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Mini Review

End-to-end gene fusions and their impact on the production of multifunctional biomass degrading enzymes

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ABSTRACT

The reduction of fossil fuels, coupled with its increase in price, has made the search for alternative energy resources more plausible. One of the topics gaining fast interest is the utilization of lignocellulose, the main component of plants. Its primary constituents, cellulose and hemicellulose, can be degraded by a series of enzymes present in microorganisms, into simple sugars, later used for bioethanol production. Thermophilic bacteria have proven to be an interesting source of enzymes required for hydrolysis since they can withstand high and denaturing temperatures, which are usually required for processes involving biomass degradation. However, the cost associated with the whole enzymatic process is staggering. A solution for cost effective and highly active production is through the construction of multifunctional enzyme complexes harboring the function of more than one enzyme needed for the hydrolysis process. There are various strategies for the degradation of complex biomass ranging from the regulation of the enzymes involved, to cellulosomes, and proteins harboring more than one enzymatic activity. In this review, the construction of multifunctional biomass degrading enzymes through end-to-end gene fusions, and its impact on production and activity by choosing the enzymes and linkers is assessed.

1. Introduction

The decline of fossil fuels, coupled with the increasing demand for energy, has shifted worldwide attention to alternative energy sources [1–3]. 70–80% of biomass is composed of cellulose and hemicellulose, which can serve as both an inexpensive and abundant source of biofuel producing sugars [4]. Their respective hydrolysis into simple sugars is catalyzed by cellulolytic and hemicellulolytic enzymes [5]. These are more accessible to substrates at high temperatures. Moreover, industrial processes are usually carried out under extremely harsh conditions, thus allowing extremophilic microorganisms to offer themselves as more suitable candidates compared to conventional ones [6]. However, this process still faces major issues, especially with the high cost of enzyme production, given the fact that a synergistic action of multiple enzymes is required for the complete hydrolysis of lignocellulosic biomass [7,8]. An interesting approach is minimizing the size of polypeptides comprising the enzymes required in the reaction mixture [9]. A series of different enzymes with varying functions degrade cellulose and hemicellulose gradually into the fermentable sugars, glucose and xylose, during enzymatic hydrolysis [10]. This cocktail of enzymes includes exoglucanases, endoglucanases, β-glucosidases, xylanases, and other necessary biocatalysts [11] (Fig. 1).

Cellulose comprises 30–60% of lignocellulose making it the most abundant organic biopolymer in nature [7,12]. It is an unbranched homopolymer which consists of glucose units joined by β-1,4-glycosidic bonds. These are connected with amorphous regions and usually exist in a crystalline form [13]. The synergistic action of three distinct enzymes, endoglucanases, exoglucanases, and β-glucosidases is necessary for the degradation of cellulose [6]. Endoglucanases prefer amorphous cellulosic regions. They randomly cleave β-1,4-glycosidic bonds in internal sites of the cellulosic polysaccharide chains, resulting in new oligosaccharide units of varying lengths [14]. Endoglucanases from thermophilic archaea and bacteria have been found to belong to the glycoside hydrolase families 5, 6, 8, 9, 12, 48, 51, and 74 [6]. Exoglucanases, also known as β-1,4-D-glucan-cellobiohydrolases or cellobiohydrolases, produce cellobiose by cleaving chain ends of cellulose. Exoglucanases belong to glycosyl hydrolase families 5, 6, 9, and 48. However, only few thermophilic bacteria have been shown to be able to produce these [15]. β-Glucosidases are involved in the conversion of cellobiose to glucose. Since the buildup of cellobiose can lead to the inhibition of the catalytic activity of endo- and exoglucanase, β-glucosidases play a critical role in the inhibition of this rate-limiting step, especially those that also confer resistance to the glucose inhibition factor [16]. β-Glucosidases belong to the glycoside hydrolase families 1, 3, 9, 30, and 116 (Fig. 1A).

