THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Engineering of industrially important microorganisms for assimilation of cellulosic biomass – towards Consolidated Bioprocessing

Citation for published version:
Valenzuela ortega, M & French, C 2019, 'Engineering of industrially important microorganisms for assimilation of cellulosic biomass – towards Consolidated Bioprocessing', *Biochemical Society Transactions*, vol. 47, no. 6. https://doi.org/10.1042/BST20190293

Digital Object Identifier (DOI):
10.1042/BST20190293

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Biochemical Society Transactions

OPEN ACCESS

Engineering of industrially important microorganisms for assimilation of cellulosic biomass – towards Consolidated Bioprocessing.

Marcos Valuenzuela-Ortega[1], Christopher E. French[1,2,*]

1. Centre for Systems and Synthetic Biology, School of Biological Sciences, University of Edinburgh, Roger Land Building, Alexander Crum Brown Road, Edinburgh EH9 3FF, UK

2. Zhejiang University-University of Edinburgh Joint Research Centre for Engineering Biology, International Campus, Zhejiang University, Haining, Zhejiang 314400, China

*To whom correspondence should be addressed: +44 (0)131 650 7098, C.French@ed.ac.uk

Conversion of cellulosic biomass (non-edible plant material) to products such as chemical feedstocks and liquid fuels is a major goal of industrial biotechnology and an essential component of plans to move from an economy based on fossil carbon to one based on renewable materials. Many microorganisms can effectively degrade cellulosic biomass, but attempts to engineer this ability into industrially useful strains have met with limited success, suggesting an incomplete understanding of the process. The recent discovery and continuing study of enzymes involved in oxidative depolymerisation, as well as more detailed study of natural cellulose degradation processes, may offer a way forward.

Importance of the problem

Many governments and organizations aim to move away from the use of non-renewable fossil carbon towards renewable production of organic compounds required for our economy; for example, a 2017 report published by the UK Industrial Biotechnology Leadership Forum [1] calls for 'greener, cleaner manufacturing processes', 'using renewable bio-based sources'. Microbial processes based on photosynthetically derived plant material play a major role in such plans. However, current processes are mainly based on easily fermentable sugars such as sucrose and glucose (derived from hydrolysis of starch) and can't be scaled up to the enormous volumes required without seriously interfering with the human food supply, especially as the world's population increases. Direct production by photosynthetic organisms is possible, but at very large scale is likely to compete with land and water requirements for human food production. The other major option is deconstruction of non-edible plant-derived materials. Many microorganisms are capable of rapid and effective degradation of these materials, especially in warm and wet conditions such as the rumen and tropical ecosystems, as well as in bioreactors, but such organisms generally do not produce useful products in quantities suitable for commercial processes, and they are currently rather difficult to engineer. This review focuses on the alternative strategy, genetically modifying industrially useful organisms to allow growth and product formation at the expense of cellulosic material.

Plant cell wall materials: the starting point

Plant cell wall material generally consists of long fibres of cellulose coated with shorter chains of hemicellulose and pectin, in most cases embedded in an amorphous matrix formed of lignin (Figure 1). Lignin is a three dimensional matrix formed by random polymerization of aromatic subunits. It is extremely difficult to degrade, and while it could in principle serve as an excellent renewable source of aromatic compounds [2], such processes are currently not economically feasible, and its major significance is in the necessity for pre-treatment to disrupt its structure before hemicellulose and

cellulose can be attacked. The situation is further complicated by covalent attachments between lignin and hemicellulose [3,4]. Hemicelluloses are branched polysaccharides, mainly with D-glucose, D-xylose or D-mannose backbones, and including other sugars such as L-arabinose, D-galactose and L-rhamnose, in some cases esterified with acetate and other small acid groups [5]. Hemicellulose in general is relatively easy to extract, dissolve, and hydrolyse. Pectins are polymers mainly composed of uronic acids. The most attractive target is cellulose, an unbranched homopolymer of D-glucose linked by β-(1,4)-glycosidic bonds [6](Figure 2a). Sequential glucose units are oriented at 180° to each other, giving a repeating unit of cellobiose. Cellulose fibrils consist of long parallel (in native cellulose) chains linked by strong intra-chain hydrogen bonds, giving a rigid linear structure, with adjacent chains packing tightly together with many inter-chain hydrogen bonds, making cellulose insoluble, with a mixture of highly ordered (crystalline) and amorphous regions.

Acid hydrolysis of cellulose requires extremely aggressive conditions, and commercial processes appear to rely mainly on enzymic degradation. The canonical cellulose hydrolysis process is based mainly on studies of the fungus *Trichoderma reesei (Hypocrea jecorina)*, which was isolated in New Guinea during WWII, and is still a major source of commercial cellulase blends [7,8]. Initially **endoglucanases** (EG, EC3.2.1.4) bind to the surface of cellulose fibres and hydrolyse random bonds, generating a free reducing (C1) and non-reducing (C4) end (Figure 2b). **Exoglucanases** (**cellobiohydrolases,** CBH) attack either the reducing (EC3.2.1.176) or non-reducing (EC3.2.1.91) end; often both types are present and act in a complementary way, as in CBHI and CBHII of *T. reesei*. These move processively along the chain, releasing cellobiose, which is then hydrolysed by **β-glucosidases** (BG, EC3.2.1.21), to release glucose, which is necessary to relieve product inhibition of CBH. Alternatively, short cellodextrins can be degraded by **cellodextrin glucohydrolases** (EC3.2.1.74), releasing glucose [9,10], or cellobiose or cellodextrins can be taken up and depolymerized intracellularly by hydrolysis or phosphorolysis [9,11]. Cellulases may be secreted, or displayed on the cell surface. For example, cellulolytic *Clostridium* spp.possess large cell-surface complexes known as **cellulosomes**, which consist of a cell-wall anchored **scaffoldin** protein (CipA in the model organism *Clostridium thermocellum*) with multiple **cohesin** domains binding a large number of mixed biomass degrading enzymes via **dockerin** domains [12](Figure 3). Such complexes appear to be extremely efficient for biomass degradation, presumably due to co-localization of synergistic cellulase activities and co-anchoring to the bacterial cell wall and the insoluble substrate.

All of these hydrolytic enzymes are classified as Glycosyl Hydrolases (GH) in the CAZY (Carbohydrate-Active Enzymes) database [13], with different structural families having a variety of related activities. For example, the major endoglucanases of *T. reesei*, EGI (Cel7B) and EGII (Cel5A), belong to family GH7 and GH5, and the major exoglucanases, CBHI (Cel7A) and CBHII (Cel6A), belong to GH7 and GH6, respectively. The difference between the related GH7 enzymes EGI and CBH1 is instructive; EG1 possess a broad active site suitable for attack on an insoluble cellulose microfibril, whereas the active site of CBHI is in a tunnel, allowing a single cellulose chain to be inserted [14]. Since they must act on an insoluble polymeric substrate, many cellulases possess a **Carbohydrate Binding Module** (CBM) at the N- or C-terminal end, which enhances their activity by allowing binding to the surface of the cellulose fibril. These CBM are also classified in a number of different structural families [15] and may also contribute to amorphogenesis (conversion of crystalline to amorphous cellulose, facilitating enzyme access and hydrolysis) [16]. CBMs may occur at either end of the catalytic domain. For example, EGI (Cel7B) and CBHI (Cel7A) have a carbohydrate binding module of family CBM1 at the C-terminus, EGII (Cel5A) and CBHI (Cel6A) have a CBM1 module at the N-terminus.

Current commercial processes based on cellulosic material are dominated by cellulosic ethanol, the so-called 'second generation' biofuel [17]. While a number of companies operate pilot to medium-

scale plants [18], production is dwarfed by that based on maize starch or sucrose, and production costs appear to be much higher. This is further exacerbated by the distributed nature of the biomass starting material, especially where readily available materials such as agricultural wastes are used, leading to high costs for transportation [19]. Standard processes appear to involve pre-treatment to disrupt lignin, followed by enzymic hydrolysis of hemicellulose and cellulose, using commercially available cellulase blends which appear to be derived mainly from *T. reesei*. The resulting sugars are then fermented to ethanol, mainly by *Saccharomyces cerevisiae*, one of very few organisms capable of producing ethanol at sufficiently high titres to make distillation commercially viable. Variants of the process include SSF (Simultaneous Saccharification and Fermentation), in which hydrolysis and fermentation occur simultaneously, reducing product inhibition of cellulases, and SSCF (Simultaneous Saccharification and Co-Fermentation), in which both hexoses and pentoses are fermented simultaneously by a suitably capable organism. This provides a paradigm for development of other commercial processes based on cellulosic materials, but the costs of pre-treatment and enzymes represent a considerable burden on economic viability [19]. Therefore, a great deal of research has gone into engineering microorganisms to produce their own cellulases and hemicellulases, and then ferment the resulting hexose and pentose sugars to produce useful products. This is called '**consolidated bioprocessing**' (CBP)[20]. One approach is engineering of cellulose-degrading microorganisms to add product-formation pathways; the other is engineering of industrial organisms to allow partial or complete degradation and assimilation of cellulosic biomass. This review will focus on the latter approach, which is also interesting from a biochemical point of view in that it has the potential to test our understanding of the normal process of biomass degradation in nature.

