BACTERIAL RECYCLING OF PULP

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Life on Earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component. The carbon cycle is closed primarily as a result of the action of cellulose-utilizing microorganisms present in soil and the guts of animals. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere and is of interest in relation to analysis of carbon flux at both local and global scales. The importance of microbial cellulose utilization in natural environments is further enhanced by the status of ruminants as a major source of dietary protein. Finally, microbial cellulose utilization is also an integral component of widely used processes such as anaerobic digestion and composting.

The broad distribution of cellulolytic capability could suggest conservation of a cellulose-degrading capability acquired by a primordial ancestor early in evolutionary development; however, this would seem unlikely, given that the capacity for cellulose biosynthesis did not evolve until much later, with the development of algae, land plants and the bacterium *A. xylinum*. More likely is the convergent evolution toward a cellulolytic capability under the selective pressure of abundant cellulose availability following the development of cellulose biosynthesis. Evidence for such convergent evolution is discussed below.

A number of species of the most primitive group of fungi, the anaerobic *Chytridomycetes*, are well known for their ability to degrade cellulose in gastrointestinal tracts of ruminant animals. Although taxonomy of this group remains controversial, members of the order Neocallimastigales have been classified based on the morphology of their motile zoospores and vegetative thalli; they include the monocentric genera *Neocallimastix, Piromyces,* and *Caecomyces* and the polycentric genera *Orpimomyces* and *Anaeromyces*. Cellulolytic capability is also well represented among the remaining subdivisions of aerobic fungi. By contrast, the much more diverse subdivisions *Ascomycetes, Basidiomycetes*, and *Deuteromycetes*, contain large numbers of cellulolytic species. Members of genera that have received considerable study with respect to their cellulolytic enzymes and/or wood-degrading capability include *Bulgaria, Chaetomium,* and *Helotium (Ascomycetes); Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula (Basidiomycetes);* and *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium,* and *Trichoderma (Deuteromycetes).* The thermophilic and hyperthermophilic procaryotes represent a unique group of microorganisms that grows at temperatures that may exceed 100°C. Several cellulolytic hyperthermophiles have been isolated during the past decade. Surprisingly, no cellulolytic thermophilic archaea have been described, although archaea that can grow on cellobiose and degrade other abundant polysaccharides, such as starch, chitin, and xylan, have been

isolated. Only two aerobic thermophilic bacteria have been described that produce cellulases: *Acidothermus cellulolyticus* and *Rhodothermus*.

Uptake and phosphorylation of cellulose hydrolysis products. The driving force for uptake of glucose and its oligomers appears to vary among cellulolytic species, although there is to date no evidence for involvement of classical phosphoenolpyruvate (PEP)-dependent phosphotransferase systems. Nongrowing cells of *C. thermocellum* display uptake of cellobiose and cellodextrins by a common, ATP-dependent system, while glucose enters via a separate mechanism that is also ATP dependent. Strobel showed a sharp decline in the transport rate accompanying the addition of inhibitors that decreased intracellular ATP concentrations but not in response to inhibitors that abolished the proton motive force. These results are consistent with cellobiose and cellodextrin transport via an ATP-binding cassette protein, which is also a feature of the model for cellodextrin transport for *C. cellulolyticum* proposed by Desvaux et al. The cellulolytic actinomycete bacterium *Streptomyces reticuli* produces an ABC protein that assists in the transport of cellobiose, cellotriose, and possibly other cellodextrins. An ABC protein associated with a xylan metabolism operon has been identified based on DNA sequence data in R. flavefaciens. By contrast, *F. succinogenes* utilizes a Na^+ electrochemical gradient for uptake of glucose and cellobiose, and the Na^+ requirement for growth on cellulose suggests that Na^+ may be the driving force for uptake of cellodextrins as well.

Important distinguishing features of cellulosic biomass among potential feedstocks for biological processing include low purchase price, potential for supply on a very large scale, recalcitrance to reaction, and heterogeneous composition. For production of fuel and bulk chemicals, a feedstock having low cost and availability on a large scale is required and thus there is ample incentive to develop and apply technology that can cost effectively accommodate the recalcitrant, heterogeneous character of cellulosic biomass. For products with higher value and lower volume (e.g., fine chemicals or pharmaceuticals), there is much less incentive to use a low-cost feedstock, the scale of availability feedstock is not a significant issue, and feedstocks other than cellulosic biomass can be used which present fewer processing challenges. Because of these factors, it is natural in our view to focus on commodity products when considering biological processing of cellulosic feedstocks, and we do so here. The economics of cost-competitive commodity processes are dominated by feedstock cost and thus require high product yields. As a result, there is a very strong incentive to conserve the reducing equivalents present in fermentable carbohydrate feedstocks, which is the defining feature of anaerobic metabolism. Biological production of commodity products from cellulosic biomass is thus likely to involve microbial metabolism that is effectively anaerobic, although development of microbes for use in such processes can in principle begin with either aerobes as well as anaerobes or facultative anaerobes. These ideas are developed more fully elsewhere.

