12

Heterologous Expression and Extracellular Secretion of Cellulases in Recombinant Microbes

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1. Introduction

Lignocellulose, starch, sucrose, and macroalgal biomass are different forms of plant biomass that have been exploited for bioethanol production. Among them, lignocellulose, found in both agricultural and forest waste, has attracted great attention because of its relative abundance in nature (Lynd et al. 2002). Lignocellulose is a complex polymer made up of cellulose, hemicellulose, and lignin. Efficient conversion of lignocellulose into bioethanol involves a series of steps, namely, the collection of biomass; pretreatment to dissolve lignin; size reduction to reduce the number of recalcitrant hydrogen bonds; enzymatic saccharification to yield simple sugars; and, finally, fermentation of the sugars to ethanol. The main hurdle in this process is the lack of low-cost technology to overcome the recalcitrance associated with lignocellulose (Lynd et al. 2002; Himmel et al. 2007; Xu et al. 2009). Pretreatment is needed to dissolve the lignin, and enzymes such as xylanases are needed to hydrolyze the hemicellulosic fraction that otherwise would prevent cellulases from accessing the cellulose (Wen et al. 2009) (Fig 1A). The half-life of crystalline cellulose at neutral pH is estimated to be one hundred million years (Wilson 2008). A cocktail of saccharification enzymes—with endoglucanases, exoglucanases and β-glucosidases forming the major portion—is needed to disrupt the chemical stability of cellulose. The physical stability of lignocellulose, rendered by hydrogen bonds formed between adjacent cellulose polymers, is still a major obstacle to the efficient hydrolysis of cellulose. An additional challenge in cellulose hydrolysis is the relatively poor kinetics exhibited by cellulases (Himmel et al. 2007). Cellulases have lower specific activities than do other hydrolytic enzymes, because their substrate (cellulose) is insoluble, crystalline, and heterogeneous (Fig 1B) (Zhang and Lynd 2004; Wilson 2008). Activity of each of the cellulases in complex enzyme cocktails is inhibited by intermediates—such as cello-oligosaccharides and cellobiose, produced during cellulose hydrolysis—leading to discontinuity in the process. For example, exoglucanase action yields cellobiose, which inhibits endoglucanase (Fig 1C) (Lee et al. 2010).

