Synergy in *Trichoderma reesei* cellulases

Abstract

Synergy has been observed between the various cellulolytic enzymes of the fungus *Trichoderma Reesei.* Termed cellulases, these enzymes come together to form a potent cocktail of biomass degrading enzymes. The biofuel industry has seen a recent surge partly due to the advances in attaining a more applicable understanding of the mechanisms of synergy that occur when said enzymes are used to degrade biomass into fermentable sugar. To that end, this review summarizes the various theories postulating the molecular mechanisms of synergy at play in *T. reesei* endocellulases and exocellulases. Herein, we suggest possible reasons for explaining the overlapping characters of the enzymes *Tr*Cel7A, *Tr*Cel6A and *Tr*Cel7B, and how they may be driving synergistic mechanisms.

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List of Abbreviations
BC - bacterial cellulose, **BG** - β-glucosidase, **BMCC** - Bacterial microcrystalline cellulose, **CBH** - cellobiohydrolase, **CBM** - cellulose binding domain, **CD -** catalytic domain, **CEF** – cellulose enriched fraction, **CF** - clean fractionation, **CMC** - carboxymethylcellulose; endoactivity indicator, **CrI -** crystallinity index, **DP** - degree of polymerization, **DS/DSE** - degree of synergy/ degree of synergistic effect, **EG** - endoglucanase, **GH** - glycoside hydrolase, **Glu** - glutamate residue; amino acid, **HS-AFM -** High-Speed Atomic Force Microscopy, $\mathbf{k_{cat}}$ - rate constant of catalytic events; catalytic constant, **kDa -** kilo-daltons, $\mathbf{k_{off}}$ **-** dissociation rate, $\mathbf{k_{on}}$ - association rate, **PASC** - phosphoric acid swollen cellulose, **SEM -** Scanning Electron Microscopy, **SSA** - specific surface area, **TEM** - Transmission Electron Microscopy

1. Introduction

With the increased understanding of the impact of climate change, there is a universal effort in the bioengineering field to generate alternative energy by converting biomass to biofuels. There is a surge of research in the focus area of cellulases and specifically their ability to break down cellulose in the way of producing renewable energy. Great efforts have been put forth in understanding these enzymes, increasing their productivity and investigating the molecular mechanisms leading to synergy between the enzymes.

Cellulose is the most abundant organic molecule found on the planet and is mainly found in plant and algae cells walls. It is naturally found as a crystalline and linear polymer consisting of thousands of D-glucose residues connected by β-1,4-glycosidic bonds (1). Through extensive inter and intramolecular hydrogen bonds and Van der Waals forces, glycosidic bonds form crystalline structures seen in Figure 1.

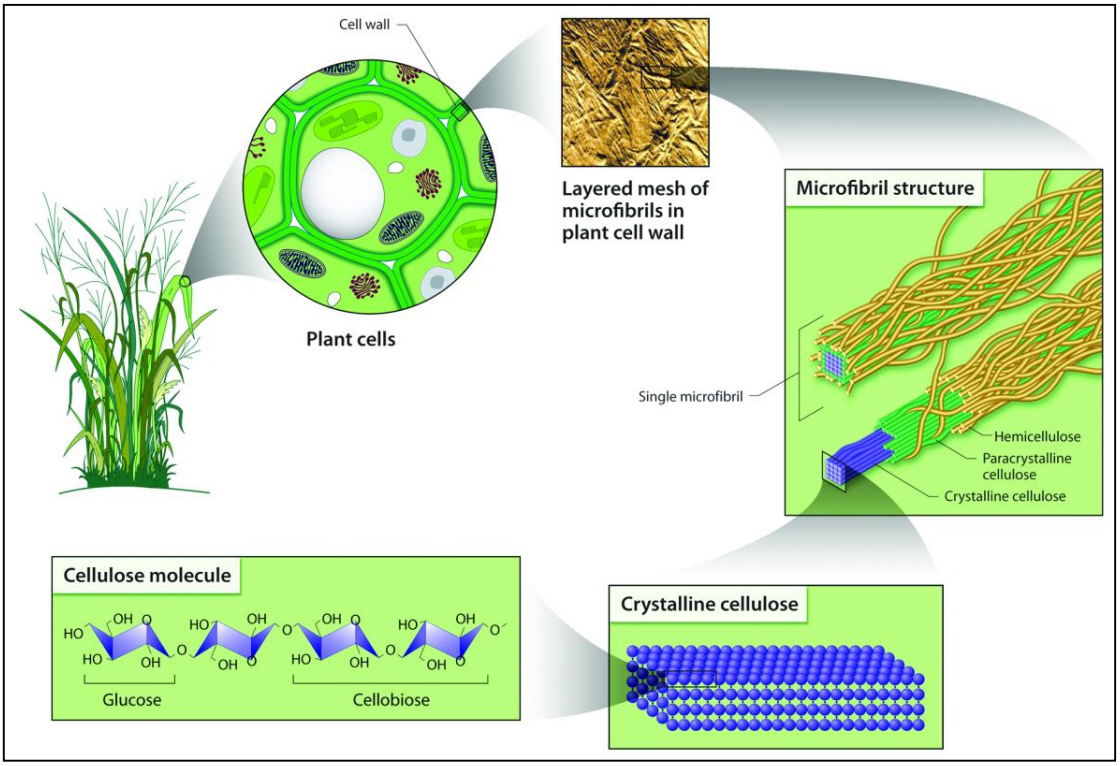

Figure 1. Structure of plant cell wall and organization of cellulose (2)

Although the crystalline structure of cellulose seems straight forward and organized, there are still difficulties in breaking it down. The cellulose chains have two different ending groups; a reducing end and a non-reducing end (Figure 2) and there are specific

enzymes that have specific preferences as to where they attack this molecule to break it down.

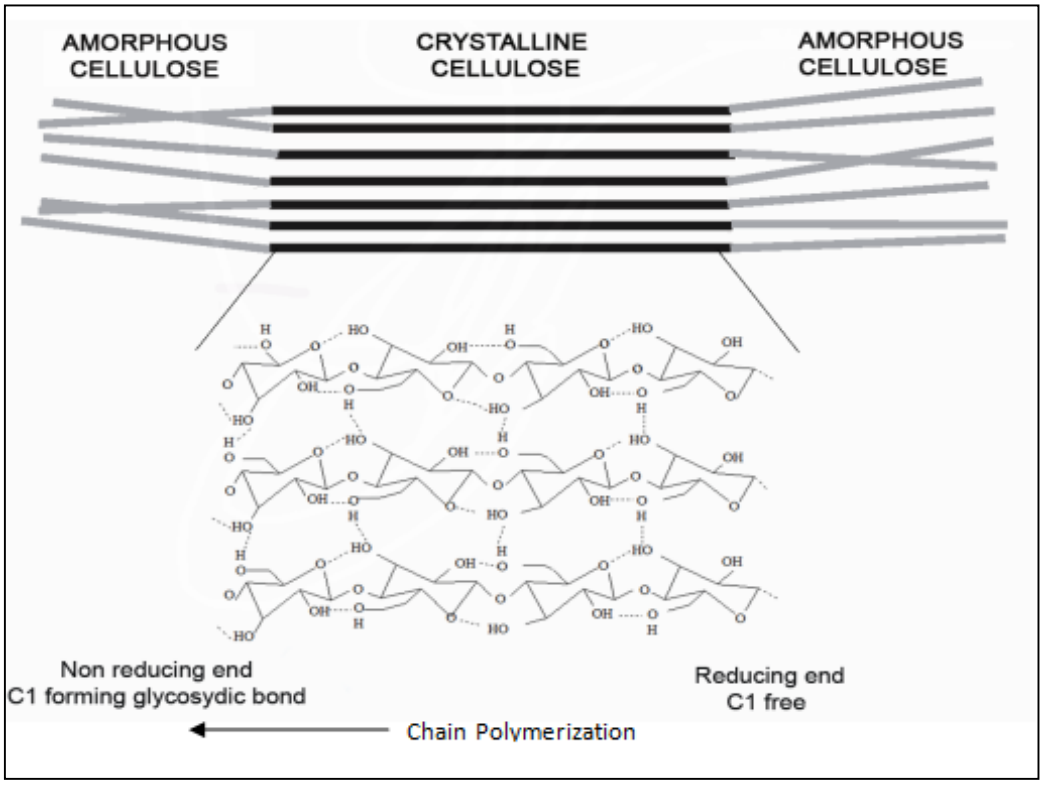

Figure 2. Shows the structure of cellulose on a molecular level with its two different end groups (2)

Three types of cellulases have target zones on cellulose. There are endocellulases that attack the molecular cellulose at internal glycosidic bonds as opposed to exocellulases that attack the ends of the cellulose chains, generating the cellobiose and other soluble carbohydrates. There are also beta-glucosidases that cleave the β-1,4-glycosidic bonds of cellobiose to make glucose. The exocellulases are divided into two groups; those that prefer the reducing ends and those that prefer the non-reducing ends. Each enzyme´s goal is to hydrolyze the glycosidic bonds in their preferred point of attack. In this report, we have chosen to refer to endoglucanases as endocellulases and cellobiohydrolases as exocellulases.

These enzymes work together to generate cellobiose or glucose and, with the right combination, they are more productive in groups than alone. Synergy is the phenomenon used to explain this increased productivity enzymes of varying character are used in a cocktail. In the following report, we are investigating, through existing research, the synergy between two cellulases from the fungus *Trichoderma Reesei*. The two enzymes

under study are cellobiohydrolases (exocellulases) *Tr*Cel7A and *Tr*Cel6A. The goal of this paper is to discuss existing research and suggest novel mechanisms of synergy between *Trichoderma Reesei* cellobiohydrolases and the question at hand is if *Tr*Cel6A, traditionally thought of as an exocellulase, could possibly have some endocellulase qualities. We would also like to discuss the synergy observed between cellulosomes and free enzymes and the intriguingly high amount observed.

2. Cellulase substrates

Cellulose

Synergy between cellulases is highly influenced by the characteristics of the substrate they are degrading. Therefore, it is important to gain an insight into the various aspects of cellulose structure and heterogeneity.

