harboring pSYL2 (lane 2) or pWUR113 (lanes 3, 4), digested with the indicated restriction enzyme (*BamH*I or *Bcl*I). The blot was hybridized with with a ^{32}P-labelled internal fragment of the *cbpA* gene that contained a single *Bcl*I site (see text) (panel **A**) followed by stripping and rehybridizing ^{32}P-labelled pSYL2 vector (panel **B**). Controls; C1, pSYL2 (100 ng) digested with *BamH*I; C2, :pWUR113 (100 ng) digested with *BamH*I

Production of CbpA by *C. acetobutylicum*

In order to determine if CbpA or CbpA-like proteins were produced by *C. acetobutylicum* wild-type or transformants harboring pSYL2 or pWUR113 grown on glucose, Western blot analyses using anti-CbpA antibodies (20) were carried out. In extracellular medium of early stationary phase (24 hours) glucose-grown cultures of *C. acetobutylicum* wild-type and

transformants, no protein of the expected size of CbpA (approximately 180 kDa) reacting with anti-CbpA was observed (Fig. 5.4). In the extracellular medium from transformants harboring pWUR113, extra proteins reacted with the anti-CbpA antibodies compared to the wild type or the transformant harboring the vector pSYL2. The extra bands observed in the Western blot corresponded to proteins of a smaller size than expected, and probably correspond to proteolytic degradation products of CbpA (Fig. 5.4).
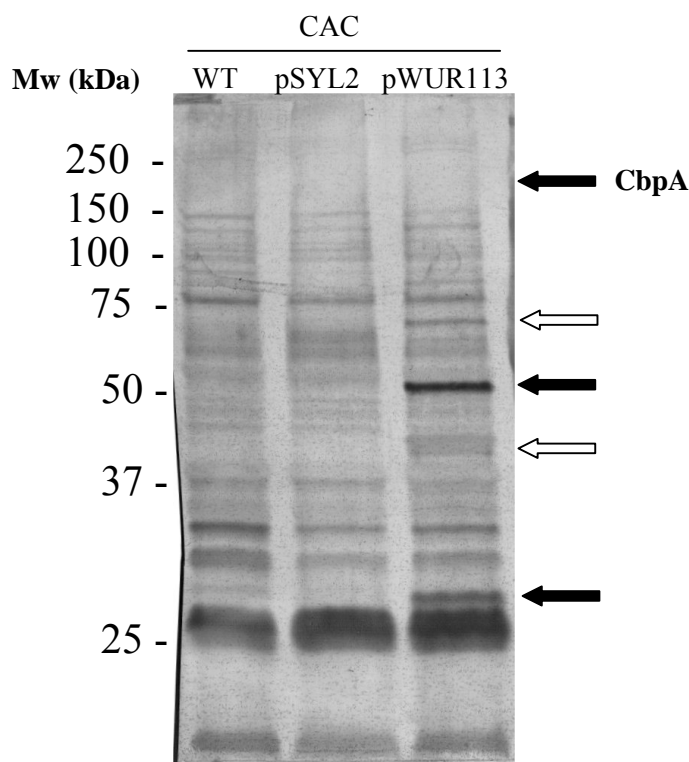
Figure 5.4 Detection of CbpA in extracellular growth medium of *C. acetobutylicum* grown on glucose ATCC 824 wild-type (WT) and transformants harboring pSYL2 or pWUR113. In each lane, 10 μg of protein were loaded. The expected position of CbpA is indicated, as are the extra bands visible in the lane corresponding to the transformant harboring pWUR113 (dark arrows indicate dominant proteins and white arrows indicate minor proteins).

C. acetobutylicum wild-type or transformants harboring pSYL2 or pWUR113 were not able to utilize microcrystalline cellulose (Avicel or Sigmacell) for growth. Also, when Avicel or Sigmacell containing cultures were supplemented with Celluclast 1,5L, the transformants showed the same growth and production characteristics as the wild-type (data not shown).

The transformants harboring pSYL2 or pWUR113 did not show increased extracellular endoglucanase (CMCase), lichenase or laminarinase activities compared to the wild-type when grown on glucose or cellobiose. Under the conditions of our assay, activity on Avicel could not be detected in cultures either from the wild-type or the transformants grown on glucose or cellobiose (not shown).

Because in the amino acid sequences of CbpA from *C. cellulovorans* and the putative Cbp from *C. acetobutylicum* there are highly conserved regions (Fig. 5.2), cross-reaction of the anti-CbpA polyclonal antibodies is to be expected. The mature Cbp, encoded by the gene CAC0910, has a calculated molecular mass of approximately 150 kDa, and on the Western blot shown on Figure 5.4, bands corresponding to proteins of that size could be seen in all lanes.

To analyze the production of the potential Cbp by *C. acetobutylicum* in more detail, the wild-type strain was grown on glucose, cellobiose, xylose or lichenan as sole carbon sources and concentrated extracellular medium samples were subjected to Western blot analysis using the anti-CbpA antibodies. As a control, *C. cellulovorans* cellobiose- or cellulose-grown cultures were also subjected to the same conditions. Strongly reacting bands corresponding to mature CbpA (approximately 185 kDa) could be seen in *C. cellulovorans* samples (Fig. 5.5). The anti-CbpA antibodies reacted very strongly, not only with a protein corresponding to CbpA, but also with a number of smaller proteins present in the extracellular medium, that could possibly be generated by proteolysis of CbpA or that could correspond to other proteins containing very homologous antigenic domains. In all growth substrates tested for *C. acetobutylicum*, extracellular proteins of approximately the predicted size of the mature *C. acetobutylicum* Cbp (approximately 150 kDa) reacting with anti-CbpA were produced (Fig. 5.5). In cultures grown on xylose or lichenan, a number of different extra anti-CbpA-reacting proteins were produced, which could have homologous domains to the Cbp.

Discussion

C. acetobutylicum ATCC 824 was not able to utilize crystalline cellulose or CMC for growth. The addition of Celluclast 1.5L, a cellulase from the fungus *Trichoderma reseei*, to the medium (Table 5.1) was enough to enable *C. acetobutylicum* to grow on microcrystalline cellulose but not on CMC, consistent with previous studies in *C. beijerinckii* (9). However, in the genome of this bacterium, a set of genes coding for cellulosome components is present (14). A major component in the cellulosome is the Cbp, which plays an essential role in the binding of cellulosomes to cellulose, and therefore in the activity of this complex.
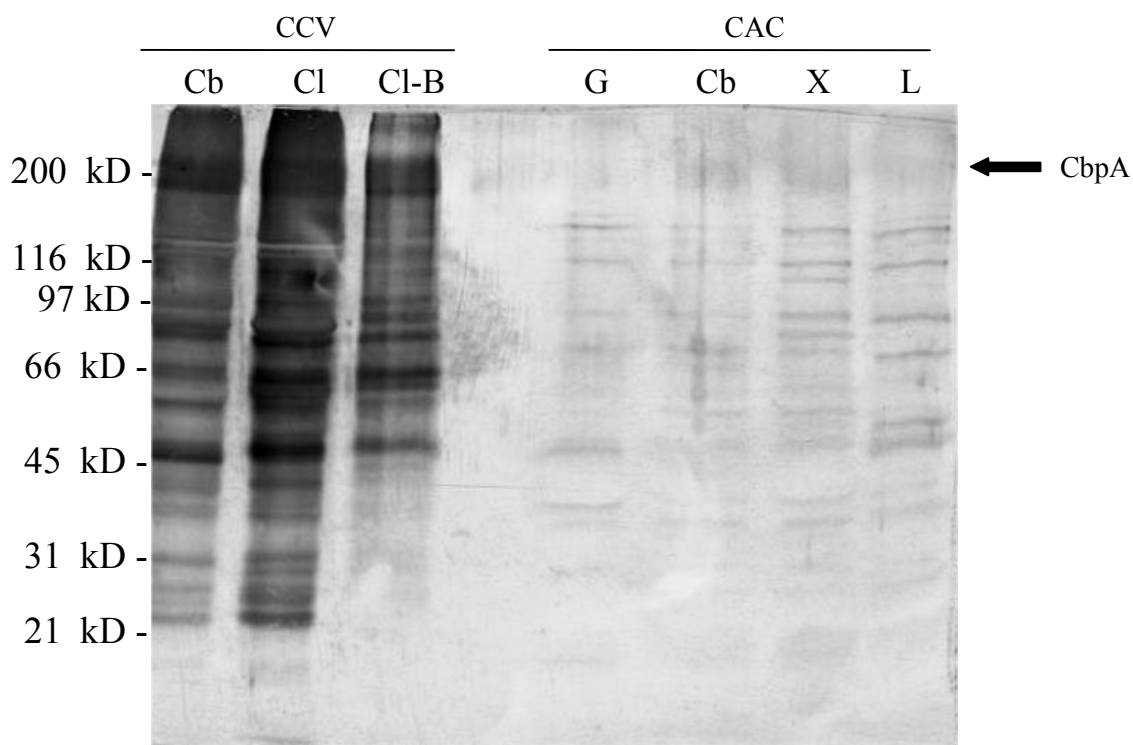
Figure 5.5 Detection of Cbp and CbpA in extracellular growth medium of *C. acetobutylicum* (CAC) and *C. cellulovorans* (CCV) cultures, respectively. The Western blot shown was generated using polyclonal antibodies raised against purified CbpA. Lanes: *1*, cellobiose (Cb)-grown *C. cellulovorans*; *2*, cellulose (Cl)-grown *C. cellulovorans*; *3*, cellulose-bound proteins from *C. cellulovorans* cellulose-grown; *4*, glucose (G)-grown *C. acetobutylicum*; *5*, cellobiose (Cb)-grown *C. acetobutylicum*; *6*, xylose (X)-grown *C. acetobutylicum*; *7*, lichenan (L)-grown *C. acetobutylicum*. In each lane, 5 μg of protein was loaded, except in lane 3, to which less than 1μg of protein was applied.

Since *C. acetobutylicum* does not seem to produce active cellulosomes, the possibility that it does not produce a functional Cbp could not be excluded as an explanation for the lack of true cellulolytic properties. The cloning of a gene coding for a functional Cbp into *C. acetobutylicum* was carried out to investigate this possibility. The gene selected, the *cbpA* gene from *C. cellulovorans*, was cloned under the control of its own promoter. The regulation at the transcriptional level of the expression of cellulosomal genes has not been studied in detail yet, and no specific regulatory sequences have been identified in the promoter or coding regions of these genes. In the promoter of the *cbpA* gene or in the one upstream of the CAC0910 gene that encodes the *C. acetobutylicum* Cbp no putative CRE or XylR sites were detected.

Although the amino acid sequences from the Cbps from *C. acetobutylicum, C. cellulovorans* and other cellulolytic clostridia are highly homologous, the nucleotide sequence of the *cbp* gene from *C. acetobutylicum* shows very low homology to the nucleotide sequences of the corresponding genes in other organisms. However, the codon usage of *C. acetobutylicum* and the one in the *cbpA* gene from *C. cellulovorans* were compatible. A DNA fragment containing the promoter and the

major part of the *cbpA* gene from *C. cellulovorans* was transformed successfully into *C. acetobutylicum*. As expected, because of the low DNA similarity, the construct did not integrate into the chromosome of *C. acetobutylicum* (Fig. 5.3).

The transformants harboring the *cbpA* gene showed no increase in cellulolytic properties with respect to the wild-type or the transformant strains harboring the empty vector. Although full-length CbpA could not be detected in extracellular medium of transformants harboring the *cbpA* gene (Fig. 5.4), a number of proteins of a smaller molecular weight cross-reacted strongly with the anti-CbpA antibodies, possibly indicating degradation or processing of the protein produced.

The anti-CbpA antibodies reacted strongly with CbpA present in extracellular medium of cellobiose or cellulose-grown *C. cellulovorans* and with a large number of smaller proteins (Fig. 5.5, Lanes CCV), probably indicating proteolysis of the CbpA protein. This could be a sign of low stability of CbpA in the medium supernatant. In the case of the extracellular medium of *C. acetobutylicum* grown on different substrates (Fig. 5.5, lanes CAC), there are a number of proteins that reacted with anti-CbpA, but the reaction is less strong than that with the *C. cellulovorans* proteins, possibly due to lower expression levels or lower affinity. The *cbp* gene from *C. acetobutylicum* encodes a mature protein of approximately 150 kDa, and in extracellular medium of glucose-, cellobiose-, xylose- or lichenan-grown cultures from this strain, proteins with this size reacted with the anti-CbpA antibodies. Although this could indicate that *C. acetobutylicum* produces and secretes Cbp, this should be confirmed by using antibodies specific for this Cbp. Growth on xylose or on lichenan resulted in the production of more extracellular anti-CbpA reacting proteins compared to growth on glucose. These anti-CbpA-reacting extra proteins most probably contain homologous domains to those present in the CbpA; CBM3, SLH or Cohesin domains (Fig. 5.1). In the genome of *C.acetobutylicum* there are several genes encoding putative cellulosomal proteins that contain one or more of the mentioned domains (8, 14). Most of these also contain glycoside hydrolase modules. In a previous study, we have shown that growth on xylose or lichenan induced the production of extracellular cellulases and hemicellulases in *C acetobutylicum* (8).

C. acetobutylicum harboring the *cbpA* gene from *C. cellulovorans* was not able to utilize cellulose and did not show improved cellulolytic properties compared to the wild-type strain. From these results the conclusion can be made that the presence of a functional *cbpA* gene is not enough to enable *C. acetobutylicum* to utilize cellulose, and that the cause(s) for its lack of true cellulolytic properties is probably to be found somewhere else in the genome. The study of the genes coding for the cellulosomal subunits and their transport, processing and assembly and their regulation is a necessary step towards enabling *C. acetobutylicum* to utilize cellulose or cellulosic materials, and making the production of butanol from biomass economically viable.

Acknowledgements

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Chapter 6

Production by *Clostridium acetobutylicum* ATCC 824 of CelG, a Cellulosomal Glycosyde Hydrolase belonging to Family 9

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Abstract

The genome sequence of *Clostridium acetobutylicum* ATCC 824, a noncellulolytic solvent-producing strain, predicts the production of various proteins with domains typical for cellulosomal subunits. Most of the genes coding for these proteins are grouped in a cluster similar to that found in cellulolytic clostridial species, such as *Clostridium cellulovorans*. CAC0916, one of the open reading frames present in the putative cellulosome gene cluster, codes for CelG, a putative endoglucanase belonging to family 9, and it was cloned and overexpressed in *Escherichia coli*. The overproduced CelG protein was purified by making use of its high affinity for cellulose and was characterized. The biochemical properties of the purified CelG were comparable to those of other known enzymes belonging to the same family. Expression of CelG by *C. acetobutylicum* grown on different substrates was studied by Western blotting by using antibodies raised against the purified *E. coli*-produced protein. Whereas the antibodies cross-reacted with CelG-like proteins secreted by cellobiose- or cellulose-grown *C. cellulovorans* cultures, CelG was not detectable in extracellular medium from *C. acetobutylicum* grown on cellobiose or glucose. However, notably, in lichenan-grown cultures, several bands corresponding to CelG or CelG-like proteins were present, and there was significantly increased extracellular endoglucanase activity.

Introduction

Among the members of the bacterial genus *Clostridium* several species, including *Clostridium acetobutylicum*, have the capacity to produce organic solvents, as well as organic acids, during fermentation of a wide range of carbohydrate substrates. *Clostridium* strains have been used to develop industrial production of acetone, butanol, and ethanol, the so-called ABE fermentation. Although this process was abandoned several decades ago due to its inability to compete with the petrochemical synthesis of these solvents (9, 13), new possibilities for a more sustainable solvent production via ABE fermentation of cheap substrates have been suggested (5). These substrates include lignocellulosic materials, such as domestic organic waste, that contain sugars which can be utilized during ABE fermentation (4). However, cellulose, the main component of lignocellulose, cannot be degraded by solvent-producing clostridia, and use of cellulose as a substrate for ABE fermentation requires addition of expensive cellulolytic enzymes (19). Hence, there is a need to develop cellulose-degrading clostridia that are able to produce solvents. One way to approach this is to clone and express genes involved in cellulose degradation in appropriate solvent-producing

clostridia (20). However, this requires a basic understanding of why solventogenic clostridia do not ferment cellulose, while other, non-solvent-producing clostridial strains do so effectively (25).

Recently, the complete genome of the well-known solventogenic strain *C. acetobutylicum* ATCC 824 has been sequenced (21). This organism is able to utilize a broad range of mono- and disaccharides, starches, and other substrates, such as pectin, inulin, whey, and xylan. However, it does not ferment or bind to cellulose (10), although, like other solvent-producing strains, it produces extracellular cellulolytic enzymes during growth on different substrates (18). Analysis of the *C. acetobutylicum* ATCC 824 genome revealed the presence of a number of genes coding for enzymes involved in cellulose degradation. Some of these genes appear to be grouped in a gene cluster similar to the cluster found in true cellulolytic clostridia, such as *Clostridium cellulovorans* and *Clostridium cellulolyticum*. The cellulosome is an extracellular high-molecular-mass complex consisting of a number of catalytic components (glycohydrolases) that tightly bind to one large noncatalytic scaffolding protein that attaches to cellulose and to the cell surface, thereby minimizing the diffusion of hydrolytic products (25, 29). The production of cellulosomes is typical of cellulolytic clostridial species, and until now there was no evidence that any solvent-producing strain produced them or had the genes coding for cellulosomal components in their chromosomes.

Since *C. acetobutylicum* ATCC 824 does not grow on cellulose, the question arises whether the genes in the presumed cellulosomal gene cluster indeed code for secreted and cellulose binding glycoside hydrolases and whether these genes are expressed under the appropriate conditions. In this study we focused on one of the genes that is present in the putative cellulosome gene cluster, the open reading frame (ORF) CAC0916, which codes for a putative glycoside hydrolase belonging to family 9 (6, 7). This gene was cloned in *Escherichia coli*, and the overproduced purified protein was functionally characterized. Expression studies of this gene in *C. acetobutylicum* grown on different substrates were performed and revealed that in lichenan- and xylose-grown cultures CelG-like proteins were secreted.

