IJLST

ISSN: 0974-5335
IJLST (2011), 4(5):31-36

Cellulosomes - A Robust Machinery for Cellulose Degradation

Vijay Kothari[1], Parini Surti[2], Devanshi Kapadia[2],

1 Assistant Professor, Institute of Science, Nirma University, Ahmedabad, INDIA
2 Graduate Student, Institute of Science, Nirma University, Ahmedabad, INDIA
vijay23112004@yahoo.co.in ; vijay.kothari@nirmauni.ac.in

ABSTRACT:
Cellulose is the most abundant polymer in nature. It is the chief component of huge amount of agricultural waste generated every year. Cellulosomes are the multicomponent, multienzyme complexes of anaerobic cellulolytic bacteria, which employ the synergistic activity of various resident enzymes that efficiently degrade cellulose and other components of plant cell wall. The cellulolytic enzyme complexes of anaerobes, *Clostridium thermocellum* and *Clostridium cellulovorans* have been studied widely. To understand the complex phenomena between the plant cell wall polymers, cellulolytic microbes and their enzymes, a highly integrated approach is required. Designer cellulosomes may prove a useful tool for economic conversion of cellulosic biomass to biofuels. Efficient cellulosome application will provide dual benefits of cellulosic waste disposal as well as conversion of cellulose into fermentable sugars for ethanol production for solving fuel crisis.

Key words: Cellulosomes, Cellulase, Designer cellulosomes, Carbohydrate Binding Module (CBM), Consolidated Bioprocessing (CBP), Waste management.

INTRODUCTION

Cellulose, the most abundant polymer in nature, is a linear, insoluble biopolymer composed of repeating β–D-glucopyranose residues linked by β-1, 4 glycosidic bonds. The repeating unit of cellulose is a disaccharide-cellobiose, unlike glucose in case of starch. It is highly polymerized and composed of both amorphous and crystalline forms. Therefore, it is difficult to degrade resulting in accumulation within the environment. Extensive research is on for efficient conversion of bulky cellulosic waste into biofuel. The complexity of the cellulose structure is increased due to the presence of microfibrils composed of crystalline nanodomains which further assemble into plant cell walls and tunica of some sea animals [1].

Aerobic bacteria produce noncomplexed cellulases as they weakly adhere to cellulose. Certain anaerobic bacteria use cellulose as a carbon source and degrade it into several other metabolites. Hence they are ecologically very important [2]. They are ubiquitous in anaerobic soil environments, sewage and in wood chips. *Clostridium spp.*, *Acetovibrio spp.*, *Bacteroides spp.*, etc. degrade cellulose via a large extracellular complex of enzymes, called cellulosome acting in a consortium. They not only act on cellulose, but also on other plant cell wall polymers such as hemicellulose, lignin, pectin, xylans, etc. [3].

The enzymatic machinery to degrade cellulose is possessed only by microorganisms and not by higher plants or animals, though plants must possess such enzymes that occasionally cleave lignocellulose in the plant cell wall to induce structural changes during its developmental stages [4]. The cellulase system mainly comprises of following three enzymes:

i. Endogluconase : for the initial breakdown of cellulose molecule
ii. Exogluconase : removes cellobiose units from the non reducing ends
iii. Beta-glucosidase : for hydrolysis of cellobiose to glucose

Box 1. Glossary

Cellulose: A linear, insoluble biopolymer composed of repeating β–D-glucopyranose residues linked by β-1, 4 glycosidic bonds.

Cellulosome: A multienzyme complex capable of cellulose degradation.

Designer cellulosomes: Artificial cellulosomes, constructed from cellulosome components of different organisms to improve efficiency of natural cellulosomes.

Consolidated bioprocessing (CBP): A system wherein production of cellulase, hydrolysis of the substrate and fermentation is accomplished in a single step process, using cellulolytic microorganisms.

