

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,400

Open access books available

117,000

International authors and editors

130M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Lignocellulosic Biomass Utilization Toward Biorefinery Using Mesophilic Clostridial Species

Yutaka Tamaru and Ana M. López-Contreras

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/56480>

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 butanol⁵. 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 3:6:1⁴. *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 *Bacillus 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

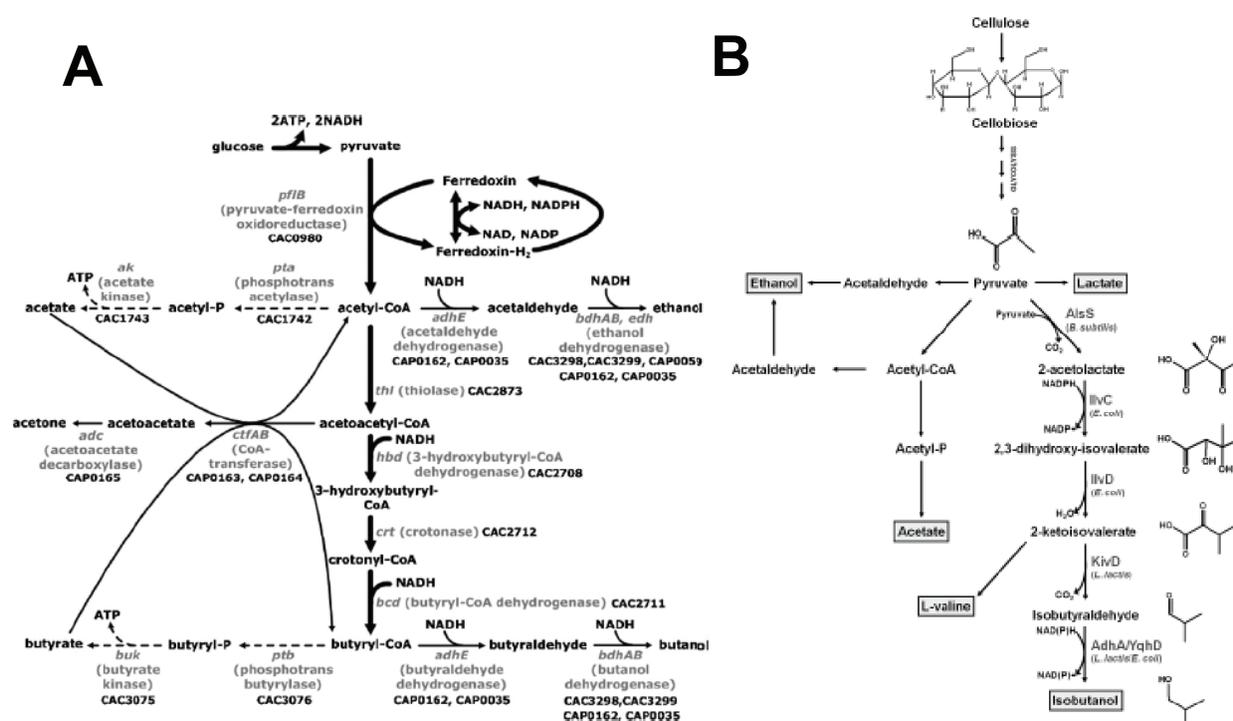


Figure 1. (A) Metabolic pathways in *C. acetobutylicum* [3]. Reactions which predominate during acidogenesis and solventogenesis are indicated by dotted and solid arrows, respectively. Thick arrows indicate reactions which activate the whole fermentative metabolism. Gray letters indicate genes and enzymes 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-producing *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.

