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Cellulolytic Microorganisms from Thermal Environments

7

Tatiana A. Vishnivetskaya, Babu Raman, Tommy J. Phelps, Mircea Podar and James G. Elkins

Abstract

Conversion of liguocellulosic biomass to liquid fuels using biological processes offers a potential solution to partially offset the world's dependence on fossil fuels for energy. In nature, decomposition of organic plant biomass is brought about by the combined action of several interacting microorganisms existing in complex communities. Bioprospecting in natural environments with high cellulolytic activity (for example, thermal springs) may yield novel cellulolytic microorganisms and enzymes with elevated rates of biomass hydrolysis for use in industrial biofuel production. In this chapter, various cellulose-degradmg microorganisms (in particular, thermophilic anaerobic bacteria), their hydrolytic enzymes, and recent developments in the application of biomass fermentations for production of sustainable bioenergy are reviewed. In this context, results from ongoing research at the Oak Ridge National Laboratory in the isolation and subsequent phylogenetic and metabolic characterization of thermophilic, anaerobic, cellulolytic bacteria from the hot springs of Yellowstone National Park are presented.

Introduction

Each year photosynthetic fixation of CO_2 yields over 4 billion tons of plant biomass (Detroy and Stjulian, 1983; Beguin and Aubert, 1994; Leschine, 1995). Depending on plant species and cell type, plant cell walls typically consist of between 35% to 50% cellulose, 20% to 35% hemicellulose, and 10% to 25% lignin on a dry weight basis (Saha, 2004; Somerville, 2006). In fact, cellulose is the most abundant organic biopolymer in terrestrial environments. Crops and other sources of biomass can be used for the production of fuel and energy, as they represent stored solar energy that is converted from sunlight during the fixation of carbon dioxide into organic compounds such as polysaccharides. Therefore, plant biomass is a substantial natural resource for renewable energy. There are several different routes for harvesting the energy stored in plant biomass:

1. Plant material (e.g. wood) can be burned directly to produce energy, adequate for domestic heating and cooking.
2. Organic material, including cellulosic biomass, can be converted to synthesis gas (a mixture, primarily of carbon monoxide and hydrogen) through the process of thermal gasification, which can then be converted to biofuels either chemically by Fischer-Tropsch synthesis or biologically using microbial processes (Inderwildi *et al.*, 2008; Valdez-Vazquez and Poggi-Varaldo, 2009).
3. Plant material in the form of animal manure can be decomposed to the flammable gas methane through methanogenesis (das Neves *et al.*, 2009).
4. Plant biomass can be enzymatically hydrolysed and the resulting sugars fermented to produce liquid ethanol, which can be used as a transportation fuel either directly or in combination with gasoline (Lynd *et al.*, 2002); and lastly
5. Plant material can be fermented by obligate or facultative anaerobes to higher alcohols

such as butanol and propanol, or converted to other chemicals such as acetone, diacetyl, etc.

Butanol could also serve as a direct replacement for gasoline due to its favourable physical properties (Garncarek and Kociolek-Balawejder, 2009).

In light of the growing concern regarding the long-term supply of fossil fuels, applied research aimed at unlocking the energy stored in plant biomass for anthropogenic use has increased (Lynd *et al.*, 2002; Saha, 2004). However, the recalcitrance of lignocellulosic materials to enzymatic hydrolysis still poses the largest technological hurdle in developing a photosynthetic-based bioenergy economy. Plant biomass is made in part from cellulose, a homopolymer consisting of a linear chain of linked d-glucose units. As a natural substrate, cellulose is insoluble in water (Chang *et al.*, 2006). Cellulose is associated with other plant polymers such as hemicelluloses, lignin, pectin, and proteins (Somerville, 2006). This interwoven, complex association hinders the ability of hydrolytic enzymes (e.g. cellulases) to act on cellulose fibrils, thereby inhibiting degradation. In order to improve enzyme accessibility and aid hydrolysis, plant biomass is usually pretreated using a combination of heat, pressure, and harsh chemicals to remove hemicellulose and proteins, and partially hydrolyse the crystalline cellulose (Wyman, 1999). Pretreated biomass is then hydrolysed using a cocktail of enzymes (for example, from the fungus *Trichoderma reesei*) and the liberated sugars are anaerobically converted to ethanol using fermentative microorganisms, such as *Escherichia coli*, *Zymomonas mobilis* or yeast. Along with readily fermented hexoses, cellulosic biomass also contains pentose sugars such as l-arabinose and d-xylose, derived from the hemicellulose component of plant cell walls. Industrial production of bioethanol from lignocellulolytic material is therefore dependent on the availability of robust microorganisms that are capable of fermenting both C_6 and C_5 carbohydrates present in the feedstock. For example, some wild-type bacteria (*Z. mobilis*) and yeast (*Saccharomyces cerevisiae*) are able to rapidly and efficiently convert glucose (C_6) substrates into ethanol but cannot ferment xylose (Dien *et al.*, 2003). These and other organisms have been engineered to obtain strains that are capable of fermenting both hexoses and pentoses to produce ethanol (Yanase *et al.*, 2007; Bettiga *et al.*, 2009).

Traditional ethanol production in a simultaneous saccharification and co-fermentation (SSCF) process configuration requires a dedicated step for cellulase production (Lynd *et al.*, 2005). An alternative process under development for the conversion of lignocellulosic biomass is termed consolidated bioprocessing (CBP). It involves the use of either native cellulolytic organisms (e.g. *Clostridium thermocellum*) or recombinant organisms which have been engineered to produce cellulolytic and other hydrolytic enzymes (e.g. yeast). This allows for the integration of several biologically mediated processes, specifically saccharolytic enzyme production, enzymatic hydrolysis of pretreated plant biomass carbohydrates, and fermentation of hexose and pentose sugars, into a single step process. Eliminating or minimizing the need for external addition of expensive cellulases offers significant economical advantages for the CBP process over the conventional SSCF. Cost comparison analysis of different bioprocess configurations has highlighted the potential impact of the CBP process in establishing an economically viable and sustainable ethanol industry. (Lynd *et al.*, 2002, 2005). Thermophilic and extreme thermophilic cellulolytic fermentative organisms potentially offer additional economical advantages in an industrial bioethanol production scheme. For example, fermentation processes operating at elevated incubation temperatures (80°C or higher) may allow for partial distillation of ethanol to occur. Removal of ethanol from the fermentation broth reduces its inhibitory effects on cell growth and metabolism, consequently leading to increased ethanol productivity (Hamilton-Brehm *et al.*, 2010). Natural environments such as thermal hot springs with decaying plant material (discussed in the ensuing section) are a rich resource of thermophilic cellulolytic organisms with potential use in high-temperature processes. For example, bioprospecting in these locations could lead to the discovery of novel microbial species or communities that are capable of both degrading plant polymers and fermenting the suite of sugars present in lignocellulosic biomass to ethanol.

Natural environments as a source of cellulose-degrading microorganisms

Cellulose-degrading microbial communities contribute significantly to the global carbon cycle, partly by playing an essential role in biomass degradation and turnover. Bacterial and fungal degradation of cellulose occurs through the action of extracellular enzymes that are either cell-free or associated with the outer cell envelope. Microbially mediated cellulose depolymerization provides a virtually endless resource of easily digestible metabolites and products, including soluble sugars, organic acids, alcohols, CO_2, and H_2 (Leschine, 1995). These compounds are readily utilized as carbon and energy sources by other, non-cellulolytic commensal members of the microbial community inhabiting the cellulolytic environment. CO_2 released into the atmosphere by microbes is converted back to sugars and further to lignocellulosic biomass during photosynthesis and plant growth (Leschine, 1995; Desvaux *et al.*, 2001; Doi, 2008).

Cellulose decomposition occurs in soils, sediments, aquatic environments as well as environments impacted by human activities or associated with animals (sewage sludge, compost piles, wood-chip piles, paper mills, wood processing plants). Soil and sediments are a huge reservoir of carbon in the form of dead and recalcitrant plant biomass, where cellulose is degraded either aerobically or anaerobically. Aerobic cellulose decomposition may be achieved through the activities of a single species or consortia of mesophilic fungi and bacteria. For instance, the cellulose and hemicellulose components of wood are completely degraded and metabolized by single fungal species of either *Phlebia radiate* (white-rot fungi), *T. reesei* or *Penicillium* species (Busto *et al.*, 1996; Fujii and Shintoh, 2006; Cho *et al.*, 2009). Mesophilic aerobic cellulolytic bacteria, such as *Bacillus pumilus*, *Cellulomonas* spp., *Cellvibrio gilvus*, *Pseudomonas fluorescens*, and *Streptomyces* spp., are often found in soils, on plant materials, in humus, animal faeces, and leaf litter (Deshpande *et al.*, 2009; Dinis *et al.*, 2009).

While most of the cellulose in nature is degraded aerobically, 5–10% is degraded anaerobically by cellulose-fermenting microorganisms (Leschine, 1995). Anaerobic cellulose decomposition, which requires mixed populations, benefits from being performed by a community of physiologically diverse microorganisms (Leschine, 1995). Anaerobes are metabolically diverse and can perform various fermentations and respirations, employing varied electron acceptors (e.g. carbon dioxide, inorganic sulfur compounds, and inorganic nitrogen compounds). A typical ecosystem undergoing anaerobic degradation of lignocellulosic plant matter by microbial communities is schematically represented in Fig. 7.1. In the absence of oxygen, cellulose is decomposed to acetate, H_2 and CO_2, which are then able to undergo methanogenesis through a complex microbial food chain (Leschine, 1995). Cellulose is initially hydrolysed through the action of extracellular enzymes of cellulolytic bacteria. The released sugar monomers and oligomers are fermented by cellulolytic and other saccharolytic microorganisms with production of CO_2, H_2, organic acids (e.g. acetate, propionate, butyrate), and alcohols (e.g. ethanol). Exogenous H_2 is immediately consumed by methanogens or acetogens, which use H_2 to reduce CO_2 to, respectively, CH_4 or acetate. Acetate can then serve as a terminal electron acceptor and the predominant end products of the methanogenesis appear to be CH_4, CO_2, and H_2O (Fig. 7.1). Cellulose-decomposing microbial communities comprising several symbiotic anaerobic bacteria are found in the gut flora of herbivorous mammals (e.g. the rumen of cattle and sheep), leaf eating birds (e.g. hoatzin, the only known representative), and plant eating insects (e.g. termites, grubs, larvae). In addition to anaerobic bacteria and archaea, the microbial communities in the intestinal tract of large herbivorous mammals also contain a number of anaerobic fungi, which are classified into six genera, namely *Anaeromyces*, *Caecomyces*, *Cyllamyces*, *Neocallimastix*, *Orpinomyces* and *Pyromyces* (Nicholson *et al.*, 2005).

While the majority of common natural habitats where cellulose degradation occurs (e.g. soils, sediments, compost and even gut environments) are temperate or even low-temperature niches, thermal (55–95°C) aquatic environments that contain decaying plant material are also a good source of lignocellulose-degrading microorganisms (see

Figure 7.1 Schematic representation of anaerobic degradation of lignocellulosic plant biomass by microbial communities in soils and freshwater sediments. Cellulolytic, xylanolytic and ligninolytic microbes degrade the major polysaccharide components of plant cell walls to monomers and oligomers. Commensal microorganisms assimilate the soluble sugars and other end products (ethanol and lactate usually do not accumulate), which are eventually converted to methane and carbon dioxide.