The cell walls of plant and algae cells are fortified with fibrous materials, including cellulose, providing stability and protection from bacterial and fungal pathogens (3). Cellulose is a tough, fibrous, linear, water insoluble polysaccharide. It is a major constituent of wood and plant fibre, located primarily in cell walls. It is an unbranched polymer made up of repeating units of the disaccharide cellobiose. Cellobiose consists of two units of D-Glucose. A molecule of cellulose can consist of up to 15000 repeating units of D-Glucose (1) with a reducing and non-reducing end (4). Cellulose differs from other polymers of glucose by the links formed between repeating units, namely through β-1,4 glycosidic linkages. β-1,4 linkages allow for high molecular stability due to the number of hydrogen bond crosslinks that form between adjacent D-glucose molecules (see Figure 3) (1).

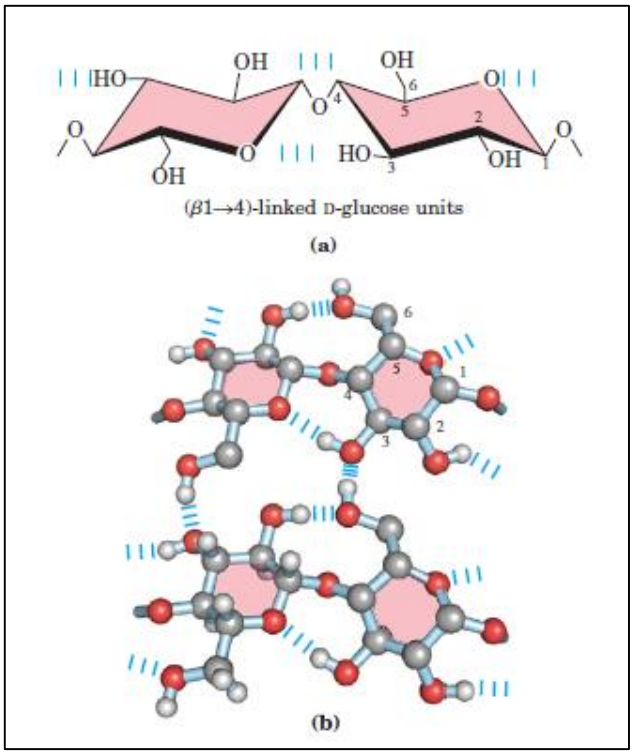

Figure 3. (a) A cellobiose unit with β-1,4 glycosidic linkages between D-Glucose molecules. (b) Hydrogen bond cross-links between adjacent D-Glucose molecules (1)

Cellulose is produced biosynthetically by an ordered cellulose synthase complex in plant cell membranes called rosette terminal complexes (5). The D-Glucose molecules in repeating cellobiose units are rotated 180° towards each other, conferring symmetry on the cellulose molecule due to equal numbers of hydroxyl residues present on each side of the polysaccharide chain (6). Symmetry lends itself to the formation of a crystalline structure in nanometre sized microfibrils of cellulose due to the presence of numerous ordered hydrogen bonds and van der Waal's interactions (6), occurring both inter and intra chain. The D-Glucose molecules are in the chain conformation and neighbouring molecules lie in an extended chain, forming 2 interchain and 2-3 intrachain hydrogen bonds, the interchain hydrogen bonds are weaker due to the longer distance between hydrogen and electronegative atoms (6) (Figure 4). The fibrils form sheets, which are then stacked upon one another giving cellulose a 3-D structure (6).

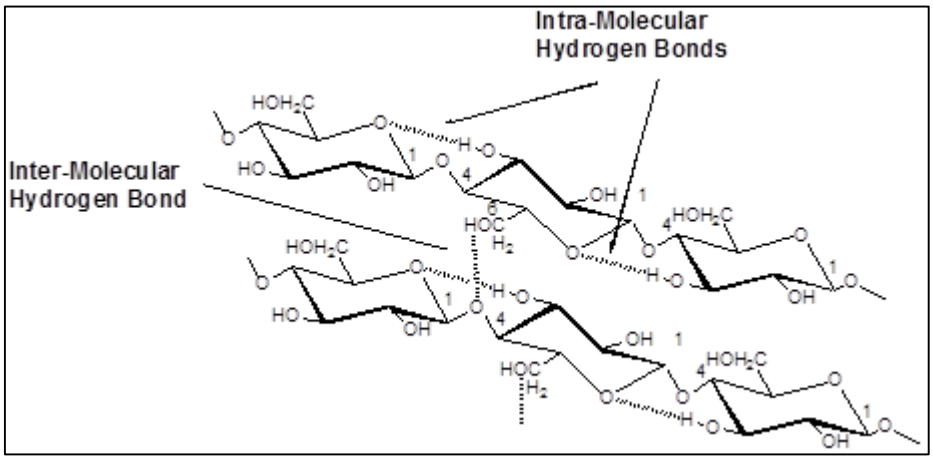

Figure 4. Inter and intra chain hydrogen bonding in cellulose chains (7)

The crystalline cellulose superstructure formed in this way has a high tensile strength. A form of crystalline cellulose known as cellulose I is most abundant in biomass and can be found in two forms, namely cellulose Iα and Iβ. The two forms differ in their hydrogen-bonding pattern (6) and in that, Iβ occurs mostly in higher plants. A two-phase cellulose model is widely accepted, wherein cellulose chains contain both ordered (crystalline) and less ordered (amorphous) sections (6). Occurrence of amorphous regions create obstacles for the enzymes breaking down the chain and even though it is perceived as the reason for slowing down the process of degradation of the substrate, it is a feature of cellulose that appears in nearly all types of cellulolytic substrates.

A matrix of heteropolymers constituted of other sugar monomers like xylan, arabinose, galactose and mannose, surrounds cellulose microfibrils in plant cell walls (4). These often shorter chained polysaccharides, collectively termed hemicellulose, are amorphous in structure and therefore do not possess the tensile strength and structural stability of cellulose (3). Another component of plant cell walls is lignin, a phenolic polymer. Together, lignin and hemicellulose form a matrix of long strands around the cellulose portion of the cell wall. This amalgamation of polysaccharides and phenolic polymers is referred to as lignocellulosic biomass. Overall, typical biomass mixtures (for example, switchgrass) are composed of 40 – 50 % cellulose, 13 - 28 % lignin and 28 – 37 % hemicellulose (3). Biomass from varying sources materials has varying percentages of these polymers. The relative amounts of lignin and cellulose in certain plant species and crops make them more suitable for the production and extraction of biofuels (3).

Saccharification of cellulose by enzymes secreted by fungi degrades it to cellobiose or glucose units, providing them with sugars to use as an energy source, usually by way of hydrolytic cleavage (8). There are several families of enzymes, including exocellulases and endocellulases. Synergy between these and other enzymes has been observed and reported (9). Commercially, the cost and time efficient breakdown of cellulose to its constituent sugars is of great importance to the future of the biofuel industry, therefore understanding how fungal enzymes employ synergistic mechanisms could lead to the improvement of biofuel yields from plant biomass. Fungi apply the “free enzyme paradigm”, meaning that they secrete enzymes into solution, which then diffuse towards the substrate (4). This is in contrast to the “cellulosome” strategy employed by some anaerobic bacteria that digest plant matter, whereby enzymes form extracellular complexes via adhesion to one another. These complexes may contain several substrate-binding domain and catalytic domains (10). The presence of lignin, hemicellulose, other biopolymers and the slow breakdown of cellulose makes plant cell walls resistant to degradation, a property termed biomass recalcitrance (11).

Crystallinity Index

Crystallinity index (CrI) is the amount of crystalline versus amorphous cellulose in a sample. CrI is a contributing factor to biomass recalcitrance. The crystalline portion of cellulose is tightly packed, preventing the penetration of enzymes and smaller molecules like water (4). The overall structure of cellulose is heterogeneous, with ordered cellulose interspersed with amorphous regions (12). The CrI of cellulosic substances fluctuates

depending on the method of measurement. Even though values between 0% - 95% are known, it is generally estimated to lie between 40% and 95% in cellulose from natural sources (13). Usually, synergistic degradation by enzymes is most notable on substrates with a higher degree of crystallinity (14).

Degree of polymerization

The degree of polymerisation (DP) is a way of defining the relative abundance of terminal and interior β-glycosidic bonds available for digestion by endo or exocellulases. Several methods are employed to measure the DP of a substance, where cellulose must first undergo dissolution without the reduction of chain length (6). DP is the measurement of the number of monomeric units in a polymer (2). Generally, the solubility of cellulose decreases as the length of the polymer increases due to the density of inter and intramolecular hydrogen bonds in a longer cellulose chain (6). Exocellulases prefer substrates with a lower DP because of the larger number of chain ends present, whereas substrate DP does not seem to have an influence on the activity of endocellulases creating chain ends (6). Table 1 shows a compilation of CrI and DP values for commonly used substrates.

Substrate	Crystallinity index (%)	Degree of polymerization
Carboxymethyl cellulose (CMC)	NA	100 – 2000
Cellodextrins	NA	2 – 6
Avicel	50 – 60	300
BC	76 – 95	2000
PASC	0 – 4	100
Cotton	81 – 95	1000 – 3000
Filter Paper	0 – 45	750
Wood Pulp	50 – 70	500 – 1500
Fluka Avicel PH-101	56 – 91	200 – 240
Fluka cellulose	48 – 82	280
Sigma α-cellulose	62	2140 – 2420

Table 1. A compilation of CrI and DP values for cellulose based substrates (2)

3. Cellulases

Trichoderma reesei – cellulase producing model organism

A key portion of the Carbon cycle on Earth is the degradation of biomass by fungi. Fungi evolved separately from plants and are taxonomically more closely related to animals. Since they have evolved parallel to plants, they have developed enzymes that allow them to digest lignocellulosic substances and cellulose. Fungi developed the ability to digest lignin some 300 million years ago, decreasing the amount of coal deposits laid down then onwards significantly (15). Many fungi strains have evolved a potent mix of enzymes highly efficient at breaking down lignocellulosic substrates. Most fungi apply the free enzyme paradigm in their degradation strategy, producing high concentrations of enzymes that readily digest the non-cellulose portions of plant matter. Subsequently, cellulases produced by fungi digest cellulose at a high synergistically propagated efficiency and speed. This makes cellulases isolated from fungi key in furthering commercially viable processes in the development of biofuels (4).

Trichoderma reesei is a well-researched species of fungi that specializes in the free enzyme degradation of plant material. Many genetically engineered clones of *T. reesei* have been developed to study cellulase related genes and the enhanced ability of these cellulases when they are employed in a cocktail by *T. reesei* (4). *T. reesei* was first studied at the US Army Natick Laboratories by Reese and Mandel in the 50s, when they coined their theory of fungi breaking down carbohydrates using a cocktail of enzymes (4). Radiation and chemical mutagenesis techniques have since been employed to create strains of *T. reesei* with increased yields of cellulase and beta glucosidase proteins. One of the most enhanced strains reported at the time was the RUT-C30 strain, producing 2.2 g/L of cellulase protein (4). The expression and induction of RUT-C30 has come a long way, with current derivatives of RUT-C30 reporting as much as 100 g/L of cellulase enzymes produced (4).

