Introduction

Each year, photosynthetic fixation of CO₂ yields over 4 trillion tons of plant biomass (interior
and leaf, 380, leaf, 380). In addition, when plant cells are
processed for industrial and energy purposes, additional biomass can be exploited. This includes cellulosic fibers
and lignin, which can then be converted to biofuels or
cellulose-based products. Plant materials can be
converted to biofuels by fermentation, which involves
the breakdown of carbohydrates into sugars and
ethanol. The resulting sugars can then be used to
produce biofuels.

3. Plant material, including cellulose-containing
biomass, can be converted to ethanol. Ethanol
production involves the conversion of plant sugars
into ethanol, which can then be used as an alternative
fuel source.

4. Plant biomass can be converted to biofuel by
fermentation. This process involves the breakdown
of carbohydrates into sugars and ethanol, which can
then be used as an alternative fuel source.

5. Plant material, including cellulose-containing
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production involves the conversion of plant sugars
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6. Plant material, including cellulose-containing
biomass, can be converted to ethanol. Ethanol
production involves the conversion of plant sugars
into ethanol, which can then be used as an alternative
fuel source.
such as butanol and propanol, or converted to other chemicals such as aceton, isopropanol, etc.

Butanol could also serve as a direct replacement for gasoline due to its favourable physical properties (Garcia-Solera and Roselló-Balasiewicz, 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 up from cellulose, a homopolymer consisting of a linear chain of β-D-glucose units. As a natural substrate, cellulose is insoluble in water (Chiang 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 react on cellulose fibers, 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 (Wynn et al., 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 Zymomonas mobilis or yeast. Anaerobically fermented hexoses, cellulose 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 lignocellulosic material is therefore dependent on the availability of robust microorganisms that are capable of fermenting both C6 and C5 carbohydrates present in the feedstock. For example, some wild-type bacteria (Z. mobilis) and yeast (Saccharomyces cervisiae) are able to rapidly and efficiently convert glucose (C6) substrates into ethanol but cannot ferment xylose (Dien et al., 2003). Those and other organisms have been engineered to obtain strains that are capable of fermenting both hexoses and pentoses to produce ethanol (Yamas et al., 2007; Gerth et al., 2009).

Traditional ethanol production in a simultaneous saccharification and co-fermentation (SSCF) process configuration requires a dedicated step for cellulose production (Lynd et al., 2005). An alternative process under development for the conversion of lignocellulosic biomass is to termocondensed bioprocessing (CBP). It involves the use of either native cellulosic organisms (e.g. Clostridium thermocellum) or recombinant organisms which have been engineered to produce cellulolytic and other hydrolytic enzymes (e.g. xylanase). This allows for the integration of several biologically mediated processes into a single saccharification 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 economic advantages for the CBP process over the conventional SSCF. A 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).

Thermophiles and extreme thermophiles are the most promising cellulosic 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-Krevis et al., 2010).

Natural environments such as thermal hot springs with decaying plant material (discussed in the ensuing section) are rich source of thermophilic cellulosic organisms with potential use in high-temperature processes. For example, bioengineering 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 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 edible sugars, organic acids, alcohols, CO2, and H2 (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. CO2 released into the atmosphere by microbes is converted back to sugars and further to lignocellulosic biomass during photosynthesis and plant growth (Leschine, 1995; DeWaxs et al., 2001; Dui, 2008).

