Review

Cellulosomes – Promising Supramolecular Machines of Anaerobic Cellulolytic Microorganisms

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Abstract

Cellulose is the main structural component of plant cell wall and thus the most abundant carbohydrate in nature. However, extracting the energy from this abundant source is limited by its recalcitrant nature. The hydrolysis of plant cell wall requires synergystic action of different enzymes, including multiple cellulases, hemicellulases, pectinases, etc. Meanwhile aerobic cellulolytic microorganisms release large quantities of synergistically acting free enzymes in their environment, most anaerobic microorganisms evolved more efficient strategies to optimize the process of plant cell wall degradation, for example production of extracellular multi-enzyme complexes (cellulosomes). Cellulosomes consist of at least one core structural protein, named scaffoldin, which firmly binds numerous enzymatic subunits, and usually also plays a major role in substrate binding. Although the general structure of cellulosomes seems universal, differences in number and identity of complex components do exist among microorganisms. The article surveys the current knowledge about cellulosomes, focusing on three best investigated cellulolytic clostridia, one representative of ruminal bacteria and novel findings concerning anaerobic fungi. Efforts in construction of artificially engineered cellulosomal systems (designer cellulosomes) as well as their biotechnological potential are also discussed.

Keywords: Cellulose degradation, microorganisms, enzyme complexes, cellulosomes, biotechnology

1. Introduction

Cellulose is the major building component of plant matter and as such makes the most abundant renewable source of organic polymers in the biosphere. From the biotechnological perspective, cellulosic material thus represents the most valuable source of biomass in which an enormous quantity of energy has been deposited by photosynthesis.[1] In response to high worldwide demand for energy, unstable and uncertain petroleum sources and concern over global climate change, many countries have initiated extensive research and development programs in sustainable and renewable energy resources.[2] The European Union has developed a vision in which 25% of E.U.'s transportation fuels will be produced in biorefineries by 2030.[3] It is therefore not surprising, that one of the major industrial challenges over the past years has become conversion of cellulose biomass into biofuels, such as ethanol. Furthermore, as cellulose waste represents the largest single waste by-product of our society, increasing scientific interest is oriented toward combined cellulose waste management and energy resources.[4,5] However, the use of such waste is limited by heterogenous and recalcitrant nature of (ligno)cellulosic material, which makes it difficult to be degraded in a cost-effective manner.[6,7] Current procedures for cellulosic biomass degradation combine physico-chemical treatment with enzymatic hydrolysis, the second being a feasibility-limiting step because of high costs and limited performance of currently available enzyme preparations.[7] Recently, some new strategies have been introduced to make the hydrolysis of cellulose more efficient. One of the possible future approaches is based on the use of multi-enzyme complexes, naturally found in many anaerobic cellulolytic microorganisms, the cellulosomes. Cellulosomes are supramolecular assemblies that exploit the synergistic interactions afforded by optimal proximity and organization of their enzyme components, making them more efficient in degradation of cellulosic substrates.[8] Increasing knowledge of such natural degradation systems combined with genetic engineering tools enables designing of cellulosomes with improved properties.[9–11] This article surveys current knowledge about cellulosomal systems of selected anaerobic microorganisms as well as achievements in construction of designer cellu-

losomes and some of their possible biotechnological applications.