Other species of fungi like *Aspergillus nidulans or Postia placenta* can produce cellulases in a great variety, numbering into the hundreds. Comparatively, *T. reesei* produces a smaller amount and variation of enzymes, yet produces much better results when degrading biomass. This ability of *T. reesei* is attributed by the fungus's high capacity of enzyme production, induction of cellulase translating genes and high amount of secretion (4).

3 Classes of cellulolytic enzymes

Cellulases are divided into three sub-categories of enzymes: 1,4-β-D-glucan glucanohydrolase or endocellulase, 1,4-β-D-glucan cellobiohydrolase or exocellulase cellobiohydrolase, and lastly, 3-D-glucoside-glucohydrolase or beta-glucosidase cellobiase.

Exocellulase

Also known as cellobiohydrolases, these are monomeric proteins with a molecular weight ranging from 50 to 65 kDa, although there are smaller variants (41.5 kDa) in some fungi, such as *Sclerotium rolfsii*. Low levels of glycosylation (around 12% to none at all) are found in these enzymes; and their optimum pH is 4 to 5, with an optimum temperature from 37 to 60 °C (2) depending on the specific enzyme-substrate and the origin of the enzymes. Cellobiohydrolase enzymes account for 40 - 70% of the total component of the cellulase system and are able to hydrolyse crystalline cellulose (2). Exocellulases catalyse the successive hydrolysis of residues from the reducing and non-reducing ends of cellulose, an activity called processivity discussed in detail in "Processivity in *T. reesei* cellulases". These include *T. reesei* cellobiohydrolase I and II (*Tr*Cel7A and *Tr*Cel6A), releasing cellobiose molecules as the main product (16).

Endocellulase

These are monomeric enzymes with a molecular weight that ranges from 22 to 45kDa, although some fungi such as *Sclerotium rolfsii* and *Gloeophyllum sepiarium* have endo-cellulases twice this size (18). They cleave glycosidic bonds on the internal regions of cellulose generating oligosaccharides with different sizes and creating new chain ends that can in turn be attacked by exocellulases (16). Most endocellulases have a preference for amorphous regions of cellulose (4).

Beta-glucosidase cellobiase (BG)

These hydrolyse soluble cellobiose and other cellodextrins with a DP up to 6 to produce glucose in the aqueous phase, this results in the alleviation of cellobiose inhibition of exocellulases. These enzymes have molecular weights ranging from 35 to 90 kDa, and can be monomeric or exist as homooligomers, as is the case β-glucosidase of the yeast *Rhodotorula minuta*. Their optimum pH ranges from 3.5 to 5.5, and their optimum temperature ranges from 45 to 75 °C (4). The main purpose of β-D-glucosidase in cellulose hydrolysis is that it catalyses the hydrolysis of terminal non-reducing residues in beta-D-

glucosides generating the release of glucose. This is achieved by splitting the cellobiose into two units of glucose (17).

In order to effectively convert cellulosic biomass to fermentable sugars, a complete cellulase package comprising of components from each class of cellulases is necessary because the individual cellulase components are far less effective at hydrolysing cellulose substrates than an entire system of different cellulases working together as a team (17).

Structural features of some *T. reesei* enzymes

Cel6A of T. reesei (TrCel6A)

The fungal species *Trichoderma reesei* produces the cellobiohydrolase *Tr*Cel6A, also referred to as CBH II, its original name, belonging in the Glycoside Hydrolase Family 6 (GH6) of enzymes. *Tr*Cel6A is a cellobiohydrolase that binds to cellulose and cleaves at the 1,4-β-D-glycosidic bond of glucose subunits in cellulose during hydrolysis. *Tr*Cel6A is a processive enzyme, that when associated with a single cellulose chain goes through repeated catalytic cycles hydrolysing multiple glycosidic bonds, producing cellobiose as a product. *Tr*Cel6A attacks the cellulose chain at the non-reducing ends and achieves this due to its complex three domain structure, consisting of a catalytic core domain (CD) linked by a relatively long O-glycosylated polypeptide to a small carbohydrate binding molecule (CBM). The O-glycosylated linker of *Tr*Cel6A are 27 residues (4).

The CD, usually 70% of the protein, has different dimensional arrangements in different cellulases. In cellobiohydrolases, it has a tunnel shape for processive exo degredation and in endocellulases, it has a cleft shape for internal attack degradation (2). The CD is N-glycosylated and its role in hydrolysis is cleaving the glycosidic bonds in cellulose (2).

The CBD facilitates hydrolysis with the substrate and CD and is responsible for both initiating hydrolysis and the processivity of the protein. CBDs are usually O-glycosylated, containing 30-200 amino acids and are single, double or triple domains in enzymes. The CBD authorizes the latching on of the protein to substrate and increases the rate of hydrolysis (2) (see Figure 5).

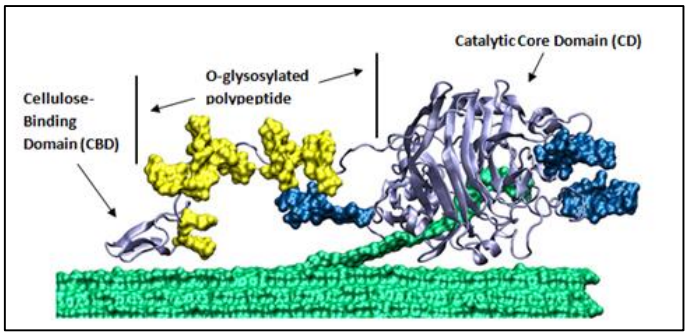

*Figure 5. TrCel6A and Tr*Cel7A *three domain enzyme structure (4)*

Cel7A of T. reesei (*TrCel7A*) and comparisons between Cel7A and Cel6A

*Tr*Cel7A, cellobiohydrolase I (CBH I), is the most abundant enzyme secreted by *Trichoderma reesei.* It is a modular 52 kDa enzyme with a catalytic domain in the family 7 of Glycoside Hydrolases and has a family 1 cellulose binding domain, which are interconnected by a highly glycosylated linker. Like *Tr*Cel6A, the CD of *Tr*Cel7A has a tunnel shaped active site with binding sites, linked by a relatively long O-glycosylated polypeptide to a small CBM. During enzymatic hydrolysis of cellulose, *Tr*Cel7A attacks at the reducing ends of the cellulose polymer (4).

The tunnel of *Tr*Cel7A is roughly twice as long as *Tr*Cel6A. *Tr*Cel7A, like all GH7 enzymes, employs a double displacement retaining catalytic mechanism. The structure of *Tr*Cel6A allows it to cleave the cellulose chain from its reducing end (4). It has been determined that *Tr*Cel7A is more processive than *Tr*Cel6A, due to its preference to hydrolyse reducing ends, the longer binding tunnel in its structure, and the location of cleavage that is skewed towards the tunnel exit (4). Another structural difference is that *Tr*Cel7A has a CD with a tubular structure that can accommodate ten subunits of the cellulose substrate, whereas *Tr*Cel6A has an open configuration (4).

Cel7B of T. reesei (*Tr*Cel7B)

*Tr*Cel7B is an endocellulase and it has overall fold and appearance of *Tr*Cel7A but exhibits a very open binding site “cleft” instead of the tunnel that *Tr*Cel7A possesses (4). There are also differences in the loops; *Tr*Cel7B is missing some of the loops that *Tr*Cel7A has. But at the same time a specific loop A1 has a similar length to that of *Tr*Cel7A (18). These differences lead to the small differences between the structures of the two enzymes. The deletions in *Tr*Cel7B make its binding site more open and cleft-like. The additions to *Tr*Cel7A,

yield a tunnel forming loop. These structural differences distinguish the small and subtle differences between CBHs and EGs even though their jobs are so uniquely different.

Processivity in *T. reesei* cellulases

Definition and types of processivity

Processivity is an important characteristic of polymer active enzymes (19) and can be defined as the ability to produce several single molecules of disaccharides (cellobiose) out from the polymer crystal without dissociating from the enzyme in between those subsequent hydrolytic steps (8). From the point of view of synergy, processivity may play a role in explaining some of the mechanisms of synergy between different types of enzymes. Processive enzymes are one of the most abundant enzymes secreted by biomass-degrading microorganisms. They stay closely attached to single polymer chain and thus have a significant hydrolytic potential (20).

Association and dissociation

To get the best understanding of the processivity of cellulose, it is worth it to summarize basic kinetic term explained in relation to cellulose and cellulases, see Figure 6 for reactions related to terms.

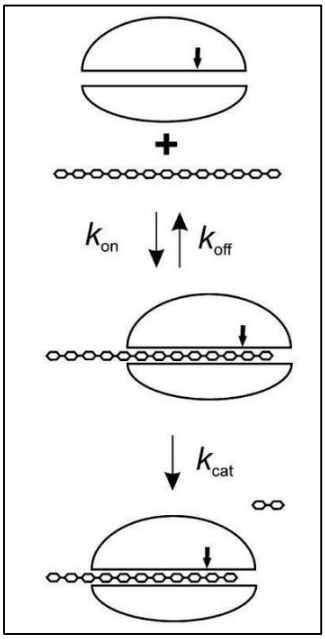

Figure 6. Association, disassociation and catalytic constants for TrCel7A (19)

k_{cat} represents the rate constant leading from one productive complex through catalytic events to another productive complex one unit further on polymer chain (19); catalytic constant.

k_{on} is a rate constant that specifies the rate of forming a threaded complex without providing separate information about the steps that take place towards forming this complex (21).

k_{off} is a dissociation rate constant of the enzyme substrate complex (19). This rate constant is found in most literature sources as the key rate-limiting factor in processive hydrolysis (21).

