**Construction of efficient multifunctional enzymes for Bioethanol producion: Various approaches and Futuristic Developments**

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**Abstract:**

Increase consumption of non renewable sources i.e. the fossil fuels has caused worldwide global warming and pollution. Bioethanol is one kind of biofuel produced from biological raw materials and lignocellulosic biomass. Bioethanol production can reduce burden on the non-renewable energy sources. The prime components of the plant lignocellulosic biomass are made up of cellulose, hemicellulose and lignin. Cellulose and hemicellulose can be hydrolysed to monomeric sugars by the plant cell wall degrading enzymes i.e. cellulases and hemicellulases. These monomeric sugars for example glucose and xylose can be utilized by the yeast for production of bioethanol. Moreover, the cellulases and hemicellulases extensively used for large number of industrial purposes. However, due to low catalytic efficiency and high production cost of multiple enzymes, significant efforts have been made for improving the production and performance of the present enzymes. Therefore, present chapter provides an overview of current scenario of plant cell wall degrading enzymes. The chapter discusses different approaches of protein engineering tool to study the underlying catalytic mechanism of these enzymes and techniques for improving their activity. The chapter also discusses recent development in production of efficient multifunctional chimeras for degradation of complex polysaccharide in a single reaction. Furthermore, the chapter also highlights the synthetic biology approaches that have been used for designing artificial designer cellulosomes containing distinct cellulases and xylanases domains which showed enhanced synergistic action against plant based biomass. Finally, the chapter covers various challenges and futuristic applications of these engineered enzymes in the field of renewable energy production.

**Keywords (4-5):** Lignocelluloses bimoass, bioethanol, protein engineering, designer cellulosome

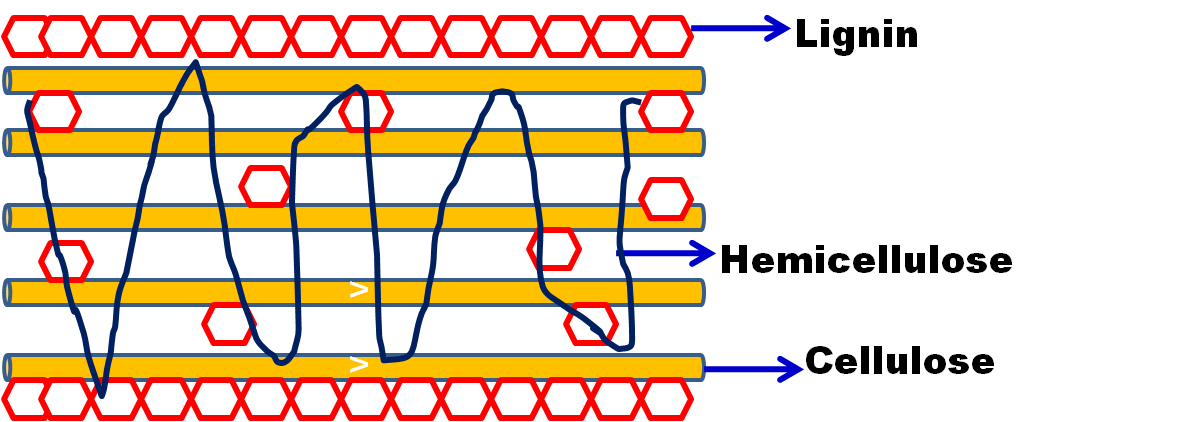
* 1. **Introduction**

The extensive use of fossil fuels is the leading cause of global warming and pollution worldwide. The use of renewable energy sources is one of the solution to decrease the increasing harmful effects.

Nowadays, the main focus of the mankind is to make life and activities on earth more at ease by adding essential products through biological mechanism. In the current scenario, each year millions of tons of biological raw materials and lignocellulosic biomass are wasted by burning them without providing benefits to living organisms. Therefore, it is highly important that these raw materials and lignocellulosic biomass should be efficiently utilized for generating renewable energy source in the form biofuel by developing advanced scientific strategy. The plant based lignocellulosic biomass are made up of complex polysaccharides mostly cellulose, hemicelluloses and lignin. These complex polysaccharides are breakdown by different set of enzymes called cellulase, hemicellulase and laccase in simpler monomers. The monomers mostly glucose and xylose are utilized by yeast in fermentation process to produce alcohol known as bioethanol. The bioethanol is the alternative form of energy that can be used as energy source instead fossil fuels. The enzymatic process involved in depolymerization of complex polysaccharides is one of the critical step in the production of bioethanol. Moreover, the low catalytic efficiency and production multiple enzymes involved in complete degradation of carbohydrate polymer is one of the major concern in the scientific community.

Nowadays, major advancement in enzyme technology has resulted in development of efficient enzymes having high catalytic efficiency and multifunctionality. These efficient engineered enzymes are utilized for complete degradation of carbohydrate polymer into monomers.

* + 1. **Lignocellulosic biomass and its components**



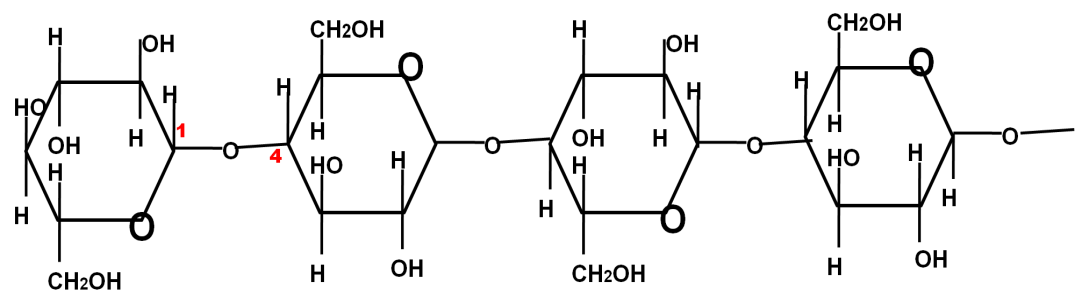
**Figure: 1** Diagrammatic representations of components of the plant based lignocellulosic biomass

Plant based lignocellulosic biomass are cheap and readily available. From the biofuel production view lignicellulosic biomass is of particular interest. The major polymeric components of lignocellulosic biomass are cellulose, hemicelluloses and lignin as shown in Figure. 1 [1]. Generally, cellulose fibrils are encrusted with hemicelluloses forming an open system, the remaining open spaces are filled up with lignin (Figure. 1). The different components of lignocelluloses biomass are discussed below.