2. Cellulolytic Strategies of Microorganisms

Numerous microorganisms have been reported to be importantly engaged in recycling of carbon by mineralization of biomass in different ecosystems such as the rumen, the gut of termites or other insects, soil, mud, compost or biogas reactors being some of them.[12] Meanwhile many microbes are capable of hydrolyzing soluble forms of polysaccharides, only a few of them can degrade non-soluble cellulose components of cell walls, specifically parts composed of crystalline cellulose. The latter represents the most recalcitrant form of cellulose due to its highly-ordered structure, in which adjacent chains of cellodextrines (polymers of D-glucose) in cellulose sheets are precisely arranged and tightly bound by hydrogen bonds.[13] Furthermore, the cellulose microfibrils in plant cell walls are usually enwrapped in a meshwork of hemicellulose and pectin (sometimes also lignin). Degradation of such substrate thus essentially requires not only different cellulases, but also a battery of many other enzymes (xylanases, mannanases, pectinases, etc.) to act in synergy. Microorganisms have developed different strategies to exploit the energy bound in plant cell walls. Aerobic cellulolytes (fungi, bacteria) typically produce high concentrations of numerous individual extracellular enzymes with binding modules for different types of substrates, whereas anaerobic microorganisms have developed more economical ways to release the energy from plant biomass. Thermophilic cellulolytic bacteria of the family *Syntrophomonodaceae*, for example, combine more than one of catalytic domains with a carbohydrate binding module (CBM) in one complex polypeptide. Such multi-functional cellulases have been found, for example, in *Anaerocellum thermophilum* and *Caldocellulosiruptor spp*.[14,15] Assembly of all the components needed for plant cell wall degradation within one multi-protein complex represents an even more elaborate system, which was first discovered in the thermophilic bacterium *Clostridium thermocellum.*[16] Such complexes were later also found in a variety of other cellulolytic members of bacterial order *Clostridiales,* many of them belonging to the family *Clostridiaceae*.[17] Among them, the cellulosomes of *C. thermocellum*, *C. cellulovorans* and *C. cellulolyticum* were investigated in great detail.[11] Some clostridial relatives found in rumen are known to produce cellulosome systems as well (*Ruminococcus albus* and *Ruminococcus flavefaciens* being two of the most investigated). Moreover, the evidence of cellulosomal type complexes was lately also revealed in some anaerobic fungi, namely *Piromyces sp*., *Orpinomyces sp*. PC-2 and *Neocallimastix sp.*[18]

It should be emphasized, that beside cellulosomal enzymes, most of the abovementioned microorganisms also produce diverse collections of free enzymes that cooperate in cell-wall degradation, but will not be discussed in this article.

3. General Properties of Bacterial Cellulosomes

The cellulosome is a discrete, extracellular protein complex, which comprise a set of multi-modular components, some of which are structural and some of which are catalytic (Fig. 1). The core structural subunits of cellulosomes are called scaffoldins, as they function as scaffolds, on which all the other (mostly catalytic) subunits are firmly attached.[19] The various enzymatic subunits anchor to cohesin modules of scaffoldin by their dockerin modules.

High-affinity, calcium dependent interactions between cohesin modules of the scaffoldin and enzyme-born dockerin modules facilitates the assembly of cellulosomes.[7] Although there is considerable degree of amino acid homology between cohesins from different species, the cohesin-dockerin interactions appear to be species-specific.[23] The cohesins present in scaffoldin of *C. thermocellum*, for example, did not interact with the dockerins present in *C. cellulolyticum* cellulosomal enzymes and vice versa.[20]

Another important module scaffoldins typically contain is carbohydrate binding module (CBM), which mediates the attachment of the whole protein complex onto the cellulosic substrate and helps to disrupt the crystal surface at the solid-liquid interphase. CBMs have been classified in 59 families according to amino acid sequence homology. In terms of function, the CBMs have been divided into three general categories: type-A CBMs bind strongly to insoluble polysaccharide surfaces by their hydrophobic faces, composed mainly of aromatic amino acid residues. On the other hand type-B CBMs interact with soluble glycan chains and are also called ‘chain binders’ because they have a range of affinities that increase with polysaccharide chain length. Type-C CBMs bind to small saccharides.[21,23]

Cellulosomes derived from different bacteria show a divergent type of architecture, owing to the number of interacting scaffoldins as well as the content and specificities of their resident cohesin.[21] In some cellulosome systems, for example, the primary scaffoldin is supplemented by another type of cohesin-containing scaffoldin that anchores the complement of the cell enzymes onto the cell surface. Additional adaptor scaffoldins might also exist, which can either further amplify the number of enzymes that can be incorporated into the cellulosome or alter its repertoire of components.[22] Owing to the different specificities of the cohesin-dockerin pairings, the cohesins of the primary (enzyme-encorporating) clostridial scaffoldins

