Consolidated bioprocessing of cellulosic biomass: an update

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Biologically mediated processes seem promising for energy conversion, in particular for the conversion of lignocellulosic biomass into fuels. Although processes featuring a step dedicated to the production of cellulase enzymes have been the focus of most research efforts to date, consolidated bioprocessing (CBP) – featuring cellulase production, cellulose hydrolysis and fermentation in one step – is an alternative approach with outstanding potential. Progress in developing CBP-enabling microorganisms is being made through two strategies: engineering naturally occurring cellulolytic microorganisms to improve product-related properties, such as yield and titer, and engineering non-cellulolytic organisms that exhibit high product yields and titers to express a heterologous cellulase system enabling cellulose utilization. Recent studies of the fundamental principles of microbial cellulose utilization support the feasibility of CBP.

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Introduction

Energy conversion, utilization and access underlie many of the great challenges of our time, including those associated with sustainability, environmental quality, security and poverty. New applications of emerging technologies will be required to respond to these challenges [1,2•]. As one of the most powerful of these technologies, biotechnology could give rise to important new energy conversion processes. Resources for the biological conversion of energy to forms useful to humanity include plant biomass and derivatives thereof (e.g. synthesis gas and animal wastes), sunlight [3,4], inorganic compounds [5•], and fossil resources (e.g. oil, coal and natural gas) [6]. Energy carriers that can be made from these resources include organic fuels, electricity [4,5•] and hydrogen [3].

Among forms of plant biomass, lignocellulosic biomass ('biomass') is particularly well-suited for energy applications because of its large-scale availability, low cost and environmentally benign production [7]. In particular, many energy production and utilization cycles based on cellulosic biomass have near-zero greenhouse gas emissions on a life-cycle basis [8–10]. The primary obstacle impeding the more widespread production of energy from biomass feedstocks is the general absence of low-cost technology for overcoming the recalcitrance of these materials [7].

Biomass processing schemes involving enzymatic or microbial hydrolysis commonly involve four biologically mediated transformations: the production of saccharolytic enzymes (cellulases and hemicellulases); the hydrolysis of carbohydrate components present in pretreated biomass to sugars; the fermentation of hexose sugars (glucose, mannose and galactose); and the fermentation of pentose sugars (xylose and arabinose). These four transformations occur in a single step in a process configuration called consolidated bioprocessing (CBP), which is distinguished from other less highly integrated configurations in that it does not involve a dedicated process step for cellulase production. Thermochemical processing options appear more promising than biological options for the conversion of the lignin fraction of cellulosic biomass, which can have a detrimental effect on enzymatic hydrolysis but also serves as a source of process energy and potential coproducts that have important benefits in a life-cycle context [7,11•].

Fundamental and applied topics relevant to CBP were comprehensively reviewed in 2002 [12••]. Here, we provide an updated perspective focusing on recent developments. Ethanol production is emphasized, as this has been the focus of most work pursuant to CBP to date. In principle, however, the CBP strategy is applicable to any fermentation product.

The motivation for consolidated bioprocessing

As addressed in more detail elsewhere [12••], CBP offers the potential for lower cost and higher efficiency than processes featuring dedicated cellulase production. This results from avoided costs for capital, substrate and other raw materials, and utilities associated with cellulase production. In addition, several factors support the possibility of realizing higher hydrolysis rates, and hence reduced reactor volume and capital investment, using CBP. These include enzyme–microbe synergy (discussed below), as well as the use of thermophilic organisms and/or complexed cellulase systems. Moreover,

