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Review Article

ADVANCES IN CELLULOSIC ENZYME TECHNOLOGIES FOR ENHANCED STABILITY AND CATALYSIS

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ABSTRACT

Cellulosic ethanol has been the most promising second-generation biofuel in terms of raw material availability. The production process mandates efficient removal of lignin followed by a three-step sequential enzymatic conversion of cellulose to glucose. Cellobiase (E.C. 3.2.1.21), a β-glucosidase (BGL), obtained preferably from filamentous fungi catalyzes the final rate limiting step of this reaction, namely, the hydrolysis of cellobiose to glucose. It is therefore the most sought after model for cellulosic enzyme research. Efficient conversion of cellulosic biomass to glucose requires enhanced stability and superior catalysis. This in turn mandates strong producer organism able to secrete a high titer of the enzyme into the extracellular medium, optimized media formulation and improvised technologies for catalysis. Particularly, stabilization of the big cellobiose aggregates remains a significant technological bottleneck in this regard. Large aggregates of cellulosic enzymes are indispensible for industrial scale catalysis; however, these enzymes are prone to spontaneous dissociation by sheer dilution. Conventional immobilization and cross-linking approaches involving glutaraldehyde or entrapment in alginate beads have either proven cost-ineffective or have resulted in retention of poor specific activity for efficient catalysis. Over the last decade, new generation enzyme technologies such as synthetic multi-enzyme cellulosome complex, use of protein stabilizing osmolytes and reducing agents to maximize substrate exposure has opened up new avenues for cross-linker free stabilization and enhanced catalysis. The review is a fresh update of the producer strains, media optimizations and enzyme technologies to boost the production of cellulosic ethanol.

Keywords: Lignocellulosic bioethanol, Extracellular cellobiase, Filamentous fungi, Enzyme stabilization, Cellobiase aggregates.

1. INTRODUCTION

Sustainability is the most vital aspect of natural resource management of Mother Earth. This involves achieving operational efficiency and minimization of toxic impact on the environment, keeping in mind socio-economic considerations, all of which are mutually inter-dependent. However, a prolonged dependence on traditional fossil fuel energy resources is grossly unsustainable, owing to its super-swift-depletion worldwide following decades of overuse and misuse [1]. An acute energy crisis is thus looming large on the face of the globe due to the unprecedented increase in the consumption of fossil fuels, and it has been estimated that the projected depletion time of crude oil reserves at the current rate of consumption is only a meager forty years [2]. Associated with this is the uncontrolled, but steady emission of harmful greenhouse gases (GHGs) from the usage of fossil fuels, resulting in acute global warming [1], thus jeopardizing the environmentalists' dream of a self-sustainable, greener and technologically-sound Mother Earth.

Consequently, a collective quest for the discovery of carbon neutral solid, liquid and gaseous fuels as alternative, renewable energy resources for potential use for transportation purposes of vehicles has been kindled over the past few decades [1, 2]. One of the most promising and scientifically-sound developments in this regard has been the discovery of 'biofuels' or fuels

derived from geologically recent carbon fixation [3]. Production of such biofuels from pre-existing biomass is one of the safer and greener alternatives to reduce consumption of crude oils, much needed for the abatement of environmental pollution.

2. BIOFUEL: SCOPES AND TYPES

In order to be considered a biofuel, a fuel must contain over eighty percent renewable materials. The scope of this term encompasses a wide range of alternative fuel sources, and has evolved from the first generation (1G) bioethanol produced from sugar, starch or vegetable oil to the more advanced, energy efficient and economic third generation (3G) biodiesels derived from algae (fig.1). Bioethanol can be produced from three categories of agro-based raw materials-simple sugars, complex starch and the inedible, intricate plant biomass called lignocelluloses [3]. As the price of these raw materials usually varies largely, it can, in turn, significantly affect the cost of the large-scale production of bioethanol [3].

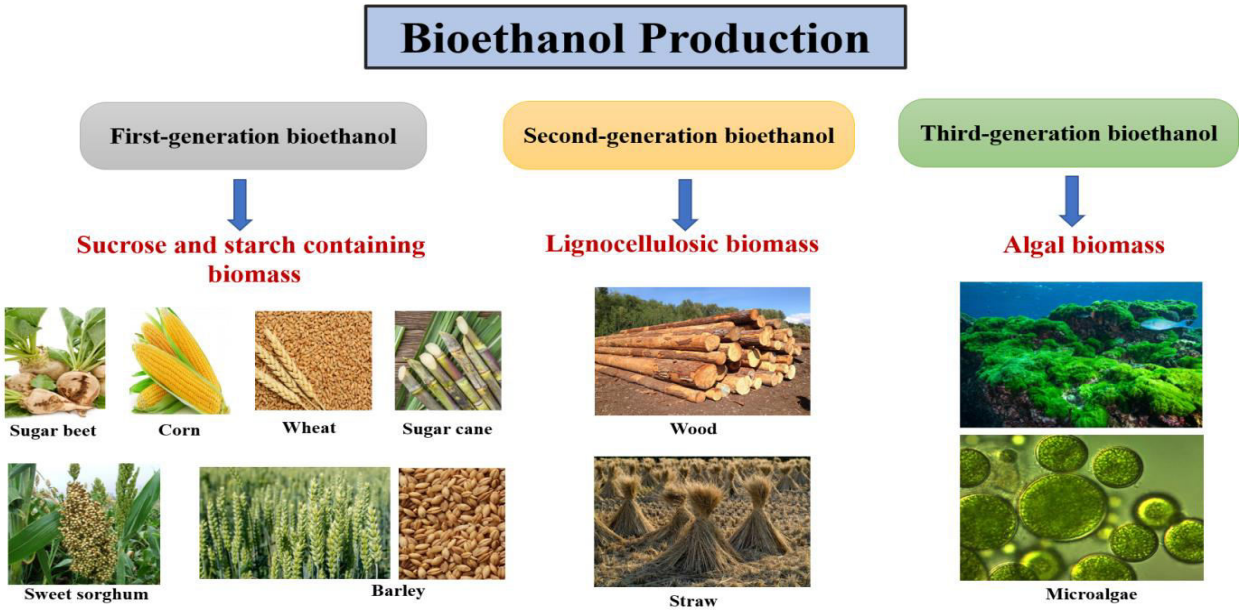


Fig 1: First generation, second generation and third generation biofuels as classified according to nature of raw materials used

Use of simple sugars and starch, akin to 1st generation bioethanol directly interferes with water conservation strategies, occupies a substantial area of agriculturally productive lands for the production of the fuel, consumes a significant part of world's forest reserves and puts a tremendous pressure on world food markets [1, 2]. For example, production of starch-based bioethanol from corn was much popular for quite a while due to the efficiency of the technology, but soon met with some inevitable socio-economic objections. The cultivation of corn encroached upon the farmlands dedicated for growing higher food crops, and thus threatened to breach the UN food security norms. *Jatropha* spp., another potential biofuel crop also met with the same fate. Under such circumstances, an improved thought for probing into an alternative source of bioethanol production was much needed. This set the ideal platform for the development of the most promising 2G biofuel-cellulosic (ligocellulosic) ethanol. As cellulose (lignocellulose) is the most abundant biomolecule on the earth, therefore the idea of its utilization for the production of cellulosic bioethanol is a sustainable, eco-friendly option for the biofuel industry. Also, such 2G bioethanol derived from lignocellulosic agriculture and forest residues, and from non-food crop feedstocks resolves some of the major conflicts raised by 1G bioethanol production [1, 2]. It releases significantly lesser amounts of GHGs as compared to corn-bioethanol. Additionally, the raw material required for its industrial production is present in surplus even in agro-wastes which are not considered as fodders, and therefore no additional farmland is required. So, the production of bio-based products and bioenergy from cheap, renewable sources like lignocelluloses is beneficial not only to the environment, but also to local economy and national energy security across the globe

[4]. Cellulose is derived mostly from plant sources acting as the largest contributor of the total cellulose pool of the biosphere [5]. Cellulose is a structural homopolysaccharide, present as an essential cell wall constituent of a vast niche of the Plant kingdom, ranging from the red algae to conifers. It is a linear homo-polymer of β-D-anhydroglucopyranoside residues, linked by β-(1,4)-glycosidic bonds, connecting several hundred to many thousands of glucose units. Although plant biomass is mostly cellulose (35-48%), it is occluded by hemicelluloses (22-30%) and lignin (15-27%), together constituting a complex intricate network of a biomolecule, known as lignocellulose [6, 2]. Being a low-cost energy resource based on its energy content ($3-4/GJ), cellulose (lignocellullose) has multifarious commercial applications, starting from the production of nanomaterials on one hand to applications in biofuel production, textile polishing and finishing, pulp and paper industry, and lifestyle agriculture on the other [4, 5, 7].