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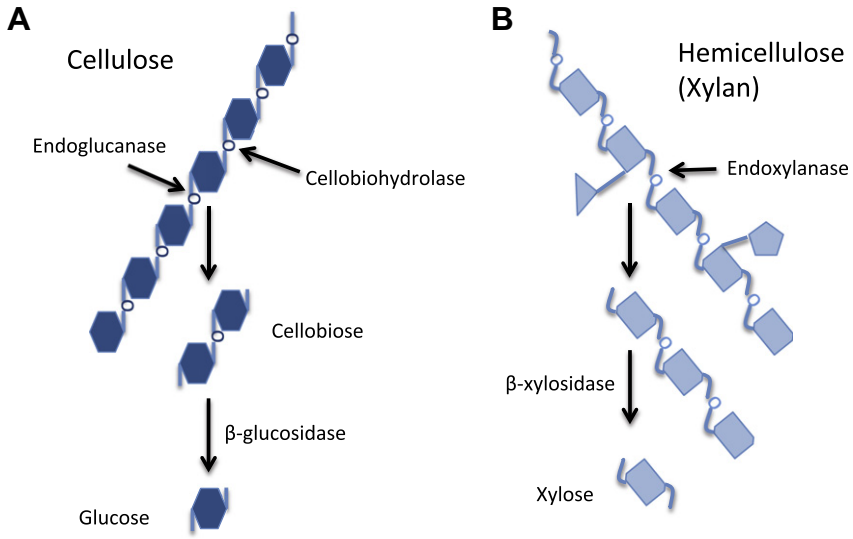

Fig. 1. Procedural mechanistic action of cellulases and xylanases on cellulose and hemicellulose, respectively. (A) Hydrolysis of the individual cellulose fibers to break them into smaller sugars by synergistic action of endoglucanase and cellobiohydrolase. β-Glucosidase hydrolyzes cellobiose into glucose. (B) Endoxylanase hydrolyzes complex hemicellulose into smaller oligosaccharides. Finally, β-xylosidase releases xylose from the non-reducing end of xylan-oligosaccharides.

Besides cellulose, hemicellulose represents the second most abundant polysaccharide in nature. It comprises 20–40% of lignocellulosic biomass [12]. The major component of hemicellulose is xylan. It is a polysaccharide consisting of a backbone of xylopyranosyl units linked by β-1,4-glycosidic bonds [6]. Xylanases belong to the glycosyl hydrolase families 8, 10, 11, and 43. It is the primary enzyme of attack, degrading internal β-1,4-xylan into xylose. β-Xylosidases in turn, remove these alternating xylose residues. The hydrolysis of xylobiose and other oligosaccharides from non-reducing termini is accompanied by the action of enzymes such as acetyl xylan esterase, α-arabinofuranosidase, and α-glucuronidase (Fig. 1B).

2. Generation of fusion enzymes

Due to this complex structure of cellulose and hemicellulose, and the need of several enzymes for their complete hydrolysis, nature has invented multiple solutions for lignocellulosic degradation. One is cellulosomes, produced by anaerobic bacteria. These are extracellular multienzyme complexes considered as natural and multifunctional [17]. They are located on the scaffoldin, the core protein. The latter in turn contains carbohydrate binding modules (CBM), non-catalytic domains, and cohesion domains [18]. Others include enzymes with a broad specificity to substrates and loose catalytic centers, and single polypeptides that harbor domains with multiple functionalities [19,20].

Through evolution, enzymes that have tightly bound functions have paved the way for the rise of bifunctional enzymes [21]. Primitive precursors are the most probable base of evolution for these complexes. Metabolically, assembling separate genes coding for enzymes that have similar or related functionalities is considered advantageous since these open reading frames are controlled by common regulatory elements. Advantages at the protein level can also be witnessed. Catalytic processes occurring in one enzyme can now have a direct effect on the remaining enzymes present in the complex [22].

Thus, a proposed feasible method for reducing the number of enzymes required for biomass conversion in industry is the engineering of multifunctional enzymes that can catalyze more than one reaction [23]. The alteration of existing proteins has been the main focus to biocatalyst designs. In this way, one takes advantage of properties already existing in enzymes and subsequently constructs chimeras [24]. The strategies used by nature to evolve enzymes, are mimicked by the construction of hybrid or chimeric ones using molecular biology techniques.

2.1. Fusion

Gene fusion technology has become an attractive, widely used, tool in biotechnology. More than one technique is followed for gene fusion. However, these usually fall into one of two categories. The first is termed insertional, where one domain is incorporated in-frame through the middle of the other domain [25]. Usually flexible, and exposed, the N- and C-termini of one protein are fused to internal segments of another protein via two ligation sites, resulting in rigid structures, which are more stable and resistant to proteolytic cleavage [26–28]. This is due to the fact that the domains are connected via one or two linkers, which would decrease denaturation by prohibiting undesired folding [25].