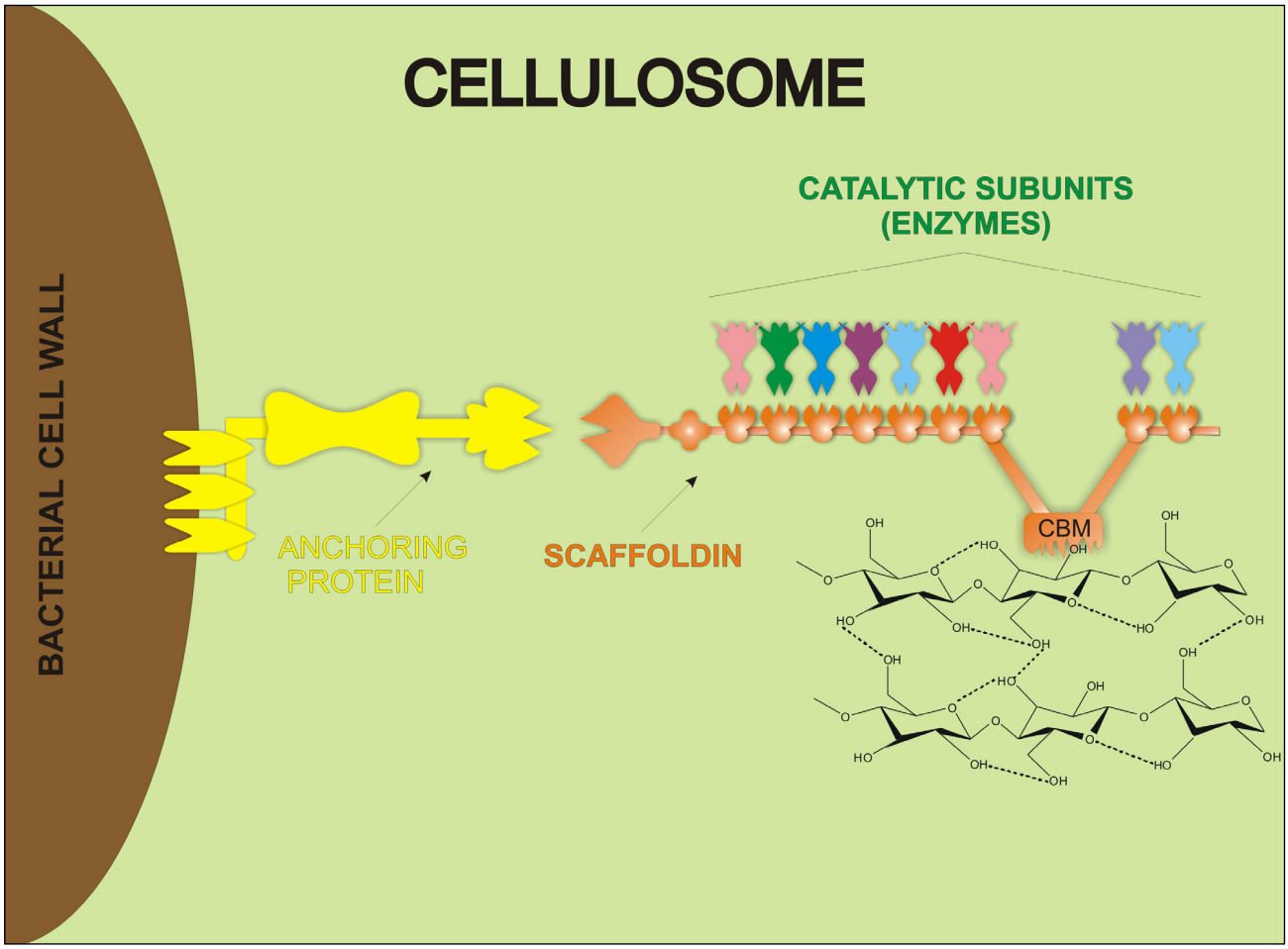

Figure 1: Schematic representation of basic cellulosome model (artwork by M. Vodovnik).