Engineering of non-cellulolytic bacteria for cellulose degradation

Much early research focused on engineering of *E. coli*, a tractable model organism easily engineered to produce a wide variety of useful products, including ethanol. In addition to glucose, *E. coli* is natively capable of assimilating D-xylose, L-arabinose and most other biomass-derived sugars, thus grows well with hemicellulose hydrolysate, but does not normally assimilate cellobiose or cellooligosaccharides, though it is straightforward to isolate mutants which can ferment cellobiose [21], or to introduce this activity. *E. coli* expressing a GH3 cellodextrinase intracellularly is able to grow well with cellobiose or cello-oligosaccharides as carbon source, indicating a native ability to take up cello-oligosaccharides at least as large as cellohexaose (A. Salinas, C.K. Liu and C. French, manuscript in preparation). While there is a vast literature on the expression, secretion, and surface display of various classes of cellulases in *E. coli* and other Proteobacteria, there seem to be relatively few reports which have the explicit aim of generating a strain capable of degrading cellulose and assimilating the resulting sugars. One reason may be that *E. coli* is relatively poor at secreting proteins to the extracellular medium in the large amounts which are required for hydrolysis of biomass polysaccharides, although a recent report [22] describes a native cellulolytic *E. coli* strain isolated from a rumen culture; closer examination of the genome of this strain would surely be of interest.

Much of the relevant work in *E. coli* uses strains engineered for ethanol production by expressing the pyruvate decarboxylase and alcohol dehydrogenase of *Zymomonas mobilis* or *Saccharomyces cerevisiae*. These strains produce ethanol from hemicellulose hydrolysate, though they can't achieve the impressive titres seen in *S. cerevisiae*, making distillation more costly. For example, an ethanologenic strain secreting a multifunctional GH5 endoglucanase/exoglucanase/xylanase derived from a rumen metagenomic library was able to produce up to 3 g/l ethanol from CMC or 2 g/l from Avicel, though this required co-culture with a β-glucosidase-producing strain [23], or 8 g/l ethanol

from pretreated plant biomass, as compared to 25 g/l ethanol when a commercial cellulase blend was used for saccharification [24]. Other biofuels produced in a similar way include biodiesel and terpenoids. *E. coli* strains expressing a *Bacillus* endoglucanase, *Clostridium* endoxylanase (both as fusions with *E. coli* protein OsmY to aid secretion), and *Cellvibrio* β-xylosidase and β-glucosidase were able to grow on plant biomass pre-treated with the ionic liquid 1-ethyl-3-methylimidazolium acetate, producing 71 mg/l biodiesel (fatty acid ethyl esters), 28 mg/l n-butanol, or 2 mg/l pinene (a terpenoid hydrocarbon) [25]. In a later study, up to 10 mg/l limonene, another terpenoid, was produced from cellulosic material pretreated with the same ionic liquid, using a mutant strain resistant to the toxic effects of this solvent, expressing a variant cellulase active in this reagent [26]; this is interesting, though substantially less than the 550 mg/l for the original strain grown on glucose, or 300 mg/l for the mutant growing on glucose in the presence of the ionic liquid. In another engineered strain, up to 0.07 g/l of the bioplastic polyhydroxybutyrate (PHB) was produced from CMC [27], a very low titre, though the authors point out that this host system was not optimized for PHB production.

Closely related organisms such as *Citrobacter freundii* have the native ability to assimilate cellobiose, and can be engineered using the same vectors and techniques as *E. coli*; coexpression of an endoglucanase and exoglucanase from *Cellulomonas fimi* in *C. freundii* resulted in strains capable of some degree of growth at the expense of Avicel (microcrystalline cellulose), though a small amount of yeast extract was also required [28]. Another potentially interesting organism is *Pseudomonas putida*, which naturally possesses a wide range of metabolic pathways and shows good tolerance to many toxic compounds. Generally, *P. putida* strains assimilate only a limited range of sugars, including glucose but not other biomass-derived sugars such as xylose and cellobiose. A recent report described engineering of *P. putida* for simultaneous assimilation of glucose, cellobiose and xylose, via co-expression of the *E. coli* xylose transporter, xylose isomerase and xylulokinase with an intracellular *Clostridium* cellulolyticum β-glucosidase [29]; interestingly, cellobiose was apparently taken up via the glucose transporters, so that a dedicated cellobiose uptake system was not required. Deletion of glucose dehydrogenase was also required to prevent oxidation of xylose. *P. putida* has also been engineered for surface display of cellulases; three thermophilic cellulases from *Ruminiclostridium (Clostridium) thermocellum* were displayed on the outer membrane of *P. putida* using a system based on autotransporters, with the aim of generating a cost-effective system for manufacture of cellulases for standard saccharification processes [30]. The *P. putida* system was found to be superior to *E. coli* for this purpose [31]. While the aim here was not to develop an organism for consolidated bioprocessing, this does show the feasibility of expressing active cellulases in *P. putida*.

Another target is *Bacillus* spp, especially *B. subtilis*. These are used in manufacture of enzymes for starch processing and laundry detergents, and are well studied and genetically tractable. In particular, *B. subtilis* is naturally competent and can take up large pieces of DNA and integrate them into the chromosome. This forms the basis for DNA assembly techniques such as OGAB (Ordered Gene Assembly in *Bacillus*). *Bacillus* spp. are also proficient for high level secretion of degradative enzymes, though cell-wall associated proteases can cause problems when secreting heterologous proteins. Though *Bacillus* spp. are not normally considered effective cellulolytic organisms, many strains do produce various cellulases [32], making them an interesting starting point. In particular, being relatives of *Clostridium* spp., they are an interesting option for reconstruction of cellulosomes. For example, a recent report describes use of OGAB to assemble a reduced cellulosome system with intact scaffoldin and its cell surface anchor protein, together with 6 assorted cellulosomal biomass degradation enzymes, and demonstrated correct assembly, enzyme activity, and degradation of grass biomass [33]. While *B. subtilis* is not well suited to production of cellulosic ethanol, it seems a

good candidate for engineering of processes for manufacture of other products from cellulosic materials. A related organism is *Geobacillus*, a thermophilic genus capable of assimilation of many products of biomass degradation; an engineered ethanol producing strain of *G. thermoglucosidasius* formed the basis of the cellulosic ethanol technology of TMO Renewables [34]. Tools for engineering of *Geobacillus* sp. have been created [35,36], and a recent report [37] describes engineering of *G. denitrificans* to express endoglucanase and exoglucanase, as a first step towards generating a novel thermophilic host for consolidated bioprocessing. Considering the potential advantages of thermophilic processes, this seems to be an avenue worth pursuing, though engineering of more complex product formation pathways than ethanol may be more challenging, since all enzymes will need to sourced from thermophilic hosts or engineered for increased thermostability.

The lactic acid bacteria, *Lactococcus* and *Lactobacillus* spp., are also members of the low-GC Gram positive group (Phylum Firmicutes) and are used in the large scale manufacture of lactic acid [38]. Lactic acid production from cellooligosaccharides up to cellooctaose has been reported [39] using *Lactococcus lactis* strain expressing a clostridial endoglucanase and cellodextrinase; CMC hydrolysis was detected, but growth or lactic acid production on cellulose or CMC was not reported. Cellulosomes have been assembled on *Lactobacillus plantarum*, including two endoglucanases and two xylanases from *Clostridium papyrosolvens*, using a combinatorial approach based on 'adaptor scaffoldins' to increase surface display levels. Saccharification of wheat straw was demonstrated, but the amounts of sugars released were apparently insufficient to allow growth of the recombinant organisms [40]. Another interesting host is *Corynebacterium glutamicum*, a high-GC Gram positive bacterium used in the manufacture of L-glutamate and other amino acids [41]. Cell surface display of a *C. thermocellum* endoglucanase and β-glucosidase gave some degree of saccharification of pretreated biomass (up to 57 mg of reducing sugars per g rape stem biomass)[42]. In another report [43] secretion or surface display of a bacterial endoglucanase and β-glucosidase allowed some degree of growth with cellobiose or CMC, with production of up to 6 mM L-lysine, far below commercially useful levels, but a good starting point. Another high-GC Gram positive bacterium, the oil-producing *Rhodococcus opacus*, has also been engineered for cellulose degradation by expression of six cellulases from *Cellulomonas fimi* and *Thermobifida fusca*, with different strains each expressing 1 to 3 cellulases [44]. A consortium of strains was able to achieve 20% hydrolysis of birchwood cellulose over 18 days, yielding 2 g/l cellobiose, after which a further cellobiose-utilizing strain was able to grow and produce lipids over 4 days using the resulting supernatant. The authors noted that substantial improvements were required for a useful process.