3. BOTTLENECKS IN INDUSTRIAL PRODUCTION OF CELLULOSIC BIOETHANOL

3.1. Efficient removal of lignin

Inspite of being the most promising feedstock for its easy availability and low cost, there has been a few technological constraints in the large-scale commercial production of bioethanol from lignocellulosic materials [3]. In fact, enzyme-catalyzed conversion of lingo-cellulosic plant biomass to simple sugars for the production of biofuels is highly inefficient a process, and is still considered as one of the key steps, limiting industrial biofuel production at a cheap cost [6]. One of the significant reasons hindering efficient bioconversion is the structural rigidity of the recalcitrant lingo-celluloses which hugely restrict the physical access of soluble microbial lignocellulases for depolymerizing the cellulose in order to release glucose for the production of bioethanol [8, 9]. As lignin is recalcitrant to microbial degradation, a pre-treatment process to loosen up the lignin is often mandatory for altering or removing structural and compositional impediments of lingo-celluloses by hydrolysis in order to improve the rate of enzymatic action and increase the yields of fermentable sugars [9]. Pre-treatment processing conditions must be tailor-made in accordance to the specific chemical and structural composition of the different sources of lignocellulosic biomass. Even if the toughest lignin is efficiently removed, the hemicellulose encapsulation reduces optimal enzyme exposure and hinders the breakdown of cellulose. An efficient method of removing this hemicellulose is the ammonia freeze explosion pre-treatment, which simultaneously reduces both lignin and hemicelluloses, as well as decrystallizes cellulose for an optimum hydrolysis [9]. Therefore, hydrolysis of complex lignocellulose into simpler fermentable sugars, sugar acids and phenolics has become a mandatory pre-requisite for a faster down-stream conversion into glucose [4, 9-11].

3.2. Enzymatic conversion of lignocellulose to glucose

It was in 1819 that the French chemist Henri Braconnot first discovered that cellulose could be hydrolyzed into sugars by treatment with sulfuric acid [3]. However, it was found subsequently that acidic hydrolysis suffered from a number of intrinsic disadvantages including denaturation of enzymes in the subsequent downstream fermentation process since the residual acid cannot be neutralized effectively [3]. With the rapid development of enzyme technologies over the past few decades, acid hydrolysis process of lignocellulose has gradually been replaced by enzymatic hydrolysis [3, 12]. Biocatalysts exert lesser corrosive effects to industrial processing equipments, and due to their extreme substrate specificity, they generally produce lesser toxic wastes to ensure better environmental sustainability [13].

Enzymatic hydrolysis of lignocellulosic material into fermentable sugars is carried out by a complex mixture of lignocellulolytic enzymes. Lignocellulolytic enzymes constitute mostly extracellular enzymes, including ligninolytic ones (peroxidases and oxidases) and hydrolytic ones (cellulases, hemicellulases, amylases, pectinases, chitinases, esterases, proteases and mannases) [11, 14]. Most of these enzymes find indispensible applications in diverse areas and are excellent study models for the research fraternity [15]. The various sectors routinely employing lingo-cellulolytic enzyme preparations include breweries, textile, animal feed, food, pulp and paper industries, and laundry, besides they being used as additive in detergents and for improving the digestibility of animal feeds [4, 13, 16]. The lignocellulosic wastes generated are converted by cellulases to commercially-important products like glucose, soluble sugars, enzymes, alcohol, and single cell proteins (SCPs) [17]. In the natural environmental niche, synergistic associations among different cellulolytic microbes play a significant role in the hydrolysis of lignocellulosic polymers [7]. The complex structure of lignocellulose is biologically

simplified into its monomeric building blocks, the β-D-glucosyl unit, which is subsequently fermented to different products such as ethanol (biofuel), acetic acid, lactic acid, antibiotics and others [5]. So, after efficient removal of lignin and hemicelluloses, the core cellulosic biomass is subjected to saccharification involving sequential and synergistic actions of three cellulolytic enzymes [8]; (1) endo-glucanases (EGs, E.C. 3.2.1.4), which cleave the cellulose chains internally at random sites, acting mainly on the amorphous parts of the cellulose fiber to generate oligosaccharides and new chain ends; (2) exoglucanases, including cellodextrinases (E.C. 3.2.1.74) and cello-biohydrolases (CBHs; E.C. 3.2.1.91 for the cellobiohydrolases acting on the non-reducing end, and E.C. 3.2.1.176 for the cellobiohydrolases acting on the reducing end), producing glucose, cellobiose and higher cellooligosaccharides; and (3) β-glucosidases (BGLs, E.C. 3.2.1.21, cellobiase) which catalyze the last rate limiting step to hydrolyze inhibitory cellobiose and short and soluble cello-oligosaccharides into additional glucose units [6, 16, 18, 19]. This process is collectively referred to as 'enzymatic saccharification' of cellulose. The final end product glucose is then converted to ethanol via yeast-mediated alcoholic fermentation.

A hallmark of this cellulase-enzyme system is synergy that ensures an efficient hydrolysis. This means that the catalytic activity of the entire enzyme-system is higher than the sum total of the respective activities of individual enzymes in the system [19]. The last step of the saccharification represents the technically most challenging step, and hence it constitutes the bottleneck for efficient saccharification. Therefore isolation and maintenance of a good producer strain of cellobiase is as critical as any other of the other processes during the actual fermentation.

4. PRODUCER MICROORGANISMS OF CELLULOSIC ENZYMES

The key elements in the saccharification process of lignocellulosics are the cellulolytic microorganisms [17]. To efficiently hydrolyze and degrade the insoluble biopolymer of cellulose, the microorganisms must secrete the cellulases, possibly excepting the BGLs, that are either extracellular or bound to the producer cell-surface [4]. Cellulolytic activity is hugely common among many genera in the Domain Bacteria, and within the fungal, protozoal, plant and a few animal groups in the Domain Eukarya [5, 19]. No cellulolytic genus has yet been identified in the Domain Archaea [19].