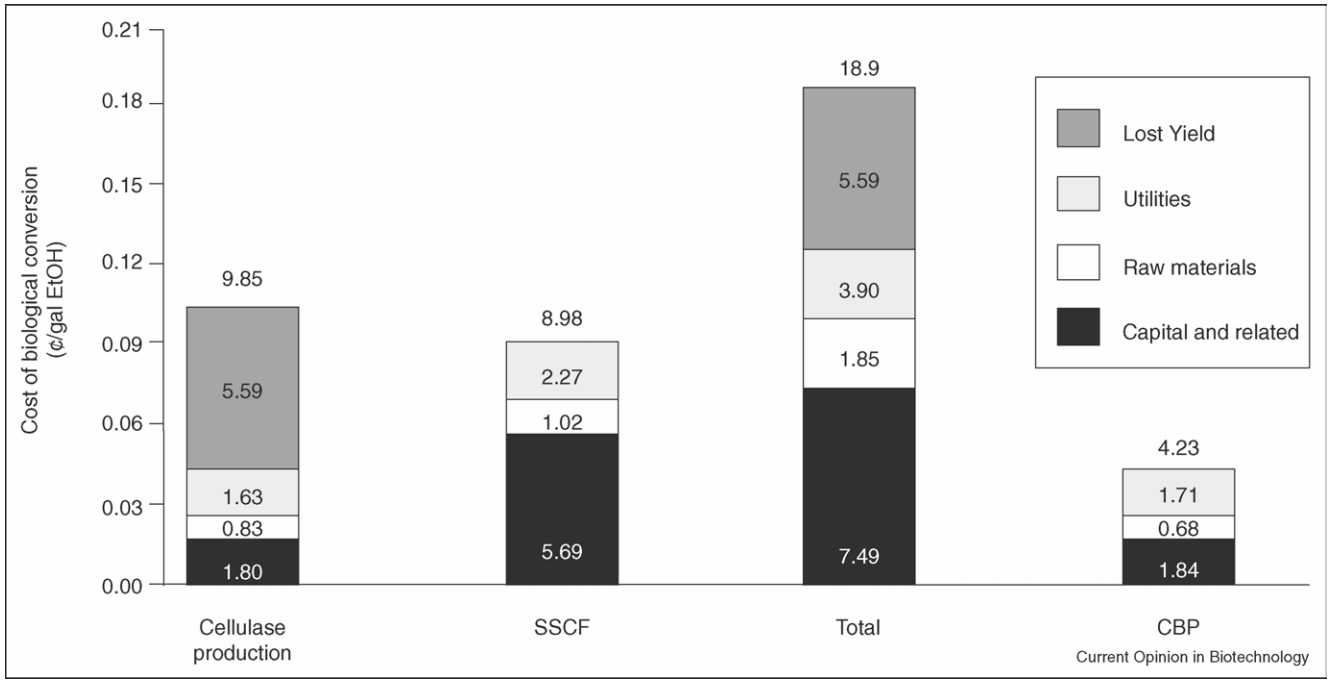
cellulose-adherent cellulolytic microorganisms are likely to successfully compete for products of cellulose hydrolysis with non-adhered microbes, including contaminants, which could increase the stability of industrial processes based on microbial cellulose utilization.

Over the past few years, much effort has been devoted to reducing the cost of producing cellulase enzymes [13]. Following greater than tenfold cost reductions, cellulase production costs have recently been reported in the range of 10 to 20 cents per gallon (¢/gal) of ethanol produced [14•]. These exciting developments could well enable a variety of formerly infeasible industrial processes, but do not diminish the potential of CBP to offer significantly lower costs than other configurations. Figure 1 compares the projected costs for biological processing associated with ethanol production for a CBP process and for an advanced process featuring at-site dedicated cellulase production in combination with simultaneous saccharification with co-fermentation of hexose and pentose sugars (SSCF). Adding together the 9.90 ¢/gal ethanol for dedicated cellulase production and 9 ¢/gal for SSCF gives a total cost for biological processing of 18.9 ¢/gal, which is more than fourfold larger than the 4.2 ¢/gal projected for CBP. For the advanced process scenarios analyzed here, identical in every respect except for the configuration assumed for biological processing and consequences thereof, the projected wholesale selling price of ethanol from a cellulosic feedstock costing $40/dry ton is 77 ¢/gal ($1.08/gal gasoline equivalent) for the dedicated cellulase production/SSCF scenario and 63 ¢/gal ($0.88/gal gasoline equivalent) for CBP. The difference between these projected prices, 20 ¢/gal gasoline equivalent, is particularly important for fuel production with its low manufacturing margins and price-driven competitive markets. For comparison, the average wholesale price of gasoline was $0.98/gal for the period 2001 to 2004 and $1.32/gal for the first quarter of 2005 [15].

