Chapter 6

Lignocellulosic Biomass Utilization Toward Biorefinery Using Meshophilic Clostridial Species

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Additional information is available at the end of the chapter

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1. Introduction

Lignocellulosic biomass such as agricultural, industrial, and forestry residues as well as dedicated crops constitute renewable and abundant resources with great potential for a low-cost and uniquely sustainable bioconversion to value-added bioproducts. Thus, many organic fuels and chemicals that can be obtained from lignocellulosic biomass can reduce greenhouse gas emissions, enhance energy security, improve the economy, dispose of problematic solid wastes, and improve air quality. In particular, liquid biofuels are attractive candidates, since little or no change is needed to the current petroleum-based fuel technologies. However, the biorefining process remains economically unfeasible due to a lack of biocatalysts that can overcome costly hurdles such as cooling from high temperature, pumping of oxygen/stirring, and, neutralization from acidic or basic pH. Therefore, bioconversion of the lignocellulosic components into fermentable sugars is an essential step in the biorefinery.

In nature, a variety of microorganisms including bacteria and fungi have the ability to degrade lignocellulosic biomass to C-5 and/or C-6 sugars. Moreover, new concepts have been proposed to enable the overall goal of cost reduction. These include genetically modifying the cell wall composition of energy crops in order to make their conversion easier, and combining the processes of glycoside hydrolases (GHs) and polysaccharide lyases (PLs) production, saccharification, and fermentation. Several clostridial species produce an extracellular enzyme complex called the cellulosomes and free extracellular enzymes called non-cellulosomes [1,2]. The cellulosomes are particularly designed for efficient degradation of plant cell wall polysaccharides such as cellulose, hemicellulose, and pectins. The component parts of the multi-component complex are integrated by virtue of a unique family of integrating modules, the cohesins and the dockerins, whose distribution and specificity dictate the overall cellulosome architecture. On the other hand, several

clostridial species are able to ferment carbohydrates to acetone, butanol, and ethanol (ABE). Industrial application of this process, also known as ABE fermentation, has a long history, but the process economics after 1960 became unfavorable compared to the petrochemical process, and its commercial exploitation was gradually abandoned. The inefficiency of the fermentation still hampers commercial reintroduction of this renewable butanol production process. However, improving the yields and productivities of the solvent products is key to its successful reintroduction.

2. Solvent-producing clostridia

Biological production of butanol (*n*-butanol, 1-butanol) has a long history as an industrially significant fermentation process [3]. An excellent review article by Jones and Woods on the history of acetone–butanol–ethanol (ABE) fermentation processes is available [4]. After Pasteur discovered bacterial butanol production from his landmark anaerobic cultivation in 1861, fermentative ABE production prospered during the early 20th century, and after ethanol became the second largest industrial fermentation process in the world. In 1945, two thirds of industrially used butanol was produced by fermentation in U.S. However, the ABE fermentation process lost competitiveness by the 1960s due to the increase of feedstock costs and advancement of the petrochemical industry except in Russia and in South Africa, where the substrate and labor costs were low. The ABE fermentation processes in South Africa and Russia continued to operate until the late 1980s and early 1990s [5]. It has recently been reported that the Russian fermentation industry is concentrating on the conversion of agricultural biomass into butanol[5]. The successful industrial-level butanol fermentation in these countries can provide guidelines to our current efforts to produce butanol in large-scale. Commercial solvent titres peak at about 20 g/L from 55 to 60 g/L of substrate, resulting in solvent yields of approx. 0.35 g/g sugar consumed [6]. The butanol:solvent molar ratio is typically 0.6 with an A:B:E ratio of 3:6:1[4]. *C. acetobutylicum* strain EA2018 was also developed using chemical mutagenesis and found to produce higher butanol:solvent ratios (0.7) than the parental strain (0.6) [7]. This strain has been licensed to several commercial producers in China (GBL market data). The acetone pathway has also been knocked out in this strain resulting in higher butanol:solvent ratios (0.8) but no overall increase in higher butanol titre was observed [8]. Butanol is the preferred solvent since it attracts the highest price in the chemical market. Between butanol and ethanol, butanol is a choice of fuel as compared to ethanol, mainly because of its higher energy density, lower volatility and reduced corrosiveness. In addition, butanol has relatively better compatibility for current car engines and infrastructures, offering more convenience and versatility in applications [9,10]. Thus, butanol production from lignocellulosic materials has attracted much attention from contemporary researchers in the discipline of bioenergy.