Both cellobiohydrolases (I and II) adsorb strongly and slowly to the substrate. Dissociation constant onto Avicel (further detail on Avicel in the chapter "Pretreatment") was found to be $K_D=10^4$-10^5/M (22). At equilibrium, the constant is related to association and dissociation rate coefficients:

$$K_D = \frac{k_{off}}{k_{on}}$$

It is also confirmed that the adsorption of cellulase is significantly affected by the reaction conditions and characteristics of substrate used (23). Therefore, it is advised to add both (or more) enzymes of cellulases cocktail simultaneously to the substrate and in non-saturated concentrations to obtain maximum synergistic effects (22). It is so because of the widely observed competition between cellulases during the adsorption process (22). Moreover, interactions between absorbed and non-absorbed proteins was shown to strongly influence the degree of uptake of endo and exocellulases, when tested together (24). Dissociation rate (k_{off}) is perceived a rate-limiting step in the hydrolysis of cellulose (21).

Pretreatment of cellulases

Pretreatments can effect synergy between various enzymes by changing the nature of the substrates in a way that may increase or decrease enzymatic efficiency. Pretreatment is classically defined as large-scale industrial processes to break down biomass before subjecting it to further degradation. In this report, we are redefining pretreatment as all techniques of preparing substrate before enzymatic digestion.

To obtain biofuel, the complex web of plant material in biomass is broken down via three processes: pretreatment, hydrolysis, and fermentation. Pretreatment of cellulose involves size reduction by boiling or steaming and chemical treatments with acids, bases, salts and solvents (25). Pretreatments disrupt the lignin seal of cellulose to boost enzyme activity by increasing access to holocellulose, increasing surface area and affecting the degree of cellulose crystallinity (25). All pretreatments lead to developed porosity of substrates and

increased cellulose hydrolysis. In acidic pretreatments, the amorphous hemicelluloses hydrolyse quicker than cellulose to soluble sugar and some oligomers, through the disruption of xylosidic bonds and cleavage of acetyl ester groups (25). Amorphous regions in cellulose are degraded by acid treatments, leading to swelling and enlarged cellulose fibrils (25).

Pretreatment is usually an acid treatment to strip the hemicellulose and lignin before they are treated with the enzyme mixtures, to prevent them from limiting the diffusion of the enzyme into the reaction sites which slows the rate of hydrolysis (25).

Various types of pretreatment

Laboratory techniques

Phosphoric acid swollen cellulose (PASC)

Phosphoric acid swollen cellulose (PASC) is prepared from Avicel and is considered an amorphous substrate (26). PASC is prepared, in short, by treating Avicel with 85% orto-phosphoric acid and acetone. It is washed multiple times with water and blended to homogeneity (27). In comparison to Avicel, PASC has lower DP, lower molecular weight, lower CrI and bigger specific surface area (SSA) (26).

Bacterial microcrystalline cellulose (BMCC) with hydrochloric or acetic acid

Bacterial microcrystalline cellulose (BMCC) is obtained from bacterial cellulose (BC), mostly from *Acetobacter xylinum* (28). In the process of its preparation, pieces of BC pellets are boiled in sodium hydroxide, washed with water or acetic acid, pelleted by centrifugation and re-suspended and boiled in hydrochloric acid (29). This recipe with minor changes is used in most of the literature sources in this report. BMCC is a product with reduced DP and higher crystallinity in comparison to BC (29). As a substrate with high CrI and simple morphology, it was used as a model substrate to study the mechanism of crystal erosion (28).

Ammonia treatment

Ammonia treatment is used to convert cellulose I to cellulose III (30). In this supercritical treatment, cellulose I is inserted into a steel pressure vessel (on dry ice) and ammonia is added to the vessel until the samples are immersed. After bringing the vessel to room temperature, the temperature is increased to 140°C in an oil bath for 1 hour. The ammonia gas is leaked out and sample is washed with dry methanol (31). A substrate pretreated in this way is said to have more suitable lanes for cellulase movement (30).

Homogenized Avicel

Homogenization of Avicel leads to a substrate with particles of reduced size, there is however no decrease in CrI but increased degree of hydration of some parts of the substrate (28).

Industrial techniques

Clean fractionation

Clean fractionation (CF) is a pretreatment method that employs water, methyl isobutyl ketone (MIBK) and either ethanol or acetone with sulphuric acid to separate biomass into three fractions enriched mainly in cellulose, hemicelluloses, or lignin. An insoluble cellulose-enriched fraction (CEF), in which most of lignin and hemicelluloses were removed, is a main focus substrate for hydrolysis (10). CEF has a higher CrI and low amount of hemicellulose and lignin (10).

4. Synergy in cellulases

Quantification of synergy

When enzymes from various biomass degrading organisms digest lignocellulosic substrates their activities often show synergism. Cellulases also have a synergistic mode of action between two or more enzymes that efficiently degrade cellulose. Synergy is defined as the sum of the overall hydrolysis of the mixture of enzymes being greater than the sum of degrees of hydrolysis of the individual enzymes (26). Synergy can be measured in a few different ways by quantifying rate of hydrolysis, product formation or substrate usage. In most cases, it is measured using mole fractions of the different enzymes in mixture. It is termed degree of synergy (DS) or degree of synergistic effect (DSE).

As synergism is expressed as a ratio between combined cellulase activity and the sum of individual components, values more than 1 are considered synergistic. Values more than 2 are common whereas those up to 5 have been reported (6).

The Endo-exo synergy model

The synergism between endocellulases and exocellulases is the most widely studied type of synergism in cellulases (6). More detailed description of those enzymes can be found in the section on cellulases "Structural features of some *T. reesei* enzymes".

The traditional mechanism of endo-exo synergism is that various endocellulases attack random internal sites on cellulose strands generating new chain ends which then serve as attack points for processive hydrolysis for exocellulase (*Tr*Cel7A) (29) (9). The degree of synergy between those enzymes is said to vary depending on the ratio of concentrations of endocellulases and cellobiohydrolase, and the substrate used (38) (14).

Even though this endo-exo synergy mechanism is the most widely accepted (29), during the last few years, some novel, alternative theories appeared in literature.

One alternative explanation is that endocellulases remove obstacles from the path of exocellulases, aiding the processive run of *Tr*Cel7A (32) (33).

It is generally thought that the dissociation of *Tr*Cel7A (k_{off}) is a rate limiting step of hydrolysis (19). Simply meaning, that when cellobiohydrolase is unproductively bound to substrate, the overall hydrolysis rate is lower (19) (34). It is therefore proposed that, when endocellulase is present in the hydrolysis mixture (non-saturating concentrations of both

enzymes, simultaneous addition to substrate (24)), it creates cuts, not in random places as in traditional approach to endo-exo synergy, but just in front of the obstacle so that when *Tr*Cel7a is approaching the amorphous region, it has a dissociation site and may dissociate from the strand (9) (32). Endoglucanases are, thus, perceived to have a preference for amorphous regions (4). This approach is compared in Figure 7 with the traditional approach, where endoglucanase performs random cuts leading to the production of new ends.

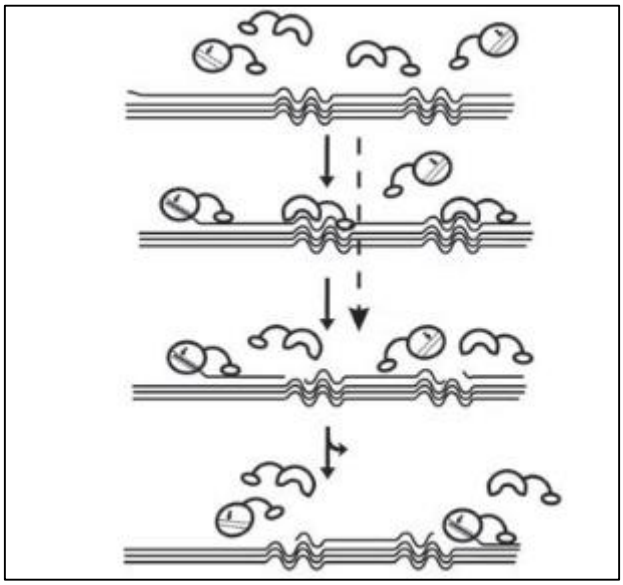

Figure 7. The alternative explanation of synergy by Jalak et. al (9). From top to bottom: Left: new approach postulates that endocellulase binds in front of the amorphous region (obstacle) and creates dissociation sites so that exocellulases do not get unproductively bound to the substrate. Right: Traditional approach postulates that endocellulase binds randomly to create new attack points for exocellulase.

As shown in Figure 7 (on the left), according to the new approach, cellobiohydrolase is not trapped unproductively at the end of its processive run but is released from the substrate chain and can be used to bind to another binding site on the substrate. However, this doesn't happen for all catalytic events because not all the endocellulases will systematically create dissociation sites that enable all exocellulases to dissociate from the chain.

There is also an idea, that another rate-limiting factor in hydrolysis is the length of the obstacle-free path. This means a length of crystalline cellulose lacking amorphous areas which can act as obstacles to a processive enzyme, along with many other putative obstacles. The enzyme can get stuck at these points because it doesn't have a chain end with a dissociation site to dissociate from. The longer the substrate chain without the obstacle, the higher possibility of the cellobiohydrolase being stuck by this obstacle (34). Endocellulase is, therefore, perceived to cleave the cellulose chains, making them shorter, what makes the

possibility of *Tr*Cel7a completing the run without being blocked by an obstacle and being unproductively bound to the substrate higher (34). This is much the same as the way that amorphous cellulose acts as an obstacle.

A study also presents the idea that the action of the enzymes on the crystal structure of substrate surface leads to erosion of the cellulose surface (29) (35). In this way, there are randomly created solitary chains which may become obstacles in the way of processive run of *Tr*Cel7a. Authors see the function of endocellulase as the enzyme which attacks those solitary chains and enables further action of cellobiohydrolase on them. However, it can be seen as additional function in hydrolysis as the endocellulase is also actively producing new chains for binding of *Tr*Cel7a (29).

As in all mechanisms presented above the cellobiohydrolase present is the *Tr*Cel7a, there is one study that uses *Tr*Cel6a together with endocellulase in different concentrations (26). Even though this group does not come up with any novel mechanism of endo-exo synergy, their experiments show that degree of synergy increases with a decrease in ratio of endocellulase to exocellulase (28).

Another study by Josefsson et. al. proposed a mechanism of synergy whereby the attachment of both endocellulase and cellobiohydrolase causes the swelling of cellulose microfibrils, and thus made it easier for both endocellulase to attack and cellobiohydrolase to start its processive run (36). They propose this as an extension of the typical endo-exo model of synergy.