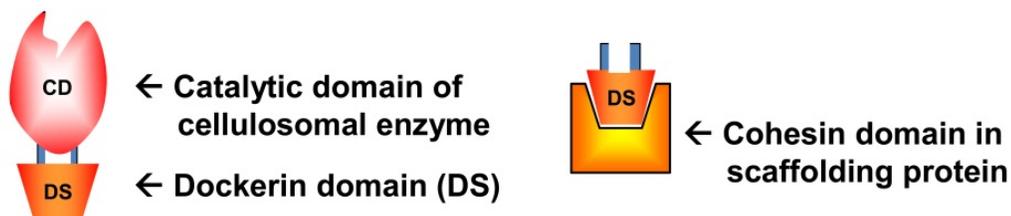
Since 2002, over 100 genome sequencing projects of *Clostridium* species have been done or are being done mainly by the United States Department of Energy Joint Genome Institute

Species	Habitat
<i>Clostridium acetobutylicum</i> *	Soil
<i>Clostridium aldrichii</i>	Wood digester
<i>Clostridium cellobioparum</i>	Soil
<i>Clostridium cellulof fermentans</i>	Soil
<i>Clostridium cellulolyticum</i> *	Rot grass
<i>Clostridium cellulovorans</i> *	Wood chips
<i>Clostridium herbivorans</i>	Pig intestine
<i>Clostridium hungate</i>	Soil
<i>Clostridium josui</i>	Compost
<i>Clostridium papyrosolvens</i>	Paper mill
<i>Clostridium thermocellum</i> *	Compost, Soil

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

Table 1. Cellulolytic clostridial species from natural biomass decaying ecosystems

A Model of cohesin-dockerin interaction



B Model for *C. cellulovorans* cellulosome

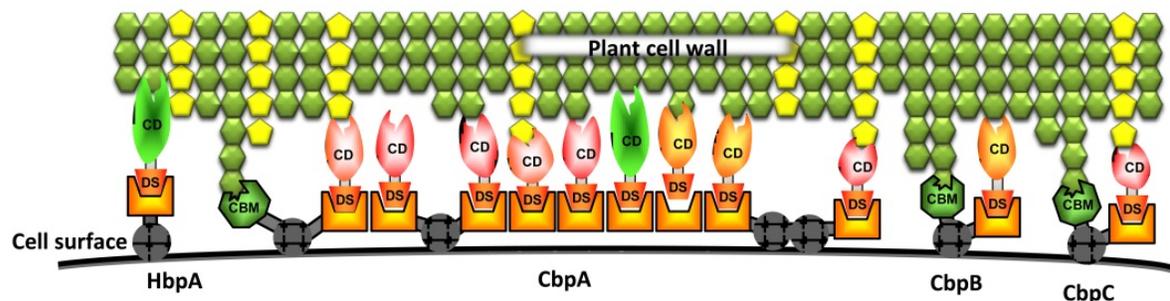


Figure 2. Model for *C. cellulovorans* cellulosomes. (A) Model of cohesin–dockerin interaction. (B) Recent model of cellulosomes attached to its substrate and cell surface.

(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
<i>C. cellulovorans</i> 743B	DF093537-DF093556	5.10	4220	57	31.1
<i>C. acetobutylicum</i> ATCC 824	AE001437	3.94	3672	12	30.9
<i>C. cellulolyticum</i> H10	CP001348	4.07	3390	65	37.4
<i>C. thermocellum</i> 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.