Overall, it seems clear that engineering of non-cellulolytic bacteria for cellulose breakdown is possible, but in essentially all systems reported to date, sugar release is slow and product titres are far below those obtained in processes based on soluble sugars, suggesting that major improvements will be required if such systems are to be used for commercial processes. This defines the challenge for the next stages of this work.

Engineering of yeasts for cellulose degradation.

S. cerevisiae is a model eukaryote used on an enormous scale in industry for bread, alcoholic beverages, and bioethanol, and has attracted increasing interest from pathway engineers, especially when processes are to be scaled up for commercial production [45,46]; for example, isobutanol, artemisinin, farnesene, squalene and other compounds have been or are being manufactured at commercial scale in engineered strains of *S. cerevisiae*. *S. cerevisiae* can natively use glucose, as well as maltose, isomaltose, sucrose, and some other saccharides, but not cellobiose, xylose, or arabinose. Thus one early target was the generation of strains capable of using these sugars, preferably simultaneously with use of glucose, since this can significantly increase production levels

from enzymically processed biomass. Strains capable of assimilating all of these sugars have been developed, though co-utilization of D-xylose and L-arabinose seems to require strain evolution after engineering [47,48], perhaps due to regulatory issues as well as metabolic cross-talk [49]. Such strains have been exploited in commercial biomass fermentation processes, though there are still considerable barriers to large scale implementation [50].

The more challenging target is direct hydrolysis of biomass via secretion or surface display of polysaccharide degrading enzymes. Secretion is the simplest approach, using either a single strain or a consortium, each producing one of the necessary enzymes; however, where the rate of glucose release is low, glucose may tend to be used for cell maintenance rather than ethanol production [51]. Secretion levels may be increased by multiple gene integration at delta (Ty1 retrotransposon) sites and promoter engineering [52], as well as direct engineering of the *S. cerevisiae* protein secretion system [53,54,55,56,57,58,59]. Interestingly, the response to different manipulations is frequently reported to be different in different target proteins, suggesting that different factors are limiting in each case. Following secretion, enzymes may be released, or displayed on the cell surface via 'anchors' such as Sed1, a- or α-agglutinin, either directly, or indirectly via cohesin-dockerin interactions to a surface-displayed scaffoldin to create a 'mini-cellulosome' [60]. Some reports have compared surface display to secretion, usually finding a slight advantage for display [61], especially where multiple enzymes are displayed on a single cell rather than using a consortium each displaying one enzyme [62]. A synthetic cellulosome-like system has also been reported, with enzymes immobilized via disulphide bonds, and this was reported to enhance stability [63]. It is also important to consider testing in industrially competent hosts rather than only in laboratory strains [64,65].

Results are often reported as the amount of ethanol released from CMC (a soluble cellulose derivative), PASC (amorphous cellulose) or Avicel (microcrystalline cellulose) by pre-grown cells. Typical values range from 0.8 g/l to 2.9 g/l (Table 1). By contrast, commercial cellulosic ethanol processes are reported to generate ethanol at 5% to 7% v/v (39.5 to 55.3 g/l)[19]. Since distillation costs are much higher at low ethanol concentraions, this suggests that major improvements are needed before such processes can be used commercially. No reports seem to describe production of commercially useful levels of ethanol from cellulose without supplemental cellulases. However, a number of reports describe improved ethanol production from complex, realistic biomass substrates, due to some degree of assimilation of substrates which are normally not used [66], and/or reduced requirement for exogenous cellulases [64]. Even small yield increases may be economically significant in processes with low profit margins in which the costs of substrate and enzymes form a major part of total costs.

Other yeasts have also been a target of such research. *Pichia pastoris (Komagataella phaffii)* is a widely used host for recombinant protein production, and is capable of secreting heterologous proteins at high levels; as such it has often been used to generate recombinant cellulases for research and potentially commercial use [67,68] including innovative approaches such as self-cleaving fusions [69] and minicellulosomes [70]. A few reports describe development of strains capable of growth and product formation at the expense of cellulose;. For example, a strain secreting a *T. reesei* endoglucanase together with an endoglucanase and β-glucosidase of *Aspergillus niger*, was able to grow at the expense of cellobiose and CMC, but not Avicel [71]; the authors suggest that this opens the way for production of recombinant proteins using cellulose-based media. Another report describes construction of a strain able to produce ethanol from pentoses and hexoses, expressing a mini-cellulosome including clostridial xylanase and endoglucanase [72]; approximately 1.1 g/l ethanol was produced from CMC, and a similar titre from *Miscanthus* biomass.

Given its good protein secretion abilities, and the availability of tools for expression of heterologous proteins, *P. pastoris* seems a strong candidate for further work. Another industrially useful species is the oil-producing yeast *Yarrowia lipolytica*. A consortium secreting *T. reesei* EGII and CBHII and a chimeric version of CBHI showed some ability to grow with Avicel as sole carbon source in a minimal medium [73], a particularly difficult challenge. A single strain secreting *T. reesei* EGI, EGII and CBHII together with *Neurospora* CBHI, and overexpressing endogenous β-glucosidases, was able to grow with pretreated industrial cellulose pulp, or less successfully with Avicel, as sole carbon source [74], though with much lower rates than native cellulolytic organisms. Since this species is already able to produce valuable products, this seems a good starting point towards a consolidated bioprocessing system.

Thus, overall, it seems that construction of systems able to ferment native cellulose effectively without supplemental cellulases is yet to be achieved. However, current systems are capable of reducing the quantities of cellulases required, and of increasing yields of ethanol and other products from complex substrates such as food wastes which include a cellulosic component; even quite minor improvements have the potential to make large differences in process economics where volumes are large and profit margins small.

New developments

It has become apparent that a variety of proteins beyond classical cellulases are involved in biomass degradation. **Expansins** are plant proteins related to the GH45 family of cellulases, but lacking a critical catalytic residue; they decrease the crystallinity of cellulose and are presumed to act in loosening the plant cell wall structure so that new material can be added [75]. Expression of plant expansins in bacteria has been problematic, so many studies have used bacterial expansin-like proteins such as YoaJ (EXLX1) of *Bacillus subtilis*, despite their relatively low activities [76]. *T. reesei* also encodes an expansin-like protein, designated **swollenin** (SWO1), and similar proteins are found in many other cellulolytic fungi, suggesting an important role in biomass breakdown. Bacterial expansins and swollenins can synergistically enhance sugar release from biomass by commercial enzymes [77,78] though in some cases little effect is seen [79]. Thus, they appear to be interesting candidates for addition to recombinant systems, with some published results suggesting beneficial synergy [80,81](Table 1).