**1.1.1.1 Cellulose**

Cellulose is the major component plant based biomass. The cellulose content in most plant based biomass is on an average 33% [2]. The cellulose is extracted from biomass materials for biofuel production [2]. Cellulose is an organic polymer consisting of linear chain of glucose molecules connected together by β (1→4) linkage as shown in Figure 2 [3].

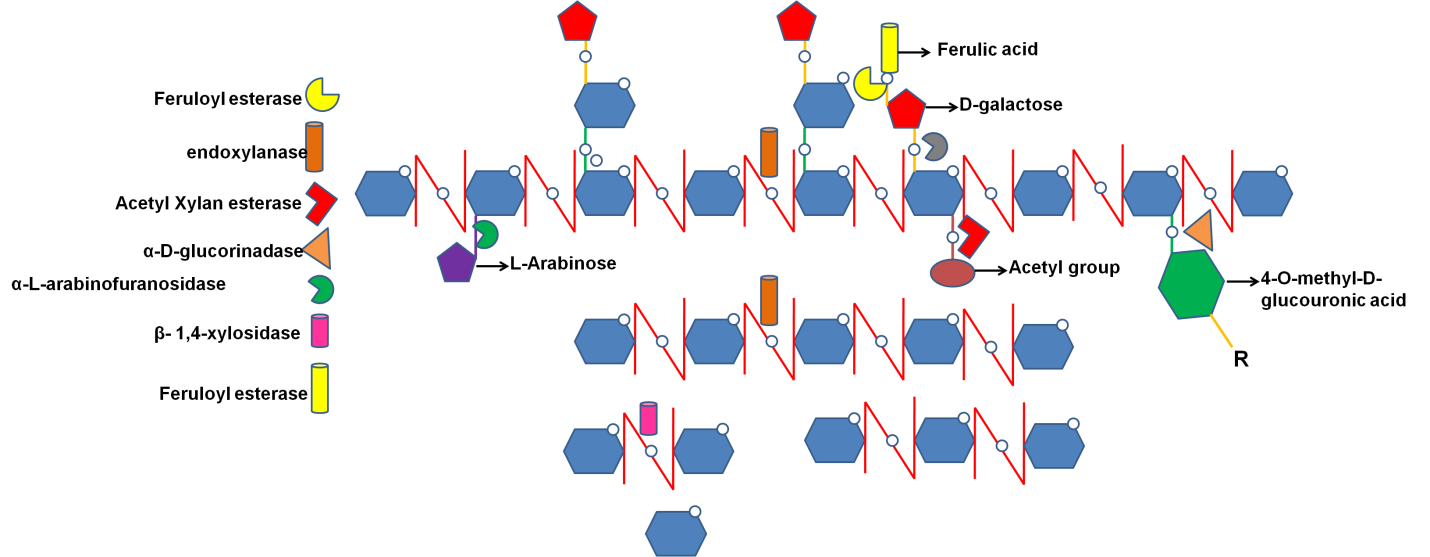
The insoluble microfibrils of the cellulose forms cable-like structures (Figure 1) which are composed of approximately 24 hydrogen-bonded chains containing β-(1,4)-linked glucose molecules as shown in Figure 2 (Guerriero*et al..* 2010, Fernandes*et  al.,* 2011, Nsor*et al.,* 2017). The glucan chains which are polysaccharides made up of glucose units forms parallel and consecutive chain of glucose molecules. The repeating unit of cellulose is [cellobiose](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/cellobiose), a disaccharide of glucose in which there is a 180° rotation of each monomer in relation to its neighbour glucose molecule as shown in Figure 3 [3]. The hydrolysis of cellobiose is an measure of β-glucosidase activity.  This arrangement helps the glucan chains to form a flat, inflexible and ribbon-like crystalline structure merged together by Van der Waals forces and hydrogen bonds to form microfibrils. Hydroxyl groups present in cellulose macromolecules are connected with large number of intra- and intermolecular hydrogen bonds, which resulted in various ordered crystalline arrangements such as flat ribbon like conformations of cellulose chain [4].



**Figure 2:** Demonstration of Cellulose chain in plant based lignocellulosic biomass

**1.1.1.2 Hemicellulose**

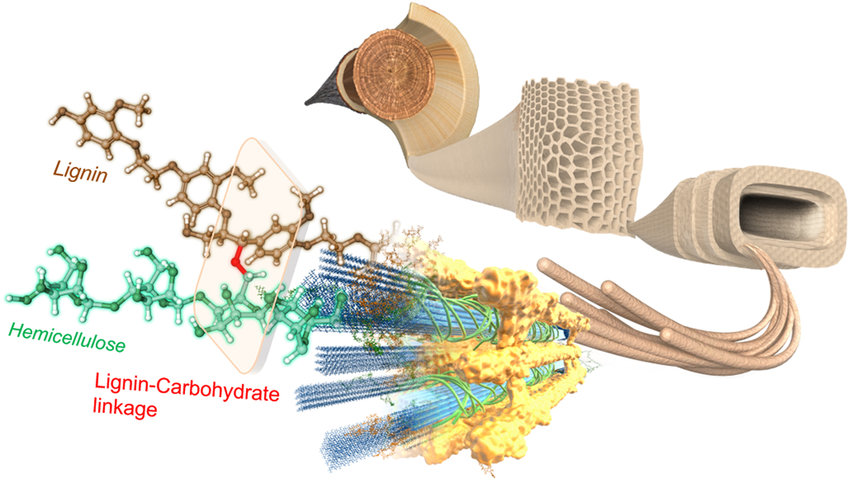
Hemicelluloses is the second most abundant polysaccharide representing 25-30% plant based biomass [5]. The hemicelluloses are made up of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids [5]. The xylan is the most abundant hemicelluloses known. The hemicelluloses are not homogenous like cellulose, mostly they are heterogenous for example the hardwood hemicelluloses contains xylan however the softwood contains glucomannans [6]. The heteropolysaccharidesxylan backbone is made up of homopolymaric chain of xylopyrranose units connected via β-1,4 linkages as shown in Figure 3. Based on the source of xylan, backbone chain of xylan constitutes branches of arabinose, glucuronic acid or its 4-O-methyl ether, and acetic, ferulic, and p-coumaric acids (Figure 3).



