J Ind Microbiol Biotechnol (2009) 36:319–332
DOI 10.1007/s10295-008-0521-8

REVIEW

Biosolutions to the energy problem

Arnold L. Demain

Received: 27 November 2008 / Accepted: 16 December 2008 / Published online: 10 January 2009
© Society for Industrial Microbiology 2009

Abstract We are in an energy crisis caused by years of neglect to alternative energy sources. There are many possible solutions and a number of these are based on microorganisms. These include bioethanol, biobutanol, biodiesel, biohydrocarbons, methane, methanol, electricity-generating microbial fuel cells, and production of hydrogen via photosynthetic microbes. In this review, I will focus on the first four possibilities.

Keywords Ethanol · Butanol · Biodiesel · Energy crisis · Petroleum · Microbial fermentations

Introduction

We are living in an unprecedented time in the history of mankind with respect to economic development based on available energy sources. As the debate continues in US political and economic circles, alternative renewable energy sources again become the epicenter of this issue. The history of this paradigm has been up and down for more than 30 years, being subject to oil crisis volatility in a cyclical manner. Economical and technological approaches have been suggested to overcome oil dependence from foreign sources and strong scientific commitment has been devoted to this paramount challenge. Today more than ever before, unprecedented global environmental issues strongly related to the social and economic impact of the energy sector are dominating the international agenda. It is clear that the petroleum-based economy is getting closer and closer to the end of its lifecycle. Therefore, it is very important to anticipate and to avoid any shortfall in future supply, and to provide access to new bioenergy alternatives for the marketplace.

A. L. Demain (✉)
The Charles A. Dana Research Institute for Scientists Emeriti (R.I.S.E.), HS-330, Drew University, Madison, NJ 07940, USA
e-mail: ademain@drew.edu

The USA energy problem

For more than 30 years, petroleum and its derivatives have dominated the economy, becoming important commodities in multiple industrial sectors which transform them into products and services to satisfy population needs. In the USA, this dependency has already reached its saturation point, putting at risk our economy, energy security, homeland security as well as the environment [43, 95].

The economy, energy security climate protection, and minimizing the threat of global climate change, constitute the major factors in favor of an ambitious and stable renewable energy policy in the USA and abroad [27]. The US economy is firmly attached to imported oil and its derivatives at present. Severe economic and social problems could be generated as a consequence of any unexpected disruption in the oil supply. Therefore, as a major oil importer, this represents for the US an unstable situation that becomes more prominent during geopolitical unrest which jeopardizes energy security and the stability of the world's oil supply. In addition, increasing energy demands from China, India and other nations raise the pressure to compete for oil supplies. It is clear that global oil reserves and new petroleum discoveries will not be enough to meet the annual demand worldwide. In addition, the strong variation in market prices makes the whole scenario very unstable.

Petroleum import is the largest single part of the US trade deficit. Our transportation and industries rely heavily

on technologies which use fossil energy such as coal, petroleum and natural gas [44, 49, 50]. The US has 5% of the world's population but only 2% of the world's oil reserves. However, it consumes 25% of the world's petroleum, most of which is imported.

The USA uses 20 million barrels of petroleum daily. 60% of this (12 million barrels) is imported [41]. Over 62% of all the petroleum used in the USA is for transportation and industry. In 2004, it was imported at a cost of over $100 billion. In 2005, the proportion going for transportation and industry increased to 71% which cost $140 billion. Our transportation needs require 14–15 million barrels of petroleum per day [12, 49, 50]. In 1990, the US consumed 112 billion gallons of gasoline. By 2003, this had increased to 132–137 billion gallons per year [44] and today the number is double that.

The accelerated consumption of petroleum is forcing a severe climate change, diminishing the quality of our environment. US transportation alone generates tremendous amounts of "green house gases (GHG)", mainly carbon dioxide. Carbon dioxide is expected to increase from around 1.9 billion metric tons in 2004 to about 2.7 billion metric tons in 2030.

In 2006, the world used 85 million barrels of oil per day which is expected to increase to 105 million barrels per day by 2015. Renewable energy only supplies 2.5%. A total of 220 billion gallons of gasoline and other petroleum products (e.g., diesel) are used for transportation. Liquid fuels such as gasoline, diesel and jet fuel, all used for transportation, constitute 70% of the total. At present, 79% of global energy use comes from fossil energy sources: 35% from crude oil, 23% from natural gas, and 21% from coal. The remaining sources are hydraulic and nuclear energy (9%), and firewood, wind energy, and solar energy (12%). Microbes can help solve the energy problem in a number of ways [106]. A number of these are described below.