Table 7.1). The range of substrates these thermophilic microorganisms can utilize depends on the temperature, biotope, and inorganic chemistry of the aqueous phase. For example, many anaerobic thermophilic bacteria of the order Thermotogales do not utilize crystalline cellulose as a substrate, but are capable of fermenting simple and complex sugars to H_2, CO_2, and acetate (Blumer-Schuette *et al.*, 2008). The ability to utilize crystalline cellulose as well as hemicellulose as a growth substrate is limited to terrestrial thermophilic bacteria of the phylum Firmicutes with growth temperature optima around 70°C. Cellulolytic, anaerobic thermophiles have been isolated from terrestrial hot springs of Kamchatka (Far East Russia), Iceland, New Zealand, Nagano Prefecture in Japan and the Great Artesian Basin in Australia. (Taya *et al.*, 1988; Hudson *et al.*, 1990; Svetlichnyi *et al.*, 1990; Bredholt *et al.*, 1995; Mladenovska *et al.*, 1995; Miroshnichenko *et al.*, 2008; Ogg and Patel, 2009). The cellulolytic bacteria isolated to date belong to the order Clostridiales, including the extremely thermophilic *Anaerocellum thermophilum, Caldicellulosiruptor kristjanssonii, Caldicellulosiruptorkronotskyensis, Caldicellulosiruptor hydrothermalis, Caldicellulosiruptor lactoaceticus,* and the thermophilic *Caloramator australicus, Clostridium thermocellum* and *Thermoanaerobacter thermohydrosulfuricus* (Freier *et al.*, 1988; Svetlichnyi *et al.*, 1990; Mladenovska *et al.*, 1995; Koskinen *et al.*, 2008a; Miroshnichenko *et al.*, 2008; Ogg and Patel, 2009).

One of the most abundant distributions of thermal sites is within Yellowstone National Park (YNP), located in north-western USA. Bacterial and archaeal diversity within the different YNP hot springs is well documented, and several novel thermophilic microorganisms have been isolated (Hugenholtz *et al.*, 1998; Reysenbach *et al.*, 2000; Meyer-Dombard *et al.*, 2005; Nakagawa

Table 7.1 Thermophilic microorganisms capable of degrading lignocellulosic biomass

Species	Temperature range (optimum), °C	pH range (optimum)	Fermentation end-products[1]	Original source	Reference
Aerobic					
Acidothermus cellulolyticus	37–70 (55)	3.5–7.0 (5.0)	NA	Hot springs, Norris Geyser basin, YNP	Mohagheghi *et al.* (1986)
Caldibacillus cellulovorans	(68)	(7.0)	NA	Artificial compost heap	Bergquist *et al.* (1999)
Rhodothermus marinus	54–77 (65)	(7.0)	NA	Submarine alkaline hot spring, Iceland	Alfredsson *et al.* (1988), Hreggvidsson *et al.* (1996)
Anaerobic					
Anaerocellum thermophilum[2] DSMZ 6725	40–83 (75)	(7.2)	Lac, Ac, H_2, CO_2	Valley of Geysers, Kamchatka, Russia	Svetlichnyi *et al.* (1990); Yang *et al.* (2010)
Caldicellulosiruptor lactoaceticus	50–78 (68)	5.8–8.2 (7.0)	Lac, Ac, Et, H_2, CO_2	Alkaline hot spring, Hveragerdi, Iceland	Mladenovska *et al.* (1995)
Caldicellulosiruptor saccharolyticus	45–80 (70)	5.5–8.0 (7.0)	Ac, H_2, CO_2	Hot spring, Taupo-Rotorua region, New Zealand	Rainey *et al.* (1994)
Caldicellulosiruptor kristjanssonii	45–82 (78)	5.8–8.0 (7.0)	Lac, Ac, Et, H_2, CO_2	Slightly alkaline hot spring, Iceland	Bredholt *et al.* (1999)
Caldicellulosiruptor owensensis	50–80 (75)	5.5–9.0 (7.5)	Lac, Ac, Et, H_2, CO_2	Dry sediment, Owens Lake, California, USA	Huang *et al.* (1998)
Caldicellulosiruptor kronotskyensis 2002[3]	45–82 (70)	6.0–8.0 (7.0)	Lac, Ac,	Terrestrial hot springs, Kamchatka, Russia	Miroshnichenko *et al.* (2008)
Caldicellulosiruptor hydrothermalis 108[3]	50–80 (70)	6.0–8.0 (7.0)	Lac, Ac	Terrestrial hot springs, Kamchatka, Russia	Miroshnichenko *et al.* (2008)
Clostridium stercorarium	(65)	(7.3)	Et, Ac	Compost heap	Madden (1983)
Clostridium thermocellum JW20	37–69 (60)	6.1–7.5 (7.0)	Et, Ac, Lac, H_2, CO_2	Cotton bale, Louisiana, USA	Freier *et al.* (1988)
Desulfurococcus fermentans (crenarchaeote)	63–89 (81)	4.8–6.8 (6.0)	Ac, H_2, CO_2	Uzon caldera, Kamchatka, Russia	Perevalova *et al.* (2005)
Fervidobacterium islandicum	50–80 (65)	6.0–8.0 (7.2)	Lac, Ac, Et, H_2, CO_2	Thermal spring, Varma River, Hveragerdi, Iceland	Huber *et al.* (1990)
Spirochaeta thermophila	40–73 (67)	509–7.7 (7.0)	Ac, CO_2, H_2, Lac	Saline hot spring, Shiashkotan, Russia	Aksenova *et al.* (1992)
Thermoanaerobacter cellulolyticus	50–85 (75)	6.0 – 9.5 (8.1)	Ac, H_2 CO_2, Lac	Alkaline hot spring, Nagano Prefecture, Japan	Bredholt *et al.* (1995)
Thermotoga neapolitana	5 –90 (80)	5.5 – 9.0 (7.0)	NR	Submarine thermal vent, Naples	Jannasch *et al.* (1988), Sunna *et al.* (1997)

[1]End-products are those reported and abbreviated as follows: Lac, lactate; Ac, acetate; Et, ethanol. [2]*Anaerocellum thermophilum* was recently reclassified as *Caldicellulosiruptor bescii* (Svetlichnyi *et al.*, 1990; Yang *et al.*, 2010). [3]Type strain. NA, not applicable; NR, not reported.

et al., 2005). For example, two studies describe the isolation of mesophilic and thermophilic xylan-degrading bacteria from YNP. In the first, several mesophilic bacteria of the genus *Clostridium* showing high xylanolytic activity were isolated from agricultural and forest soil samples and water-sediment samples from acidic springs. This included the *Clostridium acetobutylicum* isolate 3BYR, which exhibited very high extracellular xylanase, xylosidase, alpha-l-arabinofuranosidase and alpha-glucuronidase activities, and utilized up to 80% of oat spelt xylan as a carbon source during growth (Trudeau *et al.*, 1992). In the second study, thermophilic, anaerobic, xylanolytic strains of *Thermoanaerobacterium xylanolyticum* sp. nov. and *Thermoanaerobacterium saccharolyticum* sp. nov. were isolated from Frying Pan Springs. These organisms are non-cellulolytic and grow chemoorganotrophically at 60°C and pH 6.0, fermenting xylan and starch to acetate, ethanol, lactate, CO_2, and H_2 (Lee *et al.*, 1993). compared with the above xylan-degrading microbes, studies dedicated to isolation of cellulolytic bacteria from YNP are scarce. Cellulolytic organisms isolated from YNP include the anaerobic thermophile *C. thermocellum* strain YS (Bayer *et al.*, 1983) from hot spring soil samples and the aerobic, acidophilic thermophile, *Acidothermus cellulolyticus*, from acidic hot springs (Mohagheghi *et al.*, 1986). We hypothesized that the anaerobic thermal springs of YNP probably contain a wide variety of cellulose-degrading microbial communities and focused our efforts on enriching these communities on natural pretreated biomass (see later), with the eventual aim being the isolation of novel cellulolytic microorganisms with high rates of lignocellulosic biomass utilization.

Enzyme systems of cellulolytic microorganisms

Plant cell wall composition

Plant cell walls are composed of three major polysaccharide polymers, namely, cellulose, hemicellulose and pectin. Cellulose is a stable unbranched homopolymer with linear chains of β-1,4-linked glucose units stacked tightly in parallel into microfibrils via strong inter- and intra-chain hydrogen bonding. The structurally rigid cellulose microfibrils are largely crystalline in nature, with semi-crystalline and amorphous regions in between (Chang *et al.*, 1981). Hemicellulose and pectin, on the other hand, are heterogeneous branched polymers and are chemically and structurally more complex than cellulose. Pectin is a family of galacturonic acid-rich polysaccharides including homogalacturonan, xylogalacturonan, and rhamnogalacturonan I and II polymers (Mohnen, 2008). Hemicellulose consists primarily of xylan or other backbone structures (e.g. galacto-glucomannan, arabinogalactan, lichenins), classified based on their monomeric composition. In addition, hemicellulose sugars are often acetylated or methylated and also linked with lignin, which is another heterogeneous plant polymer of linked aromatic monomers (Knox, 2008). To counter the chemical and structural complexity of plant cell wall polysaccharides, cellulolytic organisms have evolved enzyme systems with a wide array of cellulases and hemicellulases for hydrolysis and degradation of plant biomass (Schwarz *et al.*, 2004; Dashtban *et al.*, 2009).

Plant cell wall-degrading enzymes

Plant biomass-degrading enzymes were originally classified based on the substrates they hydrolysed and their mode of action. More recently, cellulases and other carbohydrate active enzymes (CAZymes; www.cazy.org) have been classified based on their sequence homology, structural fold and mechanistic themes (Henrissat and Davies, 1997; Cantarel *et al.*, 2009). As of September 2010, the CAZy database classification included 118 glycoside hydrolase (GH), 16 carbohydrate esterase (CE), 22 polysaccharide lyase (PL) and 61 carbohydrate binding module (CBM) families. Cellulases hydrolyse the β-1,4-glucosidic bonds in cellulose polymers either internally (endo-acting), or at the reducing/non-reducing ends (exo-acting) of the polysaccharide chain. Owing to a high degree of crystallinity and structural stability of the cellulose polymer, the combined synergistic action of several cellulases with differing specificities (e.g. glycoside hydrolases belonging to families GH48 and GH9 in bacteria, based on CAZy classification) is needed for its degradation (Wilson, 2009).

In contrast, hemicellulose degradation requires the action of several types of enzymes that act upon bonds between different carbohydrate moieties or the bonds between carbohydrate and non-carbohydrate moieties in this chemically heterogeneous plant polymer. For example, while xylanases (e.g. GH10, GH11 family enzymes) and mannanases (e.g. GH26) cleave the main backbone, other hemicellulases such as arabinofuranosidase (e.g. GH43) and glucuronidase cleave the bonds between backbone sugars and side-chain substituent sugars (Shallom and Shoham, 2003). Carbohydrate esterases are another group of enzymes that cleave the ester bond between the hemicellulose sugars and acetyl group (e.g. acetyl esterases belonging to the CE2, CE3 families) or lignin monomeric compounds such as ferulic and *p*-coumaric acids (e.g. feruloyl esterases belonging to the CE1 family) (Wong, 2006). Lastly, the polysaccharide lyase family of enzymes is active against glycosidic bonds present in anionic polysaccharides such as the pectin component of the plant cell wall (Michaud *et al.*, 2003).