Organism development strategies

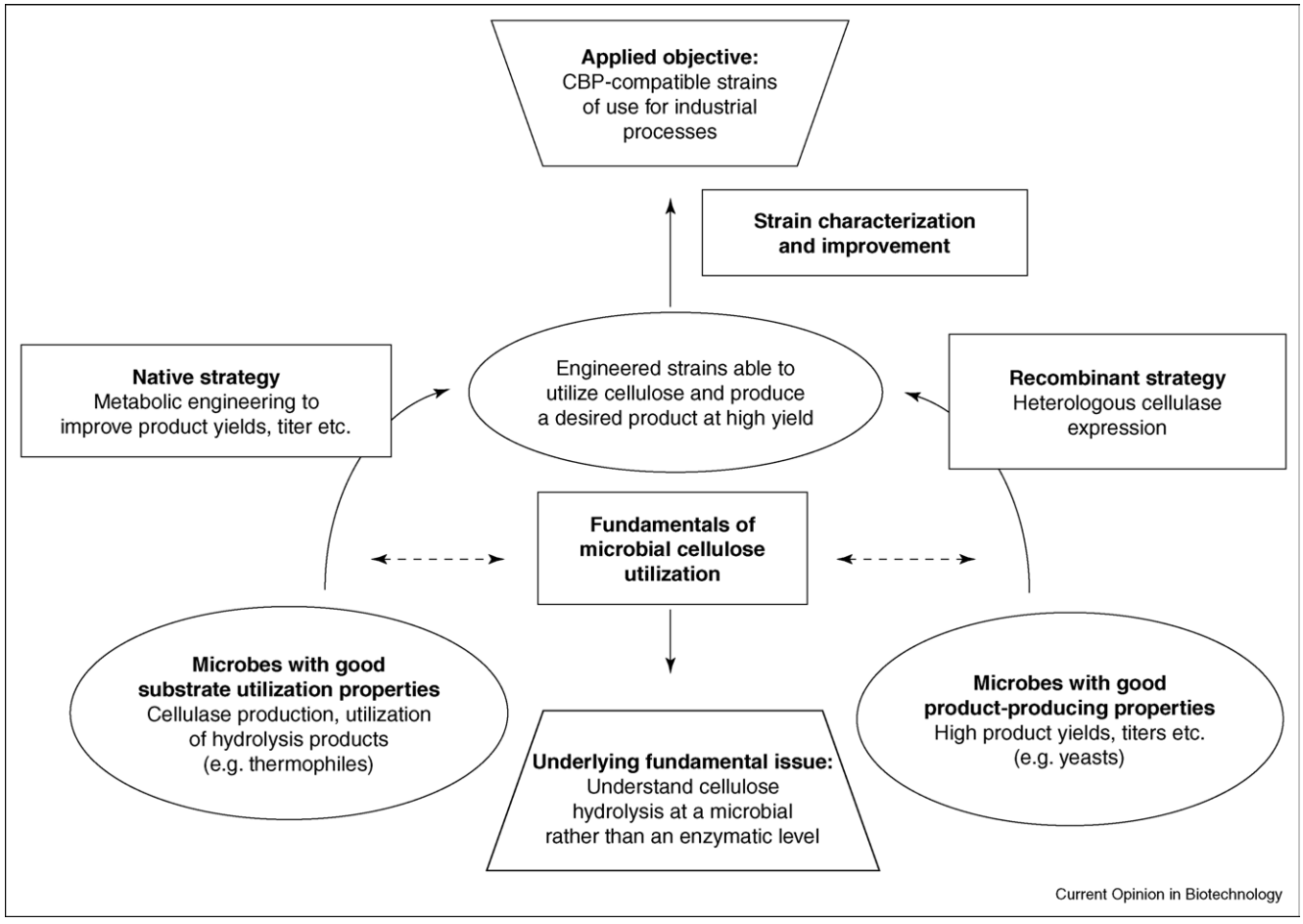
Microorganisms with the combination of substrate-utilization and product formation properties required for CBP are not currently available, but could probably be developed given sufficient effort. Such developments can be pursued by two strategies (Figure 2). The native cellulolytic strategy involves engineering naturally occurring cellulolytic microorganisms to improve product-related properties, such as yield and titer. The recombinant

Figure 1



The comparative cost of ethanol production by consolidated bioprocessing (CBP) and by simultaneous saccharification and co-fermentation (SSCF) featuring dedicated cellulase production. Processes are simulated assuming aggressive performance parameters intended to be representative of mature technology. Cellulase production: yield, 400 FPU (filter paper unit)/g carbohydrate; productivity, 400 FPU/L/h. Hydrolysis and fermentation yields are set at 95% of the theoretical value for both CBP and SSCF. The SSCF reaction time is 7 days. Savings in the SSCF reactor accompanying reaction times less than 7 days are completely offset by the increased enzyme costs associated with higher cellulase loadings required to achieve shorter reaction times. The CBP reaction time is set at 1.5 days, consistent with the expectation of higher hydrolysis rates (see text). If the reaction time were increased to 3 days, the cost of CBP increases from 4.4 ¢/gallon to 5.5 ¢/gal. (Simulations were carried out using ASPEN-based models based on the modeling framework developed at the National Renewable Energy Laboratory [55,56], with modifications for mature technology as described elsewhere [2].)