Gasoline versus ethanol as fuels

Bioethanol and other biofuels could be very important to foster energy independence and reduce gas emissions. A very strong debate on gradual substitution of petroleum by use of renewable alternatives such as biofuels dominates the political and economic agenda worldwide [25, 75].

As a biofuel, ethanol has many positive features when compared to gasoline but also a few negatives (Table 1).

Table 1 Properties of bioethanol versus gasoline

Positive
Enhances US energy security
Offers a favorable trade balance
Excellent transportation fuel. High heat of vaporization, high octane and low flame temperature yield good engine performance
Cleaner and more efficient burning
Higher octane rating
Replaces tetraethyl lead as octane enhancer
Causes a disproportionate increase in octane rating when mixed with gasoline
Decreases smog formation due to low volatility
Decreases greenhouse gas emission due to recycling by growth of plants
Decreases particles and toxic emissions
Decreases level of ozone precursors emitted thereby averting air pollution
Less toxic to humans; contains no sulfur
Used as oxygenate replacing methyl *tert*-butyl ether (MTBE) which has been phased out by many states. Also more efficient than MTBE as oxygenated fuel; only half the volume is necessary to produce the same effect as MTBE. Ethanol is biodegradable in contrast to MTBE
Negative
Only 2/3 the energy content
Requires engine modification when mixed with gasoline at over 15% of total fuel
Cannot be shipped via pipelines

Ethanol from corn

Worldwide bioethanol production between 2004 and 2007 doubled to 13.2 billion gallons [102]. The current international market for bioethanol is $15 billion. Internationally, it has been mainly produced from sugar or corn starch. Corn-based ethanol production became commercially viable in the USA in the 1980s. Several cooperatives and private ventures began corn to ethanol production. Since the early 1980s, 1–10% ethanol has been used to blend with gasoline. Ethanol production has increased over the years. In 1979, production was only 10 million gallons. In 1987, 340 million bushels of corn were used to produce 850 million gallons of anhydrous ethanol which was 3% of our auto fuel requirement. 2.8 billion gallons were produced in 2003 [13], 3.4 billion gallons in 2004 and by 2005, it had increased to 3.8 billion gallons [33, 53, 71, 73, 79, 110, 114, 115]. In 2006, it rose to 4.8 billion gallons and by 2007, the figure reached over 6 billion gallons. This impressive growth was derived from a growing number of production facilities, resulting in an impact on the US economy, agriculture, energy and the transportation sectors.

Reports that ethanol from corn had a negative energy balance are incorrect since they ignored the co-products that could displace whole corn and soybean meal that are used in animal feed. The energy saved partially offsets the energy required for ethanol production [31]. Biofuels could

help to eventually yield the equivalent of 8 million barrels of petroleum per day in the USA [44]. About 17–20% of the 2006 corn crop, equivalent to 2.15 billion bushels, was utilized to produce ethanol. By 2008, 3–4 billion bushels were used. Although corn could yield up to 13 billion gallons per year, US production of ethanol already utilizes 23% of the corn crop and is causing economic problems such as elevated costs of corn for human consumption and for animal feed. Furthermore, ethanol from corn has only supported 2% of the national transportation requirements. If the projected ethanol demand were to be fulfilled only with corn, it would require more corn than the US currently produces, and would dangerously compete with food crops. Such a limited availability of corn and a restricted capacity to expand its production due to infrastructure constraints puts a ceiling on corn to ethanol production goals [31, 44]. The US government via NREL has estimated that ethanol from corn will not go over 10–15 billion gallons per year [56]. Therefore, corn has no real future for solving US, energy problems. In the future, lignocellulosic biomass, presents an opportunity to counteract US dependence on fossil energy sources and open up major new crops for the agricultural economy [44, 73, 115].

In 2000–2004, the average wholesale price for gasoline was $0.91 per gallon [44]. Since then, it rose above $4 per gallon. On the other hand, the cost of producing ethanol from corn is $1.10 per gallon. The 2006 price for ethanol from corn was $1.40 per gallon involving a $0.51–0.54/gallon Federal subsidy. However, this has to be compared to huge tax breaks given to the US oil companies.

Ethanol from sugar cane

Historically, Brazil has been a leading nation in renewable liquid fuels. Ethanol is produced from sugar cane there. Since the 1990's; an aggressive program has achieved the use of pure ethanol in 90% of new cars and blends of 20–22% for older models. During 2005, the blend was increased to 25%. The renewable fuels program in Brazil has been an excellent example worldwide. Production increased up to 3.8 billion gallons of ethanol in the 2003–2004 crop year which represented 40% of Brazil's domestic fuel consumption [88]. The price of ethanol is 65% that of gasoline. The side product, sugar cane bagasse, can be used to generate electricity.