CBP requires a microbial culture that combines properties related to both substrate utilization and product formation. Desired substrate utilization properties include the production of a hydrolytic enzyme system allowing high rates of hydrolysis and utilization of resulting hydrolysis products under anaerobic conditions with a practical growth medium.

Desired product formation properties include high product selectivity and concentrations. A cellulolytic culture with this combination of properties has not been described to date.

Development of microorganisms for cellulose conversion via CBP can be pursued according to two strategies. The native cellulolytic strategy involves naturally occurring cellulolytic microorganisms to improve product-related properties such as yield and tolerance. The recombinant cellulolytic strategy involves engineering noncellulolytic microorganisms that exhibit high product yields and tolerance so that they become able to utilize cellulose as a result of a heterologous cellulase system. Subsequent subsections review in detail the progress for each of these strategies with respect to developing cultures capable of utilizing cellulose in a CBP configuration. In the remainder of this subsection, we comment on strategic aspects related to hemicellulose utilization.

The recombinant cellulolytic strategy for organism development pursuant to cellulose conversion via CBP begins with noncellulolytic microorganisms having excellent product formation properties and involves heterologous expression of a functional cellulase system. Such heterologous expression has been undertaken for a variety of purposes with a variety of microorganisms. We focus here on studies aimed at, or at least anticipating, enablement of growth on cellulose. Thus, we do not, for example, catalogue the vast and important literature associated with cloning cellulase components in *E. coli* for the purpose of enzymological studies. Heterologous expression of cellulase pursuant to growth enablement has been investigated to date primarily in *S. cerevisiae*, enteric bacteria, and *Z. mobilis*. We focus on the body of work involving these three organisms because this encompasses the most advanced embodiment of the recombinant cellulolytic organism development strategy to date. Some work on heterologous cellulase expression has been undertaken with additional hosts, whose potential utility should not be dismissed. The possibility of restoring functional expression of cryptic cellulase genes in *C. acetobutylicum* is also intriguing. General properties of yeast, enteric bacteria, and *Z. mobilis* as industrial biocatalysts, discussed elsewhere, are sufficiently established that an actively cellulolytic strain based on any of these hosts would probably find industrial application. We do not comment further on such properties here except to note that both the suitability of these organisms for use in industrial processes and the tools for genetic manipulation are much better established than is the case for naturally cellulolytic anaerobes. As discussed in the concluding discussion, these advantages must be weighed against the difficulty of enabling rapid growth on pretreated lignocellulose by organisms that are not naturally cellulolytic.

The last decade has seen marked advances in the depth and breadth of scientific understanding with respect to the structure, function, and genetics associated with the components of cellulase systems. Such advances include solving the 3-D structures of over two dozen cellulases, leading to a much better understanding of reaction mechanisms; the availability of many new protein sequences; meaningful new classification schemes based on structural features; and a better understanding of the regulation of cellulase genes, especially in fungal systems. Significant progress has also been made since 1990 with respect to understanding interactions among cellulase components. This includes a better understanding of synergistic interactions for an increasing number of noncomplexed cellulase systems, as well as a better and broader

understanding of the structure and composition of cellulosomes. We expect that expanding knowledge of the molecular details of cellulose hydrolysis will continue at an accelerated pace during the coming decade. This expectation is supported by the progress made in the last decade, the powerful new tools that continue to become available, and the talent and dedication with which a substantial cadre of scientists is pursuing these issues.