Cellulosomes were first observed on the surface of *Clostridium thermocellum* [5]. The complex appeared as a large protuberance consisting of scaffoldin proteins. Surrounding these proteins are attached different subunits of various enzymes. These subunits are arranged in an orderly manner and thus form a large extracellular complex with mass ranging from 650 kDa to 2.5 MDa [3]. These are similar in mass to proteosomes and ribosomes. They are the largest extracellular multienzyme complexes that are best studied in *Clostridium cellulovorans* and *Clostridium thermocellum.* These bacteria sense the insoluble form of cellulose and trigger the transcription and post translational modifications of some cellulase proteins. The cellulolytic system in *C. cellulovorans* is thought to be regulated by the carbon catabolite repression (CCR) mechanism whereas in *C. thermocellum*, it is

the availability of crystalline cellulose as a substrate that regulates it.

The structure of cellulosomes in general seems to be very complex, comprising of a non-catalytic subunit- the scaffoldin protein, and an enzymatic subunit. The scaffoldin subunit contains cohesion module connected to carbohydrate binding module (CBM), a dockerin and a surface layer homology module (SLH). Cohesin modules are the major building blocks of scaffoldins, which are responsible for organizing the cellulolytic subunits into the multi-enzyme complex. There occurs calcium dependent, cohesin-dockerin interaction between these two subunits. The type I cohesin domain of scaffoldin binds to the enzymatic subunit on its type I dockerin domain and type II cohesins binds the cellulosomes to the cell through type II dockerins (Fig.1). Another domain of scaffoldin, CBM (carbohydrate binding module) enables cellulosome to bind to its respective substrate. In the enzymatic subunit the various cellulolytic enzymes are incorporated randomly [6]. The cellulosomes from different anaerobic mesophilic bacteria differ in their enzyme compositon, arrangements, and stoichiometry.

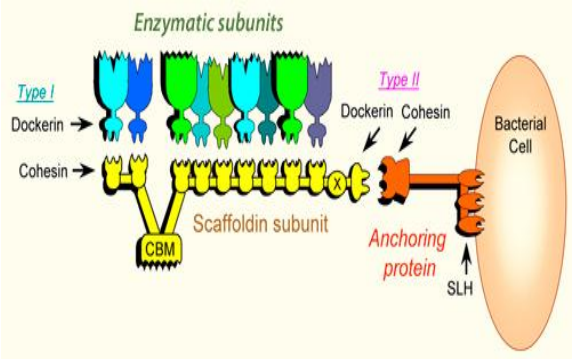

Fig 1. Schematic of cellulosome system [7, 8]

NATURALLY OCCURRING CELLULOSOMES

Cellulosomes have been reported from a large number of bacteria (Box 2). Bacterial cellulosomes can be divided into two groups: the first group contains simple cellulosomes of mesophilic organisms such as *C. cellulovorans*, *C. acetobutylicum*, etc. having only one scaffoldin with only type I dockerins and 6 to 9 type I cohesins. There is no strong evidence of these cellulosomes to be present on the cell surface. The second group contains cellulosomes found in *C. thermocellum*, *Acetovibrio cellulolyticus*, etc. They are complex due to the interaction of many scaffoldins together. Also they are transiently or permanently attached to the cell surface [9].

The protein complexes and interactions are well studied in case of human proteasomes and ribosomes, but the cooperation of the enzymes within the cellulosome seems more complex. Bacteria possess cellulosomes of varying enzyme compositions, arrangements and stoichiometry. Degradation efficiency of cellulosomes is correlated with their enzyme diversity [10]. In depth understanding of the catalytic efficiency of its enzymatic subunits is also hindered by the heterogeneity, complexity and size of natural cellulosomes. To have a better understanding of natural cellulosomes, concept of designer cellulosomes has been introduced which are comparatively simpler, homogenous and better defined.

Box 2. Cellulosome possessing anaerobic bacteria [8]

- *Acetivibrio cellulolyticus*
- *Bacteroides cellulosolvens*
- *Clostridium acetobutylicum*
- *Clostridium cellulolyticum*
- *Clostridium cellulovorans*
- *Clostridium josui*
- *Clostridium papyrosolvens*
- *Clostridium thermocellum*
- *Ruminococcus albus*
- *Ruminococcus flavefaciens*