The second, end-to-end fusion, allows the N-terminal to C-terminal linkage of two different domains. Its advantage in comparison to insertional fusion is the fact that it is not strictly dependent on protein conformations, structures, and folding. This makes it an easy and simple process, and therefore often used in a wide variety of scientific fields including biological screening, monitoring gene expression, recombinant protein purification, displaying cell surface proteins, protein folding, protein regulation, metabolic engineering, and protein localization [22,29–31].

With the advancement of technology, Overlap Extension PCR (OE-PCR) is now the backbone of gene fusions, allowing not only exact DNA manipulation, but also modification of the ends of DNA fragments for further processes [32,33]. The construction of fusion genes using the end-to-end method usually requires a set of primers for each gene. The gene at the 5′-end is amplified using a forward primer containing a cleavage site of the restriction enzyme to be chosen. This is required for further ligation in the desired vector. The reverse primer, on the other hand, introduces a complementary region of the second gene of the construct. The primers of the second gene are generated in the opposite fashion, with the reverse primer having a restriction enzyme cleavage site

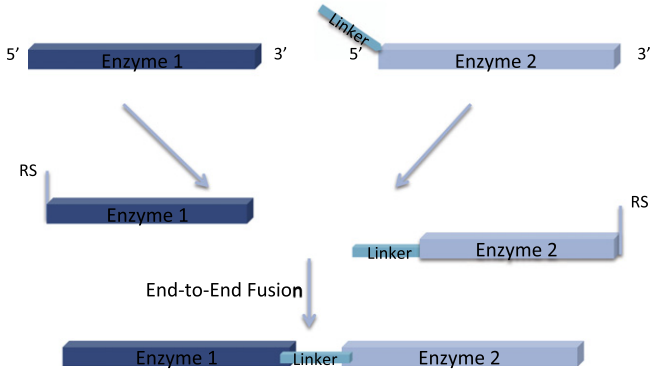

Fig. 2. Schematic representation of an end-to-end gene fusion approach. PCR is used for the incorporation of a restriction site (RS) at the 5′-end of gene encoding Enzyme 1, as well as a restriction site at the 3′-end of the fusion partner. In addition, both genes are fused by PCR and connected via a linker sequence.

and the forward having a complementary region to the first gene. OE-PCR allows any DNA elements to be joined freely, and at any location, independent of restriction sites (Fig. 2).

When OE-PCR is hindered by the size of the genes that should be fused, two-step ligation experiments can be carried out. The main disadvantage is the amino acid scars that are left because of Type II restriction sites. However, this can be circumvented with cohesive ends for genetic assembly through the use of Type IIS restriction enzymes [34,35].

However, the close proximity of proteins to each other might sometimes result in unfavored folding as well, resulting in loss of activity of one or both catalytic domains. A way of altering this is by the addition of linker sequences that allow for extended conformation and stability [36]. The job of linkers is to guarantee independent actions of functional modules present alongside each other. They can be introduced during cloning reactions and OE-PCR. Subsequently, they can be embedded by traditional cloning procedures or by inverse PCR, respectively. Kim et al. [37] were able to construct 64 different chimeras in a high-throughput fashion by overlap PCR, with a linker region of 177 bp serving as an overlapping part.

2.2. Linkers

Not only are linkers simple covalent connectors, they also establish communication between different modules existing within proteins; thus giving promising insight into protein–protein interactions [38]. Linker sequences are often required when constructing functional fusion proteins. They allow for maximal flexibility by adopting extended conformation [39]. In increasing order, Gln, Glu, Thr, Phe, Arg, and Pro are the most preferred amino acids found in linkers [40]. In contrast to previous studies, Ala was shown to have no linker preference, probably due to its high occurrence in proteins. The fact that proline does not have an amide hydrogen to donate through hydrogen bonding, is a cause for isolating the linker structurally from the domain. This in turn, favors the presence of proline in linkers. Shen et al. [41] described that proline rich linker sequences have rigid, extended conformations, while polyserine linkers are more flexible. Howard et al. [42] concluded that glycine residues flank these sequences, the majority of which is found at their start or terminus. With linkers being targets for protease degradation, their rigidity plays a key factor in avoiding proteolytic cleavage [43].