**Figure 3.** Xylan structure with different substituents and sites of attack by xylanases

**1.1.1.3 Lignin**

Lignin is the largest non-carbohydrate polymer present in plant based biomass. It is constitute (15-40%) of lignocellulosic biomass [7]. Lignin is responsible for protecting the plant cell wall from microbial degradation, providing mechanical strength and hydrophobicity to the plant cell wall. The lignin is made up of from polymerization of different aromatic groups Syringyl (S), guaiacyl (G), and hydroxyphenyl (H) to form monolignols [7]. The lignin is responsible for recalcitration in plant based biomass. The cellulose accessibility is disrupted by higher content of lignin in lignocellulosic biomass thus reducing the avaibality of sugar residues for enzymatic saccharification reaction [7]. The lignin is connected to the carbohydrate polymer i.e. cellulose and hemicelluloses to form lignin carbohydrate complex or LCC through different linkages known as lignin carbohydrate complex linkages or LCC linkages (Figure. 4).The LCC linkages are mostly Benzyl ether and phenyl glycoside linkages. The Benzyl ether linkages connect the primary OH groups of carbohydrates such as glucose, galactose,mannose and arabinose to the α-position of lignin, while the Phenyl glycoside linkage is not well studied unlike Benzyl ether linkages [7].



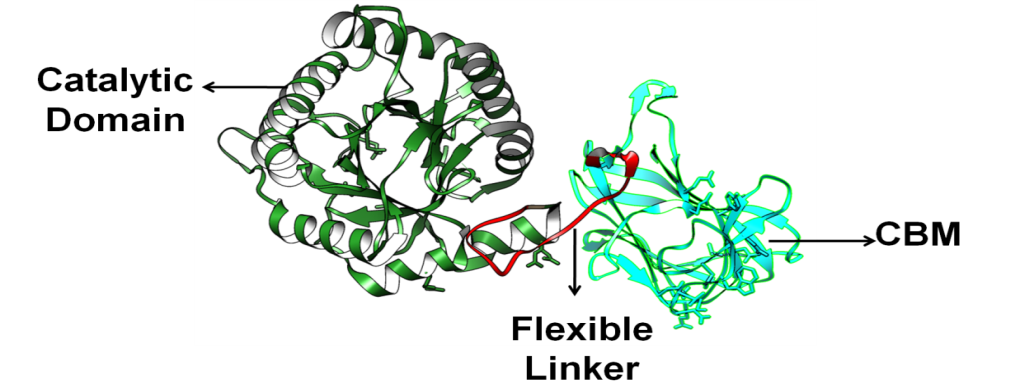
# Figure 4 A three-dimensional view of the lignin–carbohydrate complex (LCC) in the wood cell wall (Nishimura et al., 2018)

* + 1. **Enzymes for Lignocellulosic Hydrolysis**

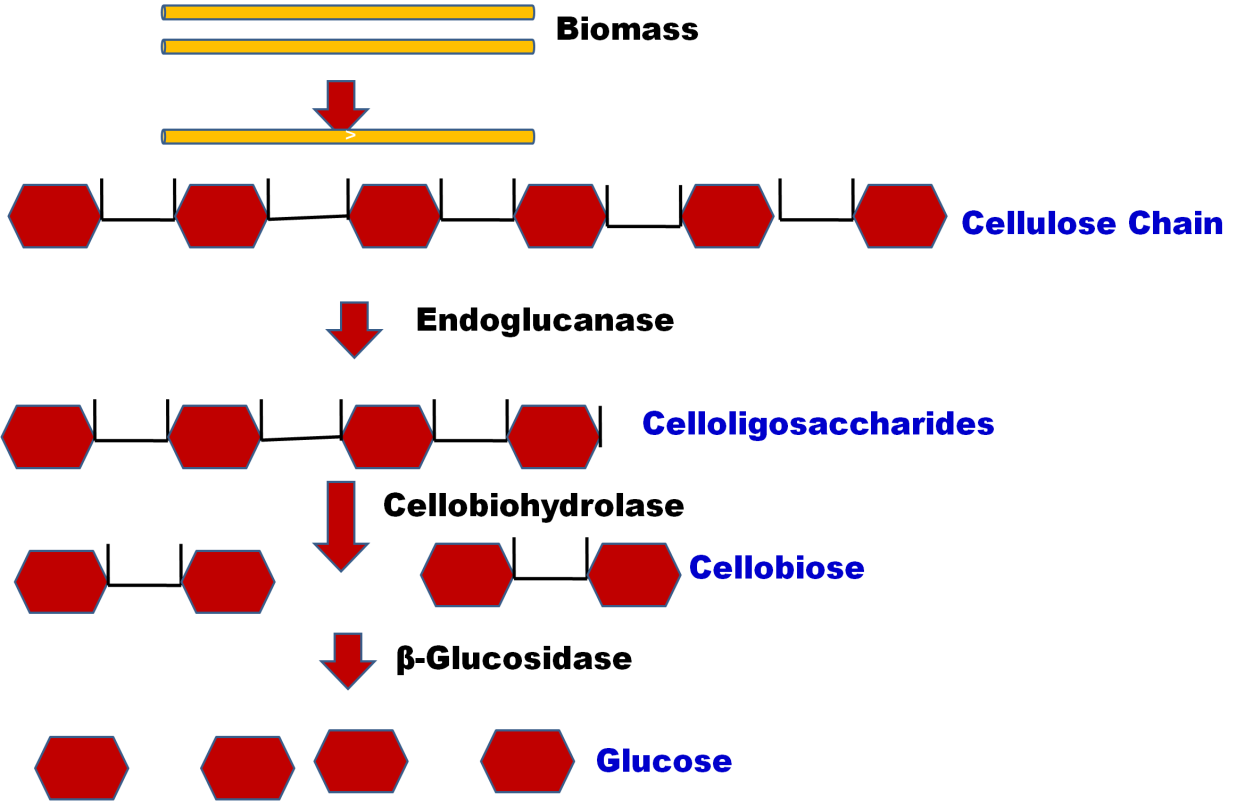
The plant based lignocellulosic biomass is recalcitrant in nature. Several factors are involved for biomass recalcitrant for example, high lignin content, protection of cellulose by lignin, cellulose sheathing by hemicelluloses and high crystalinity and degree of polymerization [8]. The accessibility of plant carbohydrates for enzymatic saccharification is prevented by biomass recalcitration. However, various pretreatment strategies showed improved accessibility of plant carbohydrates thus increasing the enzymatic hydrolysis step for releasing total reducing sugars for further bioethanol production [8]. Moreover, the complete degradation of the plant carbohydrates mostly cellulose and hemicelluloses are achieved by utilizing multiple number of enzymes discussed in the subsequent sections [9].

* + - 1. ***Cellulases***

Cellulose can be depolymerized to give glucose by the combined action of endoglucanase, cellobiohydrolase and β-glucosidase [9]. These Cellulase enzymes involved in hyrolysis of cellulose polymers into cellooligosacharides and glucose belong to different glycoside hydrolase families (GH) ([www.cazy.org](http://www.cazy.org)) [9]. Cellulase enzymes consist of two separate modules, the catalytic module, which contains the active site and the non-catalytic carbohydrate binding module (CBM) as shown Figure 5. Both modules are connected by flexible linkers (Figure 5), containing mostly serine and threoninine [11].