Cellulosomes in C. cellulovorans

Genome sequence of *C. cellulovorans* has been studied [11]. In *C. cellulovorans*, the scaffoldin protein CbpA is 1,848 amino acids long, with a molecular weight of 189 kDa. It is rich in valine and threonine residues and is encoded by a 5,544 bp long *cbpA* gene. The tertiary structure of scaffoldin reveals that this molecule is curled due to the presence of one cysteine residue near C-terminal and two others at N-terminal along with a signal peptide of 28 amino acids [12]. Scaffoldin interacts with the cellulosomal enzymes through nine hydrophobic domains termed cohesins. The cellulose binding domain (CBD), also called as CBM [13] of scaffoldin displays a special characteristic which allows the cellulosome to bind to chitin besides crystalline cellulose. In addition to these cohesin and CBM domains, four hydrophilic domains (HLDs)/surface layer homology domains (SLHs) in CbpA are responsible for binding of cellulosome to cell surface [14].

The cellulase complex mainly consists of three subunits: p170, p100, and p70, with molecular weight of 170, 100, and 70 kDa, respectively [15]. Out of these three, the p100 and p70 subunits are major enzymatic subunits of cellulosomes. p170 is the core subunit having highest affinity for cellulose. It is not an anchor protein but it is responsible for the coordination among different enzymes of cellulosome

[16]. The p100 subunit has CMCase (carboxy methyl cellulase) activity. The *exgS* gene of p70 subunit encodes an exoglucanase ExgS of molecular weight 77.7 Da and 703 amino acid length [17]. The different enzymes in the cellulase complex are encoded by eleven different genes which have been cloned and sequenced [18, 19]. These are: cellulase genes *engB*, *engE*, *engM*, *engY*, *engK*, *engL*, *engH* (endogluconase) and *exgS* (exoglucanase), mannanase gene *manA* [18], the pectate lyase gene *pelA* [20], and the xylanase gene *xynA* [14]. The cellulase complex interacts with scaffoldin by its dockerin domain. The dockerin domain is a duplicated sequence of 20 amino acids each. It is considered as the hallmark of all cellulose degrading enzymes [18].

Cellulosomes in C. thermocellum

C. thermocellum can grow anaerobically under thermophilic conditions, and can hydrolyse partially digested plant tissues. It has a very peculiar cellulase system comprising of: (i) endo beta gluconases, (ii) four exogluconases, (iii) a cellobiose phosphorylase, (iv) cellodextrin phosphorylase and, (v) two β-glucosidases that helps it to solubilize the crystalline cellulose completely. This high activity is known as the true cellulase activity-'Avicelase'. The cellulase complex has a high specific activity. The hydrolysis of the crystalline cellulose is possible due to the sulfhydryl groups present on it, which are required for the structural stability as well as for the saccharification of the crystalline cellulose. Ca^{++} and Fe^{++} ions are known to stimulate and decrease the cellulase activity, respectively. Latter does so by acting as chelating agent. These are some of the unusual properties of cellulosomes in *C. thermocellum* [21].

The availability of energy and enhanced growth rate leads to the repression of genes responsible for cellulase production. On the contrary, low growth rate and the presence of crystalline cellulose as a substrate enhance the production of the cellulase proteins [22]. *C. thermocellum* prefers cellulose as a substrate to other sugars such as glucose, fructose or sorbitol. In presence of these sugars, transcription rate of the cellulolytic enzymes is very low and the bacterium grows on these substrates only after a relatively longer period of adaptation [23].

Cip A is a macromolecular scaffoldin protein. It is composed of nine modules of type I cohesins which act like substrates for the type I dockerins (primarily glycoside hydrolases but also carbohydrate esterases and polysaccharide lyases). For every type I dockerin of the enzyme, there is a corresponding complementary type I cohesin on the Cip A, forming the enzyme-substrate interaction. There is sequence similarity among the nine type I cohesin Cip A modules but the type I dockerin modules exhibit certain modifications in their surface receptor sequence [24].