Cellulose degradation 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). Some canker and vessels of a large number of woody plants, not only the forms of dead and recalcitrant plant biomass, where cellulase is degraded (e.g. aerobic or anaerobic). Aerobic cellulose degradation may be achieved through the activities of a single species or consortia of mesophilic fungi and bacteria. For instance, the cellulose and hemicelulose components of wood are completely degraded and metabolized by single fungal species of either F前往us radiata (white-tot fungus), T. resin or Pencillicium species (Berto et al., 1995; Fujii and Shiokoh, 2006; Cho et al., 2005). Mesophilic aerobic cellulolytic bacteria, such as Celllobacter pireum, Cellidium spp., Cellulobioryx glucins, Pseudomonas farcians, and Streptomyces spp., are often found in soils, on plant materials, in human, animal faeces, and leaf litter (Demonte et al., 2009; Data 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. Microorganisms 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, H2, and CO2, which are then able to undergo methanogenesis through a complex microbial food chain (Leschine, 1995). Cellulose is initially hydrolyzed through the action of extracellular enzymes of cellulolytic bacteria. The released sugars monomers and oligomers are fermented by cellulolytic and other saccharolytic microorganisms with production of CO2, H2, organic acids (e.g. acetate, propionate, butyrate), and alcohols (e.g. ethanol). Exogenous H2 is immediately consumed by methanogens or acetogens, which use H2 to reduce CO2 to CH4, or acetate, CH3COOH, or acetate. Acetate can then serve as a terminal electron acceptor and the predominant end products of the methanogenesis appear to be CH4, CO2, and H2O (Fig. 7.1). Cellulose-decomposing microbial communities comprising several symbiotic anaerobic bacteria are found in the gut flora of herbivorous mammals (e.g. rumen of ruminant species). In addition to anaerobic bacteria and archaebacteria, 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, Catenomyces, Cylindroomyces, Novellamyces, Paracynomyces and Pyrenomyces (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 either temperate or even low-temperature niches, the aerobically (5–5%) aquatic environments that contain decaying plant material are also a good source of lignocellulosic-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 poly saccharides components of plant cell walls to monomers and oligomers. Commensal microorganisms assimilate the solvable 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, moisture, and inorganic chemistry of the anaerobic phase. For example, many anaerobic thermophilic bacteria of the order Thermoglossum do not utilize crystalline cellulose as a substrate, but are capable of fermenting simpler complex sugars to H₂, CO₂, and acetate (Blumer-Schade et al., 2005). The ability to utilize crystalline cellulose as well as hemicellulose as a growth substrate is limited to terrestrially 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, Nagono Prefecture in Japan and the Great Artesian Basin in Australia. (Tyszka et al., 1988; Hadoon et al., 1996; Svetlichnyi et al., 1996; Bredieß et al., 1999; Madanovskaya et al., 1999; Miroshnichenko et al., 2000; Ogg and Patel, 2009). The cellulolytic bacteria isolated to date belong to the order Clostridiales, including the extremely thermophilic Anaerocellum thermophilum, Caldicellulosiruptor rzezychowskii, Caldicellulosiruptor hydrothermalis, Caldicellulosiruptor bescii, and the thermophiles Clostridium thermocellum and Thermoclostrum thermohalophilum (Freter et al., 1988; Svetlichnyi et al., 1990; Mikhailenkova et al., 1995; Kozikina et al., 1998; Miroshnichenko et al., 2000; 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 YNP hot springs is well documented, and several novel thermophilic microorganisms have been isolated (Hugenholtz et al., 1998; Reysenbach et al., 2000; Meyer-Dumbad et al., 2005; Nakagawa et al., 2009).
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, xyloglucan, and rhamnogalacturonan and rhamnogalacturonan I and II polysaccharides (Mollen, 2008). Hemicellulose consists primarily of xylan or other backbone structures (e.g. galacto- glucomannans, arabinoxylans, xylans), classified based on their monosaccharide 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 (Kloos, 2008). To counter the chemical and structural complexity of plant cell wall polysaccharides, cellular microorganisms have evolved enzyme systems with a wide array of cellulases and hemicellulases for hydrolysis and degradation of plant biomass (Schwarz et al., 2004; Daubert et al., 2009).