Linker-rich prolines and hydroxyamines are usually found in microbial cellulases and xylanases. The suggestions that they are *O*-glycosylated, aid them in maintaining an extended conformation, and protection against proteolysis [44]. Argos [45] also suggested that flexibility and stability are contributed by the small polar residues usually seen in linkers. Stable linkers are needed when producing chimeric enzymes, without affecting the latter's function. This stability is mainly dependent on the linker length and composition. Linker length usually affects domain–domain orientations, protein stability, and folding rates [46,47]. Constructs with the same linker composition, but with varying lengths, were shown to yield higher activity with the longest chain length linkers [48]. During recombinant protein production, proteolytic cleavage occurs at unprotected and flexible sites. This can be the case when long linkers are used, and would ultimately result in a lower yield of active protein. On the other side, while shorter linkers can overthrow such problems, they can still cause a loss of function by bringing modules into close proximity. Catalytic activities require the proper natural conformation of each domain. Thus, the importance of suitable linker peptides is reflected in the necessary space required to attain an active conformation [36].

2.3. Bifunctionality

When an enzyme comprises two explicit catalytic dimensions in one polypeptide chain, and can catalyze two continuous reactions, it is labeled as a bifunctional enzyme [49]. In addition, its catalysis involves complex multi-substrate reactions, in which a vast amount of intermediary enzymatic forms are associated. Accordingly, new features of bifunctional enzymes may arise, such as the detection of intermediates that do not appear in the solution, but are nevertheless products of the first catalyzed reaction, and subsequent substrates for the following one [50].

One of the most important features to keep in mind when fusing different genes is to choose ones that have functional similarities and close temperature and pH profiles (Fig. 3). An interesting

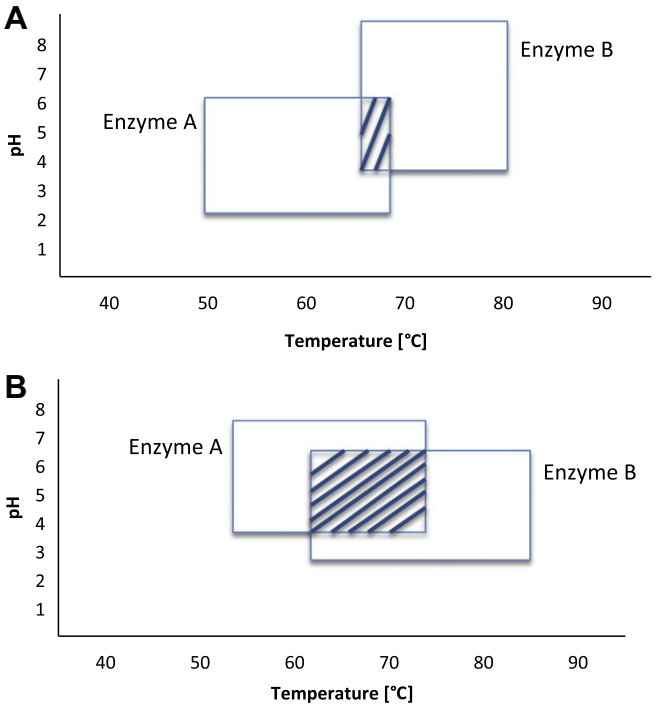

Fig. 3. Activity profiles of putative fusion proteins. When choosing enzymes for fusion constructs, it is important to take into consideration the pH and temperature characteristics of both enzymes to find strong points of similarity between both. Enzymes in (A) have small profile similarities as compared to (B) where enzymes are proximally closer in their characteristic range. Thus, enzymes in (B) are better candidates for fusion construction.

Table 1
Fusion constructs for cellulose and hemicellulose hydrolysis.

Donor-organisms/fusion enzymes	GH family	Linker	Activity[b]
Thermotoga maritima [54]			
Cel/Glu[a]	Cellulase 5	–	CMC (+), pNPG (+)
Glu/Cel	β-Glucosidase 5	–	pNPG (−), CMC (+)
Paenibacillus sp. [9]			
Cel/Xyl	Endoglucanase 5	–	CMC (−), XYN (−)
Xyl/Cel	Xylanase 11	–	XYN (−), CMC (+)
Cel/Xyl		+	CMC (+), XYN (−)
Xyl/Cel		+	XYN (−), CMC (−)
Thermotoga maritima [53]			
Cel-Xyl	Cellulase 5	–	CMC (+), XYN (+)
Xyl-Cel	Xylanase 10	–	XYN (−), CMC (−)
Clostridium thermocellum [23]			
Xyl/Af-Xd	Xylanase 10 Arabinofuranosidase/Xylosidase 43	+	CMC (+), XYN (+), 4NPA (+), 2NPX (+)
Bacillus amyloliquefaciens/Bacillus subtilis [56]			
Gla-Xyl	β-Glucanase 16	–	β-glucan (+), XYN (+)
	Xylanase 11	+	β-glucan (+), XYN (+)