4.1. Fungal producers

Among all the lignocellulolytic microorganisms thrown a light upon, the fungi have been the subject of most extensive experimentation and research, due to their rich diversity and secretion of copious amounts of lingocellulolytic enzymes extracellularly [11, 20]. They are beneficial microbes that help in the recovery of energy from degraded ecosystems [21]. With a sound understanding of the enzyme production capabilities of these fungi, their industrial applications can be mapped easily [21]. Everyday large amounts of recalcitrant lingocellulosic wastes are dumped into the environment by mostly agricultural and industrial sources, leading to environmental pollution. Solid state fermentation (SSF) using these agro-industrial wastes as substrates for fungal growth is an efficient method to degrade such lignocellulosic masses by the cellulolytic fungi, in the process producing enzymes at low cost [22]. Such cellulolytic fungi can be easily isolated from natural resources like cattle dung and cattle dung-contaminated soil samples [23]. Scientists have found that these natural fungal isolates, once grown by SSF technique on non-pretreated saw dust, could produce copious cellulases to be suitably applied for bioethanol production [23]. The use of fungi like *Penicillium* and wine yeast would help biotechnologists to identify genes that could improve the production yield and nitrogen efficiency. Adaptive evolution over the years is also producing high-yielding strain (HYS) that have the potential to be directly applied in breweries at large. Among the fungal divisions, the widely-diverse subdivisions of Ascomycetes, Basidiomycetes (polypores), and Deuteromycetes harbor most of the cellulolytic species. More than hundred cellulolytic fungi have been reported till date, and this number is still increasing. Among the most well-studied aerobic fungi, belongs the yeasts and the mold genera *Trichoderma, Penicillium*, *Aspergillus*, *Termitomyces*, *Poria*, *Humicola*, *Acremonium, Chaetomium, Coriolus, Phanerochaete, Schizo-phyllum, Serpula, Fusarium, Geotrichum* and *Paecilomyces,* whereas the best studied anaerobic fungal cellulase producers are species of the *genera Piromyces* and *Neocallimastix* [19].

Sixty aerobic, non-*Saccharomyces* yeast strains have been isolated from grape musts in Uruguayan vineyards, including *Metschnikowiapulcherrima,* the best source of thermo stable (temperature optimum at 50°C) and acid-stable (pH optimum at 4.5) BGL [24]. Among the aerobic filamentous fungi, the soft-rot fungi *Trichoderma reesei* (teleomorph: *Hypocrea jecorina*), *Penicillium* spp. (like *P. purpurogenum, P. pinophilum, P. brasilinum*, *P.*

citrinum, *P. occitanis*, *P. decumbens*and*P. echinulatum*), *Aspergillus* spp. (like *A. niger*, *A. nidulans* and A. *oryzae*) and *Termito-mycesclypeatus* deserve special mention [6, 18. 25, 26].

Trichoderma reesei is the best-studied cellulolytic fungus, and are the most efficient producers of cellulases and hemicellulases [27, 28]. In more than one way, the extracellular cellulase enzyme-system of *T. reesei* has been instrumental for detailed studies on different cellulase systems from different sources [19]. The cellulase enzymatic system of *T. reesei* has five EGs and two CBHs, as well as some additional cellulolytic enzymes, such as the low-catalytic swollenin, vital for applications in the pulp and paper industries [19]. *T. reesei* secretes large amounts of all three types of cellulases - EGs, exoglucanases and BGLs, required for the degradation of crystalline cellulose [28].

Aerobic fungal cellulose degraders produce large amounts of extracellular enzymes [19]. Many *Penicillium* species can produce extracellular lignocellulases. The fungus *Penicillium purpurogenum* produces intracellular BGL, with maximum enzyme activity observed when sucrose is used as the substrate [29]. Various cultural parameters are thereby optimized for the cultivation of *P. purpurogenum* to enhance the production of BGL, which resulted in a maximum extracellular release of the enzyme after 96 h of cultivation at 30°C by the addition of amino acids like histidine and cysteine [29]. BGL of *P.purpurogenum* shows acid-stability (pH optimum at 2), thus being widely applied for debittering in breweries [25; 29]. *P. pinophilum* shows an extensive array of cellulase enzymes, containing eight EG components. An enzyme-preparation of particular lingocellulolytic impor-tance is the 1:1 (v/v) blended enzyme extract of *Chrysoporthe cubensis*: *P. pinophilum* [29]. A similar repertoire of extracellular cellulases is secreted by *P. brasilinum*, from whose culture medium, three different EGs and two CBHs were purified [29]. While *P. citrinum* MTCC 6489 strain produces high amounts of xylanase and cellulases, extracellular endo - β - 1, 4 - xylanase was obtained from *P. occitanis* Pol6, grown on oat-based xylan medium [29]. Although both the *Penicillium* species strains CR-316 and CR-313 secrete high levels of cellulases, *Penicillium* CR-316 produces thermostable cellulases, making the strain industrially viable [29]. Comparative genomics analysis of *P. chrysogenum* and *P. decumbens* (*P. oxalicum*) showed that *P. decumbens* has more genes for cellulases. Besides, it was also found that *P. decumbens* has more diverse components of the lignocellulolytic enzyme-system and hemicellulases, and their productions are induced in the medium when cellulose is the carbon source in wheat bran rather than glucose, in comparison to the much popular *T. reesei* [29].

4.2. Bacterial producers

Cellulases are produced in abundance by bacteria, particularly by the members of the Order Actinomycetales, of the anaerobic Order Clostridiales and of several aerobic Orders [19]. The cellulolytic machineries however differ between the aerobic and anaerobic bacteria [19]. For the first time, in the early 1980s, the scientists observed the multi-enzyme complex present in the thermophilic, anaerobic bacterium *Clostridium thermocellum*, which is meant for the degradation of lignocellulosic biomass, and termed it 'cellulosome' [19, 30]. Since then, cellulosome-producing bacteria have been isolated from a large variety of environments, indicative of their wide presence [30]. Like, apart from Clostridia, the cellulosome has been reportedly found in other anaerobic bacteria, including members of the *Ruminococcus* species, which are symbionts present in ruminant animals, with cellulo-somes attached onto their surface [2, 19]. Cellulosomes are the bacterial nanomachines required for dismantling of complex plant polysaccharides like lignocelluloses [30]. Not only that in a given species the cellulosomes exhibit intrinsic heterogeneity, but also between different species, there exist huge differences in the structure and composition of these cellulosomes [30]. Generally, a cellulosome comprise of a complex of 'scaffoldin' along with various enzymatic subunits. It is the structural subunit containing a huge diversity of secreted cellulases and other plant cell-wall digesting enzymes bound to a common protein scaffold [30, 31]. These scaffold proteins have 'cohesin' modules that bind conserved 'dockerin' modules on the enzymes [31]. Co-localization of these modules on the scaffold allows them to function synergistically [31]. The inter-subunit interactions in these multi-enzyme complexes are mediated by these cohesin and dockerin modules [30, 31]. The cellulosome is catalytically more efficient than the free enzyme system, because it has the unique property of properly orienting the whole enzymatic machinery onto the substrate surface to promote an effective interaction [19]. Most of the cellulosome systems studied so far have been shown to harbor cellulases from many glycosyl hydrolase (GH) families [19]. Because of their highly efficient structural

organization and associated hydrolytic activities, cellulosomes have much promising application in the degradation of lignocellulosic biomass, leading to its conversion to valuable products, like biofuels [30]. Genetic engineering techniques can also be used to improve the biodegradative action of cellulosomes by reconstituting cellulosomes with potent enzymes from different microbial sources [2].

5. FUNGAL CELLOBIASES: COMPLEX AGGREGATES OF GLYCOSIDASES WITH HIGHER STABILITY

Cellobiases from filamentous fungi are preferred over their bacterial counterparts owing to the high titre, specific activity, low K_m and high thermo stability of the former [8, 32]. The enzyme is secreted into the culture medium as big aggregates which form spontaneously [33]. However, catalysis and thermal stability of the enzyme are highly dependent on its intrinsic self-association [33] and this factor highly limits the enzyme efficiency. Additionally product inhibition by glucose represents another aspect to address during enzymatic conversion of cellulosic substrates [34]. In anaerobic bacteria and some fungi, cellobiase is an integral part of the cellulolytic enzyme complex structured in the form of a scaffold known as 'cellulosome', where upto eleven different enzymes are tethered in a definite array [35]. The arrangement of different proteins in this scaffold represents another layer of structural complexity in terms of optimizing its stability and performance. Therefore, a significant amount of biofuel research in recent times has been concentrated towards technological advancements for better understanding the functioning of these complex enzyme aggregates.