Important tools for understanding microbial cellulose utilization have in many cases become available only recently or have not yet been developed. Such tools include systems that allow foreign genes to be expressed in cellulolytic microorganisms, which are established for the aerobic *T. reesei* but not for most cellulolytic anaerobes. The recent development of an electrotransformation system for *C. cellulolyticum* makes possible new studies of microbial cellulose utilization using homologous recombination-mediated gene knockout. Such studies can be expected to yield exciting comparative results as similar systems become available for more cellulolytic microorganisms that are not currently transformable. Methods to independently quantify cells and cellulase can be expected to result in a second set of new insights, particularly in the areas of bioenergetics, metabolic control, and kinetics. New methods are required to fractionate and characterize glycocalyces and would be quite informative if developed. Continuous culture on cellulosic substrates is just now beginning to be applied in ways that give insights extending beyond summary description of substrate conversion and product formation, and it can be expected to yield rich insights in the coming years, especially when coupled with new analytical methods. Studies in which heterologous cellulase expression confers the ability to grow on nonnative substrates have begun to appear only in the last few years and represent an exciting frontier with the potential to become an important tool for fundamentally oriented investigations while also being relevant to applied goals.

The substantial potential of quantitative analysis to contribute to our understanding of cellulose hydrolysis at both subcellular and cellular levels has been realized to date to a very limited extent. In contexts such as specific activity of cellulases and adsorption, it is at present often difficult to draw quantitative conclusions that extend beyond the reach of a particular study. This limitation may be addressed by paying more attention to methodological standardization and by undertaking more interspecific comparative studies under controlled conditions. These measures can be expected to shed light on several fundamental issues of considerable interest about which there is currently substantial uncertainty, the relative efficacy of complexed and noncomplexed cellulase systems being a case in point. Quantitative studies at different levels of aggregation have great potential as a framework to test and develop our understanding but have seldom been undertaken.

Explanations for the features and molecular diversity of cellulase enzyme systems are logically sought in an understanding of the niches and adaptive strategies of the microorganisms in which these systems evolved. Conversely, results of molecular studies substantially enhance the depth and clarity with which the adaptive strategies of cellulolytic microorganisms can be understood. This potentially important complementarity can be more fully exploited in the future as understanding of microbial cellulose utilization advances. The vast majority of studies investigating cellulose hydrolysis and cellulase enzyme systems have proceeded within the context of

an enzymatically oriented intellectual paradigm. In terms of fundamentals, this paradigm focuses on cellulose hydrolysis as primarily an enzymatic rather than microbial phenomenon. In terms of applications, the enzymatic paradigm anticipates processes featuring production of cellulase in a step separate from that in which the cellulosic feedstock is hydrolyzed for the purpose of conversion to a desired product. This paradigm is clearly manifested in statements accompanying the early work of pioneers in the field.

In response to the needs of the enzymatic paradigm, research focused primarily on microorganisms that actively secrete cellulases. Since higher levels of cellulase secretion are observed in aerobic microorganisms than in anaerobes, it was logical for studies inspired by the enzymatic paradigm to focus on cellulase production using aerobes as well as their noncomplexed cellulase enzyme systems.

IN VITRO ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS DERIVED FROM LEAVES OF *CAMELLIA JAPONICA* CULTIVARS (THEACEAE) AGAINST *ESCHERICHIA COLI* STRAIN

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Camellia L., with about 280 species, is a genus of high economic, ecological and phylogenetic value in the family Theaceae [Vijayan et al., 2009; Yang et al., 2013; Li et al., 2019]. It is endemic to South and East Asia and China, which contains more than 80% of the species and is the centre of species diversity [Gao et al., 2005]. Among *Camellia* species, *C. japonica* L. has the highest economic value due to its beautiful ornamental flowers and medicinal properties. It is native to South Korea, Japan and China. *Camellia japonica*, which comprises thousands of cultivars, is the most widely cultivated species. It has also been used in traditional medicine in Japan, China, and Korea [Yoon et al., 2017]. Extensive studies have been conducted to evaluate the phytochemical composition of different parts of *C. japonica* plants, including saponins in fruits and seeds, and triterpenes in flowers and seed oil [Akihisa et al., 1997; Yoon et al., 2017; Pereira et al., 2022]. The leaves of *C. japonica* have been reported to possess antioxidant, antifungal and cytotoxic properties [Park et al., 2002; Thao et al., 2010; Onodera et al., 2016; Teixeira and Sousa, 2021]. The aim of the present study was to determine the antibacterial activity of six plant cultivars, i.e. *Camellia japonica* 'Kramer's Supreme', 'C.M. Wilson', 'La Pace', 'Mrs Lyman Clarke', 'Benikarako', 'Fanny Bolis' against *Escherichia coli* (Migula) Castellani and Chalmers (ATCC®25922™) strain. *Escherichia coli* is considered one of the clinically important bacteria, which are