Modular nature of enzymes

Carbohydrate-active enzymes, especially in anaerobic bacteria, have a modular structure. For example, cellulases and hemicellulases consist of several structurally and functionally distinct units called *domains* or *modules* (Gilkes *et al.*, 1991; Schwarz *et al.*, 2004). Apart from the catalytic module(s) which cleave the glycosidic bond, the additional non-catalytic domains participate and contribute to the overall hydrolytic activity of the enzyme. For example, the carbohydrate binding modules (CBM) help in targeting the catalytic module, and in attachment of the free enzymes, to the insoluble plant polysaccharide substrates (Shoseyov *et al.*, 2006). Other non-catalytic modules include the immunoglobulin-like (Ig), fibronectin type-III (Fn3), dockerins involved in cellulosome assembly and the surface-layer homology (SLH) module (Doi and Tamaru, 2001). SLH modules interact with cell wall polysaccharides and aid in the attachment of free enzymes or cellulosome anchor proteins to the cell surface (e.g. *C. thermocellum*; see discussion below). Recent evidence suggests that apart from the modular composition, their arrangement on the polypeptide chain also has a bearing on the role these accessory modules play in the overall functional activity of a given enzyme (Bayer *et al.*, 2004).

Complexed and non-complexed enzyme systems

Cellulolytic organisms have evolved to produce a host of enzymes for degrading plant cell wall polysaccharides. Broadly, two types of enzyme systems have been observed in cellulolytic microorganisms, namely the *non-complexed* and *complexed* systems (Bayer *et al.*, 2006; Doi, 2008). Aerobic fungi (e.g. *T. reesei*), aerobic thermophilic bacteria (e.g. *Acidothermus cellulolyticus*, *Thermobifida fusca*) and anaerobic, extremely thermophilic bacteria (e.g. *Caldicellulosiruptor saccharolyticus*, *A. thermophilum*) contain the non-complexed enzyme system, in which several individual secreted endo- and exo-glucanases act synergistically in degradation of plant cell wall cellulose (Svetlichnyi *et al.*, 1990; Rainey *et al.*, 1994). Complexed enzyme systems are found in anaerobic fungi (e.g. *Anaeromyces*, *Caecomyces*, *Cyllamyces*, *Neocallimastix*, *Orpinomyces*, and *Pyromyces*), and both mesophilic (e.g. *Bacteroidetes cellulosolvens*, *Clostridium cellulovorans*, *Clostridium cellulolyticum*, *Ruminococcus flavefaciens*) and thermophilic bacteria (*C. thermocellum*) (Ali *et al.*, 1995b; Ljungdahl, 2008). These microorganisms form large extracellular enzyme complexes on the cell surface, termed the cellulosome, consisting of a non-catalytic scaffolding protein(s) with many bound catalytic components exhibiting cellulolytic, hemicellulolytic and other hydrolytic activities (Bayer *et al.*, 1998; Miron *et al.*, 2001; Doi and Kosugi, 2004; Xu *et al.*, 2004; Fendri *et al.*, 2009).

Cellulosomal architecture

In cellulosomes, a structural component called scaffoldin contains binding sites (cohesins) that interact with the docking modules (dockerins) borne by the enzymatic units. The highly specific and stable cohesin-dockerin type interaction mediates the assembly of the catalytic components onto the scaffoldin (Type I interaction) and also the attachment of the scaffoldin itself (with bound subunits) onto the cell surface via anchor proteins

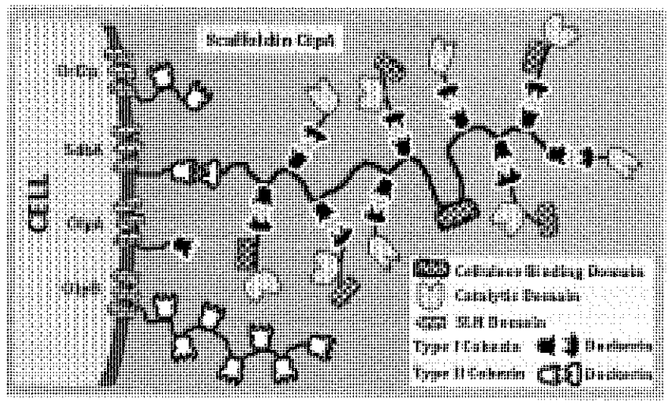

Figure 7.2 Simplified schematic representation of *Clostridium thermocellum* cellulosomal architecture. The cellulosome backbone scaffoldin protein CipA contains 9 type-I cohesins decorated with catalytic units bearing the type-I dockerin. CipA also contains a type-II dockerin which interacts with the type-II cohesins of the anchor proteins (e.g. SdbA, OlpB, Orf2p), which in turn are anchored to the bacterial cell surface via interaction of the SLH domains with the cell wall-associated polysaccharides. Also shown is another cell surface anchor protein, OlpA, containing a type-I cohesin. CipA and many catalytic units also contain cellulose (and other carbohydrate) binding domains which aid in attachment of the cellulosome complex to various plant cell wall components. Figure adapted from Raman *et al.* (2009).

(Type II interaction) (Beguin and Lemaire, 1996; Belaich *et al.*, 1997; Doi and Tamaru, 2001). A simplified schematic of the cellulosomal architecture in *C. thermocellum* is shown in Fig. 7.2; further details abont this system are provided in a later section. More complex architectures involving multiple adapter scaffoldin proteins in cell wall anchoring has also been reported (Xu *et al.*, 2004; Rincon *et al.*, 2005). In cellulolytic organisms with complexed enzyme systems, the scaffoldin component usually contains a carbohydrate binding module (CBM) which aids in substrate recognition and binding. Thus the cellulosome complex provides for both proximity-based synergy of catalytic units via their assemblage on the scaffoldin, as well as targeting-based synergy between microbe and biomass via CBM-mediated formation of the cell-cellulosome-substrate tri-complex (Lu *et al.*, 2006). However, the mechanism(s) of microbial adhesion to cellulose and its relative importance in substrate utilization in cellulolytic organisms is not clearly understood and reqnires further research (Bayer *et al.*, 1998; Demain *et al.*, 2005).

Cellulolytic enzymes of anaerobic thermophilic bacteria

In this section, we will focus on the enzyme systems of three anaerobic cellulolytic bacteria that are either thermophilic (*C. thermocellum*, abbreviated *Cthe*) or extremely thermophilic (*A. thermophilum*, *Athe* and *Ca. saccharolyticus*, *Csac*), and whose genomes have been sequenced (http://genome.ornl.gov/microbial/cthe/; van de Werken *et al.*, 2008; Kataeva *et al.*, 2009). Table 7.2 compares the distribution of glycoside hydrolases, esterases, lyases and carbohydrate binding modules across these three organisms. Genome-level comparison reveals a similar total number of glycoside hydrolase domains encoded in *Cthe*, *Csac* and *Athe* (Table 7.2). Complete cellulose degradation requires the combined action of exo-acting cellobiohydrolases and endoglucanases (Warren, 1996). While all three organisms encode GH48 family bacterial reducing-end cellobiohydrolase, *Cthe* has a higher distribution of other cellulose-specific hydrolytic enzymes (e.g. belonging to the GH9 and GH8 families) as compared with *Csac* and *Athe* (Table 7.2). These

observations are consistent with *C. thermocellum* being known as one of the most efficient cellulose-degrading microorganisms (Lynd and Zhang, 2002). In comparison, the extreme thermophiles display a wider distribution of glycoside hydrolase domains across several different families, including several GH13 amylases and other α-glucan acting enzymes (e.g. belonging to the GH4, GH29 and GH65 families), reflecting their broader snbstrate specificity (Table 7.2).

All three organisms encode enzymes for hydrolysing the non-cellnlosic components of the plant cell wall, namely, hemicellulose and pectin. In addition to xylanases (e.g. belonging to the GH10 and GH11 families), Cthe contains more mannanase (GH26) and arabinofuranosidase

Table 7.2 Comparison of glycoside hydrolase (GH), carbohydrate esterase (CE), polysaccharide lyase (PL) and carbohydrate binding module (CBM) family domain distribution across the genomes of three anaerobic cellulolytic bacteria, the thermophilic *Clostridium thermocellum* ATCC 27,405(*Cthe*) and extremely thermophilic *Caldicellulosiruptor saccharolyticus* DSM 8903 (*Csac*) and *Anaerocellum thermophilum* DSM 6725 (*Athe*)

Domain Family	*C. thermocellum*	*Ca. saccharolyticus*	*A. thermophilum*
GH	# Domains		
1	2	1	1
2	1	4	4
3	2	2	1
4		2	2
5	12	6	6
8	1		
9	16	2	2
10	6	6	5
11	1		1
13	2	7	7
15	1	1	1
16	2	1	
18	3		
20		1	
23	2	2	2
26	3	1	
28		2	2
29		1	1
30		1	1
31		1	1
36		1	1
39	1	2	2
42		1	1
43	6	4	3
44	1	1	1
48	2	1	3
51	1	1	1
53	1		
65		2	2
67		1	1
74	1	1	1
78		1	
81	1		
88		1	
94	3	2	2
105		1	1
Total	71	61	56

Domain Family	*C. thermocellum*	*Ca. saccharolyticus*	*A. thermophilum*
CBM	# Domains		
3	23	10	16
4	7		
6	11	1	1
9	2		
11	1		
13	2		
16	3		
20		1	2
22	5	5	5
25	2		
28		1	1
30	1		
32	1	1	
34	1		
35	7		
36			1
41		1	1
42	4		
44	1		
48	1	3	3
50	2		
54	1	1	
Total	75	24	30

Domain Family	*C. thermocellum*	*Ca. saccharolyticus*	*A. thermophilum*
CE	# Domains		
1	3	1	
2	1		
3	2		
4	3	3	2
7	1	1	1
8	1		
9	1	1	1
12	2		1
NC	1		
Total	15	6	5

PL	# Domains		
1	2		
3			1
9	1		1
11	1		1
NC		1	
Total	4	1	3

(GH43) domains and carbohydrate esterases (e.g. CE1, CE4) than *Csac* and *Athe*. Among pectin-degrading enzymes, both *Cthe* and *Athe* contain polysaccharide lyases (e.g. PL9, PL11) which attack the pectin backbone, and esterases (e.g. CE8, CE12) which cleave the side-chains, while *Csac* contains glycoside hydrolases (e.g. belonging to the GH28 and GH105 families) that are active against pectin. Consistent with the biomass-degrading capacity encoded in their genomes, *Ca. saccharolyticus* and *A. thermocellum* can grow on cellulose, xylan, pectin and a range of other biomass-derived sugar monomers and oligomers (de Vrije *et al.*, 2009; Vanfossen *et al.*, 2009; Yang *et al.*, 2009a). In contrast, *C. thermocellum* can only grow on cellulose and the derived cello-oligosaccharides (Demain *et al.*, 2005). Therefore, the repertoire of enzymes encoded by *C. thermocellum* for hemicellulose and pectin degradation is only for unwrapping and exposing of its preferred substrate of cellulose, which is intertwined with these polysaccharides in plant cell walls.