Figure 2



Organism development strategies and related fundamentals.

cellulolytic strategy involves engineering non-cellulolytic organisms that exhibit high product yields and titers so that they express a heterologous cellulase system that enables cellulose utilization. Each strategy involves considerable uncertainty, and different strategies could prove advantageous for different products. In light of this and other considerations [12••], we believe that both the native and recombinant strategies for CBP organism development merit investigation.

The utilization of cellulose by microorganisms involves a substantial set of fundamental phenomena beyond those associated with enzymatic hydrolysis of cellulose [12••], many of which are incompletely understood. Advances in understanding the fundamentals of microbial cellulose utilization will both enable and draw from advances in organism development for CBP.

Native cellulolytic strategy

Naturally occurring cellulolytic microorganisms are starting points for CBP organism development via the native strategy, with anaerobes being of particular interest [12••]. The primary objective of such developments is to engineer product yields and titers to satisfy the requirements of an industrial process. Metabolic engineering of mixed-acid fermentations in relation to these objectives has been successful in the case of mesophilic, non-cellulolytic, enteric bacteria [16]. Far more limited work of this type has been undertaken with cellulolytic bacteria, primarily because of the absence of suitable gene-transfer techniques. Recent developments, however, appear to be removing this limitation for some organisms.

Among cellulolytic anaerobes, gene-transfer systems have been described for *Clostridium cellulolyticum* [17,18] and for *Clostridium thermocellum* [19•]. Electrotransformation (ET) protocols for these organisms, first described in 2000 for *C. cellulolyticum* and in 2004 for *C. thermocellum*, involve rather specialized conditions and, in the case of *C. thermocellum*, apparatus. ET-mediated transfer and expression of foreign genes has also been reported for *Thermoanaerobacterium thermosaccarolyticum* [20] and *Thermoanaerbacter-*

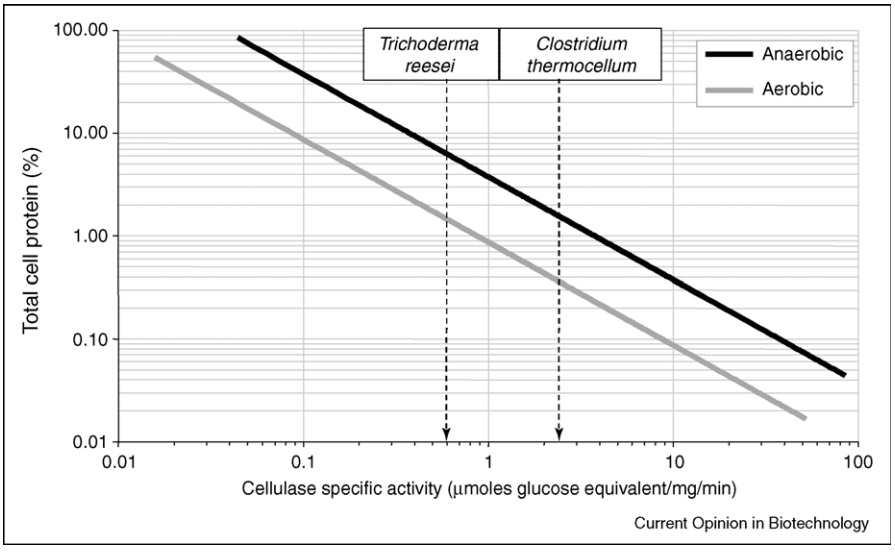
ium saccharolyticum [21,22], non-cellulolytic pentose-utilizing anaerobic thermophiles with substrate-utilizing capabilities that compliment those of *C. thermocellum* (which does not ferment pentoses). Efforts to engineer the end-product metabolism of cellulose-utilizing anaerobes are limited to the 2002 report of Guedon *et al.* [23], in which expression of pyruvate decarboxylase and alcohol dehydrogenase resulted in increased growth, decreased production of lactate, and increased production of acetate and ethanol. In *T. saccharolyticum*, production of lactic acid has been completely eliminated by targeted gene knockout [24•] and elimination of acetic acid production has also recently been achieved (J Shaw, unpublished). ET efficiencies for *C. thermocellum* ($\sim 10^5$ transformants per experiment using replicative plasmids [19•]) appear high enough to allow similar targeted gene knockouts to be obtained in this organism.