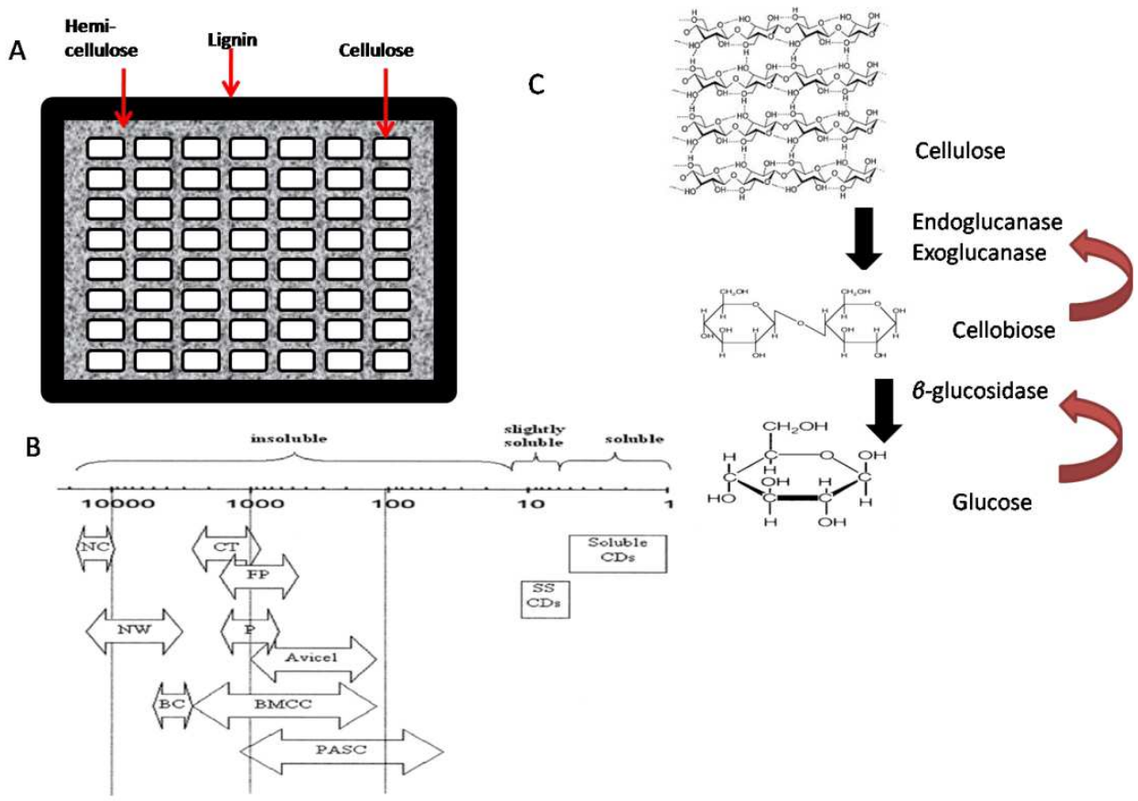

Fig. 1. (A) Schematic representation of the barriers to access the cellulose present in plant cell wall. Adapted from Biotechnology and Bioengineering (Zhang and Lynd 2004). (B) Degree of solubility of various forms of cellulose. CD, cellodextrin; SS CDs, Semi soluble CD; CT, cotton linters; FP, filter paper; P, wood pulp; BC, bacterial cellulose; BMCC, bacterial microcrystalline cellulose; PASC, phosphoric acid swollen cellulose; NW, natural wood; NC, natural cotton. Reproduced with the permission from Biotechnology and Bioengineering (Zhang and Lynd 2004). (C) Schematic representation of enzymatic hydrolysis of cellulose.

Despite these hurdles, several species of *Clostridium*, *Trichoderma*, and *Aspergillus* can efficiently degrade cellulose. Exploitation of the innate potential of the microbial world might be an economical alternative to overcome the recalcitrance associated with lignocellulose (Alper and Stephanopoulos 2009). Two major strategies have been employed to hydrolyze lignocellulose by using microbial consortia. In the first strategy, native cellulolytic organisms like *Clostridium* spp. are engineered to produce bioethanol. In another approach, cellulolytic ability is imposed on efficient ethanol producers such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Zymomonas mobilis* (Xu et al. 2009). This chapter focuses mainly on the cellulolytic systems that have been engineered into recombinant microorganisms.

2. Enzymatic hydrolysis of lignocellulose

In native cellulolytic organisms, enzymes needed for cellulose hydrolysis—xylanase, endoglucanase, exoglucanase, and β-glucosidase—are expressed either separately or in

complexes called cellulosomes (Fig 2). Noncomplexed cellulase systems are characteristic of cellulolytic aerobic bacteria (such as *Bacillus* spp.) and fungi (such as *Trichoderma* spp.) (Lynd et al. 2002). Endoglucanase hydrolyzes amorphous cellulose randomly, leading to the formation of cello-oligosaccharides of varying chain length. Exoglucanases are highly selective enzymes and act on either the reducing or the nonreducing end of cello-oligosaccharides to liberate glucose or cellobiose, respectively. β-Glucosidase hydrolyzes cellobiose into its glucose monomers (Lynd et al. 2002). Cellobiose inhibits both exoglucanase and endoglucanase. Hence, β-glucosidase plays an important role in the overall process, because it prevents the accumulation of cellobiose (Shewale 1982).

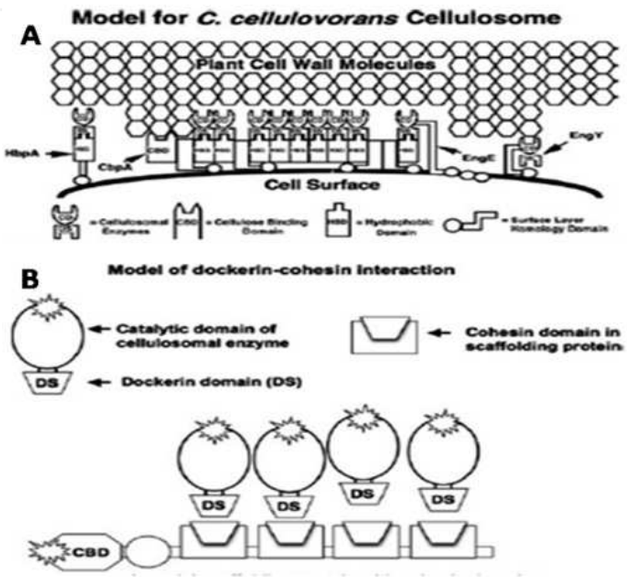

Fig. 2. (A) A model of cellulosome. (B) Synthetic scaffoldin favors arrangement of cellulases with higher activity in close proximity and hence would favor a proper synergy.
Reproduced with a permission from Annals of New York Academy of Science (Doi 2008).

Anaerobic bacteria such as *Clostridium* spp. usually produce complexed cellulases called cellulosomes. In cellulosomes, individual enzymes attach to a scaffoldin with their dockerin domains, while exposing the cellulose-binding domain. This complex enables proper synergy among endoglucanase, exoglucanase, and β-glucosidase. (Bayer et al. 1998). Several chimeric scaffoldins have been engineered to position enzymes of higher activity together, and thereby increase the overall hydrolysis efficiency (Fig 2B) (Wen et al. 2009). Even though the large size of the cellulosomes restricts them to only the most readily accessible regions of cellulose, cellulosomes can hydrolyze cellulose more efficiently than free cellulases can (Wilson 2009).