DESIGNER CELLULOSOMES

Designer cellulosomes are artificial cellulosomes, constructed to improve the degrading efficiency of existing cellulosomes. This can be achieved by studying the functions of cohesins, analyzing the synergy between various cellulosomal enzymes and enhancing the enzyme specificity towards different substrates [25]. Early observations of cohesin-dockerin interactions aroused the necessity to design minicellulosomes. When individual components of different cellulolytic bacterial enzymes associate with each other, they act in a cooperative manner giving rise to a minicellulosome [26]. The cohesin-dockerin interaction is different among different species. Recently, a hybrid minicellulosome was constructed exploiting one such cohesin-dockerin interaction property, containing upto four different components. Cellulases incorporated in it (Cel48F and Cel9E of *C. cellulolyticum*) are among the most potent, but scarce in nature [27]. Reports demonstrated that binding of these enzymes on the chimeric scaffoldin enhanced the catalytic activity of cellulosome, further revealing that the most active complex of cellulosomes which degrade crystalline cellulose contains Cel48F and Cel9E as critical components [28, 29]. It can be hypothesized that naturally microorganisms produce cellulosomes which act in concert with non-cellulosomal lignocellulolytic enzymes and act together on the substrate efficiently.

Chimeric cellulosomes

It was reported that construction of a trifunctional chimeric cellulosome [29], contained -

1. cellulose binding module and two cohesins of divergent specificity forming a chimeric scaffoldin
2. dockerin complementary to the divergent cohesin i.e. cellulase
3. divergent cohesin-dockerin device

Recombinant cellulosomes involve one or more strains of the same bacterial species from which different components of an enzyme are taken. These proteins can be secreted from cells into the medium. Association of these proteins with each other in the medium to form a complex can be observed. If such experiments prove successful then they can be carried out *in vivo* for cellulose uptake in an organism [26].

C. acetobutylicum contains the genes for cellulosome system but not reported to degrade crystalline cellulose. Experiments have been successful in transforming this inactive cellulosome to a partially active one. It is efficient in its action on carboxymethyl-cellulose (CMC) and other phosphoric

acid swollen cellulose [3]. This *in vivo* construct of minicellulosomes now converts biomass into solvents of industrial use. Thus the aspect of designer cellulosomes is not limited to creation of newer cellulase systems but also to improvements in the pre-existing cellulosome containing organisms [30].

APPLICATIONS

Fibrous biomass degradation

Farmers feed starchy grains to their economically important cattle so as to accelerate the growth of ruminants in them [31]. But this practice is expensive as grains are also needed for human consumption. The fibrous biomass taken up by cattle is not completely digested to its respective monosugars and hence there arises a need to increase the digestibility of fibers. A potential solution was suggested by introducing the cellulolytic genes into the tobacco and rice plants that were served as feed to cattle thereafter. The genes encoding thermostable xylanaseA (*xynA)* and a modified version of endoglucanase 1 (*EgI)* were expressed in tobacco and rice plants. This was an *Agrobacterium* mediated transfer and it was observed that this addition of microbial cellulases enhanced the feed digestibility and intake in cattle. Cellulase enzyme genes were transferred and the transgenic plants were then tested for their viability since there was a risk of the plant cell wall getting digested. But the transgenic plants thus obtained, fertilized normally suggesting that the expression of foreign genes in plants can produce enzymes in an active state in the cells. This expression of cellulase genes in plants promotes the utilization of tough and unruly biomass as feed.

Decomposing lignocellulosic wastes

Agrowaste contain lignocellulosic material, a combination of cellulose, hemicellulose and lignin as major components [32]. For their efficient degradation, a synergistic action of cellulolytic and lignolytic enzymes is required. Submerged fermentation processes are being carried out to produce lignocellulolytic enzymes that can act on paddy straw which contains a very high amount of lignocellulose. Since silica is present in paddy straw, it cannot be used as fodder for cattle. Degradation of substances like paddy straw has been a challenge to satisfactory disposal of agricultural waste [33], which can well be achieved by smart application of cellulosome machinery.

Towards alternative fuel source

On worldwide basis, 1.3×10^{10} metric tons of wood is produced by terrestrial plants per year. Cellulose feedstock available from agriculture and other sources are approximately 180 million tons per year [21]. Thus a large amount of inexpensive cellulose is available for conversion into cost effective products. Also the increasing prices of petroleum and other fuels necessitate intensification of efforts for development of better and novel fuel alternatives. Using cellulose as a raw material for fuel production will also help to get rid of huge cellulosic wastes from various sources**.** As the world supply of oil is limited, and sooner or later, it is going to get depleted, there is an urge for the quest of an alternative fuel source which is inexpensive, eco-friendly and plentiful in nature. This worldwide problem of fuel depletion and cellulosic waste disposal can be resolved with efficient large scale conversion of cellulose into ethanol.

