Review

Designer cellulosomes – catalytic nanomachines with significant potential in biotechnology and circular economy

Maša Vodovnik [1*]

Abstract

Cellulosomes are multienzyme complexes originally found on the surface of certain anaerobic cellulolytic bacteria and fungi specialized in the degradation of plant cell walls. Recently, the efficiency in lignocellulose conversion and architectural features of these intricate complexes inspired the construction of artificial chimeric complexes for targeted substrate degradation. The simultaneous advancements in synthetic biology, protein engineering and the pursuit of greater sustainability across various industries have highlighted the immense potential of these artificially designed enzymatic complexes for diverse applications. Notably, they hold significant promise for industries specializing in the valorization of plant biomass waste and the production of bio-based renewable energy. The article discusses the main architectural features, design and construction steps, and various biotechnological applications of these intriguing nanomachines.

Keywords

Designer cellulosomes; lignocellulose valorization; protein engineering; nanobiotechnology, bio-based products

1 University of Ljubljana, Biotechnical Faculty, Department of Microbiology, Chair of Microbiomics, Diversity and Biotechnology

*** Corresponding author:**
E-mail address: masa.vodovnik@bf.uni-lj.si

Citation: Vodovnik, M., (2024). Designer cellulosomes – catalytic nanomachines with significant potential in biotechnology and circular economy. Acta Biologica Slovenica 68 (1)

Received: 18.12.2024 / **Accepted:** 09.01.2025 / **Published:** 09.01.2025

https://doi.org/10.14720/abs.68.01.21452

Sintetični celulosomi – katalitični nanostroji z velikim potencialom v biotehnologiji in krožnem gospodarstvu

Izvleček

Celulosomi so multiencimski kompleksi, primarno identificirani na površini nekaterih anaerobnih celulolitičnih bakterij in gliv specializiranih za razgradnjo rastlinskih celičnih sten. Zaradi izjemne učinkovitosti pri razgradnji odpornih lignoceluloznih materialov ter njihovih edinstvenih arhitekturnih značilnosti so postali navdih za razvoj sintetičnih hibridnih analogov zasnovanih za ciljno razgradnjo različnih (predvsem odpadnih) lignoceluloznih substratov. Hiter razvoj sintezne biologije, napredki v proteinskem inženirstvu ter vse večja potreba po trajnostnih rešitvah obstoječih izzivov so vodili do spoznanja, da imajo ti umetno zasnovani katalitični nanostroji velik potencial v mnogih industrijskih panogah, ki slonijo na učinkoviti razgradnji lignocellulozne biomase. Še posebno se pričakuje, da bi lahko ti umetni kompleksi pomembno doprinesli v industrijskih aplikacijah, ki temeljijo na valorizaciji odpadne rastlinske biomase, zlasti v sektorjih osredotočenih na pridobivanje obnovljive energije in bele biotehnologije. Članek se osredotoča na glavne arhitekturne značilnosti, zasnovo in ključne korake pri konstrukciji sintetičnih celulosomov ter njihov potencial za različne biotehnološke aplikacije.

Ključne besede

celulosomi; valorizacija lignoceluloze; proteinski inženiring, sintezna biologija, nanobiotehnologija, bio-osnovani produkti