Several clostridial species such as *Clostridium acetobutylicum*, *C. beijerinckii*, *C. pasteurianum*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* are known to be solventogenic, producing acetone, butanol, and ethanol, but they present relatively low tolerance to butanol [5,11,12]. Among wild-type clostridial species, typical end concentrations of butanol are around 12 g/L from fermentation of glucose [12]. The fermentation efficiency was

reported to be hampered due to the accumulated butanol (e.g., >7.4 g/L) [12], which could lead to cell growth inhibition and premature cessation of fermentation [13]. Such negative inhibition leads to low achievable butanol concentration and will thus increase the downstream costs associated with product purification [13]. Attempts have been made to improve the butanol concentration up to 17.8 g/L by genetically manipulating the wild-type clostridial species [12]. Nevertheless, genetically modified bacteria are usually unstable due to plasmid excision [14], leading to the deterioration of butanol-producing capability within batches of experiments. Hence, the search for novel and enhanced wild-type microbes with improved butanol tolerance is of great necessity for industrial applications [15].

3. Metabolic engineering of mesophilic clostridia

Synthetic biology has recently been used to introduce biosynthetic capacity for butanol into non-natural hosts. The choice between using or engineering natural function versus importing biosynthetic function has been reviewed [16]. Commonly used host strains include *Escherichia coli* and *Saccharomyces cerevisiae* that are relatively easy to genetically manipulate but do not tolerate more than 2% 1-butanol [17]. In addition, these strains do not display broad substrate ranges and cannot compete with natural or engineered clostridia for the production of 1-butanol from a broad range substrates including pentose sugars and sugars derived from cellulosic feedstocks.

For successful metabolic engineering of *C. acetobutylicum*, it is necessary to have efficient genetic engineering tools for metabolic pathway manipulation. In 2001, the complete genome sequence of *C. acetobutylicum* was published [18]. The *C. acetobutylicum* ATCC 824 genome consists of a 3.94 Mbp chromosome and a 192,000 bp megaplasmid pSOL1. A total of 3,740 and 178 ORFs were identified on the chromosome and megaplasmid, respectively. *C. acetobutylicum* has distinctive families of proteins involved in sporulation, anaerobic energy conversion, and carbohydrate degradation, which are well matched to the physiological characteristics of *C. acetobutylicum*. For butanol formation, two mechanisms have been identified in this strain; one is related to solventogenesis (ABE forming process) and the other is alcohologenesis (butanol and ethanol forming process). The key genes involved in solventogenesis are shown in **Figure 1A**. The genes involved in alcohologenesis remain unidentified. It is currently believed that the enzymes encoded by the *adhE* (aldehyde/alcohol dehydrogenase; CAP0035), *pdc* (pyruvate decarboxylase; CAP0025), and *edh* (ethanol dehydrogenase; CAP0059) genes are associated with this metabolism [12].

Several *Bacullus subtilis*–*C. acetobutylicum* and *E. coli*–*C. acetobutylicum* shuttle vectors were developed in the early 1990s [19,20]. Mermelstein et al. made a breakthrough in metabolic engineering of *C. acetobutylicum* ATCC 824 [21]. Since *C. acetobutylicum* ATCC 824 possesses a strong restriction system encoded by Cac824I (recognizing 50-GCNGC-30), which prevents efficient transformation of recombinant plasmid prepared in *E. coli*. Thus, they developed a *B. subtilis*–*C. acetobutylicum* shuttle vector pFNK1, which allowed higher transformation efficiency. Using this shuttle vector, the acetoacetate decarboxylase (*adc*), and the phosphotransbutyrylase (*ptb*) genes were successfully expressed at elevated levels in strain