The Exo-exo synergy model

Exo-exo synergism is a concept that was first demonstrated by Fagerstam and Pettersson in 1980. In *T.reesei*, *Tr*Cel7A and *Tr*Cel6A, it was originally thought of that these two exocellulases both attacked on the non-reducing ends of cellulose. But this would create competition between them (37) and it has been shown that together they produce more cellobiose than individually (30). Barr and his colleagues suggested that the preference of *Tr*Cel7A and *Tr*Cel6A was due to their structure. The tunnel that encloses the active site of TrCel7A is divided in 10 subsites (-7 to +3), each one binds one glycosyl unit. An external subsite (+4) is positioned close to the tunnel exit (38). Hydrolytic cleavage of the glycosidic bond occurs between the subsites -1 and +1 when the glycosidic oxygen is pointing towards the the catalytic acid. Considering that the glycosyl units of a cellulose chain alternate their orientation in 180°, after each bond cleavage and product release the chain has to be moved

two subsites ahead (filling again subsites +1 and +2) in order to place the glycosidic bond in scissile orientation again. That explains the processive release of cellobiose as the main product of the TrCel7A activity (38) (37). Barr's suggestion that the reducing end of a small ligand binds in site -7 is consistent with *Tr*Cel7A attacking cellulose from the reducing end and that the exact opposite was occurring in *Tr*Cel6A (37). In *Tr*Cel6A, the shorter tunnel is divided in 4 subsites (-2 to +2), whereas an extra subsite (+4) is positioned at its (38). Ligands bind with the non-reducing end, indicative of *Tr*Cel6A attacking cellulose from the non-reducing end (37). This difference in specificity though slight, leads to synergism because of their ability to become more productive together and avoid competition.

As current opinion is this that *Tr*Cel7A prefers the reducing ends, degrading the crystal leading to fibrillation, thinning of the crystal, or narrowing of the crystal end, whereas *Tr*Cel6A prefers the non-reducing ends (37), hydrolyzing the crystal from the non-reducing end, less processively than TrCel7A, thereby sharpening the crystal tip (39). The synergy between these two cellobiohydrolases can be described as two simultaneous attacks from opposite sides of the chains of glucose molecules hydrolyzing and releasing units of cellobiose (see Figure 9). The synergy only occurs when the cellulases are more efficient together, then they are apart. This synergy between *Tr*Cel7A and *Tr*Cel6A was demonstrated through an experiment conducted by T. Uchihashi et al., where cellulose was imaged in the presence of *Tr*Cel6A alone and then compared to mixture where *Tr*Cel7A was added. After TrCel7A was additionally added, enzyme molecules started moving from many points on the cellulose surfaces, and the degradation of crystalline cellulose was dramatically faster than the case with TrCel7A alone (39) as shown in Figure 8.

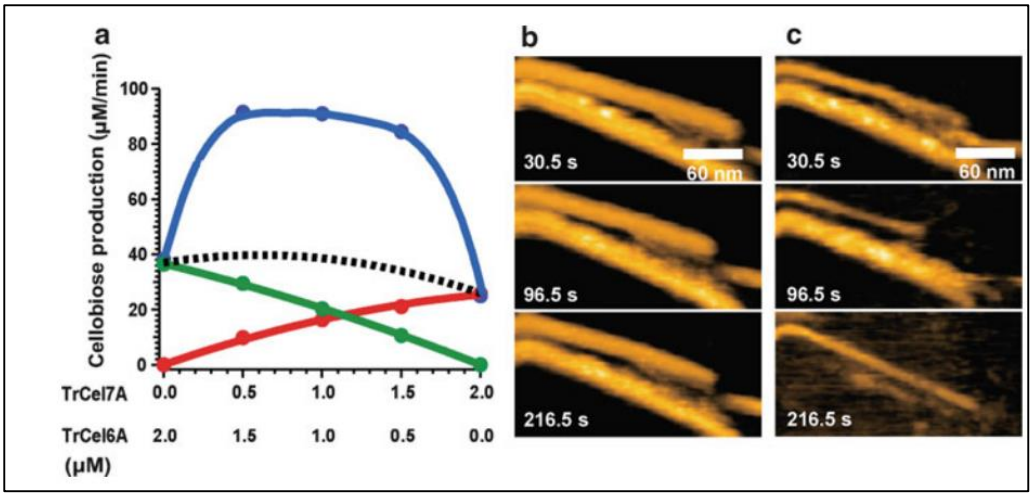

Figure 8. Synergy between TrCel7A and TrCel6A in cellobiose production from cellulose. TrCel6A activity alone is represented by the green line and TrCel7A activity alone is represented by the red line. The blue line represents the synergy between TrCel6A and TrCel7A when combined. The dotted line represents the sum of the cellobiose production of the green and red line combined. The AFM images represent injection of TrCel6A alone on cellulose (b) and the addition of TrCel7A on cellulose(c). The AFM images show the progression of the cellulases over time from 0-216.5 seconds and show a distinct difference between the enzymes acting alone on cellulose (b) and combined (c).

The order of the additions of enzymes did not change the synergistic outcome. No matter which cellulase was added secondly, either *Tr*Cel7A or *Tr*Cel6A, they were always more effective at breaking down cellulose together than apart.

Igarashi believes that *Tr*Cel7A and *Tr*Cel6A work together to relieve traffic jams in the cellulose (30) and that *Tr*Cel7A sharpens ends of the cellulose and latches on and covers more ground (30) (10). The term “traffic jams” describes a situation in which enzymes are stuck due to an obstacle in front of them and are unable to quickly dissociate from the substrate chain. To clarify, once an exocellulase on a processive run is stalled due to an obstacle on the cellulose chain, it then continues to block other exocellulases also. Somewhat like a car becoming stuck in a pot hole on a highway may back up traffic. But in this case, the highway is much smaller in width than the car (cellulase) and it results in the processive run of other exocellulases on nearby strands also becoming stalled. Obstacles, on the other hand, may be non-crystalline (amorphous) regions of cellulose. Igarashi *et al*. provided a visual support for this theory using HS-AFM (30). Whereas another theory states that they simply have different preference and don´t attack the same areas (37).

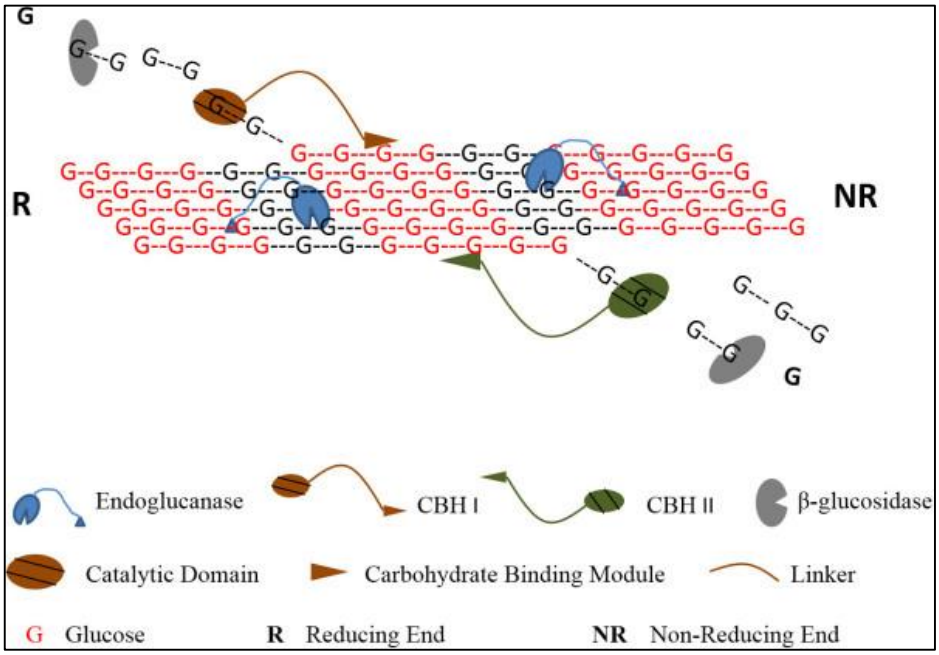

Figure 9. The traditional model of Exo-exo synergy (40)

Free enzyme and cellulosome synergy

In a study by Resch et. al., cellulosomes isolated from secretions by the bacterium *Clostridium thermocellum* were studied in combination with free enzymes from a Novozyme formulation known as Ctec2. Synergy is observed when biomass pretreated with clean fractionation (CF) was digested by a combination of cellulosomes and free enzymes. The mechanism is further investigated by the use of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to deduce a mechanism of action. The visual data from TEM shows a wrinkled texture and removal of fibrillated cellulose from the surface of treated samples when digested by free enzymes and the formation of delamination pockets and abrasions on the sample surface when digested by cellulosome mix. The combination of the two enzyme types shows a combination of the wrinkled and delamination/abrasion morphology. Similarly, the visual data from SEM show a combination of the two effects (10).

They use the synergy observed in substrate conversion and the visual data and propose a mechanism whereby the cellulosomes attach to deeper rows of cellulose fibre and splay them, whereas the free enzymes digest ends exposed both beneath and on the surface faces of cellulose crystallites (10) (see Figure 10).

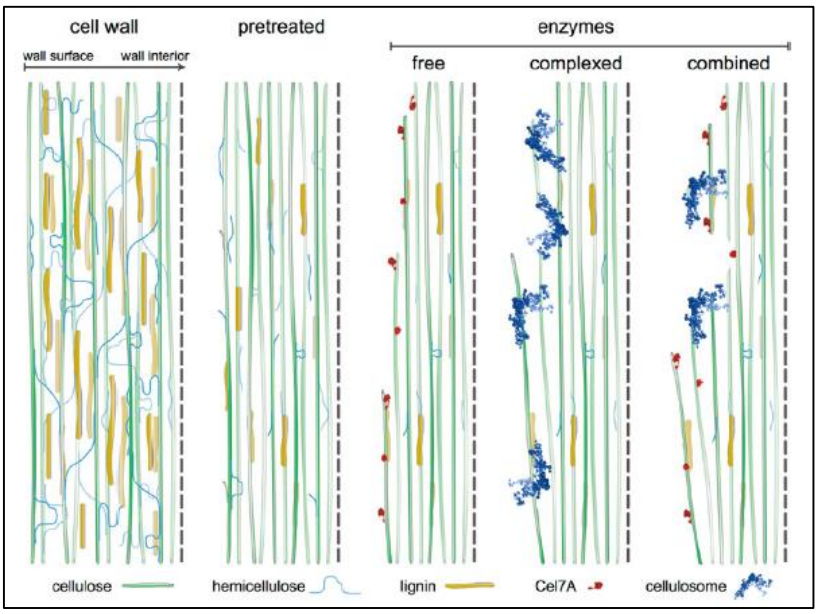

Figure 10. A model for the synergy observed between cellulosomes and free enzymes (10)

Another study by Another study by Brunecky et. al., used the enzyme CelA from the bacterium *Caldicellulosiruptor bescii* in combination with Ctec2 and observed synergy also. CelA employs what they call an 'intermediate' strategy as it is secreted as a free enzyme yet contains multiple catalytic domains. They also used visualization techniques to observe the degradative action of the combined enzymes, both free and semi-cellulosome. Observations in TEM include the formation of cavities. They go on to postulate that synergy is achieved through a mechanism more than the splaying and free enzyme degradation. As CelA attaches, it stays bound via its CBM and its catalytic domain continues to form a cavity through a long linker module (41) (see Figure 11).