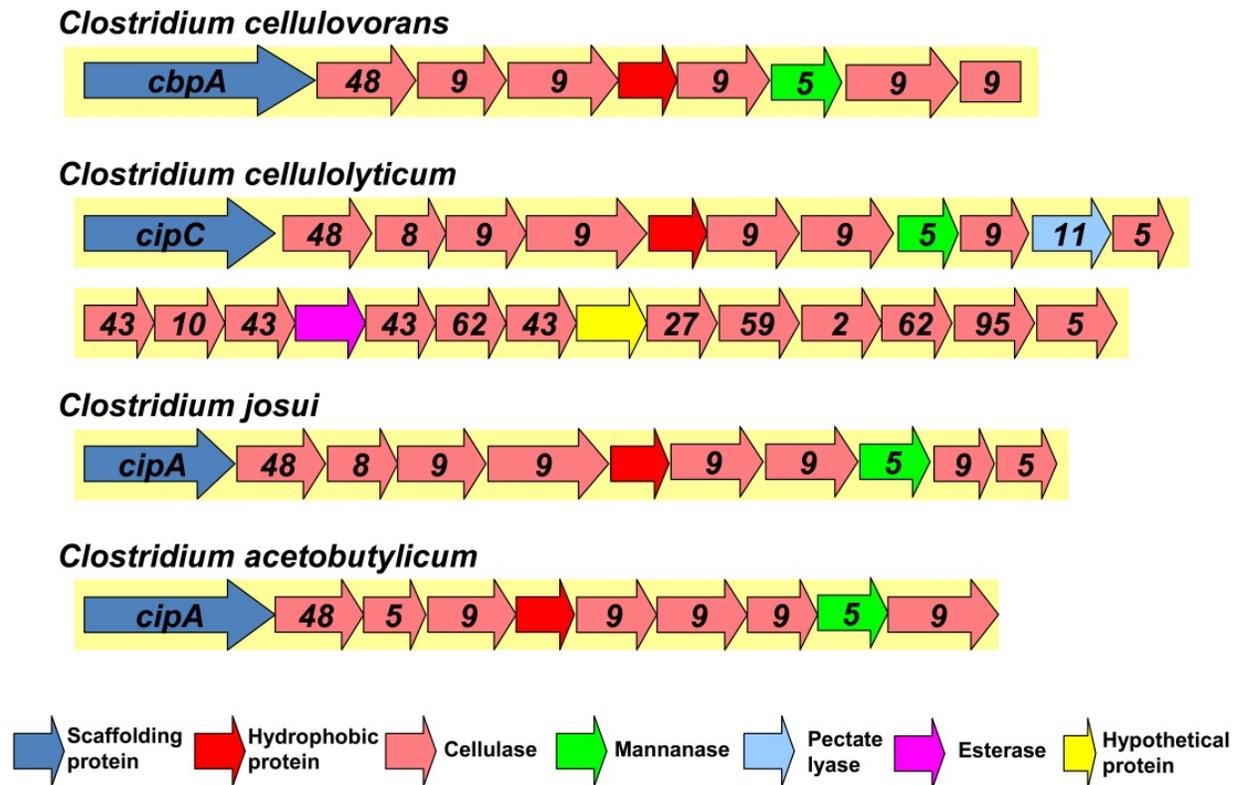


Figure 3. Cellulosome-related gene clusters in the genome of mesophilic clostridia.

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 ($\Delta adhE \Delta ldhA \Delta frdBC \Delta fnr \Delta pta$) 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 cellulose-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*.

Author details

Yutaka Tamaru*

Department of Life Science, Graduate School of Bioresources,
Department of Bioinformatics, Life Science Research Center,
Laboratory of Applied Biotechnology, Industrial Technology Innovation Institute, Mie University,
Tsu, Japan

Ana M. López-Contreras

Food and Biobased Research, Wageningen University and Research Centre, Wageningen,
The Netherlands