6. BOTTLENECKS AND PROMISES IN CELLULOSIC ENZYME TECHNOLOGIES

Understanding the cellulosic enzymes at the molecular level may help in unraveling some of the integral features that equip them with their catalytic prowess [21]. Cellulases have non-catalytic carbohydrate-binding modules (CBMs) and/or other functionally known or cryptic modules at the N- or C-terminal ends of the catalytic module [4]. It is the combined action of three major enzymes of the cellulase enzyme-system which determines the overall efficiency of cellulose degradation [7]. However, cellobiase (BGL) has the highest biotechnological value of all, since it regulates the final turnover of glucose, and at the same time reduces the inhibitory effect of cellobiose on EGs and CBHs [18]. Also, as BGL's substrate is soluble, the hydrolysis reaction is performed in the liquid phase, rather than on the surface of the insoluble cellulose particles, as in the case of EGs and CBHs [18]. To ensure improved industrial yields, individual cellulases are improved by either rational designing or by directed evolution [4]. As BGL activity has often been found to be rate-limiting during enzymatic hydrolysis of cellulose, the commercial cellulase enzyme preparations are often supplemented with BGL-activity [18]. Cellulolytic enzymes often suffer from low substrate affinity, thermolability and end product (glucose) inhibition. A higher temperature is needed during the enzymatic saccharification process to enhance the reaction rate, and minimize chances of microbial contamination. Therefore, finding an efficient cellulolytic enzyme preparation still eludes the biofuel industry.

6.1. Enzyme conjugation to nanoparticles

Over the last few decades, advancement in technology and newer scientific interventions has been able to provide some valuable insights about facilitating these enzymatic bioconversions. In order to minimise the recalcitrance of crystalline cellulose to enzymatic degradation, advanced technologies like conjugation of cellulases to synthetic nanoparticles (NPs) have been developed so as to increase the substrate accessibility as well as the catalytic efficiency of the enzyme [8]. Scientists have conjugated cellulase from the mold *Trichoderma viride* to polystyrene NPs, and tested the efficiency of the hydrolytic activity of this NP-conjugated complex on cellulosic substrates from purified and natural sources [8]. They found that the complexed enzyme displayed a higher efficiency in its action on microcrystalline cellulose [8]. Similarly, the NP-conjugated complex was observed to be more efficient in degrading natural cellulose structures in the thickened walls of cultured wood cells, both the results keeping hopes ablaze about the potential applications of cellulose-NP complexes in biofuel production from physically-intractable materials [8].

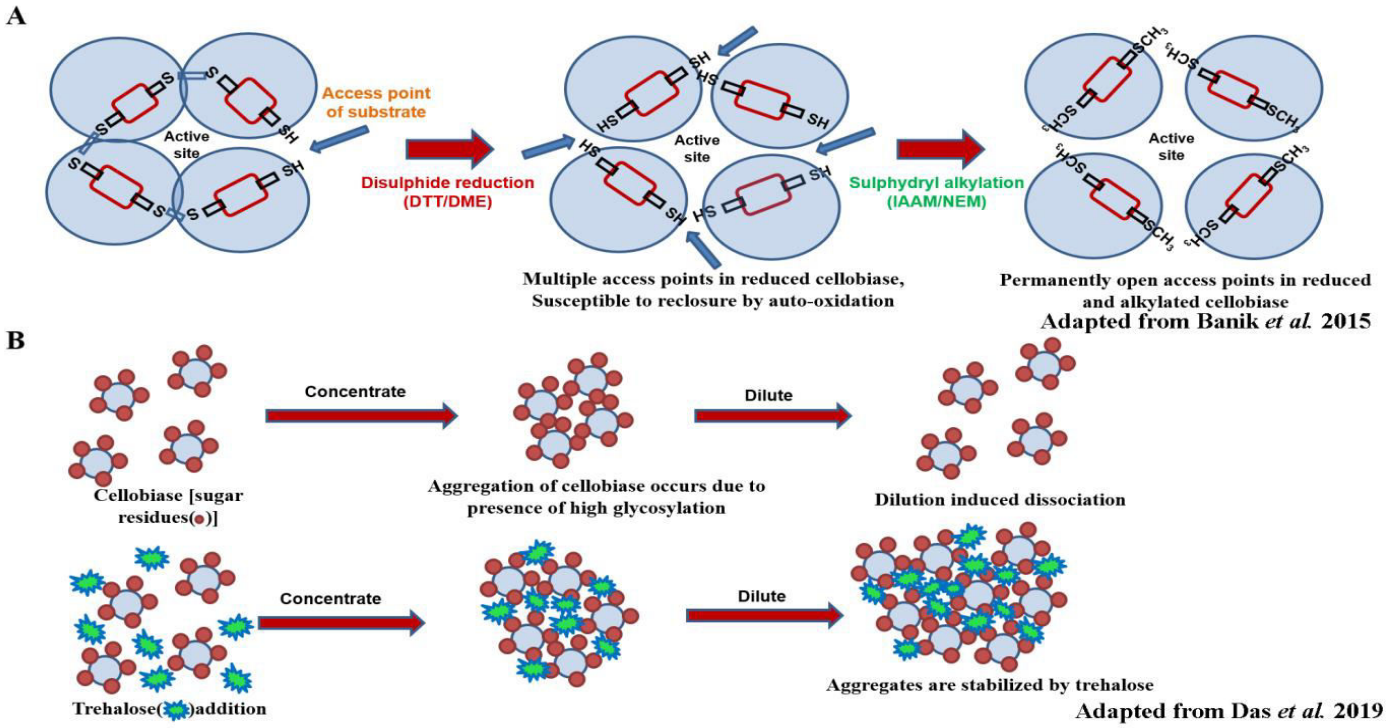
6.2. Stabilization of cellobiase assemblies for enhanced stability and catalysis

As stated earlier, fungal cellobiase is released extracellularly into the culture medium in co-aggregation with other enzymes, and the big aggregates subsequently associate again through reversible concentration driven protein-protein interaction. Both homoaggregation as well as heteroaggregation is vital for stability and catalysis of the protein [33, 36]. Therefore,

during the subsequent purification steps, activity and stability of the enzyme is severely hampered, mostly due to dissociation of the aggregates caused by sheer dilution. Therefore, stabilization of these unique natively aggregated fungal enzyme assemblies has been a major area of research for biotechnologists. Over the last few decades, quite a few cross-linking and immobilization based stabilization strategies have been reported (table 1) alongside a few immobilization independent approaches to increase enzyme catalysis (fig. 2A) and stability (fig 2B).