Introduction

As the world strives for alternatives to fossil fuels and more sustainable methods for managing agricultural and industrial waste, leveraging the evolutionarily refined natural systems capable of efficiently degrading accumulating plant biomass is becoming increasingly important (Gayathri et al., 2021). Among the most elaborated systems capable of efficient degradation of lignocellulose have been found on the surface of some anaerobic microorganisms. Cellulosomes are large, multi-enzyme complexes primarily produced by anaerobic cellulolytic bacteria, particularly from the order *Clostridiales*, inhabiting different environments rich in lignocellulosic materials, such as soil, compost and rumen (Bayer et al., 2004). The pioneering work that established the cellulosome paradigm began over four decades ago with Bayer and Lamed (Bayer et al., 1983), who first described the extracellular cellulolytic complex of *Clostridium thermocellum*. Subsequent research uncovered similar or even more intricate complexes in other anaerobic (hemi)cellulolytic bacteria, with the most extensively characterized examples being the cellulosomes of *Clostridium cellulolyticum, Clostridium cellulovorans, Acetivibrio cellulolyticus*, and *Ruminococcus flavefaciens* and fungi (Artzi et al., 2017). Recently, a minimalistic cellulosome has also been described in the butanologenic bacterium *Clostridium saccharoperbutylacetonicum* (Levi Hevroni et al., 2024). Cellulosomes in these species are specialized for degrading the recalcitrant plant cell wall polysaccharides, including cellulose, hemicellulose, and pectin. Their exceptional efficiency in breaking down lignocellulosic biomass is due to their highly organized structure and synergistic action of multiple enzymes positioned close to one another (Alves et al., 2020). With the recent rise of white and green biotechnology followed by circular economy movements, these efficient cellulolytic complexes attracted significant attention as potential catalysts for the valorization of lignocellulose waste, not only because of their catalytic efficiency but also due to their specific architectural features. The modular architecture of structural and catalytic subunits composing these complexes was recognized as potential building blocks for the construction of designer catalytic complexes that can be adapted for specific types of plant biomass (feedstocks), potentially expressed in different hosts and used in various industrial applications (Asemoloye et al., 2023).

Cellulosome architecture

The unique architecture of cellulosomes is critical to their function. The primary components of each cellulosome include one or more structural proteins, scaffoldins, and several catalytic subunits. Primary scaffoldin acts as the backbone of the cellulosome architecture, anchoring the complex to the substrate on the one hand and providing firm docking sites for enzymatic subunits on the other. The enzymatic machinery of cellulosomes comprises a diverse set of carbohydrate-active enzymes (CAZYmes) involved in the degradation of plant cell walls, particularly glycoside hydrolases, carbohydrate esterases, and polysaccharide lyases (Artzi et al., 2017). Furthermore, proteases and their inhibitors (SERPINS) have also been found within some complexes. The spatial organization of the enzymes within the complex minimizes diffusion limitations, enhancing the overall efficiency of the complex (Alves et al., 2021).

The main interaction holding together the subunits in cellulosome is the high-affinity interaction between cohesin modules on the scaffoldins and dockerin modules on the enzyme subunits (Type I cohesin-dockerin interaction) (as depicted in Fig. 1). This interaction allows dynamic assembly of cellulosome components and adaptation to the enzyme repertoire based on substrate availability. In addition, a different type of cohesin is also present on the secondary, anchoring scaffoldin (Type II), known to bind the main scaffoldin on one side and attach to the bacterial cell wall from the other (Artzi et al., 2017). Some cellulosomes, for example, the complexes identified in *R. flavefaciens*, are known for even more elaborate structures, involving several scaffoldins binding with each other, resulting in multiplied enzyme-binding sites (Vodovnik et al., 2013; Stern et al., 2016). Furthermore, adaptor scaffoldins have also been discovered that enable switching the substrate specificity of the complex by accommodating different sets of the synthesized enzymes.

Most of the scaffoldins (and some enzyme polypeptides) also contain a carbohydrate-binding module (CBM), which plays a crucial role in targeting and binding to specific carbohydrates, enhancing the catalytic efficiency of the complex by bringing the enzymes in close proximity to the substrate. According to structural properties, CBMs are classified in different families. Some CBM families (for example, CBM 1,2 and 3) target crystalline or amorphous cellulose through hydrophobic stacking interaction and hydrogen bonding with glucose residues. Others, such as CBM families 6 and 22, bind to hemicellulose components like xylan, mannan or arabinoxylan. Their specificity depends on the sugar composition of the hemicellulose substrate. In addition, pectin-binding CBMs targeting homogalacturonan or rhamnogalacturonan (CBM family 28) have also been identified. Typically, the main scaffoldin identified within the cellulosomal scaffoldins belongs to the family CBM3a, which is known to recognize regular repeating structures on insoluble carbohydrate surfaces, such as crystalline cellulose or chitin. The catalytic (GH) and structural (CBM, dockers, cohesins) modules in cellulosomes are connected by linker regions, which provide flexibility and enable dynamic movement on the surface of the substrate (Alves et al., 2021).