Product tolerance is a key property impacting the feasibility of CBP through the native cellulolytic strategy. Recent studies have been carried out to select and characterize ethanol-tolerant strains of *C. thermocellum* [25•]. Growth of a selected strain was found to occur at ethanol concentrations exceeding 60 g/L, a titer sufficient not to put thermophiles at a disadvantage relative to more conventional ethanol producers in the context of lignocellulose conversion [26]. Reported ethanol titers produced by *C. thermocellum*, as well as other thermophiles, are limited however to $\leq$26 g/L [12••]. We think it likely that the discrepancy between tolerance to added ethanol and the maximum titers produced can be resolved in the course of metabolic engineering to maximize ethanol yields. Organic acids and their salts are more inhibitory than ethanol for both thermophiles (*T. thermosaccharolyticum* [27]) and *E. coli* [28], and elimination of organic acids has been shown to allow substantially higher neutral product titers in several instances [28] (J Pierce, personal communication) (P Soucaille, personal communication). In addition, titers have been brought to levels consistent with tolerance to added products through a sustained effort in the case of production of both ethanol [28] and 1,3-propanediol (J Pierce, personal communication) by *E. coli*.

Recombinant cellulolytic strategy

Non-cellulolytic microorganisms with desired product formation properties (e.g. high yield and titer) are starting points for CBP organism development by the recombinant cellulolytic strategy. The primary objective of such developments is to engineer a heterologous cellulase system that enables growth and fermentation on pretreated lignocellulose. Given the complexity and effort required to achieve this objective, it is appropriate to examine its feasibility. On the basis of the sufficiency of expression of growth-enabling heterologous enzymes [29•], the level of enzyme expression required to achieve a specified growth rate may be calculated as a function of enzyme specific activity. For growth enabled by cellulase with specific activities in the range available, required expression levels are well within the range reported in the literature (1–10% of cellular protein; Figure 3) [30,31]. Protein expression at this level has been reported in both *Saccharomyces cerevisiae* [30] and *E. coli* [32], although not to date for active cellulases. The feasibility of CBP

Figure 3



Required protein expression to achieve growth on cellulose in relation to cellulase specific activity. Cellulase requirements are calculated using the following expression: percentage cell protein = $\{(\mu/Y_{X/S})/[(U/mg\ cellulase) \times (0.00018\ g/\mu mol) \times (60\ min/h)]\} \times (1\ g\ cells/0.5\ g\ cell\ protein)$, where μ = specific growth rate on the substrate (0.02 h^{-1}) and $Y_{X/S}$ = cell yield on glucose (0.45 g cells/g substrate for aerobic growth and 0.1 g cells/g substrate for anaerobic growth). Data for *T. reesi* were obtained from [57] and for *C. thermocellum* from [58].

through the recombinant approach is further supported by models that combine fermentation bioenergetics and the kinetics of pretreated substrate hydrolysis with declining reactivity over the course of the reaction [33]. It is likely that evolutionary approaches will be valuable in augmenting the rational design of heterologous cellulase expression systems [34•].

To date, the heterologous production of cellulases has been pursued primarily with bacterial hosts producing ethanol at high yield (engineered strains of *E. coli*, *Klebsiella oxytoca* and *Zymomonas mobilis*) and the yeast *S. cerevisiae*. Cellulase expression in strains of *K. oxytoca* resulted in increased hydrolysis yields (but not growth without added cellulase) for microcrystalline cellulose (Avicel, FMC, Philadelphia), and anaerobic growth on amorphous cellulose [35,36]. Although dozens of saccharolytic enzymes have been functionally expressed in *S. cerevisiae* [12••], anaerobic growth on cellulose as the result of such expression has not been definitively demonstrated. Recently, Kondo and coworkers expressed cellulases [37••], xylanases [38], and amylases [39] on the cell surface of different *S. cerevisiae* strains. High cell density suspensions of the recombinant strains fermented amorphous cellulose, raw starch, and birchwood xylan to ethanol with yields of 0.45, 0.44 and 0.3 g ethanol/g substrate, respectively. Several cellobiohydrolases have been functionally expressed in *S. cerevisiae* [40–44]. The specific activity of recombinant cellobiohydrolase was shown to be comparable to that of the native enzyme [43] (R den Haan *et al.*, unpublished), suggesting that increased expression levels should be a target for further research.