The evolution of *C. thermocellum* as a voracious cellulose degrader is further highlighted by the preferential distribution of cellulose-specific carbohydrate binding modules in its genome. *Cthe* contains an abundance of CBMs (75 CBM domains from 18 families), including 23 cellulose-binding CBM3 family domains, as compared with *Csac* and *Athe* (Table 7.2). *Cthe* also has several domains (e.g. from the CBM4, CBM6, CBM35 families) that bind to various hemicellulose components, including xylan, glucomannan, and arabinoxylan. In contrast, Csac and Athe have representative domains in CBM families (e.g. CBM20, CBM48) that bind to starch, glycogen, etc., concordant with the presence of many α-glucan acting enzymes in their genomes, as discussed earlier.

While most of the CAZymes in *C. thermocellum* contain a dockerin and are cell-bound as part of the cellulosome, the organism also has a small number of cell-free enzymes including a GH48 family enzyme (Zverlov *et al.*, 2005). In comparison, *A. thermophilum* and *Ca. saccharolyticus* do not produce cellulosomes and contain cell-free, non-complexed enzyme systems. Table 7.3 lists all CAZymes from *Cthe*, *Athe* and *Csac* that have at least two domains belonging to the GH, CE, PL or CBM families. In *Cthe*, cellulosomal enzymes are bound to a scaffoldin subunit that contains a family 3a CBM and aids in the collective binding of the complex to crystalline cellulose. Yet, more than half of the cellulosomal catalytic units also contain CBM domain(s) of related substrate specificity, in addition to the dockerin module (Table 7.3). For example, several GH9 cellulosomal cellulases contain a family 3c CBM that is thought to play a 'helper' role, acting in concert with the catalytic domain, in transiently binding and feeding a loose non-crystalline cellulose chain into the active site of the enzyme (Gal *et al.*, 1997; Irwin *et al.*, 1998).

Cthe has several multifunctional enzymes that contain catalytic domains with complementing activities oriented towards hemicellulose degradation, potentially to counter the structural and chemical complexity of this heterogeneous polysaccharide. Examples include GH10 and GH11 xylanases paired with esterases belonging to the CE1 (XynZ, *Cthe*_1963) and CE4 (XynA, *Cthe*_2972) families, respectively. Some of the cellulosomal enzymes display a more complex modular architecture, as in the example of a GH9 cellulase paired with GH44 and CBM30, CBM44 domains (CelJ, *Cthe*_0624). Non-cellulosomal multi-modular enzymes in *Cthe* contain multiple repeated domains from the same CBM family, as in the case of GH16 (LicA, *Cthe*_2809) paired with 4 CBM4 domains (Table 7.3). A similar trend is also observed in the cell-free enzymes of *Athe* and *Csac*, which contain multiple CBM3 domains.

Although *Athe* and *Csac* have fewer multifunctional enzymes than *Cthe*, genome analysis reveals a higher order of modular architecture of enzymes in these extremely thermophilic organisms. Several enzymes contain two catalytic domains and multiple CBM domains, in some cases with differing substrate specificities (see Table 7.3). Examples of multi-domain enzymes sharing similar architecture in the two organisms include: (i) GH5 catalytic domain paired with a GH44 (*Athe*_1859 and *Csac*_1077, ManA) along with two CBM3 domains (Saul *et al.*, 1990; Gibbs *et al.*, 1992); and (ii) two GH43 arabinofuranosidase domains appended with a family 6 and -22 CBM domain (*Athe*_0182 and *Csac*_2411, XynF). Of particular interest is a very similar cellulase encoded by *Athe* and *Csac* that contains GH9 cellulase and GH48 cellobiohydrolase family catalytic domains along with three CBM3 domains (*Athe*_1867 and *Csac*_1076, CelA). This organization suggests synergism between these modules for concerted action on crystalline cellulose (Teo *et al.*, 1995; Zverlov *et al.*, 1998). There is no evidence for a similar enzyme with paired cellulase domains in the cellulosome producing *Cthe*.

Table 7.3 Multimodular enzymes of *C. thermocellum* (cellulosomal and non-cellulosomal; *Cthe*), *A. thermophilum* (non-cellulosomal; *Athe*) and *Ca. saccharolyticus* (non-cellulosomal; *Csac*) containing at least two domains belonging to the GH, CE, PL or CBM families. Enzymes containing at least three domains are highlighted in grey.

C. thermocellum : Cellulosomal Enzymes	
Locus	Modular Architecture
Cthe_0015	CBM42-Doc1-GH43
Cthe_0043	GH9-CBM3-Doc1
Cthe_0246	Doc1-CBM35-PL11
Cthe_0412	CBM4-Ig-GH9-Doc1
Cthe_0413	CBM4-Ig-GH9-CBM3-Doc1
Cthe_0433	GH9-CBM3-Doc1
Cthe_0543	GH9-CBM3-Doc1
Cthe_0578	GH9-CBM3-Doc1
* Cthe_0624	CBM30-Ig-GH9-GH44-Doc1-CBM44
Cthe_0625	GH9-CBM3-Doc1
Cthe_0661	GH43-CBM13-Doc1
Cthe_0745	GH9-CBM3-Doc1
* Cthe_0797	GH5-Doc1-CE2
^ Cthe_0798	CE3-CE3-Doc1
Cthe_0821	GH5-CBM32-Doc1
* Cthe_0912	CBM22-GH10-CBM22-Doc1-CE1
Cthe_1271	GH43-CBM6-CBM6-Doc1
* Cthe_1472	GH26-GH5-CBM11-Doc1
Cthe_1838	CBM22-GH10-Doc1
* Cthe_1963	CE1-CBM6-Doc1-GH10
Cthe_2137	GH39-CBM35-CBM35-Doc1
Cthe_2138	CBM42-GH43-Doc1
* Cthe_2139	GH5-CBM42-GH43-Doc1
Cthe_2147	CBM3-GH5-Doc1
* Cthe_2179	PL1-Doc1-CBM35-PL9
Cthe_2193	GH5-CBM6-Fn3-CBM13-Doc1
Cthe_2194	CE1-CBM6-Doc1
Cthe_2196	GH43-CBM6-Doc1
Cthe_2197	GH2-CBM6-Doc1
Cthe_2360	GH9-CBM3-Doc1
Cthe_2590	CBM22-GH10-Doc1
Cthe_2760	GH9-CBM3-Doc1
Cthe_2761	GH9-CBM3-Doc1
Cthe_2811	GH26-CBM35-Doc1
Cthe_2950	PL1-Doc1-CBM35
* Cthe_2972	GH11-CBM6-Doc1-CE4
Cthe_3012	GH5-CBM6-Doc1
^ Cthe_3141	CE12-Doc1-CBM35-CE12

C. thermocellum : Non-cellulosomal Enzymes	
Locus	Modular Architecture
Cthe_0040	GH9-CBM3-CBM3
Cthe_0071	GH48-CBM3
Cthe_0795	GH13-CBM34
Cthe_1257	CBM3-CBM4
Cthe_1800	CBM50-CBM50-Unk-GH18
Cthe_2191	CBM48-GH13
Cthe_2809	SLH-GH16-CBM4-CBM4-CBM4-CBM4

A. thermophilum	
Locus	Modular Architecture
Athe_0089	GH11-CBM36
^ Athe_0182	GH43-CBM22-GH43-CBM6
Athe_0183	CBM22-CBM22-GH10
Athe_0558	CBM48-GH13
Athe_0594	GH5-CBM28-SLH
Athe_0609	CBM41-CBM48-GH13-CBM20
Athe_0618	CBM22-CBM22-GH10
Athe_1853	PL11-CBM3
* Athe_1857	GH10-CBM3-CBM3-GH48
* Athe_1859	GH5-CBM3-CBM3-GH44
* Athe_1860	GH74-CBM3-CBM3-GH48
* Athe_1865	GH9-CBM3-CBM3-CBM3-GH5
^ Athe_1866	GH5-CBM3-CBM3-CBM3-GH5
* Athe_1867	GH9-CBM3-CBM3-CBM3-GH48

Ca. saccharolyticus	
Locus	Modular Architecture
Csac_0671	CBM48-GH13
Csac_0678	GH5-CBM28-SLH
Csac_0689	CBM41-CBM48-GH13-CBM20
Csac_0696	CBM22-CBM22-GH10
Csac_0784	CBM48-GH13
* Csac_1076	GH9-CBM3-CBM3-CBM3-GH48
* Csac_1077	GH5-CBM3-CBM3-GH44
* Csac_1078	GH10-CBM3-GH5
Csac_1079	GH9-CBM3-CBM3-CBM3
Csac_1085	GH74-CBM3
Csac_2410	CBM22-CBM22-GH10
^ Csac_2411	GH43-CBM22-GH43-CBM6

Entries marked with ^ and * contain two catalytic domains of the same or different families, respectively.

Module classification according to the carbohydrate active enzyme (CAZy) database (www.cazy.org; Cantarel *et al.*, 2009); GH, glycoside hydrolase; CE, carbohydrate esterase; PL, polysaccharide lyase; CBM, carbohydrate binding module; Doc1, dockerin type I; Ig, immunoglobulin-like fold; Fn3, fibronectin III; SLH, surface layer homology domain; Unk, module of unknown function.

Information gathered from the carbohydrate active enzymes database, as of September 2010 (www.cazy.org; Cantarel *et al.*, 2009). NC, not classified.

It is possible that organisms with free, non-complexed enzyme systems evolved to encode multiple catalytic domains with complementing activities in a single polypeptide chain to compensate for the lack of 'proximity effect' achieved in a complexed enzyme system, such as the cellulosome. The multifunctional enzymes in *Athe* and *Csac* are also encoded in contiguous genomic regions (*Athe*_1857–60, 1865–67; *Csac*_1076–79) of potential operon structure, probably for their coordinated expression to synergistically degrade plant biomass. Diversity in the modular structure of enzymes in cellulolytic organisms probably reflects the complexity of the substrate chemistry encountered in plant cell walls, and is potentially important in the survival and persistence of these bacteria in their natural environment. The synergism promoted by multi-domain enzymes suggests an intermediate level in evolution between simple free enzymes (containing only one catalytic domain) and cellulosomal systems in which several enzymes with diverse catalytic functions are complexed together.

Among thermophilic bacteria, the cellulosome system of the anaerobic Gram-positive bacterium *C. thermocellum* has been extensively studied (Bayer *et al.*, 2008; Ding *et al.*, 2008). The primary scaffoldin CipA contains nine type-I cohesins and one type II dockerin. CipA is anchored to the cell surface via three well-characterized anchor proteins, SdbA, Orf2p and OlpB, containing 1, 2 and 7 type-II cohesins respectively [see Fig. 7.2, (Bayer *et al.*, 1998)]. The *C. thermocellum* genome encodes more than 70 type-I dockerin-containing components with diverse hydrolytic activities and specificities (Zverlov *et al.*, 2005). In general, cellulosome-containing organisms have an overabundance of dockerin-containing subunits compared with the number of cohesins on the scaffoldin. As a consequence, It is probable that multiple cellulosomal subpopulations exist, even within an organism. Indeed, chromatographic separation of cellulosomes from *C. papyrosolvens*, *C. thermocellum* and *C. cellulovorans* identified distinct cellulosomal subpopulations with differing subunit composition and enzyme activities in these organisms (Ali *et al.*, 1995a; Pohlschroder *et al.*, 1995; Han *et al.*, 2005). These findings suggest that the process of cellulosome assembly is not an entirely random process and the cellulosome composition is probably regulated to make the organism better suited for attacking specific growth substrates.