Table 1: Cross-linking/immobilization/other technologies used to stabilize cellulolytic enzyme aggregates

Brief description of technology used	Enzyme targeted	Reference
Cross-Linked Enzyme Aggregates (CLEAs)	Cellulase, Xylanase	[37]
Peptide chain extensions	Cellulase	[38]
Alteration of surface electrostatics	Cellulase	[39]
NPG (Nanoporous gold) immobilization of enzyme by physisorption	Xylanase	[40]
Cellulase nanoparticles	Cellulase	[41]
Immobilization of enzyme in an inorganic-organic hybrid support (TiO_2-lignin)	Cellulase	[42]
Immobilization of cross-linked enzyme aggregates (CLEA) on the amine-functionalized Fe_3O_4 @silica core-shell magnetic nanoparticles (MNP)	Cellulase	[43]
Immobilization of enzyme on magnetic nanoparticles encapsulated in polymeric nanospheres	Cellulase	[44]
Immobilization of enzyme on modified PVA (Polyvinyl alchohol) coated chitosan beads	Cellulase	[45]
Artificial multifunctional chimeras	Cellulase, Xylanase	[46]
Salt bridge design	Cellulase	[47]

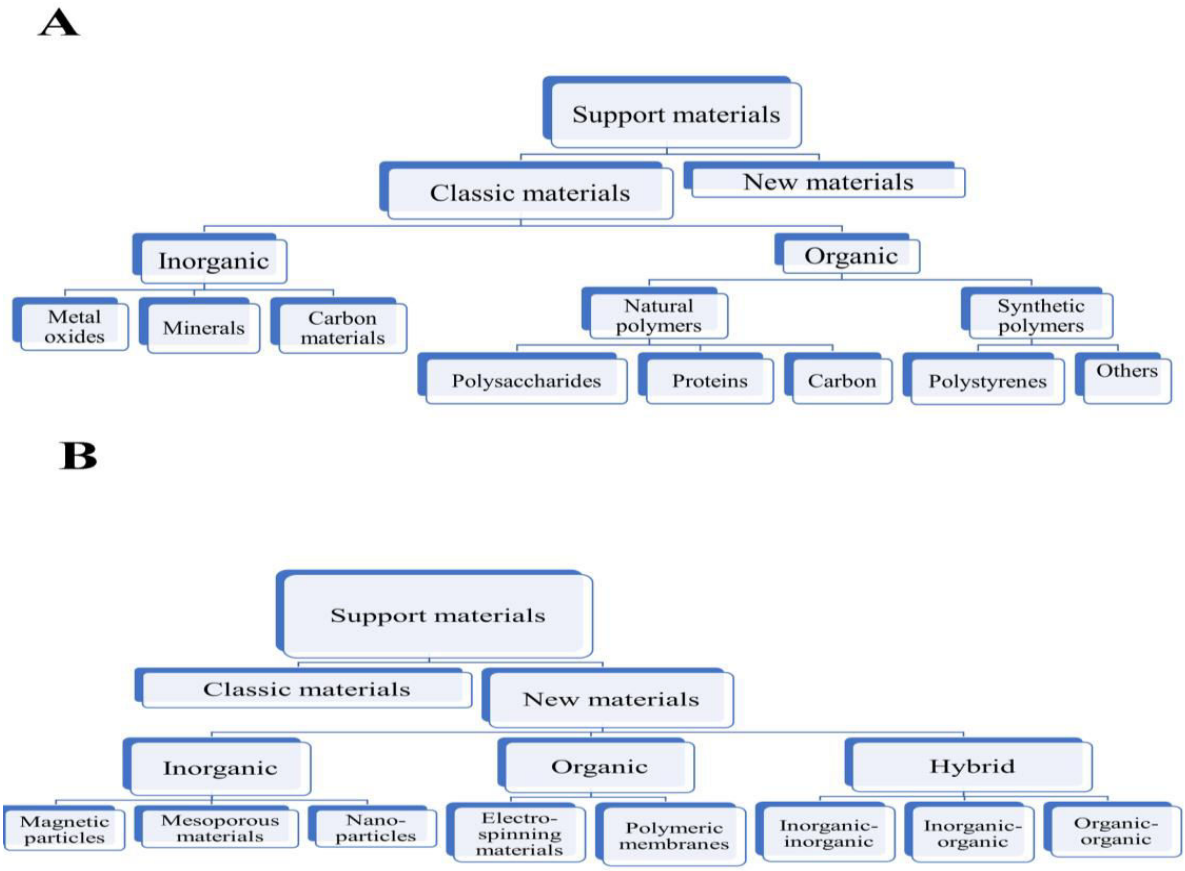


A) Addition of reducing agents decrease K_m of cellobiose [48] B) Trehalose stabilizes big aggregates of cellobiases against spontaneous dilution induced dissociation (49)

Fig 2: Newer technologies for *in situ* enhancement of cellobiose activity

6.3. Immobilisation/Cross-linking of cellobiase

With growing need in numerous application areas like food, pharmaceuticals, textile, chemical etc, industrial enzyme market expanded rapidly throughout the last few decades. In view of this, the process of immobilezation was in demand as it rendered the biocatalyst several advantages over the free one. First and foremost, the cost of operation is reduced significantly as the immobilized enzymes can be used repeatedly or recycled. In some cases, stability of the enzyme is also increased. Immobilization also imparts more control and usability of the enzyme as in downstream processing, immobilized enzymes are more easily separated. [50-52]. In addition to these, some enzymes had shown higher efficiency and regio/stereo-specificity or selectivity through immobilization [53]. Although the process of immobilization of enzymes dates back to 1940s, immobilized whole cells were in use long before, around 1815. Initially, single enzymes were immobilized, but later on from 1985, multiple enzymes were immobilized simultaneously for the ease of production and processing. Over the time, various methods were applied for immobilization and numerous support matrix were exploited for different catalytic processes. Support matrix plays very important role in providing suitable physical and chemical properties to the enzymes like hydrophilicity, compressibility, derivatization and compatibility etc. [53-57]. They can be broadly categorized into two classes-organic and inorganic [58]. Several natural as well as synthetic varieties are available for both of them (fig. 3).



A) Classic materials B) New materials [42, 58]

Fig 3: Support materials for enzyme immobilization

Porous matrices are preferred over non porous one as the enzyme loading is higher and immobilized enzymes are more protected from the environment. Considering the performance of the enzyme in hydrophilic environment, organic matrices are exploited in industries to a greater extent compare to inorganic matrices although the latter is more stable physically and chemically [59]. Interaction between the support matrix and the enzyme can be irreversible or reversible [60]. Covalent coupling, entrapment and crosslinking are irreversible

in nature whereas adsorption and disulfide linkages are reversible. The process of immobilization and support matrix vary depending on the type of the enzyme, the nature and conditions of the catalysis reaction. The interactions between the support matrix and the enzyme are also dependent on the method of immobilization [42]. This is especially important in case of covalent binding.

Betaglucosidase has been immobilized on several surfaces such as sponge [61], chitosan beads [62], clay minerals or mineral-organic substances, adsorbed via covalent binding to solid supports, agarose gels, nylon polymers, alginate gels, silica gel, Eupergit C, carbon nanotubes, SiO_2 nanoparticles [63], polyacrylamide gel, hybrid nanomaterials of magnetic iron oxide nanoparticle with graphene oxide [64] etc. Recently wrinkled and mesoporous silica nanoparticles [65] and super-paramagnetic nanoparticles [66] were also explored for their effectivity. Polyacrylamide has been in use as matrix since long because of its low cost, chemical stability, uniform physical state and porosity. One thermo stable betaglucosidase from *Bacillus subtilis* was investigated after immobilization in polyacrylamide gel for extraction of phenolics from sugarcane juice. Results showed concentration of reducing sugar was more in case of free enzyme (9.438mg/l) compared to the immobilized one (8.134mg/l). Reduced activity of the immobilized enzyme may be due to several reasons like denaturation and leakage of the enzyme from gels because of use and diffusion effects [67]. In polyacrylamide gels, enzyme is exposed to a strong electrostatic field due to highly electronegatively charged acryl acid groups created in the macro-environment. This also affects the enzyme activity. Moreover, immobilization may change the structure and steric interactions of the enzyme in three-dimensional network [68]. When betaglucosidase from Novozyme's cellulosic ethanol enzyme kit was immobilized on 29 nm mesoporous silica particles both via physical adsorption and covalent binding, it resulted in increase in the activity and substrate affinity of the enzyme compared to the adsorbed enzyme. Physical absorption was done with 50mM citrate buffer at pH 4.7 and covalent linking was performed via glutaraldehyde. NaCl helped in adsorption whereas Triton X-100 decreased the adsorption. This explained the electrostatic attractive forces between the enzyme and the support surface. Whereas Triton-X 100 might weaken van der Waals interaction and impose additional physical hinderance via formation of small aggregates [69]. Super paramagnetic iron oxide nanoparticles are small synthetic particles of core size less than 10nm. They are well dispersed in liquid and can easily be removed applying magnetic field. Olive betaglucosidase immobilized on super paramagnetic iron oxide nanoparticles showed higher thermal stability compared to the free enzyme [70]. Alginate, a polysaccharide of glucuronic and mannuronic acids, is a popular support matrix for immobilization due to its compatibility and processivity. Immobilized betaglucosidase showed high activity at 50°C and 80°C but substrate affinity and reaction velocity were lower compared to the free enzyme. The active sites might be less accessible to the substrate molecules and decreased affinity of the enzyme towards the substrate at higher temperatures may also be attributed to the internal diffusion of the immobilized enzyme.