**Figure: 6** Structure overview of a cellulase enzyme

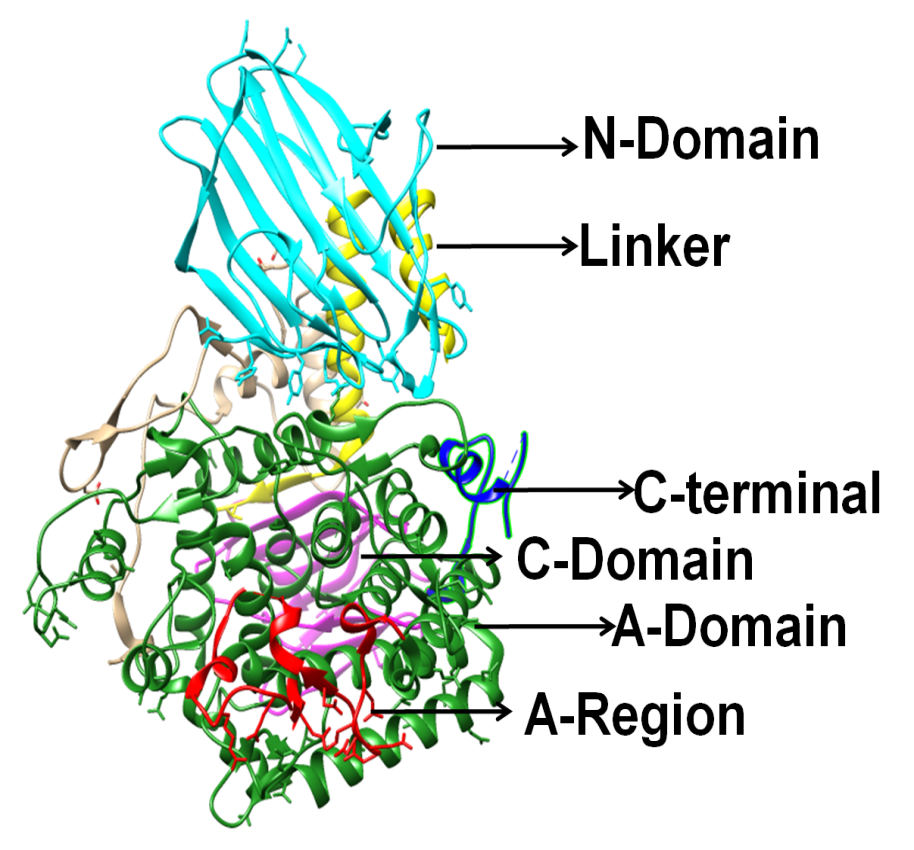
Moreover, cellulolytic enzymes act synergistically, in which endo-β-1,4-glucanase acts randomly on the cellulose chain and produces cellodextrins which are the larger cellooligosacharides as a hydrolyzed products [12] (Figure 6). Cellobiohydrolase acts at the end of the cellodextrin and releases cellobiose as the main product [12] (Figure 6). Finally, β-glucosidase hydrolyzes the cellobiose to form two molecules of glucose [12] (Figure 6). The following sections describe different cellulases and their mode of actions.



**Figure 7:** Action of different cellulases in depolimerization of cellulose chain

* + - 1. ***Hemicellulases***

Hemicelluases plays important role in degradation of plant based biomass. Hemicellulases depolarize xylans, xyloglucans, arabinoxylans and glucomannans of plant based biomass [5]. The hemicellulases are modular structure with catalytic and functional domains as shown in Figure. 7. In a previous study on extracellular β-L-arabinofuranosidase (HypBA2) belonging to the glycoside hydrolase (GH) family 121 from *Bifidobacterium longum* [13]it was found that the multidomain protein consist of mostly N-terminal domain (amino acid residues 38-123, Brown), a N-domain from residue 124-303,Cyan followed by linker region from residue 304-347, yellow a catalytic region comprising of (α/α)6 barrel domain (348–771, green) followed by a C-terminal domain (772–870, blue).

