Energy and Emission Control Technologies

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REVIEW

Consolidated bioprocessing for biofuel production: recent advances

This article was published in the following Dove Press journal:
Energy and Emission Control Technologies
6 May 2015
Number of times this article has been viewed

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Abstract: Consolidated bioprocessing (CBP), the combination of saccharolytic enzyme production and secretion, hydrolysis of polysaccharides, and fermentation of available sugars within a single-unit operation, improves cellulose conversion efficiency and decreases lignocellulosic biomass processing costs for producing biofuels and value-added products. *Clostridium thermocellum*, an anaerobic, thermophilic bacterium is a significant biocatalyst aspirant for CBP, due to its native cellulolytic and ethanologenic capabilities. This review highlights strain development/modification, metabolic engineering, and process improvements associated with CBP in the context of using *C. thermocellum* as a model biocatalyst to reduce operating expenditures and inhibitory effects for enhanced biofuels production. In addition, opportunities in using a microbial consortia and biofilms are discussed. As an overview of recent advances in CBP technologies to convert lignocellulosic biomass to biofuels, this review gives researchers a platform for future development of efficient and sustainable CBP approaches.

Keywords: *Clostridium thermocellum*, cellulosome, anaerobic, cellulolytic, ethanol

Introduction

Expected population growth with corresponding energy usage, limiting resources, and environmental stresses will require development of sustainable technological advances to meet future energy demands that do not compromise available resources.[1] Sustainable developments, such as renewable energy systems, equalize energy supply and demand while improving food, economic, energy, and environmental security.[2] In 2012, 19% of the global final energy consumption came from renewable forms of energy, with 10.2% (50.3 EJ yr^{-1}) supplied from various forms of biomass.[3,4] Lignocellulosic biomass, organic biological matter derived from agricultural, municipal, and forestry operations, is a renewable resource that can be used for the production of reliable forms of energy, such as biofuels.[5,6] The attractiveness of using lignocellulosic biomass as raw material in biofuel production processes is attributed to abundance, relatively low expense, and environmentally negligible production and potential for global distribution.[7,8] While lignocellulosics possess roughly 40%–50% cellulose, 20%–40% hemicellulose, and 20%–30% lignin by weight, the major challenge is the presence of lignin, effective breakdown of the recalcitrant cellulose structure, and efficient bioconversion to liquid fuels.[9,10] The conversion of biomass through biochemical techniques, including pretreatment, hydrolysis, and fermentation, has the potential to produce heat, electricity, fiber, chemicals, and biofuels while improving economic and environmental sustainability.[11] To this end, significant research has focused on consolidated bioprocessing (CBP), a conversion approach, though still in its infancy stages of development, that combines

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all rate-limiting processes, pretreatment, saccharification, and fermentation, within a single reactor to decrease operating cost, increase conversion efficiencies, and reduce by-product inhibition. While a plethora of microbes possess cellulolytic and solventogenic capabilities, *Clostridium thermocellum* is a strong candidate for CBP, due to its natural ability to rapidly solubilize cellulose by way of its cellulosome, a multi-enzyme complex, and produce ethanol. Additionally, *C. thermocellum* is an anaerobic thermophile, which eliminates the need for aeration, decreases contamination concerns, and improves temperature-dependent functionality of hydrolytic enzymes.[12–14] This review focuses on highlighting process improvements, recombinant microbial catalyst and enzyme development, and metabolic engineering associated with CBP in the context of using *C. thermocellum* as a model biocatalyst and providing an overview in support of further research in sustainable biofuel production from lignocellulosic biomass. In addition, recent opportunities in using microbial consortia and biofilms are discussed.

Lignocellulosic biomass structure

Lignocellulose comprises of three main polymers, cellulose, hemicelluloses, and lignin, linked in a dense complex matrix with varying compositions depending on type, species, and source of biomass, in addition to pectin, extractives, and structural ash.[15–18] Cellulose, the main component of the plant cell wall bestowing structural support, is composed of β-D-glucopyranose moieties linked through β-(1,4) glycosidic bonds.[7,8] While disaccharide cellobiose is the repeating unit of the cellulose chain, the degree of polymerization in nature ranges from 2,000 to 20,000 units, and when combined through covalent and hydrogen bonding as well as Van der Waals forces form microfibrils.[15,18,19] Hydrogen bonding within a cellulose microfibril determines stability or straightness of the chain, while hydrogen bonds between cellulosic units introduce order, such as crystalline structures, or disorder with amorphous structures.[20] In addition, due to the intra- and interchain hydrogen bonding, cellulose is insoluble in most solvents, including water, and considered extremely resistant to enzymatic hydrolysis.[7] The cellulose microfibril structures are integrated with hemicellulose, various noncrystalline sugars, and other polymers, such as pectin. Hemicellulose is the second most abundant polymer and contrasts with cellulose by lacking chemical homogeneity, possessing a lower molecular weight and short lateral chains that are relatively easily hydrolyzed.[8,15,18,21] While differing in composition, hemicelluloses are highly branched, heterogeneous polymers of pentoses, such as xylose and arabinose; hexoses, such as mannose, glucose, and galactose; and acetylated sugars.[8,22,23] Among the main polymers in lignocellulosic materials, hemicellulose is the most thermochemically susceptible and alleged to cover the cellulose microfibrils; therefore, to significantly increase cellulose digestibility, at least 50% of this polymer must be removed.[24] Lignin, the adhesive binding various components of lignocellulosic biomass together, is a highly complex three-dimensional amorphous heteropolymer network of phenyl propane units, such as *p*-coumaryl, coniferyl, and sinapyl alcohol, which provides strength as well as opposition to microbial attack and oxidative stress for plant cell walls.[24,25] While lignin is a valuable coproduct for the generation of heat, power, and solid fuel, the noncarbohydrate polymer creates a major limitation in enzymatic and microbial hydrolysis of lignocellulosic biomass.[7,26] Chang and Holtzapple[27] showed that biomass digestibility was enhanced with increasing lignin removal and in addition to being a physical barrier, the disadvantageous effects of lignin consisted of nonspecific adsorption of hydrolytic enzymes, interference with, and nonproductive binding of cellulolytic enzymes to lignin–carbohydrate complexes, and toxicity of lignin derivatives to microorganisms. Determining the comparative copiousness of cellulose, hemicellulose, and lignin polymers is essential for selecting optimal energy conversion systems to reduce processing time and cost.[10,28–30]

Advancements in bioprocessing

During the past decade, technological advances have promoted commercial feasibility of biochemical conversion processing for the production of liquid fuels from lignocellulosic biomass.[2] This renewable energy production approach includes such operations as biomass size reduction, pretreatment, hydrolysis, and fermentation to produce value-added products.[31] Size reduction or comminution mechanically lacerates biomass to facilitate enzymatic accessibility to lignocellulosic materials for further pretreatment and digestibility.[32] Pretreatment strategies strive to enhance biomass porosity to increase sugar yields during subsequent hydrolysis and degradation failure of pentoses, as well as to improve the breakdown and recovery of lignin- and hemicellulose-derived sugars, while minimizing by-product inhibition of later unit operations.[8,33] Following pretreatment of lignocellulosic biomass, hydrolysis, also known as saccharification, converts complex carbohydrates into simple five- and six-carbon monomers, such as arabinose, fructose, galactose, glucose, mannose, and xylose, by using acids or highly specific external sources of cellulases and hemicellulases.[34–36] In

contrast with acid hydrolysis, enzymatic hydrolysis has advantages in reduced energy requirements and imperturbable environments without the formation of inhibitory byproducts.[37] Furthermore, enzymatic hydrolysis has proven to be advantageous, due to nominal toxicity, decreased utility expenditures, and truncated corrosion compared to acid or alkaline hydrolysis.[38–42] Subsequent to saccharification, fermentation procedures can be conducted as batch, fed-batch, continuous, and solid-substrate processes depending on the kinetic properties of choice microbes, source of lignocellulosic hydrolyzate, and process economics to generate biofuels.[43,44] Extensive understanding of the genomes, physiologies, and metabolisms of *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli* has resulted in their industrial utilization in bioconversion operations.[45,46] Natively, *S. cerevisiae* is incompetent in the fermentation of pentose sugars, such as xylose and arabinose, nor proficient at functioning at optimal exogenous hydrolytic enzymatic temperatures between 50°C and 60°C.[5,45] Therefore, metabolic and evolutionary engineering was required to enhance usability of various hydrolyzed monomers and tolerance to environmental stressors.[47] Generally, strain engineering of saccharolytic and fermentative microbes are conducted to express cellulases and hemicellulases, secrete proteins, produce biofuels, and tolerate solvents and lignin byproducts, such as furans, weak organic acids, and phenolics, from fermentation procedures.[48]