Conversion of cellulose to ethanol

Ethanol can be produced from corn and starch, which poses competition to raw materials for food industry. Cellulose being not edible is a better raw material to yield ethanol, by employing anaerobic bacteria [34]. Cellulose catabolism is carried out in two steps:

(1) Enzymatic depolymerisation of insoluble cellulose, and
(2) Cellular utilization of the products of hydrolysis (cellobiose and cellodextrins)

Bacteria adhere to cellulose via (i) cellulosome organelle, (ii) noncatalytic cellulose-binding proteins, (iii) glycosylated moieties of the bacterial glycocalyx or of specific binding proteins, and (iv) fimbriae or pilus-like structures.

The cellulose-enzyme-microbe (CEM) complex formation takes place differently in case of aerobic and anaerobic bacteria. Since aerobic bacteria adhere very weakly to the cellulose surface, they do not give rise to any complexed products. In case of anaerobic bacteria, complexed fermentable products are formed due to the complete adherence and CEM complex formation. These can be further degraded to other secondary products. The CEM complex ensures proper access of the bacteria to cellulose and thus it prevents cellulose to be attacked from a number of other microbes and also protects the proteins of the resident bacteria from the effects of the proteolytic enzymes. Products of cellulose hydrolysis (generated by cellulolytic organisms) are taken up by other bacteria and fungi, and phosphorylated by various metabolic pathways to yield simple metabolic products. Cellobiose and cellulodextrins are produced primarily which are converted to ethanol by reduction [2].

Consolidated bioprocessing (CBP)

CBP is a system wherein production of cellulase, hydrolysis of the substrate and fermentation is accomplished in a single step process, using cellulolytic microorganisms [35]. It is an economically attractive strategy for the production of third

generation biofuels. It offers advantages of lower energy input, simple feedstock preparation and low cost production of biofuels. For high product selectivity and concentration, development of microorganisms for cellulose conversion via CBP can be made effective in two ways.

1. Modifying the naturally occurring cellulolytic microorganisms to improve yield and tolerance of products.
2. Engineering non-cellulolytic microorganisms, so that they are able to utilize cellulose by a recombinant cellulolytic strategy [2].

But for this to occur, there has to be a proper understanding of the metabolism in cellulolytic bacteria and the mechanism of conversion of cellulose into biofuels such as ethanol.

RATE LIMITING FACTORS FOR CELLULOSE UTILIZATION

Despite cellulosome being an efficient system for cellulose degradation, there are certain factors that determine the rate of conversion of cellulose polymer to its respective short chain monomers. These include particle size, pore volume, accessible surface area, degree of polymerization and crystallinity [36]. A homogenous substrate, containing same sized particles cannot be obtained and therefore it is difficult to get a pure culture of cellulolytic microbes. All these parameters are interconnected and alteration in any one of them would affect others. Pretreatment is often offered as a solution, but in some experiments the crystallinity increases with an increase in hydrolysis of cellulose which is not desirable [37].

FUTURE ASPECTS

Cellulosomes can prove to be a useful tool for production of biofuel. Much remains to be investigated about cellulosomes and their applicability. Questions such as - how the assembly of cellulosomal subunits takes place at gene level, how does the complex interaction between the cohesin and dockerin occur at molecular level, what are the factors regulating the gene expression of cellulosomes, how does the formation of polycellulosomes takes place, what is the mechanism by which these cellulosomes attach to the cell surface - awaits answer. Ways to improve the existing natural cellulosomes are certainly needed to be explored, so as to increase the efficiency of cellulose degradation. Also studies regarding the diversity of cellulosomes should be carried out in order to isolate and characterize various microorganisms possessing cellulosomes. Fabrication of versatile designer cellulosome complexes with desired functionalities, may well open doors to novel biotechnological applications.

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