The attachment of cellulosomes to the bacterial cell walls is another critical aspect of their function in biomass degradation. Several strategies are used to anchor scaffoldins to the bacterial cell wall, including noncovalent interaction via S-layer homology (SLH) domains hydrophobic anchoring to the lipid bilayer, or sortase-mediated covalent anchoring of the scaffoldins to peptidoglycan (Artzi et al., 2017).

Designer cellulosomes

Natural cellulosomes, although highly efficient in natural environments, have several constraints that limit their industrial utility, the main three being: (1) non-optimal enzyme composition (the enzyme repertoire of natural cellulosomes is limited to what the host organism naturally expresses), (2) environmental constraints (natural cellulosomes are often adapted to specific environmental conditions, such as strict anaerobiosis, which may not align with industrial requirements), (3) lack of flexibility (the fixed enzyme arrangement in natural cellulosomes restricts their adaptability to various substrates) (Lamote et al., 2023). These challenges have been tacked by the advances in molecular and synthetic biology, which paved the way for the construction of artificial complexes with customized enzyme combinations with improved flexibility, efficiency, specificity and stability in industrial conditions (Joseph et al., 2018). Designer cellulosomes (depicted in Fig. 1) are modular complexes with previously designed architecture and composition constructed using synthetic biology and protein engineering tools (Vazana et al., 2013). The construction of such engineered complexes is achievable by leveraging the species-specificity inherent

in cohesin-dockerin interactions. Apart from the controlled incorporation of target catalytic activities, the engineering of the cellulosomes also allows for the expression and stability optimization of target complexes. The optimization of different subunits can be performed either by rational engineering approach, relying on previous knowledge of the structure-function relationship, or directed evolution, by constructing a library of random mutants followed by rigorous selection based on desired properties of the proteins. Other approaches to increase the stability of designer cellulosomes in industrially-relevant conditions, such as glycosylation, have also been studied (Khan et al., 2020).

Several such designer complexes targeted for different biotechnological applications, particularly focused on valorization of (hemi)cellulose biomass, have already been constructed and displayed improved activity, stability, and adaptability across diverse substrates (Wen et al., 2010; Ponsetto et al., 2024).

Construction of designer cellulosomes

The construction of the designer cellulosomes is a multi-step process that is based on previous knowledge regarding the structure-function relationship of different protein components. The main steps in the construction of designer cellulosomes involve (1) selection of target substrate to be degraded (transformed in value-added products); (2) selection of optimal enzyme activities and stochiometry for optimal degradation of the target substrate (based on its composition, i.e. hemicellulose or cellulose prevalence, crystallinity, branching..); (3) complex design: selection of the modules and design of the cellulosome components (scaffoldin design, including the number and specificity of cohesin domains based on the number and type of enzymes to be incorporated and enzymatic subunits design, involving target catalytic domains and compatible dockerin modules); (4) construction of vectors encoding designed modular proteins, molecular cloning to construct