Engineering efforts to increase the efficiency of cellulases and to enhance their kinetic properties have focused mainly on improving the specific activity by improving the thermal or the pH stability of the enzymes (Wen et al. 2009). However, a more important parameter to consider is the efficiency of access to the cellulose interior. While the active-site plays an essential role in other hydrolytic enzymes, the cellulose-binding domain constitutes the key module for cellulases (Bayer et al. 1998). In fact, the cellulose-binding domain determines the type of cellulase. Several efforts to establish a kinetic model for

cellulose hydrolysis have failed because of the heterogeneous nature of the cellulosic substrate and the need for multiple enzyme activities (Kadam et al. 2004). In addition to enzyme-substrate proximity, enzyme-enzyme synergy should be considered as a factor for the efficient hydrolysis of cellulose. Whether any relationship or correlation between the crystallinity of lignocellulose and the rate of enzymatic hydrolysis exists remains unclear (Zhang and Lynd 2004). Moreover, the mechanism of cellulose hydrolysis remains incompletely understood, because some groups of cellulases have both exoglucanase and endoglucanase activities.

The low processivity of cellulases demands that the enzymes be replenished several times during the saccharification process. The economic feasibility of enzymatic hydrolysis of lignocellulose to simple sugars is limited by the poor kinetic properties of the enzymes. The use of cellulase-secreting microbes could be an economical alternative to the enzymatic saccharification process. With microbes, the enzymes can be continuously produced, secreted, and used to hydrolyze cellulose into simple sugars that could be directly fermented to ethanol (Fig 3). Thus, microbial fermentation of lignocellulose offers greater promise for economical bioethanol production.

3. Native cellulolytic organisms

The quest for cellulolytic organisms has recently gained increased interest because of the potential to circumvent the cost of enzymes used for cellulose hydrolysis. An ideal host for cellulosic ethanol production should possess certain traits, such as a broad substrate range (utilizing both pentoses and hexoses), high productivity, and tolerance to both ethanol and toxic compounds of lignin (Fischer et al. 2008). In order to identify desirable organisms for cellulosic ethanol production, naturally evolved cellulose-degrading microbes have been characterized from several sources, including the rumen of cattle and the gut of insects, and even from marine environments (Hess et al. 2011). However, most of these microbes cannot be cultivated with synthetic media in the laboratory. Hence, DNA isolates were directly sequenced and putative carbohydrate-hydrolyzing genes were identified (Hess et al. 2011). With this metagenomic approach, identification of microbes suitable for cellulosic fuel production has not been possible, because our current knowledge of the genes is limited.

Well-characterized native cellulolytic organisms include *Cellulomonas fimi*, *Fibrobacter succinogenes*, *Ruminococcus albus*, and *C. thermocellum*. Among these, *C. thermocellum* is of considerable importance, because it is recognized as a "cellulose-using specialist" (Zhang and Lynd 2005). Cellulolytic organisms produce many isoforms of the three different cellulases. *T. reesei,* for example, can secrete five endoglucanases, two cellobiohydrolases, and two β-glucosidases. Apart from cellulases, these organisms also secrete adhesion proteins like glycocalyx, which enables strong adhesion of the cellulolytic organisms to cellulose (Lynd et al. 2002).

Despite the diversity of cellulolytic organisms, none of these organisms are known to produce ethanol efficiently (Xu et al. 2009). Even as the search for a cellulolytic organism with the ability to produce ethanol continues, another strategy would be to engineer efficient ethanol production into cellulolytic organisms such as *Clostridium* spp. (Lynd et al. 2005). However, a lack of proper genetic tools for manipulating these uncommon laboratory strains and very limited knowledge of their genotypes have resulted in a need to engineer the cellulolytic ability into efficient ethanol producers such as *S. cerevisiae, E. coli* and *Z. mobilis*.