**Plant wall-degrading enzymes**

Plant biomass-degrading enzymes were originally classified based on the substrates they hydrolysed and their mode of action. More recently, cell walls and other carbohydrate-active enzymes (CAZymes; www.cazy.org) have been classified based on their sequence homology, structural fold and mechanistic themes (Heinritz and Davies, 1997; Castorena et al., 2009). As of September 2010, the CAZY database classification included 118 glycoside hydrolases (GH), 16 carbohydrate esterases (CE), 22 polysaccharide lyases (PL) and 61 carbohydrate binding modules (CBM) families. Cellulases hydrolyse the β-1,4-glycosidic bonds in cellulose polymers either endo-acting, or at the reducing/non-reducing ends (co-acting) of the cellulose 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 GH14 and GH15 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-carbo-hydrate substituents. These enzymes include plant polysaccharides. For example, while xylanases (e.g. GH10, GH11 family enzymes) and mannanases (e.g. GH26) cleave the main backbone, other hemicelluloses such as arabinogalactans (e.g. GH43) and glucomannans cleave the bonds between backbone sugars and side-chain substituent sugars (Shaham 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 CE1, CE3 families or lignin monomeric components such as ferulic and p-coumaric acids (e.g. feruloyl esterases belonging to the CE1 family). Weng, 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 (Michael 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 catalytic modules (CMs) (Gilbert et al., 1994; 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 (Shooswy et al., 2006). Other non-catalytic modules include the immunoglobulin-like (iG), fibronectin type-III (Fn3, dockers) involved in cellulosome assembly and the surface-layer homology (SLH) module (Doi and Tamura, 2001). SLM modules interact with cell wall polysaccharides and aid in the attachment of free enzymes or cellulosomes anchor proteins to the cell surface (e.g. C. Thermohalophilicum, see discussion below). Recent evidence suggests that apart from the modular composition, their arrangement on the polysaccharide 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., 2000; Doi, 2000). Aerobic fungi (e.g. T reversi), aerobic thermophilic bacteria (e.g. Acidothermus cellulolyticus, Thermobifida fusca) and archaea, extremely thermophilic bacteria (e.g. Caldicellulosiruptor saccharolyticus, A. Thermohalophilicum) contain the non-complexed enzyme systems, in which a single individual secreted endo- and exo-glucanases act synergistically in degradation of plant cell wall cellulase (Svedlichkova et al., 1998; Rainey et al., 1994). Complexed enzyme systems are found in anaerobic fungi (e.g. Anaeromyces, Catenaria, Calylynnium, Neocallospora, Orpinomyces and Pyro- myces), and both mesophilic (e.g. Bacteroides cellulosolvens, Clostridium cellulosolvens, Clostridium cellulolyticum, Ruminococcus flavefaciens) and thermophilic bacteria (C. Thermohalophilicum; Ali et al., 1999b; Ieyabl, 2008). These microorganisms form large extracellular enzyme complexes on the cell surface, termed the cellulosomes, 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; Fendt et al., 2009).

**Cellulosomal architecture**

In cellulosomes, a structural component called scaffolds contains binding sites (cohesins) that interact with the docking modules (dockers) borne by the enzymatic units. The highly specific and stable cohesin-docker type interaction engenders the assembly of the catalytic components onto the scaffold (Type I interaction) and also the attachment of the scaffold itself (with bound subunits) onto the cell surface via anchor proteins.
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 Cte), or extremely thermophilic (A. thermophila, Atl and Ca. saccharolyticus, Csa), and whose genomes have been sequenced (http://genome.cme.berkeley.edu/. van de Weerken et al., 2008; Kataeva et al., 2009). Table 7.2 compares the distribution of glycoside hydrolases, esterases, lipases and carbohydrate binding modules across these three organisms. Genomewide level comparisons reveals a similar total number of glycoside hydrolase domains encoded in Cte, Csa and Atl (Table 7.2). Complete cellulose degradation requires the combined action of existing cellulose-hydrolases and endoglucanases (Warren, 1998). While all three organisms encode GH48 family bacterial reducing-end cellobiohydrolase, Csa has a higher distribution of other cellulose-specific hydrolitic enzymes (e.g. belonging to the GH9 and GH3 families) as compared with Cte and Atl (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 substrate specificity (Table 7.2).

All three organisms encode enzymes for hydrolyzing the non-cellulosic components of the plant cell wall, namely hemicellulose and pectins. In addition to xyloses (e.g. belonging to the GH10 and GH11 families), Cse contains more mannans (GH16) and arabino-furanolase.
Table 7.3 Multimodular enzymes of C. thermocellum (cellulosomal and non-cellulosomal; Cth), A. thermopropionibacterium (non-cellulosomal; Atrh) and Ca. saccharolyticus (non-cellulosomal; Cth) containing at least two domains belonging to the GH, CE, PL or CBM families. Enzymes containing at least three domains are highlighted in grey.