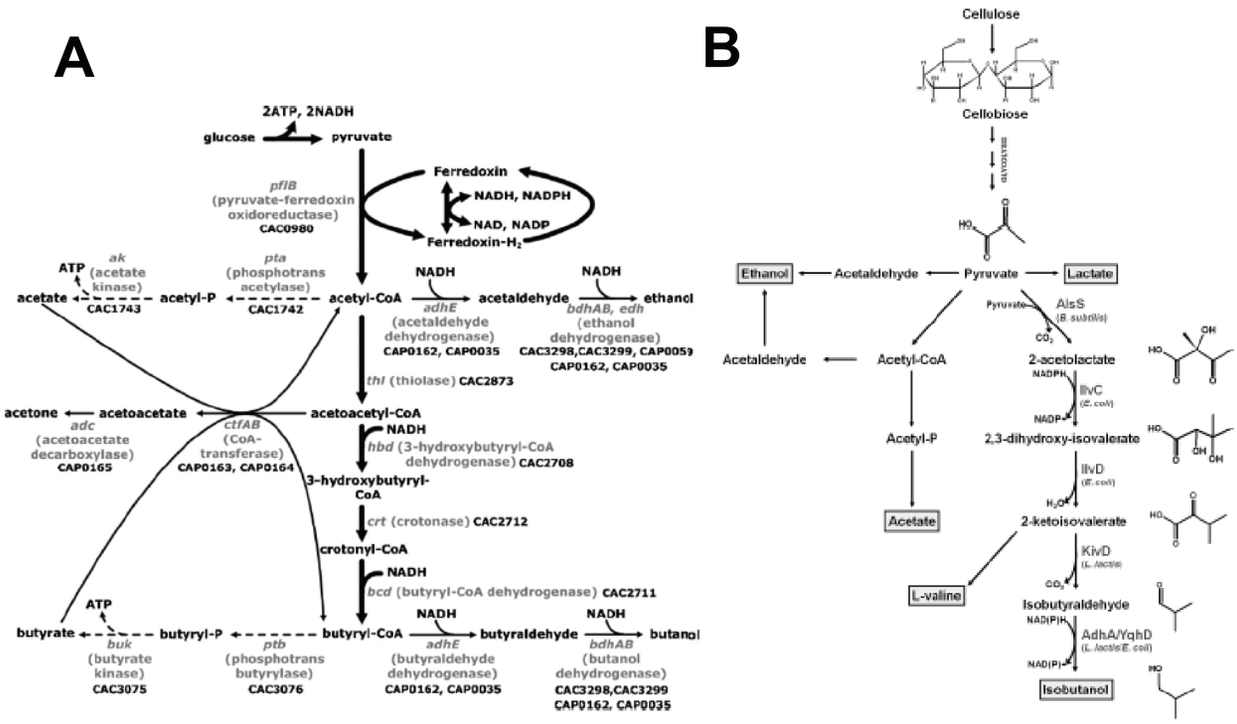

Figure 1. (A) Metabolic pathways in *C. acetobutylicum* [3]. Reactions which predominate during acidogenesis and solventogenesis are indicated by dotted and solid arrows, respectively. Thick arrows indicate reactions which activate the whole fermentative metabolism. Gray letters indicate genes and enzymes for the reactions. CAC and CAP numbers are the ORF numbers in genome and megaplasmid, respectively. (B) The pathway for isobutanol production in *C. cellulolyticum* [59] from cellulose. In order to achieve direct isobutanol production from pyruvate, the genes encoding *B. subtilis* α-acetolactate synthase, *E. coli* acetohydroxyacid isomeroreductase, *E. coli* dihydroxy acid dehydratase, *Lactococcus lactis* ketoacid decarboxylase, and *E. coli* and *L. lactis* alcohol dehydrogenases were cloned, respectively.

ATCC 824. The development of an *in vivo* methylation system was an important step [22]. Methylation of the shuttle vectors with w3TI methyltransferase (encoded by *B. subtilis* phage w3T) prior to transformation greatly reduces or prevents the degradation of the transforming plasmid DNA by the attack of a strong restriction system (Cac824I) present in *C. acetobutylicum* [22]. The copy number of commonly used plasmids in *C. acetobutylicum* is around 7–20 copies per cell, which seem to be suitable for metabolic engineering purposes [23]. Significant advances for *C. acetobutylicum* have been made to methods for gene integration [24]. Superior performance has also been demonstrated from genetically engineered derivatives of *C. acetobutylicum* ATCC 824 [25,26]. Methods based on a group II intron system for gene knockout have been described [27,28]. More recently an improved method, based on allele coupled exchange (ACE), has been described for stable integration of larger DNA fragments [29]. It is now possible to construct multi-step biosynthetic pathways paving the way for new synthetic clostridia.