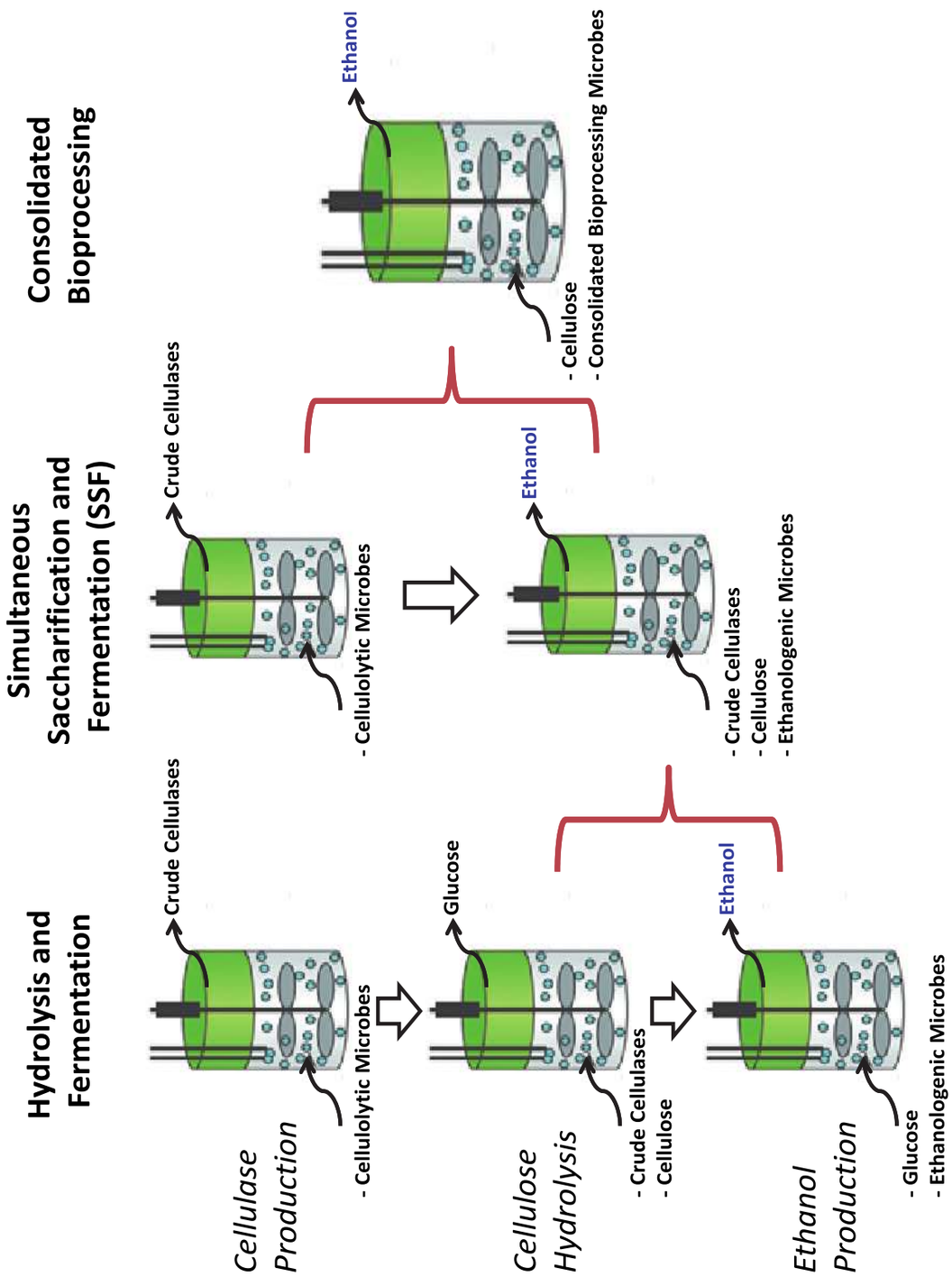

Fig. 3. Schematic representation of the benefits of consolidated bioprocessing over simultaneous saccharification and fermentation.

4. Recombinant cellulolytic organisms

Because the specific activity of cellulase enzymes is at least two-fold lower than that of other hydrolytic enzymes such as starch-hydrolyzing enzymes (Zhang and Lynd 2004; Wilson 2008), even native cellulolytic organisms must produce a high titer of cellulase to efficiently hydrolyze cellulose. The need for synthesis of a large quantity of cellulases is a "metabolic burden," even to native cellulolytic organisms (Zhang and Lynd 2005). Thus, heterologous expression of cellulolytic enzymes in industrial ethanol-producing hosts such as *S. cerevisiae, E. coli* and *Z. mobilis* is especially challenging. Despite these obstacles, several recombinant strains have been engineered for efficient cellulosic ethanol production.

4.1 *S. cerevisiae*

Yeast is an efficient industrial host with a high productivity of ethanol and with well-developed genetic tools. However, yeast does not possess endogenous cellulolytic ability. Several heterologous cellulases have, therefore, been expressed in yeast for direct conversion of cellulose into ethanol. Endoglucanase genes from *Bacillus* spp. were successfully integrated (randomly, at approximately 44 sites) into the chromosome of yeast, resulting in the direct conversion of cellodextrin into ethanol (Cho et al. 1999).