were termed type I cohesins, whereas those of the anchoring scaffoldins were termed type II cohesins. However, three phylogenetic groups of cohesins have later been proposed according to sequence homology (type I and II showing homology to cohesins of *C. thermocellum* primary/anchoring scaffoldins, and type III showing homology to *R. flavefaciens* ScaA and ScaB cohesins).[23] The structures of three different type I cohesins determined by X-ray crystallography revealed a jelly-roll topology forming a flattened nine-stranded β-sandwich. The structures of three different clostridial type II cohesins have also been solved and show the same jelly-roll topology as the type I cohesins but contain several additional structural elements, i.e. an α-helix and two 'β-flaps' that disrupt the normal course of β-strands 4 and 8. Characterization of *R. flavefaciens* cohesins type III is currently still in progress.[24] The dockerin type is set according to that of the cohesin with which it interacts.[25] Type I dockerins possess two calcium binding segments which are required for cohesin recognition and species specifity. The solution structure determination by NMR indicates that dockerins undergo substantial conformational change upon binding to cohesins. The crystal structure of the complex showed that cohesin recognition was predominately through helix-3 of the dockerin, through the near-perfect duplication of the 22-residue sequence in helix-1 could result in reverse binding of the dockerin. However, type I cohesins display less conformational flexibility than type II cohesins of *C. thermocellum*.[26]

Major factors that contribute to the efficiency of cellulosomal complexes in plant cell wall degradation are believed to be: 1) retention of a high local concentration of enzymes with different substrate specificities necessary for cleaving plant cell-wall polysaccharides and 2) maintaining of close contact between enzymatic machinery and the insoluble substrate.

Such organization might help to limit the diffusion and non-productive adsorption of break-down products and maximize the potential for synergy between different enzyme catalytic and binding specificities. Moreover, competitiveness in occupying a limited number of binding sites on the substrate is avoided by binding of the whole complex of enzymes to the substrate through a single strong binding domain.[22,17]

4. Cellulosomes of Selected Anaerobic Bacteria

4. 1. *Clostridium thermocellum*

C. thermocellum is thermophilic, strictly anaerobic, spore-forming bacterium, which hydrolyzes a wide range of polysaccharides from lignocellulosic biomass. It is readily isolated from soil, compost, hot springs and other places, where lignocellulosic biomass is abundant. This bacterium has attracted high interest as an extremely effective cellulose decomposer at elevated temperatures, which makes it an advantageous candidate for many biotechnological applications.[27] The extraordinary efficiency of its hydrolytic machinery was shown to be linked to formation of a huge (Mw > 2 MDa) extracellular enzyme complex.[28,29] In some strains, the cellulosomes aggregate to larger supercomplexes, called polycellulosomes, with Mw up to 100 MDa. The major structural protein of *C. thermocellum* cellulosomes is scaffoldin Cip A, which consists of nine enzyme-binding cohesin domains, CBM_{3A} with broad binding specificity for different sites on crystalline cellulose, a hydrophilic X-domain and a dockerin II domain. The latter binds to cohesin II domains of three other structural proteins (SdbA, ORF2p, OlpB) which participate in attachment of the complex on the surface of the cell.[30]

Later genomic survey of *C. thermocellum* revealed altogether more than 71 genes with dockerin molecular signatures, scattered through the genome. Almost all of them encode catalytic modules, with some exceptions of proteins with structural or unknown function.[31] Although *C. thermocellum* ferments exclusively sugars derived from cellulose, the evidence indicate that it has the potential to degrade a number of other polysaccharides, most of which supposedly play a role in effective removement of hemicellulose meshwork around the cellulose crystals.[32]

4. 2. *Clostridium cellulolyticum*

C. cellulolyticum, a mezophilic cellulolytic clostridium, was isolated from decayed grass (Petitdemange *et al.*, 1984)[33]. It is an oligotrophic microorganism that is able to grow on cellulose, cellobiose and glucose as well as on some other sugar monosaccharides and polysaccharides.[33,34]