In order to understand the substrate-related changes in the subunit composition of the global cellulosome population in *C. thermocellum* ATCC 27405, we isolated cellulosomes from cultures grown on a variety of model substrates (cellobiose, amorphous cellulose, crystalline cellulose) and dilute-acid pretreated switchgrass. Using quantitative proteomics methods (multidimensional LC-MS/MS and ^{15}N-metabolic labelling) we comprehensively measured the relative changes in levels of cellulosomal subunit proteins on a per-scaffoldin basis. In total, 80% of all dockerin containing proteins (59/73) and all of the cohesin-module containing components in *C. thermocellum* were detected in the cellulosomal preparations, including many subunits not reported previously (Raman *et al.*, 2009). Cellulosomes from amorphous cellulose, cellobiose and pretreated switchgrass-grown cultures displayed the most distinct differences in composition as compared with cellulosomes from crystalline cellulose-grown cultures (Raman *et al.*, 2009). Several cellulosomal components showed differential protein abundance in response to the presence of non-cellulose plant biomass components in the growth medium. It is important to understand the relative contribution and importance of the various enzymatic components in plant cell wall degradation. Such knowledge would help in engineering a strain that is best suited to grow on a specific feedstock of interest, and also for constructing non-native designer cellulosomes with tailored enzyme composition in organisms for industrial ethanol production.

Cellulolytic fermentations in production of sustainable energy

Sustainable energy can be defined as the provision of energy for our current needs without compromising the ability of future generations to meet their energy needs. Sustainable energy sources include all renewable resources, such as solar, wind, hydro, geothermal and tidal power, as well as biofuels derived from plant biomass. Biofuels such as hydrogen and ethanol can be produced from renewable plant biomass or organic waste materials using microbial fermentations. For example, Karakashev and colleagues have enriched strictly anaerobic, extremely thermophilic H_2 producing bacteria affiliated with the genera *Bacillus* and *Clostridium* in a lab-scale digester treating household solid organic wastes at 80°C and pH 8.1 (Karakashev *et al.*, 2009). The mixed culture was able to utilize glucose, galactose, mannose, xylose, arabinose, maltose, sucrose, pyruvate and glycerol as carbon sources. Growth on glucose produced acetate, CO_2 and H_2 at a maximal rate of 1.9 mol H_2/mol glucose (Karakashev *et al.*, 2009). Bacterial species isolated from natural sources and which could be used in hydrogen and ethanol production are listed in Table 7.1.

Fermentation by pure cultures

Most cellulolytic bacteria produce acetate and H_2 as the main fermentation end products during growth on cellulose-, hemicellulose- and pectin-containing biomass, with varying amounts of ethanol, lactic acid, and CO_2. For example, *Ca. saccharolyticus* ferments cellulosic biomass to H_2 (3.8 mol/mol glucose), acetate, and lactate as major end products, and ethanol and CO_2 as minor fermentation products (Mladenovska *et al.*, 1995; van de Werken *et al.*, 2008; Ivanova *et al.*, 2009). *Ca. lactoaceticus* strain 6A ferments cellobiose (4 g/l; approximately 22 mM equivalents of glucose) to 17.8 mM H_2, 19.3 mM lactate, 10.3 mM acetate, 9.8 mM CO_2, and 0.3 mM ethanol. Other species of the genus *Caldicellulosiruptor*, such as *Ca. kristjanssonii* strain 177R1B(T), produce 8 mM acetate/g/l Avicel, H_2 and CO_2 as major fermentation products, together with small amounts of lactic acid and 0.55 mM ethanol/g/l Avicel (Bredholt *et al.*, 1999). Attributes such as a broad substrate specificity, high hydrogen-producing capacity, and ability to co-utilize glucose and xylose make bacteria of the genus *Caldicellulosiruptor* attractive candidates for the production of renewable bioenergy in the form of hydrogen (van Niel *et al.*, 2002; Kadar *et al.*, 2003; O-Thong *et al.*, 2008; van de Werken *et al.*, 2008; Vanfossen *et al.*, 2009).

Thermophilic bacteria of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* are also known to produce considerable amounts of ethanol from soluble sugars and other polymeric substrates (e.g. starch) (Lee *et al.*, 1993; Koskinen *et al.*, 2008b). Hence, these bacteria are attractive candidates for the production of biofuels from lignocellulosic biomass. However, the known representatives from these genera are non-cellulolytic, although they can grow chemoorganotrophically by utilizing xylan and starch, and also on a number of di- and monosaccharides, including glucose and xylose. For example, a thermophilic (60°C and pH 6.0), anaerobic strain of the xylan-degrading bacterium, *Thermoanaerobacter ethanolicus* (similar to strains 39E and JW200) has been isolated from Frying Pan Springs in YNP (Lee *et al.*, 1993). *T. ethanolicus* JW200 produced approximately 5 g/l of ethanol from 20 g/l of sucrose, while *Thermoanaerobacter* strain 65-2 produced 6.5 g/l of ethanol from 40 g/l of molasses (Lee *et al.*, 1993; Koskinen *et al.*, 2008b). A mutant strain of *T. ethanolicus* (39E H8) has been adapted to grow in the presence of up to 8% (v/v) ethanol, compared with less than 1.5% (v/v) alcohol tolerance for the wild-type strain (39E). The ethanol tolerant mutant strain had an increased percentage of transmembrane long-chain C-30 fatty acids and lacked the primary alcohol dehydrogenase (ADH), which is probably involved in ethanol consumption, whereas the secondary ADH functioned primarily to produce ethanol (Burdette *et al.*, 2002). Along with ethanol, the fermentation products included acetate, lactate, H_2 and CO_2.

Fermentation by co-cultures

There are a few studies, reviewed below, on the development of an anaerobic fermentation system for the production of H_2 by a community of extremely thermophilic organisms, including *Caldicellulosiruptor* spp. Such a reactor was tested for H_2 production from hydrolysed paper sludge, which is a solid industrial waste containing mainly cellulose, by *Ca. saccharolyticus* and *Thermotoga elfii* (Kadar *et al.*, 2003). The maximal volumetric H_2 production rate from paper sludge hydrolysate was 5 to 6 mmol/l/h, which was lower than the production rate in media with glucose, xylose, or a combination of these sugars (9–11 mmol/l/h),

possibly due to the presence of inhibiting components in paper sludge hydrolysate (Kadar *et al.*, 2004). In culture during sugar fermentation, *Ca. saccharolyticus* grown on sucrose and *T. elfii* grown on glucose yielded 3.3 mol of H_2 and 2 mol of acetate per molC6 sugar unit. The H_2 production level was about 83% of the theoretical maximum. *Ca. saccharolyticus* and *T. elfii* reached maximum cell densities of 1.1×10^9 and 0.8×10^9 cells/ml, and their maximum H_2 production rates were 11.7 and 5.1 mmol/g dry weight/h, respectively (van Niel *et al.*, 2002). Co-culture of *Ca. saccharolyticus* and *Thermotoga neapolitana* simultaneously and completely utilized all pentoses, hexoses and oligomeric saccharides present in miscanthus hydrolysate (a total sugar concentration up to 17 g/l) with production of 2.9–3.4 mol H_2/mol hexose, corresponding to 74 to 85% of the theoretical yield (de Vrije *et al.*, 2009). O-Thong *et al.* (1998) compared microbial communities in H_2 producing reactors (70°C), fed either with xylose or lignocellulosic hydrolysate, and identified the presence of *Thermoanaerobacterium* spp. only in reactors fed with the hydrolysate. Other major groups of hydrogen producers detected included *Caldicellulosiruptor* spp., other Firmicutes, and archaea (O-Thong *et al.*, 2008).

It has been suggested that a co-culture composed of cellulolytic and saccharolytic bacteria could convert cellulosic materials to ethanol more effectively (Erbeznik *et al.*, 1997). For example, the co-culture of *T. ethanolicus* JW200 with *C. thermocellum* JW20 allows for the utilization of the wide range of substrates present in plant biomass, including xylose, that is not otherwise utilized by *C. thermocellum* (Erbeznik *et al.*, 1997). These strains, isolated from a Louisiana cotton bale, showed cellulose degradation and subsequent growth at 60°C and pH 6.1–7.5 in the presence of as much as 10% w/v cellobiose (300 mM), 6% glucose (333 mM), 5% xylose (333 mM), and 6% fructose (333 mM). The fermentation products upon cellulose degradation were ethanol (0.92 μmol/ml), acetate, lactate, H_2 (1.4 μmol/ml), and CO_2 (Freier *et al.*, 1988). The latter study elucidated the effect of externally added H_2 in increasing the formation of ethanol during growth on cellulose or cellobiose (Freier *et al.*, 1988). Another example of a co-culture involves an extremely thermophilic cellulolytic Gram-negative anaerobe strain H173 isolated from a New Zealand hot spring and an ethanologenic extreme thermophile, *Thermoanaerobacter wiegelii* strain Rt8.B1. Strain H173 could completely degrade up to 10 g/l of Avicel at 70°C and pH 7.0 (Hudson *et al.*, 1990). Two growth phases have been observed for this bacterium – an initial phase during which a cellulase complex was produced and carbohydrates were fermented to form acetic and lactic acids, followed by a second phase during which the cellular metabolism stopped but the cellulase complex continued to actively break down cellulose (with accumulation of glucose in the medium). When strain H173 was co-cultured with *T. wiegelii* strain Rt8.B1, glucose was fermented to ethanol (12.7 mM) and acetate (11.9 mM), and no reducing sugars accumulated in the culture medium (Hudson *et al.*, 1990; Cook *et al.*, 1996).

Another enrichment, dominated by bacteria closely affiliated with *T. thermohydrosulfuricus*, produced 1.21 molethanol/mol glucose, and 0.68 molH_2/mol glucose at 78°C (Koskinen *et al.*, 2008a). Even higher ethanol production by a co-culture of *Clostridium uzonii* AK15 and *Thermoanaerobacterium aciditolerans* AK17 has been reported (Koskinen *et al.*, 2008b). In the co-culture, *C. uzonii* AK15 was the main hydrogen producer (up to 1.9 molH_2/mol xylose, 33% of the theoretical maximum), while *T. aciditolerans* AK17 produced ethanol from both glucose and xylose, up to 1.6 molethanol/mol glucose (80%) and 1.1 molethanol/mol xylose (66%), respectively. The hydrogen yields by *T. aciditolerans* AK17 were significantly lower, 1.2 molH_2/mol glucose (30% of theoretical maximum) and 1.0 molH_2/mol xylose (30%). Stable and continuous ethanol and hydrogen co-production was achieved in a continuous-flow 60°C bioreactor by a co-culture of *C. uzonii* AK15 and *T. aciditolerans* AK17, with an ethanol yield of 1.35 mol/mol glucose and a H_2 yield of 0.08 mol/mol glucose at a production rate of 6.1 mmol/l/h. *T. aciditolerans* AK17 showed high ethanol tolerance, up to 4% (v/v), and over time the AK17 became the dominant bacterium in the bioreactor (Koskinen *et al.*, 2008b). Since bacteria of the genus *Thermoanaerobacter* cannot degrade cellulose, in co-culture they survive by cross-feeding on soluble carbohydrates (e.g. glucose, cellobiose, and small cello-oligomers) released through the action of the cellulase complex of cellulolytic bacteria. However, if the same culture is subcultured on glucose, cellobiose, or xylose, which are growth substrates for *Thermoanaerobacter* spp., this stimulates the growth of the *Thermoanaerobacter* spp. cell population and accounts for the apparent ability of cellulolytic bacteria to utilize xylose (Erbeznik *et al.*, 1997).