With the advent of cell-surface display technologies, it has become possible to express artificial cellulosomes (rather than free cellulases) in yeast. Cellulosomes facilitate the assembly of different cellulolytic enzymes in close proximity, and thereby favor a proper synergy between the enzymes (Tsai et al. 2010). Surface display of endoglucanase from *T. reesei* and *β*-glucosidase from *A. aculeatus* in yeast helped in the successful conversion of barley *β*-glucan into ethanol with 93% of the theoretical yield and without any pretreatment (Fujita et al. 2002). Co-displaying the exoglucanase from *Aspergillus* spp. along with the endoglucanase and *β*-glucosidase in yeast has resulted in the direct conversion of amorphous cellulose into ethanol (Fujita et al. 2004; McBride et al. 2005). Very recently, recombinant yeast has been further modified to express *β*-glucosidase within the cell. A high-affinity transporter for cellobiose and cellodextrin has also been cloned into the recombinant yeast. This strain co-metabolizes xylose and cellobiose more efficiently (Ha et al. 2011).

Although several studies have demonstrated efficient ethanol production from amorphous cellulose, attempts to engineer yeast to hydrolyze crystalline cellulose have been unsuccessful because of low exoglucanase activity (la Grange et al. 2010). The exoglucanase and *β*-glucosidase activities in recombinant cellulolytic yeast strains are insufficient to support growth with cellulose as a sole carbon source. Hence, a synthetic yeast consortium has been developed with four engineered yeast strains, each expressing either the scaffoldin from *Clostridium* spp. and *Ruminococcus* spp. or the three enzymes, namely, exo- and endoglucanases from *Clostridium* spp. and *β*-glucosidase from *Ruminococcus* spp. (Fig 4) (Tsai et al. 2010). However, investigators have been unable to completely decipher the efficiency of the synthetic consortium, because the ratio of the different cellulases needed for a proper synergy has not been established. A cocktail δ-integration tool has been developed in yeast to predict the optimum ratio of different cellulases, but with little success (Yamada et al. 2010).

Another major problem with recombinant cellulase expression is that heterologous cellulases are made to function at a suboptimal temperature. The optimal temperature for the growth of recombinant hosts is 37°C, but cellulases are more active at temperatures

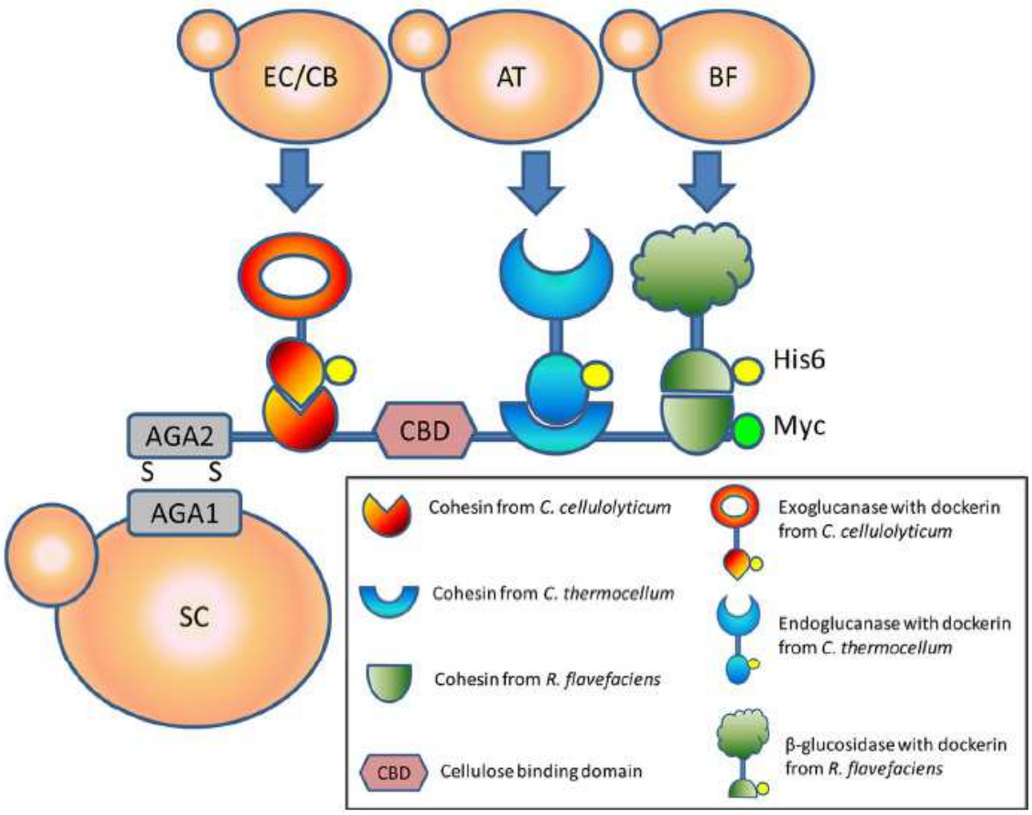

Fig. 4. Schematic representation of the synthetic yeast consortium developed for efficient cellulose utilization. Reproduced with a permission from Applied and Environmental Biotechnology (Tsai et al. 2010). CBD, Cellulose Binding Domain; SC, trifunctional scaffoldin; EC/CB, Exoglucanase; AT, Endoglucanase; BF-β-glucosidase;

above 50°C. Therefore, the thermotolerant yeast *Kluyveromyces marxianus* has been engineered to display thermostable endoglucanase and β-glucosidase on its surface. This engineered, thermostable yeast ferments β-glucan directly to ethanol at 48°C (Yanase et al. 2010).