Isobutanol is a more promising fermentation product because it is less toxic than 1-butanol. Unlike ethanol, isobutanol can also be blended at any ratio with gasoline or used directly in current engines without modification [30]. It is an attractive biofuel but cannot substitute for

1-butanol in the chemical market. One synthetic approach for isobutanol production involves the introduction of genes encoding enzymes that convert either acetyl-CoA or pyruvate to isobutanol. Alternatively, genes encoding enzymes that convert 2-keto acids intermediates (from amino acid synthesis) into isobutanol and branched-chain alcohols; 2-methyl-1-butanol and 3-methyl-1-butanol can be introduced [31,32,33]. Several companies are currently involved in scale-up and demonstration. Gevo Inc. (http://www.gevo.com) has engineered *E. coli* to produce isobutanol [34] and recently acquired a commercial-scale ethanol plant in Minnesota for retrofit to produce isobutanol. The company has also received Environmental Protection Agency certification to blend isobutanol in fossil fuels. DuPont has also engineered several biocatalysts for isobutanol [35] and assigned the technology to Butamax™ Advanced Biofuels (http://www.butamax.com), a joint venture between BP and Dupont. Butamax™ is collaborating with Kingston Research Limited, another BP–Dupont joint venture, to build a demonstration plant in the UK. Previously, the cellulosome-producing *C. cellulolyticum* has also been genetically engineered for improved ethanol production [36]. With this respect, most of the research concerning the construction of an organism for consolidated bioprocessing has focused on ethanol production. Despite this, it has been asserted that higher alcohols (i.e., alcohols with more than two carbons), such as isobutanol, are better candidates for gasoline replacement because they have energy density, octane value, and Reid vapor pressure that are more similar to those of gasoline [37].

4. Cellulosome-pruducing *CLostridium cellulovorans*

The anaerobic clostridia are found in the soil, on decaying plant materials, in rumens, in sewage sludge, in termite gut, in wood-chip piles, in compost piles, and at paper mills and wood processing plants (**Table 1**). Most of these bacteria occur in natural habitats such as soil and decaying plant materials, but some are enriched by human activities, such as in compost piles, in sewage plants, and at wood processing plants. Other natural habitats include the anaerobic rumen of various ruminants and the gut of termites, where they process plant materials for the host organism's nutrition. The biotechnological potential of polysaccharolytic enzymes has resulted in the isolation and characterization of a large number of anaerobic, Gram-positive, spore-forming bacteria, the majority of which have been allocated to the genus *Clostridium*. Among some clostridia, the cellulosomes produced by *Clostridium* species are particularly designed for efficient degradation of plant cell wall polysaccharides. The component parts of the multicomponent complex are integrated by virtue of a unique family of integrating modules, the cohesins and the dockerins (**Fig. 2A**), whose distribution and specificity dictate the overall cellulosome architecture [38]. The cellulosomes are characterized by the presence of two general components: (1) the nonenzymatic scaffolding protein(s) with enzyme-binding sites called cohesins and (2) a variety of cellulosomal enzymes with dockerins, which interact with the cohesins in the scaffolding protein.

Since 2002, over 100 genome sequencing projects of *Clostridium* species have been done or are being done mainly by the United States Department of Energy Joint Genome Institute

Species	Habitat
*Clostridium acetobutylicum**	Soil
Clostridium aldrichii	Wood digester
Clostridium cellobioparum	Soil
Clostridium cellulofermentans	Soil
*Clostridium cellulolyticum**	Rot grass
*Clostridium cellulovorans**	Wood chips
Clostridium herbivorans	Pig intestine
Clostridium hungate	Soil
Clostridium josui	Compost
Clostridium papyrosolvens	Paper mill
*Clostridium thermocellum**	Compost, Soil

Single asterisk (*), species whose genome sequencing is complete.

Table 1. Cellulolytic clostridial species from natural biomass decaying ecosystems