Immobilisation of the enzyme has also been tested on macro porous carrier *Amberlite IRA 900 Cl* via adsorption with subsequent crosslinking by glutaraldehyde. Although reaction velocity was increased, there was a significant increase in K_m as compared to the native enzyme. This may be attributable to the immobilization of the enzyme molecules at sites or positions with decreased substrate accessibility. On the other hand, ionic and covalent bonds and diffusive effects created by immobilization increased the stability of the enzyme against heat inactivation [71]. The enzyme also retained high cellulolytic activity at acidic pH. In other studies, crude culture filtrate of *Aspergillus niger* was immobilized on various carriers via adsorption, covalent bonding, ionic bonding and entrapment [61]. When properties of immobilized betaglucosidase were compared with free enzyme, covalently linked immobilized enzyme showed higher thermal stability but lower substrate affinity and reaction velocity. In addition, the immobilized enzyme was also found to be more resistant to the inhibitory effect of different chemicals. However, enzyme activity recovery was not much in case of several matrices like chitosan, sol-gel beads, sodium alginates, Eupergit C and S-layer [62]. Betaglucosidase immobilized on magnetite again showed low activity recovery and poor accessibility towards substrate. Contrary to the usual trend of immobilized enzymes, enzymes immobilized on super paramagnetic particles showed lower K_m value or higher substrate affinity compared to the free enzyme. It also rendered the immobilized enzyme reusable with enhanced thermostability and higher shelf life. Similar observations were obtained when betaglucosidase was immobilized on non-porous magnetic

particles activated with cyanuric chloride and poly-glutaraldehyde. Immobilized enzyme showed greater thermostability and better performance in hydrolysing lignocellulosic material.

Traditional approaches of stabilization of enzyme aggregates through the use of cross-linking agents such as glutaraldehyde were initially in vogue [72, 73]. However, use of glutaraldehyde was a non-ecofriendly costlier option, and had to be abandoned soon. A cellobiase preparation covalently coupled to cyanogen bromide was reported to have enhanced thermostability with a lowering of K_m [74]. Immobilization of cellobiase in Concanavalin A-sepharose beads followed by entrapment in polypropylene glycol-alginate beads stabilized the aggregates for about four consecutive days [75]. In a more recent study, bubbling immobilisation of cellobiase in sodium alginate with chitosan as carrier achieved a significant reduction in K_m with enhanced catalysis at higher temperature [76]. Optimized immobilization was subsequently achieved through Response Surface Methodology tool [77]. A new and promising cross-linking independent approach of stabilizing the cellobiase aggregates has been reported employing trehalose, a small molecule osmolyte [49]. Trehalose is thought to act by docking onto cellobiase aggregates through replacement of hydrogen bonded water from solvent accessible surface area. In this way, the big enzyme aggregates are shielded by a viscous trehalose matrix which prevents their subsequent dissociation on dilution.

7. GENETICALLY ENGINEERED CELLULOSIC ENZYMES

A thrust area to improve performance of cellulolytic enzymes over the last decade has been the use of genetic engineering tools to design cellulolytic enzymes with improved catalysis and high thermostability, the two indispensible attributes for successful, revenue-earning commercialization of lignocellulose-biorefineries [4]. These thermostable and alkali-tolerant lignocellulolytic enzymes are gaining much attention as biocatalysts due to their robust activities at both high temperature and alkaline pH [13]. The development and optimization of suitable fermentation processes for the production of such thermo-alkali-stable lignocellulolytic enzymes, including thorough research on producer micro-organisms (i.e. strain screening and strain improvement via mutation and other recombinant techniques) and their performances, optimization of bioreactors, culture media (i.e. fixing concentrations of particularly C and N) and other fermentation parameters like temperature, pH, inoculum size, speed of agitation, rate of aeration, and dissolved oxygen tension (DOT), along with different modes of operation (*i.e.* submerged fermentation, SmF and solid state fermentation, SSF) are nowadays dealt with in much details for a successful, eco-friendly and low-cost industrial application [13]. Therefore future prospects in lignocellulolytic enzyme research are directed towards bio-prospecting of robust and new fungal enzymes to overcome the challenges in the degradation of recalcitrant, stubborn wastes and complex physiological regulations of enzymes, use of multiple high-yielding fungal strains or a mixture of purified enzymes from such different sources, use of unique and novel inducers to maximize enzyme production, gene cloning to screen for new generation of enzymes, and application of various other genetic engineering techniques to widen the horizon of applicability of these enzymes [14]. With the development of modern tools such as genomics and proteomics, the entire protein content of the cellulosomes and their respective expression levels can now be studied and appropriately controlled. Over the past two decades, integrated genomics have been used to find new cellulosomal genes, providing information that has led to a better understanding of the structure-function relationships of the cellulosome systems [19]. An 18-subunit protein complex called a 'rosettasome' was found by scientists to be amenable for genetic engineering, so that they can bind to dockerin-containing enzymes in order to function like an 'artificial' cellulosome [31]. Rosettasomes are thermo stable, Group II chaperonins isolated from the hyper-thermo-acidophilic archaeon Sulfolobusshibatae [31]. By artificially fusing a cohesin module from Clostridium thermocellum to a circular permutant of a rosettasome subunit, it was demonstrated that the cohesin-rosettasomes (these engineered multi-enzyme structures are termed 'rosettazymes') bind dockerin-containing exo- and endo-gluconases. These bound enzymes were reported to show increased cellulolytic activity compared to free state in solution, and that this increased activity depends on the number and ratio of the bound glucanases [31]. On the other hand, the lactic acid bacterium (LAB) Leuconostocmesenteroides produce an intracellular thermostable (temperature optimum at 500C), acid-stable (pH optimum at 5.5-6.0) and low Km (0.07 mM for p-nitrophenyl-β-D-glucopyranoside or pNPG, high substrate affinity) BGL when grown on an arbutin-containing medium [78].

8. USE OF ENGINEERED STRAINS FOR CELLULOLYTIC ENZYME PRODUCTION

As the cellulase enzymes are produced in very small quantities by the wild-type (WT) organisms, strain improvement in industries are extensively carried out by extensive application of mutation and selection processes [17] (table 2). Such improved HYS can cut down the cost of the production processes by the virtue of an increased yield, alongside providing some specialized characteristics to the products.