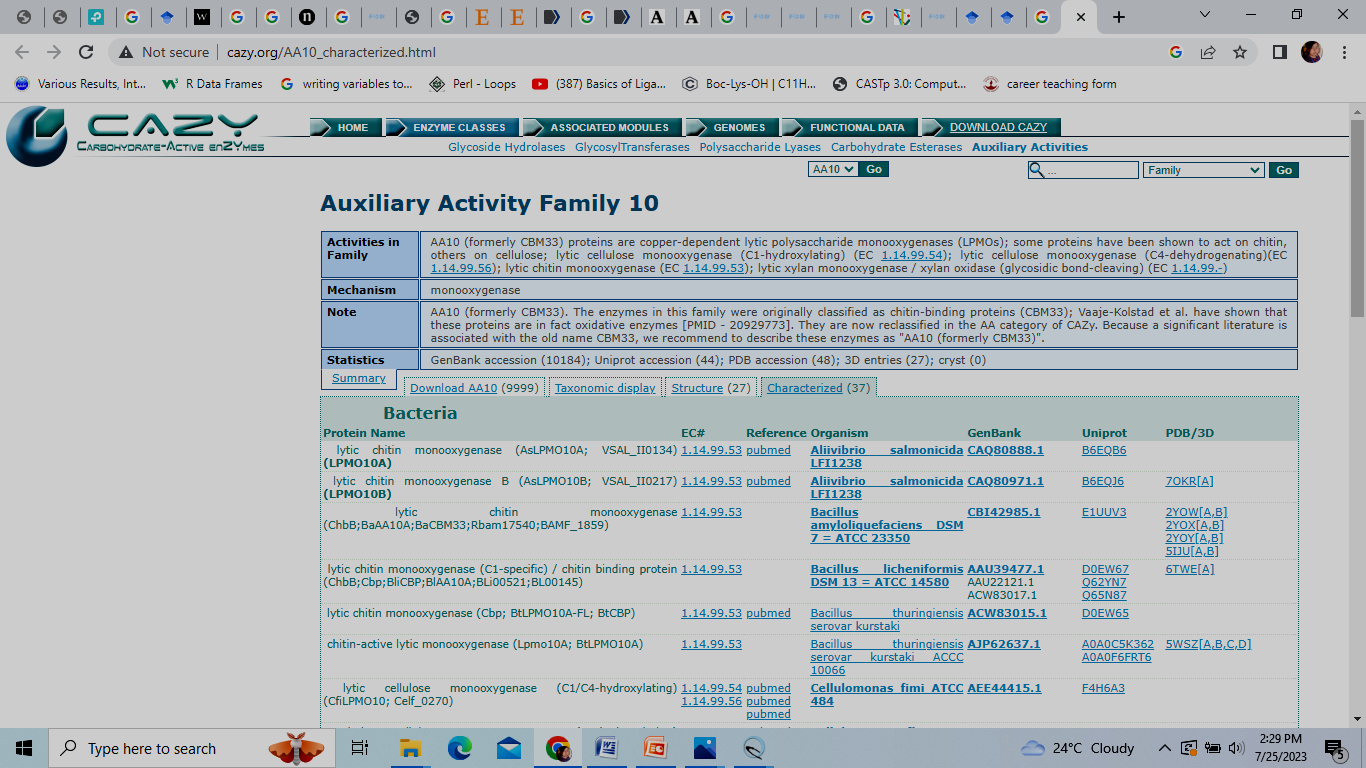


**Figure 8:** Structure of multimodular of extracellular β-L-arabinofuranosidase (HypBA2) belonging to the glycoside hydrolase (GH) family 121 from *Bifidobacteriumlongum*

The complete breakdown of hemicellulosic matter in plant biomass requires synergistic action of multiple enzyme due to its branched and organized structure as shown in Figure. 3

* + - 1. ***Lytic polysaccharide monooxygenases (*LPMOs*)***

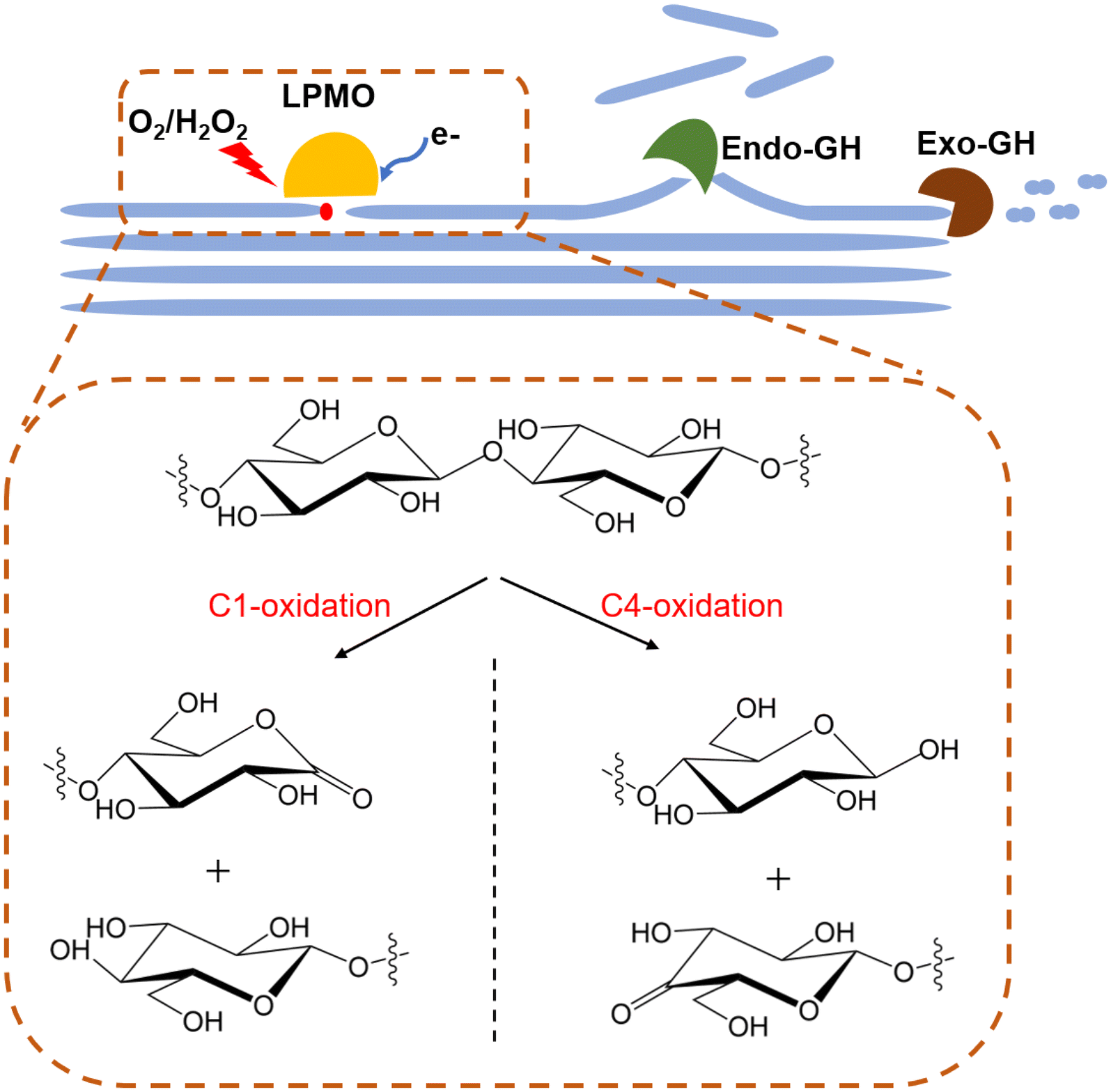
Lytic polysaccharide monooxygenases (LPMOs) are the monocopper based enzymes that are broadly dispersed in nature that are responsible for catalysis of the hydroxylation of glycosidic bonds in polysaccharide through oxidative reaction i. e.[14]. The LPMOs are auxillary enzymes and are distributed in CaZy database in families AA9, AA10, AA11, AA13, AA14 and AA15 as demonstrated in Figure 9. In family AA10 the bacterial LPMOs are found which are responsible for cleaving cellulose and Chitin [15].