Perhaps the most interesting and significant development in the last few years has been the discovery and characterization of enzymes involved in oxidative depolymerisation of cellulose and related polymers such as chitin [82,83]. These were originally believed to be hydrolytic cellulases (family GH61, in fungi) or carbohydrate-binding modules (family CBM33, in bacteria), but were discovered to be copper-containing enzymes capable of oxidative attack on either C1 or C4 of a β-1,4-glycosidic bond, followed by spontaneous scission of the chain with release of products bearing an oxidized C1 or C4 end (Figure 4). Oxygen and a reducing agent, such as ascorbate, are required for activity. Family GH61 was reclassified as **AA9**, and CBM33 as **AA10**, and several more families have since been discovered [84,85,86]. In the current literature, these enzymes are designated as **Lytic Polysaccharide Monooxygenases (LPMO)**, though it is not entirely clear whether this is the most accurate depiction of their activity, since it now appears that H_2O_2 is a preferable substrate to O_2. Recent reports indicate that the copper centre is first reduced to Cu(I) by the reductant, followed by binding of O_2, which can be reduced to H_2O_2 [87](Figure 4). In the absence of a polysaccharide substrate, H_2O_2 is released, but in the presence of substrate, attack on C1 or C4 of a glycosidic bond occurs, followed by hydroxylation, giving an intermediate product which spontaneously degrades, cleaving the chain. When O_2 is used as oxidant, stoichiometric quantities of reductant are required, whereas when H_2O_2 is supplied, only catalytic quantities of reductant are needed [88]. In nature,

lignin degradation products may act as reductants [89,90], and cytochrome domains of cellobiose dehydrogenase (family AA3) can directly donate electrons to the copper centre of LPMO [91,92], as can PQQ-dependent pyranose dehydrogenase [93]. LPMO are widely distributed in fungi and bacteria, and also appear to occur in some plants [94] and insects [95]; thus they appear to play an important and previously unappreciated role in natural biomass degradation. LPMO are reported to enhance saccharification in cellulase blends, especially when H_2O_2 rather than O_2 is supplied as oxidant [96]. Use of LPMO is this way may be difficult or impossible in standard SSF processes for ethanol production, which are operated anaerobically, but this does not preclude their use in processes for manufacture of other products. However, so far there seem to be few reports of incorporation of LPMO-encoding genes into biomass degrading cassettes for improving in-vivo biomass degradation capability. In one report inclusion of an LPMO and cellobiose dehydrogenase in pentafunctional minicellulosomes in *S. cerevisiae,* increased ethanol production from PASC and Avicel (Table 1), and cells were also able to grow slowly with PASC as sole carbon source [97]; in *Y. lipolytica*, co-expression of *T. reesei* swollenin and LPMO, as well as a xylanase, in a previously engineered cellulase secreting strain of *Y. lipolytica*, increased sugar release from, and growth on, various cellulosic substrates [98].

Alternative strategies and complicating factors

Alternative strategies based on engineering of native cellulolytic organisms may offer advantages [16]. However, these systems also have their problems. A different approach is the use of thermochemical processing to convert biological materials to **syn gas**, a mixture of H_2, CO and CO_2, which can then be assimilated by anaerobic organisms such as *Clostridium ljungdahlii*, or aerobic organisms such as *Cupriavidus necator* (formerly *Ralstonia eutropha*)[99]. These organisms can be genetically modified to produce desirable products [100,101] This method has the potential advantage that it can accept a very wide range of input materials, converting them to a simple and easily separated product stream which is then fed to the engineered organisms. However, this approach does require energy intensive processing and specialized fermenter technology for gas feeding [102]. A further complicating factor may arise in the future: sugars obtained from biomass may become too valuable to be used for conversion to low value bulk chemicals. Glucose from cellulose, in particular, may be converted to starch-like products which could be used as animal feed, freeing higher value grain-based materials for human food use. This may become increasingly important as the human population rises and becomes wealthier.

Perspectives

- Importance: cellulosic biomass represents a huge renewable source of sugars which could potentially be used as a feedstock for microbial processes to produce biofuels, feedstock chemicals and other valuable products, replacing non-renewable fossil carbon without interfering with the human food supply.
- Current understanding and challenges: engineering industrially useful microorganisms to degrade cellulosic material by expression and secretion or surface display of cellulases has proven challenging, with sugar yields generally too low and hydrolysis rates too slow to maintain good growth or product formation. *E. coli* and *S. cerevisiae*, while attractive hosts in many ways, do not naturally secrete high levels of extracellular enzymes, and alternative hosts should be considered.
- Future directions: The recent discovery of oxidative depolymerizing enzymes, and more detailed knowledge of amorphogenic proteins such as expansins/swollenins, indicates that our understanding of natural biomass degradation is still incomplete and requires further

research. Improved techniques for high throughout DNA assembly, strain generation and strain characterization, coupled with more detailed study of natural biomass degradation processes, and increasing interest from government and industry, may soon lead to large improvements.

Abbreviations

AA: Auxiliary Activity; BG: β-glucosidase; CAZY: Carbohydrate Active Enzymes database; CBH: cellobiohydrolase; CBM: Carbohydrate-Binding Module; CBP: consolidated bioprocessing; CDH: cellobiose dehydrogenase; CMC: carboxymethyl cellulose; EG: endoglucanase; FPU Filter Paper Units; GH: Glycosyl Hydrolase; LPMO: lytic polysaccharide monooxygenase; PASC: phosphoric acid swollen cellulose; PQQ: Pyrolloquinoline quinone; SSF: Simultaneous Saccharification and Fermentation; SSCF: Simultaneous Saccharification and Co-fermentation

Competing interests

The Authors declare that there are no competing interests associated with the manuscript.

Acknowledgements

References

1. Industrial Biotechnology Leadership Forum (2017) Growing the UK Industrial Biotechnology Base. Enabling technologies for a sustainable circular bioeconomy: a national industrial biotechnology strategy to 2030. http://beaconwales.org/uploads/resources/UK_Industrial_Strategy_to_2030.pdf

2. Chio, C.L., Sain, M., and Qin, W.S. (2019) Lignin utilization: A review of lignin depolymerization from various aspects. *Renewable and Sustainable Energy Reviews* **107**, 232-249 https://doi.org/10.1016/j.rser.2019.03.008

3. Terret, O.M., and Dupree, P. (2019) Covalent interactions between lignin and hemicelluloses in plant secondary cell walls. *Current Opinion in Biotechnology* **56**, 97-104 https://doi.org/10.1016/j.copbio.2018.10.010

4. Nishimura, H., Kamiya, A., Nagata, T., Katahira, M., and Watanabe, T. (2018) Direct evidence for α ether linkage between lignin and carbohydrates in wood cell walls. *Scientific Reports* **8**, 6538 https://doi.org/10.1038/s41598-018-24328-9

5. Donev, E., Gandla, M.L., Jonsson, L.J., and Mellerowicz, E.J. (2018) Engineering non-cellulosic polysaccharides of wood for the biorefinery. *Frontiers in Plant Science* **9**, 1537 https://doi.org/10.3389/fpls.2018.01537

6. Rongpipi, S, Ye, D., Gomez, E.D., and Gomez, E.W. (2019) Progress and opportunities in the characterization of cellulose - an important regulator of cell wall growth and mechanics. *Frontiers in Plant Science* **9**, 1894 https://doi.org/10.3389/fpls.2018.01894

7. Bischof, R.H., Ramoni, J., and Seiboth, B. (2016) Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei. Microbial Cell Factories* **15**, 106 https://doi.org/10.1186/s12934-016-0507-6

8. Gupta, V.K., Steindorff, A.S., de Paula, R.G., Silva-Rocha, R., Mach-Aigner, A.R., Mach, R.L., and Silva, R.N. (2016) The post-genomic era of *Trichoderma reesei*: what's next? *Trends in Biotechnology* **34**, 970-982 https://doi.org/10.1016/j.tibtech.2016.06.003

9. Liu, W.J., Bevan, D.R., and Zhang, Y.H.P. (2010) The Family 1 glycoside hydrolase from *Clostridium cellulolyticum* H10 is a cellodextrin glucohydrolase. *Applied Biochemistry and Biotechnology* **161**, 264-273 https://doi.org/10.1007/s12010-009-8782-x

10. Rutter, C., and Chen, R., (2014) Improved cellobiose utilization in *E. coli* by including both hydrolysis and phosphorolysis mechanisms. *Biotechnology Letters* **36**, 301-307 https://doi.org/10.1007/s10529-013-1355-7

11. Parisutham, V., Chandran, S.P., Mukhopadhyay, A., Lee, S.K., and Keasling, J.D. (2017) Intracellular cellobiose metabolism and its applications in lignocellulose-based biorefineries. *Bioresource Technology* **239**, 496-506 https://doi.org/10.1016/j.biortech.2017.05.001

12. Barth, A., Hendrix, J., Fried, D., Barak, Y., Bayer, E.A., and Lamb, D.C. (2018) Dynamic interactions of type I cohesin modules fine-tune the structure of the cellulosome of *Clostridium thermocellum*. *Proceedings of the National Academy of Sciences, USA* **115**, E11274-E11283 https://doi.org/10.1073/pnas.1809283115

13. Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, E.M., and Henrissat, B. (2014) The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Research* **42**, D490-495 https://doi.org/10.1093/nar/gkt1178

14. Schiano-di-Cola, C., Rojel, N., Jensen, K., Kari, J., Sorensen, T.H., Borch, K., and Westh, P. (2019) Systematic deletions in the cellobiohydrolase (CBH) Cel7A from the fungus *Trichoderma reesei* reveal flexible loops critical for CBH activity. *Journal of Biological Chemistry* **294**, 1807-1815 https://doi.org/10.1074/jbc.RA118.006699