Separate hydrolysis and fermentation and simultaneous saccharification and fermentation are two bioconversion technologies generally commissioned for commercial implementation (Figures 1A and 1B).[16] Enzymatic hydrolysis can also be performed simultaneously with the cofermentation of glucose and xylose as separate operations in a process known as simultaneous saccharification and cofermentation (Figure 1C).[47] Saccharification and fermentation and saccharification and cofermentation have advantages over traditional separate hydrolysis and fermentation, often supporting increased alcohol yields by eradicating by-product inhibition and improved rates of hydrolysis, as well as reduced cost of operation by jettisoning the need for a secondary reactor, lowering enzyme loadings, and decreasing processing time and risk of contamination.[48,49] Technological advancements to improve operating procedures and biomass solubilization effectiveness naturally led to the holistic CBP approach consisting of three biotransformations.[48] To achieve CBP benefits, cellulase generation, saccharification of biomass, and fermentation of reducing sugars take place in conjunction by a monoculture or coculture of microorganisms, such as bacteria like *C. thermocellum* and fungi including *Neurospora crassa*, *Fusarium oxysporum*, *S. cerevisiae*, and *Paecilomyces* sp., for the generation of biofuels (eg, ethanol, butanol) and other value-added products.[12,35,50–52] Alleviation of production expenditures and reduced chemical usage can be achieved by CBP, as the microbes used reduce the need for a multitude of external enzymes, which are not substrate specific.[16,48] Additionally, this approach decreases the need for numerous reactors and offers simplicity in operating procedures by minimizing the compatibility requirements of enzyme and fermentation systems.[53–55] In fact, a comparative cost analysis conducted on ethanol production, when considering capital, raw materials, utilities, and yield loss expenditures, resulted in a projection of \$0.04 gal^{-1} for CBP, while a saccharification and cofermentation was projected at \$0.19 gal^{-1}.[54] Eclectic selections of microorganisms have been reconnoitered for CBP (Table 1); however, a microbial consortium that combines optimum substrate use and product formation properties, such as rapid hydrolysis rates and enhanced product concentrations and selectivity is preferable in CBP systems.[56] In addition, anaerobic metabolism is imperative to CBP, due to superfluous expenditures associated with aerating large culture volumes, as well as product and lignocellulosic energy inefficiencies, as a result of aerobic respiration.[13] Thermophilic microorganisms are also necessary to improve hydrolysis rates, which is a major challenge when conducting CBP at optimum ambient conditions for mesophilic microbes of interest.[48] Although, the potential and rationale for CBP of biofuels exists, long fermentation periods and meager biofuel yields resulting from by-product formation (eg, organic acids), sensitivity of microorganisms to alcoholic solvents, and limited growth in hydrolysis supernatant resulted in significant research focused on improving the robustness of microorganisms utilized in CBP by employing recombinant strategies on cellulolytic and solventogenic organisms.[57,58] While biochemical conversion systems demonstrate prodigious potentials for producing biofuels with minimal environmental impacts, the present challenge is efficiently converting lignocellulosic material to available sugars and fermenting crude sugars to biofuels with a robust microorganism and relatively high operating costs.[59,60]

Native cellulolytic and ethanologenic microbes

C. thermocellum, an extensively researched strict anaerobic, acetogenic, cellulolytic, and thermophilic bacterium, possesses the ability to degrade cellulose and ferment mixed products from cellulose solubilization, while producing ethanol, acetate,

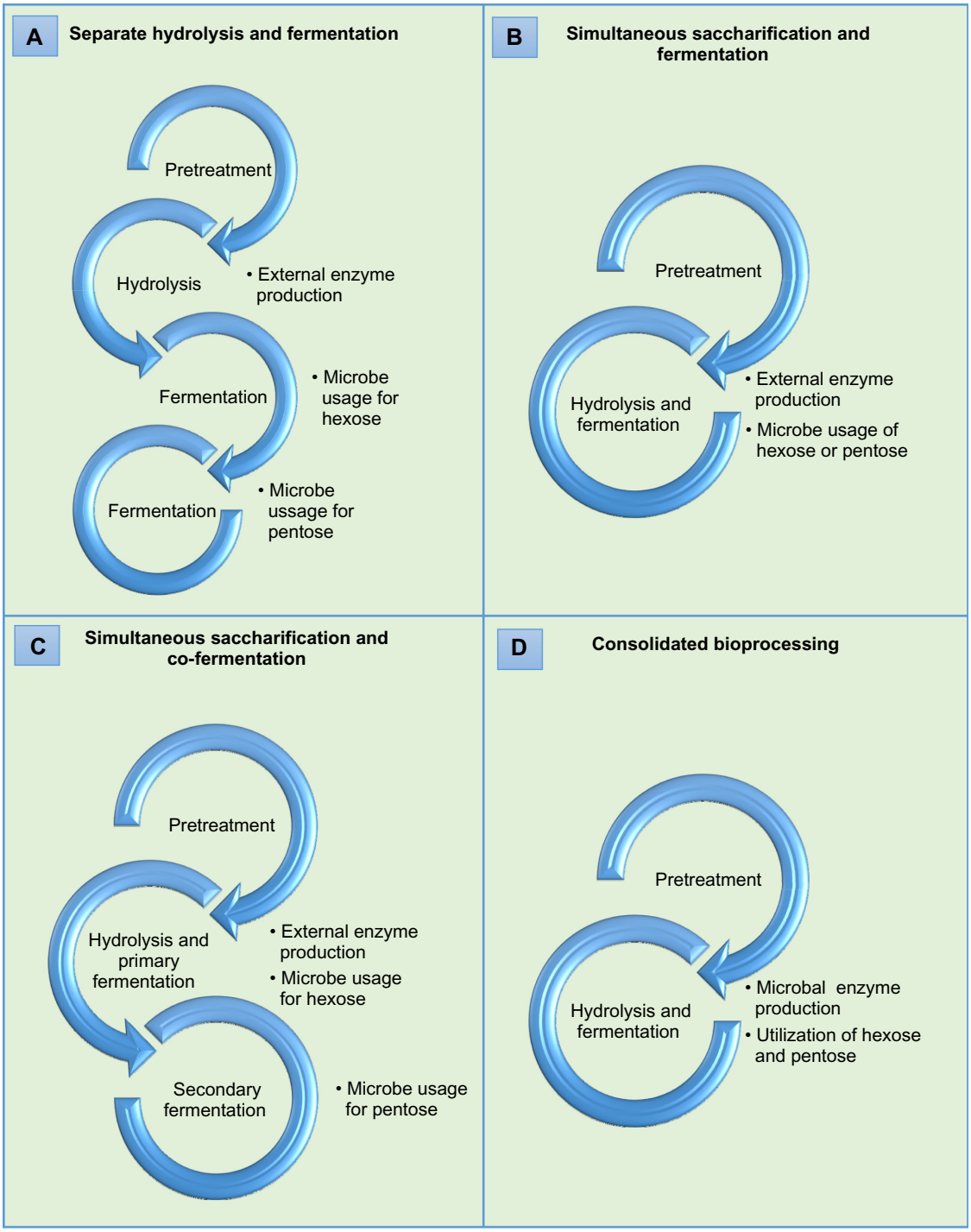

Figure 1 Bioprocessing approaches.
Notes: Bioprocessing approaches for the conversion of lignocellulosic biomass to biofuels and other value-added products with (**A**) separate hydrolysis and fermentation; (**B**) simultaneous saccharification and fermentation; (**C**) simultaneous saccharification and co-fermentation; and (**D**) consolidated bioprocessing.

hydrogen (H_2), carbon dioxide (CO_2), lactate, and formate under various growth conditions.[61–67] Cellulose degradation rates approaching 2.5 g L^{-1} h^{-1}, thermally stable biomass degrading enzymes, and proclivity to synthesize value-added products makes *C. thermocellum* of great interest to CBP development.[61,68,69] In addition, an optimum growth temperature of 60°C precludes that of most contaminating microorganisms, which allows for *C. thermocellum* cultures to be maintained and viable when using nonsterile substrates for pure-culture fermentation processes.[70] High fermentation temperatures and anaerobic conditions also reduce operational expenditures for ethanol recovery and reactor cooling, which are typically associ-

Table 1 Cellulolytic and hemicellulolytic, ethanologenic (including genetically modified) microorganisms utilized for consolidated bioprocessing