[a] Enzymes: Cel – cellulase, Glu – β-glucosidase, Xyl – xylanase, Af-Xd – bifuncational Arabinofuranosidase/Xylosidase, Gla – β-glucanase.
[b] Substrates: CMC – Carboxymethylcellulose, pNPG – 4-nitrophenyl-β-D-glucuronide, XYN – Xylan, 4NPA – 4-nitrophenyl-α-L-arabinofuranoside, 2NPX – 2-nitrophenyl-xylopyranoside.

approach would be to choose enzymes belonging to different glycosyl hydrolase families, and test the similarities and differences in structure and function of the complete complex. Zhang et al. [51] concluded that synergistic actions between thermostable cellulases and xylanases belonging to GH10 were better than those with xylanases from GH11. Furthermore, they showed that the addition of xylanase could significantly enhance the activity of cellulase. Cellulose hydrolysis occurred faster, since the addition of xylanase removed the hemicellulose barrier, which in turn exposed more cellulose chains, thus allowing increased accessibility to cellulose chains. Another aspect that might occur is when the active site of one reaction can affect the active site of the second, in whichever orientation they are found [52]. In fusion constructs, the size of the enzymes plays an important factor in affecting the overall activity. For an increase in catalytic efficiency, the larger domain is fused mainly at the C-terminus. A larger domain at the N-terminus might interfere with thermostability by affecting the folding and conformation. The thermostability of *Aspergillus niger* xylanase is decreased, when a glucanase moiety from thermophilic species *Thermotoga maritima* is fused at the N-terminus. On the other side, the glucanase fused at the C-terminus resulted in an increase of total thermostability of the xylanase [36].

The construction of a chimeric protein, however, sometimes results in the reduced activity of one or more of the catalytic domains. Hong et al. [53] assembled a bifunctional cellulase–xylanase by end-to-end fusion, from *T. maritima*. Although they were able to successfully construct enzyme fusions, only one conformation was shown to be functional. This activity loss could be due to inappropriate protein interactions and misfoldings that affect activity levels and destabilize protein structure. A chimeric protein composed of cellulase followed by xylanase and separated by a glycine-serine linker by Adlakha et al. [9] was shown to be favorable over its opposite conformation, as well as both conformations with no added linker in between. This indicates again that the sequential incorporation and the linker both play a critical role in the characterization of chimeric proteins. A bifunctional cellulase-β-glucosidase also showed activity when β-glucosidase was fused downstream cellulase, but not vice versa [54] (Table 1).

This further clarified the potential need and importance of having a peptide linker to confer stability between both domains. Not only is the linker a criterion, but the orientation also plays an important factor for optimal activity of both enzymes. An et al. [55] designed end-to-end fusions of cellulase and xylanase. Both activities were seen when cellulase was fused downstream of xylanase, but not in the opposite direction. As a conclusion, intra-chain interactions are achieved through proper linker composition, and stabilization of primary structures.

Once the basis of end-to-end fusion is laid down, the possibilities are endless. There has been a recent effort into developing trifunctional or multifunctional enzymes. These could contain all the necessary enzymes in the complete hydrolysis of three or more substrates. Fan et al. [23] were able to construct a multidomain enzyme possessing xylanase, cellulase, arabinofuranosidase and xylosidase activities. Interestingly enough, the multifunctional enzymes were able to retain the enzymatic properties of the parental enzymes as well as exhibiting synergistic effects during hydrolysis. An alternative way to construct more complex multiprotein domains is through cellulosomes. Designer cellulosomes have been constructed by integrating more than one gene encoding for different enzymes, along with scaffoldins and dockerins mimicking natural cellulosomes. This could also open the door for multifunctional fusion enzymes being good candidates for designer cellulosomes.

With the advancement of biological tools and technologies, it is possible to mimic nature, and artificially speed up processes that take long times to evolve. An interesting approach is constructing multifunctional enzymes to better study the relationship and synergistic effects between functionally related enzymes. Although linkers play a major role in stabilizing constructs, they are not the sole reason behind successful fusion and consequent activity. Choosing the conformation of the genes involved might also prove decisive. A number of studies have been done to change conformations as well as linkers between the same set of genes. However, more insight and research is still needed to come up with a concessive idea about the optimum mode of action. It will be an important tool for reducing reaction times, as well as enzyme dosage, while maintaining high enzymatic activity at the same time. If a set of rules is found, fusion constructs will be a very interesting approach for vast industrial processes, especially bioethanol production, through the efficient degradation of lignocellulosic biomass.

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