<table>
<thead>
<tr>
<th>C. thermocellum (Cellulosomal Enzymes)</th>
<th>Locus</th>
<th>Modular Architecture</th>
<th>C. thermocellum (Non-cellulosomal Enzymes)</th>
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<tr>
<td>Cth0501</td>
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Entries marked with "x" 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; Condit et al., 2009); GH, glycoside hydrolase; CE, carbohydrate esterase; PL, polysaccharide lyase; CBM, carbohydrate binding modules. All enzymes are classified as potentially glycoside hydrolases of the GH family, based on their catalytic activity domain.

Information gathered from the carbohydrate active enzymes database, as of September 2016 (www.cazy.org; Condit 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 cellulose. The multifunctional enzymes in Atta and Cocca are also encoded in contiguous genomic regions (Atta 1857-60, 1865-67; Cocca 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 multienzyme systems suggests an intermediate level in evolution between simple free enzymes (containing only one catalytic domain) and cellularly embedded systems in which several enzymes with diverse catalytic functions are coupled together.

Among thermophilic bacteria, the cellulolytic systems 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 six type-I cohesins and one type-II dockerin. CipA is anchored to the cell surface via three well-characterized anchor proteins, SilA, SipZ, and OipL, containing 1, 4, and 7 type-II cohesins respectively (see Fig. 7.2, Bayer et al., 2008). These cohesins encode more than 70 type-I dockerin-containing components with diverse hydrolytic activities and specificities (Zverlov et al., 2005). In general, cellulose-degrading organisms have an abundant Amsterdam containing subsatins compared with the number of cohesins on the scaffoldin. As a consequence, it is probable that multiple cellulose-degrading subpopulations within an organism. Indeed, chromosome separation of cellulases from C. papyrosolvens, C. thermocellum and C. cellulovorans identified distinct cellulolytic subpopulations with differing subunit composition and enzymatic activities in these organisms (Ali et al., 1995a; Polischuk et al., 1995; Han et al., 2005). These findings suggest that the process of cellulose assembly is not an entirely random process and the cellulolysis 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 cellulolysis system in C. thermocellum ATCC 27405, we isolated cellulases from cultures grown on a variety of model substrates (cellulose, amorphous cellulose, crystalline cellulose and dilute-acid pretreated switchgrass. Using quantitative proteomics methods (multi-dimensional LC-MS/MS and INN-methodological labelling) we comprehensively measured the relative changes in levels of cellulolytic subunit proteins on a per scaffoldin basis. In total, 80% of all dockerin-containing proteins (59,773) and all of the cohesin-module containing components in C. thermocellum were detected in the cellulolysis preparations, including many subunits not reported previously (Raman et al., 2009). Cellulases from amorphous cellulose, cellulose and pretreated switchgrass-grown cultures displayed the most distinct differences in composition as compared with cellulases from crystalline cellulose-grown cultures (Raman et al., 2009). Several cellulolysis components showed differential protein abundance in response to the presence of non-cellulosic 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 digestive cellulolytic systems 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 fermentation processes. Given that human beings have achieved purely anaerobic, extremely thermophilic H₂ producing bacteria affiliated with the genus *Bacillus* and *Clostridium*, which are capable of fermenting household solid organic wastes at 80°C and pH 8.1 (Karakashvili et al., 2009), the mixed culture was able to utilize glucose, galactose, mannose, xylose, arabinoose, maltose, nacrose, pyranose and gluconolactone as carbon sources. Growth on glucose produced acetate, H₂ and CO₂ at a maximal rate of 1.9 mol H₂/mol glucose (Karakashvili 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 H₂ as the main fermentation end products during growth on cellulose, hemicellulose and pectin-containing biomass, with varying amounts of ethanol, lactic acid, and CO₂. For example, *C. saccharolyticum* ferments cellulolysis biomass to H₂ (3.8 mol/mol glucose), acetate, and lactate as major end products, and ethanol, acetate, and CO₂ as minor products (Makowski et al., 1995; van der Weken et al., 2008; Bravone et al., 2008). *C. lactoaceticum* strain 6A ferments cellulose (4 g/l, approximately 22 mM equivalents of glucose) at 8-9 mol/mol glucose and 10.3 mol acetate, 9.8 mol CO₂, and 0.3 mol ethanol. Other species of the genus *Clostridium*, such as *C. kivunzi* strain T171/1(T), produce 8 mol acetate/g (g) Avicel, H₂ and CO₂ as major fermentation products, together with small amounts of lactic acid and 0.55 mol ethanol/g Avicel (Bendich et al., 1999). Attributes such as a bacterial substrate, high coproducts, high extracellular CO₂, and hydrogen as co-utilization of glucose and xylose make bacteria of the genus *Clostridium* attractive candidates for the production of renewable bioenergy in the form of hydrogen (van Nielen et al., 2003, Kadar et al., 2008; O'Brien et al., 2008; van der Weken et al., 2008; Venkatesou et al., 2009). Thermophilic bacteria of the genera *Thermoanaerobacterium* and *Thermoanaerobacter* are also known to produce considerable amounts of ethanol from soluble sugars and other polymeric substrates (e.g. starch) (Lee et al., 1993; Kokkinis et al., 2008). 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 xylose and starch, and also on a number of d- and meso-saccharides, including glucose and xylose. For example, a thermophilic (60°C and pH 6.0) anaerobic strain of the xylo-xylo-aerobic bacterium, *Thermoanaerobacter ethanolicus* (similar to strains SVE and JV2/II0) 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 maltose (Lee et al., 1993; Yourkakis et al., 2008). A mutant strain of *T. ethanolicus* (398 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 (398). The ethanol tolerant mutant strain had an increased percentage of transmembrane long-chain C-50 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 (Bendette et al., 2002). Along with ethanol, the fermentation products included lactate, acetate, H₂ and CO₂.