Thermophilic cellulolytic bacteria from hot springs of Yellowstone National Park

This section describes research conducted at the Oak Ridge National Laboratory (ORNL, Oak Ridge, TN, USA) within the framework of the BioEnergy Science Centre (BESC), one of the three Bioenergy Research Centres sponsored by the United States Department of Energy. To our knowledge, the hot springs located within Yellowstone National Park (YNP) have not been extensively studied with respect to isolation of cellulolytic bacteria. Hence, our initial research efforts have been directed to the enrichment, identification and isolation of thermophilic microorganisms that are capable of degrading lignocellulosic biomass, from samples collected at select sites within YNP.

Sampling sites and anaerobic batch enrichments

A total of 134 samples consisting of spring water, sediments, microbial mats, and plant material (100 ml each) were collected from 7 different

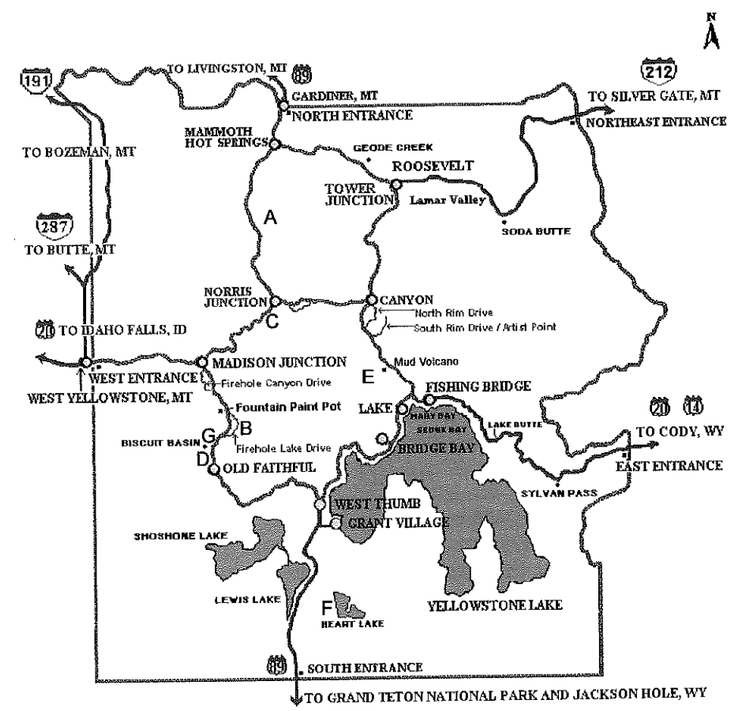

Figure 7.3 Locations (A through G) of sampling sites are shown on a map of Yellowstone National Park (WY, USA). The primary anaerobic enrichments established from these sites are indicated in Table 7.4.

locations (10 sites) within YNP (Fig. 7.3). Samples varied in their ambient temperature, from 50 to 93.7°C, and pH, from 3 to 9.5 (Table 7.4 and Fig. 7.4A). Immediately after collection, samples were reduced by adding a few crystals of sodium dithionite and 1 ml of 2.5% cysteine HCl, pH 7.2. The samples were kept at room temperature during transportation to ORNL. In the laboratory, enrichment cultures inoculated with 2% (v/v) of an environmental sample were initiated using 10 ml of a fermentative cellulolytic anaerobes (FCA) liquid growth medium at pH 7.0 (Hamilton-Brehm *et al.*, 2010), containing either dilute-acid pretreated switchgrass or *Populus* shavings (0.1% w/v) as the sole carbon and energy source. These primary anaerobic enrichments, incubated at temperatures ranging from 55 to 85°C in increments of 5°C, were screened for turbidity, pH changes, gas production, and end product formation. Tubes positive for growth were transferred to 50 ml of fresh FCA medium (N_2:CO_2 headspace; 80%:20%) and subcultured at least three consecutive times (Table 7.4).

Physiological characteristics of batch cellulolytic enrichments

Enrichment cultures inoculated with samples collected from Obsidian Pool, and thermal features around the Witch Creek/Heart Lake, and White Creek areas (Fig. 7.3) displayed gas production and visible turbidity upon repeated transfer in medium containing pretreated switchgrass. Stable enrichments from these sites were selected for further analysis using the same FCA medium with 0.2% (w/v) of dilute-acid pretreated substrates. End-products formed during growth on pretreated switchgrass included H_2, CO_2, acetate, and traces of ethanol. Acetate was the major end-product with concentrations reaching up to 0.73, 0.24, 0.18 g/l for the Obsidian Pool, Heart Lake, and White Creek enrichment cultures, respectively, after 120 hours of incubation at 75°C. Among the three, the Obsidian Pool enrichments displayed the fastest growth on pretreated switchgrass, and hydrolysed approximately 50% of the starting pretreated biomass within 24 hours. The Obsidian Pool enrichment also hydrolysed crystalline

Table 7.4 Anaerobic batch enrichments established in this study using dilute-acid pretreated switchgrass or *Populus* as sole carbon and energy source

			Enrichments – growth[2]	
Location[1]	Site	Environmental conditions	Switchgrass (*Panicum virgatum*) (60°C/80°C)	Populus (*Populus deltoides*) (60°C/80°C)
A	Amphitheater Stream	50–74.3°C, pH 3	–/++	–/–
B	Octopus Spring	80.6°C, pH 7.5	+++/+++	++/++
	White Creek area	73–92.1°C, pH 7	+/–	+/–
	Five Sisters	69.5–85°C, pH 8.2–8.5	–/+	–/+
	Fire Hole Spring	78°C, pH 7.5–8	–/+	–/–
C	Artist Paintpots	55.5–93.7°C, pH 6–9.5	++/–	+/+
D	Black Sands Basin	59–87°C, pH 7–9	–/+	–/–
E	Obsidian Pool area	61.9–90°C, pH 5.5	++/++++	–/++++
F	Witch Creek area	74–92°C, pH 7–8.5	+++/++	–/–
G	Rabbit Creek	64.7–92°C, pH 4–9	+++/+++	++/++

[1]Location of sites is shown on YNP map, Fig. 7.3.

[2]Growth in liquid enrichments was characterized based on turbidity using a scale from + (poor growth) to ++++ (very good growth) and – (no growth).

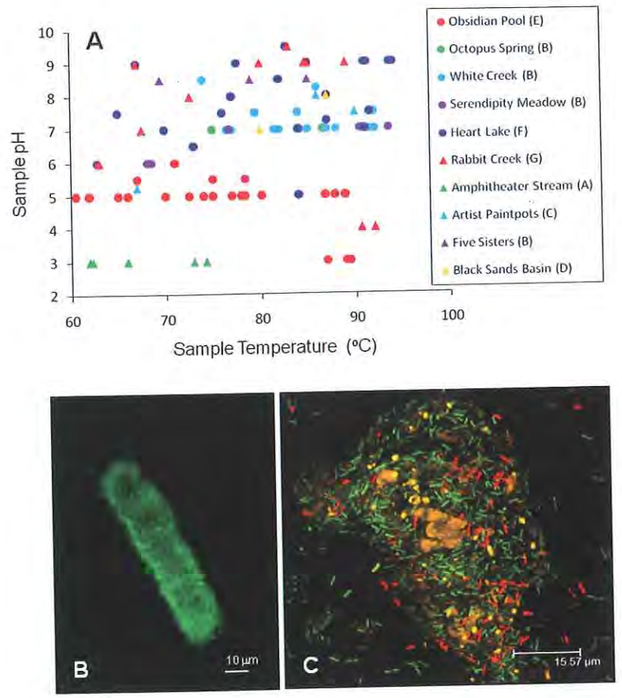

Figure 7.4 (A) Temperature and pH values of samples collected across various sites within YNP. (B) Epifluorescent microscopy imaging of SYTO9-stained cells shows attachment of bacterial cells to particles of switchgrass used in enrichment cultures. (C) Laser confocal microscopy visualization of Live/Dead dye-stained bacterial cells confirmed the predominance of live cells (green) attached to the insoluble substrate (dead cells are red).

cellulose (Avicel), Birchwood xylan and pretreated *Populus*, but the overall cell densities were lower during growth on *Populus* as compared with either switchgrass or Avicel.

Staining the cultures with SYTO9 (Invitrogen, Carlsbad, CA) and imaging via epifluorescent or laser confocal microscopy revealed cells attached to particles of insoluble biomass (Fig. 7.4B,C). The cell morphology and overall cell densities in the established enrichments were examined using phase contrast and epifluorescent microscopy (Axioskop 2 plus, Zeiss). Enrichments were classified on the basis of observed morphotypes into cultures with high variety of morphotypes (≥3) and those with a prevalence of a particular morphotype (e.g. rods or cocci). Enrichment cultures with stable growth, multiple morphotypes and a cell density of ~10^8 cells/ml were selected for isolation attempts. An isolation method using flow cytometry was developed in order to circumvent plating on solid or semisolid media and for isolation of cells grown on insoluble biomass substrates (Elkins *et al.*, 2008). The cultures were chilled before sorting and oxygen exposure was minimized to preserve cell viability. Cells from enrichment cultures were dislodged from particles of insoluble biomass by shaking and sorted with an InFlux Cell Sorting Flow Cytometer (Cytopeia, Seattle, WA, USA) into 48-well plates. Each individual well in the plate, containing

0.6% (w/v) of pretreated switchgrass in 0.5 ml of reduced FCA medium, was inoculated with a single cell and the plate was incubated under anaerobic conditions at the respective enrichment culture growth temperature. Positive growth in the wells was detected using a pH indicator for acid production and was confirmed by microscopy. Wells displaying growth were considered putative isolates and were subcultured into Balch tubes containing fresh FCA medium with 0.1–0.5% (w/v) dilute-acid-pretreated switchgrass or Avicel. Successfully recovered cultures displaying growth on cellulosic substrates were resorted (and the above procedure repeated) and verified for purity by microscopy and sequencing of multiple PCR amplified 16S rRNA gene recombinants. A total of 155 putative isolates were recovered from multiple isolation attempts.

Community analyses of batch enrichments

The total community genomic DNA (cgDNA) was extracted using the MoBio PowerSoil™ kit (MoBio Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene fragments were amplified using 8f and 1492r *Bacteria*-specific primers (Lane, 1991) and cloned using standard procedures. The plasmids from positive transformants were amplified using Rolling Circle Amplification (RCA) (TempliPhi process, Illustra™ GE Healthcare, UK). Subsequently, a total of 615 recombinants from enrichment cultures differing in growth substrate and temperature were sequenced (Vishnivetskaya *et al.*, 2009). Phylogenetic analysis of the 16S rRNA genes (Fig. 7.5) showed that the majority of the sequences were affiliated with the phylum Firmicutes (80.9% of clones), followed by Dictyoglomi (12.9%), Nitrospira (3.4%), and others (2.7%). The bacteria of the phylum Firmicutes fell into five groups. The most abundant group (54.4%) was represented by *Caldicellulosiruptor* spp., followed by *Caloramator* spp. (11.5%), *Thermoanaerobacter* spp. (8.8%), *Thermovenabulum ferriorganovorum* (4.1%), and *Clostridium xylanovorans* (2.0%). Similar results were obtained for enrichment cultures established using water from the hot springs of Uzon Caldera, Kamchatka, with ambient temperatures from 68 to 87°C and pH from 4.1 to 7.0 (Kublanov *et al.*, 2009). The latter study showed, using DGGE analysis of 16S rRNA gene fragments, that the bacterial communities developing on carbohydrates (cellulose, carboxymethyl cellulose, chitin, or agarose) contained the genera *Caldicellulosiruptor* and *Dictyoglomus*.