Microorganism	Genotype	Substrate	Ethanol yield (g/L)	Time (h)	References
Cellulolytic					
Clostridium cellulolyticum H10	Wild type	Cellulose MN301	0.51	460	195,196
Clostridium cellulolyticum CC-pMG8	pdc adhB	Cellulose MN301	0.83	460	195
Clostridium japonicus Ueda107	Wild type	Avicel	0.0002	48	197
Clostridium japonicus MSB280	pdc adhB	Avicel	0.0035	48	197
Clostridium thermocellum ATCC 27405	Wild type	Crystalline cellulose	2.66	240	198
		Sugarcane bagasse	3.5	240	199
		Paper pulp sludge	14.1	240	199
Clostridium thermocellum DSM 1313	Wild type	Avicel	1.3	72	69
Clostridium thermocellum M1570	Δldh Δpta	Avicel	5.6	72	69
Clostridium phytofermentans ATCC 700394	Wild type	Filter paper	2.9	672	200
		AFEX corn stover	2.8	234	201
Geobacillus sp. R7	Wild type	Prairie cord grass	0.035	168	202
Trametes hirsuta	Wild type	Rice straw	3	96	203
Hemicellulolytic					
Thermoanaerobacterium saccharolyticum	Wild type	Xylose	3.4	10	166
Thermoanaerobacterium saccharolyticum ALK2	Δldh Δpta Δack	Xylose	5.5	10	166
Thermoanaerobacterium saccharolyticum M1442	Δldh Δpta Δack	Hardwood hydrolyzate	7.4	68	204
Geobacillus thermoglucosidasius TM242	Δldh Δpfl pdh-unreg	Xylose	9.6	12	205
Clostridium phytofermentans ATCC 700394	Wild type	Birch wood/Xylan	0.46	24	200
Thermoanaerobacterium aotearoense SCUT27	Wild type	Xylose	1.5	24	206
Thermoanaerobacterium aotearoense Δldh Mutant	Δldh	Xylose	3.5	24	206
Thermoanaerobacter mathranii BG1	Wild type	Xylose	2.3	48	207
Thermoanaerobacter mathranii BG1L1	Δldh	Xylose	2.2	48	207

ated with fermentations using mesophilic, aerobic bacterium.[68] Decreases in solubility of product gases (H_2 and CO_2) at higher fermentation temperatures also allow enhanced removal efficiency of product gases to be achieved, based on Henry's law.[71] The ability to hydrolyze most recalcitrant forms of cellulose as effectively as the commercially significant fungus, *Trichoderma reesei*, in combination with the robustness of thermostable enzymes, limited contamination and continuous distillation of fermentation products at elevated temperatures has resulted in the persistent interest in *C. thermocellum* as a biocatalyst in CBP for ethanol generation (Table 2).[72]

Fermentation by *C. thermocellum*

C. thermocellum hydrolyzes cellulosic substrates into shorter chain sugars, primarily cellobiose for conversion to ethanol, acetate, and lactate as primary end products through various metabolic pathways (Figure 2).[73] Instead of a phosphotransferase system, *C. thermocellum* mediates the transportation of cellobiose using an ATP-binding cassette.[74–76] Phosphorylation of cellobiose and glucose to glucose 1-phosphate takes place prior to conversion through glycolysis as the predominate pathway, with less common flux through the pentose phosphate pathway or the Entner–Doudoroff pathway.[64,73,76] Most of the pyruvate is converted to acetyl-CoA, reduced Fd (FdH_2), and CO_2 with a significant amount abridged to lactate by lactate dehydrogenase.[77] Subsequently, reduction of acetyl-CoA to acetaldehyde and then to ethanol catalyzed by NAD^+-linked dehydrogenase transpires or acetate is formed yielding stoichiometric amounts of ATP.[77,78] Utilizing the three-branched pathway to metabolize hexoses without genetic manipulation or the addition of external factors, *C. thermocellum* has been shown to produce 0.6 mol to 1.0 mol of ethanol per mol of hexose with an ethanol tolerance of 5 g L^{-1}, due to a high degree of membrane fluidity based on lipid content.[77,79,80] Aside from ethanol, acetate, and lactate, other compounds including pyruvate, malate, uracil, soluble glucan, and free amino acids, which normally are products disassociated with *C. thermocellum*, have been observed in the liquid fraction of lignocellulosic fermentations.[81]

Varying substrate complexity and cultivation systems render a multitude of product ratios for a particular strain (Table 2), in addition to versatile functionality and inherent by-product tolerance. For instance, S14 advantageously possesses the capability to cultivate in an inclusive temperature and pH growth range and produce considerable quantities of cellulosomal enzymes in the medium.[82] Furthermore, cellulosomes of S14 were shown to have an increased activity

Table 2 *Clostridium thermocellum* strains utilized for consolidated bioprocessing

Strain	Fermentation	Substrate	Ethanol (mM)	Acetate (mM)	Lactate (mM)	Total products (mM)	References
AS-39	Liq	Cellobiose	47	23	–	–	208
	SmC	Cellulose	52	23	–	–	208
27405	SmC	Milled filter paper	17.4	9.1	–	–	209
	SmC	Pretreated switchgrass	4.3	8.5	–	–	155
	SmC	Pretreated *Populus*	18.0	13.5	–	–	155
	Liq	Cellobiose	27	51	7	–	81
	SmC	Avicel	37	45	6	–	81
	SmC	Microcrystalline cellulose	23.7	25.2	27.0	–	82
	Liq	Cellobiose	36	55	55	–	210
	SmC	Avicel	56.7	20.2	14.5		198
	SSC	Avicel	48.4	52.5	12.9	–	198
	SSC	Avicel	29	59	5	100	199
	SmC	Avicel	11	122	4	–	199
	Liq	Cellobiose	100	142	68	–	71
	SmC	α-cellulose	75	105	150	–	71
	SmC	Whatman paper	65	90	130	–	71
	SmC	Delignified wood	89	113	114	–	71
	SmC	Paper pulp sludge	–	–	–	72.9	83
	SSC	Paper pulp sludge	–	–	–	306.7	83
	SmC	Sugarcane bagasse	–	–	–	49.6	83
	SSC	Sugarcane bagasse	–	–	–	74.9	83
	SmC	Avicel	11.7	8.3	0.8		62
CS7	SmC	Milled filter paper	17.1	5.4	–	–	209
CS8	SmC	Milled filter paper	18.0	7.3	–	–	209
JN4	SmC	Avicel	6.2	7.6	13	–	211
JW20	SmC	Paper pulp sludge	–	–	–	79.9	83
	SSC	Paper pulp sludge	–	–	–	157.4	83
	SmC	Sugarcane bagasse	–	–	–	62.1	83
	SSC	Sugarcane bagasse	–	–	–	74	83
	SmC	Cellulose	13.2	20.5	4.8	–	212
DSM 1237	SmC	α-cellulose	70	35	8	–	85
DSM 1237	SmC	Whatman paper	18.5	15.2	–	–	213
DSM 1313	SmC	Avicel	28.7	46.4	27.6	–	69
	Liq	Cellobiose	14.8	18.6	2.8	–	160
	SmC	Avicel	15.2	18.6	0.6	–	160
	SmC	Cellulose	20.8	12.7	4.2	–	66
LQRI	SmC	Paper pulp sludge	–	–	–	75.7	83
	SSC	Paper pulp sludge	–	–	–	116	83
	SmC	Sugarcane bagasse	–	–	–	53.3	83
	SSC	Sugarcane bagasse	–	–	–	70.6	83
	Liq	Cellobiose	26	24	–	–	208
	SmC	Cellulose	28	27	–	–	208
	SmC	MN300	31.2	22.5	–	–	167
	SmC	Solka floc	30.8	27.3	–	–	167
	SmC	SO_2-treated wood	16.9	13.6	–		167
	SmC	Steam-exploded wood	14.9	15.2	–		167
	SmC	Untreated wood	2.2	6.4	–	–	167
M1570	SmC	Avicel	121.8	2.7	1.2		69
S14	SmC	Microcrystalline cellulose	41.2	63.0	8.2	–	82
SS19	SmC	Filter paper	296.5	–	–	–	214
SS21	SmC	Filter paper	311.3	–	–	–	215
SS22	SmC	Filter paper	289.6	–	–	–	215
YS	Liq	Cellobiose	38	19	–	–	208
	SmC	Cellulose	36	12	–	–	208

Abbreviations: Liq, liquid cultivation, soluble carbon; SmC, semi-solid cultivation, liquid insoluble carbon; SSC, solid-substrate cultivation.