Table 2: Recombinant strains used for increased cellulolytic enzyme production

Recombinant strain	Gene altered	Reference
Trichoderma reesei	Cel7A	[80]
Saccharomyces cerevisiae	CBH1	[81]
Trichoderma reesei	cel3A	[82]
Aspergillus oryzae	eg1	[83]
Pichia pastoris	CBH3	[84]
Saccharomyces cerevisiae	CBH2	[85]
Pichia pastoris	bgl1	[86]
Trichoderma reesei	xyn2	[87]
Pichia pastoris	XylA	[88]
Pichia pastoris	BCC7197	[89]
E. coli	Xynsig	[90]
Hansenula polymorpha	xyn2	[91]
Hansenula polymorpha	xlnD	[91]
Pichia pastoris	PcXylB	[92]
E. coli	xynB	[93]

Genetically-engineered strains derived from *A. niger* are now finding much applications in industrial processes [6, 19]. Cellulolytic enzymes are abundantly expressed in many different *Aspergillus* species, like endoglucanases A (*eglA*), B (*eglB*), and C (*eglC*), which are members of GH families 12, 5, and 74, respectively; cellobiohydrolases A (*cbhA*) and B (*cbhB*), both of which are members of GH family 7; and a GH3 BGL (*bglA*) in the industrially important *A. niger*. Numerous genes for lytic polysaccharide monooxygenase (LPMO) enzymes from the GH61 family are also traced in *A. niger*, *A. nidulans* and *A. oryzae* [18]. A study has reported an increase in enzymatic efficiency by co-culturing two different fungal strains [79]. This study indicated that the cellulases obtained from a compatible mixed culture obtained after simultaneous mixing of both *A. niger* and *P. chrysogenum* have more lignocellulolytic activity as compared to their respective pure cultures when grown on the solid wastes of sugar and paper industries including baggase, paper waste and cotton waste by SSF technique [79]. It was revealed that newspaper, an industrial carbon waste, at pH 5.0 and 40°C temperature was the best source of carbon for the enhanced production of cellulase in the compatible mixed culture after 8 days of incubation, at the same time reducing much of the environmental pollution [79].

Genomic analysis reveals that orthologs of multiple components like Cre1, ACEI and LaeA have been found to control cellulase production in *T. reesei* [29]. Nowadays, much of the research in this field has been focused on mutation and selection of better *T. reesei* strains for the commercialization of cellulase enzymes, and both WT and genetically-engineered strains derived from *T. reesei*are being used in industrial processes [6,19].To date, 17 cellulase-encoding genes have been discovered and cloned from *T. reesei*, of which only four cellulases (Cel5A, Cel6A, Cel7A and Cel7B) are secreted in large quantities (90-95% of the total secreted cellulases) into the culture medium [28]. CBHs constitute about 80% of the total cellulolytic proteins in the secretome of *T. reesei* [19]. A cre1$^-$ *T. reesei* mutant created was found to over-expresses Cel61A (encoding an EG) twice as much as the WT under cellulose-inducing conditions [29]. The use of DNA microarray technology for the identification of new *T. reesei* genes involved in cellulosic biomass conversion via high throughput analysis of expression of cDNA libraries generated by suppression subtractive hybridization (SSH) is also already underway [27]. Presently, the hyper-secreting mutant Rut C-30 strain of *T. reesei* is generated by three steps of random mutagenesis (successive treatments with UV light, *N*-nitroguanidine, and again UV light), which is a highly efficient extracellular enzyme protein producer (around 19 g L^{-1}) [28]. Although another mutant strain, CL847, was found to yield higher amounts of extracellular protein (about 40 g L^{-1}), the proteins secreted from Rut-C30 has been reported to have a higher percentage of cellulase [28]. Up to seven BGL genes have been found in the *T. reesei* genome. Their expression is regulated by a unique system different from that of other cellulases. It was found that, BglR, a specific regulator of BGLs, is an activator for efficient BGL expression in *T. reesei* [29]. However, as they are mostly intracellular proteins, the action of a lesser percentage of extracellular BGLs is insufficient to completely degrade the cellobiose to glucose [28]. As a result, cellobiose accumulates in the media to cause feedback inhibition of the endo-and exo-glucanases [6, 28]. So, to overcome this problem,

commercial cellulases are often composed of multi-component enzyme mixtures produced by different fungal strains. Complete hydrolysis of cellulose by purified *T. reesei* BGL is made possible only after its supplementation with a commercial BGL preparation from *A. niger* (Novozymes SP188) [6, 28]. Studies on possible differences between wild-type *T. reesei* BGL1 and supplemented SP188 BGLs shows that SP188 BGLs (K_m for cellobiose = 0.57 mM, low substrate affinity) has a lower specific activity than *T. reesei* BGL1 (K_m for cellobiose = 0.38 mM, around 1.5-fold high substrate affinity), and is also more sensitive to glucose inhibition [6]. Presently, several *T. reesei* cellulose cocktails with improved BGL activities have been commercialized by MNCs like Novozymes (C-Tec cocktail) and Genencor International Inc. (Accellerase cocktail) [28]. However, use of such cellulase mixtures turned out problematic in their applications in pulp and paper industries, because of their non-specificity and hyper-degradative power, much detrimental to the strength properties of the fibers being processed [19]. Hence, recent advance-ments in modern biotechnological tools have resulted in commercial mono-component cellulase preparations targeted for specific process applications, like EGs meant for textile processing finding their use in pulp and paper processing as well [19].

Orthologs of Cre1, ACEI and LaeA also control the cellulase production in *P. decumbens*, just like *T. reesei* [29]. Interestingly, CreA and its orthologs majorly repress cellulolytic gene expression in *P. decumbens*, making their deletions important for constructing industrially-important cellulose hyper-producing strains [29]. On the other hand, species or genus-specific regulators like ACEII, ENVOY, Xpp1, and GRD1 are diminished in *P. decumbens*. While lactose induces the expression of lignocellulolytic genes in *P. decumbens* at lower concentration, sophorose, the transglycosy-lation product of BGL, cannot induce the expression of cellulolytic genes in the same fungi [29]. Experiments involving mutagenesis are also widely carried out nowadays in order to alter the expression of regulatory genes of *P. decumbens* for maximizing cellulase produc-tion. Although random mutagenesis is often applied, it is not only cumbersome and time-consuming, but most of the times, also screens out for undesired phenotypes. However, in this regard, the HYS JU-A10-T of *P. decumbens* is industrially well-acclaimed [29]. An over-expression of cellulose regulator B (ClrB) activator was associated with a drastic increase in cellulase production in *P. decumbens*. As functional redundancy was seen to be exhibited by cellodextrin transporters (Cdt) in *P. decumbens*, the deletion of any one among CdtC, CdtD, and CdtGcellodextrin transporter proteins does not affect cellulase expression in it. Similarly, strain carrying carbon catabolite repressor, removal of *creA/cre1* was partially unrepressed in both *P. decumbens* and *T. reesei*, thus improving FPA activity by 1.5-fold under induction condition in *P. decumbens*. Further, deletion of *creA* and over-expression of the activator ClrB in the same fungus was found necessary to avoid the dependence on inducer for maximum cellulose production. Other double-gene mutations including overexpression of *clrB* with *bgl2* deletion and alternating, the double deletion of both *creA* and *bgl2* were found able to induce the expression and over-secretion of cellulase enzyme. A triple-mutant strain of *P. decumbens*, RE-10, created by three-step genetic engineering for the purpose of induction of cellulolytic gene expression in comparison to the WT *P. decumbens*, is the best genetically-engineered strain till date. However, the activity of extracellular BGL of this RE-10 mutant is undetectable in comparison to WT strain, a phenomenon observed only in presence of cellulose, but not wheat bran, and no homolog of *bglR* exists in its genome. The EG Cel61A with a CBM oxidatively degrade cellulose using redox-active cofactor, which could only be found secreted by this high-cellulase producing RE-10 mutant. Four GH61 family protein-encoding genes have been identified in the genome of *P. decumbens*, two of which were detected in the RE-10 strain as well, which are candidate-genes for studies on probing into the alternative lignocellulose-degrading mechanism in molds, and also on how to supplement industrial cellulases with improved hydrolytic activity. Moreover, several hemicellulases are also elaborated by RE-10, including α-l-arabinofuranosidase, endo-β-1,4-xylanase, β-xylosi-dase, and β-1,3-glucanosyltransgly-cosylase [29]. Mutants of *P. echinulatum* can produce large amounts of cellulases and its thermostable enzyme-complex (temperature optimum at 50°C). This enzymatic complex of *P. echinulatum* is capable of exhibiting higher glucose-dase activities as demonstrated by filter paper activity (FPA, representing overall cellulose activity) assays in comparison to the enzymatic complex of *T. reesei* Further studies with the *P. echinulatum* 9A02S1 mutant strain have shown that presence of lactose and cellulose both induces their production of cellulases, but when lactose was used as the sole C-source, cellulase secretion was not found,