As might be expected, conferring the ability to grow on non-native substrates as a result of heterologous enzyme expression is more advanced for soluble substrates than for cellulose. Strains of *E. coli* [18,28], *S. cerevisiae* [45], and *Z. mobilis* [46] able to ferment xylose to ethanol were developed in the 1990s. More recently, several strains of *S. cerevisiae* with expanded substrate utilization capability have been developed: a xylose-isomerase-expressing strain that grows well (maximum specific growth rate $\mu_{max} = 0.09\ h^{-1}$) on xylose [47•]; a strain expressing the genes of the bacterial L-arabinose utilization pathway and overexpressing the yeast galactose permease allowing the fermentation of arabinose [48]; and a β-glucosidase-expressing strain able to grow anaerobically on cellobiose at rates comparable to glucose [29]. Strains of *S. cerevisiae* able to ferment starch were developed in earlier work and achieve impressive yields and titers without added amylase [49].

Selected fundamentals impacting the feasibility of CBP

Experimental evidence supporting the feasibility of CBP comes from a recent study of cellulose utilization by *C. thermocellum* [50••]. It was shown in this study that the average degree of polymerization of cellulose hydrolysis products taken up by *C. thermocellum* is about four glucose units; thus, the mechanism of cellulose hydrolysis differs from that of fungal cellulase systems for which cellobiose is the primary hydrolysis product. Both physiological studies and experiments with ^{14}C-cellulose indicate that bioenergetic benefits specific to growth on cellulose are realized as a result of the efficiency of oligosaccharide uptake combined with intracellular phosphorolytic cleavage of β-glucosidic bonds [51], and that these benefits exceed the bioenergetic cost of cellulase synthesis [52]. This work provides insight into how naturally occurring cellulolytic microorganisms are able to achieve rapid growth on cellulose (e.g. $\mu_{max} = 0.16\ h^{-1}$ on crystalline cellulose [12••]) in spite of the modest ATP available from anaerobic fermentation.

A further phenomenon impacting the potential feasibility and performance of CBP is enzyme–microbe synergy — that is, the possibility that the effectiveness of the cellulase is enhanced when it is present as cellulose–enzyme–microbe (CEM) complexes as compared with cellulose–enzyme (CE) complexes. CEM complexes have a central role in microbial cellulose hydrolysis by anaerobic microbes [12••], whereas cellulose hydrolysis in envisioned industrial processes featuring dedicated cellulase production is carried out exclusively by CE complexes that do not involve the presence of a living, cellulolytic microorganism. In observations dating back to 1956, cellulase pioneers Reese and Mandels [53,54] observed that rates of hydrolysis are substantially higher when mediated by growing cellulolytic cultures as compared to enzymatic preparations, even under optimized conditions. Preliminary work underway in the Lynd laboratory indicates that cellulase-specific hydrolysis rates are several fold higher for *C. thermocellum* cultures than for cell-free cellulase preparations (Y Lu *et al.*, unpublished). Definitive quantitative evaluation of enzyme–microbe synergy is an important objective for future research, and could provide further evidence supporting the desirability of CBP.

Although studies aimed at understanding the fundamentals of microbial cellulose utilization have necessarily focused on naturally occurring cellulolytic bacteria, insights from such studies are of importance to both the native and recombinant organism development strategies. In particular, it may be highly desirable, and perhaps necessary, to incorporate features of naturally occurring cellulolytic microbes (e.g. high specific activity complexed cellulase systems, uptake and phosphorolytic cleavage of oligosaccharides, and adherence of cells to cellulose) when developing recombinant cellulolytic microbes for use in CBP.

Conclusions

CBP has the potential to provide the lowest cost route for biological conversion of cellulosic biomass to fuels and

other products in processes featuring hydrolysis by enzymes and/or microorganisms. To realize this potential, microorganisms must be developed that utilize cellulose and other fermentable compounds available from pretreated biomass with high rate and high conversion, and which produce a desired product at high yield and titer. Both of these capabilities are possessed by known microorganisms, but to date have not been combined in a single microorganism or microbial system. Several lines of evidence support the feasibility of such combinations using biotechnology, which could proceed through two distinct strategies each with several potential host organisms. Success in this endeavor could provide a leap forward with respect to the low-cost conversion of renewable biomass into fuels as well as a variety of industrial chemicals, thereby realizing societal benefits.

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