Materials and Methods

Bacterial strains, media, and growth conditions

C. acetobutylicum ATCC 824 was kindly supplied by P. Soucaille (INSA, Toulouse, France). Stock cultures were maintained as spore suspensions in sterile 10% (vol/vol) glycerol at -20°C. Spore suspensions were heat shocked for 10 min at 75°C in a water bath prior to inoculation. For production of precultures, vegetative cells were grown overnight at 37°C in clostridial growth

medium (23, 32), which contained (per liter) 0.75 g of KH_2PO_4, 0.75 g of K_2HPO_4, 0.348 g of $MgSO_4$, 0.01 g of $MnSO_4.H_2O$, 0.01 g of $FeSO_4.7H_2O$, 1 g of NaCl, 2 g of asparagine, 5 g of yeast extract, 2 g of $(NH_4)_2SO_4$, and 50 g of glucose. For growth experiments the same medium was used except that the glucose was replaced by one of the following carbon sources at a concentration of 2% (wt/vol): glucose (Merck, Darmstadt, Germany); or cellobiose, xylose, lichenan, or laminarin (all obtained from Sigma, Zwijndrecht, The Netherlands). All experiments were performed in an anaerobic chamber (Coy Laboratory Products, Grass lake, Mich.) under an atmosphere containing 20% CO2, 4% H_2, and 76% N_2, unless indicated otherwise.

C. cellulovorans DSM 3052 (28) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection and was cultivated in medium 320 with cellulose MN 300 (Macherey-Nagel, Düren, Germany) or cellobiose (Sigma) as the carbon source. For vector construction and protein production *E. coli* strains XL1 blue (Stratagene, Amsterdam, The Netherlands) and M15(pREP4) (Qiagen, Germantown, Md.), respectively, were used. These strains were grown in Luria-Bertani broth as described previously (24), and the medium was supplemented, when appropriate, with ampicillin (50 μg/ml), isopropyl-β-thiogalactopyranoside (IPTG) (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) (40 μg/ml), or kanamycin (50 μg/ml).

Transformation procedures, DNA manipulation and PCR

All general DNA manipulations in *E. coli* were carried out essentially as described previously (24). Restriction endonucleases and modification enzymes were purchased from Roche Diagnostics (Mijdrecht, The Netherlands), Eurogentec (Maastricht, The Netherlands), or Qiagen. DNA isolation from *E. coli* was performed with a Wizard Plus SV miniprep kit (Promega Inc., Leiden, The Netherlands). Genomic DNA from *C. acetobutylicum* ATCC 824 was isolated as described previously (22).

Oligonucleotides used for mutagenesis or PCR were purchased from Eurogentec. The DNA fragments containing the desired mutations were cloned first into pGEMT-Easy (Promega Inc.) and subsequently into the expression vector pQE60 (Qiagen). The mutations were verified by sequencing by using an automated laser fluorescent ALF DNA sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and fluorescently labeled M13 universal and reverse primers. The complete coding sequence of the *celG* gene from *C. acetobutylicum* ATCC 824 was obtained by PCR as an approximately 2.1-kb *Nco*I/ *Bam*HI fragment. The primers used were based on the sequence of the *celG* gene (described with gi:15894203 at the National Center for Biotechnology Information web site) and included the forward primer 5'-

CGC<u>CATGG</u>AGAAGTTGTTAGCTACTTTG-3'		and		the		reverse		primer
5'GGC<u>GGATCC</u>TTACTGTTTACCTGAAATAA 3', to which *Nco*I and *Bam*HI sites (underlined)
were added, respectively. The *celG* gene without a signal peptide sequence was amplified by using
the		same		reverse		primer		and		forward		primer
5'-CG<u>CCATGG</u>CTAAGAGTACAGAGGATAAAAAC-3' (*Nco*I site underlined), which resulted
in a PCR fragment that was approximately 2 kb long. The *Nco*I sites were added in such a way that
they allowed in-frame fusion of the coding region to the pQE60 expression sequences.

 Analytical methods.

	Avicelase activity reaction products were analyzed with a Dionex BioLC high-performance
chromatography system (Dionex Corporation) by using a Carbopac PA-100 anion-exchange column
(25 cm by 4 mm) equilibrated in 0.05 M NaOH and pulsed amperometric detection. The samples
loaded (20 µl) were eluted isocratically with 0.05 M NaOH for 2 min, followed by a 20-min linear
gradient of 0.05 to 0.95 M NaOH at a rate of 1 ml/min. Peaks were identified by analyzing a
standard mixture containing 1 mg of a cellooligosaccharide mixture (Sigma) per ml by using the
same chromatographic conditions.

Preparation of culture samples for enzyme assays and western blotting

	Cells were sedimented by centrifugation at $10,000 \times g$ at 4°C for 15 min. The culture
supernatant was collected, filtered through a 0.22-µm-pore-size filter, and concentrated
approximately 30-fold by ultrafiltration through a PM10 membrane (Millipore, Etten-Leur, The
Netherlands) at 4°C. Low-molecular-weight compounds and medium components were removed
from the concentrated material by dilution with 4 volumes of ice-cold 50 mM sodium citrate buffer
(pH 5.7), followed by reconcentration by ultrafiltration. This process was repeated three times, and
the resulting fractions were used for enzymatic activity determinations or for Western blot assays.

	For preparation of cell extracts of *C. acetobutylicum*, cells (1 ml) were harvested by
centrifugation, resuspended in 0.1 ml of 50 mM Tris HCl (pH 8.0) buffer, and sonicated with a
Branson Sonifier. Cell debris was removed by centrifugation ($10,000 \times g$ for 20 min). The insoluble
fraction was resuspended in 0.1 ml of the same buffer.

	Cellulose-bound proteins in *C. cellulovorans* cultures were harvested by centrifuging an
aliquot of the culture and washing the pellet, consisting of cellulose, cellulose-bound proteins, and
cells, briefly with a buffer containing 8 M urea. Proteins bound to the cellulose were denatured and
became soluble, whereas the bacterial cells remained mostly intact. The protein-containing soluble
fraction was obtained by centrifugation.

Cellulose-binding proteins in *C. acetobutylicum* concentrated extracellular medium samples were extracted twice with 0.3% (wt/vol) Avicel or once with 0.5% (wt/vol) Avicel as indicated below for 45 min at 37°C. Proteins bound to cellulose were eluted as described above.

Cellulase activity assays

E. coli transformants grown on agar plates were screened for endoglucanase activity by the Congo red staining method described previously (30), with the following modifications: carboxymethyl cellulose (CMC) (high viscosity; Sigma) was added to the medium at a concentration of 0.1% (wt/vol), and carboxymethyl cellulase (CMCase) activity was detected after 2 to 4 days of incubation at 37°C by washing the cells out of the plates with sterile demineralized water and staining the CMC with Congo red. Zymograms were prepared as described previously (26).

Glycolytic activities were determined as described previously (18). The following substrates (obtained from Sigma, unless indicated otherwise) were used (final concentrations): 0.5% (wt/vol) CMC (low viscosity), 0.2% (wt/vol) lichenan, 0.5% (wt/vol) laminarin, 0.5% (wt/vol) oat spelt xylan, and 0.5% (wt/vol) Avicel (Merck) in 50 mM citrate buffer (pH 5.7). Lichenan and oat spelt xylan were incubated with the enzyme samples in a water bath at 39°C for 10 to 60 min; Avicel, laminarin, and CMC were incubated for up to 30 h. The amounts of the reducing sugar formed were measured by the 3,5-dinitrosalicylic acid method (11). One unit of activity corresponded to the formation of 1 μmol of reducing sugar (D-glucose) per min, unless indicated otherwise. Protein concentrations in the samples were determined by the Bradford assay (Bio-Rad, Veenedaal, The Netherlands).

Purification of recombinant CelG. *E.coli*

M15(pREP4) harboring the overexpression plasmid pWUR65 was grown at 37°C in Luria-Bertani medium containing ampicillin and kanamycin. Induction was performed by adding 1 mM IPTG and incubating the culture overnight at 18°C. Subsequently, the cells from 100 ml of the culture were collected by centrifugation, resuspended in 10 ml of PC buffer (50 mM KH_2PO_4, 10 mM sodium citrate; pH 7.0), and sonified with a Branson Sonifier. Clear cell extracts were obtained by centrifugation at $10,000 \times g$ for 20 min at 4°C. CelG was purified from the cell extracts by using a previously described cellulose-binding approach (12, 27), as follows. Cell extracts were stirred for 40 min at room temperature with 0.3 g of Avicel microcrystalline cellulose. The Avicel was removed from the suspension by centrifugation, and extraction with Avicel was repeated three times. The three pellets of Avicel were pooled and subsequently washed once with 5 ml of 1 M NaCl in PC buffer and twice with 5 ml of demineralized water. The bound proteins were eluted by

stirring the Avicel overnight in 300 ml of demineralized water at 4°C and were concentrated by ultrafiltration by using an Omegacell device with a polyethersulfone membrane of 10-kDa cutoff (Pall Corporation, East Hills, N.Y.). By using fast-performance liquid chromatography (FPLC), the concentrated sample was applied to a Superdex 200 column (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) equilibrated with 50 mM phosphate buffer (pH 7). Eluted fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and used for assays.

Immunological procedures

Aliquots containing 100 μg of purified CelG were mixed with an equal amount of adjuvant (Specoll) and injected into a New Zealand white rabbit. Antiserum was collected 9 weeks after the first immunization. Western blot analyses were carried out with antiserum diluted 1,000-fold by using a standard protocol (24), and the detection limit for purified CelG under the conditions assayed was approximately 3 ng.

Results

Analysis of the sequence of the protein encoded by ORF CAC0916

Analysis of the complete genome sequence of *C. acetobutylicum* ATCC 824 indicated that in its chromosome there is a putative gene cluster containing 10 unidirectionally transcribed genes predicted to encode secreted proteins with cohesin or dockerin domains (21) (Fig. 6.1). The dockerin domain is typical of cellulosomal components and is usually located at the C terminus of the protein; it consists of two duplicated sequences containing 22 amino acids each and is highly conserved in all the proteins that bind to the scaffolding protein to form active cellulosomes.

The gene cluster contains three ORFs, CAC0913, CAC0916, and CAC0917 which code for putative secreted glycoside hydrolases with a catalytic domain belonging to family 9; the products of these ORFs are designated CelH (previously designated CelG; designated CelH due to its high level of similarity to EngH from *C. cellulovorans*), CelG, and CelL, respectively (Fig. 6.1). CelG has an N-terminal signal peptide sequence consisting of 23 amino acids typical of gram-positive bacteria (31). The mature CelG consists of a family 9 catalytic domain, a carbohydrate-binding module belonging to family 3 (6), and a dockerin domain located at its C terminus. The overall amino acid sequence, including the domain structure, was homologous to the amino acid sequences of EngH from *C. cellulovorans* (40% identity and 55% similarity) and CelJ from *C. cellulolyticum* (39% identity and 53% similarity) (Fig. 6.2). Comparison of the catalytic domain of CelG with the

catalytic domains of the other enzymes belonging to the same family present in the *C. acetobutylicum* cluster revealed a higher level of similarity to CelL (64% identity and 75% similarity) than to CelH (43% identity and 58% similarity). The catalytic domains of different glycoside hydrolases belonging to family 9 produced by *C. acetobutylicum*, *C. cellulolyticum*, and *C. cellulovorans* contain highly conserved regions, including active-site residues, as indicated by similarity to the region experimentally determined for CelD from *Clostridium thermocellum* (3, 14) (Fig. 6.3).
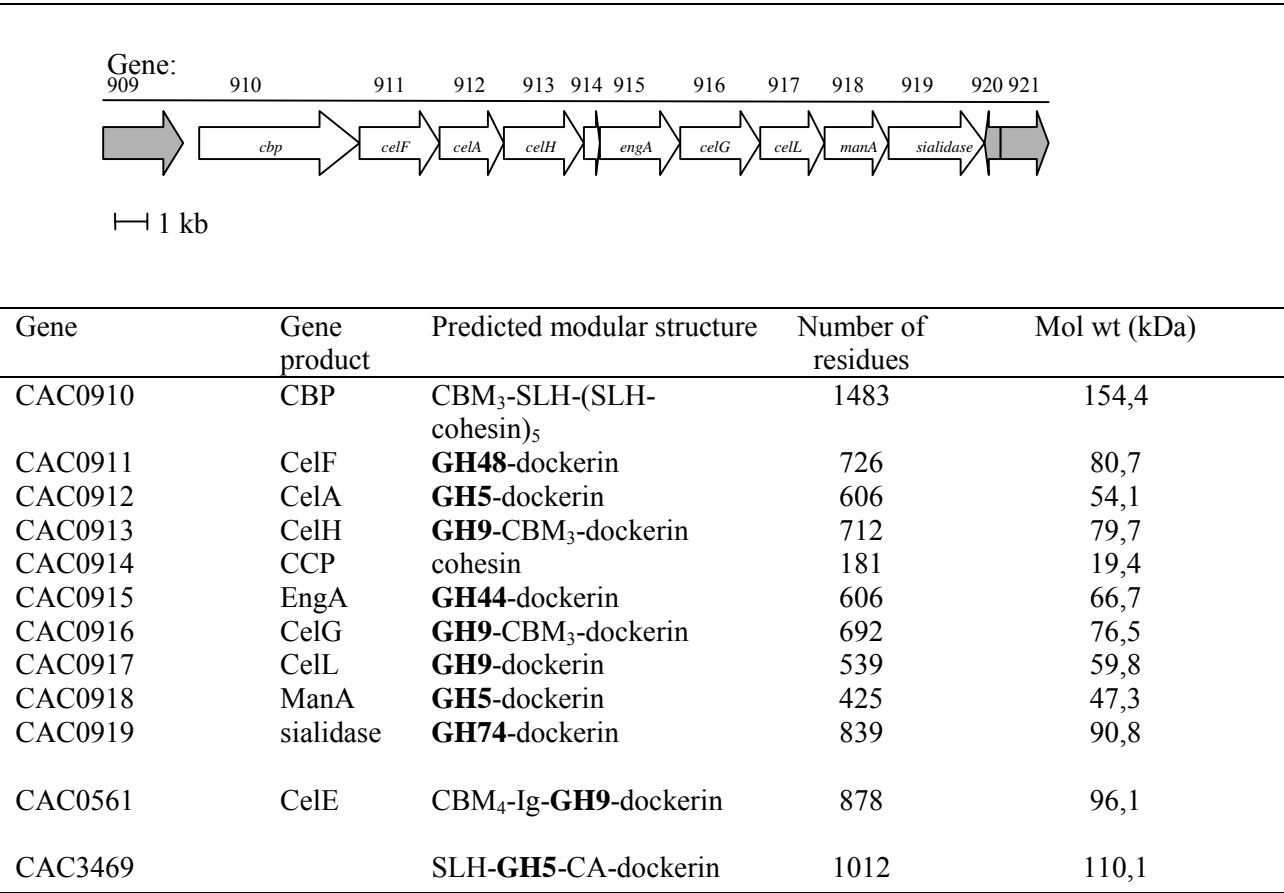

Gene	Gene product	Predicted modular structure	Number of residues	Mol wt (kDa)
CAC0910	CBP	CBM$_3$-SLH-(SLH-cohesin)$_5$	1483	154,4
CAC0911	CelF	**GH48**-dockerin	726	80,7
CAC0912	CelA	**GH5**-dockerin	606	54,1
CAC0913	CelH	**GH9**-CBM$_3$-dockerin	712	79,7
CAC0914	CCP	cohesin	181	19,4
CAC0915	EngA	**GH44**-dockerin	606	66,7
CAC0916	CelG	**GH9**-CBM$_3$-dockerin	692	76,5
CAC0917	CelL	**GH9**-dockerin	539	59,8
CAC0918	ManA	**GH5**-dockerin	425	47,3
CAC0919	sialidase	**GH74**-dockerin	839	90,8
CAC0561	CelE	CBM$_4$-Ig-**GH9**-dockerin	878	96,1
CAC3469		SLH-**GH5**-CA-dockerin	1012	110,1

Figure 6.1 Schematic representation of the putative cellulosome gene cluster present in the chromosome of *C. acetobutylicum* ATCC 824 and description of the ORFs found in the genome that contain cohesin or dockerin domains. Abbreviations: CBP, cellulose-binding protein; CCP, cohesin-containing protein; GH, glycoside hydrolase; CBM, carbohydrate-binding module; Ig, immunoglobulin-like domain; CA, cell adhesion domain; SLH, surface layer homology domain. The catalytic modules are indicated by boldface type. The number of residues includes the signal sequence. The molecular weights were determined from the peptide sequences.

The carbohydrate-binding module between the catalytic and dockerin domains is involved in cellulose binding, and it is present in two other proteins that are predicted to be encoded by the gene cluster, the cellulose-binding protein (CAC0910) and CelH (CAC0913) (Fig. 6.1). This module is highly conserved in these three proteins and other cellulosomal proteins from *C. cellulovorans*, *C. cellulolyticum*, and *Clostridium josui* (data not shown).
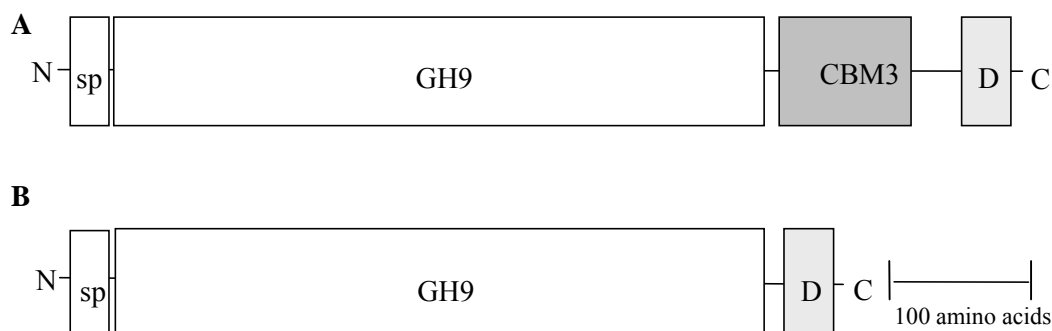
Figure 6.2 Predicted domain structure comparison of glycoside hydrolases belonging to family 9 encoded in the gene clusters from *C. acetobutylicum*, *C. cellulovorans*, and *C. cellulolyticum*. (**A**) Structure found in *C. acetobutylicum* CelJ, *C. acetobutylicum* CelG, *C. cellulovorans* EngH, *C. cellulolyticum* CelG, and *C. cellulolyticum* CelJ. (**B**) Structure found in *C. acetobutylicum* CelL, *C. cellulovorans* EngL, and *C. cellulolyticum* CelM. sp, signal peptide; CBM3, carbohydrate-binding module family 3; D, dockerin domain.