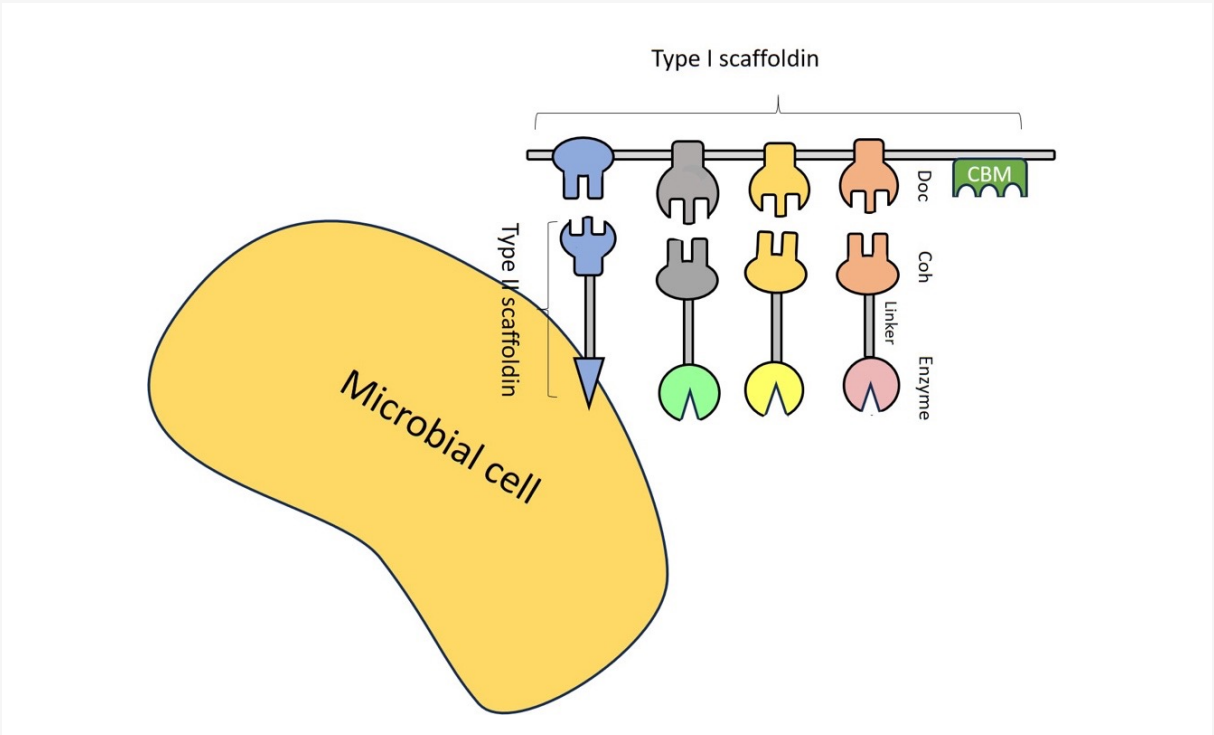

Figure 1. Graphic representation of the basic architecture of a designer cellulosome. Doc: dockerin, Coh: cohesin, CBM: carbohydrate-binding module. Differences in colours represent different sources of protein modules within the complex (i.e. the sequence of each module can originate from different species).

Slika 1. Grafični prikaz osnovne arhitekture načrtovanega celulosoma. Doc: dokerin, Coh: kohezija, CBM: modul za vezavo ogljikovih hidratov. Razlike v barvah predstavljajo različne vire beljakovinskih modulov v kompleksu (tj. zaporedje vsakega modula lahko izvira iz druge vrste).

coding sequences of the complex subunits; (5) expression of vector constructs in heterologous hosts (selected based on targeted application) and assembly of the subunits into the final complex, (6) *in vitro* determination of stability and activity of target designer complexes (Lamote et al., 2023).

The selection of protein modules includes three main aspects, starting with scaffoldin and protein modules selection, which is followed by carbohydrate-active enzymes (CAZYmes) and carbohydrate-binding modules (CBMs) selection (based on the target substrate and the desired degree of degradation). In addition, accessory proteins, such as adaptor scaffoldins, may also be added (Stern et al., 2016). Enzymes with different specificities have already been included in such designer complexes. Typically, at least one endo- and exoglucanase pair is needed to target cellulose-rich biomass (Arfi et al., 2014; Vazana et al., 2010), while different types of enzymes targeting hemicellulases (xylanases, arabinases, mannanases), expansins (Chen et al., 2016) or even lignin-targeting laccases may also be added (Fierobe et al., 2001; Fierobe et al., 2005). Further, the selection of cohesin-dockerin pairs and possible linkers is also performed, which does not depend on the substrate composition. The efficiency of designer cellulosomes is influenced by many parameters, including the selected dockerins and linkers, as well as the docking enzyme ratio on the scaffoldin. Recently, the Versatile platform was successfully applied to construct a range of different docking enzymes and scaffoldin variants, facilitating the targeted study of specific parameters influencing the activity of designer cellulosomes. This platform includes a tile repository composed of dockerins, cohesins, linkers, tags and catalytic modules and allows for the fast and efficient construction of designer cellulosomes, enabling the creation of a practically infinite number of complexes (Vanderstraeten et al., 2022).

After selecting the basic components, the final complex architecture is designed by deciding the order of different modules in scaffoldin(s) and designer enzymes, which considers enzyme synergy and potential spacial constraints (Caspi et al., 2009).