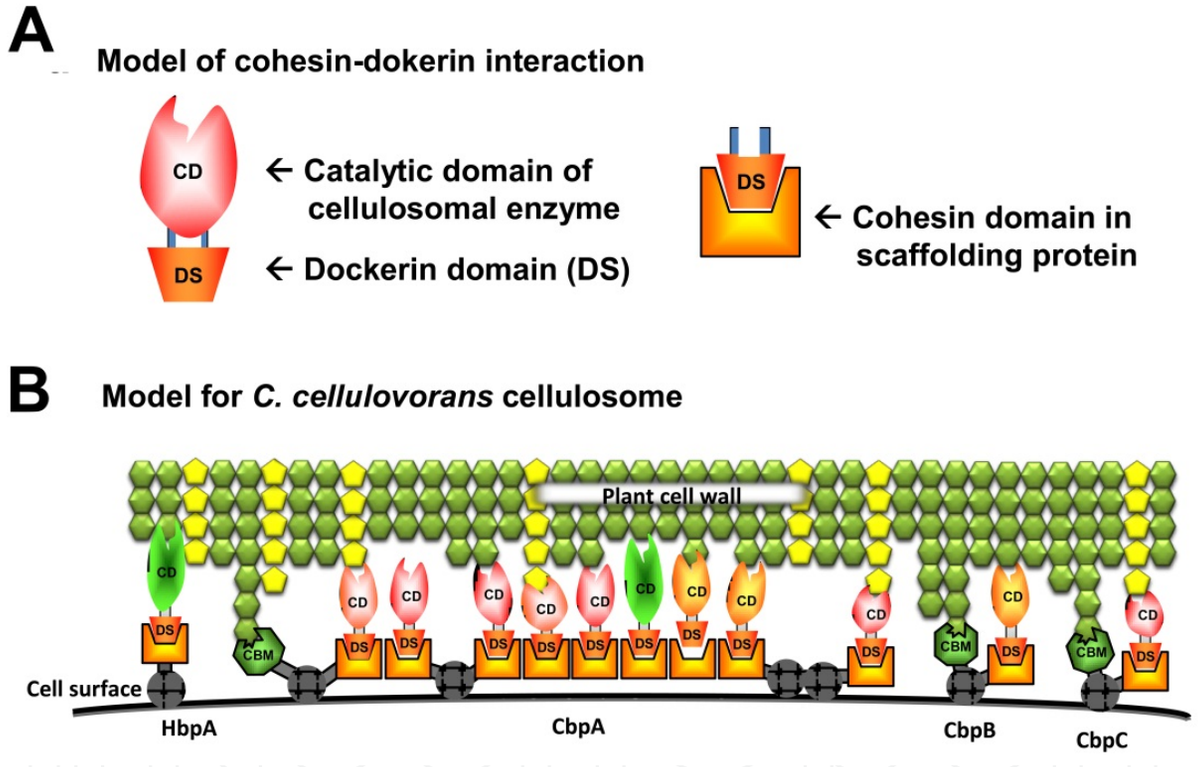

Figure 2. Model for *C. cellulovorans* cellulosomes. (A) Model of cohesin–dockerin interaction. (B) Recent model of cellulosomes attached to its substrate and cell surface.

(DOE-JGI). The whole genome sequences of cellulosome-producing *Clostridium* species, i.e., thermophilic *C. thermocellum* ATCC27405 and mesophilic *C. cellulolyticum* H10 were sequenced by the JGI in 2007 and 2009, respectively. In 2009 the complete genome of *C. cellulovorans* was sequenced using the next-generation DNA sequencers to compare not only cellulosomal genes but also noncellulosomal ones among cellulosome-producing clostridia [39]. *C. cellulovorans* is able to degrade native substrates in soft biomass such as corn fiber and rice straw efficiently by producing the cellulosomes. The whole genome sequence of *C.*

cellulovorans comprised 4,220 predicted genes in 5.10 Mbp. As a result, the genome size of *C. cellulovorans* was about 1 Mbp larger than that of other cellulosome-related clostridia, mesophilic *C. acetobutylicum* and *C. cellulolyticum*, and thermophilic *C. thermocellum*. A total of 57 cellulosomal genes were found in the *C. cellulovorans* genome (**Table 2**) and coded for not only CAZymes but also lipases, peptidases, and proteinase inhibitors [40,41]. Cellulosomal genes among clostridial genomes were identified and classified as cohesin-containing scaffolding proteins and dockerin-containing proteins. So far, the scaffolding proteins for constructing cellulosomes were found in *C. acetobutylicum* [42], *C. cellulolyticum* [43], *C. cellulovorans* [44], *C. josui* [45], and *C. thermocellum* [46].