quite in contrary to the results observed for *T. reesei* [29].

9. OPTIMIZATION OF PRODUCTION MEDIA

Optimization of carbon and nitrogen sources is a critical aspect in obtaining an industrial titre of cellobiase. Experiments using residues from cheap agro-wastes like mustard stalk and straw (MSS) with a low lignin percentage and high cellulose and hemicellulose contents indicated that these raw materials could be utilized for a huge yield of glucose by the action of lignocellulolytic enzymes by *Termitomycesclypeatus* in submerged culture [10]. Enzyme productions increased by 2-10 times through addition of commonly available cheap agro-residues such as wheat bran and rice straw (MWR) in 1:1:1 ratio and by using alkali-treated MSS (TMSS) into the fermentation media. The enzymes obtained from the MWR and TMSS media were found to saccharify 10% (w/v) wheat bran up to 53% and 58% in 24 h, results indicating that MSS from India has a huge potential as a cheap and renewable raw material for production of bioethanol. In a few Basidiomycota like *Termitomyces clypeatus*, extracellular release of cellobiase and other glycosidases is found to increase significantly under secreting conditions induced by addition of sodium succinate in the medium [94]. The titre of extracellular cellobiase was further boosted through inclusion of glycosylation inhibitors like 2-Deoxy-glucose in the culture medium [36; 26]. This under-glycosylated cellobiase was also found to be resistant towards proteolytic as well as endoglycosidase-H digestion and showed higher stability and specific activity than its native counterpart [26]. Analyses of metabolic enzymes, together with molecular analyses through proteomic profiling and transcriptomics data revealed that 2-Deoxy-glucose exerted its effect through catabolite repression [69], and subsequent induction of metabolic stress in the fungus [95]. Concomitant with the reformulation of production media, a few studies have also reported that *in situ* inclusion of small molecule additives like salts, detergents, amino acid and vitamins can also substantially boost activity and catalytic efficiency of extracellular cellobiase activity (table 3).

Table 3: Additives used in the production media for increased enzyme titer

Additives used	Carbon source	Nitrogen source	Enzyme produced	Reference
Lichenan	Lichenan	Peptone	Bacterial cellulase	[97]
β-glucan	β-glucan	Peptone	Bacterial cellulase	[97]
Cotton seed	CMC	$NaNO_3$ and cotton seed	Endoglucanase, glucoamylase, and xylanase	[98]
L-cystine	CMC	$NaNO_3$	Endoglucanase, filter paperase and β-glucosidase	[98]
Valine	CMC	$NaNO_3$	Endoglucanase and glucoamylase	[98]
Thiamine	CMC	$NaNO_3$	Endoglucanase and glucoamylase	[98]
Riboflavin	CMC	$NaNO_3$	Endoglucanase and glucoamylase	[98]
$CoCl_2$	CMC	$NaNO_3$	Endoglucanase and glucoamylase	[98]
$MnCl_2$	CMC	$NaNO_3$	Endoglucanase and glucoamylase	[98]
Lactose	Lactose	Peptone	Fungal cellulase	[99]
$CaCl_2$	CMC	3,5-Dinitrosalicyclic acid	Endoglucanase and β-glucosidase	[100]
$MgCl_2$	CMC	3,5-Dinitrosalicyclic acid	Endoglucanase and β-glucosidase	[100]
KCl	CMC	Peptone	Endoglucanase	[101]

Most of them are either thought to act by loosening up the aggregates for better substrate availability or increase production of enzymes by supplementing nutrient resource [96]. In one such study, the activity of the enzyme from *T. clypeatus* was found to increase in the presence of common reducing agents like dithiothreitol (DTT) and β-mercaptoethanol (ME), with a resultant decrease in K_m (thereby signifying an increase in substrate affinity) from 0.4 mM to 0.3 mM in case of DTT, and from 0.4 mM to 0.35 mM in case of ME [48]. Thus it was postulated that the reduction of disulphide bonds allowed for a better cellobiase-substrate interaction. Catalysis was further observed to be enhanced if the reduced enzyme was pre-alkylated in accordance with the hypothesis that reduced thiols can be aerially oxidized to reform the di-sulphides, thus hindering active site accessibility of the substrate.

However, despite all technological advancements, the huge cost of purified cellulases required for the hydrolysis of pre-treated lignocellulosic materials poses

the biggest obstacle for the successful commercialization of biomass biorefineries, because a large amount of this enzyme is consumed for biomass saccharification [4]. So, for a successful decrease in cellulase usage, increase in volumetric productivity and reduction in capital investment, consolidated bioprocessing (CBP) is nowadays practiced through successful integration of cellulase production, cellulose hydrolysis and ethanol fermentation in only a single step [4]. Also, over the last few years, the traditional way of cellulose degradation has been remarkably re-framed by the discovery of a novel class of enzymes, particularly abundant in the saprophytic fungi known as lytic polysaccharide monooxygenases (LPMO)[18]. Together with cellobiose dehydrogenase (CDH, E.C. 1.1.99.18), LPMO forms an oxidative enzymatic system that speeds up the enzymatic hydrolysis of lignocellulose [18]. These enzymes require copper for their activity, and cleave cellulose oxidatively using electrons from CDH or even ascorbate [18]. Often LPMO genes might even outnumber cellulase genes, although the exact mode of action of these encoded enzymes still remains obscure. The discovery of LPMO thus shatters the age-old notion that only hydrolytic enzymes are the main pioneers in the degradation of recalcitrant cellulose and hemicelluloses to simpler fermentable sugars.

10. CONCLUSION

Ethanol-from-cellulose (EFC) holds a great potential due to the widespread availability and relatively low cost of cellulosic materials. However, although several EFC processes are technically feasible, cost-effective processes are often difficult to achieve. Therefore, we are yet to go a long way to ensure stable and cheap supply of raw material from cellulosic substrates. Based on the present scientific and technical knowledge, third generation biofuels derived from microalgae are considered to be a technically viable and efficient alternative energy resource**.** The current limitations in conversion of cellulose to ethanol are believed to be alleviated in near future with development of modern tools and cutting-edge research. This will facilitate the production and use of various new generation cellulosic ethanol and the latest algal biomass-based third generation biofuels.Therefore, with the technology in hand, the need of the hour is an increased collaboration between public and private sectors for economical production, implementation of fair Government policies and promotion of mass awareness. Then, we may not be far from gifting our future generation a cleaner, greener and sustainable Mother Earth.

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Conflict of interest

All authors have seen and agreed upon the final version of the manuscript. There is no conflict of interest with any authority.

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