Production of CelG by *E. coli* and characterization of CelG

The *C. acetobutylicum celG* gene was cloned into the expression vector pQE60 with and without the coding sequence for its predicted 23-residue signal peptide sequence, resulting in plasmids pWUR64 and pWUR65, respectively. Upon induction with IPTG, *E. coli* M15(pREP4) harboring pWUR64 and pWUR65 produced an additional protein of the expected size (approximately 76 and 74 kDa, respectively). However, the strain harboring pWUR64 produced a much smaller amount of CelG than the strain harboring pWUR65 (data not shown). For this reason we continued our work with *E. coli* M15(pREP4) harboring pWUR65 that coded for the mature CelG.

Upon induction with 1 mM IPTG for 3 h at 37°C, most of the CelG produced by *E. coli*(pREP4)(pWUR65) was found in the insoluble fraction of the cells due to formation of inclusion bodies. However, when the incubation temperature was decreased to 18°C and the induction time was extended to overnight incubation, the major part of the mature CelG became soluble and could be purified from the cell extract by making use of its capacity to bind to cellulose. This was realized by incubation of the cell extract with microcrystalline cellulose, which resulted in binding of the most of the CelG, which could be recovered by extensive washing of the cellulose with demineralized water (Fig. 6.4). The recovered CelG was almost pure, and it was purified further to homogeneity by column chromatography using Superdex 200 resin. Both in the cell extract and in the purified fractions the protein appeared as two bands of approximately the same size.

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                           20        *        40        *        60
CAC_CelH   34 ------ETNTN--FNYGEALQKSLMFYEFERSGKLPQDKRDNWRGDSGLDDGKDVGLDLT : 85
CCV_EngH   32 ------ETTATPTFNYGEALQKSIMFYEFQRSGKLPTDIRSNWRGDSGTKDGSDVGVDLT : 85
CLY_CelG   36 ---------AG-TYNYGEALQKSIMFYEFQRSGDLPADKRDNWRDDSGMKDGSDVGVDLT : 85
CAC_CelG   24 -----AKSTEDKNYNYVDAFSKSILFYEANWCGPDAGNNRLKWRGPCHEDDGKDVGLDLT : 78
CAC_CelL   19 KPLTITADTNDQKHNYVDAFSKSIMFYEANWCGADAGNNRLKWRSGCHENDGKDVGLDLT : 78
CCV_EngL   27 ----------APKFDYSDAFGKSIMFYEANWSG-KVENNRFDWRGDAFLKDGADVGLDLS : 75

                  ↓    ↓  *        80        *        100       *        120
CAC_CelH    : GGWYDAGDHVKFNLPASYTLTMLGWSLYQNRDSYEKSGQLKYLTSDMKWCSDFLMKCHPA : 145
CCV_EngH    : GGWYDAGDHVKFNLPMSYTVAMLAWSLSEDKAAYEKSGQLDYLVKEIKWATDYLMKCHTA : 145
CLY_CelG    : GGWYDAGDHVKFNLPMSYTSAMLAWSLYEDKDAYDKSGQTKYIMDGIKWANDYFIKCNPT : 145
CAC_CelG    : GGFHDAGDHVKFGLPQAYAASTLGWAYYEFKDSFVKKGQDKYMLNILKHFTDYFLKCYPN : 138
CAC_CelL    : GGFHDAGDHVKFGLPQAYSASTLGWAYYEFKDTFVKKGQDKYMLNILKHFTDYFLKCYPD : 138
CCV_EngL    : GGFFDAGDHVKFGLPQAYAASTLGWGYFAFKDAYKSNGQDAYMLKILKHFTDYFLKCYPD : 135

                 *        140       *        160       *        180
CAC_CelH    : PDVFYYQVGDSGLDHKWWGPAEVMQ---MDRPSFKVDD-DNPGSAVTAEASAALAATALN : 201
CCV_EngH    : PNEYYYQVGDGGADHKWWGPAEVMQ---MARPAYKVDL-QKPGSSVVAETAAALASTAFA : 201
CLY_CelG    : PGVYYYQVGDGGKDHSWWGPAEVMQ---MERPSFKVDA-SKPGSAVCASTAASLASAAVV : 201
CAC_CelG    : KTTFYYQCGDGTTDHSYWGPPELQTLDITTRPTLYAATPDKPASDVCGSTAAALALMYIN : 198
CAC_CelL    : NNTFYYQCGDGNTDHSYWGPPELQT---TSRPTLYKATPSTPASDVCGNTAAALALMYIN : 195
CCV_EngL    : NNTFYYQVGDGDLDHAYWGPPEIQP---TARPTKFKATPSDPASDVCGNTAAALAIMYLN : 192

                 *        200       *        220       *        240
CAC_CelH    : FKNDDADYANKCLSHAKDLFNFADKTRSDKGYTAANGYYSSTS-FYDDLSWAACWLYMAT : 260
CCV_EngH    : LKDIDKAYSEQCIQHAKELYNFADTTKSDAGYTAANTYYNSWSGYYDELSWAAAWLYMAT : 261
CLY_CelG    : FKSSDPTYAEKCISHAKNLFDMADKAKSDAGYTAASGYYSSSS-FYDDLSWAAVWLYLAT : 260
CAC_CelG    : YKDVDAEYANKCLTAAKDLYDFG---KTYKGLSESGGFYGSTG-YLDDLSWGAVWLYAAT : 254
CAC_CelL    : YKDVDSAYANKCLTAAENLYNLG---KNYKGMSQSGGFYTSSG-YLDDLSWGAIWLYEAT : 251
CCV_EngL    : YKDVDATYANKCLTAAKNLYSLG---KNYKGLSHGQSYYVSTS-YYDDLAWGAVWLYQAT : 248

                 *        260       *        280       *        300
CAC_CelH    : NDKSYLDKAEAYVDNWSREQQTDIISYKWGMCWDDVHYGAQVLLAELTNKQIYKDSVERN : 320
CCV_EngH    : NDASYLEKAESYVPFWKVEQQTTTIAYRWAHCWDDVHFGAQLLLARLTGKSIYKESVERN : 321
CLY_CelG    : NDSTYLDKAESYVPNWGKEQQTDIIAYKWGQCWDDVHYGAELLLAKLTNKQLYKDSIEMN : 320
CAC_CelG    : NDNSYMTDIDSFLTAKGIGG-DNSYSNHWTHCWDDVFGGVFVKLAQLTNNPKYKAIAEEN : 313
CAC_CelL    : NNNNYLSDAQSFLQSANIN---EYYQYNWTHCWDDVLGGVFVKMAQITGDAKYKNIVKGN : 308
CCV_EngL    : NDNSYMTDANSFLSQINADG-DKVYTSYWTHCWDNVWTGVIYKMAEVTGKANYKASVENN : 307

                 *        320       *        340       *        360
CAC_CelH    : LDYWTVGYDGNKVQYTPKGLAWISSWGPLRYS--LATAFLADTYSKWSGCDASKAKAYED : 378
CCV_EngH    : LDYWTTGYDGNKIKYTPKGLAWMDSWGSLRYA--TTTAFLADVYASSDVCSISKVDTYKN : 379
CLY_CelG    : LDFWTTGVNGTRVSYTPKGLAWLFQWGSLRHA--TTQAFLAGVYAEWEGCTPSKVSVYKD : 378
CAC_CelG    : LNYWIK-----DAPTTPGGLKYIASWGTLRYT--AAECMLALVYKTS-----KNEEYLN : 361
CAC_CelL    : IDYWTN-----SVTTTPGGLKFRTGWGTLRYTDVAAECMLALVYKTS-----NDSEAMN : 358
CCV_EngL    : LNYWMN-----NIKTTAGGLKFATDWGSLRYS--STQAMLALIQYNRT-----KEQKYLD : 355

                 *        380       *  ↓     400       *        420
CAC_CelH    : FAKSQVDYALGSSGR--SYVVGFGVNPPEHPHHRTAESSWF-----DDKTVPGYSRHTLY : 431
CCV_EngH    : FAKSQADYALGSTGR--SFVVGFGENAPKKPHHRTAHSSWS-----DQQVNPTDHRHVLY : 432
CLY_CelG    : FLKSQIDYALGSTGR--SFVVGYGVNPPQHPHHRTAHGSWT-----DQMTSPTYHRHTIY : 431
CAC_CelG    : FAKSQIDYMLGSNPRNSSYEVGFGNNYPKNPHHRAASGRMEGAPGYEKKTDP--EKHLLY : 419
CAC_CelL    : LAKSQIDYILGTNPQGMSYEVGFGSKYPKYPHHRAASGRNESAG--EKKTEP--EKHILY : 414
CCV_EngL    : FATRQIDYILGDNPRSSSYVVGFGNNYPKFPHHRGASGRLE-PG--EMKTMP--EKHILT : 410

                 *      ↓  440      ↓   *        460
CAC_CelH    : GAMVGGPD-QNDKFDDDVANFNQNEPACDYNAGLVATLSSM : 471
CCV_EngH    : GALVGGPD-ASDGYTDAIDNFTNNEVACDYNAGFVGLLARQ : 472
CLY_CelG    : GALVGGPD-NADGYTDEINNYVNNEIACDYNAGFTGALAKM : 471
CAC_CelG    : GALVGGPDGTSDDYSDDVNQYVNSEVAIDYNAGFVGALAGM : 460
CAC_CelL    : GALVGGPD-QNDSYIENIEEYQHSEVAIDYNAGFVGALAGI : 454
CCV_EngL    : GALVGGPD-GNDQYKDDINNYQQTEVAIDYSAGFVGAMAGI : 450
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Figure 6.3 Alignment of the catalytic domains of glycoside hydrolases belonging to family 9 encoded in the gene clusters of *C. acetobutylicum* (CAC), *C. cellulolyticum* (CLY), and *C. cellulovorans* (CCV) that contain a carbohydrate-binding module belonging to family 3. The arrows indicate possible active-site residues, as determined by sequence similarity with CelD from *C. thermocellum*, in which the residues were determined by site-directed mutagenesis.

Both proteins showed activity, as determined by zymogram analysis with CMC or lichenan as the substrate (results not shown). As expected from previous studies of similar enzymes (1), the major band, which was larger, corresponded to the full-length mature CelG and the smaller band may have corresponded to a truncated form in which the dockerin domain had been removed.
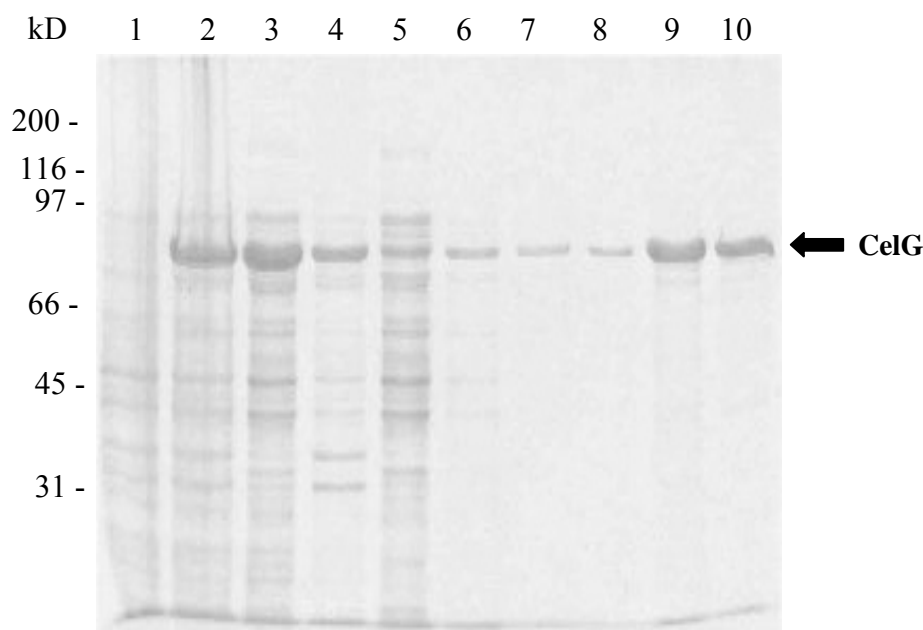
Figure 6.4 Purification of CelG from *E. coli*(pREP4)(pWUR65). SDS-PAGE of noninduced cells (lane 1), induced cells (lane 2), cell extract of induced cells (lane 3), and the insoluble fraction (lane 4) showed that predominantly soluble CelG was produced. Further purification of CelG following extraction of the cell extract by Avicel was revealed by SDS-PAGE of the unbound fraction (lane 5), subsequent washes in 1 M NaCl (lane 6) or demineralized water (twice) (lanes 7 and 8), and preparations eluted with 8 M urea (lane 9) or demineralized water (lane 10).

The purified CelG showed the highest CMCase activity at pH 6 and at 50°C (data not shown). The CMCase activities of related family 9 glycoside hydrolases from *C. cellulolyticum* (1) were also highest under similar conditions. CelG showed the maximum activity with CMC and less activity with lichenan, xylan, and Avicel (Table 6.1). The products obtained from the degradation of Avicel after 20 h of incubation with CelG were analyzed by high-performance liquid chromatography. A mixture of glucose, cellobiose, cellotriose, and cellotetraose was found to be the main product (results not shown).

Table 6.1 Specific activities of purified *E. coli*-produced CelG on different substrates

Activity	Sp act (U/mg of protein)[a]	%
CMCase	7400	100
Lichenanase	2300	31
Xylanase	20	0.3
Avicelase	7	0.1
Laminarinase	< 0.01	--

[a] One unit of activity was defined as 1 nmol of reducing sugar released per min.

Extracellular cellulase production by *C. acetobutylicum* ATCC 824 grown on different carbon sources

C. acetobutylicum ATCC 824 is able to utilize a wide range of carbon sources, including pentose sugars, such as xylose, and also insoluble sugar polymers, such as xylan (17). Lichenan is a polymer formed by β-1,4-1,3 linked glucose units, and although it has limited solubility, it does not have a crystalline structure like that of cellulose. Laminarin, a polymer of β-1,3-linked glucose units, is easily soluble in water. Lichenase and laminarinase activities have been found in the extracellular medium of cultures of *Clostridium beijerinckii* (20) and hence were also used as substrates for growth of *C. acetobutylicum*. While growth on glucose, cellobiose, xylose, or lichenan was readily established (Fig. 6.4), on laminarin no growth was observed independent of preculture conditions. Since lichenan is mostly insoluble at the concentration used, growth was determined by measuring the amounts of protein in cell extracts of culture samples taken after different incubation times, while in all other cultures growth was monitored by measuring the optical density at 600 nm (Fig. 6.5).

At the late exponential growth phase cells were removed, and the extracellular medium was concentrated by ultrafiltration and dialyzed. The glycoside hydrolase activities present in the concentrated extracellular media were determined (Table 6.2). In the extracellular medium of cultures grown on lichenan the endoglucanase (CMCase) activity was significantly higher than the ones in the extracellular media of the cultures grown on the other substrates. The laminarinase and xylanase activities in cultures grown on lichenan or xylan were also higher than those in the cultures grown on glucose and cellobiose as the substrates. Under the assay conditions used, no reducing sugars were detected as degradation products of Avicel.
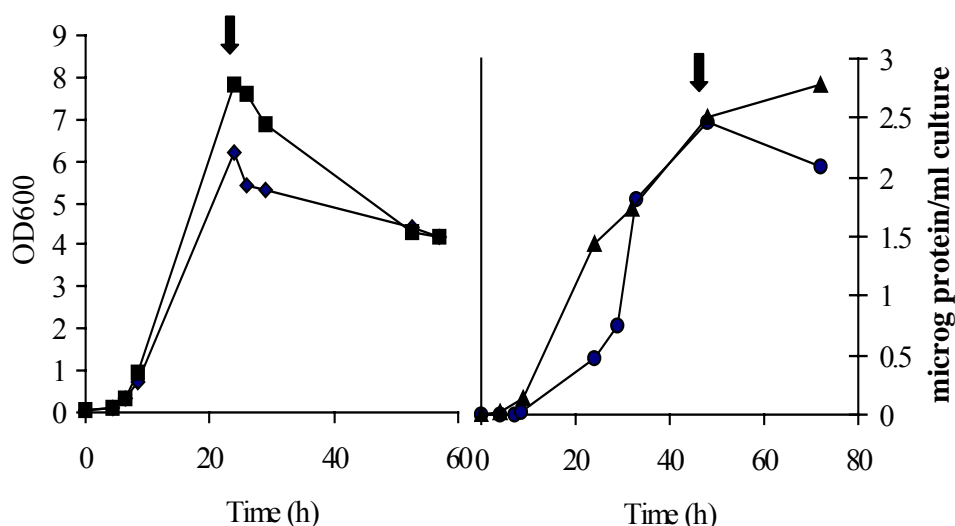
Figure 6.5 Growth curves for cultures of *C. acetobutylicum* ATCC 824 on different substrates. The optical densities at 600 nm (OD600) were determined for cultures grown on cellobiose (■), glucose (◆), and xylose (●), while growth on lichenan (▲) was determined by monitoring the protein concentration in cell extracts. The concentration of protein in the cell extract from a lichenan-grown culture incubated for 48 h equaled that of a culture which was grown on glucose and had an optical density at 600 nm of approximately 2.0. The arrows indicate the times when cultures were collected to determine enzymatic activities (Table 6.2).

Expression of CelG by *C. acetobutylicum*

To determine the production and localization of CelG by *C. acetobutylicum*, Western blot analyses were performed by using polyclonal antibodies raised against the purified *E. coli*-produced CelG. The concentrated extracellular medium samples of cultures grown on glucose, cellobiose, xylose, and lichenan used for the activity assays shown in Table 6.2 were also used for the immunological assay (Fig. 6.6). In addition, extracellular medium samples from *C. cellulovorans* grown on cellulose and cellobiose were also prepared and used as controls, since this organism is able to grow on cellulose and its cellulolytic system has been extensively studied (8, 29). Moreover, CelG shows high levels of similarity to several of the glycoside hydrolases produced by *C. cellulovorans*, especially EngL and EngH (Fig. 6.2), which are components of the cellulosome complex that are encoded by the cellulosome gene cluster. EngH has a cellulose-binding module that exhibits a high level of similarity to the module present in CelG and is a similar size (approximately 79 kDa), while EngL does not have this binding module and is smaller (approximately 50 kDa) (29). Due to the high levels of similarity among these proteins, particularly

95

in their highly conserved catalytic domains (Fig. 6.3), cross-reactivity of the *C. acetobutylicum* anti-CelG antibodies with proteins present in extracellular medium from *C. cellulovorans* cultures could be expected.