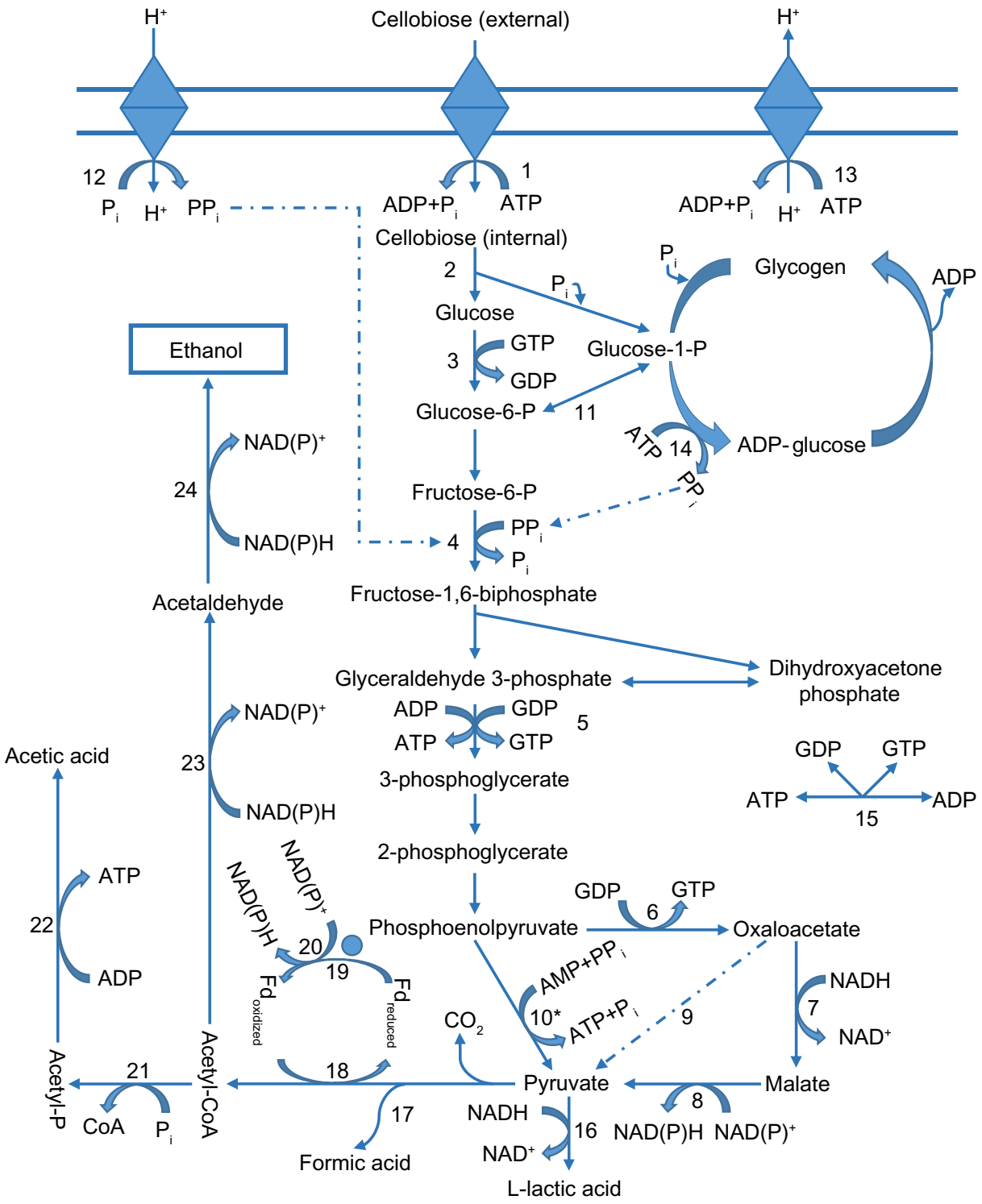

Figure 2 Metabolic functionality of *Clostridium thermocellum* cellulosome for product formation.

Notes: The lone numbers correspond to enzymes, ABC transporter (1); cellobiose phosphorylase (2); glucokinase (3); phosphofructokinase (4); phosphoglycerate kinase (5); phosphoenolpyruvate carboxykinase (6); malate dehydrogenase (7); malic enzyme (8); oxaloacetate decarboxylase (9); pyruvate phosphate dikinase (10); phosophoglucomutase (11); membrane-bound pyrophosphatase (12); ATP-energized protein pump (13); ADP-glucose synthase (14); NDP-kinase (15); lactate dehydrogenase (16); pyruvate:formate lyase (17); pyruvate:Fd oxidoreductase (18); ferredoxin hydrogenase (19); NAD(P)H hydrogenase (20); phosphotransacetylase (21); acetate kinase (22); acetaldehyde dehydrogenase (23) and alcohol dehydrogenase (24). The closed circle represents conversion between H_2 and H^+ by (19 and 20). Broken arrows indicate hypothesized source and point of use of pyrophosphate in glycolysis. The asterisk designates a strain dependent reaction. Figure adapted from: *Applied and Environmental Microbiology*; Zhou J, Olson DG, Argyros DA, et al. Atypical glycolysis in Clostridium thermocellum; 2013;79(9):3000–3008; doi: 10.1128/AEM.04037-12; and amended with permission from the American Society for Microbiology; Copyright © 2013, American Society for Microbiology.[72] And adapted from Springer; *Applied Microbiology and Biotechnology*; End-product induced metabolic shifts in Clostridium thermocellum ATCC 27405; 92; 2011;199–209; Rydzak T, Levin D, Cicek N, Sparling R; Copyright © Springer-Verlag 2001; with kind permission of Springer Science+Business Media.[210]

on microcrystalline cellulose and lignocellulosics, such as rice straw, with higher resistance to cellobiose inhibition, compared to the cellulosomes of ATCC 27405.[82]

In addition to substrate utilization, supplemental nutrients, such as salts, vitamins, and reducing agents, are of great importance to product formation when utilizing *C. thermocellum* and other anaerobes.[83,84] Islam et al[85] demonstrated enhanced cellulose fermentation and end-product synthesis by *C. thermocellum* DSM 1237 with varied nutrient compositions of α-cellulose, yeast extract, urea, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $FeSO \cdot 6H_2O$, and vitamins under carbon-excess conditions. The researchers also illustrated two major effects, a general growth enhancement effect and a carbon flux shifting effect, which rendered volumetric ethanol and H_2 yields that increased by 2.3-fold and 2.04-fold, respectively, when compared to the basic nutritional blend.

Shifting metabolic product synthesis in *C. thermocellum* can also be induced with the supplement of acetone, ethanol, formate, or sodium azide or by increasing the hydrostatic bioreactor pressure by incorporating exogenous gases.[14,16,86,87] More specifically, the addition of acetate when initiating fermentative procedures increases ethanol and decreases formate production, while the addition of ethanol increases hydrogen and acetate concentrations. Formate proves advantageous not only at increasing hydrogen and ethanol but also at decreasing acetate. Alternatively, the initial incorporation of hydrogen was determined to decrease carbon dioxide and increase formate production, while the addition of headspace carbon monoxide considerably enhanced the production of ethanol and inhibited the generation of H_2, CO_2, and acetate.[16] Shifts in end-product formation can also be accomplished through strain modifications, such as with the evolved M1570 (Δhpt, Δldh, Δpta), which produced a 40:1 molar ratio of ethanol to organic acid while making 5.61 g L^{-1} ethanol.[69]

Cellulosome structure and capability

C. thermocellum expresses a collection of cellulolytic enzymes, which accumulate into large, complex, multi-protein structures on the cell surface, known as cellulosomes.[70,88] Cellulosomes with molecular weights ranging from 2.0 to 6.5×10^6 and encompassing 14–26 polypeptide subunits, depending on strain and growth conditions, were first recognized while conducting research on adhesion factors linking bacterium to cellulose.[89–99] Specifically, an electron micrograph demonstrated that the cytoplasmic membrane of *C. thermocellum* was bordered by a thin peptidoglycan layer, a regular S-layer, and an amorphous outer layer formed by the protuberate cellulosome.[68,100] By way of the cellulosome, *C. thermocellum* indeed attaches to cellulose, while over 20 enzymes, including endoglucanases, cellobiohydrolases, and xylanases, within the cellulosome efficiently degrade cellulose to glucose and cellulodextrins, followed by subsequent transportation for cellular metabolism, in earlier stages of cellulolysis.[71,101] More specifically, during optimal growth conditions, *C. thermocellum* forms a monolayer biofilm without an extracellular polymeric matrix, parallel to the carbon fibers of the lignocellulosic substrate, caricaturing the topography to facilitate extracellular hydrolysis.[102,103] During the latter stages of a cell culture, it has been reported that the cellulosome detaches from the bacterium cell; however, the possibility exists that the cellulosome remains attached to high-molecular weight cellulose compounds.[96] The effectiveness of cellulosomes to catalyze the hydrolysis of cellulose depends significantly on the maintenance of structural integrity, as partially disassociated cellulosome complexes result in major activity loss, especially as it relates to crystalline forms of cellulose.[104] Nonetheless, economic production of cellulolytic enzymes and reducing enzyme to biomass ratio required for commercialization of biofuels derived from lignocellulosic biomass could be achieved when utilizing cellulolytic CBP microbes for enzyme production, secretion, and recycling.[104,105] While advancements are still required in enzyme recycling, solutions can be found exploiting the tethering capability of the *C. thermocellum* cellulosome.[12,105]