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**Figure 9:** Demonstration of LPMOs are auxillary enzymes and are distributed in CaZy database

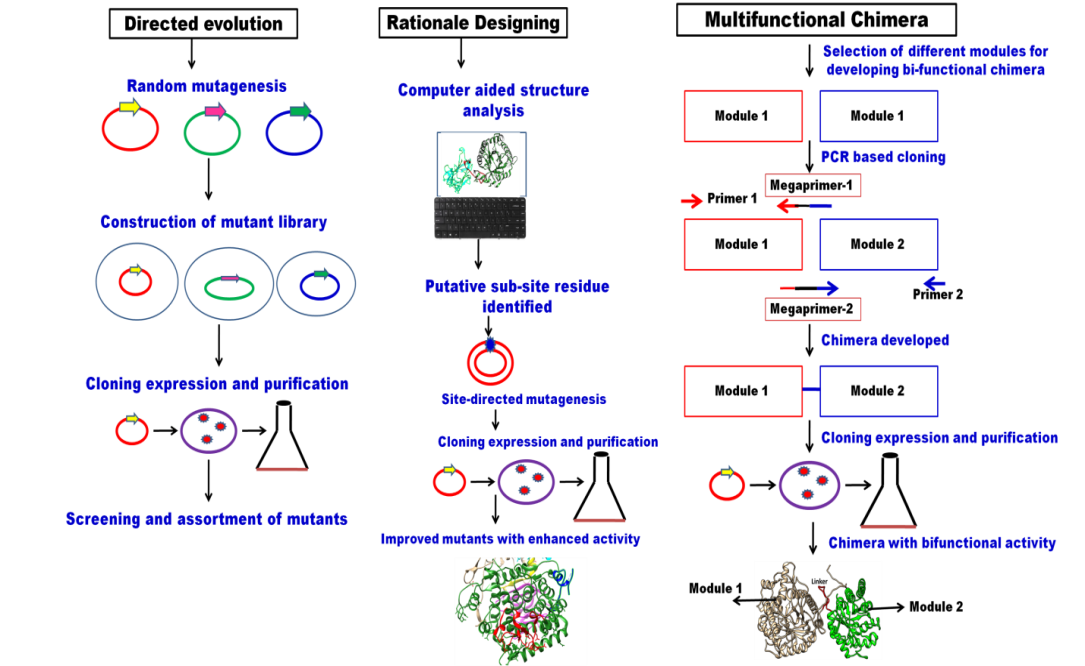
LPMOs produces holes on the crystalline surface of cellulose through oxidation reaction as shown in Figure 9. Thus LPMOs produce varied degree of cello-oligosaccharides [15]. Hence, the released cello-oligosaccharides are acted more efficiently by cellulases. The LPMOs acts synnergistically with cellulases for efficient breakdown of cellulose polymer in enzymatic hydrolysis reaction. Thus, nowadays the LPMOs act as a potent candidate in plant based biomass degrading enzyme cocktail [16].



**Figure 9:** Diagramatic representations of LPMOs and glycosidase in synergestic degradation of carbohydrate polymer (Zhou et al., 2020)

* 1. **Protein engineering existing plant cell wall degrading enzymes**
     + 1. ***Strategies for protein engineering***

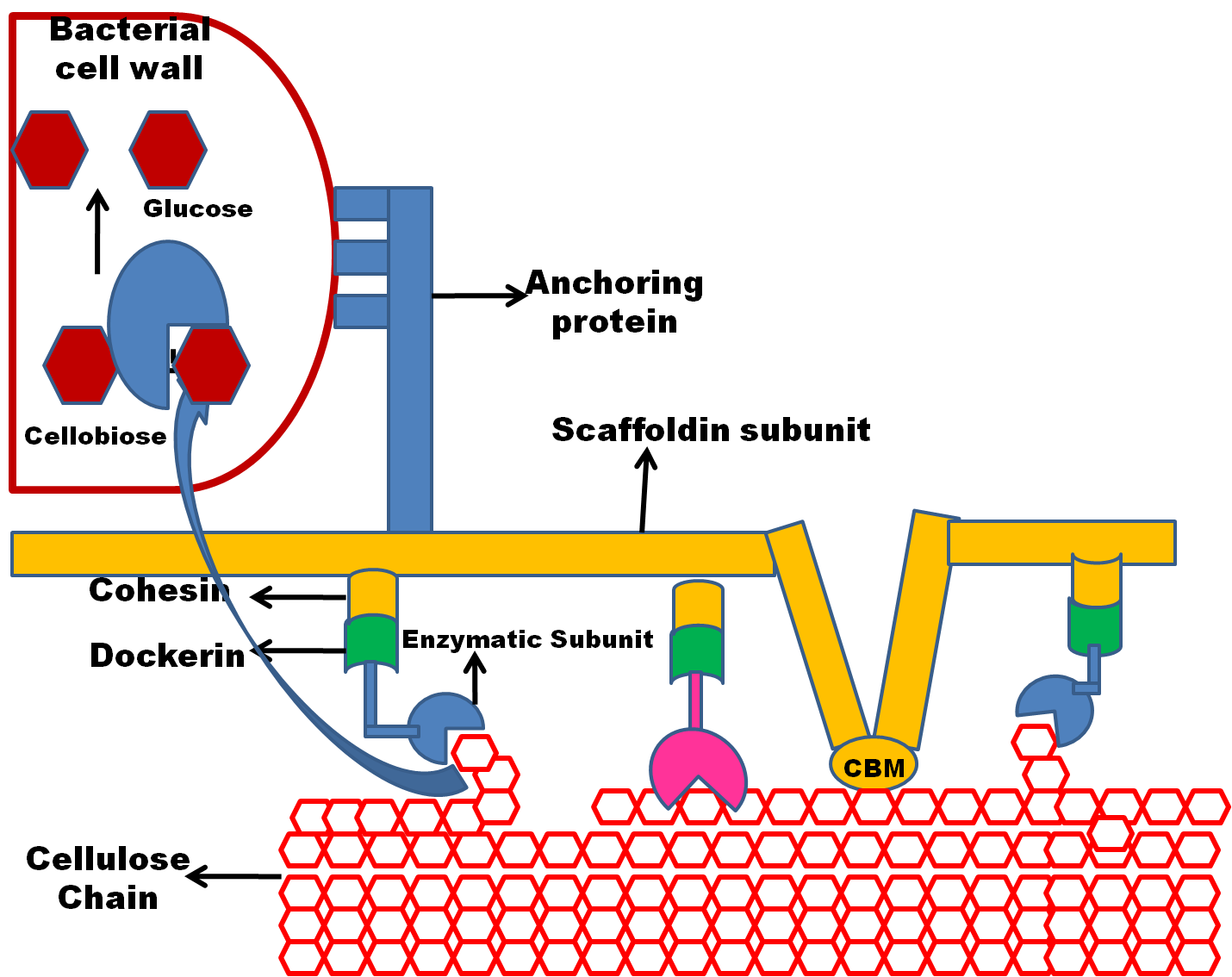
Cell wall degrading enzymes are extensively used for a large number of industrial purposes and consequently, significant efforts have been made for improving their production and performances. Protein engineering has been used as a tool to study the underlying catalytic mechanism of these enzymes, as well as improving their activity. Protein engineering includes the mutagenesis of potential active residues and their kinetic analysis. Inactive mutants are often used to study the protein-ligand complexes at the three-dimensional level. The protein can be engineered by three strategies mainly, directed evolution, rational designing and developing multifunctional chimeras. In directed evolution random mutagenesis of the target genes were performed and the variant with the improved activity were selected [2] as shown in **Figure.10**. In rational designing under protein engineering, the three-dimensional complexes of enzymes have been used to design new strategies for modification and exploitation of the glycoside hydrolases for engineering the enzymes and modifying their functions [17] as shown in **Figure.10**. The multifunctional chimeras were constructed by using molecular biology techniques for fusing two or more modules in a single polypeptide chains [18] as shown in **Figure.10**. The multifunctional chimera developed can reduce the cost of production of several required for complete degradation of lignocellulosic biomass [19]