Table 6.2 Cellulolytic or hemicellulolytic activities in extracellular media of cultures of *C. acetobutylicum* ATCC 824 grown on different carbon sources

Activity	growth substrates[a]:			
	Cellobiose	Glucose	Xylose	Lichenan
Lichenase	119.2	72.1	88.3	90
Xylanase	33.3	17.7	42.6	43.4
CMCase	0.3	0.1	0.2	4.5
Laminarinase	0.08	0.02	0.1	0.2
Avicelase	<0.02	<0.01	<0.01	<0.04

[a] Samples were taken at the end of the exponential growth phase. The average estimated protein concentrations in the extracellular medium calculated from the concentrations determined in the concentrated samples were 0.9 μg of protein/ml in cellobiose cultures, 3 μg of protein/ml in glucose cultures, 0.8 μg of protein/ml in xylose cultures, and 1.2 μg of protein in lichenan cultures. One unit of activity corresponded to the formation 1 μmol of reducing sugar per min. The specific activity corresponded to units per milligram of protein in the concentrated medium sample.

    C. cellulovorans grown on cellobiose or cellulose produced a larger amount of total extracellular protein (as calculated from concentrated samples, between 25 and 26 μg of protein/ml in both cultures) than the *C. acetobutylicum* cultures produced (up to 3 μg of protein per ml [Table 6.2]). Several extracellular proteins with affinity to anti-CelG were present in the supernatants of cultures of *C. cellulovorans*, and the amount was larger in the cellulose-grown culture (Fig. 6.6). The presence of multiple bands in the Western blot indicates that there were several proteins with similarity to CelG in the extracellular medium. In the *C. cellulovorans* cellulosome gene cluster there are four genes coding for proteins containing catalytic domains belonging to glycoside hydrolase family 9 (29), and these proteins or their degradation products are likely to cross-react with the anti-CelG antibodies. The crossreacting protein with a molecular mass of approximately 75 kDa most likely corresponds to EngH, which is highly homologous to CelG (Fig. 6.2 and 6.3), and the band at approximately 56 kDa could correspond to EngL, which is highly homologous to the protein encoded by the gene CAC0917 (Fig. 6.1) (29). Interestingly, in the lane loaded with the cellulose-bound fraction of the *C. cellulovorans* cellulose-grown culture (Fig. 6.6, lane 3) there is only one major band corresponding to a protein with a molecular mass of approximately 75 kDa that could correspond to EngH (29).
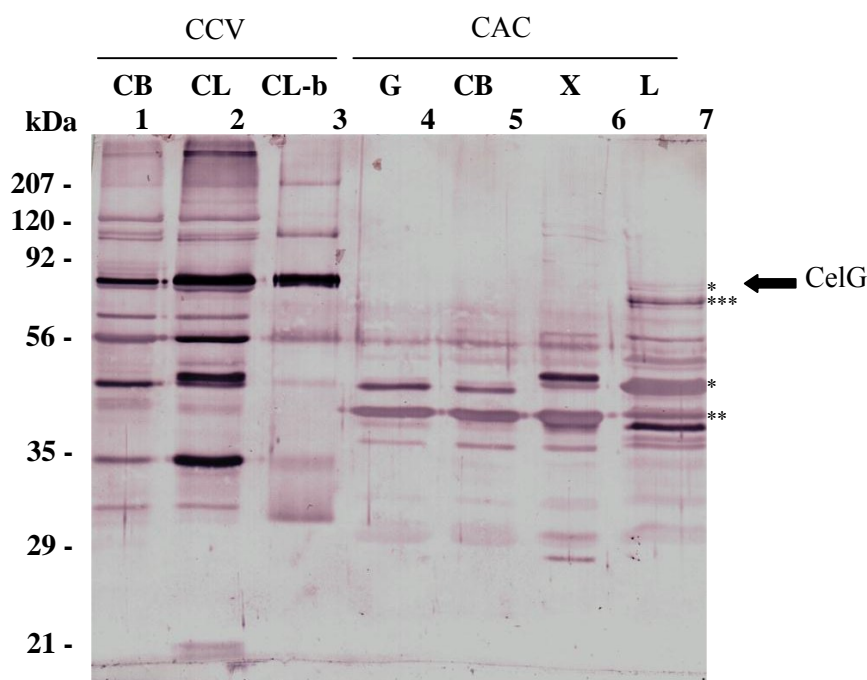
Figure 6.6 Detection of CelG in extracellular growth medium of *C. acetobutylicum* (CAC) and *C. cellulovorans* (CCV) cultures. The Western blot was generated by using polyclonal antibodies raised against purified CelG. Lane 1, cellobiose (CB)-grown *C. cellulovorans*; lane 2, cellulose (CL)-grown *C. cellulovorans*; lane 3, cellulose-bound proteins from cellulose-grown *C. cellulovorans*; lane 4, glucose (G)-grown *C. acetobutylicum*; lane 5, cellobiose (CB)-grown *C. acetobutylicum*; lane 6, xylose (X)-grown *C. acetobutylicum*; lane 7, lichenan (L)-grown *C. acetobutylicum*. 2.4 μg of protein was loaded in each lane, except in lane 3, where less than 1 μg of protein was applied. The arrow indicates the position of the mature *E. coli*-produced CelG. Bands that correspond to proteins in the extracellular medium of lichenan-grown cultures that bind to Avicel are indicated as follows: three asterisks indicate binding of the protein, two asterisks indicate binding of approximately 50% of the protein, and one asterisk indicates binding of approximately 10% of the protein.

Western blot analyses showed that cultures of *C. acetobutylicum* grown on glucose and cellobiose produced several proteins that cross-reacted with the anti-CelG antibodies, but all of them were considerably smaller than CelG (Fig. 6.6, lanes 4 and 5, respectively). No proteins that were the size of CelG were detected even when as much as 30 μg of protein was applied (data not shown). However, in the supernatants from the xylose- and lichenan-grown cultures, there were additional large proteins that reacted with the anti-CelG antibodies. In the medium from the lichenan-grown culture, one of anti-CelG reacting proteins had a molecular mass (approx. 75 kDa) corresponding to that of the mature CelG produced by *E. coli*. Remarkably, a protein that was only slightly smaller than CelG was found to react strongly with the anti-CelG antibodies and could correspond to a degradation product of the secreted CelG or a further processed active form of this enzyme.

No protein corresponding to CelG could be detected in Western blots loaded with 10 μg of proteins from cytoplasmic or membrane fractions of *C. acetobutylicum* cells from the same cultures as the extracellular media used in the experiment whose results are shown in Fig. 6.6 (data not shown).

In an attempt to extract cellulose-binding proteins from concentrated extracellular media from cultures of *C. acetobutylicum* grown on glucose or cellobiose and cellobiose-grown *C. cellulovorans* cultures, Avicel (0.5%, wt/vol) was added to the samples. Following extraction for 1 h at 37°C, centrifugation, and subsequent resolubilization of the bound proteins in buffer with 8 M urea, no band corresponding to CelG from *C. acetobutylicum* was visible in the Western blots, while in samples from *C. cellulovorans* cultures several proteins (including one with the size of EngH, producing the major band) were found to cross-react with anti-CelG (data not shown).

To determine the binding capacity to Avicel of the anti-CelG-reacting proteins present in the extracellular medium of lichenan-grown cultures, an assay was carried out in which a concentrated sample was extracted with 0.3% (wt/vol) Avicel. The concentrated extracellular medium sample and the extracted sample were subjected to SDS-PAGE and Western blot analysis by using the anti-CelG-antibodies. While some bands were not affected by the Avicel extraction, several bands in the Western blot disappeared completely (100%), or their intensities decreased in the extracted sample (approximately 50 or 10%) compared to the intensities in the concentrated medium sample (Fig. 6.6). The approximately 70-kDa anti-CelG-binding protein (Fig. 6.6, lane 7) bound significantly to Avicel and was not detectable in the extracted sample (data not shown). The protein having the molecular mass of mature CelG (approximately 74 kDa) was also extracted, although to a lesser extent (Fig. 6.6), indicating that there was binding to Avicel.

Discussion

While *C. acetobutylicum* does not grow on cellulose, genomic analysis predicts the presence of genes coding for proteins that contain glycoside hydrolase as well as cohesin or dockerin domains (Fig. 6.1). A gene cluster containing 10 genes involved in cellulosome formation exhibits high levels of similarity, both in gene organization and predicted amino acid sequence, to the clusters found in true cellulolytic strains, such as *C. cellulolyticum*, *C. josui*, and *C. cellulovorans* (2, 15, 29). In these known cellulosome gene clusters the first gene codes for a nonenzymatic scaffolding protein (CBP), and this gene is followed by a gene coding for a glycoside hydrolase belonging to family 48 and then by genes coding for glycoside hydrolases belonging to different families and other small cohesin-containing proteins. Another conserved feature is the presence of

at least three genes coding for enzymes containing a catalytic domain belonging to family 9 glycoside hydrolases, and this suggests that these enzymes could play an important role in cellulosomal function. Outside the gene cluster there is a single ORF (CAC0561) present in the genome of *C. acetobutylicum* which codes for a putative cellulosomal glycoside hydrolase belonging to family 9 (Fig. 6.1). In order to study expression of the putative cellulosomal genes and to analyze glycolytic activity in *C. acetobutylicum*, we focused on CelG, the protein coded by the ORF CAC0916.

A *celG*-overexpressing *E. coli* strain was constructed that produced large amounts of intracellular CelG which could be easily purified by making use of the high affinity for cellulose of its cellulose-binding domain (CBM3). Purified CelG had characteristics similar to those of other enzymes belonging to the same family (Table 6.1). It exhibited the highest activity with CMC and somewhat lower activity with lichenan, and its relative activities with xylan and Avicel were low, all of which are typical features of endoglucanases.

C. acetobutylicum was grown on different substrates, and the extracellular hydrolytic activities produced were determined. In concentrated extracellular media from lichenan-grown cultures there was significantly higher endoglucanase (CMCase) activity than in concentrated extracellular media from any of the other cultures. In xylan- or lichenan-grown cultures the extracellular xylanase activity was higher than the activity in cellobiose- or glucose-grown cultures (Table 6.2). In general, the extracellular glycoside hydrolase activities measured were lowest in glucose-grown cultures, suggesting that there is a catabolite repression mechanism. In all cases some residual laminarinase activity was observed; this activity was highest in lichenan- and xylosegrown cultures, but apparently it was too low to support growth on laminarin.

To determine expression of the *celG* gene, polyclonal antibodies raised against purified CelG from *E. coli* were used. The concentrated medium samples used in the activity assays were used to detect the presence of CelG by Western blot analysis (Fig. 6.6). Only in the sample from the culture grown on lichenan did a distinct protein of the size of the mature CelG (approximately 74 kDa) react with the anti-CelG antibodies (Fig. 6.6). In the samples from lichenan- and xylose-grown cultures, additional proteins that were smaller than CelG and were not present in the glucose- or cellobiose-grown cultures reacted with the anti-CelG antibodies. These proteins were likely to be degradation products of CelG or other smaller proteins, indicating that xylose and lichenan induce the production of CelG-like proteins. In the case of *C. cellulovorans* it is known that although the composition of the cellulosomes can be regulated by the growth substrate, major cellulosomal subunits are constitutively expressed (16). This could explain the finding that in cellobiose- or cellulose-grown cultures of *C. cellulovorans* basically the same set of anti-CelG-binding proteins

were present (Fig. 6.6, lanes 1 and 2). Strongly reacting bands appeared; one of these bands was approximately the same size as mature CelG (approximately 74 kDa) and probably represented EngH, and another had a molecular mass of approximately 56 kDa and could have corresponded to EngL, an enzyme with a catalytic domain of glycoside hydrolase family 9 and a dockerin domain but with no carbohydrate-binding module (Fig. 6.2) (29). In the cellulose-bound fraction of the *C. cellulovorans* cellulose-grown culture (Fig. 6.6, lane 3) the major anti-CelG-binding protein appeared to be the same size as CelG, and no strong reaction with smaller proteins was observed.

This study is the first functional analysis of a gene encoding a putative cellulosomal enzyme from *C. acetobutylicum*. Possible explanations for the lack of cellulolytic activity in *C. acetobutylicum* include (i) the absence of functional genes coding for necessary specific cellulosomal subunits, (ii) the presence of mutations in genes coding for cellulosomal components that could lead to the production of unstable or inactive cellulosomes, or (iii) deregulated expression of the cellulosome components or the transport systems needed for efficient export and assembly in the extracellular medium. In this study we have shown that one of the genes in the putative cellulosome gene cluster codes for an active endoglucanase enzyme with a functional cellulose-binding domain. This enzyme was not detectable in the extracellular medium of glucose- or cellobiose-grown cultures of *C. acetobutylicum*. However, growth on xylose or on lichenan gave rise to enhanced production of CelG-related proteins by *C. acetobutylicum*. In the extracellular medium of lichenan-grown cultures, a weak band corresponding to a protein that was the same size as the purified mature *E.coli*-produced CelG was detectable by Western blotting, and it bound to a certain extent to Avicel (Fig. 6.6). In the same sample, a slightly smaller protein that reacted with anti-CelG bound to Avicel, suggesting that it possibly contains a carbohydrate-binding module with affinity for cellulose, which could indicate that this protein is a processed active form of CelG (its molecular mass could correspond to that of mature CelG without the dockerin domains, approximately 68 kDa). The extracellular endoglucanase activity found in lichenan-grown cultures was higher than that in the other cultures (Table 6.2), which is consistent with the presence of CelG in the medium, although probably the increased activity cannot be attributed to CelG alone. These induction results suggest that the regulation of the cellulosomal enzymes in *C. acetobutylicum* is different from that in *C. cellulovorans*, in which expression of CelG-like genes was observed in both cellulose- and cellobiose-grown cultures. Whether this difference in regulation can be related to the inability of *C. acetobutylicum* to utilize cellulose needs to be investigated. A detailed study of the genes involved in cellulosome formation and their expression patterns and characterization of their products are required to determine whether and under what conditions *C. acetobutylicum* is able to utilize cellulose or cellulosic substrates as an approach for cost-effective production of solvents.

Acknowledgments

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Chapter 7

Substrate-induced Production and Secretion of Cellulases by

***Clostridium acetobutylicum* ATCC 824**

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Abstract

Clostridium acetobutylicum ATCC 824 is a solventogenic bacterium that grows heterotrophically on a variety of carbohydrates, including glucose, cellobiose, xylose and lichenan, a linear polymer of β-1,3 and β-1,4 linked β-D-glucose units. *C. acetobutylicum* does not degrade cellulose, although its genome sequence contains a number of cellulase-encoding genes and a complete cellulosome-like gene cluster. In the present study it is demonstrated that a low but significant induction of cellulase activity occurs during growth on xylose or lichenan. The *celF* gene, located in the cellulosome-like gene cluster and coding for a unique cellulase that belongs to the glycoside hydrolases family 48, has been cloned in *E. coli* and antibodies were raised against the overproduced CelF. Western blot analysis indicates catabolite repression by glucose or cellobiose and up-regulation by lichenan or xylose of the extracellular production of CelF by *C. acetobutylicum.*

Introduction

C. acetobutylicum ATCC 824 produces acetone, butanol and ethanol by fermentation and has been used for the industrial production of these solvents (7). This organism is able to utilize a wide range of carbohydrate substrates, including the polymers starch and xylan, and mono- and disaccharides such as glucose, xylose and cellobiose. However, like the rest of the well-studied solvent-producing clostridial strains, it does not degrade cellulose, the main sugar component in lignocellulosics, which are among the most interesting substrates for the fermentation (20).

Cellulose is a highly recalcitrant substrate for enzymatic degradation due its physical properties. Cellulose molecules are composed of chains of β-1,4 linked glucose units. The chains are insoluble and form fibrils, in which cellulose chains form parallel bundles that are very stable due to interchain hydrogen bonds and Van der Waals interactions between the pyranose rings (2). The microbial degradation of cellulose is carried out by a number of different enzymes that cleave the β-1,4- glycosidic bonds between the β-D-glucose units. Based on structural features, these enzymes have been divided into 14 different families of glycoside hydrolases (3). According to their mode of action, cellulases are subdivided in endo- and exoglucanases (also called cellobiohydrolases). Endoglucanases (E.C. 3.2.1.4) randomly cleave the cellulose chains at exposed positions and create new ends, while exoglucanases (E.C. 3.2.1.91) degrade the polymeric chain either from the reducing or the non-reducing end, producing cellobiose as main product. These two types of enzymes can be distinguished by their substrate specificity. Endoglucanases show high

activity on soluble cellulose derivatives, such as carboxy-methylcellulose (CMC) and very low (or not at all) on microcrystalline cellulose, while exoglucanases show relatively high activity on microcrystalline cellulose. During an efficient degradation of cellulose, both types of enzymes act synergistically, and all cellulolytic organisms known so far produce at least one, but in most cases several of each type of glucanase (18).

Sequence analysis of the genome of *C. acetobutylicum* ATCC 824 indicates the presence of a gene cluster containing ten unidirectionally transcribed genes predicted to encode secreted proteins with cohesin or dockerin domains (22). These domains are typical of components of a large extracellular complex specialized in the degradation of cellulose called cellulosome, that is produced by a number of anaerobic microorganisms, including clostridial species (29, 30). In an active cellulosome, glycoside hydrolases bind to a large non-enzymatic scaffolding protein that binds to cellulose, called Cbp or Cellulose integrating protein (Cip). Dockerin domains, usually located at the C-terminus of the enzymatic cellulosomal subunits, consist of two duplicated sequences of, on average, 22 amino acids each. Dockerin domains bind to cohesin domains, about 100 amino acids long, present at the C-terminal end of the Cbp. In the chromosome of *C. acetobutylicum* most of these genes are located in large cluster, such as those found in the cellulolytic species *C. cellulovorans* and *C. cellulolyticum* (29). All these clusters start with a gene encoding the Cbp followed by a gene encoding for a putative glycoside hydrolase from family 48, annotated as CelF, that is unique in the genome of *C. acetobutylicum*. Orthologs of CelF produced by other cellulolytic clostridia have been characterized as cellobiohydrolases (exo-cellulases) and identified as major components of the cellulosome (10, 13, 25).