Definitively, the primary and largest structural subunit of the multi-functional *C. thermocellum* cellulosome, known as the cellulosome-integrating protein (CipA), is a glycoprotein with a mass of 210–250 kDa and functions as a nonhydrolytic scaffoldin.[94,98] The modular protein consists of a carbohydrate-binding module (CBM-3a), also referred to as the cellulose-binding domain, with a comprehensive binding specificity for crystalline cellulose, a hydrophilic module of unknown functionality, termed X, a modified dockerin domain and nine internal repeated sequences or hydrophobic cohesion domains positioned relative to the amino terminus, which bind catalytic subunits.[106,107] Specifically, amalgamation of the catalytic subunits into the cellulosome transpires by way of calcium-dependent interactions between the scaffoldin cohesions and Type I dockerin domains of the catalytic subunits.[108,109] Furthermore, the cellulosome binds to cellulose via the CBM-3a, as well as through the CBMs of the catalytic subunits. In fact, Kataeva et al[110] demonstrated synergism between the catalytic subunit, CelD, and the CBM of the *C. thermocellum* scaffoldin. Type II dockerin domains

attached to the C-terminus of the bacterium scaffoldin do not bind to the scaffoldin cohesion domains, instead, these dockerin domains bind to specific Type II cohesion domains on the cell surface, known as anchoring proteins.[111–114] These proteins (SdbA, Orf2p, and OlpB) contain an S-layer homology module for correlating with the cell surface and consequently anchoring the cellulosome to the cell.[115] While 23 cellobiohydrolase (exoglucanases) genes for cellulosomal components that contain dockerin domains have been recognized, endoglucanases have also been ascertained, as well as lichenase, chitinase, mannanase, and five xylanases, with two containing xylan esterase modules that remove feruloyl residues.[104,116–120] Although *C. thermocellum* is incapable of fermenting xylan, xylose, and other five-carbon sugars, the latter enzymes are believed to breakdown glycans encasing cellulose microfibrils to improve accessibility of the cellulose to be degraded by cellulosomes.[115]

The efficient degradation of lignocellulosic biomass is due to the juxtaposition of directing the enzymatic complex to the substrate, known as the targeting effect, and the spatial propinquity of the different types of cellulases to one another, known as the proximity effect.[121] While the cellulosome assembles in a temperately selective manner, this multi-enzyme system is one of the most efficient natural biocatalyst for degrading lignocellulosic biomass, therefore research groups have attempted to capture its function physically, incorporate it into other microorganisms, and mimic its behavior in synthesized enzyme complexes.[109,122–124]

Recombinant cellulolytic approach

Microbial strain engineering to facilitate high cellulolytic activity, the ability to use numerous carbon sources, and resistance to toxic compounds released during the pretreatment of lignocellulosic biomass are important for the development of cost-effective fermentation of lignocellulosics for the directed generation of biofuels and value-added chemicals.[125] To this end, recombinant cellulolytic approaches originating from natural anaerobic mesophilic and thermophilic bacteria and fungi possessing cellulosomes (Table 3) have been investigated.[117,126,127] In fact, natural cellulosomes detectible on the cell surface increases the localized concentration of enzymes allowing for enhanced consumption of cellobiose and cellodextrins by a microorganism, which minimizes inhibition related to product formation.[88] Through the use of electron microscopy, Fourier-transform infrared spectroscopy, and X-ray diffraction analysis, synergistic enzymatic activity of the cellulosomes are considered advantageous over aerobic bacteria and fungi, which exhibit fluctuating enzymatic functionality and

Table 3 Studied bacteria and fungi with cellulosomal structures

Microorganisms	Temperance	Cellulosome structures		Source	References
		Protein	Domain		
Aerobic bacteria					
Vibrio vulnificus	Mesophilic	Scaffoldin	Fungal-type dockerin	Ala Wai Canal	216,217
Anaerobic bacteria					
Acetivibrio cellulolyticus	Mesophilic	Scaffoldin	CBM, Type I cohesion and Type II dockerin	Sewage	218,219
		Surface-anchoring proteins	Type II cohesion		
Bacteroides cellulosolvens	Mesophilic	Scaffoldin/surface-anchoring proteins	CBM and Type II cohesion	Sewage	219–221
Bacteroides sp. *P-1*	Thermophilic	Multi-enzyme complexes		Rotting biomass	222
Clostridium cellulovorans/cellulolyticum	Mesophilic	Scaffoldin Enzymes	CBM and Type I cohesion Type I dockerin	Wood fermenter/compost	217,223–227
Clostridium josui	Thermophilic	Scaffoldin Enzymes	CBM and Type I cohesion Type I dockerin	Compost	228
Clostridium thermocellum	Thermophilic	Scaffoldin	CBM, Type I cohesion and Type II dockerin	Sewage and soil	93,99,194, 229–231
		Surface-anchoring proteins Enzymes	Type II cohesion Type I dockerin		
Ruminococcus albus/flavefaciens	Mesophilic	Enzymes	Type I dockerin	Rumen	232–234
Anaerobic fungi					
Orpinomyces joyonii/PC-2	Mesophilic	Enzymes	CBM, fungal-dockerin	Rumen	235–237
Neocallimastix patriciarum	Mesophilic	Enzymes	Fungal-dockerin	Rumen	237,238
Piromyces equi/E2	Mesophilic	Enzymes	Fungal-dockerin	Rumen/feces	236–238

Abbreviation: CBM, carbohydrate-binding module.

specificity associated with external enzyme secretion.[16,128] *C. thermocellum* is the most cellulolytic thermophile and distinctively possesses exceptionality with regard to size, complexity, and genomic arrangement of its cellulosome, vindicating one of the highest rates of cellulose degradation and utilization.[14,88,117,129,130] As a result, the recombination of cellulosomal components of *C. thermocellum* into minicellulosomes has been engineered onto the surface of *Aspergillus niger*, *Bacillus subtilis*, *Clostridium acetobutylicum*, *E. coli*, *Lactococcus lactis*, *Thermoanaerobacterium saccharolyticum*, and *S. cerevisiae* by way of anchoring proteins or cohesion–dockerin interactions.[5,131–137]

Engineering of bacteria

With regard to bacteria, fragments of the scaffoldin protein CipA from *C. thermocellum* containing a single cohesin module, two cohesin modules, one cohesin and a cellulose-binding module, or only a cellulose-binding module were functionally displayed on the cell surface of *L. lactis*.[134,135] To alleviate challenges associated with cell toxicity from protein overexpression, a *nisA* inducible promoter was used as well as the C-terminal-anchoring streptococcal M6 motif.[135] While significant variation in efficiencies were exhibited among the designed minicellulosomes, accredited to structural characteristics in protein conformation, scaffold size, and presence of noncohesion modules, the surface display of functional scaffold proteins proved imperative for developing recombinant microorganisms capable of using multiple metabolic pathways to directly convert carbonaceous biomass into biofuels and other value-added chemicals.[135] Similarly, *B. subtilis* was engineered to display multiple thermophilic cellulase enzymes (Cel8A) from *C. thermocellum* on the cell surface by way of proteins containing a *staphylococcus aureus* cell wall sorting signal covalently anchored to peptidoglycan by coexpression with the *Bacillus anthracis* sortase A (SrtA) transpeptidase.[136] Additionally, a Cel8A–dockerin fusion protein was stably anchored onto *B. subtilis* utilizing noncovalent cohesion–dockerin interactions to increase lignocellulosic degradation potential during CBP.[136]

The capability of *T. saccharolyticum* to unhesitatingly solubilize hemicellulose with the heterologous expression of a functional cellulosome makes this Gram-positive anaerobic thermophile of interest in CBP. In fact, expression and localization of a full-length CipA from *C. thermocellum* was observed by developing an inducible system based on the native *xynA T. saccharolyticum* promoter, regulated by xylan and xylose.[131] As part of the study, an exorbitant quantity of xylose and SigmaCell 101, a microcrystalline cellulose, was hydrolyzed when the Δ*cipA* mutant *C. thermocellum* was cocultured with the CipA-expressing *T. saccharolyticum* strain.[131]

In addition to native ethanologenic species, other solventogenic microbes, such as *C. acetobutylicum*, that proficiently convert sugars to ethanol and butanol are of interest in CBP; however, these species are known to inefficiently grow on and metabolize crystalline cellulose, which has prompted the engineering of secretion and assemblage of cellulosomes by allele-coupled exchange technology.[133] Specifically, BioBrick2-standardized fragments were used to assemble a range of synthetic genes encoding *C. thermocellum* cellulosomal scaffoldin proteins (CipA variants) and glycoside hydrolases (GHs, Cel8A, Cel9B, Cel48S, and Cel9K) as well as synthetic cellulosomal operons that direct the synthesis of Cel8A, Cel9B, and a truncated form of CipA. Successful heterologous protein expression, secretion, and self-assembly of cellulosomal subunits by recombinant *C. acetobutylicum* was demonstrated by utilizing supported allele-coupled exchange technology as a platform for synthesizing novel cellulosomes for CBP.