**Figure 10:** Diagrammatic representation of protein engineering strategies

* + - 1. ***Engineered multi enzyme complexes***

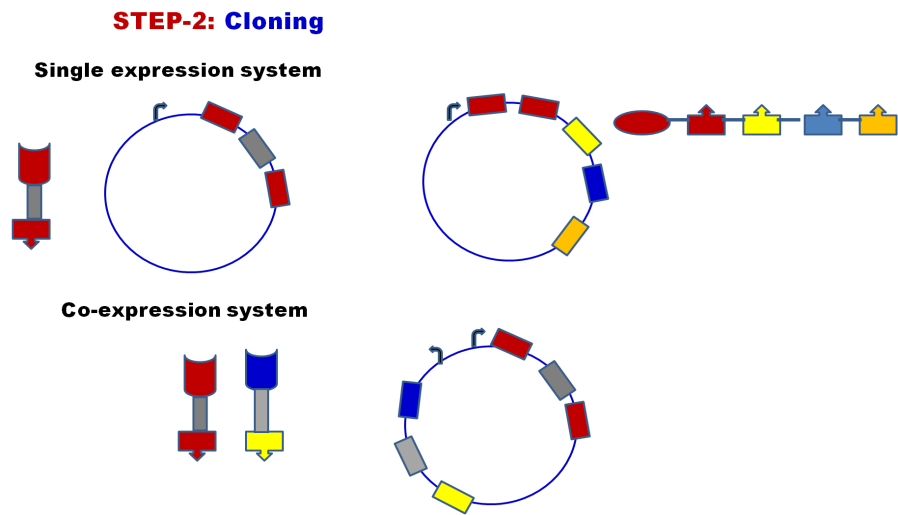
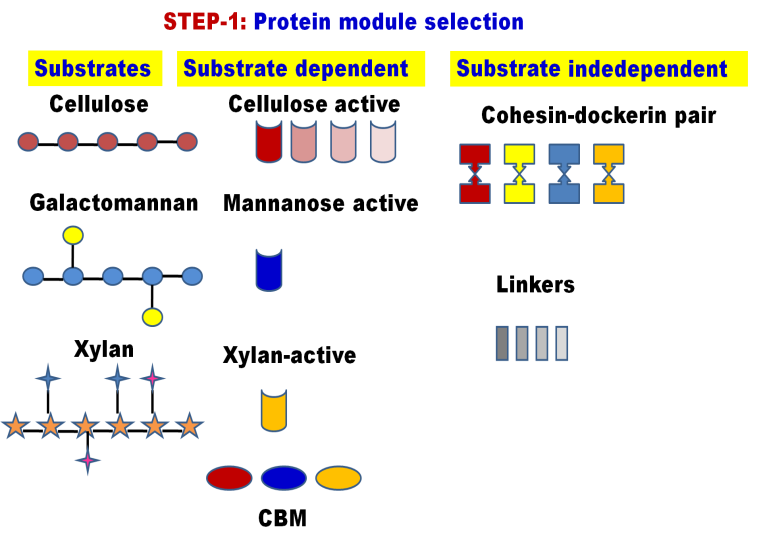
Multi enzyme complex or cellulosome are large complex entity and are mostly found in anaerobic bacteria [20]. The multi enzyme complex or cellulosome is made up different carbohydrate degrading enzymes such as cellulase and xylanase [20]. The different domains in the cellulosome are attached by dockerin domain to a cohesion domain of main scaffoldin protein as shown in **Figure 11.** This complex structure is connected to the surface of microorganism. The cellulosome facilitates the microorganism to degrade the insoluble form of cellulose to soluble form so that the later could be absorbed by bacterial cell wall.



**Figure 11:** Structure and organization of cellulosome

* + - * 1. ***Construction of designer cellulosome***

Designer cellulosome or engineered multi enzyme complexes are developed by attaching carbohydrate active enzyme to a scafoldin discussed in the previous section with the help of strong cohesion and dockerin interaction [21]. Designer cellulosomes have efficient hydrolytic activity due to improved enzyme substrate closeness [21]. The designer cellulosome contains various domains which includes cellulases, hemicellulases, LPMOs and laccases [22]. The designer celluosome are constructed by controlled incorporation of desired catalytic activities based on the target substrate. The designer cellulosome can be constructed using various steps discussed below in the flow chart (Figure 12). The first step includes selection of domains and designing of designer cellulosome enzymes and scafoldin protein. The second step consist of cloning of the cellulosome components by using their coding sequences. In the next steps the different components are assembled in the final multi enzyme complex. Lastly, in the final step the catalytic activity was determined for new assembled designer cellulosome.