15. Armenta, S., Moreno-Mendieta, S., Sanchez-Cuaipo, Z., Sanchez, S., and Rodriguez-Sanoja, R. (2012) Advances in molecular engineering of carbohydrate-binding modules. Proteins: Structure, Function and Bioinformatics 85, 1602-1617 https://doi.org/10.1002/prot.25327

16. Bernardes, A., Pellegrini, V.O.A., Curtolo, F., Camilo, C.M., Mello, B.L., Johns, M.A., Scott, J.L., Guimaraes, F.E.C., and Polikarpov, I. (2019) Carbohydrate binding modules enhance cellulose enzymatic hydrolysis by increasing access of cellulases to the substrate. Carbohydrate Polymers **211**, 57-68 https://doi.org/10.1016/j.carbpol.2019.01.108

17. Robak, K., and Balcerek, M. (2018) Review of second generation bioethanol production from residual biomass. Food Technology and Biotechnology 56, 174-187 https://doi.org/10.17113/ftb.56.02.18.5428

18. Lynd, L.R., Liang, X., Biddy, M.J., Allee, A., Cai, H., Foust, T., Himmel, M.E., Laser, M.S., Wang, M., and Wyman, C.E. (2017) Cellulosic ethanol: status and innovation. *Current Opinion in Biotechnology* **45**, 202-211 https://doi.org/10.1016/j.copbio.2017.03.008

19. Liu, C.G., Xiao, Y., Xia, X.X., Zhao, X.Q., Peng, L.C., Srinophakun, P. and Bai, F.W. (2019) Cellulosic ethanol production: Progress, challenges and strategies for solutions. *Biotechnology Advances* **37**, 491-504 https://doi.org/10.1016/j.biotechadv.2019.03.002

20. Jouzani, G.S., and Taherzadeh, M.J. (2015) Advances in consolidated bioprocessing systems for bioethanol and biobutanol production from biomass: a comprehensive review. *Biofuels Research Journal* **5**, 152-195 https://doi.org/10.18331/BRJ2015.2.1.4

21. Joseph, A.M., Sonowal, R., and Mahadevan, S. (2017) A comparative study of the evolution of cellobiose utilization in *Escherichia coli* and *Shigella sonnei*. *Archives of Microbiology* **199**, 247-257 https://doi.org/10.1007/s00203-016-1299-0

22. Pang, J., Liu, Z.Y., Hao, M., Zhang, Y.F., and Qi, Q.S. (2017) An isolated cellulolytic *Escherichia coli* from bovine rumen produces ethanol and hydrogen from corn straw. *Biotechnology for Biofuels* **10**, 165 https://doi.org/10.1186/s13068-017-0852-7

23. Loaces, I., Bottini, G., Moyna, G., Fabiano, E., Martínez, A., and Noya, F. (2016) EndoG: A novel multifunctional halotolerant glucanase and xylanase isolated from cow rumen. *Journal of Molecular Catalysis B: Enzymatic* **126**, 1-9 https://doi.org/10.1016/j.molcatb.2016.01.004

24. Loaces, I, Schein, S., and Noya, F. (2017) Ethanol production by *Escherichia coli* from *Arundo donax* biomass under SSF, SHF or CBP process configurations and in situ production of a multifunctional glucanase and xylanase. *Bioresource Technology* **224**, 307-313 https://doi.org/10.1016/j.biortech.2016.10.075

25. Bokinsky, G., Peralta-Yahya, P.P., Georg,e A., Holmes, B.M., Steen, E.J., Dietrich, J., Lee, T.S., Tullman-Ercek, D., Voigt, C.A., Simmons, B.A., and Keasling, J.D. (2011) Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. *Proc Natl Acad Sci U S A.* **108**, 19949–54 https//doi.org/10.1073/pnas.1106958108

26. Frederix, M., Mingardon, F., Hu, M., Sun, N., Pray, T., Singh, S., Simmons, B.A., Keasling, J.D., and Mukhopadhyay, A. (2016) Development of an *E. coli* strain for one-pot biofuel production from ionic liquid pretreated cellulose and switchgrass. *Green Chemistry* **18**, 4189 https://doi.org/10.1039/c6gc00642f

27. Gao, D.F., Luan, Y.Q., Wang, Q., Liang, Q.F., and Qi, Q.S. Construction of cellulose-utilizing *Escherichia coli* based on a secretable cellulase. *Microbial Cell Factories* **14**, 159. https://doi.org/10.1186/s12934-015-0349-7

28. Lakhundi, S.S., Duedu, K.O., Cain, N., Nagy, R., Krakowiak, J., and French, C.E. (2016) *Citrobacter freundii* as a test bed for recombinant cellulose degradation systems. *Letters in Applied Microbiology* **64**, 35-42 https://doi.org/10.1111/lam12668

29. Dvorak, P., and de Lorenzo, V. (2018) Refactoring the upper sugar metabolism of *Pseudomonas putida* for co-utilization of cellobiose, xylose, and glucose. *Metabolic Engineering* **48**, 94-108 https//doi.org/10.1016/j.ymben.2018.05.019

30. Tozakidis, I.E.P., Brosette, T., Lenz, F., Maas, R.M., and Jose, J. (2016) Proof of concept for the simplified breakdown of cellulose by combining *Pseudomonas putida* strains with surface displayed thermophilic endocellulase, exocellulase and β-glucosidase. *Microbial Cell Factories* **15**, 103 https//doi.org/10.1186/s12934-016-0505-8

31. Obeng, E.M., Brosette, T., Ongkudon, C.M., Budiman, C., Maas, R., and Jose, J. (2018) The workability of *Escherichia coli* BL21 (DE3) and *Pseudomonas putida* KT2440 expression platforms with autodisplayed cellulases: a comparison. *Applied Microbiology and Biotechnology* **102**, 4829-4841 https://10.1007/s00253-018-8987-4

32. Orencio-Trejo, M., De La Torre-Zavala, S., Rodriguez-Garcia, A., Aviles-Arnaut, H., and Gastelum-Arellanez, A. (2016) Assessing the performance of bacterial cellulases: the use of *Bacillus* and *Paenibacillus* strains as enzyme sources for lignocellulose saccharification. *Bioenergy Research* **9**, 1023-1033 https://doi.org/10.1007/s12155-016-9797-0

33. Chang, J.J., Anandharaj, M., Ho, C.Y., Tsuge, K., Tsai, T.Y., Ke, H.M., Lin, Y.J., Tran, M.D.H., Li, W.H., and Huang, C.C. (2018) Biomimetic strategy for constructing *Clostridium thermocellum* cellulosomal operons in *Bacillus subtilis*. *Biotechnology for Biofuels* **11**, 157 https://doi.org/10.1186/s13068-018-1151-7

34. Raita, M., Ibenegbu, C., Champreda, V., and Leak, D.J. (2016) Production of ethanol by thermophilic oligosaccharide utilising *Geobacillus thermoglucosidasius* TM242 using palm kernel cake as a renewable feedstock. *Biomass and Bioenergy* **95**, 45-54 https://doi.org/10.1016/j.biombioe.2016.08.015

35. Sheng, L.L., Kovacs, K., Winzer, K., Zhang, Y., and Minton, N.P. (2017) Development and implementation of rapid metabolic engineering tools for chemical and fuel production in *Geobacillus thermoglucosidasius* NCIMB 11955. *Biotechnology for Biofuels* **10**, 5 https://doi.org/10.1186/s13068-016-0692-x

36. Reeve, B., Martinez-Klimova, E., de Jonghe, J., Leak, D., and Ellis, T. (2016) The *Geobacillus* plasmid set: a modular toolkit for thermophile engineering. *ACS Synthetic Biology* **5**, 1342-1347 https://doi.org/10.1021/acssynbio.5b00298

37. Daas, M.J.A., Nijsse, B., van der Weijer, A.H.P., Groenendaal, B.W.A.J., Janssen, F., van der Oost, J., and van Kranenberg, R. (2018) Engineering *Geobacillus denitrificans* to introduce cellulolytic activity; expression of native and heterologous cellulase genes. *BMC Biotechnology* **18**, 42 https://doi.org/10.1186/s12896-018-0453-y

38. Tarraran, L., and Mazzoli, R. (2018) Alternative strategies for lignocellulose fermentation through lactic acid bacteria: the state of the art and perspectives. *FEMS Microbiology Letters* **365**, fny126 https://doi.org/10.1093/femsle/fny126

39. Gandini, C., Tarraran, L., Kalemasi, D., Pessione, E., and Mazzoli, R. (2017) Recombinant *Lactococcus lactis* for efficient conversion of cellodextrins into L-lactic acid. *Biotechnology and Bioengineering* **114**, 2807-2817 https://doi.org/10.1002/bit.26400