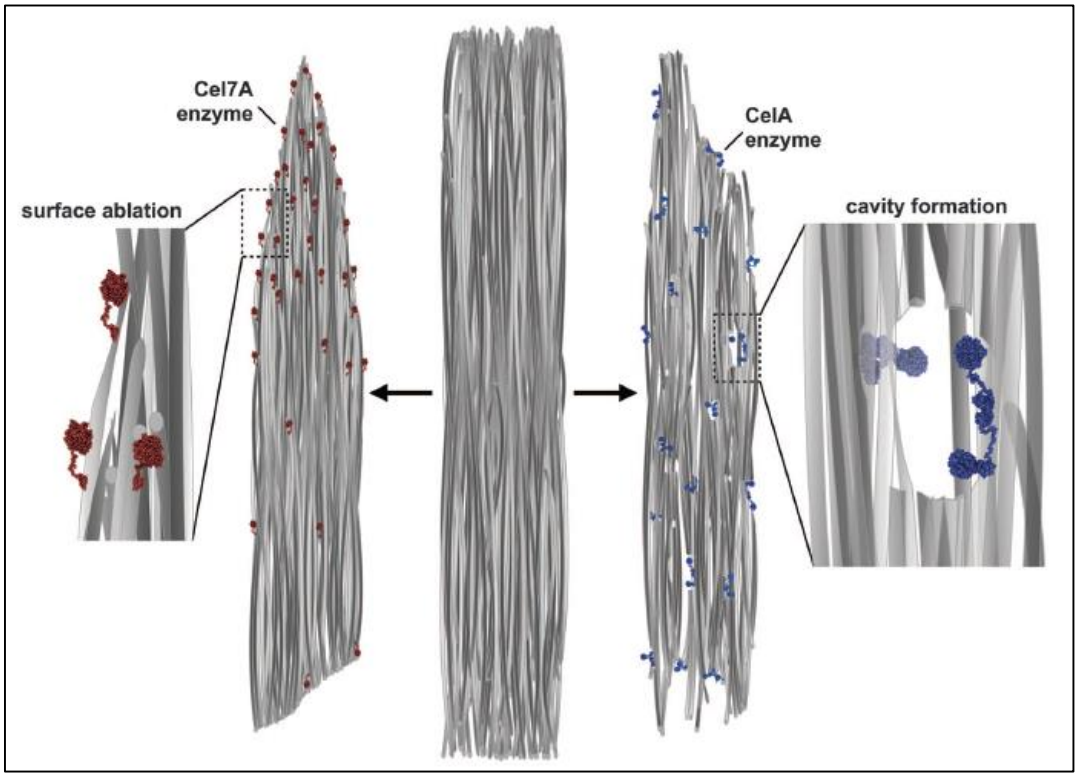

Figure 11. Another model for the synergy observed between cellulosomes and free enzymes (41)

5. Discussion

Most of the mechanisms of synergy between the various enzymes secreted by *T. reesei* are well established yet remain a subject of discussion because it is possible that a full understanding of synergistic contribution has not been elucidated that accounts for all synergy observed. The mechanisms of synergy are of particular interest, as better understanding of how enzymes synergise at a molecular level can lead to application of said information to formulating more effective enzyme cocktails for industrial scale biomass degradation.

The character of cellulolytic enzymes mentioned in this report is usually derived from experiments which quantify a parameter such as apparent processivity or probability of endo-mode initiation. Some research shows that these parameters overlap for endocellulases and exocellulases (19). For example, Kurasin *et al.* imply that both exocellulases and endocellulases possess processive ability and that exocellulases also show a surprisingly high probability of endo-mode initiation (19). Therefore, the aforementioned mechanisms of synergy have been organised according to association, dissociation and micro versus macro scale. Micro scale describes the intermolecular mechanisms between cellulases and cellulose and between cellulases themselves that create synergy. The term “macro” is referring to the overall physical conditions that contribute to the synergy created by breakdown of cellulose by cellulases, without specifying the molecular mechanisms.

Synergy based on dissociation from the cellulose chain is demonstrated through Endo-exo synergy. There are several extensions to the classical theory of synergy between endocellulases and cellobiohydrolases (see “The Endo-exo synergy model”). The theory that endocellulases cleave the substrate at points where there is amorphous cellulose or tangles and obstacles is described well. Whereas, *Tr*Cel7A has a dissociation site and is therefore not blocked on the substrate chain, though existing research does not provide a complete explanation of observed synergy (9). A theory postulating that endocellulases can cut the substrates into smaller, shorter pieces making it less possible for *Tr*Cel7A to become trapped in obstacles could provide one explanation of why such high synergy is observed (30).

Another example of dissociation according to Igarashi *et al.* is that endocellulases relieve “traffic jams” on substrates (see “The Exo-exo synergy model”), therefore, alleviating

obstacles and enabling further action of *Tr*Cel7A (30). This leads to a thought that shorter chains could be created as such points before obstacles are cleaved. Hence, Valjamae and his team´s theory, stating that enzymes acting on cellulose leave solitary chains and craters in the cellulose structure which become obstacles for *Tr*Cel7A, helps us understand the processes occurring further (29). They go on to say that endocellulases can attack those shorter chains and enable further action of *Tr*Cel7A. There are many factors that contribute to the synergism between cellulases based on the enzymes' ability to dissociate from the cellulose chain, therefore, we hypothesize that Endo-exo synergism is grossly over simplified and is intertwined and works in parallel with Exo-exo synergism.

We postulate that the reason most studies don't fully account for observed synergism is because they only use a single mechanism to describe the synergy. As shown in Figure 12, two or more of the various mechanisms may be occurring simultaneously. The enzymes involved do not always follow strict definitions of their catalytic nature, for example an enzyme changing it's typical or primary function based on an increase in competition. This increase in competition may be achieved through increasing the enzyme load of one enzyme in ratio to another.

In contrary to dissociation, association based synergy can be exemplified by Exo-exo synergism. Traditionally, Exo-exo synergism between *Tr*Cel7A and *Tr*Cel6A has led researchers to believe that these cellulases solely attack cellulose at the ends of the chains. Few people have debated this concept, Barry *et al*. believe that exocellulases can actually possess endocellulase activity (35). They proposed that Exo-exo synergy, in fact, does not even exist without Endo-exo synergy and that competition between *Tr*Cel7B, an endocellulase, and *Tr*Cel7A drives *Tr*Cel6A to act as an endocellulase. *Tr*Cel6A´s more flexible tunnel-forming loops that impact processivity, making it less processive than *Tr*Cel7A and therefore gives it a partial endo-like characteristic (35). Uchihashi *et al*. have suggested that when *Tr*Cel6A and *Tr*Cel7A are mixed together with cellulose, their synergy is accounting for *Tr*Cel6A´s ability to change its exo-acting behaviour and nick at the middle of the crystalline cellulose chains, acting as an endocellulase, therefore making more attack points for *Tr*Cel7A (39).

Although *Tr*Cel6A has endo-activity, it is not as efficient as a traditional endocellulase, but it attacks less randomly than a traditional endocellulase (42). Barry *et al.* also believe that the competition between *Tr*Cel7A and *Tr*Cel7B drives a switch from exo to endo activity in

*Tr*Cel6A while others believe that the amorphous cellulose limits processivity of *Tr*Cel7A and therefore *Tr*Cel6A must use its endo activity to alleviate obstacles.

It is also stated that the exo *Tr*Cel6A forms a complex with only the non-reducing ends of the glucan chains, while the endo *Tr*Cel6A is able to form a complex with interior regions at the same rate as with chain ends (35). *Tr*Cel6A is furthermore assumed to adopt a relatively high dissociation rate because of its flexible tunnel loops of TrCel6A that are expected to occasionally open to form a cleft like topology that resembles those of endocellulases, showing that sometimes dissociation and association combine to play a part in apparent synergy.

Since association to the cellulose chain allows an enzyme to be processive, processivity can be an interesting area to speculate about because it plays an important role in the way exocellulases degrade cellulose chains. Endocellulases from the bacterium *Sacchagus degradans*, can be pseudo-processive and possess some similarities to those from *T. Reesei*. We propose a novel mechanism of synergy where endocellulases may also be taking on a processive role in cellulose hydrolysis. It is termed pseudo because even though the enzyme does dissociate after the hydrolytic event, it may not diffuse away and continues to cleave bonds on the same chain. As mentioned, Kurasin *et al.* show the apparent processivity of endocellulases (19).