Engineering of fungi

A. niger was utilized in CBP, as a direct result of its inherent ability to secrete feruloyl esterases that hydrolyze diferulate cross-links in lignocellulose and enhance overall hydrolysis performance.[137] In an effort to facilitate a stronger synergism between catalytic domains, a minicellulosome was incorporated into *A. niger* to prevent large-scale diffusion of enzymes and lower protein requirements for hydrolysis. In detail, a chimeric protein composed of the feruloyl esterase A from *A. niger* was associated with the Cel48S dockerin from *C. thermocellum* and produced in *A. niger*. Analyzing the chimeric enzyme for its binding capacity indicated that translational fusion to glucoamylase improved the secretion efficiency of the protein and allowed production of the first functional fungal enzyme joined to a bacterial dockerin.[137]

For *S. cerevisiae*, most cell surface display methods developed have been based on agglutinin and flocculin model systems, which incorporate cell wall proteins (α-agglutinin, Aga1, Cwp1, Cwp2, Tip1p, Srp1, Flo1p, Sed1p, Tir1p, and YCR89W) containing a glycosylphosphatidylinositol (GPI) signal motif covalently cross-linked to β-1,6-glucan.[138] More specifically, *S. cerevisiae* was genetically modified to assemble a cell surface designer cellulosome by heterologously expressing a chimeric scaffoldin protein (Scaf3p) regulated

by a phosphoglycerate kinase 1 promoter, β-xylanase 2 secretion signal, cell wall protein 2 (Cwp2), and termination sequences, for GPI-mediated anchoring to the cell wall.[138] While fluorescent microscopy confirmed that Scaf3p targeted the yeast cell surface, Far Western blot analysis demonstrated functionality of the *C. thermocellum* dockerin domain binding the Scaf3 protein. Phenotypic evidence for cohesin–dockerin interaction was also established with the detection of a two-fold increase in tethered endoglucanase enzyme activity in *S. cerevisiae* cells compared to the wild type. This work highlighted the feasibility of designing cellulolytic strains of *S. cerevisiae* through emulation of the cellulosome concept.

Tsai et al[139] functionally displayed a cell surface minicellulosome exhibiting three divergent cohesin domains (CelE, CelA, and CelG) from *C. thermocellum*, *Clostridium cellulolyticum*, and *Ruminococcus flavefaciens* on *S. cerevisiae.* The recombinant cellulosome maintained synergistically significant glucose liberation and produced ethanol when utilizing a β-glucosidase (BglA) from *C. thermocellum* tagged with dockerin from *R. flavefaciens*.[139] As a result, *S. cerevisiae* was engineered to display a succession of uni-, bi-, and trifunctional minicellulosomes consisting of a miniscaffoldin containing a CBM and three cohesion modules tethered to the cell surface through the yeast α-agglutinin adhesion receptor.[140] While the cell surface assembly of the minicellulosome was dependent on high-affinity interactions between cohesion–dockerin binding domains, the triplicating enzymatic functionality of the surface-displayed minicellulosome enhanced the capabilities of *S. cerevisiae* at hydrolyzing lignocellulosic material for ethanol production during CBP by improving enzyme–enzyme and enzyme proximity synergies, as well as conceptually explicating cellulosome synthesis and anatomical modality. Subsequently, a cellulolytic four-strain *S. cerevisiae* consortium capable of either displaying a CipA scaffoldin derived from C. *thermocellum* or secreting one of three types of cellulases, CelA from *C. thermocellum*, CBHII from *T. reesei*, or BGLI from *Aspergillus aculeatus*, was developed for CBP.[141] A 20% increase in ethanol production was observed by regulating the combination and concentration of the four *S. cerevisiae* strains, with a CipA:CelA:CBHII:BGLI ratio of 2:3:3:0.53 producing 1.80 g L^{-1} ethanol after 94 hours. Advantageously, this system allows for the optimization of ethanol production as well as limitless implementation of enzymes into the minicellulosome construct, such as hemicellulases and pectinases adhered through fusion domains.

Artificial cellulosomes

In an effort to understand the relationship between the cellulosome structure and enzyme activity, reduce enzyme loadings and related costs, and improve compatibility of multi-protein complexes with biorefinery applications for biofuel production, truncated designer cellulosomes were constructed with cohesion modules and proven highly active compared to free cellulases during lignocellulosic degradation.[142–144] One such example is the robust 18-subunit, self-assembled synthetic multi-enzymatic complex, known as the rosettazyme, which consists of a CipA cohesion module and four dockerin-containing cellulases (Cel9B, Cel9K, Cel9R, and Cel48S) from *C. thermocellum*.[122,144] More specifically, rosettazymes are thermostable, group II chaperonins (scaffold-base) from the hyperthermo-acidophilic archaeon *Sulfolobus shibatae*, which in the presence of ATP/Mg^{2+} assemble into double-ringed structures that truss dockerin-containing endo- and exo-glucanases and enhance the cellulose degradation activity of bound enzymes.[144] The versatility of the rosettazymes cohesion–dockerin interaction on the cellulosomal scaffoldin provides a limitless number of arrays for the utilization of fabricating defined enzymatic nanostructures for the production of biofuels.[143] A chimeric cohesion-fused β-glucosidase (BglA-CohII) that binds directly to the cellulosome of *C. thermocellum* through an unoccupied dockerin module of the CBM was designed to direct enzymatic activity to substrate location.[145] BglA was proven to increase microcrystalline cellulose and pretreated switchgrass degradation, maintain cellobiose activity, and integrate with the native cellulosome, permitting the *C. thermocellum* cellulosome to subsist as a homo-oligomer. These findings demonstrate the significance of enzyme targeting to enhance lignocellulosic biomass degradation.

In addition to cohesion–dockerin interaction, conjugation techniques dependent on scaffold material and enzyme type, such as metal affinity between polyhistidine tag and core-shell quantum dots, protein fusion, and zinc-finger protein (ZFP)-guided assembly, have been investigated to improve stability, storage properties, and enzymatic synergies of synthetic multi-protein complexes.[122,144] In contrast with rosettazymes, cellulose–protein fusions are single polypeptide chains with cellulose domains internal to the scaffolding protein designed to increase cellulase thermostability and activity.[146] Purposefully, CelA from *C. thermocellum* was inserted into a hyperstable α-helical consensus ankyrin protein domain scaffoldin, which gave rise to an arrangement of multiple cellulose domains at a predetermined spacing within a single polypeptide to optimize reactivity of a repetitive

cellulose lattice.[146] Nanoparticles can be model supports for cellulose immobilization to enhance enzymatic activity.[147] In a study to evaluate the effects of nanoparticles on catalytic performance of artificial cellulosomes, it was found that size was moderately significant and enzyme proximity was extremely important to enhancing enzymatic activity.[147] Proteins can also be site-specifically localized onto a double-strand DNA scaffold using DNA-binding proteins, such as ZFPs composed of three subunits recognizing specific 3-bp sequences to create artificial bifunctional cellulosomes for improving the hydrolysis of cellulose.[148] By taking advantage of ZFP modality, site-specific docking of CelA and CBM of *C. thermocellum* onto a single DNA template was achieved.[148] As a result of advancements in protein engineering and conjugation technology, artificial cellulosomes have shown great promise by mimicking the enzymatic synergism observed in native cellulosome systems; however, these complexes are still less active than naturally occurring cellulolytic microorganisms.[136] While synergistic interactions between CBMs and catalytic domains impact the efficient degradation of lignocellulosic biomass by native, recombinant, and artificial cellulosomal possessing microbes, investigation of molecular mechanisms by way of metabolic fluxes are also required to improve growth potential, uptake and utilization of monosaccharides, alcoholic yields, and resiliency to inhibitory byproducts.[149]

Genomics, transcriptomics, proteomics, and metabolic fluxes

To understand the complex reactions required to degrade and ferment lignocellulosic biomass to biofuels, significant research has focused on the genomic, transcriptomic, proteomic, and metabolic profiles of *C. thermocellum*.[45] To expand on the knowledge base for clostridial species, such as *C. thermocellum*, relevant to current biofuel production efforts, the genome was sequenced and shown to include a 3.8-Mb DNA, single-chromosome arrangement consisting of a 39.8% guanine/cytosine content and 3,173 protein-encoding genes.[150,151]

Microarray transcriptomic analysis on *C. thermocellum* demonstrated deviations in gene expression levels resulting from modifications in carbon source and structure, nutrient availability, and microbial density, which validates the microbe's ability to sense and respond to external factors by signaling peptides or transcriptional regulators to transport and metabolize nutrients.[152] When altering the fermentative substrate from cellobiose to crystalline cellulose, expression profiles of genes corresponding to energy generation, translation, glycolysis and amino acid, nucleotide, and coenzyme metabolism increased.[153] Moreover, expression profiles for cellulosomal genes, inorganic ion transport and metabolism, signal transduction, amino acid transport, phosphate transport, and resistance–nodulation–division transport increased when utilizing pretreated yellow poplar, diluted acid-pretreated *Populus*, and switchgrass as lignocellulosic carbon sources.[154,155] These discoveries highlight the importance of carbon source on the metabolic functionality and performance of *C. thermocellum* in CBP.