4.2 *E. coli*

The broad substrate range of *E. coli*, together with its ample genetic tools and its substantial fermentation capacity, renders the species to be a potential candidate for bioethanol production. *E. coli*, with chromosomally integrated genes encoding pyruvate decarboxylase and alcohol dehydrogenase, is an efficient ethanol producer (Ohta et al. 1991). Several attempts have been made to engineer cellulolytic ability in ethanologenic *E. coli*. The species also has endogenous cryptic genes for cellobiose metabolism and an endoglucanase for the hydrolysis of soluble cellulose (Park and Yun 1999; Kachroo et al. 2007; Vinuselvi and Lee 2011).

Achieving a higher extracellular titer of cellulases is a bottleneck in the development of a recombinant cellulolytic *E. coli* for ethanol production. *E. coli* does not have a proper protein secretion system (Shin and Chen 2008). Because *E. coli* is a gram-negative bacterium, it has an outer membrane rich in peptidoglycan, which acts as a barrier for protein secretion. The extracellular protein concentration observed with *E. coli* is 0.0088 g/L, one hundred-fold less than that observed with native cellulolytic organisms (Qian et al. 2008; Xu et al. 2009;

Vinuselvi et al. 2011). Gram-negative bacteria possess five different protein-export pathways (Types I–V), two of which are found in *E. coli* (Type I and Type II).
Several attempts have been made to increase the extracellular titer of recombinant proteins in *E. coli*: by exploiting the Sec/TAT signal sequence (Zhou et al. 1999; Angelini et al. 2001), by fusion of recombinant proteins with extracellular proteins such as OsmY (Qian et al. 2008), or by increasing membrane permeability (Shin and Chen 2008). Cellulase secretion in *E. coli* has been achieved through the expression of endoglucanase, along with the *out* genes of *Erwinia chrysanthemi*, under the control of a surrogate promoter (Zhou et al. 1999). Deletion of *lpp* weakens the outer membrane, allowing any proteins targeted to the periplasmic space to be secreted into the medium. Approximately 70% of the cellulases produced were secreted into the medium in an *lpp* knockout *E. coli* strain (Shin and Chen 2008). Several studies have used OsmY as a fusion partner for recombinant protein secretion in *E. coli.* However, this technique has not been exploited for cellulase secretion because of the large size of cellulases (Aristidou and Penttilä 2000; Qian et al. 2008) (Fig 5).

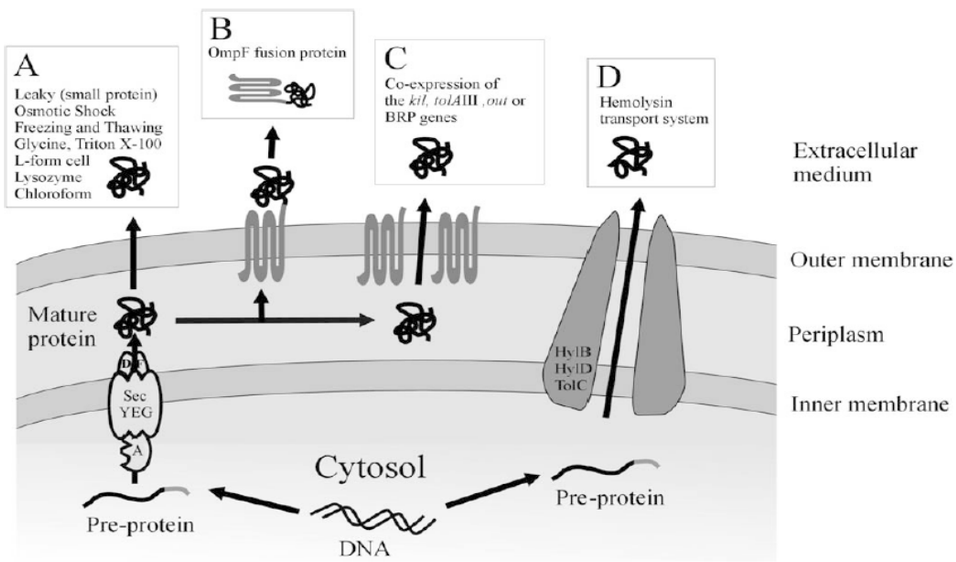

Fig. 5. Schematic representation of the strategies used for extracellular secretion of recombinant proteins in *E. coli*. (A) Membrane disruption using detergents or through lpp deletion increases membrane permeabilization and the periplasmic proteins are leaked into the extracellular space. (B) Use of OmpF fusion proteins helps in the secretion of small proteins. (C) *out* gene of *Erwinia* encodes for a bacteriocin release protein pore which helps in the secretion of the periplasmic proteins. (D) Use of SEC/TAT pathway signal sequence favors direct secretion of cellulases into the medium. Reprinted with a permission from Applied Microbial Biotechnology (Choi and Lee 2004).