Organism	GenBank Accession No.	Genome size (Mb)	No. of genes	No. of cellulosomal genes	% GC
C. cellulovorans 743B	DF093537-DF093556	5.10	4220	57	31.1
C. acetobutylicum ATCC 824	AE001437	3.94	3672	12	30.9
C. cellulolyticum H10	CP001348	4.07	3390	65	37.4
C. thermocellum ATCC 27405	CP000568	3.84	3191	84	39.0

Table 2. General features of cellulosomal clostridial genomes compared with that of *C. cellulovorans*

Among a total of 57 cellulosomal genes of the *C. cellulovorans* genome, 53 dockerin-containing proteins and four cohesin-containing scaffolding proteins were found, respectively [40]. More interestingly, two scaffolding proteins, CbpB and CbpC, consisting of a carbohydrate-binding module (CBM) of family 3, a surface–layer homology domain and a cohesin domain, were recently found and tandemly localized in the *C. cellulovorans* genome, while there were no such scaffolding proteins in other cellulosomal clostridia. Thus, by examining genome sequences from multiple *Clostridium* species, comparative genomics offers new insight into genome evolution and the way natural selection molds functional DNA sequence evolution. A recent model for the *C. cellulovorans* cellulosome reveals that the enzymatic subunits are bound to the scaffolding through the interaction of the cohesins and dockerins to form the cellulosome (**Fig. 2B**).

Carbohydrate-active enzymes (CAZymes) are categorized into different classes and families in the CAZy database (for more information please visit the CAZy web page; www.cazy.org). CAZymes that cleave, build, and rearrange oligo- and polysaccharides play a central role in the biology of bacteria and fungi and are key to optimizing biomass degradation by these species. Currently, more than 2,500 GHs have been identified and classified into 115 families [47]. Interestingly, the same enzyme family may contain members from bacteria, fungi, and plants with several different activities and substrate specifications [48]. However, fungal cellulases (hydrolysis of β-1,4-glycosidic bonds) have been mostly found within a few GH families including 5, 6, 7, 8, 9, 12, 44, 45, 48, 61, and 74 [47,49]. Cellulases have a small independently folded CBM that is connected to the catalytic domain by a flexible linker [48]. The CBMs are responsible for binding the enzyme to the crystalline cellulose, and thus enhance the enzyme activity [38]. Currently, many CBMs have been

identified and classified into 54 families; however, only 20 families (1, 13, 14, 18, 19, 20, 21, 24, 29, 32, 35, 38, 39, 40, 42, 43, 47, 48, 50, and 52) have been found in fungi. Among 53 cellulosomal genes encoding dockerin containing proteins in the *C. cellulovorans* genome, a total of 29 genes coded for cellulolytic, hemicellulolytic and pectin-degrading enzymes [40]. Compared with the genome-sequenced species within cellulosomal clostridia, the proteome of *C. cellulovorans* focusing on dockerin-containing proteins showed representation of many proteins with known functions. In the *C. cellulovorans* cellulosome, there are 16 cellulase genes belonging to families GH5, GH9 and GH48, six mannanase genes belonging to families GH5 and GH26, three xylanase genes belonging to families GH8, GH10 and GH11, an endo-beta-galactosidase gene belonging to family GH98, and two pectate lyase genes belonging to families PL1 and PL9.

5. Cellulose metabolism of *C. acetobutylicum*

Cellulosomal gene clusters were conserved only in mesophilic clostridia (**Fig. 3**) [40]. Furthermore, these cellulosomal genes were randomly distributed in the *C. cellulovorans* genome except for the cellulosomal genes related to a large cellulosomal cluster, whereas two large cellulosomal gene clusters were found in the *C. cellulolyticum* genome. Even though the organization of genes encoding cellulosome subunits differs among mesophilic cellulolytic clostridia, there is nonetheless a clear similarity, particularly when looking at the cluster of genes following the main scaffoldin gene. Such a cluster is not found in *C. thermocellum*. This would suggest that the cellulosomes of the mesophilic clostridia, including the 'ghost' cellulosome of *C. acetobutylicum*, may have arisen from a common ancestral gene cluster. However, attempts have been made to develop a *C. acetobutylicum* strain that can utilize cellulose directly. There is evidence that *C. acetobutylicum* ATCC 824 can produce an active cellulosome. The *celF* gene, encoding a unique cellulase, was found to be up-regulated in *C. acetobutylicum* ATCC 824 during growth on xylose or lichenan [50]. However, *C. acetobutylicum* ATCC 824 had no cellulolytic activity suggesting that some element of the cellulosome is missing or not expressed. In an effort to make *C. acetobutylicum* utilize cellulose more directly, the *engB* gene from *C. cellulovorans* or the gene encoding the scaffold protein from *C. cellulolyticum* and *C. thermocellum* were introduced into *C. acetobutylicum*. However, the level of expressed heterologous cellulase was rather low [51,52]. On the other hand, the *man5K* gene encoding the mannanase Man5K from *C. cellulolyticum* was cloned alone or as an operon with the gene *cipC1* encoding a truncated scaffoldin (miniCipC1) of the same origin in the solventogenic *C. acetobutylicum* [53]. The recombinant strains of the solventogenic bacterium were both found to secrete active Man5K in the range of milligrams per liter. In the case of the strain expressing only *man5K*, a large fraction of the recombinant enzyme was truncated and lost the N-terminal dockerin domain, but it remained active towards galactomannan. When *man5K* was coexpressed with *cipC1* in *C. acetobutylicum*, the recombinant strain secreted almost exclusively full-length mannanase, which bound to the scaffoldin miniCipC1, thus showing that complexation to the scaffoldin stabilized the enzyme. Moreover, the secreted heterologous complex was found to be functional: it binds to crystalline cellulose via the carbohydrate-binding module