8. References

- [1] Tamaru Y, Miyake H, Kuroda K, Ueda M, Doi RH. Comparative genomics of the mesophilic cellulosome-producing *Clostridium cellulovorans* and its application to biofuel production via consolidated bioprocessing. *Environ Technol* 2010;31:889–903.
- [2] Tamaru Y, Doi RH. Chapter 20: Bacterial strategies for plant cell degradation and their genomic information. *In* Carbohydrate Modifying Biocatalysts (Ed. Peter Grunwald). Pan Stanford Publishing Pte. Ltd. (Singapore), 2011;p.761–789.
- [3] Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. Fermentative butanol production by Clostridia. *Biotechnol Bioeng* 2008;101:209–228.
- [4] Jones DT, Woods DR. Acetone–butanol fermentation revisited. *Microbiol Rev* 1986;50:484–524.
- [5] Zverlov VV, Berezina O, Velikodvorskaya GA, Schwarz WH. Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: Use of hydrolyzed agricultural waste for biorefinery. *Appl Microbiol Biotechnol* 2006;71:587–597.
- [6] Jones DT, Keis S. Origins and relationships of industrial solvent producing clostridial strains. *FEMS Microbiol Rev* 1995;17:223–232.
- [7] Zhang Y, Yang Y, Chen J, High-butanol ratio *Clostridium acetobutylicum* culturing method and its use. Chinese Patent 1997 CN 1063483C.
- [8] Jiang Y, Xu C, Dong F, Yang Y, Jiang W, Yang S. Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. *Metab Eng* 2009;11:284–291.
- [9] Dürre P. Fermentative butanol production: bulk chemical and biofuel. *Ann NY Acad Sci* 2008;1125:353–362.
- [10] Swana J, Yang Y, Behnam M, Thompson R. An analysis of net energy production and feedstock availability for biobutanol and bioethanol. *Bioresour Technol* 2011;102:2112–2117.
- [11] Ahn JH, Sang BI, Um Y. Butanol production from thin stillage using *Clostridium pasteurianum*. *Bioresour Technol* 2011;102:4934–4937.
- [12] Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. Fermentative butanol production by Clostridia. *Biotechnol Bioeng* 2008;101:209–228.

* Corresponding Author

- [13] Ezeji TC, Qureshi N, Blaschek HP. Bioproduction of butanol from biomass: from genes to bioreactors. *Curr Opin Biotechnol* 2007;18:220–227.
- [14] Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The ClosTron: A universal gene knock-out system for the genus *Clostridium*. *J Microbiol Methods* 2007;70:452–464.
- [15] Bramono SE, Lam YS, Ong SL, He J. A mesophilic *Clostridium* species that produces butanol from monosaccharides and hydrogen from polysaccharides. *Bioresour Technol* 2011;102:9558–9563.
- [16] Alper H, Stephanopoulos G. Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? *Nat Rev Microbiol* 2009;7:715–723.
- [17] Knoshaug EP, Zhang M. Butanol tolerance in a selection of microorganisms. *Appl Biochem Biotechnol* 2009;153:13–20.
- [18] Nölling J, Breton G, Omelchenko MV, Makarova KS, Zeng Q, Gibson R, Lee HM, Dubois J, Qiu D, Hitti J, Wolf YI, Tatusov RL, Sabathe F, Doucette-Stamm L, Soucaille P, Daly MJ, Bennett GN, Koonin EV, Smith DR. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J Bacteriol* 2001;183:4823–4838.
- [19] Lee SY, Mermelstein LD, Bennett GN, Papoutsakis ET. Vector construction, transformation, and gene amplification in *Clostridium acetobutylicum* ATCC 824. *Ann NY Acad Sci* 1992;665:39–51.
- [20] Minton, N.P., Brehm, J.K., Swinfield, T.-J., Whelan, S.M., Mauchline, M.L., Bodsworth, N., Oultram, J.D., 1993. Clostridial cloning vectors. In: Woods DR, editor. *The clostridia and biotechnology*. Stoneham: Butterworth-Heinemann. p. 119–150.
- [21] Mermelstein LD, Welker NE, Bennett GN, Papoutsakis ET. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. *Bio/Technology* 1992;10:190–195.
- [22] Mermelstein LD, Papoutsakis ET. *In vivo* methylation in *Escherichia coli* by the *Bacillus subtilis* phage w3TI methyl-transferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* 1993;59:1077–1081.
- [23] Lee SY, Mermelstein LD, Papoutsakis ET. Determination of plasmid copy number and stability in *Clostridium acetobutylicum* ATCC 824. *FEMS Microbiol Lett* 1993;108:319–324.
- [24] Green EM. Fermentative production of butanol—the industrial perspective. *Curr Opin Biotechnol* 2011;22:337–343.
- [25] Harris LM, Blank L, Desai RP, Welker NE, Papoutsakis ET. 2001. Fermentation characterization and flux analysis of recombinant strains of *Clostridium acetobutylicum* with an inactivated *solR* gene. *J Ind Microbiol Biotechnol* 2001;27:322–328.
- [26] Harris LM, Desai RP, Welker NE, Papoutsakis ET. Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: need for new phenomenological models for solventogenesis and butanol inhibition? *Biotechnol Bioeng* 2000;67:1–11.
- [27] Shao, L., Hu, S., Yang, Y., Gu, Y., Chen, J., Yang, Y., Jiang, W., Yang, S., 2007. Targeted gene disruption by use of a group II intron (targetron) vector in *Clostridium acetobutylicum*. *Cell Res.* 17, 963–965.