A large set of genes related to cellulosome of *C. cellulolyticum* has been studied. Majority of the genes were found to be organized into a 26 Kb large, transcriptionally linked cluster (the '*cel*' cluster). The first gene in the cluster encodes the scaffoldin CipC, which is followed by seven genes coding for cellulases, one gene coding for mannanase and one gene coding for rhamnogalacturonan lyase. Three other genes encoding cellulosomal proteins were found to be located outside the gene cluster.[34]

Individual cellulosomes of *C. cellulolyticum* range in mass from 600 to 700 kDa. Their core structural unit is glycosylated scaffoldin CipC, which can bind up to eight enzymes.[35] Its N-terminal part is composed of cellulose binding module, which is followed by hydrophilic X2 module and seven hydrophobic cohesin modules. The C-terminal part contains a second X2 domain and a final cohesin module.[36]

The number of CipC cohesin modules indicates that no more than eight enzymes can be bound in a single cellulosome. However, at least 22 dockerin-bearing enzymes that can be produced by *C. cellulolyticum* suggest, that bacterium might produce a heterogenous population of cellulosomes with diverse enzymatic properties.[34]

4. 3. *Clostridium cellulovorans*

C. cellulovorans is another mezophilic clostridium, which was isolated from wood chip pile.[37] Its efficiency in plant cell wall degradation, together with observation of cell-surface protuberances first suggested that *C. cellulovorans* might posses (poly)cellulosomes, which was later confirmed by biochemical and molecular studies.[38] The major structural component of *C. cellulovorans* cellulosome, scaffoldin CbpA, consists of a cellulose-binding domain (CBD), four hydrophilic domains (HLD) and nine conserved hydrophobic cohesin (Coh) domains. The CBD preferably binds to crystalline cellulose portions of the substrate, although binding to amorphous cellulose, as well as some other types of substrates has also been detected.[39,40] Interestingly, the HLD domains were shown to have affinity to bacterial surface as well as to cellulose, which suggested their possible dual function. It is hypothesized that HLD domains of scaffoldin along with EngE protein bind the cellulosome to the cell surface, meanwhile HLD along with CBD cooperate in binding of the cellulosome to the substrate.[41] The cohesin domains of CbpA bind up to nine glycosyl hydrolases, capable of degrading cellulose, hemicellulose as well as pectin.[42] The enzyme composition of *C. cellulovorans* may vary depending on several factors, major ones involving carbon source.[43]

Similar to *C. cellulolyticum*, many cellulosomal genes of *C. cellulovorans* were found in one large gene cluster (*cbpA-exgS-engH-engK-hbpA-engL-manA-engM-engN*). However, the transposase gene, which was discovered on its 3' end, suggests the cluster might result from horizontal gene transfer.[30]

4. 4. *Ruminococcus flavefaciens*

R. flavefaciens is one of the most important bacterial species involved in cellulose breakdown in herbivorous animals. Although it was first isolated from rumen, related bacteria have also been detected in large intestines of horses, wild herbivores and humans.[44–46]

Ultrastructural and biochemical observations first indicated the presence of cellulosomes in *R. flavefaciens* 17.[47] However, firm evidence for complex cellulosomal organization has come from identification of proteins carrying cohesin sequences. These structural components of cellulosome are encoded within the *sca* gene cluster (*scaC-scaA-scaB-cttA-scaE*), the arrangement of which is conserved in at least four *R. flavefaciens* strains.[48–50]

The primary scaffoldin of *R. flavefaciens*, ScaA, is a 130 kDa glycosylated protein, which interacts with a variety of catalytic polypeptides, possessing xylanase, endoglucanase and esterase activities.[51] In addition, ScaA binds an adaptor scaffoldin, ScaC, which further broadens the repertoaire of subunits present in cellulosome.[52,53] The C-terminal dockerin of ScaA binds to a second heavily glycosylated structural protein, ScaB. This protein seems to provide a platform for anchoring up to seven ScaA subunits, forming a multi-scaffoldin cellulosomal complex. In addition, ScaB was shown to mediate attachment of the cellulosomal enzyme complex to the bacterial surface through its interaction with protein ScaE, which is covalently bound to the gram-positive peptidoglycan via sortase-mediated linkage. ScaE anchoring scaffoldin was also found to bind non-catalytic protein CttA, which supposedly play a role in cellulose binding.[53,54]