40. Stern, J., Morais, S., Ben-David, Y., Salama, R., Shamshoum, M., Lamed, R., Shoham, Y., Bayer, E.A., and Mizrahi, I. (2018) Assembly of synthetic functional cellulosomal structures onto the cell surface of *Lactobacillus plantarum*, a potent member of the gut microbiome. *Applied and Environmental Microbiology* **84**, e00282-18 https://doi.org/10.1128/AEM.00282-18

41. Baritugo, K.A.G., Kim, H.T., David, Y.C., Choi, J.H., Choi, J.I., Kim, T.W., Park, C., Hong, S.H., Na, J.G., Jeong, K.J., Joo, J.C., and Park, SJ. (2018) Recent advances in metabolic engineering of *Corynebacterium glutamicum* as a potential platform microorganism for biorefinery. *Biofuels, Bioproducts and Biorefining* **12**, 899-925 https://doi.org/10.1002/bbb.1895

42. Kim, S.J., Hyeon, J.E., Jeon, S.D., Choi, G.W., and Han, S.O. (2014) Bi-functional cellulases complexes displayed on the cell surface of *Corynebacterium glutamicum* increase hydrolysis of lignocellulose at elevated temperature. *Enzyme and Microbial Technology* **66**, 67-73 https://doi.org/10.1016/enzmictec.2014.08.010

43. Anusree, M., Wendisch, V.F., and Nampoothiri, K.M. (2018) Co-expression of endoglucanase and beta-glucosidase in *Corynebacterium glutamicum* DM1729 towards direct lysine fermentation from cellulose. *Bioresource Technology* **213**, 329-244 https://doi.org/10.1016/j.biortech.2016.03.019

44. Hetzler, S., Bröker, D, and Steinbüchel, A. (2013) Saccharification of cellulose by recombinant *Rhodococcus opacus* PD630 strains. Applied and Environmental Microbiology 79, 5159-5166 https://doi.org/10.1128/AEM.01214-13

45. Pretorius, I.S. (2016) Synthetic genome engineering forging new frontiers for wine yeast. *Critical Reviews in Biotechnology* **37**, 112-136 https://doi.org/10.1080/07388551.2016.1214945

46. Jensen, M.K., and Keasling, J.D. (2015) Recent applications of synthetic biology tools for yeast metabolic pathway engineering. *FEMS Yeast Research* **15**, 1-10 https://doi.org/10.1111/1567-1364.12185

47. Wang. X., Yang, J.J., Yang, S., and Jiang, Y. (2019) Unraveling the genetic basis of fast L-arabinose consumption on top of recombinant xylose-fermenting *Saccharomyces cerevisae*. *Biotechnology and Bioengineering* **116**, 283-293 https://doi.org/10.1002/bit.26827

48. Verhoeven, M.D., Bracher, J.M., Nijland, J.G., Bouwknegt, J., Daran, J.M.G., Driessen, A.J.M., van Maris, A.J.A., and Pronk, J.T. (2018) Laboratory evolution of a glucose-phosphorylation-deficient, arabinose-fermenting *S. cerevisiae* strain reveals mutations in GAL2 that enable glucose-insensitive L-arabinose uptake. *FEMS Yeast Research* **18**, foy062 https://doi.org/10.1093/femsyr/foy062

49. Gopinarayanan, V.E., and Nair, N.U. (2019) Pentose metabolism in *Saccharomyces cerevisiae*: the need to engineer global regulatory systems. *Biotechnology Journal* **14**, 1800364 https://doi.org/10.1002/biot.201800364

50. Jansen, M.L.A., Bracher, J.M., Papapetridis, I., Verhoeven, M.D., de Bruijn, H., de Waal, P.P., van Maris, A.J.A., Klaassen, P., and Pronk, J.T. (2017) *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. *FEMS Yeast Research* **17**, fox044 https://doi.org/10.1093/femsyr/fox044

51. Lee, C.R., Sung, B.H., Lim, K.M., Kim, M.J., Sohn, M.J., Bae, J.H., and Sohn, J.H. (2017) Co-fermentation using recombinant *Saccharomyces cerevisiae* yeast strains hyper-secreting different cellulases for the production of cellulosic bioethanol. *Scientific Reports* **7**, 4428 https://doi.org/10.1038/s41598-017-04815-1

52. Sasaki, Y., Mitsui, R., Yamada, R., and Ogino, H. (2019) Secretory overexpression of the endoglucanase by *Saccharomyces cerevisiae* via CRISPR-delta integration and multiple promoter shuffling. *Enzyme and Microbial Technology* **121**, 17-22 https://doi.org/10.1016/j.enzmictec.2018.10.014

53. Bao, J.C., Huang, M.T., Petranovic, D., and Nielsen, J. (2017) Moderate expression of SEC16 increases protein secretion by *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **83**, UNSP e03400-16 https://doi.org/10.1128/AEM.03400-16

54. Bao, J.C., Huang, M.T., Petranovic, D., and Nielsen, J. (2018) Balanced trafficking between the ER and the Golgi apparatus increases protein secretion in yeast. *AMB Express* **8**, 37 https://doi.org/10.1186/s13568-018-0571-x

55. Tang, H.T., Song, M.H., He, Y., Wang, J.J., Wang, S.H., Shen, Y., Hou, J., and Bao, X.M. (2017) Engineering vesicle trafficking improves the extracellular activity and surface display efficiency of cellulases in *Saccharomyces cerevisiae*. *Biotechnology for Biofuels* **10**, 53 https://doi.org/10.1186/s13068-017-0738-8

56. Kroukamp, H., den Haan, R., van Wyk, N., and van Zyl, W.H. 2013. Overexpression of native PSE1 and SOD1 in *Saccharomyces cerevisiae* improved heterologous cellulase secretion. *Applied Energy* **102**, SI 150-156 https://doi.org/10.1016/j.apenergy.2012.05.062

57. Tang, H.T., Bao, X.M., Shen, Y., Song, M.H., Wang, S.H., Wang, C.Q., and Hou, J. 2015. Engineering protein folding and translocation improves heterologous protein secretion in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **112**, 1872-1882. https://doi.org/10.1002/bit.25596

58. Tang, H.T., Wang, S.H., Wang, J.J., Song, M.H., Xu, M.Y., Zhang, M.Y., Shen, Y., Hou, J., and Bao, X.M. 2016. N-hypermannose glycosylation disruption enhances recombinant protein production by regulating secretory pathway and cell wall integrity in *Saccharomyces cerevisiae*. *Scientific Reports* **6**, 25654 https://doi.org/10.1038/srep25654

59. Wang, T.Y., Huang, C.J., Chen, H.L., Ho, P.C., Ke, H.M., Cho, H.Y., Ruan, S.K., Hung, K.Y., Wang, I.L., Cai, Y.W., Sung, H.M., Li, W.H., and Shih, M.C. (2013) Systematic screening of glycosylation- and trafficking-associated gene knockouts in *Saccharomyces cerevisiae* identifies mutants with improved heterologous exocellulase activity and host secretion. BMC Biotechnology 13, 71 https://doi.org/10.1186/1472-6750-13-71

60. Fan, L.H., Zhang, Z.J., Mei, S., Lu, Y.Y., Li, M., Wang, Z.Y., Yang, J.G., Yang, S.T., and Tan, T.W. (2016) Engineering yeast with bifunctional mini-cellulosome and cellodextrin pathway for co-utilization of cellulose-mixed sugars. *Biotechnology for Biofuels* **9**, 137 https://doi.org/10.1186/s13068-016-0554-6

61. Liu, Z., Inokuma, K., Ho, S.H., den Haan, R., Hasunuma, T., van Zyl, W.H., and Kondo, A. (2015) Combined cell surface display- and secretion-based strategies for production of cellulosic ethanol with *Saccharomyces cerevisiae*. *Biotechnology for Biofuels* **8**, 162 https://doi.org/10.1186/s13068-015-0344-6

62. Bae, J., Kuroda, K., and Ueda, M. (2015) Proximity effect among cellulose-degrading enzymes displayed on the *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **81**, 59-66 https://doi.org/10.1128/AEM.02864-14

63 Tang, H.T., Wang, J.J., Wang, S.H., Shen, Y., Petranovic, D., Hou, J., and Bao, X.M. (2018) Efficient yeast surface-display of novel complex synthetic cellulosomes. *Microbial Cell Factories* **17**, 122 https://doi.org/10.1186/s12934-018-0971-2