**Fermentation by co-cultures**

There are a few studies, reviewed below, on the development of an anaerobic fermentation system for the production of H₂ by a community of extremely thermophilic organisms, including *Caldicellulosiruptor* spp. Such a reactor was targeted for H₂ production from hydrolysed paper sludge, which is a solid industrial waste containing mainly cellulose, by *C. saccharolyticum* and *Thermotoga* effi (Kadar et al., 2003). The maximum volumetric H₂ 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 mill hydrolysate (Kadar et al., 2004). In culture during sugar fermentation, Candida saccharolytica grows on sucrose and T. elfi grown on glucose yielded 3.5 mol of H$_2$ and 2 mol of acetate per mol C6 sugar unit. The H$_2$ production level was about 83% of the theoretical maximum. Co-culture of C. saccharolytica and T. elfi reached maximum cell densities of 1.3 \times 10^8 and 0.8 \times 10^8 cells/ml, and their maximum H$_2$ production rates were 11.7 and 5.1 mmol/g dry weight/h, respectively (van Nie et al., 2002). Co-culture of C. saccharolytica and Thermotoga maritima simultaneously and completely utilized all pentoses, hexoses and oligomeric saccharides present in microcrystalline cellulose (R810) with production of 2.9–3.4 mol H$_2$/mol hexose, corresponding to 74 to 85% of the theoretical yield (de Vries et al., 2009). O-Thong et al. (1998) compared microcrystalline communities in H$_2$ producing reactors (70°C), fed with xylan or lignocellulosic hydrolysate, and identified the presence of Thermosporobacterium spp. only in reactors fed with the hydrolysate. Other major groups of hydrogen producers detected included Clostridium 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 cellululosic materials to ethanol more effectively (Ezhembek 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 xylan, that is not otherwise utilized by C. thermocellum (Ezhembek et al., 1997). These strains, isolated from a Louisiana cattle barn, 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 cellulose (300 mM), 6% glucose (333 mM), 5% xylan (333 mM), and 6% fructose (333 mM). The fermentation products upon cellulose degradation were ethanol (0.92 mol/mol), acetate, lactate, H$_2$ (1.4 mmol/mol), 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 cellulose (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 thermophilic extreme thermophilic Thermotoga strain R88.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 in which a cell complex was produced and carbohydrates were fermented to form acetic and lactic acids, followed by a second phase during which the cellulosomal apparatus stopped but the cellulose complex continued to actively break down cellulose (with accumulation of glucose in the medium). When strain H173 was xylanase, xylose 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. thermohydrodsulfuricans, produced 1.21 mol ethanol/mol glucose, and 0.06 mol H$_2$/mol glucose at 78°C (Koskinen et al., 2008a). Even higher ethanol production by a co-culture of Cupriavidus acnes AK15 and Thermosporobacterium acidotolerans AK17 has been reported (Koskinen et al., 2008b). In the co-culture, C. acnes AK15 was the main hydrogen producer (up to 1.9 mol H$_2$/mol xylose, 33% of the theoretical maximum), while T. acidotolerans AK17 produced ethanol from both glucose and xylose, up to 1.6 mol ethanol/mol glucose (90%) and 1.1 mol ethanol/mol xylose (66%), respectively. The hydrogen yields by T. acidotolerans AK17 were significantly lower, 1.2 mol H$_2$/mol glucose (30% of the theoretical maximum) and 1.0 mol H$_2$/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. acnes AK15 and T. acidotolerans AK17, with an ethanol yield of 1.15 mol/mmol glucose and a H$_2$ yield of 0.08 mol/mmol glucose at a production rate of 6.1 mmol/l/h. T. acidotolerans 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 Thermosporobacter cannot degrade cellulose, in co-culture they survive