5. Cellulosomes of Anaerobic Fungi

Strictly anaerobic fungi, first described by Orpin,[55] are found in rumen and caecum of herbivorous animals. They contribute to degradation of ingested plant food by producing multiple enzymes, including endo- and exocellulases, β-glucosidases, mannanases, xylanases, (acetylxylan, feruloyl) esterases, glucuronidases and lichenases, which effectively hydrolyze cellulose and hemicellulose. Some of these enzymes are released free in solution, whereas most of them are organized in large multi-enzyme complexes that resemble the cellulosomes found in anaerobic bacteria. Cellulosomes from anaerobic fungi seem to share many properties with cellulosomes of anaerobic cellulolytic bacteria and have comparable structures, but their components differ in amino acid sequences.[18,56,57] Cellulosome-type complex with high activity against cotton fiber was first isolated from *Neocallimastix frontalis.*[58] The complex had molecular mass around 670 kDa and consisted of at least six different polypeptides. Similar complexes, but of least ten polypeptides with cellulase and hemicellulase activities, were also detected in cultures of *Piromyces sp.* strain E2 and *Piromyces equi*, meanwhile biochemical and ultrastructural examination revealed cellulosome – (3 MDa) as well as polycellulosome- (80 MDa) type structures, located along the mycelium of *Orpinomyces sp.* PC-2[59–62]

The first amino acid sequence-based indications of cellulosomal organization in fungi were found by Fanutti *et al.,*[63] who studied the primary structure of xylanase A and mannanase A from *Piromyces sp.* In addition to the catalytic modules, the sequences revealed almost identical, 40-amino-acid long, reiterated non-catalytic domains, which were shown to bind specifically to 97 kDa and 116 kDa polypeptide components of cellulolytic complexes from *Piromyces* and *Neocallimastix patriciarum*. Based on functional analogy with bacterial cellulolytic enzymes it was proposed, that these non-catalytic sequences function as dockerin domains, which bind to two putative scaffoldins. The presence of such cysteine-rich dockerin domains was later confirmed in most of the enzymes, associated with cellulosomes of *Piromyces sp.* and *Orpinomyces sp.* It should be emphasized, that the sequence of fungal dockerin domains (FDDs, sometimes also called non-catalytic dockerin domains, NCDD) is completely different from those of bacterial dockerins.[18,57] Moreover, in contrast to bacterial cellulosomal enzymes which usually contain a single copy of dockerin domain, most of the fungal cellulosomal enzymes contain tandem arrays of two or three dockerin domains.[64] Based on subtle differences in the sequences of fungal dockerins (number and positions of cysteine residues), Steenbakkers *et al.* proposed their division in three types of subfamilies (Types 1 and 3 contain six cysteines, meanwhile Type 2 only four).[56]

No scaffoldin from fungal cellulosomes has yet been identified. However, Freelove *et al.* identified first of the presumably scarce proteins with carbohydrate binding modules (CBM) in cellulosomal complex of *P. equi* (NCP1). Mature protein NCP1 displayed a modular architecture comprising three copies of fungal dockerin domains and two C-terminal CBMs with wide substrate specificities (CBM29-1, CBM29-2).[65]

6. Designer Cellulosomes

The modular architecture of the cellulosomal subunits led to the proposal that individual modules could be linked together genetically to form chimeric components, whose combination would result in the construction of "designer cellulosomes". If the constructs displayed increased efficiency for substrate degradation, they could eventually be applied to improve the degradation of cellulosic wastes.[10]

The background of "designer cellulosome" concept is based on exploiting species-specificity of the bacterial dockerin-cohesin interaction. More specifically, genetic engineering tools enable the construction of chimeric scaffolding protein (mini-scaffoldin) with an optional CBM and selected cohesins of different species, which bind tightly and selectively the desired enzymes with matching dockerins. Such system enables to control the composition and spatial arrangement of the resultant designer cellulosomes in order to provide better efficiency of substrate degradation.[9,10] In one of the latest studies