The cellobiose metabolic operon from *Klebsiella oxytoca* has been introduced into *E. coli*, but the expression level of the cellobiose transporter and metabolic genes was poor, and hence could not support the growth of *E. coli* on cellobiose (Moniruzzaman et al. 1997). Cellulases from several species of *Clostridium*, *Bacillus*, *Cellulomonas*, and *Ruminococcus* have been expressed and characterized in *E. coli* (Hinchliffe 1984; Zappe et al. 1986; Fierobe et al. 1991; ReverbelLeroy et al. 1996; Lam et al. 1997; ReverbelLeroy et al. 1997; Lee et al. 2008; Li et al. 2009). Co-expression of endoglucanase from *B. pumilus* and *β*-glucosidase from

Fervidobacterium spp. in *E. coli* favored growth of the recombinant strain, with soluble carboxymethyl cellulose (CMC) as the sole carbon source (Rodrigues et al. 2010).

4.3 *Z. mobilis*

Zymomonas is an efficient ethanol producer, together with a higher tolerance to ethanol and to several inhibitory substances of lignin. *Zymomonas* species also possess a gene that codes for cellulase (Rajnish et al. 2008). While protein secretion is not a hurdle in *Zymomonas* spp., a major difficulty arises with the lack of amenable genetic tools for the introduction or modification of a gene (Linger et al. 2010). Two cellulases from *Acidothermus cellulolyticus* have been expressed in *Z. mobilis*, and a significant amount of secretion was observed when they were fused with predicted N-terminal signal peptides of *Z. mobilis* (Linger et al. 2010). Endoglucanases from different cellulolytic organisms such as *Cellulomonas* spp., *Enterobacter cloacae*, *Pseudomonas fluorescens*, and *Erwinia* spp., have been expressed in *Z. mobilis*. However, none of these cellulases were secreted efficiently (Lejeune et al. 1988; Misawa et al. 1988; Brestic-Goachet et al. 1989; Thirumalai Vasan et al. 2011).

5. Future of cellulosic ethanol

An Ideal Biofuel Producing Microorganism (IBPM) should possess four important traits: it should be able to carry out (1) biomass degradation and (2) product formation; (3) it should show tolerance to solvents, and (4) it should serve as a chassis organism for rapid growth in the bioreactor (French 2009). Chassis organisms, such as yeast and *E. coli*, are well characterized. Commercial bioethanol has been produced from sugarcane by yeast. In addition, *E. coli* and *Z. mobilis* are progressing as efficient ethanol producers. A current challenge is to engineer biomass degradation (cellulolytic) ability. Further, investigators seek to enhance tolerance to harsh conditions that arise during cellulose fermentation, such as substrate and product toxicity. In particular, the chassis organism should have enhanced tolerance to toxic compounds of lignin. Classical strain improvement through long-term adaptation and mutagenesis may be an effective way to increase the tolerance to harsh environments, such as ethanol or lignin, because the mechanisms of toxicity and tolerance are largely unknown (Fischer et al. 2008).

Engineering cellulolytic ability into recombinant hosts has long been a challenge. The number of cellulase genes that should be cloned into the recombinant host remains unclear (Vinuselvi et al. 2011). The main obstacle to developing a recombinant cellulolytic host is the inability of hosts to support expression and secretion of a sufficient quantity of cellulases. Although cellulase expression is well established in yeast, there is no known study demonstrating direct conversion of plant biomass into ethanol. Despite the characterization of several cellulases in *E. coli,* a cellulolytic cassette containing all three cellulases has not been established for *E. coli*. Furthermore, efficient genetic tools are still lacking for *Zymomonas*, limiting its potential to be engineered with a heterologous gene.