of the miniscaffoldin, and the complexed mannanase is active towards galactomannan. Taken together, these data showed that *C. acetobutylicum* is a suitable host for the production, assembly, and secretion of heterologous minicellulosomes. More studies are needed to characterize the existing cellulosomal gene cluster in *C. acetobutylicum* before further metabolic engineering.

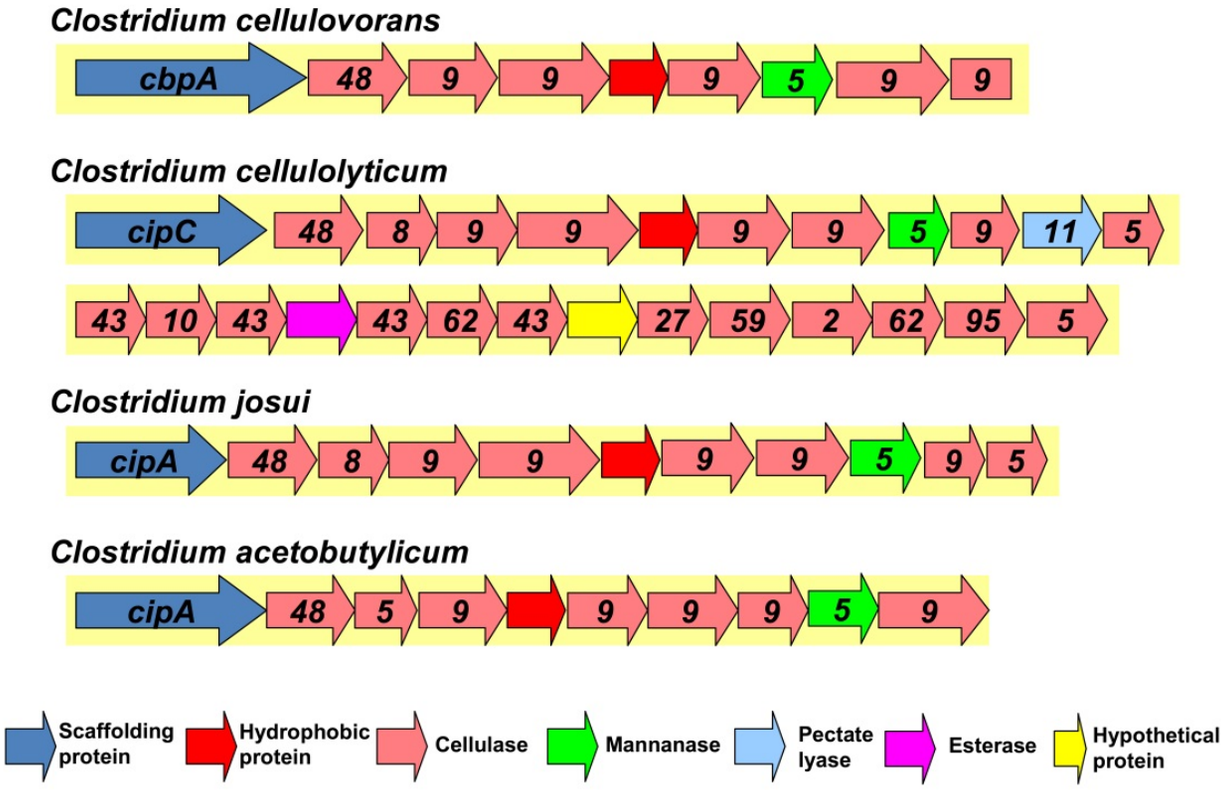

Figure 3. Cellulosome-related gene clusters in the genome of mesophilic clostridia.