64. Mo, C.L., Chen, N., Lv, T., Du, J.L., and Tian, S. (2015) Direct ethanol production from steam-esploded corn stover using a synthetic diploid cellulase-displaying yeast consortium. *Bioresources* **10**, 44460-4472 https://doi.org/10.15376/biores.10.3.4460-4472

65. Davison, S.A., den Haan, R., and van Zyl, W.H. (2016) Heterologous expression of cellulase genes in natural *Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology* **100**, 8241-8254 https://doi.org/10.1007/s00253-016-7735-x

66. Inokuma, K., Hasunuma, T., and Kondo, A. (2014) Efficient yeast cell-surface display of exo- and endo-cellulase using the SED1 anchoring region and its original promoter. *Biotechnology for Biofuels* **7**, 8 https://doi.org/10.1186/1754-6834-7-8

67. Sun, F.F., Bai, R.H., Yang, H.M., Wang, F., He, J., Wang, C.D., and Tu, M.B. (2016) Heterologous expression of codon optimized *Trichoderma reesei* Cel6A in *Pichia pastoris*. *Enzyme and Microbial Technology* **92**, 107-116 https://doi.org/10.1016/j.enzmictec.2016.07.004

68. Shi, B.S., Ke, X.J., Yu, H.W., Xie, J., Jia, Y.M., and Guo, R.F. (2015) Novel properties for endoglucanase acquired by cell surface display technique. *Journal of Microbiology and Biotechnology* **25**, 1856-1862 https://doi.org/10.4014/jmb.1503.03029

69. Araujo, J.D., Ferreira, T.C., Rubini, M.R., Duran, A.G.G., De Marco, J.L., de Moraes, L.M.P., and Torres, F.A.G. (2015) Coexpression of cellulases in *Pichia pastoris* as a self-processing proten fusion. *AMB Express* **5**, 84 https://doi.org/10.1186/s13568-015-0170-z

70. Ou, J.S., and Cao, Y.C. (2014) Incorporation of *Nasutitermes takasagoensis* endoglucanase into cell-surface displayed minicellulosomes in *Pichia pastoris* X33. *Journal of Microbiology and Biotechnology* **24**, 1178-1188 https://doi.org/10.4014/jmb.1402.02034

71. Kickenweiz, T., Glieder, A., and Wu, J.C. (2018) Construction of a cellulose-metabolising *Komagataella phaffi (Pichia pastoris)* by co-expressing glucanases and β-glucosidase. *Applied Microbiology and Biotechnology* **102**, 1297-1306 https://doi.org/10.1007/s00253-017-8656-z

72. Shin, S.K., Hyeon, J.E., Kim, Y.I., Kang, D.H., Kim, S.W., Park, C., and Han, S.O. (2015) Enhanced hydrolysis of lignocellulose biomass: bi-functional enzyme complexes expressed in *Pichia pastoris* improve bioethanol production from *Miscanthus sinensis*. *Biotechnology Journal* **10**, 1912-1919 https://doi.org/10.1002/biot.201500081

73. Wei, H., Wang, W., Alahuhta, M., Wall, T.V., Baker, J.O., Taylor, L.E., Decker, S.R., Himmel, M.E., and Zhang, M. (2014) Engineering towards a complete heterologous cellulase secretome in *Yarrowina lipolytica* reveals its potential for consolidated bioprocessing. *Biotechnology for Biofuels* **7**, 148 https://doi.org/10.1186/s13068-014-0148-0

74. Guo, Z.P., Duquesne, S., Bozonnet, S., Nicaud, J.M., Marty, A., and O'Donohue, M.J. (2017) Conferring cellulose-degrading ability to *Yarrowia lipolytica* to facilitate a consolidated bioprocessing approach. Biotechnology for Biofuels 10, 132 https://doi.org/10.1186/s13068-017-0819-8

75. Cosgrove, D.J. (2017) Microbial expansins. *Annual Review of Microbiology* **71**, 479-497 https://doi.org/10.1146/annurev-micro-090816-093315

76. Martinez-Anaya, C. (2016) Understanding the structure and function of bacterial expansins: a prerequisite towards practical applications for the bioenergy and agricultural industries. *Microbial Bitoechnology* **9**, 727-736 https://doi.org/10.1111/1751-7915.12377

77. Tomazini, A., Dolce, L.G., Neto, M.D., and Polikarpov, I. (2015) *Xanthomonas campestris* expansin-like X domain is a structurally disordered beta-sheet macromolecule capable of synergistically enhancing enzymatic efficiency of cellulose hydrolysis. *Biotechnology Letters* **37**, 2419-2426 https://doi.org/10.1007/s10529-015-1927-9

78. Rocha, V.A.L., Maeda, R.N., Pereira, N., Kern, M.F., Elias, L., Simister, R., Steele-King, C., Gomez, L.D., and McQueen-Mason, S.J. (2016) Characterization of the cellulolytic secretome of *Trichoderma*

harzianum during growth on sugarcane bagasse and analysis of the activity boosting effects of swollenin. *Biotechnology Progress* **32**, 327-336 https://doi.org/10.1002/btpr.2217

79. Eibinger, M., Sigl, K., Sattelkow, J., Ganner, T., Ramoni, J., Seiboth, B., Plank, H., and Nidetzky, B. (2016) Functional characterization of the native swollenin from *Trichoderma reesei*: study of its possible role as C-1 factor of enzymatic lignocellulose conversion. *Biotechnology for Biofuels* **9**, 178 https://doi.org/10.1186/s13068-016-0590-2

80. Chen, C., Cui, Z.L., Song, X.F., Liu, Y.J., Cui, Q., and Feng, Y.G. (2016) Integration of bacterial expansin-like proteins into cellulosome promotes the cellulose degradation. *Applied Microbiology and Biotechnology* **100**, 2203-2212 https://doi.org/10.1007/s00253-015-7071-6

81. Nakatani, Y., Yamada, R., Ogino, C. and Kondo, A. (2013) Synergetic effect of yeast cell-surface expression of cellulase and expansin-like protein on direct ethanol production from cellulose. *Microbial Cell Factories* **12**, 66 https://doi.org/10.1186/1475-2859-12-66

82. Chylenski, P., Bisarro, B., Sorlie, M., Rohr, A.K., Varnai, A., Horn, S.J., and Eijsink, V.G.H. (2019) Lytic polysaccharide monooxygenases in enzymatic processing of lignocellulosic biomass. *ACS Catalysis* **9**, 4970-4991 https://doi.org/10.1021/acscatal.9b00246

83. Bissaro, B., Varnai, A., Rohr, A.K., and Eijsink, V.G.H. (2018) Oxidoreductases and reactive oxygen species in conversion of lignocellulosic biomass. *Microbiology and Molecular Biology Reviews* **82**, e00029 https://doi.org/10.1128/MMBR.00029-18

84. Hemsworth, G.R., Henrissat, B., Davies, G.J., and Walton, P.H. (2014) Discovery and classification of a new family of lytic polysaccharide monooxygenases. *Nature Chemical Biology* **10**, 122-126 https://doi.org/10.1038/nchembio.1417

85. Vu, V.V., Beeson, W.T., Span, E.A., Farquhar, E.R., and Marletta, M.A. 2014. A family of starch-active polysaccharide monooxygenases. *Proceedings of the National Academy of Sciences USA* **111**, 13822-13827 https://doi.org/10.1073/pnas.1408090111

86. Couturier, M., Ladeveze, S., Sulzenbacher, G., Ciano, L., Fanuel, M., Moreau, C., Villares, A., Cathala, B., Chaspoul, F., Frandsen, K.E., Labourel, A., Herpeol-Gimbert, I., Grisel, S., Haon, M., Lenfant, N., Rogniaux, H., Ropartz, D., Davies, G.J., Rosso, M.N., Walton, P.H., Henrissat, B., and Berrin, J.G. (2018) Lytic xylan oxidases from wood-decaying fungi unlock biomass degradation. *Nature Chemical Biology* **14**, 306-310 https://doi.org/10.1038/NCHEMBIO.2558

87. Wang, B.J., Walton, P.H., and Rovira, C. (2019) Molecular mechanisms of oxygen activation and hydrogen peroxide formation in lytic polysaccharide monooxygenases. *ACS Catalysis* **9**, 4958-4969 https://doi.org/10.1021/acscatal.9b00778

88. Kuusk, S., Kont, R., Kuusk, P., Heering, A., Sorlie, M., Bissaro, B., Eijskink, V.G.H., and Valjamae, P. (2019) Kinetic insights into the role of the reductant in H_2O_2-driven degradation of chitin by a bacterial lytic polysaccharide monooxygenase. *Journal of Biological Chemistry* **294**, 1516-1528 https://doi.org/10.1074/jbc.RA118.006196