Thermophilic cellulolytic bacteria from hot springs of Yellowstone National Park

This morphological analysis was conducted at the Oak Ridge National Laboratory (ORNL), Oak Ridge, TN, USA within the framework of the BioEnergy Science Center (BESC), one of 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 locations (A through G) on a map of Yellowstone National Park (Figure 7.3). 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 several 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 anaerobic (FCA) liquid growth medium at pH 7.0 (Hamilton-Beck et al., 2010), containing either dilute-acid pretreated switchgrass or Populus shavings (0.1% w/v) as 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 growth, pH changes, gas production, and end product formation. Totes positive for growth were transferred to 50 ml of fresh FCA medium (N₂CO₃ headspace; 80:20% N₂:CO₂) 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 0.25% (w/v) of dilute-acid pretreated substrates. End products formed during growth on pretreated switchgrass included H₂CO₃, acetate, and traces of ethanol. Acetate was the major end-product concentration reaching up to 0.73, 0.24, and 0.15 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 hydrolyzed approximately 50% of the starting pretreated biomass within 24 hours. The Obsidian Pool enrichment also hydrolysed crystalline 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 microscopy revealed cells attached to particles of insoluble biomass (Fig. 7.4C). 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 (≤ 5) 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 ≥10⁷ 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 (Ellingsen et al., 2008). The cultures were chilled before sorting and oxygen exposure was minimized to preserve cell viability. Cells from enrichment cultures were resuspended from particles of insoluble biomass by shaking and sorted with an InFlux Cell Sorting Flow Cytometer (Cytopen, Seattle, WA, USA) into 48-well plates. Each individual well in the plate, containing