In this study, we show that *C. acetobutylicum* produced higher extracellular cellulolytic activities during growth on lichenan or xylose compared to growth on glucose or cellobiose. The production of extracellular CelF by *C. acetobutylicum* was determined by Western blotting using antibodies raised against *E. coli*-produced CelF. It was found that in lichenan- or xylose-grown cultures a significantly higher amount of CelF was present compared to glucose- or cellobiose-grown cultures.

Materials and Methods

Bacterial strains, media, and growth conditions

C. acetobutylicum ATCC 824 was kindly supplied by Dr. Soucaille (INSA, Toulouse, France). Stock cultures were maintained as spore suspensions in sterile 10% (v/v) glycerol at -20˚C. Spore suspensions were heat-shocked during 10 min at 75ºC prior to inoculation. For the production of pre-cultures, cells were grown overnight at 37 ˚C in Clostridial Growth Medium (CGM) (26, 32),

containing per liter; KH_2PO_4 0.75 g, K_2HPO_4 0.75 g, $MgSO_4$ 0.348 g, $MnSO_4.H_2O$ 0.01 g, $FeSO_4.7H_2O$ 0.01 g, NaCl 1 g, asparagine 2 g, yeast extract 5 g, $(NH_4)_2SO_4$ 2 g, glucose 20 g. For growth experiments the same medium was used containing 2 % (w/v) of the following carbon sources: glucose (Merck, Darmstadt, Germany), CMC (low viscosity), cellobiose, xylose, lichenan or laminarin (all from Sigma). Avicel (Merck, Darmstadt, Germany) was used at 6% (w/v) alone or supplemented with Celluclast 1.5L (a gift from Novozymes, Bagsvaerd, Denmark) at 2% (weight Celluclast 1.5L/weight Avicel). All experiments were performed in an anaerobic chamber (Coy Laboratory Products, USA) under an atmosphere of 20% CO_2, 4% H_2 and 76% N_2.

For vector construction and protein production the *E. coli* strains XL1 blue (Stratagene) and M15 [pREP4] (Qiagen), respectively, were used. These strains were grown in Luria-Bertani broth as described (27), supplemented, when appropriate, with ampicillin (50 μg/ml), isopropyl-β-thiogalactopyranoside (IPTG; 50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal; 40 μg/ml) or kanamycin (50 μg/ml).

Transformation procedures, DNA manipulation and PCR

All general DNA manipulations in *E. coli* were carried out essentially as described (27). Restriction endonucleases and modification enzymes were purchased from Roche Diagnostics, Eurogentec or Qiagen. DNA isolation from *E. coli* was performed with the Wizard Plus SV miniprep kit (Promega Inc). Genomic DNA from *C. acetobutylicum* ATCC 824 was isolated using the method of Pospiech and Neumann (24).

Oligonucleotides for polymerase chain reaction (PCR) reactions were purchased from Eurogentec. The DNA fragments containing the desired mutations were first cloned into pGEMT-Easy (Promega Inc), and afterwards in the expression vector pQE60 (Qiagen). The mutations were verified by sequencing using an automated laser fluorescent ALF DNA sequencer (Amersham Pharmacia Biotech), using fluorescent-labelled M13 universal and reverse primers. The complete coding sequence from the *celF* gene from *C. acetobutylicum* ATCC824 was amplified by PCR using primers based on the sequence of the *celF* gene (gi:15894198) and included as forward primer CELF1 5'-CG<u>CCATGG</u>TAAAGATAAGTAAGAA-3', and as a reverse primer CELF3 5'-GC<u>GGATCC</u>TTAACCTATTTTTGCAATTAATTTAG-3', to which *Nco*I and *BamH*I sites (underlined) were added, respectively. The resulting PCR fragment of approximately 2.1 kb was cloned into the vector pQE60, generating pWUR69. The *celF* gene without signal peptide sequence was amplified using the CELF3 primer and as forward primer CELF2 5'-CG<u>CCATGG</u>CTACAACTACAGATTCATCC-3' (*Nco*I site underlined). The resulting PCR fragment of approximately 2 kb was cloned into pQE60, generating pWUR70. The *Nco*I sites were

added in such a way that they allowed the in-frame fusion of the coding region to the pQE60 expression sequences. To obtain a 6xhistidine tag at the C-terminal of *celF* gene, primer CELF2 was used as forward primer and as reverse primer CELF4 5' GC<u>GGATCC</u>ACCTATTTTTGCAATTAATTTAG 3' (*BamH*I site underlined) was used, and the resulting fragment was cloned in pQE60, generating pWUR70-his.

Analytical methods

Solvents and acids produced during fermentations were determined by high performance liquid chromatography (HPLC) as previously described (14).

Preparation of culture samples for enzyme assays and Western blotting

Cells were sedimented by centrifugation at 10,000 x g at 4°C for 15 min. The culture supernatant was collected, filtered through a 0.20 μm sterile syringe filter (26 mm PES membrane, Corning Inc., New York, USA) and concentrated approximately 100-fold by ammonium sulfate precipitation at 85% (w/v) (6.3 M) on ice. Low-molecular-weight compounds and medium components were removed from the concentrated material by dilution with 4 volumes of ice-cold 50 mM sodium citrate buffer pH 5.7, followed by re-concentration by ultrafiltration using Macrosep devices with a molecular weight cutoff of 10 kDa (low protein binding PES membrane, Pall Filtron, East Hill, USA). The fractions obtained were used for enzymatic activity determinations or for Western blotting assays.

Cellulase activity assays

E. coli transformants grown on agar plates were screened for endoglucanase activity by the Congo red staining method of Teather and Woods (31) with the modifications described previously (15).Hydrolytic activities were determined as described previously (11). The following substrates (all from Sigma, except Avicel) were used (final concentrations): 0.5% (w/v) CMC, 0.2% (w/v) lichenan, 0.5% (w/v) laminarin, 0.5% (w/v) xylan or 0.5% (w/v) Avicel (Merck, Darmstadt, Germany) in citrate buffer 50 mM, pH 5.7. The substrates were incubated at 39º C in a water bath with the enzyme samples for 10-60 min, except for Avicel, laminarin and CMC that were incubated for periods up to 30 h. The reducing sugars formed were measured with the DNS (3,5-di-nitrosalicylic acid) method (4). One unit of activity corresponds to the formation of 1 μmol of reducing sugars (D-glucose) per min, unless stated otherwise. Protein concentrations in the samples was determined using the Bradford assay (Biorad).

Immunological procedures

Cell-free extracts of *E. coli* harboring CelF were loaded on a SDS-PAGE gel with 10% acrylamide. The protein band corresponding to CelF was cut out from the gel, homogenized, mixed 1:1 with adjuvant (Specoll) and injected into a New Zealand white rabbit. Antiserum was collected after 9 weeks of the first immunization. Western blot analyses were carried out with antiserum diluted 1000 times according to a standard protocol (27).

Bioinformatics

Search for catabolite response elements (CRE) was performed using the software package PATSCAN (12).

Results
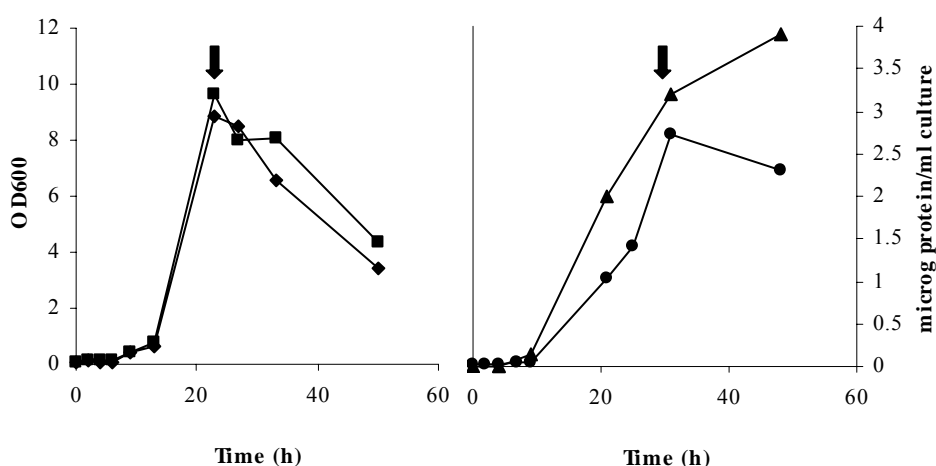
Figure 7.1 Growth of *C. acetobutylicum* ATCC824 on different substrates. Optical density (left axis) was determined of cultures grown on cellobiose (■), glucose (◆) or xylose (●), while growth on lichenan (▲) was determined by following protein concentration in cell-free extracts (right axis). The concentration of protein in the cell-free extract from a lichenan grown culture during 48 h equals that of a culture grown on glucose with an OD600 of approximately 2.0. Arrows indicate the time point when cultures were collected to determine enzymatic activities (Table 7.2).

Growth and production of cellulase activity by *C. acetobutylicum* on different substrates

C. acetobutylicum was grown on glucose, cellobiose, xylose, laminarin, lichenan and Avicel as carbon sources. On glucose, cellobiose, xylose, or lichenan (a polymer of 1,3-1,4-β-linked glucose units), *C. acetobutylicum* grew well and after 4 days of fermentation the insoluble lichenan residue had disappeared completely. Growth of the cultures was followed by OD_{600} determination, except for lichenan-grown cultures in which the amount of protein in cell-free extracts was determined (Fig. 7.1).

Laminarin, a homopolymer composed of β-D-glucose monomers linked by β-1,3-glycosidic bonds, was not degraded by *C. acetobutylicum*; on this substrate no growth was observed, independently of the pre-culture conditions. In addition, *C. acetobutylicum* was not able to utilize microcrystalline cellulose (Avicel) or CMC, although a fast and significant reduction of viscosity of the medium was observed after inoculation of the medium containing the latter substrate, possibly due to residual cellulase activity in the inoculum. When the medium containing Avicel was supplemented with a mixture of cellulolytic enzymes (Celluclast 1.5 L) growth and solvent production was observed. Due to the presence of the insoluble Avicel in the medium, growth could not be determined by OD_{600} or by protein measurement, but only by product analysis. In the case of CMC supplemented with Celluclast 1.5L, no growth was observed and only residual amounts of products were found in the medium (Table 7.1).

Table 7.1 Acids and solvents produced on different substrates at 2% (w/v), except for Avicel that was used at a concentration of 6% (w/v) and supplemented with Celluclast 1.5L (CL) at 2% (w CL/w Avicel), by *C. acetobutylicum* ATCC 824 after fermentation (the incubation time at which solvents where highest is indicated in brackets).

	products (g/l)				
Growth substrate	acetic acid	butyric acid	Acetone	butanol	Ethanol
Glucose (48 h)	1.0	1.1	0.8	3.4	0.3
Cellobiose (72 h)	1.4	2.9	0.1	2.4	0.2
Xylose (72 h)	1.9	4.3	0.2	1.3	0.2
Lichenan (144 h)	1.9	1.9	1.0	3.5	0.2
Avicel (96 h)	0.5	0.6	0.1	0.4	0.1
Avicel + CL (216 h)	2.0	1.7	2.4	7.2	0.8
CMC + CL (72 h)	0.3	0.9	0.1	0.4	0.0

The concentrations of acids (acetic and butyric acid) and solvents (acetone, butanol and ethanol) in the extracellular medium were determined at the end of the fermentation (Table 7.1). Highest production of acids coupled to lower solvent production was found after fermentation of xylose. This indicates that there was not complete switch from the acidogenic to the solventogenic growth phase on this substrate. Moreover, the utilization of xylose was not complete, and approximately 5 g/l of xylose remained in the medium after the fermentation stopped. High solvent production was observed during growth on glucose and lichenan, although the fermentation was slower on the latter substrate (Table 7.1).

At late-exponential growth phase (Fig. 7.1), cultures were collected, the extracellular medium was filtered, and proteins were concentrated by ammonium sulfate precipitation and dialyzed. Hydrolytic activities considered typical for cellulose-degrading enzymes were determined

(Table 7.2). In the extracellular medium from lichenan-grown cultures, the enzymatic activities tested were highest and reproducible and very low avicelase activity could be detected. Only in lichenan and, to a minor extent, xylose-grown cultures activity on methyl-umbelliferyl-cellobioside (MUC), a substrate used for detection of exoglucanase activity, was observed (Fig. 7.2).

Table 7.2 Cellulolytic activities in extracellular medium of cultures of *C. acetobutylicum* ATCC 824 grown on different substrates. Samples were taken at the end of the exponential growth phase. Specific activity correponds to units per miligram of protein. One unit of activity corresponds to the formation 1 μmol of reducing sugars per minute, except for activity on pNPC (p-nitrophenyl-β-D-cellobioside) where it corresponds to 1 μmol pNP released per min per mg of protein.

Activity	Growth substrate			
	glucose	cellobiose	xylose	Lichenan
CMCase	0.06	0.09	0.3	1.1
Laminarinase	0.02	0.08	0.08	0.013
Avicelase	< 0.002	< 0.006	< 0.03	0.01
PNPCase	7.4	21.8	20.3	39.8

Expression of celF by *Clostridium acetobutylicum*

The genome of *C. acetobutylicum* contains a single gene (CAC0911, gi:15894198, designated as *celF*) coding for a putative family 48 glycoside hydrolase that is located in the putative cellulosome gene cluster. The predicted protein product, CelF, contains an N-terminal signal peptide sequence of 35 amino acids typical from Gram-positive bacteria. The mature protein consists of a family 48 catalytic domain and a 22 amino acids long dockerin domain at its C-terminus. The overall amino acid sequence was highly homologous, including domain structure, to cellulosomal family 48 enzymes of cellulolytic clostridia, such as CelF from *C. cellulolyticum* (58 % identity and 73 % similarity) (7), Exoglucanase from *C. josui* (61% identity and 74 % similarity) (8, 13) and ExgS from *C. cellulovorans* (58 % identity and 71 % similarity) (25).
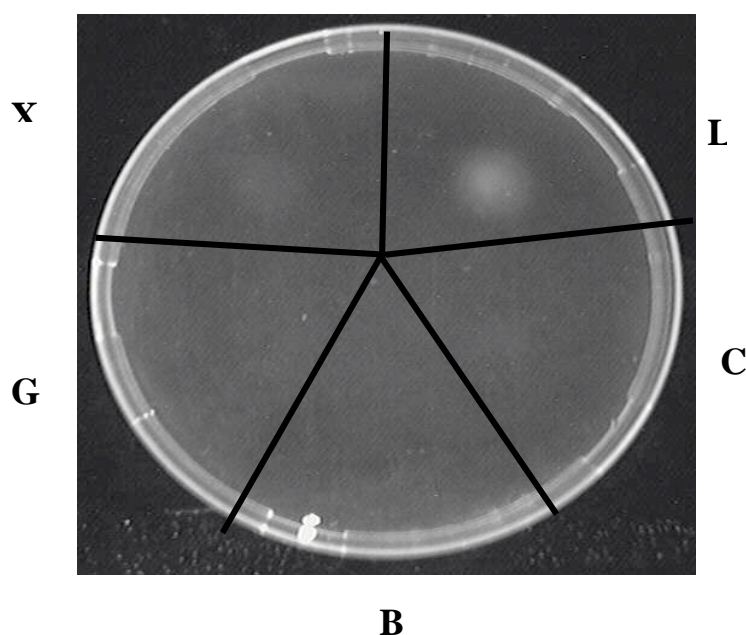
Figure 7.2 MUC hydrolysis by concentrated extracellular medium samples from C. acetobutylicum grown on different substrates. 2.5 µg of protein from glucose (G), cellobiose (C), xylose (X) or lichenan (L) medium samples were loaded in each well. In the blank well (B) 30 µl of citrate buffer (pH 5.7) were loaded.

The *C. acetobutylicum celF* gene was cloned into the expression vector pQE60 with or without the coding sequence for its predicted 35-residue signal peptide sequence, resulting in the plasmids pWUR69 and pWUR70, respectively. *E. coli* M15[pREP4] harboring either pWUR69 or pWUR70 produced a new protein of the expected size (approximately 79 kDa and 75 kDa, respectively) upon induction with IPTG. However, the strain harboring pWUR69 produced much lower amounts of recombinant protein than the strain harboring pWUR70 (results not shown). For this reason *E. coli* M15 [pREP4][pWUR70], the strain harboring the gene coding for the mature CelF, was used for further studies.

In order to determine whether *E. coli* M15 harboring either pWUR69 or pWUR70 produced CMCase activity, cells were grown on LB-agar plates supplemented with IPTG and with 0.2 % CMC. After 48 h of incubation at 37 °C and staining of CMC with Congo red, no white halos, that would indicate CMC degradation, were observed around the grown colonies. Incubation of the plates for longer time did not make a difference, indicating that there is no degradation of CMC by any of the forms of CelF (results not shown). In contrast, white halos where readily produced under the same conditions by *E. coli* harboring the gene CAC0916, which codes for a glycoside hydrolase from family 9 (15).
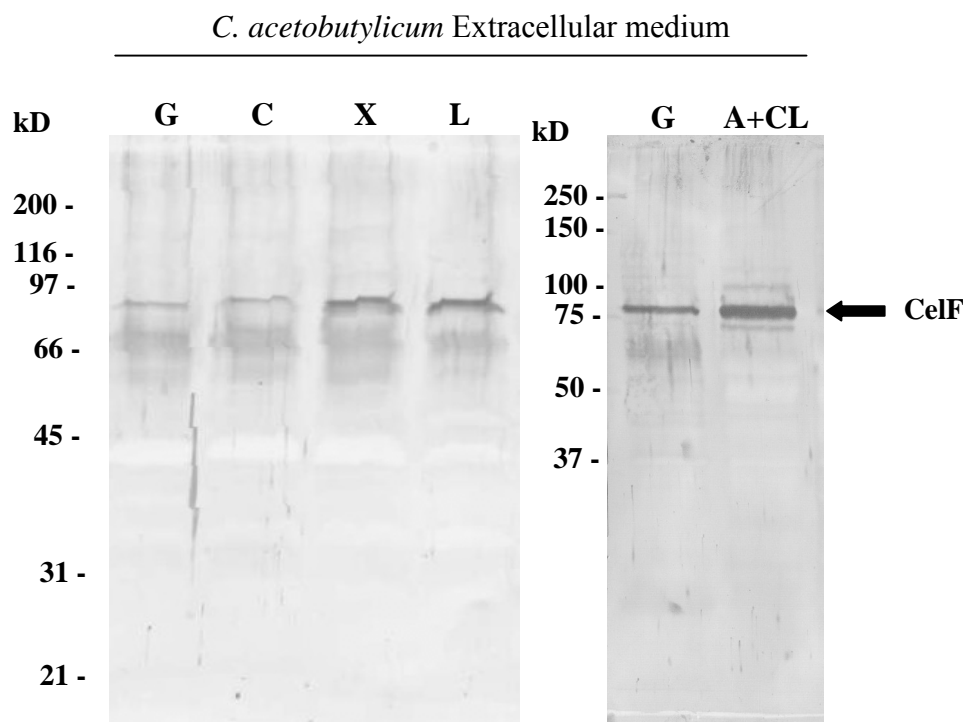
Figure 7.3 Western blot analysis of CelF using anti-CelF antibodies. CelF was detected in extracellular growth medium of C. acetobutylicum cultures grown on glucose (G), cellobiose (C), xylose (X), lichenan (L) and Avicel + Celluclast (A+CL). In each lane 5 µg of protein was loaded.