- [28] Heap JT, Kuehne SA, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP. The Clostron: mutagenesis in *Clostridium* refined and streamlined. *J Microbiol Methods* 2010;80:49-55.
- [29] Heap JT, Minton NP. Methods. International Patent Application 2009. PCT/GB2009/000380.
- [30] Dürre P. Biobutanol: an attractive biofuel. *Biotechnol J* 2007;2:1525–1534.
- [31] Connor MR, Liao JC. Microbial production of advanced transportation fuels in non-natural hosts. *Curr Opin Biotechnol* 2009;20:307-315.
- [32] Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD. Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. *Microb Cell Fact* 2008;7:36.
- [33] Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Prather KL. Engineering alternative butanol production platforms in heterologous bacteria. *Metab Eng* 2009;11:262-273.
- [34] Evanko WA, Eyal AM, Glassner DA, Miao F, Aristidou A, Evans K, Gruber PR, Hawkins AC. Recovery of higher alcohols from dilute aqueous solutions. International Patent Application, 2009. PCT/US2008/088187.
- [35] Donaldson GK, Eliot AC, Flint D, Maggio-Hall A, Nagarajan V. Fermentative production of four carbon alcohols. US Patent 2010, 7,851,188.
- [36] Guedon E, Desvaux M, Petitdemange H. Improvement of cellulolytic properties of *Clostridium cellulolyticum* by metabolic engineering. *Appl Environ Microbiol* 2002;68:53–58.
- [37] Cascone R. Biobutanol—a replacement for bioethanol? *Chem Eng Prog* 2008;104:S4–S9.
- [38] Bayer EA, Lamed R, White BA, Flint HJ. From cellulosomes to cellulosomes. *Chem Rec* 2008;8:364–377.
- [39] Tamaru Y, Miyake H, Kuroda K, Nakanishi A, Kawade Y, Yamamoto K, Uemura M, Fujita Y, Doi RH, Ueda M. Genome sequence of the cellulosome-producing mesophilic organism *Clostridium cellulovorans* 743B. *J Bacteriol* 2010;192:901–902.
- [40] Tamaru Y, Miyake H, Kuroda K, Nakanishi A, Matsushima C, Doi RH, Ueda M. Comparison of the mesophilic cellulosome-producing *Clostridium cellulovorans* genome with other cellulosome-related clostridial genomes. *Microb Biotechnol* 2011;4:64–73.
- [41] Meguro H, Morisaka H, Kuroda K, Miyake H, Tamaru Y, Ueda M. Putative role of cellulosomal protease inhibitors in *Clostridium cellulovorans* based on gene expression and measurement of activities. *J Bacteriol* 2011;193:5527–5530.
- [42] Sabathe F, Bélaïch A, Soucaille P. Characterization of the cellulolytic complex (cellulosome) of *Clostridium acetobutylicum*. *FEMS Microbiol Lett* 2002;217:15–22.
- [43] Pagès S, Bélaïch A, Fierobe HP, Tardif C, Gaudin C, Bélaïch JP. Sequence analysis of scaffolding protein CipC and ORFXp, a new cohesin-containing protein in *Clostridium cellulolyticum*: comparison of various cohesin domains and subcellular localization of ORFXp. *J Bacteriol* 1999;181:1801–1810.
- [44] Shoseyov O, Takagi M, Goldstein M, Doi R.H. Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A (CbpA). *Proc Natl Acad Sci USA* 1992;89:3483–3487.
- [45] Kakiuchi M, Isui A, Suzuki K, Fujino T, Fujino E, Kimura T, Karita S, Sakka K, Ohmiya K. Cloning and DNA sequencing of the genes encoding *Clostridium josui* scaffolding