One way to address the problems associated with heterologous cellulase expression and to reduce the metabolic burden imposed by the expression of cellulolytic enzymes in recombinant hosts would be the development of a well-defined synthetic consortium with two efficient players—native cellulolytic and solventogenic organisms—acting together. A high level of expression of multiple heterologous proteins would impose a heavy metabolic burden on the host. With a synthetic consortium, this burden could be shared by different species or by different strains of the same species. A co-culture of these strains to produce a

cellulase cocktail would, therefore, reduces the overall metabolic burden and increase the ethanol yield (Brenner et al. 2008). Synthetic biology also offers superior inducible systems, such as light-inducible promoters and the *fim* inversion system, which are capable of providing spatiotemporal changes in gene expression (Levskaya et al. 2005; Ham et al. 2006). With such systems, it is possible to regulate the expression of genes with time and, potentially, to help reduce the metabolic burden imposed on the recombinant host (Drepper et al. 2011). Using metabolic engineering and synthetic biology, Steen et al. (2010) have developed a promising way of causing *E. coli* to produce more complex biofuels—fatty esters and fatty alcohols—directly from hemicellulose, a major component of plant-derived biomass (Fig 6). This study is representative of the recent progress in cellulosic fuel production. However, the possibility of increasing the productivity of such advanced biofuels remains a significant challenge.

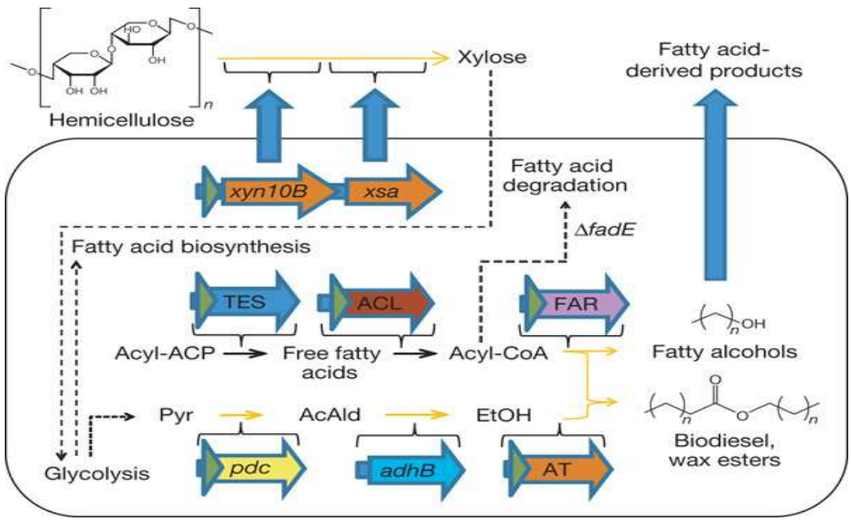

Fig. 6. Schematic representation of the new pathways engineered into recombinant *E. coli* for the production of advanced biofuels from hemicellulosic fraction of plant biomass. This recombinant strain is a representative candidate proving the potency of synthetic biology and metabolic engineering to develop a cellulosic ethanol producer. TES, thioesterase; ACL, acyl-CoA ligase; FAR, fatty acyl-CoA reductase; AT, acyltransferase; *pdc,* pyruvate decarboxylase; *adhB,* alcohol dehydrogenase; AcAld, acetaldehyde; EtOH, ethanol; pyr, pyruvate; *xyn10B* & *xsa,* xylanase. Overexpressed genes or operons are indicated; green triangles represent the *lacUV5* promoter. Reproduced with a permission from Nature (Steen et al. 2010).

6. Conclusions

Cellulosic bioethanol is gaining importance to circumvent the oil crisis and climate change. However, two major problems remain to be solved, in order to produce cellulosic ethanol economically. One problem is the high price of the cellulolytic enzymes used in the saccharification of lignocelluloses. The other problem is that the traditional saccharification and fermentation for bioethanol requires huge initial capital investment and operational cost. Consolidated bioprocessing presents a desirable way to produce bioethanol economically from lignocellulose. Microorganisms such as *Trichoderma* spp. and *C. thermocellum* effectively challenge the recalcitrance of lignocellulose, whereas microbes such

as yeast and *Z. mobilis* can produce ethanol more efficiently. Several attempts have been made to combine these two abilities into a single organism, but with little success. Recent progress in synthetic biology, metabolic engineering, and protein engineering gives hope that the goal of generating cellulosic ethanol with a single organism may not be far from reality.

7. Acknowledgements

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Bioethanol

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Recent studies have shown strong evidence of human activity impact on the climate of the planet. Higher temperatures and intensification of extreme weather events such as hurricanes are among the consequences. This scenario opens up several possibilities for what is now called "green" or low carbon economy. We are talking about creating new businesses and industries geared to develop products and services with low consumption of natural resources and reduced greenhouse gases emission. Within this category of business, biofuels is a highlight and the central theme of this book. The first section presents some research results for first generation ethanol production from starch and sugar raw materials. Chapters in the second section present results on some efforts around the world to develop an efficient technology for producing second-generation ethanol from different types of lignocellulosic materials. While these production technologies are being developed, different uses for ethanol could also be studied. The chapter in the third section points to the use of hydrogen in fuel cells, where this hydrogen could be produced from ethanol.

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