Under all growth temperatures (15-37 ºC) and induction conditions tested (0.05-1 mM IPTG), *E. coli* produced CelF as insoluble inclusion bodies, and no CelF was detectable in cell-free extracts of induced cells. As a consequence, cellulase activity could not be detected in cell-free extracts or in the insoluble fractions from cells induced under the conditions tested. CelF could only be solubilized by resuspension in a buffer containing 8 M urea. When standard dialysis or renaturation techniques were applied, CelF became insoluble as soon as the concentration of urea decreased. Despite much effort dedicated to the solubilization of CelF, including different de-naturation/re-naturation conditions, no success was achieved and the protein remained insoluble.

In order to investigate the production and localization of CelF by *C. acetobutylicum*, polyclonal antibodies were raised against the *E. coli*-overproduced CelF. *C. acetobutylicum* cultures grown on glucose, cellobiose, xylose, lichenan (the same cultures used for enzymatic activity determination) or Avicel supplemented with Celluclast 1.5L were collected and concentrated extracellular medium and cell-free extract samples were subjected to Western-blot analyses. No anti-celF-reacting proteins of the expected size were observed in any of the cell-free extracts tested when 10 µg of protein were loaded in each lane of the gel, indicating that CelF was not present in the intracellular medium. Analysis of the concentrated extracellular medium by Western blotting revealed the presence of an anti-celF-reacting protein of the expected size of mature celF (approximately 75 kDa) (Fig. 7.3). This size corresponded to that of the recombinant CelF without

signal peptide present in cell-free extracts of *E. coli* harboring pWUR70 (results not shown). In cultures grown on xylose, lichenan or Avicel supplemented with Celluclast 1.5L the amount of CelF was significantly higher than that on glucose or cellobiose (Fig. 7.3). A diffuse band corresponding to a protein of approximately 70 kDa that cross-reacted with the anti-CelF antibodies is present in almost all samples and appeared to react with serum from the non-immunized rabbit as well. Kinetic analysis showed that CelF was produced during the entire growth phase of *C. acetobutylicum* on glucose, although it decreased during the stationary phase (data not shown).

Table 7.3 Genes that encode putative extracellular proteins containing a glycoside hydrolase (GH) or a polysaccharide lyase (PL) catalytic domain and where a putative catabolite response element (CRE) has been detected in their promoter sequence or in their coding region at a distance +/- 200 bp from the start codon. The number besides the family letters indicates the family to which the catalytic domain belongs.

Gene nr	Catalytic domain	Position*	Annotated as
CAC0706	GH5	-61	Endo-1,4-β glucanase (fused to two ricin-B-like domains)
CAC0812	PL9	-164	Pectate lyase related protein
CAC2252	GH31	+134	α-Glucosidase
CAP0056	PL9	-43	Pectate lyase

* (+) and (-) indicate location in the coding region or in the promoter, respectively.

The activities determined were lowest in cultures grown on glucose compared to the rest of the growth substrates tested, suggesting a catabolite repression mechanism regulating expression of glycoside hydrolase-encoding genes. The regulation of carbon metabolism in Gram-positive bacteria is mediated by a global regulator termed catabolite control protein (CcpA) via a well-studied mechanism (21). The CcpA binds to a specific *cis*-acting DNA sequence known as catabolite response element (CRE), repressing or activating gene expression. In the genome of *C. acetobutylicum* ATCC 824 several genes encoding putative CcpA-like proteins are present; CAC3037 encodes a protein with the highest homology to the *Bacillus subtilis* CcpA (5) (42% identity and 63% similarity). When the genome sequence was screened for putative CRE sites using the consensus WTGNAANCGNWNNCW (21) 43 hits were found and when the consensus used was TGWNANCGNTNWCA (17), 6 additional hits were detected. Of the hits found, only those in which the CRE starts within 200 bp with respect to the start codon were considered significant, based on earlier studies (6). A putative CRE is present in the promoter region of ORF CAC3038 (CRE starting at position –59 with respect to the start codon), that encodes a putative isoleucyl-tRNA synthetase and is located immediately upstream of the CcpA-encoding gene. Most of the hits found were located in the promoter region or coding sequences of genes involved in sugar

metabolism. Putative CRE elements were found in the promoter regions of several genes encoding putative extracellular polymer-degrading enzymes (Table 7.3). However, no potential CRE sites were found in the genes coding for the putative cellulosomal genes, CAC0910 to CAC0919, CAC0561 and CAC3469, or in their immediate flanking regions.

Discussion

C. acetobutylicum ATCC 824 utilizes lichenan but not laminarin or cellulose for growth and solvent production. Lichenan is a linear glucan consisting of 1,3-1,4-β-linked β-D-glucose units, and has a structure that resembles the β-glucans that are abundant in plant cell walls of cereals such as barley, rye or wheat (23). This polymer is a substrate for a number of different enzymes, including lichenases (E.C. 3.2.1.73) or 1,3-1,4-β-glucanases (E.C.3.2.1.6), and is often degraded by cellulases, both exo- or endo-acting (1). Laminarin is a homopolymer of β-1,3-linked glucose units, and is degraded mainly by laminarinases (E.C. 3.2.1.39), lichenanases and 1,3-1,4 β-glucanases. All these enzymes belong to family 16 of the glycoside hydrolases, but each class contains important unique sequences, especially in the region surrounding the strictly conserved catalytic residues, that correlates with their substrate specificities (23). The genome of *C. acetobutylicum* ATCC 824 contains a single gene (CAC2807) that encodes a glycoside hydrolase from family 16; it does not belong to the cellulosome gene cluster and lacks dockerin domains. The protein coded by this gene shows high homology to *Bacillus* lichenases, and especially to LicB from *C. thermocellum* (54% indentity, 70% similarity) that seems to be a cellulosomal component. When produced by *E. coli*, LicB shows high activity on lichenan and none on laminarin (28). The absence of a gene coding for a canonical laminarinase from family 16 of the glycoside hydrolases in the genome of *C. acetobutylicum* could explain the low laminarinase activity (Table 7.2) found in the medium and its inability to utilize laminarin.

Although *C. acetobutylicum* produced CMCase activity under certain conditions (Table 7.2), it did not grow on this substrate, either because the hydrolysis products obtained could not be further converted due to the presence of the carboxy-methyl groups, or because the degradation rate was not fast enough to support growth. A similar inability to grow on CMC, even when the medium was supplemented with Celluclast 1.5L, has been observed previously for *C. beijerinckii* (16).

In lichenan-grown cultures, all enzymatic activities determined were the highest under the tested conditions and low but significant avicelase activity could be detected, suggesting induced expression of cellulolytic enzymes by lichenan. In a previous study we could not detect extracellular avicelase activity in similarly grown *C. acetobutylicum* cultures (15), but in these experiments the

protein concentrations were lower, since in the results described here the extracellular proteins were precipitated by addition of ammonium-sulfate and concentrated approximately 60-fold. In xylose-grown cultures, the activities were the second highest (Table 7.2), and activity on MUC was detectable although it was lower than in lichenan-grown cultures (Fig. 7.2). Lee *et al.* (11) reported production of endoglucanase (CMCase) and cellobiosidase (pNPCase) activities by *C. acetobutylicum* grown on various carbon sources (glucose, cellobiose, xylose or mannose) in a chemostat.

Cellulosomes are large extracellular complexes specialized in cellulose degradation. A conserved feature between cellulosomes produced by clostridial species is the presence of a family 48 glycoside hydrolase enzyme with exoglucanase activity, that seems to play an essential role in cellulosomal function. The only gene coding for a putative glycoside hydrolase from family 48 in the genome of *C. acetobutylicum*, *celF*, was overexpressed in *E. coli*. The resulting protein, CelF, was produced in inclusion bodies, appearing always in the insoluble fraction of the cell lysates. Enzymatic activity (CMCase or Avicelase activities) corresponding to this protein could not be detected in any of the fractions tested. Other clostridial cellulases from family 48, such as ExgS from *C. cellulovorans* (13) or CelF from *C. cellulolyticum* (25) have been produced by *E. coli*, which also formed inclusion bodies under standard induction conditions, but denaturation/re-naturation methods or different production conditions allowed solubilization of at least part of the corresponding protein. However, similar methods were unsuccessful in the case of CelF from *C. acetobutylicum*, and the characteristics of this enzyme could not be studied.

Polyclonal antibodies raised against the *E. coli*-produced CelF were used to detect production of this protein by *C. acetobutylicum* ATCC 824 grown on different substrates. On all substrates, CelF was specifically detected in the extracellular medium as a single band of the expected size. Celluclast 1.5L is produced by the fungus *Trichoderma reesei* (19) and it does not cross-react with the anti-CelF antibodies. CelF was found to be constitutively produced by *C. acetobutylicum*, but was induced during growth on lichenan, xylose or Avicel. In *C. cellulovorans* the major cellulosomal components are constitutively expressed, and depending on the growth substrate, specific enzymes are induced and the composition of the cellulosomes varies (9).

In the genome of *C. acetobutylicum* there is a gene encoding a CcpA-like regulator, CAC3037, and several putative CcpA-binding sites, (called catabolite repression elements, CRE). The search for CREs in the genome was performed using well-characterized consensus sequences, and various hits were found both in the chromosome and in the megaplasmid. CRE sequences are present in a number of genes, including some encoding extracellular polymer-degrading enzymes, in particular gene CAC0706, encoding a putative glycoside hydrolase from family 5, and two genes

involved in pectin degradation (Table 7.3). No CRE sites were found in the promoter or in the coding regions of genes encoding putative cellulosomal subunits.

Despite of the presence of genes involved in cellulose degradation in its genome and the production of cellulolytic enzymes, *C. acetobutylicum* ATCC 824 is not able to utilize cellulose for growth. In this study we show that CelF, a protein encoded by one of the genes present in the cellulosome gene cluster, is produced and exported to the extracellular medium during growth on glucose, cellobiose, xylose, lichenan or Avicel. On the latter three substrates there is an increased amount of CelF in the extracellular medium, correlating with increased cellulolytic activities. However, at this stage, the increased cellulolytic activities cannot be assigned to CelF only, since other genes coding for different cellulases are present in the genome. A functional study of the genes encoding proteins involved in (hemi)cellulose degradation, in particular the putative cellulosomal genes, is a essential step towards understanding the reason for the lack of true cellulolytic properties by *C. acetobutylicum* ATCC 824 and the enhancement of the utilization of lignocellulosic substrates for the production of acetone, butanol and ethanol by this strain.

Acknowledgements

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Chapter 8

Summary & Concluding Remarks

The oil crisis in the 1970s stimulated research into energy sources that could serve as alternatives to fossil fuels. Presently, the interest in alternative energy sources is not only based on economical considerations, but it is largely based on the growing awareness of the negative environmental effects associated with the production of energy from fossil fuels (e. g. CO_2 and SO_2 emissions, pollution of the marine environment). Alternative energy sources must meet three demands: environmental safety, no depletion (renewable) and releasing from foreign dependency (e. g. most of the oil comes from the Middle East, an area of high political instability). The renewable energy sources with the most potential are solar and wind energy, hydropower and biomass. Among these, biomass (composed by wood and wood-processing residues, agricultural wastes, energy crops and municipal wastes) stands out because of its abundance and its potential to be harvested on demand.

The main components of plant biomass (also called lignocellulose) are cellulose, a linear polymer of β-1,4 linked glucose residues, hemicellulose, a group of non-cellulosic linear polymers of glucose, xylose, mannose or other sugars, and lignin, a three dimensional polymer of phenolic compounds. Because of its high content in carbohydrates, plant biomass is considered to have a great potential as substrate for the fermentative production of energy carriers (1), such as ethanol or butanol, or chemicals. The ability of solvent-producing clostridia to utilize most of the sugars present in lignocellulosic hydrolysates (3) makes them very interesting for the production of solvents from biomass at industrial scale. This thesis describes research addressed at investigating the utilization of lignocellulosic substrates for the production of acetone, butanol and ethanol by clostridial strains, the ABE fermentation, a process that has been the second largest industrial fermentation after the production of ethanol by yeasts during the first half of the 20th century.

A general introduction to the ABE fermentation (including a description of the organisms, physiology of the solvent production and a short history of the process) is given in **Chapter 1** of this thesis. Potential substrates are summarized, and attention is focused on lignocellulosic substrates and microbial cellulose degradation, especially by anaerobic organisms.

In **Chapter 2**, a study of the utilization of domestic organic waste (DOW) as substrate for ABE fermentation by *C. acetobutylicum* ATCC 824 is presented. Two different samples of DOW collected at different seasons were analyzed. The polymeric fractions were composed mainly of glucose (most likely in the form of cellulose) and xylose (most likely as xylan or xyloglucan). Arabinose, mannose, galactose and rhamnose were present in lesser amounts. *C. acetobutylicum* grown in a suspension of DOW in water utilized all the soluble sugars present. The sugar polymers, however, were almost intact after the fermentation, which indicates a lack of sufficient hydrolytic

activity to degrade them. Previous hydrolysis of the substrate with cellulases resulted in increased sugar utilization and solvent production.

Suspensions of lignocellulosic insoluble materials are often viscous and very heterogeneous, as was the case for DOW, which makes handling difficult. For efficient fermentation and for simplified process technology, clear hydrolysates of lignocellulosic materials are preferred as substrates. To achieve this, a pre-treatment step is necessary to facilitate hydrolysis. The pre-treatment and hydrolysis methods to be used depend on the specific material and they may result in the formation of toxic compounds and in low sugar concentrations in the hydrolysate. The fermentation of DOW-hydrolysate prepared by steam explosion as pre-treatment step followed by enzymatic hydrolysis is described in **Chapter 3**. Three different clostridial strains were tested for growth and solvent production on concentrated and non-concentrated DOW-hydrolysate without further nutrient supplementation. In general, the solvent production on hydrolysate was low, most likely due to the low sugar concentration. In 4-fold concentrated hydrolysate growth was completely inhibited. Addition of concentrated hydrolysate to growing cultures also inhibited solvent production. When the hydrolysate was purified by anion exchange chromatography and used 4-fold concentrated, high levels of solvent production were observed, indicating the removal of some unidentified inhibitors from the hydrolysate.

An alternative approach to replace the pre-hydrolysis step of lignocellulosic substrates is the application of new strains that are able to convert the sugar polymers (especially cellulose) in the substrate directly to solvents. Since none of the solvent-producing clostridial strains known so far are cellulolytic (5), genetic modification techniques have been applied to develop cellulose-degrading strains. In **Chapter 4** the cloning of two different cellulase genes (*celA*, encoding a cellobiohydrolase from family 6, and *celD*, encoding an endoglucanase from family 5) from the anaerobic rumen fungus *Neocallimastix patriciarum* into *Clostridium beijerinckii* is described. The transformants harboring either of these genes showed significant endoglucanase activity compared to the wild-type strain, and increased conversion of lichenan, a polymer composed of β-1,3-1,4-linked glucose units that resembles β-glucan present in cereals, into solvents. However, none of the transformants, alone or in co-culture, was able to grow on crystalline cellulose (Avicel), indicating that for an efficient degradation of this polymer more enzymatic activities or higher levels of enzyme production are needed. As described in **Chapter 1**, cellulose degradation is a very complex process, and cellulolytic organisms produce a wide range of enzymes with different enzymatic activities that act synergistically in cellulose degradation. Our transformants showed enhanced utilization of lichenan, a polymeric insoluble substrate that does not have a crystalline structure like Avicel (9). Apparently, the development of strains with enhanced utilization of polymeric substrates

with low or no crystalline structure could be achieved by cloning a small number of suitable genes into the wild-type strain. However, the addition of true cellulolytic activity into a non-cellulolytic strain seems to require the cloning of a relatively larger number of genes, possibly in a specific ratio, which would make this task very laborious.

The detection of a number of genes that encode proteins involved in cellulosome formation in the chromosome of *acetobutylicum* ATCC 824 constituted a surprise from the genome sequencing (6), because this strain does not show true cellulolytic properties. Ten of these genes are grouped in a cluster that is very similar to the clusters found in cellulolytic clostridial species, such as *C. cellulovorans* and *C. cellulolyticum*. Both *C. acetobutylicum* and *C. cellulovorans* belong to Cluster I of the Clostridium genus, while the most of the cellulolytic (and cellulosome-producing) Clostridial species belong to Cluster III (8) (**Chapter 1**). It has been suggested that the gene cluster could have been acquired by these species via a horizontal gene transfer event or that there is a common ancestor for the mesophilic cellulolytic clostridia and *C. acetobutylicum* (4). *C. cellulovorans* does not produce solvents, but produces mainly butyric acid from cellulose (7). Other cellulolytic clostridia, such as *C. cellulolyticum*, do not produce butyric acid, but produce acetic acid as the major fermentation product (2). The genetic and physiological similarities between *C. cellulovorans*, whose cellulosome has been extensively characterized, and *C. acetobutylicum* make it interesting to compare their cellulolytic systems in order to discover the reason for the lack of cellulolytic properties of the latter organism.