Supporting proteomic studies expressing core metabolism proteins demonstrated growth-phase dependency for positioning of *C. thermocellum* to efficiently utilize cellulosic substrates and corresponding nutrients, which facilitates specific and consistent protein expression.[156] More specifically, metabolic proteins associated with pyruvate synthesis exhibited a decrease in expression, while proteins associated with glycogen metabolism, pyruvate catabolism, and end-product synthesis pathways increased when the microbe transitioned from exponential to stationary phase. Relative expression profiles demonstrated specific proteins utilized in carbohydrate consumption and end-product synthesis, which reinforced previous findings that hydrogen synthesis occurs by way of bifurcating hydrogenases, while ethanol synthesis is principally catalyzed by a bifunctional aldehyde/alcohol dehydrogenase. The differences in expression profiles of core metabolic proteins in response to growth phase may dictate the carbon and electron flux toward energy storage compounds and fermentation products. Cellulosome proteins also illustrate a change in expression as it relates to carbon availability. For instance, hemicellulases, such as XynA, XynC, XynZ, and XghA, are upregulated, in addition to endoglucanases CelA, CelB, CelE, CelG, and GH5, when *C. thermocellum* is grown on cellobiose; however, when grown on cellulose, GH9 are superiorly expressed with the corresponding surface-anchoring protein OlpB and endoglucanases CelS and CelK.[157] The results support the existing theory that expression of scaffoldin-related proteins is coordinately regulated by a catabolite repression mechanism, xylanase expression is prone to a growth rate-independent regulation, and transcriptional control of cellulases, such as endoglucanases, is conditional on catabolite repression. Similar results were demonstrated with pretreated switchgrass with the exception of an increased expression of GH9 and CelK as well as the decrease in xylanases due to the pretreatment-induced reduction in hemicellulose and xylan composition.[158]

A major challenge in using *C. thermocellum* for CBP biofuel production is the need to modify the organism for increased production efficiency; however, the process of properly engineering an organism is typically onerous.[159] To this end, a genome-scale model of *C. thermocellum* metabolism, iSR432, was developed by incorporating genomic sequence data, network topology, and experimental measurements of enzyme activities and metabolite fluxes for the generation of a computational apparatus for evaluating the metabolic network of *C. thermocellum* and facilitating enhanced biofuel production by way of strain engineering.[159] In fact, utilization of this model emphasized the correlation among reduction and oxidation states, as well as ethanol secretion, which permitted the prediction of gene deletions and environmental conditions that would potentially increase ethanol production.[159]

Recombinant solventogenic approach

Engineering to prolong existing metabolic pathways for the production of novel products, accelerating a rate-determining step, engineering enzymatic activities that synthesize unique biocatalytic nanostructures, and shifting metabolic fluxes toward the generation of value-added products, such as biofuels, is of extreme interest for aiding in the commercial viability of *C. thermocellum*, as a CBP biocatalysts.[16] In an effort to improve *C. thermocellum* ethanol yields by redirecting the carbon flux, a genetic system for making targeted gene knockouts was developed utilizing a toxic uracil analog, 5-fluoroorotic acid to select for deletion of the *pyrF* gene involved in organic acid production, namely *pta*, which encodes the enzyme phosphotransacetylase.[160] While the *C. thermocellum* Δ*pta* strain failed to produce acetate, the deletion marginally affected ethanol production.[161] Enzymes associated with organic acid formation were also deleted by creating a counter-selection system based on endogenous *hpt* and *T. saccharolyticum tdk* genes.[69] By combining these selection markers, the use of replicating plasmids to insert and remove markers from the host chromosome was realized.[125] Following the deletion of the L-lactate dehydrogenase (*ldh*) and *pta* genes from *C. thermocellum* M1570 with the counter-selection system and 2,000 hours of adaption, the ethanol yield of the mutant increased by 4.2-fold compared to the wild-type strain; however, ethanol yields failed to increase with decreasing acetate and lactate formation.[69] Conversely, when using the engineered *C. thermocellum* M1570 strain, CBP of downregulated caffeic acid 3-*O*-methyltransferase transgenic switchgrass was realized with a 20% increase in lignocellulosic conversion, followed by the primary production of ethanol, indicating the importance of substrate utilization when developing strains for enhanced biofuel yields.[162] In addition, Deng et al[61] examined the impact of targeted modification of enzymes associated with the malate shunt pathway in wild-type *C. thermocellum* DSM1313, including expression of pyruvate kinase gene from *T. saccharolyticum*, mutation of the phosphoenolpyruvate carboxykinase and deletion of malic enzyme gene to increase ethanol production. The researchers concluded that *C. thermocellum* with exogenous pyruvate kinase exhibited a 3.25-fold increase in ethanol yield when compared to the wild-type strain. Similarly, when the gene for malic enzyme and part of malate dehydrogenase were deleted, the anaerobic bacterium demonstrated ethanol yields more than three fold higher than that of the wild-type strain.

As a result of solvent toxicity being attributed to chaotropic effects on biological membranes, such as increased fluidity, degraded proteins and RNA, decreased energy generation and nutrient transportation, and damaged DNA and lipids, the development of strains with superior tolerance is imperative for the sustainable production of biofuels with increased yields.[134] Using an innovative engineering approach to increase ethanol tolerance, which typically ranges from 10 g L^{-1} to 20 g L^{-1} in wild-type strains, as a means of lowering production costs, Shao et al[163] isolated ethanol-tolerant strains of *C. thermocellum* that were capable of growing in medium containing up to 50 g L^{-1} ethanol. Genomic analysis of the isolated ethanol-tolerant strains revealed six common mutations, which originated in genes associated with ethanol, arginine, and pyrimidine biosynthesis pathways. Cellodextrin synthesis was also shown to be active as well as metabolically balanced.[164] Furthermore, Brown et al[165] demonstrated that a shift of cofactor specificity from NADH to NADPH for the bifunctional acetaldehyde–CoA/alcohol dehydrogenase enzyme proffers ethanol tolerance to *C. thermocellum*, resulting from an interference with electron flow. These studies provide knowledge on the mechanisms of ethanol tolerance to further metabolically engineer *C. thermocellum* aimed at higher ethanol yields to complement increased tolerance; however, the extensive realization necessitates targeted development and optimization of specific characteristics that will assist in the synergistic functionality.[45]

Microbial consortia

Thus far, recent advances regarding engineered microorganisms to produce a plethora of enzymes and biofuels from a

variety of lignocellulosic substrates are associated with low titers and consequently not commercially viable organisms.[56] Conversely, CBP conducted utilizing a microbial consortium, which is similar to natural conversion systems, is of interest due to synergies that result in extremely efficient substrate utilization and increased product yields.[56] For instance, *C. thermocellum* demonstrates a high growth rate on crystalline cellulose; nonetheless, limited ethanol yields, failure to effectively metabolize xylan, and exhibiting insufficient growth on xylose or other pentoses have resulted in hemicellulolytic thermophiles being used in conjunction with *C. thermocellum* to hydrolyze hemicellulose and ferment all sugars present in biomass.[125,166]

Cocultures with *C. thermocellum* have been reported with *Methanobacterium thermoautotrophicum* for methane and acetate as well as *Clostridium thermosaccharolyticum* for ethanol, butyrate, and acetate, which kinetically illustrated cellulose and cellobiose fermentation with enhanced ethanol yields from corn stover.[167] Once *Clostridium thermohydrosulfuricum* was isolated and discovered to ferment cellobiose, hexoses, and pentoses to ethanol, this too was coupled with *C. thermocellum* as part of a CBP study.[168] Unlike *C. thermosaccharolyticum*, *C. thermohydrosulfuricum* fails to produce butyrate and functions at thermophilic temperatures. More specifically, the microbial consortia, *C. thermocellum* and *C. thermohydrosulfuricum*, increased ethanol yields by two fold when compared to monocultures, due to the metabolic capability of *C. thermocellum*'s cellulases to hydrolyze α-cellulose and hemicellulose, enhanced utilization of mono- and disaccharides by *C. thermohydrosulfuricum*, improved cellulose consumption, increased ethanol production, and decreased acetate production.[167] This coculture was also shown to actively ferment MN300 cellulose, Avicel, Solka Floc, SO_2-treated wood, and steam-exploded wood.

More recently, Argyros et al[69] cocultured *C. thermocellum* and engineered *T. saccharolyticum* for 146 hours, which yielded 38.1 g L^{-1} ethanol from 92.2 g L^{-1} Avicel, with concentrations of acetic and lactic acids falling below detectable limits. Due to saccharification being crucial in producing lignocellulosic-derived bioethanol during CBP, extreme pH and ethanol concentrations inhibit production efficiency; therefore, several saccharides derived from lignocellulosics were investigated utilizing *C. thermocellum* and *Clostridium thermolacticum*.[169] The coculture actively fermented glucose, xylose, cellulose, and microcrystallized cellulose; in addition, the alkali environments proved conducive for ethanol production. While fermentation inhibition was observed when conditions exhibited high ethanol concentrations and extreme pH, initially low levels of ethanol resulted in an unforeseen stimulatory influence on ethanol production.[169] Conversely, a novel coculture of *C. thermocellum* and *Thermoanaerobacterium aotearoense* with pretreated sugarcane bagasse (SCB) under mild alkali conditions for bio-hydrogen production was established, which demonstrated an economically viable and synergetic advantage in bio-hydrogen production over monocultures with untreated SCB.[170] This successful microbial consortium is of interest as ethanol is another main product.