- proteinCipA and cellulase CelD and identification of their gene products as major components of the cellulosome. *J Bacteriol* 1998;180:4303–4308.
- [46] Gerngross UT, Romaniec MP, Kobayashi T, Huskisson NS, Demain AL. Sequencing of a *Clostridium thermocellum* gene (CipA) encoding the cellulosomal SL-protein reveals an usual degree of internal homology. *Mol Microbiol* 1993;8:325–334.
- [47] Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* 2009;37:D233–D238.
- [48] Dashtban M, Schraft H, Qin W. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. *Int J Biol Sci* 2009;5:578–595.
- [49] Sandgren M, Stahlberg J, Mitchinson C. Structural and biochemical studies of GH family 12 cellulases: improved thermal stability, and ligand complexes. *Prog Biophys Mol Biol* 2005;89:246–291.
- [50] López-Contreras AM, Gabor K, Martens AA, Renckens BA, Claassen PA, Van Der Oost J, De Vos WM. Substrate-induced production and secretion of cellulases by *Clostridium acetobutylicum*. *Appl Environ Microbiol* 2004;70:5238–5243.
- [51] Kim AY, Attwood GT, Holt SC, White BA, Blaschek HP. Heterologous expression of endo- β -1,4-glucanase from *Clostridium cellulovorans* in *Clostridium acetobutylicum* ATCC 824 following transformation of the *engB* gene. *Appl Environ Microbiol* 1994;60:337–340.
- [52] Perret S, Casalot L, Fierobe HP, Tardif C, Sabathe F, Bélaïch JP, Bélaïch A. Production of heterologous and chimeric scaffoldins by *Clostridium acetobutylicum* ATCC 824. *J Bacteriol* 2004;186:253–257.
- [53] Mingardon F, Perret S, Bélaïch A, Tardif C, Bélaïch JP, Fierobe HP. Heterologous production, assembly, and secretion of a minicellulosome by *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* 2005;71:1215–1222.
- [54] Olson DG, McBride JE, Joe Shaw A, Lynd LR. Recent progress in consolidated bioprocessing.. *Curr Opin Biotechnol* 2011; Dec 14.
- [55] Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev* 2002;66:506–577.
- [56] Ingram LO, Aldrich HC, Borges ACC, Causey TB, Martinez A, Morales F, Saleh A, Underwood SA, Yomano LP, York SW, Zaldivar J, Zhou S. Enteric bacterial catalysts for fuel ethanol production. *Biotechnol Prog* 1999;15:855–866
- [57] Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC. Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab Eng* 2008;10:305–311.
- [58] Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yamamoto S, Okino S, Suzuki N, Yukawa H. Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. *Appl Microbiol Biotechnol* 2008;77:1305–1316.
- [59] Higashide W, Li Y, Yang Y, Liao JC. Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. *Appl Environ Microbiol* 2011;77:2727–2733.
- [60] Nakayama S, Kiyoshi K, Kadokura T, Nakazato A. Butanol production from crystalline cellulose by cocultured *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* N1-4. *Appl Environ Microbiol* 2011;77:6470–6475.