Mingardon *et al*. (2007) for example constructed chimeric minicellulosomes combining various cellulases from *C. cellulolyticum* with non-cellulosomal endoglucanase Cel6A originating from fungus *N. patriciarum*. Enhanced activity on microcrystalline cellulose was observed when fungal cellulase was assembled in complex together with bacterial family 9 endoglucanases (but not with family 5 endoglucanase) compared to the free-enzyme system.[66]

Although the main biotechnological perspectives of such designer cellulosomes target their use in cellulose substrate processing, their current use mostly involves studies of structure, function and synergistic interaction between different cellulosomal components, oriented toward understanding of the factors, important for their efficiency.[4,5,67,68]

6. 1. "*In vivo*" Constructs with Designer Cellulosomes

Current interest in engineering microbial cells for efficient deconstruction of plant matter coupled with bioenergy production has further lead to efforts oriented toward development of recombinant microorganisms, which would be able to directly transform the energy from inexpensive cellulosic material to valuable products, such as ethanol or butanol. One of the promising approaches to reach such goal is heterologous expression of designer cellulosome components in solventogenic clostridia.[69] Recent efforts in metabolic engineering suggest that *Clostridium acetobutylicum* is one of the potential species that could successfully be engineered to exploit cellulose for solvent production. This species naturally produces acetone, butanol and ethanol by fermentation of different substrates, but it is unable to grow on cellulose due to lack of functional cellulolytic system (although its genome apparently encodes complete set of cellulosomal genes). An effort to create recombinant *C. acetobutylicum*, which would express heterologous mini-cellulosomes, was recently reported. In the first step functional recombinant scaffoldins (miniCipC1, a truncated form of *C. cellulolyticum* scaffoldin, and the hybrid scaffoldin Scaf3 from *C. thermocellum*) were constructed and expressed in selected host. The next step involved expression of functional cellulases and determination of their binding to the miniscaffoldins. The coexpression of the gene, coding for mannanase from *C. acetobutylicum* (man5K) with the gene, encoding miniCipC1 resulted in biologically functional heterocomplex, secreted by the recombinant *C. acetobutylicum*.[70,71]

7. Conclusion

Increasing knowledge of microbial systems for efficient cellulose degradation, combined with genomic data and genetic engineering tools, reveals promising opportunities for different biotechnological applications. "Cellulosomal technology" represents not only a significant potential for generation of low-cost energy, but also for many other fields, including animal feed technology, textile and paper biotechnology. Furthermore, it could successfully be applied in research and diagnostics (for affinity purification or immobilization, for instance).[72–74] All these facts indicate, that these prodigious microbial nanomachines certainly deserve scientific attention!

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Povzetek

Celuloza je temeljni gradnik rastlinske celične stene in tako predstavlja najbogatejši rezervoar ogljikohidratov v naravi. Pridobivanje energije iz tega obilnega vira je omejeno z njegovo odpornostjo proti razgradnji. Za hidrolizo celične stene je potrebno usklajeno delovanje celega spektra različnih encimov, vključujoč celulaze, hemicelulaze, pektinaze, itd. Medtem, ko aerobni celulolitični mikroorganizmi slednje v velikih količinah sproščajo v okolje, so mnogi anaerobi razvili učinkovitejše sisteme razgradnje celične stene. Eden takšnih je proizvodnja ekstracelularnih multiencimskih kompleksov, imenovanih celulosomi. Ti so v splošnem sestavljeni iz najmanj ene strukturne beljakovine, imenovane skafoldin, ki trdno veže cel spekter encimskih podenot, poleg tega pa ima običajno tudi pomembno vlogo pri pritrjanju kompleksa na substrat. Kljub temu, da je struktura celulosomov na prvi pogled univerzalna, so znane nekatere razlike med kompleksi različnih mikroorganizmov. V članku so zbrani dosedanji podatki o celulosomskih sistemih treh celulolitičnih klostridijev, predstavnika vampnih bakterij in anaerobnih gliv. Povzeti so tudi novejši dosežki genskega inženirstva v sestavljanju rekombinantnih celulosomov ter njihov biotehnološki potencial.