In addition to hemicellulolytic thermophiles, mesophiles, such as *C. acetobutylicum* and *Clostridium beijerinckii*, have enormous potential in coculturing CBP applications in conjunction with *C. thermocellum* for *n*-butanol and ethanol production from lignocellulosics. In fact, alcoholic solvents with more than two carbons, such as butanol, are ideal candidates for alternative fuels, due to compatibility with existing infrastructure, low hygroscopicity, flexible blending ratios and comparable octane value, energy density, and Reid vapor pressure similar to that of gasoline and diesel.[48,54,125,171–173] Currently, butanol is utilized for solvent extraction of fats, dye, nitro enamel, plastificator, butyl acetate, phenol formaldehyde resin, and oil-additive manufacturing.[174] With these benefits, a sequential coculture approach with *C. acetobutylicum* and *C. thermocellum* grown on solka floc or a combination of solka floc and aspen wood xylan was implemented. The results indicated an efficient utilization of all hydrolysis products derived, which produced a 1.7- to 2.6-fold increase in total fermentation products.[175] The majority of the fermentation products were acids; however, induction of solventogenesis by butyric acid was suggested as a solution. In regards to *C. beijerinckii*, a novel strategy for sequential coculturing with *C. thermocellum* was conducted to increase the production of alcoholic solvents from alkali-extracted corncobs.[176] Under combinatory optimal culture conditions for sugar and solvent production, this CBP consortium degraded 88.9 g L^{-1} of carbonaceous material and produced 19.9 g L^{-1} of total products, with 10.9 g L^{-1} ethanol in 200 hours without the addition of butyrate.[176] Nonetheless, the application of coculture CBP is vaguely understood with potential challenges of sole acid production and low product titers arising when symbiotically optimizing processing conditions, such as temperature, pH, and nutrient loads, through quorum sensing and biofilm formation for all strains as it relates to control, stability, and productivity.[176–178]

Biofilms

Comprehensive implementation of microbial biofilms, structured communities immobilized in a matrix of extracellular polymers, has been expansively applied to bioremediation; however, the potential beneficial application of biofilms in CBP for the production of biofuels has not yet been extensively investigated.[179–182] Biofilms have the potential to improve lignocellulosic biomass conversion efficiency, due to the concentration of cell-associated hydrolytic enzymes at the biofilm–substrate interface leading to increased reaction rates, a layered microbial structure allowing sequential conversion of complex substrates, and cofermentation of hexose and pentose with corresponding secretion and fungal–bacterial symbioses.[183] More impactful is the confined microenvironment within a biofilm selectively remunerating microbes with superior phenotypes deliberated from intercellular gene or signaling convergence, a process which is deficient in suspended cultures, by altering diffusion rates.[183,184] In addition, the immobilized property of biofilms, particularly when membrane attached, simplifies the separation of biofuels from microbial producer and liquid media as well as promotes retention of biomass for continued processing. With these benefits and importance of bacterial adherence in microbial lignocellulose conversion, the organization, dynamic formation, and carbon flow associated with biofilms of *C. thermocellum* was examined using noninvasive, in situ fluorescence imaging.[185] Investigation of the biofilm demonstrated the ability to extensively convert lignocellulosic substrates with a characteristic monolayered cell structure without an extracellular polymeric matrix, typically seen in biofilms. Moreover, cell division at the interface and terminal endospores appeared throughout all stages of biofilm growth. While utilizing continuous-flow reactors with an excessively high rate of dilution (2 h^{-1}), biofilm activity under low (44 g L^{-1}) and high (202 g L^{-1}) initial cellulose loadings resulted in fermentative catabolism being comparable, with 4% of metabolized sugar being utilized for cell production.[185] The study also observed 75.4% and 66.7% of the low and high cellulose loadings, respectively, being converted to primary carbon metabolites, including ethanol, acetic acid, lactic acid, and carbon dioxide.[185] Differences were also observed in ethanol/acetic acid ratios (g/g), suggesting that substrate availability for cell attachment rather than biofilm colonization rates govern the efficiency of cellulose conversion.

The importance of cellulosic surface exposure to microbial hydrolysis has received little attention regardless of implied influence on conversion kinetics; however, spatial heterogeneity of fiber distribution in pure cellulosic sheets made direct measurements of biofilm colonization and surface penetration unattainable.[102] This was circumvented by utilizing online measurements of carbon dioxide (CO_2) production in continuous-flow reactors, in conjunction with confocal imaging.[102] Results illustrated that the specific biofilm development rate of *C. thermocellum* has a significant effect on overall reactor kinetics during the period of microbial limitation. Due to biomass recalcitrance and the need to simplify enzymatic conversion, a single multi-species biofilm silicone membrane-adhered reactor was designed for CBP that featured both aerobic and anaerobic conditions.[56] Concept feasibility was successfully validated by producing ethanol with a 67% yield from un-detoxified whole-slurry dilute acid-pretreated wheat straw by the combined action of *T. reesei*, *S. cerevisiae*, and *Scheffersomyces stipitis*. The results achieved accentuates the potential of the process as a versatile inexpensive sugar platform for holistically producing biofuels and value-added chemicals from lignocellulosic biomass by specifically compiled consortia of industrially proven robust microorganisms in a CBP system.[56] Concisely, immobilized biofilms address solvent productivity, inhibitor tolerance, scalability, electron flux, and excessive fermentation periods.

Industries with interest in CBP

While academic advancements have proven beneficial in the promotion of CBP, so have the industrial advancements of Mascoma and Qteros as they relate to a holistic approach for producing biofuels and value-added products from a microbial consortium with *C. thermocellum* for the economic sustainability of transportation and rural- and agricultural-based sectors. Mascoma Corporation, an innovative renewable-fuels company, was first to report the targeted metabolic engineering of cellulose-fermenting thermophile, *C. thermocellum*, to reduce the production of unwanted organic acid byproducts and rapidly degrade and metabolize cellulose with high conversion efficiency and tolerance to commercially relevant levels of ethanol.[186] To produce high rates, titers, and yields of biofuels and biochemicals from the conversion of starch, sugars, and cellulosics, Mascoma utilizes CBP, the novel breakthrough technology, and high-performing industrial biocatalysts.[187] Mascoma has engineered yeast, TransFerm, and TransFerm Yield$^+$ for improved hydrolysis and enhanced xylose fermentation capabilities to increase yields from CBP processes.[188] In 2011, Mascoma was awarded $80 million from the Department of Energy to assist in the design, construction,

and operation of a commercial-scale hardwood cellulosic ethanol facility in Kinross, MI, USA.[189]

Qteros, a startup based in Marlborough, MA, USA, is also pursuing CBP with patented *Clostridium phytofermentans*, an anaerobic microbe with the ability to both convert recalcitrant polysaccharides into available sugars and ferment hydrolyzate into fuel-grade ethanol as the primary fermentation product.[190,191] This Q Microbe® genome has been fully sequenced, which revealed characteristics directly relevant to the efficient and cost-effective production of ethanol, such as over 105 different genes responsible for producing lignocellulosic-degrading enzymes regulated by the type of growth substrate utilized. With $100,000 awarded from the Department of Energy, the Q Microbe® has shown consistent saccharification efficiency across a broad range of feedstocks, including wheat straw, sugarcane bagasse, energy crops, such as switchgrass, and agricultural residues, such as corn stover, cob, and fiber.[192,193]

Conclusion

Biofuel production from lignocellulosic biomass is extremely attractive because of its substantial and renewable availability, relative low cost, and minimal environmental impact. Conventional sugar and starch sources exhibit high theoretical biofuel yields; however, these resources are inadequate for global utilization and impacts on food security. A potential solution lies in agricultural waste and dedicated energy crops that lack human nutritional value and require less land, water, and energy. Furthermore, biofuel production can significantly reduce greenhouse gas emissions. While biochemical conversion systems demonstrate great promise for producing biofuels, challenges of efficiently converting lignocellulosic biomass to available monomers and subsequent fermentation to biofuels with a robust microbe at low operating costs still exist. To resolve limitations in industrial implementation, CBP systems have been explored, with configurations involving *C. thermocellum* being most widely investigated. CBP has the potential to improve hydrolysis and fermentation efficiencies, eradicate contribution of exogenous saccharolytic enzymes, and eliminate inhibition effects, while requiring low energy with minimal environmental degradation to biologically convert lignocellulosic biomass to biofuels and other value-added chemicals. Hindrance of the overall ability and robustness of biocatalysts observed when incorporating genes from cellulolytic microbes for hydrolytic enzyme functionality has prompted the use of microbial consortia to achieve the advantages of a CBP operation, providing synergies that improve substrate use and increase biofuel yields. Ultimately, the economic production of sufficient quantities of biofuels will require advancements in feedstock development, conversion processes, system integration, marketing, and public policy as it relates to CBP.

Disclosure

The authors report no conflicts of interest in this work.

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