Three chapters of this thesis are dedicated to studying the cellulosome gene cluster of *C. acetobutylicum* ATCC 824. In **Chapter 5** the cloning of the *cbpA* gene, coding for the cellulose-binding protein of the cellulosome, from *C. cellulovorans* into *C. acetobutylicum* is shown. The transformant strains harboring the *cbpA* gene produced recombinant proteins of a smaller size than predicted that cross-reacted with anti-CbpA antibodies, suggesting proteolysis of the CbpA. In addition, these transformants did not show growth on cellulose or enhanced cellulolytic properties as compared to the parent strain. These results indicate that cloning of a gene coding for a functional cellulose-binding protein into *C. acetobutylicum* does not solve its lack of cellulolytic activity, and therefore a different approach was undertaken in the following studies shown in Chapters 6 and 7.

The first part of **Chapter 6** describes the characterization of an *Escherichia coli*-produced putative enzymatic subunit of the *C. acetobutylicum* cellulosome, whose gene is located in the cellulosome gene cluster. The open reading frame number CAC0916 was isolated from chromosomal DNA of *C. acetobutylicum* ATCC 824 by PCR using specific primers derived from the published genome sequence (Genebank accession number AE001437 at the National Center for Biotechnology Information web site). The *E. coli*-produced protein, CelG, was purified making use

of its high affinity to cellulose conferred by its cellulose-binding domain. CelG has a catalytic domain that is classified into the family 9 of the glycoside hydrolases classification, and the *E. coli*-produced protein showed hydrolase activity on several substrates, including microcrystalline cellulose, typical for enzymes belonging to this family. The extracellular production of CelG by *C. acetobutylicum* grown on glucose, cellobiose, xylose or lichenan was studied by Western blot analysis using polyclonal antibodies raised against the purified *E. coli*-produced CelG. Only in lichenan-grown cultures an anti-CelG cross-reacting protein of the expected size of mature CelG was detectable, although at very low levels, indicating induction by this substrate. In the extracellular medium of cellobiose- or cellulose-grown *C. cellulovorans* cultures, several anti-CelG cross-reacting proteins were present. However, the major band corresponded to a protein of similar size as mature CelG, most probably a protein highly homologous to CelG. The level of anti-CelG-reacting proteins produced by *C. cellulovorans* was significantly higher than that by *C. acetobutylicum*, suggesting different regulation mechanisms. It is tempting to conclude that the lack of cellulose degradation by *C. acetobutylicum* could be related to this lower expression level of cellulolytic enzymes compared to other cellulolytic organisms. This could be due to a defect in the induction of gene expression, either at the level of a transcription regulator, or due to some mutations in the promoter region; in addition, the low activities may reflect inefficient transport across the cytoplasmic membrane or incorrect assembly of the cellulosome subunits. However, more data are needed to confirm this hypothesis.

Another gene of the cellulosome gene cluster, *celF*, of *C. acetobutylicum* has been studied in **Chapter 7**. The *celF* gene (CAC0911) is the only gene in the whole genome that encodes a putative glycoside hydrolase from family 48, which includes enzymes with cellobiohydrolase (or exoglucanase) activity. In all cellulosome gene clusters known there is a unique gene coding for a family 48-glycoside hydrolase and its location, immediately downstream from the Cbp-encoding gene, is conserved. It has been suggested that family 48 glycoside hydrolases play an essential role in cellulosomal function and therefore we focused our attention on this particular gene. The *celF* gene was cloned and overexpressed in *E. coli*. The corresponding protein produced by *E. coli*, CelF, appeared to be insoluble under all growth and induction conditions tested, and therefore it was not possible to determine the catalytic properties of this enzyme. In a similar approach as the one described in **Chapter 6**, the production of CelF by *C. acetobutylicum* grown on different substrates was studied by Western blot analysis of culture samples using polyclonal antibodies raised against *E. coli*-produced CelF. In all the extracellular medium samples corresponding to glucose-, cellobiose-, xylose-, lichenan, or Avicel-grown cultures, CelF was detected, indicating that this protein is probably constitutively expressed. In cultures grown on xylose, lichenan and Avicel, the

amount of extracellular CelF was significantly higher than in the cultures grown on the other carbon sources. In both **Chapters 6 and 7**, extracellular (hemi)cellulolytic activities of *C. acetobutylicum* grown on the above mentioned substrates, except on Avicel due to the presence of added cellulases, have been determined. In general, the extracellular activities tested were highest in lichenan-grown cultures, followed by xylose-grown cultures. Degradation of microcrystalline cellulose (Avicel) was detected at very low levels only in lichenan-grown cultures and only when very concentrated medium was used (**Chapter 7**). Whether this activity corresponds to CelF or to other glycoside hydrolase(s) produced by *C. acetobutylicum* needs to be investigated.

In our studies we have shown that the cloning of appropriate genes in a solvent-producing strain gives rise to a modified substrate range for the utilization of polysaccharides. This implies that genetic techniques could be used for the development of strains degrading specific substrates of interest, given the wide variety of enzymes involved in polysaccharide degradation that are available. In addition, we have established that the strain *C. acetobutylicum* ATCC 824 produces inducible extracellular (hemi) cellulolytic activities and secretes at least two potential cellulosomal cellulases to the medium. These findings have generated new insights in the potential for genetic improvement of solvent-producing clostridia, even though the production of new cellulolytic solvent-producing species has not been achieved. The considerable research carried out in the last decades into physiological aspects and improvements of the process technology, together with increased genetic knowledge of solvent-producing clostridia represent important advances towards an economically viable process. Since currently there is an increasing political and social support in Europe and in the United States to stimulate the use of renewable resources for the production of chemicals and fuels, there is a chance that the ABE fermentation becomes an industrial process again in the near future.

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Nederlandse samenvatting

De oliecrisis in de jaren zeventig van de vorige eeuw stimuleerde het onderzoek naar energiebronnen die als alternatief kunnen dienen voor de fossiele brandstoffen. Tegenwoordig is de aandacht voor alternatieve energiebronnen niet alleen gebaseerd op economische overwegingen, maar voor een groot deel op het groeiende bewustzijn van de negatieve milieueffecten die gepaard gaan met de productie van fossiele brandstoffen (bv. CO_2 en SO_2 uitstoot, vervuiling van het zeemilieu). Alternatieve energiebronnen moeten aan drie eisen voldoen: veilig voor het milieu, geen uitputting van beperkte voorraden en onafhankelijkheid ten opzichte van het buitenland (de meeste olie komt uit het Midden Oosten, een gebied met grote politieke instabiliteit.) De alternatieve energiebronnen met het meeste perspectief zijn zonne- en windenergie, waterkracht en biomassa. Biomassa, een verzamelnaam voor hout, houtafval, agrarisch afval, gewassen voor energieproductie en gemeentelijk afval, heeft het voordeel dat het overvloedig aanwezig is en naar behoefte geoogst en verzameld kan worden.

De belangrijkste bestandsdelen van plantaardige biomassa zijn cellulose (een polymeer die bestaat uit glucose (suiker) deeltjes met een hoge graad van kristallisatie), hemicellulose (een groep van niet-cellulose polymeren bestaand uit glucose, xylose, mannose en andere suikers) en lignine (een kunststofachtige polymeer). Plantaardige biomassa, wat ook lignocellulose wordt genoemd, bevat veel koolhydraten en hierdoor is het een goede voedingsbron voor bacteriën en gisten. Enkele daarvan zetten koolhydraten om in brandstoffen, zoals ethanol, en andere chemicaliën. De voedingsbron waarin de bacteriën groeien wordt ook substraat genoemd. Deze thesis beschrijft het onderzoek naar het nuttig aanwenden van lignocellulose substraten voor de productie van *a*ceton, *b*utanol en *e*thanol met behulp van bacterie soorten uit de *Clostridium* familie, ook wel ABE fermentatie genoemd. Dit proces was in de eerste helft van de vorige eeuw de op één na grootste industriële fermentatie na de productie van ethanol door gisten.

Een algemene introductie over ABE fermentatie (inclusief een beschrijving van de organismen, de fysiologie en een kort historisch overzicht van het proces) wordt gegeven in **Hoofdstuk 1** van deze thesis. Verschillende soorten substraten, waaronder ook lignocellulose substraten, worden behandeld en aandacht wordt gegeven aan de microbiologische afbraak van cellulose (het belangrijkste bestandsdeel van het substraat) door anaerobe organismen. Anaerobe organismen leven in een zuurstofloze omgeving.

In **Hoofdstuk 2** wordt een studie gepresenteerd van het nuttig aanwenden van groente-, fruit- en tuinafval (GFT) als substraat voor ABE fermentatie met behulp van bacteriën van de soort *Clostridium acetobutylicum*. De suikerpolymeren in GFT bestonden voornamelijk uit glucose (waarschijnlijk in de vorm van cellulose) en xylose (waarschijnlijk xylan of xyloglucan). Arabinose, mannose, galactose en rhamnose kwamen in mindere mate voor. *C. acetobutylicum* gegroeid in een

suspensie van GFT in water verbruikte alleen opgeloste suikers en liet de polymeren bijna geheel intact.

Suspensies (vloeibare, maar wel troebele mengsels met onoplosbare deeltjes) van lignocellulose zijn vaak stroperig en erg divers van samenstelling. Dit is ook het geval voor GFT-substraat en hierdoor wordt de behandeling bemoeilijkt. Om het fermentatieproces eenvoudiger en efficiënter uit te kunnen voeren, is het belangrijk om een heldere oplossing te hebben. Om dit te bereiken wordt de lignocellulose gehydrolyseerd. Hydrolyse is het proces waarbij de polymeren in het substraat in oplosbare stukjes, bijvoorbeeld suikers, worden afgebroken. Hiervoor is ook een voorbehandeling nodig. Hoe de voorbehandeling en de hydrolyse gedaan moeten worden, hangt af van het specifieke materiaal en kan de vorming van giftige stoffen voor de bacteriën tot gevolg hebben en kan ook resulteren in lagere suikerconcentraties in het hydrolysaat, het eindproduct van deze behandeling. De fermentatie van GFT-hydrolysaat wordt beschreven in **Hoofdstuk 3**. Van drie verschillende bacterie soorten uit de *Clostridium* familie werd onderzocht hoe deze groeien op geconcentreerd en niet-geconcentreerd GFT-hydrolysaat en hoeveel van de oplosmiddelen (de ABE stoffen) ze produceren. In het algemeen was de productie van oplosmiddel in hydrolysaat laag. Dit komt waarschijnlijk door de lage suikerconcentratie. In viervoudig geconcentreerd hydrolysaat kwam de groei van de bacteriën helemaal tot stilstand. Als bij groeiende bacterieculturen geconcentreerd hydrolysaat werd toegevoegd, kwam de productie van oplosmiddel ook tot stilstand. Toen het hydrolysaat gezuiverd en viervoudig geconcentreerd werd, bleek de gewenste productie van oplosmiddel enorm toe te nemen. Dit geeft aan dat bepaalde remmende stoffen verwijderd konden worden.

Het is misschien mogelijk om de stap van pre-hydrolyse over te slaan door nieuwe varianten van de bacterie te ontwikkelen die direct uit het substraat, met name cellulose en hemicellulose, ABE kunnen produceren. Omdat geen van de tot nu toe bekende ABE-producerende clostridia in staat zijn om cellulose af te breken, hebben we genetische modificatie technieken toegepast om varianten te ontwikkelen die dit wel zouden moeten kunnen. De genen van een organisme leggen namelijk precies vast welke enzymen gemaakt worden. Enzymen zijn bepaalde eiwitten die elk een specifieke reactie kunnen activeren. De enzymen nemen niet deel aan de reacties, maar treden op als katalysator. Uiteindelijk bepalen dus de genen welke reacties op kunnen treden, zoals bijvoorbeeld het afbreken en omzetten van cellulose. Enzymen die cellulose kunnen afbreken worden ook cellulases genoemd. Het vermogen om dit te kunnen wordt cellulolytisch genoemd. **Hoofdstuk 4** beschrijft het kloneren van twee verschillende genen uit een schimmel die in de bacterie *C. beijerinckii* geplaatst worden. Deze genen coderen voor twee cellulases. De op deze manier verkregen transformanten tonen een significant hogere cellulase activiteit. Dat wil zeggen dat ze in

bepaalde mate cellulose kunnen afbreken. Ook zijn ze beter in staat om lichenan, een bepaald soort polymeer die veel in granen voorkomt, om te zetten in oplosmiddelen. Helaas waren de toegenomen activiteiten niet genoeg voor de bacterie om in cellulose te kunnen groeien. Het complexe proces van cellulose afbraak wordt beschreven in **Hoofdstuk 1** en hierin komt ook naar voren dat cellulolytische organismen een grote diversiteit aan enzymen ten toon spreiden. Al deze enzymen hebben verschillende en elkaar versterkende activiteiten op het gebied van cellulose afbraak. Onze transformanten tonen een toegenomen omzetting van lichenan in oplosmiddelen. Lichenan is een substraat van onoplosbare polymeren maar heeft niet een kristallijne structuur zoals cellulose wel heeft. Door een klein aantal geschikte genen te kloneren in de bacterie is het blijkbaar mogelijk om varianten te ontwikkelen met een toegenomen omzettingsvermogen van polymeersubstraten met weinig of geen kristallijne structuur. Echter, om ook cellulolytische eigenschappen toe te voegen aan een bacterie die dat van zichzelf niet kan, moet een groter aantal genen, en mogelijk in een bepaalde verhouding, gekloneerd worden. Dit vergt nog veel werk, en daarom hebben wij voor een andere strategie gekozen in ons verdere werk.

Een verassing was de aanwezigheid van een aantal genen die coderen voor proteïnen die betrokken zijn bij de formatie van cellulosoom in het chromosoom van *Clostridium acetobutylicum*, ook al kan de bacterie zelf geen cellulose omzetten. Cellulosoom is het gehele complex van aan cellulose bindende proteïnen met ook daaraan vast de enzymen (cellulases) die door deze binding in staat zijn om de cellulose op een efficiënte manier af te kunnen breken. Tien van de bovengenoemde genen komen voor in een cluster die erg lijkt op de clusters in *Clostridium* soorten die cellulose wel kunnen afbreken, zoals *C. cellulovorans*.

Drie hoofdstukken zijn gewijd aan het bestuderen van het genencluster die bepalend is voor het cellulosoom in de bacterie van de soort *C. acetobutylicum*. In **Hoofdstuk 5** wordt het kloneren getoond van het *cbpA* gen van *C. cellulovorans* in *C. acetobutylicum*. Dit gen codeert voor het cellulose bindende eiwit in het cellulosoom. De transformant produceerde het CbpA eiwit, maar was van een kleinere grootte dan verwacht mocht worden. Misschien komt dat omdat het werd afgebroken. Ook konden deze transformanten niet groeien op cellulose en vertoonden ze geen toegenomen cellulolytische activiteit. Deze resultaten tonen aan dat het kloneren van een gen die codeert voor cellulosebindende proteïnen in *C. acetobutylicum* niet het gebrek aan cellulolytische activiteit kan goedmaken. Daarom werd een andere benadering gekozen in de hoofdstukken 6 en 7.

Het eerste deel van **Hoofdstuk 6** beschrijft de typering van een *Escherichia coli*-geproduceerd enzymatisch deel van het cellulosoom in *C. acetobutylicum*. Het gen hiervoor bevindt zich in het genencluster die bepalend is voor het cellulosoom. Het gen dat voor dit eiwit verantwoordelijk is werd gekopieerd van het chromosoom van *C. acetobutylicum* en geplaatst in *E.*

coli. Het eiwit dat hierdoor aangemaakt werd, CelG, werd gezuiverd door gebruik te maken van de verwantschap die het heeft met cellulose, verleend door zijn cellulosebindende domein. CelG heeft typische eigenschappen van cellulases van dezelfde familie, en kan ook verschillende soorten cellulose en hemicellulose afbreken. De productie van CelG door *C. acetobutylicum* werd gecontroleerd met het gebruik van specifieke antilichamen gemaakt tegen de CelG eiwit gemaakt door *E. coli*. In vier verschillende substraten (glucose, xylose, cellobiose en lichenan) lieten we *C. acetobutylicum* groeien en hebben we de buitencellige eiwitten en cellulase activiteit gemeten. CelG was alleen aanwezig in culturen die in lichenan groeiden, en in die culturen was ook de cellulase activiteit het hoogst. Of de extra cellulase activiteit alleen van CelG komt kunnen we niet concluderen, omdat er ook andere cellulases kunnen zijn. Dit suggereert dat lichenan, en niet glucose of cellobiose, de productie van CelG stimuleert, en waarschijnlijk ook de productie van cellulases door *C. acetobutylicum*.

Een ander gen van het cellulosoom-genencluster, *celF*, van *C. acetobutylicum* wordt onderzocht in **Hoofdstuk 7**. Dit gen codeert voor een uniek enzym uit familie 48, welke ook enzymen bevat met hoge cellulolytische activiteit op kristallijn cellulose. Het idee bestaat al dat enzymen van deze familie een essentiële rol spelen voor het cellulosoom en daarom hebben wij onze aandacht toegespitst op dit specifieke gen. Het *celF* gen werd gekloneerd in *E. coli*. Het CelF eiwit dat hierdoor geproduceerd werd bleek onder alle geteste omstandigheden onoplosbaar te zijn en daardoor was het niet mogelijk om de katalytische eigenschappen van dit gen te testen. In alle culturen die op glucose, cellobiose, xylose, lichenan en Avicel groeiden, werd buitencellig CelF aangetroffen. In culturen die groeiden op xylose, lichenan en cellulose was de hoeveelheid buitencellig CelF significant hoger dan in de andere culturen. In beide **Hoofdstukken 6 en 7** werden buitencellige (hemi)cellulolytische activiteiten onderzocht van *C. acetobutylicum* groeiend op bovengenoemde substraten. In het algemeen waren deze activiteiten het hoogst in culturen die groeiden op lichenan, gevolgd door culturen groeiend op xylose. Afbraak van microkristallijn cellulose werd in zeer beperkte mate waargenomen, en alleen bij culturen groeiend op lichenan en alleen bij erg geconcentreerd medium (**Hoofdstuk 7**). Of deze activiteit het gevolg is van CelF of van andere glycoside hydrolases geproduceerd door *C. acetobutylicum*, moet verder onderzocht worden.