Dynamic changes in microbial community structure during continuous enrichment

We established a continuous-flow anaerobic enrichment culture to monitor the temporal changes in structure and composition of a thermophilic cellulolytic community. The enrichment culture was inoculated with 20 ml of spring water collected from the peripheral areas of Obsidian Pool and was maintained at a constant temperature of 65°C by using a jacketed vessel connected to a recirculating water/glycerol bath. FCA medium supplemented with 10 mM MOPS buffer (pH 7.0) was continuously added to the vessel at 8.3 ml/h. The culture initially contained 1 litre of FCA medium with 1% (w/v) Avicel as the carbon source and was supplemented with additional carbon every 7 days by adding 50 ml of nitrogen sparged FCA medium containing 4 g of Avicel. Bacterial growth was monitored by direct microscopic cell counts. Cell density increased from an initial 5.9×10^6 cells/ml to 6.3×10^8 cells/ml within two days, then decreased to 1.8×10^8 cells/ml after another 2 days, after which it remained essentially stable for the next 38 days (Fig. 7.6A). Acetate production by the thermophilic community, measured using HPLC, followed the growth curve with a maximum concentration of 5.33 mM on day 6, and then remained in the range of 4.39–3.70 mM from day 8 to day 21 (Fig. 7.6A). The pH remained circumneutral due to the continual addition of buffered FCA medium. At defined time points suspended Avicel and cell biomass were collected (Fig. 7.6A) and total community genomic DNA was extracted, amplified and cloned as described above. Sequences were aligned against the most similar sequences in the RDP (http://rdp.cme.msu.edu) and NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) databases.

Changes in the bacterial community composition at different time points are shown in Fig. 7.6B. After 48 hours of incubation, along with

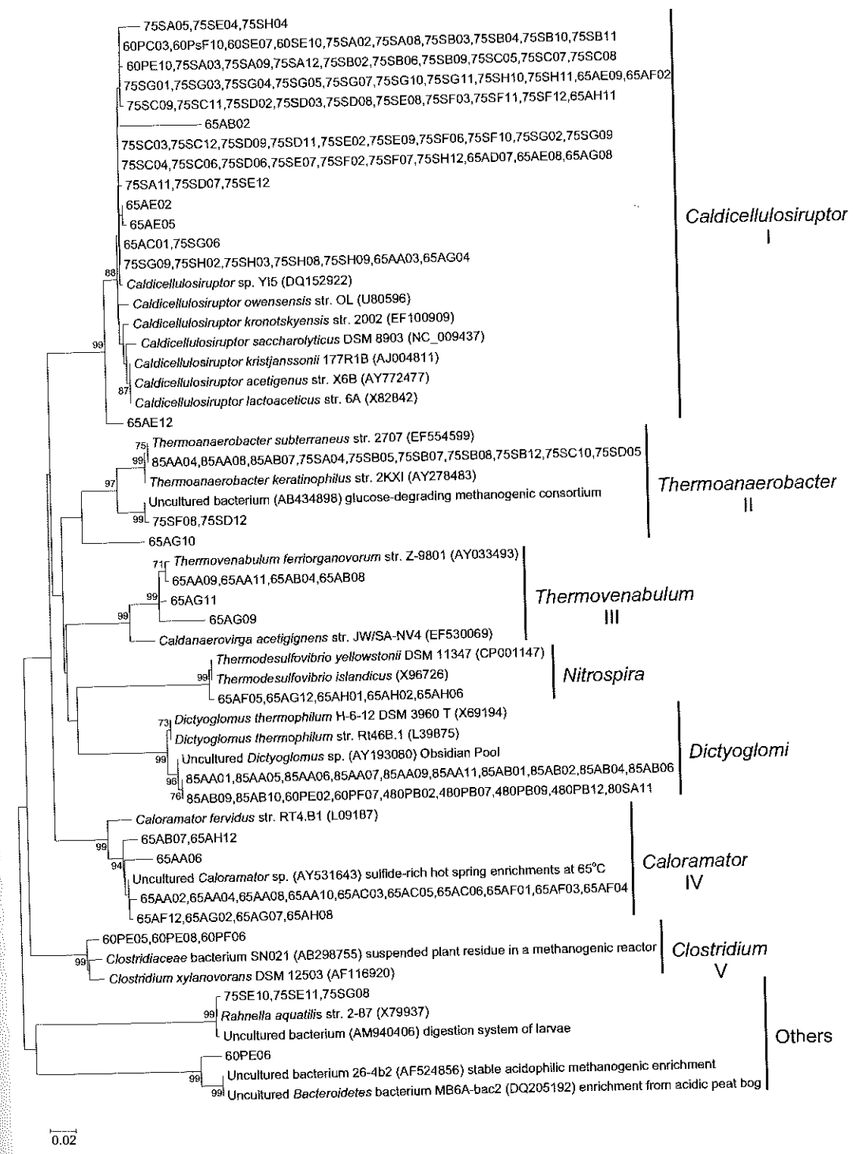

Figure 7.5 Phylogenetic analysis of environmental clones derived from batch cellulolytic enrichments. The tree was produced by a neighbour-joining method. Bootstrap values were based on 1000 replicates. The scale bar represents 0.02 changes per nucleotide position. Sequence names were designated as follows: first number, enrichment growth temperature (60°C, 65°C, 70°C, 75°C, 80°C or 85°C), followed by a letter indicating the carbon source (S, switchgrass; P, *Populus*; A, Avicel), followed by the specific location of the clone on the sequencing plate.

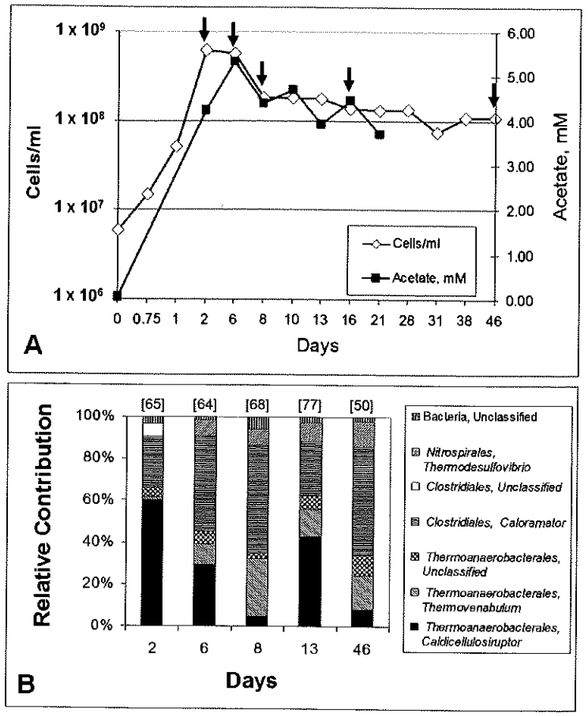

Figure 7.6 (A) Cell density and acetate production during continuous growth of Avicel enrichment of Obsidian Pool sample at 65°C for 46 days. Arrows indicate the time points at which samples used for clone library construction were collected. (B) Bacterial community composition as determined by 16S rRNA gene Sanger sequencing. The number of sequences obtained from each sample is given in parentheses. The bacterial taxonomy at order and genus levels is given on the right side of the figure.

unclassified bacteria (<80% homology, RDP database), two orders of the class Clostridia in the phylum Firmicutes, namely Thermoanaerobacterales (66.2% of total) and Clostridiales (30.8%), were detected. About 60.0% of Thermoanaerobacterales recombinants were affiliated with the genus *Caldicellulosiruptor*, which comprises hydrogen- and acetate-producing cellulolytic organisms. One of the sequences was 91% (687/751) identical to *T. ferriorganovorum*, a novel thermophilic, anaerobic bacterium isolated from a hydrothermal feature in the Uzon caldera on the Kamchatka peninsula (Zavarzina *et al.*, 2002). *T. ferriorganovorum* is capable of molecular hydrogen utilization in the presence of Fe(III) and an organic electron donor. The remaining 4.6% of recombinants were unclassified. Among Clostridiales, unclassified bacteria comprised 6.2%, while 24.6% recombinants were affiliated with the genus *Caloramator*. Another member of the genus *Caloramator*, *C. australicus* RC3T, a strictly anaerobic thermophilic bacterium, has been isolated from microbial mats colonizing thermal waters of the Great Artesian Basin, Australia (Ogg and Patel, 2009). *C. australicus* requires yeast extract for growth on energy sources such as cellobiose, cellulose, xylan and glucose (Ogg and Patel, 2009), with ethanol and acetate as the end products of glucose fermentation. In the presence of yeast extract, *C. australicus* was able to reduce iron (III), manganese (IV) and

elemental sulfur, although there was no utilization of molecular hydrogen and/or carbon dioxide in the presence or absence of iron (III).

At subsequent time points (6–46 days) we observed the disappearance of unclassified Clostridiales and an increase in the relative contribution of the genus *Caloramator* (from 24.6% to 26.0–52.0%), while the relative abundance of the genus *Caldicellulosiruptor* decreased from 60.0% to < 42.9%. Starting from day 6 we observed the appearance of sequences (7.8–12.0%) which were 99% identical to Nitrospira isolated from methanogenic sludge – *Thermodesulfovibrio yellowstonii* DSM 11,347 (also named as strain ATCC 51303) and *Thermodesulfovibrio islandicus* TGL-LS1, both obligately anaerobic, thermophilic, sulfate-reducing bacteria (Sekiguchi *et al.*, 2006). Nitrospira-like bacteria are known to be nitrite oxidizers and they tend to be slow-growing organisms. The presence of bacteria of the phylum Nitrospirae in Obsidian Pool sediment has been reported previously (Hugenholtz *et al.*, 1998).