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

<table>
<thead>
<tr>
<th>Location¹</th>
<th>Site</th>
<th>Environmental conditions</th>
<th>Switchgrass (Parvicoccus virgatum) (60°C/80°C)</th>
<th>Populus (Parvicoccus virgatum) (60°C/80°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Amphitheater Stream</td>
<td>65.7-74.3°C, pH 3</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>Octopus Spring</td>
<td>80.0-82.0°C, pH 7.5</td>
<td>+/++</td>
<td>++++</td>
</tr>
<tr>
<td>C</td>
<td>White Creek Lake</td>
<td>73.0-82.0°C, pH 7.5</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>D</td>
<td>Flow Creek</td>
<td>65.7-74.3°C, pH 3</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>Fire Hole Spring</td>
<td>78.0-79.0°C, pH 7.5-8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>Artist Paintpits</td>
<td>65.7-74.3°C, pH 3</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>Black Sands Basin</td>
<td>65.7-74.3°C, pH 3</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>Obsidian Pool</td>
<td>65.7-74.3°C, pH 3</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>Witch Creek</td>
<td>65.7-74.3°C, pH 3</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>J</td>
<td>Rabbit Creek</td>
<td>65.7-74.3°C, pH 3</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Location of sites is shown on YNP map, Fig. 7.3.
²Growth 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).
0.6% (v/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 cultivation 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% (v/v) dilute-acid pretreated switchgrass or Avicel. Successfully recovered cultures displaying growth on cellulose substrates were resuspended (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 1492E bacteria-specific primers (Lane, 1991) and cloned using standard procedures. The plasmids from positive transformants were amplified using Rolling Circle Amplification (RCA) (Trek PhD™ Process, Illestra™ GE Healthcare, UK). Subsequently, a total of 615 recombinants from enrichment cultures differing in growth substrates and temperature were sequenced (Yahata et al., 2009). Phyllogenetic analysis of the 16S rRNA genes (Fig. 7.5) showed that the majority of the sequences were affiliated with the phylum Firmicutes (89.9% of clones), followed by Deltaproteobacteria (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 Clostridium stercorarium (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 (Kulish et al., 2009). The latter study showed, using DGGE analysis of 16S rRNA gene fragments, that the bacterial communities developing on cellulose (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/glycol 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 1.1 log CFU/ml of FCA medium with 1% (v/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% of Avicel. Bacterial growth was monitored by direct microscopic cell counts. Cell density increased from an initial 5.9 × 10^5 cells/ml to 6.3 × 10^7 cells/ml within two days, then decreased to 1.8 × 10^6 cells/ml after another 2 days, after which it remained essentially stable for the next 38 days (Fig. 7.6A). Aqueous pyrolysis of 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 2.93-3.70 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, purified, and cloned as described above. Sequences were aligned among 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...
unclassified bacteria (<80% homology, RDP database), two orders of the class Clostridiales, namely Thermoclostrobacterales (66.2% of total) and Clostridiales (30.8%), were detected. About 60.0% of Thermoclostrobacterales recombinants were affiliated with the genus Caldiclostridium, which comprises hydrogen-and acetate-producing cellulolytic organisms. One of the sequences was 91% (687/751) identical to T. fujisawanorum, a novel thermophilic, anaerobic bacterium isolated from a hydrothermal vent in the Uzon caldera on the Kusshakta peninsula (Zarza et al., 2002). T. fujisawanorum 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 Calmodoncola, a strictly anaerobic thermophilic bacterium, has been isolated from microbial mats colonizing thermal waters of the Great Artiscan Basin, Australia (Ogg and Paul, 2009). C. australis requires yeast extract for growth on energy sources such as cellulose, cellulose, and glucose (Ogg and Paul, 2009), with ethanol and acetate as the end products of glucose fermentation. In the presence of yeast extract, C. australis 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 uncultured Clostridiales and an increase in the relative contribution of the genus Calmodoncola (from 24.6% to 56.0-52.9%), while the relative abundance of the genus Caldiclostridium 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 Nitrosopira isolated from methanogenic sludge - Thermoclostridium solutum DSM 11,247 (also named as strain ATCC 53850) and Thermodesulfovibrio islandicus TGL 1,213, both obligately anaerobic, thermophilic, sulfate-reducing bacteria (Sogin et al., 2002). Nitrosopira-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 sediments has been reported previously (Hageman 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, Caldiclostridium rpp. and Thermoclostridium rpp. Isolates identified as Caldiclostridium rpp. were closely related to Caldiclostridium islandicus strain DSM 11,247 (97.2-99.3% sequence identity) and to Caldiclostridium sp. strain Y13 (96.8-99.3% sequence identity). Isolates affiliated with the genus Thermoclostridium were 96.8-98.8% identical to Thermoclostridium tengconensis strain MB4 (97.0% sequence identity).

Figure 7.6 (A) Cell density and acetate production during continuous growth of Axial 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 fragment 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.
T. tengcongensis, are both anaerobic, extremely thermophilic, rod-shaped non-spore-forming bacteria. C. xerothermophilus was isolated from a sediment sample from temperate Owens Lake, California, while T. tengcongensis was isolated from Chinese hot spring. Both organisms grow at high temperatures between 59°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 cellulooses. C. xerothermophilus can utilize cellulose, xylan, starch, pectin, glucose, xylose, and other biomass sugars within a doubling time of 7.3 h during growth on glucose. The end products of glucose fermentation are acetate, lactate, H₂, CO₂, and traces of ethanol (Huang et al., 1998). T. tengcongensis cannot utilize cellulose but can grow on other substrates, including starch, cellulose, glucose, galactose, and mannose. The main end products from glucose fermentation are acetate (1.0 mol/g glucose), CO₂ (1.5 mol), ethanol (0.7 mol), and H₂ (0.5 mol), with growth inhibition by hydrogen accumulation (Xue et al., 2001).