In the next step, modular DNA encoding dockerin-containing enzymes and scaffoldin(s) is either synthesized or constructed by different cloning techniques (restriction-digestion, CPEC cloning or Versatile shuffling). Different expression systems can be used where each cellulosome subunit lays onto a separate vector (single expression system) or the sequences of multiple components are coexpressed and produced by a single host.

Further, different modes of designer cellulosome assembly are possible, such as the "*in vitro*" assembly by co-incubation of different subunits following their individual expression. The assembled complex can then be characterized by native polyacrylamide gel electrophoresis (PAGE) or affinity pull-down. An upgraded version of this process involves the direct assembly on the surface of the expression strain, usually an industrial microorganism, with the ability to directly convert the released sugars to bio-based products, such as ethanol, butanol, etc. (Lamote et al., 2023).

So far mostly yeast and solventogenic clostridia have been engineered to display the cellulosomes, particularly *Saccharomyces cerevisiae* (Tsai et al., 2009; Tsai et al., 2010; Tsai et al., 2013; Fan et al., 2020; Anandharaj et al., 2020, Ma et al., 2024; Sharma et al., 2022), *Pichia pastoris* (Dong et al., 2020) and *Clostridium acetobutylicum* (Kovács et al., 2013; Willson et al., 2016), although engineering of some other industrially relevant microorganisms, such as *Corynebacterium glutamicum* (Lee et al., 2022), has also been reported. Apart from pure solvent-producing cultures, engineered yeast consortium-producing functional mini-cellulosomes have also been reported (Goyal et al., 2021).

Biotechnological applications of designer cellulosomes

The ability to tailor cellulosomes for specific substrates and optimize their efficiency and stability has significant implications for various biotechnological applications based on cellulose waste valorization and the production of bio-based products. One of the most prospective fields that may benefit from the designer cellulosome-based catalysts involves second-generation biofuel production, which relies on converting lignocellulose biomass to solvents or hydrogen. The construction of consolidated bioprocessing systems that simultaneously efficiently degrade various lignocellulose substrates and ferment the released sugars to value-added solvents, like ethanol or butanol, holds significant promise for future sustainable bioprocessing, as it reduces process complexity, minimizes production costs, and enhances overall efficiency (Li et al., 2024). By integrating enzyme production, substrate hydrolysis, and fermentation into a single step, these systems can enable the economically viable conversion of renewable biomass into biofuels and biochemicals, contributing to the development of biorefineries and reducing dependence on fossil fuels (Wen et al., 2020). Furthermore, the trans-

formation of lignocellulose to other value-added products, including different bio-based chemicals (i.e. lactate, succinate), also holds great promise (Liu et al., 2021). In addition, applications in other, more traditional industries, such as textile, paper, feed, and food industries, where (partial) degradation of (hemi)cellulose material enhances the efficiency of processes (e.g., polishing of textiles, deinking of paper sludge, or increasing the availability of high-nutrient molecules), may also be improved through the use of these intricate nanomachines.

Challenges and Future Perspectives

Cellulosomes represent a remarkable natural solution for lignocellulosic biomass degradation, offering a powerful tool for sustainable biotechnology. Their intricate structure, enzymatic synergy, and adaptability to diverse substrates make them ideal for applications ranging from waste management to biofuel production (Xin et al., 2019). However, the construction and industrial application of designer cellulosomes presents several challenges, primarily due to the complexity of designing and assembling these highly specialized enzymatic systems. Key hurdles include the difficulty in optimizing the interactions between the scaffoldin and enzymatic components, ensuring stability and activity under industrial conditions and the need for precise control over the composition and architecture of the complexes. Furthermore, the scalability of designer cellulosome-displaying catalysts for large-scale applications, such as in biofuel production or industrial biomass degradation, remains a significant obstacle (Wen et al., 2020). By improving enzyme efficiency, tailoring cellulosome designs for specific substrates, and optimizing production systems, these biocatalytic nanomachines can significantly contribute to the development of sustainable utilization of lignocellulosic biomass (Ye et al., 2024). While challenges remain, ongoing advances in genetic engineering, synthetic biology, and process optimization promise to unlock their full potential, driving progress in renewable energy and circular bio-economy.

Acknowledgement

The author thanks the Slovenian Research and Innovation Agency for financial support and N. Lindič for help with graphic design.

Conflicts of Interest

The author declares no conflict of interest.

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