In dit onderzoek hebben we aangetoond dat het kloneren van de juiste genen in ABE-producerende clostridia aanleiding geeft tot een betere omzetting van bepaalde suikerpolymeren. Dat betekent dat genetische technieken gebruikt zouden kunnen worden voor de ontwikkeling van bacteriën die specifieke substraten kunnen afbreken, gegeven de grote beschikbare variëteit aan enzymen die betrokken zijn bij de afbraak van suikerpolymeer. Daarnaast hebben we vastgesteld dat

de stam *C. acetobutylicum* buitencellige (hemi) cellulolytische activiteiten vertoont die door lichenan gestimuleerd zijn en scheidt op zijn minst twee potentiële cellulosome cellulases af aan het medium. Deze uitkomsten hebben nieuwe inzichten gegenereerd met betrekking tot mogelijke genetische verbeteringen van oplosmiddel producerend clostridia. Het omvangrijke onderzoek dat de laatste tientallen jaren is uitgevoerd op het gebied van fysiologische aspecten en verbeteringen van het technologische proces, samen met de toegenomen genetische kennis, vertonen belangrijke vorderingen in de richting van een economisch rendabel proces. Omdat in Europa en in de Verenigde Staten belangrijke politieke en sociale ondersteuning bestaat voor het gebruik van alternatieve bronnen voor de productie van chemicaliën en brandstoffen, is de kans aanwezig dat ABE fermentatie in de nabije toekomst opnieuw een industrieel proces wordt.

Resumen en castellano

La crisis del petróleo de los años 70 estimuló la búsqueda de fuentes de energía alternativas al uso de combustibles fósiles. El interés en nuevas fuentes de energía no es solamente económico, sino que está marcado por la creciente concienciación de los efectos negativos del uso de combustibles fósiles para el medio ambiente (por ejemplo, altas emisiones de CO_2 y SO_2, contaminación marina). Las nuevas fuentes de energía han de ser no contaminantes, renovables (que no tengan una naturaleza limitada) y han de poder ser explotadas a nivel local, aliviando la dependencia de terceros países (por ejemplo, la mayoría del petróleo procede del golfo pérsico, un área de gran inestabilidad política). Las energías renovables con mayor potencial son la solar, eólica, e hidráulica, y la derivada de la biomasa. La biomasa, compuesta por residuos agrícolas y forestales, cultivos energéticos y residuos municipales, representa una fuente de energía muy interesante debido a su abundancia y a la posibilidad de ser recolectada a conveniencia.

Los componentes mayoritarios de la biomasa vegetal (también denominada lignocelulosa) son la celulosa, un polímero cristalino de unidades de glucosa, la hemicelulosa, formada por el resto de polímeros no celulósicos compuestos por carbohidratos (o azúcares) como glucosa, xilosa, arabinosa, etc, y la lignina, un polímero de compuestos fenólicos de estructura tridimensional similar a los plásticos. Debido a su alto contenido en carbohidratos, la biomasa vegetal constituye un interesante sustrato para la producción de combustibles (i. e. bioetanol) y otros productos por fermentación. En esta tesis se describen los resultados obtenidos del estudio de la utilización de sustratos lignocelulósicos para la producción de acetona, butanol y etanol llevada a cabo por especies del grupo bacteriano *Clostridium*, la denominada fermentación ABE, un proceso que en la primera mitad del siglo XX constituyó la segunda fermentación a nivel industrial, después de la producción de etanol por levaduras.

El **primer capítulo** de esta tesis expone una introducción general a la fermentación ABE, donde se describen las especies bacterianas productoras, la fisiología de éstas y un resumen de la historia del proceso. Se presta especial atención a los sustratos lignocelulósicos más interesantes y a la degradación microbiana de la celulosa.

En el **segundo capítulo** se hace un estudio del uso de residuos orgánicos domésticos (ROD) recolectados en hogares holandeses como sustrato para la fermentación ABE. Dos muestras diferentes de ROD fueron analizadas, y se observó que glucosa y xilosa son los componentes mas abundantes en la fracción polimérica de este material, posiblemente en forma de celulosa y hemicelulosa, respectivamente. *Clostridium acetobutylicum* fue capaz de crecer y producir ABE en suspensiones acuosas de ROD, aunque para ello sólo utilizó los carbohidratos solubles, y no los polímeros, que quedaron casi intactos. Cuando el sustrato en suspensión acuosa fue pre-hidrolizado con celulasas, la bacteria fue capaz de fermentar los productos de la hidrólisis de forma rápida.

Debido a las propiedades intrínsecas de los sustratos lignocelulósicos, las suspensiones acuosas de éstos resultan viscosas y difíciles de manejar, como se observó en el caso de los ROD. Por ello, para obtener una fermentación más eficiente y simplificar la operación del proceso, se prefiere el uso de hidrolizados solubles al uso de suspensiones acuosas como sustrato. La preparación de hidrolizados solubles requiere el pre-tratamiento de la biomasa para convertirla en una masa homogénea. Posteriormente, los polímeros presentes en el material, especialmente la celulosa y la hemicelulosa, son degradados en sus componentes solubles mediante hidrólisis química o enzimática. Los tratamientos que recibe el material dependen de la composición específica del mismo, y dependiendo de las condiciones empleadas (altas temperaturas, pH extremos,..), es posible que tenga lugar la formación de sustancias tóxicas para los microorganismos. El **capítulo 3** describe la fermentación de hidrolizado de ROD por tres especies diferentes de *Clostridium*. El hidrolizado fue preparado en dos etapas; el ROD fue lavado, secado y tratado por explosión a vapor, y finalmente hidrolizado enzimáticamente por celulasas (enzimas que degradan celulosa). Las tres especies de *Clostridium* crecieron en el hidrolizado, pero la producción de ABE fue muy baja debido a la baja concentración de carbohidratos del sustrato. El hidrolizado fue entonces concentrado 4 veces pero, en este último caso, las bacterias no crecieron. La adición de hidrolizado a cultivos en glucosa resultó tener un efecto negativo en la fermentación, indicando la presencia de sustancias tóxicas en el mismo. Cuando el hidrolizado fue purificado a traves de una columna de intercambio aniónico, una de las especies pudo crecer y producir niveles relativamente altos de ABE a partir del hidrolizado concentrado.

Una alternativa a la hidrólisis enzimática de los sustratos lignocelulósicos, que resulta costosa, es el uso de microorganismos capaces de transformar directamente los polímeros de carbohidratos (especialmente la celulosa) en ABE. Ya que ninguna de las especies productoras de ABE conocidas puede degradar celulosa, técnicas de genética molecular han sido empleadas para producir especies con actividad celulolítica. El **capítulo 4** describe la clonación de 2 genes que codifican dos celulasas distintas procedentes del hongo *Neocallimastix patriciarum* en *C. beijerinckii*. Las especies transformantes obtenidas producen actividad celulasa extracelular y degradan el polímero hemicelulósico lichenan mejor que la especie original. Sin embargo, ninguna de las especies transformantes es capaz, en mono- o en co-cultivo, de degradar celulosa cristalina. Esto puede ser debido a que para una degradación eficiente es necesaria la cooperación de varios tipos de celulasas diferentes, y las producidas por los transformantes no son suficientes. Como se explica en el **capítulo 1**, la degradación de celulosa cristalina en un proceso complejo, en el que diferentes tipos de enzimas con diferentes especificidades actúan de forma sinérgica. En el caso de sustratos menos cristalinos, como es el lichenan, hemos visto que es posible construir especies con

suficiente actividad enzimática para su degradación con sólo añadir un gen. Por el contrario, en el caso de sustratos can alto nivel de cristalinidad, como la celulosa, es muy posible que para desarrollar especies con capacidad hydrolítica suficiente, el número de genes a clonar sea elevado, y en una determinada proporción, lo que significa una tarea muy laboriosa. Por este motivo, en los capítulos siguientes hemos seguido otra estrategia.

Una de las sorpresas del análisis de la secuencia del genoma de la especie *C. acetobutylicum* ATCC 824 fue la existencia de una serie de genes que codifican proteínas relacionadas con la degradación de celulosa, cuando esta bacteria no es capaz de degradar dicho polímero. Especialmente interesante resulta la presencia de genes que codifican los distintos componentes de un complejo multiproteico extracelular especializado en la degradación de celulosa, denominado celulosoma. La organización de dichos genes y las proteínas que codifican son muy similares a las encontradas en otras especies, todas celulolíticas, de *Clostridium*. Diez de los genes que codifican para subunidades del celulosoma están localizadas formando un cluster, en una manera similar a la encontrada en otras especies celulolíticas bien estudiadas.

Tres capítulos de esta tesis están dedicados a estudiar el cluster formado por genes que codifican proteínas celulosomales de *C. acetobutylicum* ATCC 824. En el **capítulo 5** se describe la clonación del gen que codifica la proteína celulosomal que se ancla a la celulosa y une a las unidades enzimáticas de *C. cellulovorans* (una especie celulolítica) en *C. acetobutylicum*. La especie transformante obtenida no fue capaz de degradar celulosa cristalina, indicando que la deficiencia en actividad celulasa en *C. acetobutylicum* no puede ser suplida sólo con añadir un gen para la proteína de adhesión a la celulosa.

La primera parte del **capítulo 6** describe la caracterización una enzima celulosomal de *C. acetobutylicum* producida por *E. coli*. El gen que codifica para esta enzima, que forma parte del cluster del celulosoma, fue copiado del cromosoma de *C. acetobutylicum* y clonado en *E. coli*. La enzima producida, CelG, fue purificada fácilmente haciendo uso de la elevada afinidad por celulosa que le confiere su dominio de adhesión a celulosa. El dominio catalítico de CelG pertenece a la familia 9 de las celulasas, siendo la actividad y el rango de sustratos de esta enzima comparables al de otras enzimas de esta familia. Para determinar si *C. acetobutylicum* produce extracelularmente CelG se llevaron a cabo análisis de Western-blot con anticuerpos generados contra la enzima CelG producida por *E. coli*. Muestras de medio extracelular de cultivos de *C. acetobutylicum* crecidos en diferentes sustratos (glucosa, xilosa, celobiosa y lichenan) fueron sometidas a Western-blot. Sólo en el cultivo crecido en lichenan se detectó una proteína del peso molecular de CelG que reaccionó con el anticuerpo anti-CelG, indicando que este sustrato estimula la producción y secreción de CelG. Como control se utilizaron muestras de medio extracelular de cultivos de *C. cellulovorans*, que

produce proteínas muy similares a CelG. Se observó que el nivel de expresión de proteínas similares a CelG en C. cellulovorans es muy superior al de CelG en *C. acetobutylicum*. Es posible que la diferencia de actividad celulolítica entre estas dos especies esté relacionada con la diferencia observada entre los niveles de expresión de celulasas, pero este hecho ha de ser confirmado.

Otro gen del cluster del celulosoma, en este caso *celF*, ha sido estudiado en el **capítulo 7**. Este gen es el único que codifica para una celulasa de la familia 48, que incluye enzimas con alta actividad sobre celulosa cristalina, en todo el cromosoma de *C. acetobutylicum*. Se considera que celulasas de esta familia juegan un papel muy importante en la función del celulosoma. El gen *celF* fue clonado en *E. coli*, y la proteína correspondiente fue producida en altos niveles, pero siempre de forma insoluble e inactiva. Por este motivo no fue posible determinar las características de CelF. En un experimento paralelo al descrito en el **capítulo 6**, la presencia de CelF en medio extracelular de cultivos de *C. acetobutylicum* fue estudiada por medio de Western-blot con anticuerpos generados contra CelF producida por *E. coli*. En cultivos crecidos en lichenan, xilosa o celulosa (suplementada con celulasas) se observó una mayor cantidad de CelF que en los crecidos en glucosa o celobiosa. En los **capítulos 6 y 7** se presentan también datos de actividades (hemi)celulolíticas extracelulares en los cultivos estudiados. Las actividades determinadas en los cultivos en lichenan, seguidos por los de xylosa son las más altas, indicando que este sustrato induce la producción extracelular de (hemi)celulasas. Un nivel muy bajo, pero reproducible, de actividad sobre celulosa cristalina pudo ser detectado en cultivos en lichenan usando muestras muy concentradas.

Los resultados expuestos en esta tesis demuestran que la clonación de genes apropiados en especies de *Clostridium* productoras de ABE puede dar lugar a la creación de especies con mayor capacidad de degradación de polisacáridos. Esto implica que manipulación genetica puede ser usada para desarrollar especies que crezcan en un determinado sustrato de interés. Por otro lado, hemos establecido que *C. acetobutylicum*, aunque incapaz de crecer en celulosa, produce dos de las subunidades enzimáticas del celulosoma. Estos resultados representan nuevas perspectivas en el uso de ingeniería genética para el mejoramiento de las especies productoras de ABE. La combinación de los resultados de los numerosos estudios realizados durante las últimas décadas sobre la fisiología de la fermentación y las mejoras en ingeniería del proceso, y el incremento en el conocimiento de la genética de las especies, representan importantes avances hacia un proceso económicamente viable. Actualmente, en los países desarrollados hay un importante apoyo económico y social al uso de energías renovables para la producción de químicos y combustibles, por lo que la fermentación ABE podría volver a ser un proceso industrial en un futuro cercano.

Curriculum Vitae

Ana María López Contreras was born on the 19th of October of 1971 in Cartagena (Murcia, Spain). After moving from Los Nietos to Los Alcázares in 1982, she studied BUP and COU (Secondary school) at the Instituto de Bachillerato Luis Manzanares in Torre Pacheco. From 1989 to 1992 she completed the first cycle of the degree in Chemistry at the University of Murcia, where afterwards she followed the second cycle of the degree in Biochemistry, graduating in 1994. From January to September 1995 she carried out a practical training period at the ATO in Wageningen (The Netherlands) in the Immunochemical Technology group, at that time, leaded by Dr. Aart van Amerongen. There she studied the antifungal and antibacterial activity of synthetic peptides derived from eukariotic enzymes. Back in Spain, she carried out a Tesina de Licenciatura at the Dept. of Bioquímica, Biología Molecular B e Inmunología of the University of Murcia, on studies on the melanogenesis of mouse melanoma cells in culture. This work, directed by Dr. Francisco Solano Muñoz and Dr. José Hilario Martínez Liarte, was defended succesfully in June 1996. Soon after that, she started her PhD reseach at the ATO in the Bioconversion Group under the supervision of Dr. Pieternel Claassen, Dr. John van der Oost and Prof. Dr. Willem M. de Vos, on the genetic improvement of the acetone, ethanol and butanol fermentation. This research was continued at the Laboratory of Microbiology from January 2000 until March 2002, and the results obtained are described in this thesis. From April 2002, Ana is back at the ATO, where she works as a researcher at the Bioconversion group.

List of publications

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Caminante

Caminante, son tus huellas
el camino, y nada más;
caminante no hay camino,
se hace camino al andar.
Al andar se hace camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de pisar.
Caminante, no hay camino,
sino estelas en la mar.

Antonio Machado (1875-1939)

Caminante (Walker)

Walker, your foosteps are
the road, and nothing more;
walker, there are no roads,
you make the road while you walk.
While walking the road is made,
and when looking backwards
you see the road where you will never
step again.
Walker, there is no road,
only trails in the sea.

Antonio Machado (1875-1939)

Propositions

1. The success of (raw) lignocellulosic substrates for production of solvents by fermentation depends not only on the ability of the solventogenic organisms to degrade them, but also on the technology to process them.

2. Constructing a new cellulolytic organism is more difficult than constructing a new hemicellulolytic organism.

3. The predictions made by bioinformatics are very valuable, but they need to be confirmed by experimental data.

4. The conclusion by Nair and co-workers[1] that the orf5 (SolR) in the pSOL1 megaplasmid was a regulator of the *sol* operon turned out to be not correct. However, their results were very valuable to elucidate the mode of action of the transcriptional regulator Spo0A on the solventogenesis and to postulate the existence of an additional activator specific for the *sol* operon [2].

   [1]**Nair et al.** (1999). Regulation of the *sol* locus genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. 176, p. 319-330; [2]**Thormann et al.** (2002). Control of butanol formation in *Clostridium acetobutylicum* by transcriptional activation. J. Bacteriol. 184, p. 1966-1973.

5. The Wageningen University does not apply its knowledge on healthy food and eating habits in its own canteens.

6. The command "Free walking on roads and paths" is typical for the organized Dutch society.

7. Spain has been a (touristic) mass destination since the 10th century, when the roads to Santiago de Compostela were as full as nowadys "l'autoroute du Soleil" in high season.

Propositions belonging to the thesis "Utilization of lignocellulosic substrates by solvent-producing clostridia", by Ana López Contreras, to be defended April 4th 2003 in Wageningen.

Stellingen

1. Het succesvolle gebruik van lignocellulose substraten om oplosmiddelen te produceren door fermentatie hangt niet alleen af van de geschiktheid van betreffende organismen of deze af te breken, maar ook van de technologie om ze te verwerken.

2. Het is moeilijker om een nieuw cellulolytisch organisme te construeren dan een nieuw hemicellulolytisch organisme.

3. Voorspellingen van bioinformatici zijn zeer waardevol, maar moeten wel bevestigd worden door experimentele gegevens.

4. De conclusie van Nair e.al.[1] dat de orf5 (SolR) in het pSOL1 megaplasmide een regulator was van de *sol* operon, bleek niet correct te zijn. Maar hun resultaten waren zeer waardevol bij het vinden van de functie van de transcriptionele regulator Spo0A op de solventogenesis en doen het bestaan vermoeden van een extra activator, specifiek voor het *sol* operon[2].

   [1]**Nair et al.** (1999). Regulation of the *sol* locus genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. 176, p. 319-330; [2]**Thormann et al.** (2002). Control of butanol formation in *Clostridium acetobutylicum* by transcriptional activation. J. Bacteriol. 184, p. 1966-1973.

5. De Wageningen Universiteit past haar kennis over gezond voedsel en gezonde eetgewoontes niet toe in de eigen kantines.

6. Het gebod "Vrij wandelen op wegen en paden" is typisch voor de georganiseerde Nederlandse maatschappij.

7. Spanje is al een (toeristische) massabestemming sinds de 10[e] eeuw, toen de wegen naar Santiago de Compostela zo vol waren als tegenwoordig "l'autoroute du Soleil" in het hoogseizoen.

Stellingen behorende bij het proefschrift getiteld "Utilization of lignocellulosic substrates by solvent-producing clostridia", door Ana López Contreras, in het openbaar te verdedigen op 4 april 2003, te Wageningen.