89. Muraleedharan, M.N., Zouraris, D., Karantonis, A., Topakas, E., Sandgren, M., Rova, U., Christakopoulos, P., and Karnaouri, A. (2018) Effect of lignin fractions isolated from different biomass sources on cellulose oxidation by fungal lytic polysaccharide monooxygenases. *Biotechnology for Biofuels* **11**, 296 https://doi.org/10.1186/s13068-018-1294-6

90. Brenelli, L., Squina, F.M., Felby, C., and Cannella, D. (2018) Laccase-derived lignin compounds boost cellulose oxidative enzymes AA9. *Biotechnology for Biofuels* **11**, 10 https://doi.org/10.1186/s13068-017-0985-8

91. Laurent, C.V.F.P., Breslmayr, E., Tunega, D., Ludwig, R., and Oostenbrink, C. (2019) Interaction between cellobiose dehydrogenase and lytic polysaccharide monooxygenase. 2019. *Biochemistry* **58**, 1226-1235 https://doi.org/10.1021/acs.biochem.8b01178

92. Loose, J.S.M., Forsberg, Z., Kracher, D., Scheiblbrandner, S., Ludwig, R., Eijsink, V.G.H., and Vaaje-Kolstad, G. (2016) Activation of bacterial lytic polysaccharide monooxygenases with cellobiose dehydrogenase. *Protein Science* **25**, 2175-2186 https://doi.org/10.1002/pro.3043

93. Varnai, A., Umezawa, K., Yoshida, M., and Eijskink, V.G.H. (2018) The pyrolloquinoline-quinone dependent pyranose dehydrogenase from *Coprinopsis cinerea* drives lytic polysaccharide monooxygenase action. *Applied and Environmental Microbiology* **84**, UNSP e00156 https://doi.org/10.1128/AEM.00156-18

94. Yadav, S.K., Archana, Singh, R., Singh, P.K., and Vasudev, P.G. (2019) Insecticidal fern protein Tma12 is possibly a lytic polysaccharide monooxygenase. *Planta* **249**, 1987-1996 https://doi.org/10.1007/s00425-019-03135-0

95. Sabbadin, F., Hemsworth, G.R., Ciano, L., Henrissat, B., Dupree, P., Tryfona, T., Marques, R.D.S., Sweeney, S.T., Besser, K., Elias, L., Pesante, G., Li, Y., Dowle, A.A., Bates, R., Gomez, L.D., Simister, R., Davies, G.J., Walton, P.H., Bruce, N.C., and McQueen-Mason, S.J. (2018) An ancient family of lytic polysaccharide monooxygenases with roles in arthropod development and biomass digestion. *Nature Communications* **9**, 756 https://doi.org/10.1038/s41467-018-03142-x

96. Muller, G., Chylenski, P., Bissaro, B., Eijsink, V.G.H., and Horn, S.J. (2018) The impact of hydrogen peroxide supply on LPMO activity and overall saccharification efficiency of a commercial cellulase cocktail. *Biotechnology for Biofuels* **11**, 209 https://doi.org/10.1186/s13068-018-1199-4

97. Liang, Y.Y., Tong, S., Ang, E.L., and Zhao, H.M. (2014) Engineered pentafunctional minicellulosome for simultaneous saccharification and ethanol fermentation in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **80**, 6677-6684 https://doi.org/10.1128/AEM.02070-14

98. Guo, Z.P., Duquesne, S., Bozonnet, S., Nicaud, J.M., Marty, A., and O'Donohue, M.J. (2017) Expressing accessory proteins in cellulolytic *Yarrowia lipolytica* to improve the conversion yield of recalcitrant cellulose. *Biotechnology for Biofuels* **10**, 298 https://doi.org/10.1186/s13068-017-0990-y

99. Humphreys, C.M., and Minton, N.P. (2018) Advances in metabolic engineering in the microbial production of fuels and chemicals from C1 gas. *Current Opinion in Biotechnology* **50**, 174-181 https://doi.org/10.1016/j.copbio.2017.12.023

100. Philipps, G., de Vries, S., and Jennewein, S. (2019) Development of a metabolic pathway transfer and genomic integration system for the syngas-fermenting bacterium *Clostridium ljungdahlii*. *Biotechnology for Biofuels* **12**, 112 https://doi.org/10.1186/s13068-019-1448-1

101. Aboulnaga, E.A., Zou, H.B., Selmer, T., and Xian, M. (2018) Development of a plasmid-based, tunable, *tolC*-derived expression system for application in *Cupriavidus necator* H16. *Journal of Biotechnology* **274**, 15-27 https://doi.org/10.1016/j.jbiotec.2018.03.007

102. Asimakopoulos, K., Gavala, H.N., and Skiadas, I.V. (2018) Reactor systems for syngas fermentation processes: a review. *Chemical Engineering Journal* **348**, 732-744
https://doi.org/10.1016/j.cej.2018.05.003

Table 1. Production of ethanol from cellulosic substrates by pre-grown cells of engineered yeast strains: selected recent reports.

Host	Enzymes	Method	Substrate	Ethanol	Ref
S. cerevisiae	*T. reesei* EGII, *Neosartorya fischeri* CBH1, *Chrysosporium lucknowense* CBH2, *Saccharomyces fibuligera* BGL1 (consortium; other enzymes were tested)	Secretion	Avicel, 20 g/l	4.8 g/l*	51
S. cerevisiae	*Clostridium spp.* EG and CBH (15 combinations tested), *Neurospora crassa* cellodextrin transporter and BG	Surface display (minicellulosome); BG intracellular	CMC, 10 g/l PASC, 10 g/l Avicel, 10 g/l	1.1 g/l‡ 1.0 g/l‡ 0.8 g/l‡	60
S. cerevisiae	*T. reesei* EGII, *Talaromyces emersoni* CBHI, *Aspergillus aculeatus* BG	BG surface display, others secreted	PASC, 10 g/l	2.6 g/l	61
S. cerevisiae	*T. reesei* EGII, *Talaromyces emersoni* CBHI, *Aspergillus aculeatus* BG	Surface display (Sed1 anchor)	PASC, 10 g/l	2.9 g/l	61
S. cerevisiae	*C. thermocellum* endoglucanase, *T. emersoni* CBH1, *S. fibuligera* BG (others also tested)	Surface display (synthetic cellulosome with Aga2p/Aga1p)	PASC, 10 g/l	1.5 g/l	63
S. cerevisiae	*T. reesei* EGII and CBHII, *A. aculeatus* BG	Surface display (Aga2p/Aga1p)	PASC, 10 g/l	1.8 g/l	64
P. pastoris	*C. thermocellum* endoglucanase, *C. cellulovorans* xylanase	Surface display (minicellulosome)	CMC, 10 g/l	1.1 g/l	72
S. cerevisiae	*T. reesei* EGII and CBHII, *A. aculeatus* BG, *Aspergillus oryzae* Expansin-Like Protein	Surface display	PASC, 20 g/l	3.4 g/l *(2.5 g/l without ELP)*	81
S. cerevisiae	*T.* reesei EGII and CBHII, *A. aculeatus* BG, *Thermoascus aurantiacus* LPMO, *Humica insolens* CDH	Surface display (minicellulosome)	PASC, 10 g/l Avicel, 10 g/l	2.7 g/l 1.8 g/l *(1.5 g/l, 1.0 g/l without LPMO)*	97

*The system included 2 FPU/g supplemental commercial cellulase blend, insufficient to allow any ethanol production from Avicel in wild-type cells.

‡Data from figure 4c. Higher yields were obtained from CMC in growth and co-fermentation with 20 g/l galactose, but in this system no ethanol was produced from Avicel.

Figure Captions

Figure 1. Major components of non-food plant biomass and biological routes to their utilization

Figure 2. Cellulose and its enzymic deconstruction. (a) structure of cellulose chains in a microfibril. showing intrachain and interchain hydrogen bonds. (b) activity of endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases (BG).

Figure 3. Natural and engineered cellulosomes. (a) natural cellulosome structure in *Clostridium thermocellum*, as also expressed in *Bacillus subtilis* [33] (b,c,d) selected engineered variants expressed in *Lactobacillus plantarum* (b)[40] and *Saccharomyces cerevisiae* (c,d)[97,60]

Figure 4. Proposed reaction cycle and products of Lytic Polysaccharide Monooxygenases. Based on information in [82,83,87]

Cell wall

Cellulose chain   Oxidation of C^1 Or   Oxidation of C^4

D-glucono-
lactone end

D-gluconic
acid end

4-keto
glucose end

4-gem-diol
glucose end