The C. xerothermophilus sp. strain 01847 isolated in our study was able to grow up to 37 mM acetate and 2 mM ethanol with no detectable degradative enzymes of pretreated switchgrass fermentation (Hamilos-Literat et al., 2010). Screening of the other bacterial strains isolated from our enrichment cultures (e.g., members of the genera Thermannarathbunae and Calimicrobacterium) for novel cellulolytic and xylanolytic 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 main grain, sugar beet 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 feedstocks 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 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., 2001; Keller and Zheng, 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 (C₅ and C₆) present in lignocellulosic biomass. Strains should be capable of converting concentrated feedstock (e.g., ≥50g of carbohydrate per liter) 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 (Bliss et al., 2008). High-throughput approaches are needed for large-scale screening of naturally isolated strains, as well as strainsengineered 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, which are essential for design and optimization of biorefineries through the integration of genomics, proteomics, and metabolomics. Systems biology approaches that couple metabolic and genetic data can be used to generate hypotheses about metabolic pathways in biorefineries and test these hypotheses using experimental approaches.

The BioEnergy Science Center (BESC) web site (http://besc.energy.gov) introduces a person to the mission of the United States Department of Energy's alternative fuel research and development program. 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.

Web resources
There are several websites that discuss various aspects of cellulolytic microorganisms, bioenergy and biofuel development.

A list of cellulolytic bacteria described in the literature as thermophilic hydrolysing crystalline cellulose substrates like Avicel or filter paper can be found at the website of the Technical University of Munich (http://www.technische-muenchen.de/natinos/celobe.htm).

The genome sequences of cellulolytic thermo-phobic bacteria are available through the Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov/cgi-bin/pbma/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 these domains of life in a unique and consistent 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 (CASE) using the CASE nomenclature.

The BioEnergy Science Centre (BESC) web site (http://besc.energy.gov) 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://besc.hi-edu.org) is another web site that provides integrated and dynamic 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 experimen- tional 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
Thermophilic Cellulolytic Microorganisms


Thermostable Cellulase: Microorganisms

Thermostable cellulases are enzymes that are able to function optimally at high temperatures. These enzymes are produced by various microorganisms, including bacteria, fungi, and archaea. The thermostability of these enzymes is a result of specific amino acid sequences and structural modifications that allow them to maintain their enzymatic activity under high-temperature conditions.

The high operating temperature of thermophilic microorganisms allows for enhanced enzyme stability and activity. This property is particularly useful in industries such as pulp and paper, detergent, and biofuel production, where high-temperature conditions are required for optimal enzyme performance.

Thermostable cellulases are produced by various microorganisms, including Bacillus, Thermus, and Archaea. These microorganisms have evolved mechanisms to withstand high temperatures and maintain enzyme activity. The characterization of these enzymes has led to the development of advanced biofuel production processes and the optimization of industrial processes.

The isolation and characterization of thermostable cellulases have been achieved through various methods, including enzyme purification, activity assays, and structural analyses. These studies have provided insights into the molecular mechanisms that enable these enzymes to function optimally at high temperatures.

Thermostable cellulases have potential applications in various industries, including the pulp and paper industry, where they can be used to improve the efficiency of cellulose degradation. Additionally, these enzymes are valuable in the production of biomaterials, such as biodegradable plastics, and in the degradation of biomass for bioenergy production.

Overall, the study of thermostable cellulases has contributed significantly to the understanding of enzyme function and the potential for the development of new industrial processes. The continued research in this field is expected to lead to the discovery of more efficient and versatile thermostable cellulases, further expanding their applications in various industries.
Extreme to the Fourth Power! Oil-, High Temperature-, Salt- and Pressure-tolerant Microorganisms in Oil Reservoirs. What Secrets can they Reveal?

Hans Kristian Kottar

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 search- ing 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 extremophiles/thermophiles might provide "gane 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, CO₂ 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 immunosuppressors, 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 mounds to the sediments of the ocean floor, and even in high temperature hydrothermal vents.

At Statoil we are digging even deeper. Deep down in all 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 phylogenies. 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