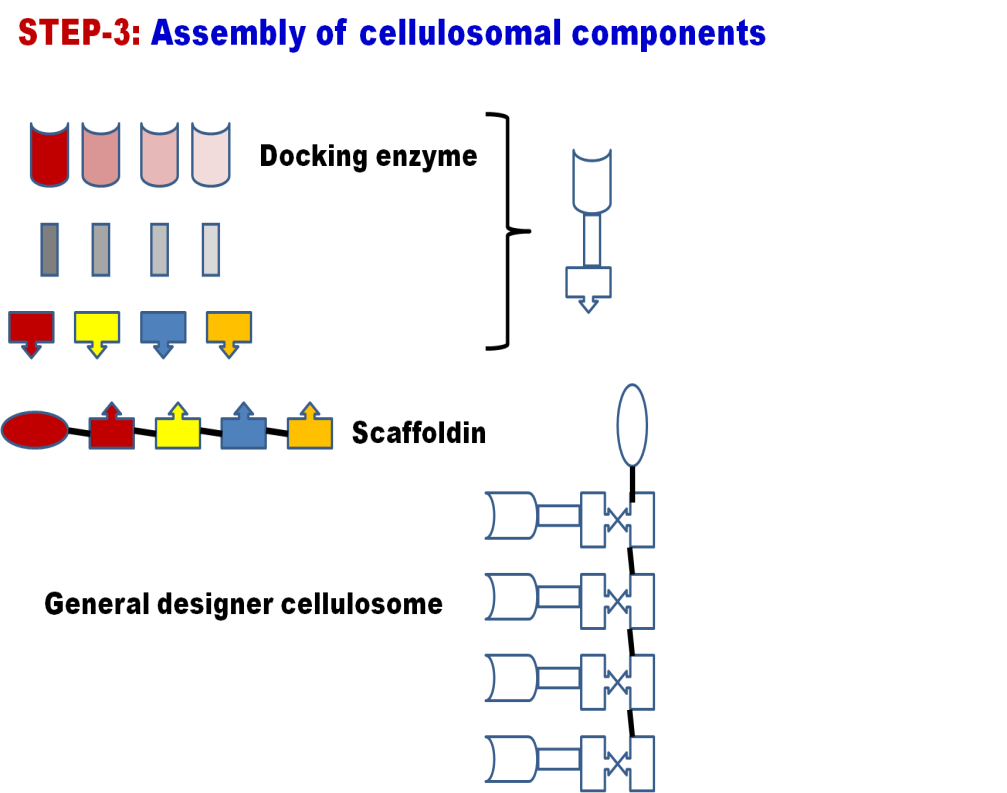
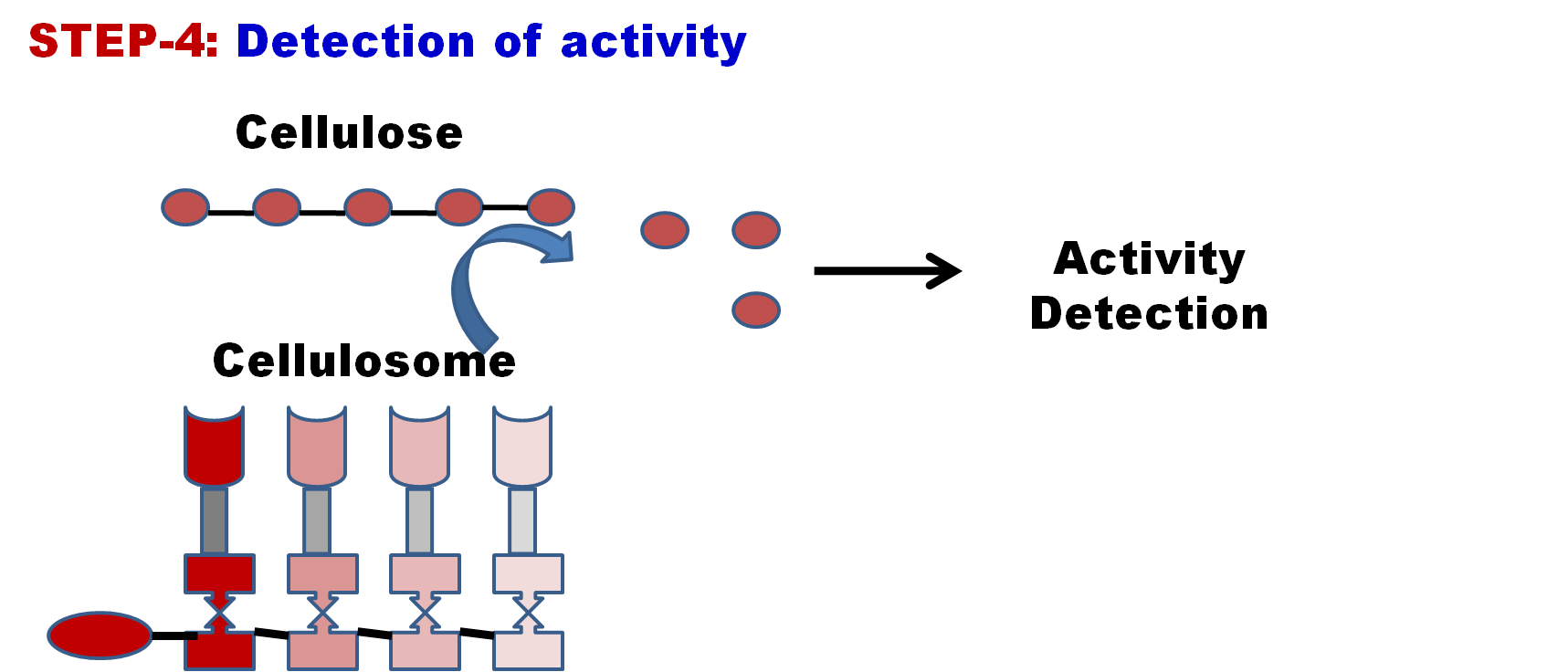


a

b

d

c



**Figure: 12** Flow chart elaboratingthe steps for design, construction and evaluation of designer cellulosome

* + - * 1. ***Applications of designer cellulosome***

Lytic polysaccharide monoxygenases in designer cellulosome

The synthetic biology approaches are used for designing efficient cellulosomal complex. The LPMOs are responsible for boosting cellulose degradation. Moreover, in anaerobic bacteria the cellulase enzymes are assembled into large complex called cellulosome. Additionally, LPMOs are mostly found in aerobic bacteria thus, it cannot benefit to the cellulosomal complex during cellulose degradation. In one of the study the LPMOs are incorporated into the cellulosomal complex through synthetic biology approach. In the study chimeric enzymes were developed by fusing LPMOs from the bacterium *Thermobifidafusca* using different dockerin in the cellulosomal system [23]. The study showed higher activity on the microcrystalline cellulose as compared to the wild-type enzymes (Table 1).

Xylan binding domain in designer cellulosome

The xylanase were employed to deconstruct the hemicelluloses component plant based lignocellulosic component [5]. Xylanases engineered in designer cellulosome system in deconstruct the cellulosic biomass more efficiently [5]**.** Xylanases are important because it is used in different industry for example textile, biorefinary, food and pharmaceutical [6]. In one of the study, an entire xylanolytic complex of the bacterium *Thermobifida fusca* has been incorporated into an artificial cellulosome using designer cellulosome approach [24].

* 1. **Conclusion**

The efficient enzyme with multi-functionality can be used for efficient degradation of cellulosic substrates. The multi-functionality can be introduced through protein engineering and designing artificial designer cellulosome. The engineered enzymes are utilized as cost effective process for enzymatic hydrolysis in deconstructing plant based lignocellulosic biomass and the technology can be transferred to biorefinery industry.

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