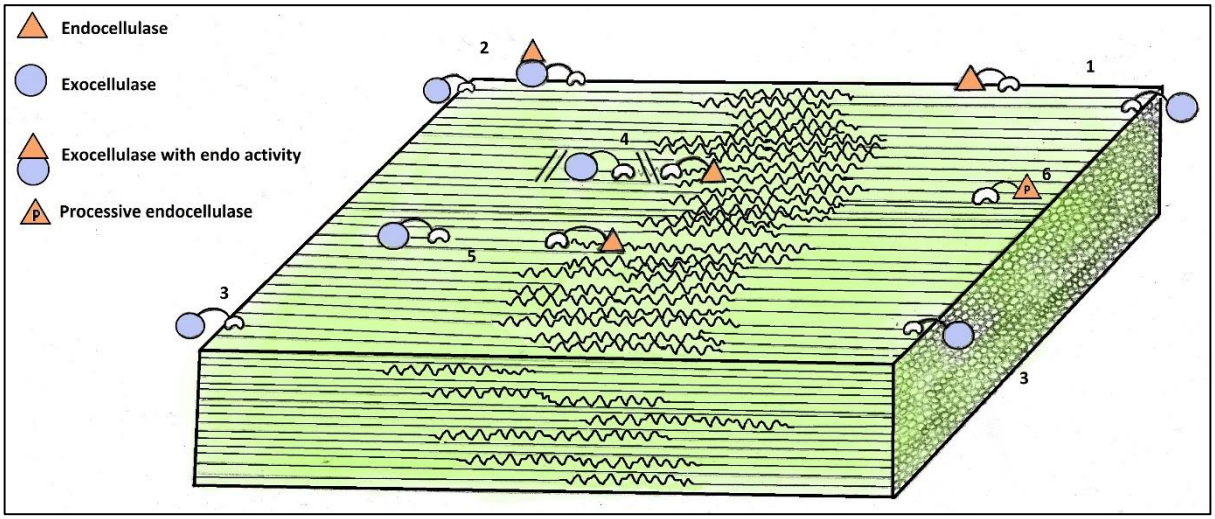

Figure 12. A summary of the various theories of synergy between cellulases. 1) Synergy observed between endocellulases and exocellulases when the endocellulases are making new attack points at amorphous regions for exocellulases. 2) Synergy observed between exocellulases and exocellulases exhibiting endo activity driven by competition between exocellulases for end attack points. 3) Synergy observed between two kinds of exocellulases attacking from reducing and non-reducing end of cellulose chains. 4) Synergy observed when endocellulases cleave amorphous regions or regions with any other obstructions to allow stuck exocellulases to dissociate and begin a processive run elsewhere. 5) Synergy observed when endocellulases cleave before exocellulases already on a processive run become unproductively stalled. 6) Synergy observed when an endocellulase has a pseudo-processive nature.

As mentioned, processivity entails *T. reesei* enzymes´ ability to latch on and cleave cellobiose units from a cellulose chain in sequence, and without dissociating from the cellulose molecule (21). Therefore, it is possible that an enzyme having a highly processive run may remain on a cellulose chain for a longer period of time cleaving more cellobiose units than if it dissociated or stalled quickly. Whereas, if a processive enzyme is stuck or stalled on the cellulose chain, it will decrease synergy. Additionally, processivity may be affected by temperature since temperature can modify an enzyme's catalytic ability (43). In conclusion, a more processive enzyme may contribute more to the amount of synergy observed between various enzymes. Again, association and dissociation seem to be overlapping in the mechanisms of synergy related to processivity.

Besides the micro or molecular scale of synergy, it can also be observed on a macro scale. Though this paper is discussing synergy between fungal cellulases, macro scale synergy is occurring in the mechanisms of synergy occurring between *T. reesei* free cellulases and cellulosmes. Some of the highest reported synergy has been observed on cellulose degradation between free enzymes and cellulosomes, this is a puzzling level of synergy making it interesting to postulate on its mechanism (10).

The study by Brunecky *et al*., using both visual and chemical analyses, postulated that the bacterium *C. bescii* secretes an enzyme CelA, with several CDs and CBMs, that creates cavities in the cellulose microfibrils (41). They go on to postulate a mechanism of synergy whereby the additional creation of cavities contributes to synergistic effect. Whereas Resch *et al.* used similar ways to quantify and observe mechanism of synergy (TEM), they mention the splaying of cellulose microfibrils by cellulosomes of *C. thermocellum* as the reason behind observed synergy (10). On observation of both the studies' TEMs it is interesting to note that the TEM of fibrils treated with CelA and *T. reesei* enzymes shows pockets forming from what they postulate are conical cavities being dug into the cellulose micro fibril and the attached excavation flaps. Yet the TEM from the Resch study also shows a similar flap and pocket formation (10). It may be possible that the synergy observed in the study by Resch *et al.* might have a cavity component to it also. Additionally, the cellulosomes expose more ends by the creations of these conical cavities to which free enzymes can adhere to and splay cellulose fibres.

Addditionally, the macro scale of synergy between enzymes on cellulose may have a component of facilitation at a higher morphological hierarchy. The study by Josefsson *et al.*

investigating the effects of swelling makes a compelling case for swelling being perceived as a component of synergy. According to it, swelling occurs when cellulases are added to the substrate and the binding of these enzymes facilitates a swelling of cellulose chains (36). They used cellulose films to demonstrate this swelling phenomenon and proposed that not only do endocellulases create new chain ends but they also work on a different level of cellulose morphology to increase synergy. As they cause swelling of cellulose, they increase the availability of new sites for creating end attack points (36).

Another macro scale contribution to synergy may be the nature of the substrate itself. According to a study by Ganner *et al.* (44), the mechanisms of synergy between cellulases are dependent on the morphology of the substrate to begin with. They propose a model wherein *Tr*Cel7B and *Tr*Cel6A are much more efficient at degrading amorphous cellulose, in contrast to *Tr*Cel7A (44). *Tr*Cel7A, on the other hand, is most proficient at degrading crystalline cellulose in the pretreated substrate. Their theory postulates that the mechanisms by which these enzymes synergise are highly influenced by the size of substrate particles and how tightly they are packed together. Such a possibility gives us an insight into why the way a substrate is pretreated before it is subjected to enzymatic degradation shows such an effect on the observed synergy.

All the enzymes and their synergistic mechanisms mentioned may also be affected by the experimental parameters under which the enzymatic processes are occurring. Factors like enzyme load or concentration, thermostability and pH of the cellulose may affect the synergy between enzymes.

For example, Andreaus *et al.*'s study shows that though binding ability (association) increases with temperature, overall activity of cellulases actually decreases (43). In their experiment, *T. reesei´s* activity and association ability were measured in respect to temperature. As temperature increased from 37-50°C association decreased slightly, but then continued to increase as temperature rose, whereas the enzymes´ activity plummeted after 50°C and eventually became inactive at 80°C (43) (see Figure 13).

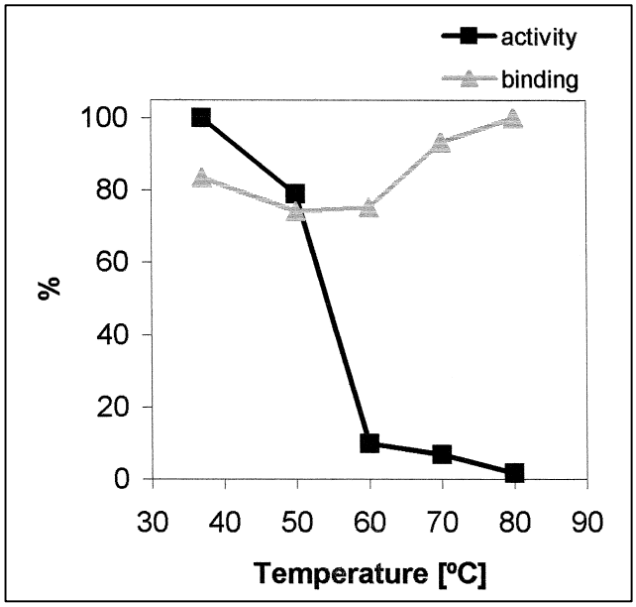

Figure 13. shows the association ability and activity of T. reesei on cotton after incubation with increasing temperature from 37-80°C (43)

In another experiment conducted by Anderaus *et al.,* they observed the correlation between increasing pH and cellulase´s ability to dissociate. Dissociation was measured in a mixture of *T. reesei* enzymes on cotton. The cellulases that were initially associated on the cotton, experienced a 10% increase in dissociation as pH rose from 5 to 10 (43) (see Figure 14).

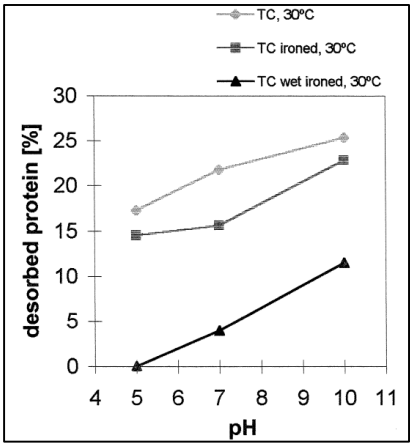

Figure 14. This figure shows the dissociation (desorption) ability in % of initially associated T.reesei on cotton with increasing pH. (Effects of temperature on cellulases binding ability (43)

Macro scale conditions affect micro scale synergy by changing the enzymes´ ability to associate and dissociate to the cellulose based on the examples mentioned above. Therefore, experimental parameters have an impact on the molecular mechanisms of synergy on a micro scale.

Another macro scale parameter is loading concentration. The highest apparent *Tr*Cel6A-*Tr*Cel7A synergy is when the loading concentrations are equal (28). On the other hand, when there are different enzyme concentrations in an experiment, it drives the traditionally exocellulase to make slight changes and exhibit endocellulase activity (30) (35). This could be due to the availability of good exo-sites for *Tr*Cel6A being occupied, forcing the exocellulase to attack its second choice, the inner parts of the crystalline cellulose, hence, the endo site. When there is an excess of enzymes, attacking the ends of celluloses, more ends are taken up, and instead competing with each other, the enzymes simply, take their second choice, attacking the inner strands (30) (35).

An experiment conducted at Roskilde University, using carboxymethyl cellulose (CMC) as an endo-activity indicator, revealed slight endo activity of both *Tr*Cel6A and *Tr*Cel7A (45). Ionic substituted CMC is an endo-activity indicator because of its high water solubility. Endocellulases cleave intermolecular β-1,4-glucosidic bonds, resulting in a drastic reduction in the DP of CMC, meaning that if an endocellulase is present in the mixture, CMC will be dissolved quickly, whereas if there are only exocellulases, CMC will remain in the mixture (46). In Figure 15, the results of the CMC experiment are shown. There is a noticeable difference between *Tr*Cel7A and *Tr*Cel6A, as endo-activity is higher in *Tr*Cel6A, which agrees with some of the published findings mentioned earlier. Therefore, it may be possible for exocellulases to become endo acting under some circumstances and erode obstacles to expose crystalline areas.

In the Roskilde University experiment, they used 1μM loads for the enzymes (45) (see Figure 15). It is observed that equal amounts of low concentrations of the *Tr*Cel6A and *Tr*Cel7A, show Exo-exo synergy but as you increase the loads or concentration of the enzymes in laboratory experiments, as mentioned previously, the exocellulases begin to compete for ends on the cellulose and make conformational changes to attack inner chains instead. The amount of the enzyme added to the mixture can have huge effects of the synergy and change the types of synergy observed (28). It has also been observed that when you alter the concentrations, not just simply adding more. This shows that a factor like concentration may be affecting association based synergy also.