6. Consolidated bioprocessing by Clostridial species

Consolidated bioprocessing, or CBP, the conversion of lignocellulose into desired products in one step without added enzymes, has been a subject of increased research effort in recent years [54]. Naturally occurring cellulolytic microorganisms are starting points for CBP organism development via the native strategy, with anaerobes being of particular interest [55]. The primary objective of such developments is to engineer product yields and titers to satisfy the requirements of an industrial process. Metabolic engineering of mixed-acid fermentations in relation to these objectives has been successful in the case of mesophilic, non-cellulolytic, enteric bacteria [56]. Far more limited work of this type has been undertaken with cellulolytic bacteria, primarily because of the absence of suitable gene-transfer techniques. Recent developments, however, appear to be removing this limitation for some organisms.

The lack of efficient genetic engineering tools including a gene knock-out system for *C. acetobutylicum* has hampered further strain improvement for a long time. As described

earlier, much effort is exerted to develop genetic engineering tools for clostridia. In the mean time, Liao and collaborators recently reported metabolic engineering of *E. coli* for butanol production [57]. The mutant *E. coli* BW25113 (Δ*adhE* Δ*ldhA* Δ*frdBC* Δ*fnr* Δ*pta*) strain overexpressing the *crt*, *bcd*, *etfAB*, *hbd* and *adhE2* genes of *C. acetobutylicum*, and *atoB* gene of *E. coli* was able to produce 552 mg/L butanol using 2% (w/v) glycerol as a carbon source. In another case, *E. coli* JM109 strain overexpressing the *crt*, *bcd*, *etfAB*, *hbd*, *adhE* and *thiL* genes of *C. acetobutylicum* was developed. This engineered *E. coli* strain was able to produce 16 mM butanol using 4% (w/v) glucose as a carbon source [58]. More recently, metabolic engineering has been used for the development of *C. cellulolyticum* H10 for isobutanol synthesis directly from cellulose [59] (**Fig. 1B**). In this study, by expressing enzymes that direct the conversion of pyruvate to isobutanol using an engineered valine biosynthesis pathway, the recombinant *C. cellulolyticum* was able to produce up to 660 mg/liter of isobutanol when grown on crystalline cellulose. To our knowledge, this was the first demonstration of isobutanol production directly from cellulose.

Butanol production from crystalline cellulose by co-cultures of the thermophilic and cellulosome-producing *C. thermocellum* and the mesophilic and butanol-producing *C. saccharoperbutylacetonicum* (strain N1-4) has been reported recently [60]. Butanol was produced from Avicel cellulose after it was incubated with *C. thermocellum* for at least 24 h at 60°C before the addition of the solventogenic strain N1-4. Butanol produced by strain N1-4 on 4% Avicel cellulose peaked (7.9 g/liter) after 9 days of incubation at 30°C, and acetone was undetectable in this coculture system. Less butanol was produced by *C. acetobutylicum* and *C. beijerinckii* in co-culture with *C. thermocellum* under the same conditions than by strain N1-4, indicating that strain N1-4 was the optimal strain for producing butanol from crystalline cellulose in this system.

7. Conclusion

It should be noted that one of the most critical factors not only for biofuel production but also for the whole biomass biorefinery concept is securing low price substrates for the processes. To compete with the conventional fossil resource-based chemical industry, the biotechnology industry needs a reliable, cost-effective raw materials infrastructure. The cost effectiveness of biomass production and the efficient storage and transport of harvested biomass resources will be critical elements for securing raw materials. Environmental impacts and sustainability are also important issues. There is a cautious prediction that agricultural crop production may not match future industrial demand. A significant amount of research has been dedicated to engineering organisms that are capable of consolidated bioprocessing (CBP). These CBP organisms are anticipated to have the ability to efficiently degrade lignocellulose, and to convert the resulting sugars to biofuels and chemical compounds at high productivities. Towards this goal, the production of biorefinery products from lignocellulose has been shown to be feasible using mesophilic clostridia. Both the successes and problems encountered in establishing new pathways in clostridial species will aid in the adaptation of the consolidated bioprocessing strategy in related mesophilic clostridial species such as *C. acetobutylicum* and *C. cellulovorans.*

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