Strain isolation and phylogenetic identification

A total of 155 strains were isolated from established anaerobic thermophilic (60°C, 75°C and 85°C) batch enrichments by sorting with a flow cytometer (see description earlier). Detailed 16S rRNA-based phylogenetic analysis of 81 strains (Fig. 7.7) showed that 57% and 43% of the total isolates were close relatives of, respectively, *Caldicellulosiruptor* spp. and *Thermoanaerobacter* spp. Isolates identified as *Caldicellulosiruptor* spp. were closely related to *Caldicellulosiruptor owensensis* strain OL (ATCC 700167) (97.2–99.3% sequence identity) and to *Caldicellulosiruptor* sp. strain Y15 (96.8–99.3% sequence identity). Isolates affiliated with the genus *Thermoanaerobacter* were 96.9–98.8% identical to *Thermoanaerobacter tengcongensis* strain MB4 (JCM 11007). *Ca. owensensis*

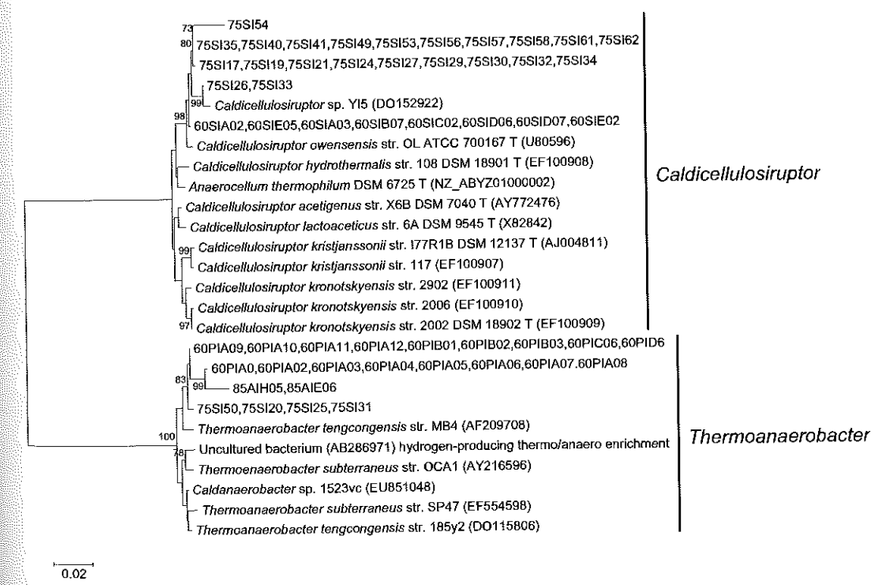

Figure 7.7 Phylogenetic analysis of single cell isolates obtained upon sorting of batch cellulolytic enrichments by flow cytometry. The scale bar represents 0.02 changes per nucleotide position. Isolate names were designated and the phylogenetic tree was constructed as described in the legend to Fig. 7.5.

and *T. tengcongensis*, are both anaerobic, extremely thermophilic, rod-shaped non-spore-forming bacteria. *Ca. owensensis* was isolated from a sediment sample from temperate Owens Lake, California, while *T. tengcongensis* was isolated from a Chinese hot spring. Both organisms grow at temperatures between 50°C and 80°C, with an optimum at 75°C, and in a pH range of 5.5–9.0 with an optimum around pH 7.5. However, the two organisms differ in their ability to utilize cellulose. *Ca. owensensis* can utilize cellulose, xylan, starch, pectin, glucose, xylose, and other biomass sugars with a doubling time of 7.3 h during growth on glucose. The end products of glucose fermentation are acetate, lactate, H_2, CO_2, and traces of ethanol (Huang *et al.*, 1998). *T. tengcongensis* cannot utilize cellulose but can grow on other substrates, including starch, cellobiose, glucose, galactose, and mannose. The main end products from glucose fermentation are acetate (1.0 molacetate/mol glucose), CO_2 (1.5 mol), ethanol (0.7 mol), and H_2 (0.3 mol), with growth inhibition by hydrogen accumulation (Xue *et al.*, 2001). The *Caldicellulosiruptor* sp. strain OB47 isolated in our study was able to generate up to 37 mM acetate and 2 mM ethanol with no detectable lactate during the course of pretreated switchgrass fermentation (Hamilton-Brehm *et al.*, 2010). Screening of the other bacterial strains isolated from our enrichment cultures (e.g. members of the genera *Thermoanaerobacter* and *Caloramator*) for elevated cellulolytic and saccharolytic activity is in progress. Comparing the metabolic characteristics of various cellulolytic microorganisms will provide evidence whether the isolates derived from YNP enrichments are attractive candidates for further research and development towards their use in biofuel production.

Future trends

Continually rising crude oil prices and environmental concerns have revived interest in renewable sources of sustainable energy such as biofuels. While the first-generation ethanol industry depended on starch or sugars derived from maize grain, sugarcane and other crops, and thus potentially competed with the food supply, second-generation ethanol derived from cellulosic biomass promises to deliver a renewable biofuel from non-food feedstock. However, many challenges remain in establishing an economically viable and sustainable lignocellulosic bioethanol industry, and these need to be addressed. With regards to biomass, the challenges include engineering transgenic plants to increase feedstock productivity and decrease biomass recalcitrance without loss of structural integrity and tolerance traits. With regards to cellulolytic microorganisms, challenges include engineering robust organisms that can degrade plant polymers at high rates and convert all biomass-derived sugars to the fuel of interest at high yields, while being tolerant to pretreatment-derived inhibitors, fermentation products and other process conditions (Blaschek and White, 1995; Lynd *et al.*, 2002; Keller and Zengler, 2004; Himmel *et al.*, 2007).

Bioethanol production relies on the availability of individual organisms or consortia with high capability for degradation of plant polymers and broad substrate specificity for co-utilization of a wide range of sugars (C_5 and C_6) present in lignocellulosic biomass. Strains should be capable of converting concentrated feedstock (e.g. ≥ 50 g of carbohydrate per litre) at high rates with maximal productivity (Lynd and Zhang, 2002; Zhang *et al.*, 2007). At the same time, organisms should also be robust enough to function adequately under conditions typically encountered in an industrial environment. This would be achieved either by genetic modification of ethanologenic strains that are currently available or through the discovery of novel cellulolytic microorganisms.

Natural ecosystems offer a wealth of resources for bioprospecting, and our group will continue to tap into the thermal features within Yellowstone National Park to isolate novel organisms with high lignocellulolytic potential. The application of the novel flow cytometry-based technique for isolating bacterial strains from enrichment cultures, as described here, will allow a screening of isolates for robustness, fast growth and high cellulolytic activity (Elkins *et al.*, 2008). High-throughput approaches are needed for large-scale screening of naturally isolated strains, as well as strains engineered for high product yields and other desired process characteristics. Many of the challenges could be efficiently tackled through the successful application of biotechnological techniques.

Several tools are currently being developed for improving bioenergy production systems. Systems biology analysis using 'omics' tools probably plays a key role in the development of robust organisms tailored for industrial biofuel production. High-throughput genome sequencing probably highlights the cellulolytic and metabolic potential of newly isolated organisms for degradation and utilization of lignocellulosic biomass-derived polymers and sugars. Functional genomics employing proteomics, transcriptomics, metabolomics and other 'omics' methods probably reveal the active or inactive enzymatic and metabolic networks in these organisms. Development of novel technologies is necessary for identifying factor(s) that are limiting bacterial growth and performance, particularly under conditions that mimic biofuel production processes. These technologies will probably guide both bioprocess development and organism development with pointers to relevant gene targets for genetic manipulation. In order to engineer strains with desired attributes for biofuel production, it is necessary to develop practical and reproducible genetic tools that permit gene expression and gene knockout in a variety of cellulolytic and ethanologenic microorganisms. Currently, many cellulolytic organisms, in particular thermophilic anaerobes, are not easily engineered through the use of traditional microbial genetic systems, posing a challenge and opportunity for biotechnology tool development (Blaschek and White, 1995; Lynd *et al.*, 2002).

Web resources

There are a number of web sites that discuss various aspects of cellulolytic microorganisms, bioenergy and biofuel development:

A list of cellulolytic bacteria described in the literature as continuously hydrolysing crystalline cellulose substrates like Avicel or filter paper can be found at the web site of the Technical University of Munich (http://www.weihenstephan.de/mbiotec/cellmo.htm).

The genome sequences of cellulolytic thermophilic bacteria are available through the Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi?page=home) and NCBI (http://www.ncbi.nlm.nih.gov/Genomes/) web pages. IMG serves as a community resource for comparative analysis and annotation of all publicly available genomes from three domains of life in a uniquely integrated context. NCBI provides several genomic biology tools and resources, including organism-specific pages that include links to many web sites and databases relevant to that species.

The description of the families of structurally related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds can be found at the CAZy database (http://www.cazy.org/). This website also provides links to commercially available carbohydrate-active enzymes (CAE) using CAZy nomenclature.

The BioEnergy Science Centre (BESC) web site (http://bioenergycenter.org/) introduces a person to the mission of the United States Department of Energy in developing alternative fuel solutions such as converting plant biomass into biofuels. BESC is a multi-institutional partnership that is connecting the world's leading scientific minds and resources for finding bioenergy solutions. The goal of BESC is to help develop viable, plentiful and clean alternative biofuel sources for generations to come.

Knowledge Base (http://besckb.ornl.gov) is another web site that provides integrated data and information representing systems biology analysis of key plants, microbes, and molecules within BESC. It is a resource for exploring metabolic networks, regulatory pathways, signalling maps, and gene expression, with links to primary genome sequence annotation and underlying experimental data, for several plant and bacterial species of importance in biofuels production.

The Food and Agriculture Organization (FAO) draws attention to high energy prices, environmental degradation, sustainability of current energy systems and the competition of food crops versus energy crops (http://www.fao.org/bioenergy/home/en/).

A list of worldwide organizations, conferences, papers and other information related to petroleum, bioenergy, natural gas, biomass, fossil-fuel and solar energy is provided at

http://www.petrolpump.co.in/energy-resources-directory/b.htm.

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8 Extreme to the Fourth Power! Oil-, High Temperature-, Salt- and Pressure-tolerant Microorganisms in Oil Reservoirs. What Secrets can they Reveal?

Hans Kristian Kotlar

Abstract

In the deep biosphere, extraordinary new types of microorganisms, sedimented or buried 200 – 500 million years ago, can be found. These organisms can be identified and characterized. The information obtained can be developed into novel tools for searching for new oil in sensitive regions like the Arctic, Antarctica and jungle areas.

Relatively few enzymes are used in large-scale industrial applications. Enzymes isolated from these extremophile/thermophile organisms might provide 'game changing' new possibilities. They may furnish incentives for the development of entirely new technical processes. These microbes provide opportunities for new technologies in second generation biofuel production. Several companies are working on alternative routes for the production of fuels using biomass as the raw source material.

Traditional heavy oil extraction methods have major difficulties in justifying their high energy usage, CO_2 emissions and soil and environment pollution. The first company implementing a large-scale process based on biotechnology principles in enhanced oil recovery will gain huge strategic and economic benefits.

The knowledge of this huge subsurface population of diverse microorganisms provides excellent opportunities for bioprospecting. There should be a multitude of spin-offs outside the oil industry. The world is desperately in need of new enzymes, new antibiotics, new immunosuppressant, new anticancer agents, etc. This chapter reviews just some of the areas we have been working on at Statoil. Hopefully some of these investigations could one day solve some of the problems we will face in the future. One day these extremophiles could be on the payroll of many different companies.

The deep biosphere

Different research organizations have been searching for the 'gold of the ocean' and have made important discoveries of organisms that can produce new compounds seen as potential antibiotics and anti-cancer medicines. They have been searching from the high mountain moors to the sediments of the ocean floor, and even in high temperature hydrothermal vents.

At Statoil we are digging even deeper. Deep down in oil reservoirs we are finding a whole new world of microorganisms that, until recently, we had very little knowledge of (Fig. 8.1). Deep in the subsurface there are microorganisms with unique properties. Some day they could be on the payroll of many different companies.

These microorganisms live at depths of between 2 and 4 km in the lithosphere, and their communities represent ancient phylotypes. The traditional understanding of the generation of oil reservoirs is through the sedimentation of river deltas from the Cambrian through to the Jurassic period, i.e. 500–200 million years ago, especially in the Jurassic. Microorganisms living throughout this period