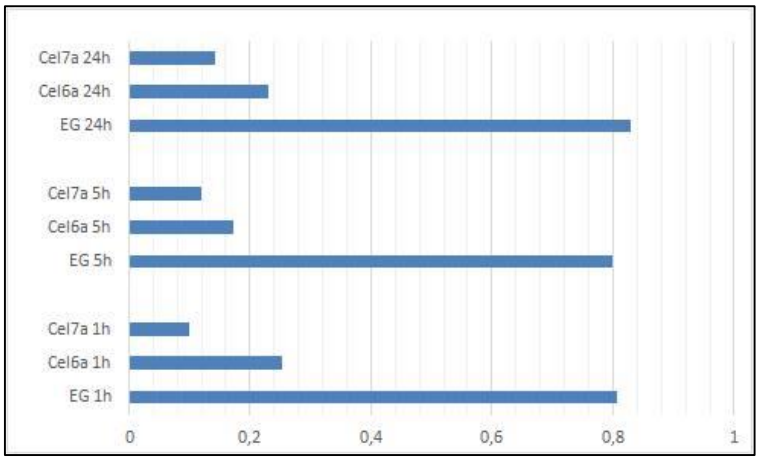

Figure 15. Experiment carried out at Roskilde university (2015) to show endo activity of TrCel6A, TrCel7A and an endoglucanase. Physical conditions: 25°C, 1µM TrCel7A and TrCel6A, 4g/L CMC, EG as positive control, time incriments of 1hour, 5 hours, and 24hours (45).

By comparing two different substrates, lignocellulose and Avicel cellulose under different conditions, we can further observe macro scale affecting the micro scale synergy. In a study conducted by Zheng *et al*., mixtures of cellulases were combined with two types of cellulose and adsorption was observed at different temperatures (47). Cellulases' association abilities were 15-fold lower than those on the microcrystalline Avicel cellulose at 4°C. Furthermore, the association rates appeared to be very different for lignin and cellulose as temperature increased. Zheng *et al*. postulated that the difference could be attributed to the selectivity of the CBD of the cellulases. At 50°C, the cellulases associated to cellulose rapidly at a peak after just 15 minutes, which followed by a quick dissociation, presumably caused by the initiation of hydrolysis of the solid substrate. Whereas, lignocellulose demonstrated a delayed association of cellulases at 50°C. It took over 12 hours for the cellulases to react and reach an association equilibrium (47). The sluggish association on lignocellulose could be trigged by the elevated temperature and the porous structure of the substrate. This is an example of macro scale changes affecting micro scale synergy and the enzymes preference of substrate and its ability to associate and dissociate (47).

In conclusion, there are far more molecular mechanisms between cellulases that contribute to the amount of synergy that is being observed. There are no strict boundaries between Endo-exo, Exo-exo, and the processive nature of *T. reesei* cellulases. Association and dissociation of cellulases can help in understanding not only molecular scale synergy but also macro scale synergy observed in studies. These enzymes have a flexible nature regarding several aspects. For example, the ability of these enzymes to switch specificity according to competition and / or availability of substrate (and substrate nature) or their slightly different

preference for attack sites. This fundamental change from the traditional approach is exemplified by *Tr*Cel6A's ability to switch roles from exo to endo and create nicks in crystalline cellulose, exposing new attack points for other exocellulases, contributing to high synergy. Furthermore, cellulosomes have a puzzlingly high synergy with free enzymes, that surpasses all other mixtures and maybe due to the high number of end creations and new attack points generated. Shifting experimental parameters may lead to an even better understanding of the unknown mechanisms of enzymes contributing to synergy.

Future perspective

To further understand synergistic mechanisms, experimental designs should include a variation of the parameters mentioned in the discussion. For example, the endo activity of *Tr*Cel6A could be tested with different parameters including: a CMC experiment with low and high concentrations of *Tr*Cel6A and *Tr*Cel7A at 1:1, altering their concentrations between *2:8,3:7 etc*. Moreover, for each ratio of the enzymes, temperature would be varied by 5°C in the interval from 25-80°C in the distinct experiments. Another parameter that can be varied is the pH. The next set of experiments should be conducted with pH varying from 3-8 by 1 unit. Those experiments would help in finding optimal environment for the synergy of *Tr*Cel6A and *Tr*Cel7A. Testing different substrates from high to low CrI in previously found optimal conditions, could possibly elucidate which substrate is the most efficiently degraded by the mixture of the enzymes and thus, is the best substrate for the bioethanol production.

Once the optimal experimental parameters for the highest observed synergy are determined, the mechanisms of synergy can be investigated.

HS-AFM (high-speed atomic force microscopy) imagining could monitor molecular mechanisms of TrCel7A and TrCel6A by recording the thinning of the cellulose, using CMC as an endocellulase indicator. If the cellulose images reveal a thinning at outer ends of the cellulose, exocellase activity is apparent, if there is thinning of the cellulose in the middle regions as well, it would suggest one of the two enzymes would be acting as an endocellulase. What is more, after performing all those experiments, we could compare the obtained results with the results from the literature.

Cellulosomes and free enzyme experiments similar to those conducted by Ganner *et al.* could reveal the mechanisms through which such high synergy is achieved when these two enzyme paradigms are combined (44). Admittedly, applying new technologies, like HS-AFM,

could greatly enhance the knowledge of the process of hydrolysis and synergy in cellulases by providing the figures which present the movement of the enzymes on the substrate chain. Therefore, we propose to conduct a hydrolysis of cellulose while inspecting the process under HS-AFM to elucidate the mechanism of this process. As shown by Igarashi *et al*., the movement of *Tr*Cel7A on cellulose chain is visible in details (time-resolution- 300ms/frame) (30).

Glossary

Amorphous regions of cellulose: are regions of cellulose which are perceived as stumbling blocks for the processive activity of cellulases on the cellulose chain; are less ordered sections of cellulose

Association constant (K): is a special case of equilibrium constant; it is associated by the reactions of association and dissociation of cellulases on cellulose chain and is represented by subsequent rates which contribute to the formula for association constant; K is defined by association rate divided by dissociation rate

Biofuel: is a fuel that is produced through some contemporary biological processes, such as agriculture and anaerobic digestion; one of the reasons for advanced research of synergy in cellulases is a production of biofuels by fermentation of glucose from hydrolysis of cellulose;

Biomass recalcitrance: is a natural resistance of plant cell walls to microbial and enzymatic deconstruction

Carbon cycle: is a cycle in which carbon is exchanged between all the spheres on the earth; it describes the movement of carbon as it is recycled or reused throughout the biosphere; it includes plant respiration

Catalytic domain (CD): a part of complex molecular architecture of cellulases; it is a tunnel shape for processive exo- degradation and in endocellulases, it has a cleft shape for end attack degradation; it is N-glycosylated; is responsible for both initiating hydrolysis and the processivity of the protein and it cleaves the glycosidic bonds in cellulose

Cellobiose: is a disaccharide; it's a reducing sugar composed of two β-glucose units linked by β-1,4 glycosidic bonds; is hydrolyzed to two single glucoses by beta-glucosidase

Cellulose binding domain (CBM): part of complex molecular architecture of cellulases; it facilitates hydrolysis with the substrate; it is O-glycosylated and contains 30-200 amino acids; it's a single, double or triple domain in enzymes; it authorizes the latching on of the protein to substrate and increases the rate of hydrolysis;

Cellulosome paradigm: system in some anaerobic bacteria wherein enzymes are organized into large extracellular macromolecular complexes

CMC: carboxymethylcellulose; endoactivity indicator

Crystalline cellulose: is an ordered section of cellulose chain; one form of crystalline cellulose known as cellulose I is most abundant in biomass and can be found in two forms- cellulose Iα and Iβ, which differ in their hydrogen binding pattern

Crystallinity index (CrI): the amount of crystalline versus amorphous cellulose in a sample

Decomplexation rate: same as dissociation rate

Degree of polymerization (DP): a way of defining the relative abundance of terminal and interior β-glycosidic bonds available for digestion by endo- or exoglucanases; it is the measurement of the number of monomeric units in a polymer

Degree of synergy (DS): the ratio of the activity of synergistic mixture to the sum of the activities of individual components; synergy is observed when the DS value is bigger than 1

Free enzyme paradigm: concept in which fungi secrete various enzymes into solution which then diffuse towards the substrate

Glycosidic bonds: a covalent bond between a carbohydrate and another group; D-glucose units are linked by β-1-4 glycosidic bonds

Holocellulose: a mixture of hemicellulose and cellulose in wood, fibrous residues that remains after extractions

Hydrolysis: cleavage of the bonds by addition of water molecule

Microfibril: is a very thin fiber, consisting mostly of glycoproteins and cellulose

Mutagenesis (chemical): a process by which a genetic information of an organism is changed, a results of this change is a mutation; a causative agent of mutagenesis is called a mutagen

Obstacle free path: a length of crystalline cellulose lacking amorphous areas which can act as obstacles to a processive enzyme

Polymer: a macromolecule, composed of many repetitive units

Pretreatment: is classically defined as large-scale industrial processes to break down biomass before subjecting it to further degradation; in this report, we are redefining pretreatment as all techniques of preparing substrate before enzymatic digestion;

Processive run: is continuous run of a cellobiohydrolase on the cellulose chain without dissociating from this chain

Processivity: ability to produce several single molecules of disaccharides out from the polymer crystal without dissociating from the enzyme in between the subsequent hydrolytic steps

Reducing/ non-reducing ends: two ends of crystalline cellulose chain which are attach points for TrCel7a and TrCel6a, respectively; reducing end of a saccharide is not involved in glycoside bond forming and thus, capable of converting to the open-chain form while non-reducing end is incapable of that conversion

Splaying: spreading out, widening, broadening

Synergy: is defined as the sum of the overall hydrolysis of the mixture of enzymes being greater than the sum of degrees of hydrolysis of the individual enzymes;

Traffic jams: they happen when enzymes are stuck due to an obstacle in front of them, probably because of the surface heterogeneity, and are unable to quickly dissociate from the substrate chain

Xylosidic bonds: bonds linking the xylose units on xylan polysaccharide backbone

Specific surface area: is a property of cellulotic substrate, which is total surface area by mass (m^2/g); it's value corresponds to some of the features of the substrate as sorption or weight gain by swelling; a gross cellulose accessibility

Cellulose III: the result of ammonia supercritical treatment of cellulose I

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