Agricultural and forest lignocellulosic biomass

Since there is not enough corn available to make a significant dent in our dependence on petroleum for energy, further increases in ethanol production will come from biomass. It is essential for the biofuels sector to move away from food and grain crops into alternative renewable feedstocks such as non-food lignocellulosic biomass. Furthermore, lignocellulosic ethanol is five times better than corn ethanol in energy balance. Ethanol from biomass could be made with as little as 10% of the energy it would provide [49, 50].

Available biomass reserves are about 180–200 million dry tons per year. This could yield 16 billion gallons of ethanol based on an overall yield of 80 gallons per dry ton [14, 101]. The US Department of Agriculture and the Department of Energy have estimated that 1 billion tons of such biomass could be produced annually from agriculture and another 0.3 billion tons from forestland; this would yield 80 billion gallons of bioenergy, about 30–40% of current usage (DOE/USDA "Billion tons" study: http://feedstockreview.ornl.gov/pdf/billion_ton_vision.pdf). This could be accomplished with relatively modest changes in land use and agricultural and forestry practices [87]. The world content of lignocellulosic biomass is 10–50 billion tons [101]. In Asia, rice straw would be an excellent substrate for bioethanol production. At present, it constitutes half of the agronomic biomass of the world, is burned, and causes health and environmental problems.

Lignocelluose in waste products is inexpensive, plentiful and renewable. It is the most abundant renewable natural substrate for conversion into fuel. Its content of cellulose and hemicellulose can be converted to oligosaccharides, hexoses and pentoses plus lignin. The content of cellulose and hemicellulose in these biomass feedstocks represents a sustainable alternative, i.e., a cheap and renewable energy source to increase biofuel production and to improve energy balance with less contribution to greenhouse effects. Tremendous amounts of cellulose are also available as municipal and industrial wastes which contribute to our current pollution problems. Once cellulosic bioethanol becomes a reality, the next emphasis will be on such waste products [20].

The cost of straw ($27 per ton) is much less than that of petroleum ($340 per ton). It is also much less costly than corn and wheat ($135/ton) or sugar ($340/ton) [99]. Between 1999 and 2005, the cost of lignocellulosic biomass increased from $30 to 40 per dry ton while gasoline prices skyrocketed.

Recovery of protein for animal feed from biomass conversion would lower the cost of ethanol by $0.11–0.13 per gallon [44]. Switch grass leaf and stems contain 10% protein. Corn stover, rice straw and wheat straw contain 4–6% protein.

Various reports indicate that ethanol from lignocellulosic biomass reduces greenhouse gas emissions by about 80% as compared to gasoline whereas corn to ethanol reduces them by 20–30%. Bioenergy crops are able to

balance CO_2 emissions by converting atmospheric CO_2 into organic carbon in biomass [26]. Agricultural residues are renewable, chiefly unexploited, and inexpensive. They include leaves, stems and stalks (but not the fruit and seeds) from sources such as corn fiber, corn stover, sugar cane bagasse, rice hulls, woody crops and forest residues. Also, there are multiple sources of waste from industrial and agricultural processes, e.g., citrus peel waste (CPW), sawdust, paper pulp, industrial waste, municipal solid waste (MSW), and paper sludge. In addition, dedicated energy crops for biofuels could include perennial grasses such as switchgrass, and other forage feedstocks such as miscanthus, bermuda grass, elephant grass, etc. These possess desirable environmental qualities, utilize water more efficiently, have high crop yields (above five dry tons per acre), prevent soil erosion, contribute to soil fertility and require minimal supplies of fertilizers and pesticides. Most importantly, perennial grasses can be adapted to various geographical diverse soils and weather across the US [44]. If agronomic practices and breeding programs on perennial switchgrass can be improved, yields could be increased to more than 14 dry tons per acre. Continuous improvement could yield 114 million acres of switchgrass by 2050 which could generate 165 billion gallons of ethanol, equivalent to 108 billion gallons of gasoline [11].

Five year field trials of switchgrass conversion to bioethanol were very successful and showed that perennial crops like switchgrass are much better than annual crops such as corn [96]. Perennial crops require fewer agricultural inputs than annual crops. Switchgrass conversion to ethanol was calculated to produce over 500% more renewable energy than energy consumed in the process and has significant benefits with respect to greenhouse gas production and conservation.

In addition to cellulose and hemicellulose, another key biomass component is lignin, a complex aromatic polymer which comprises 15–25% of plant biomass and contains 40% of the energy. The potential of lignin as an energy source (boiler fuel) for power generation (heat and electricity) is considerable. Lignin as a by-product from fractionation and conversion operations is a renewable fuel itself with minimal greenhouse gas problems mainly because the CO_2 released will be taken up by the plant biomass during growth [44, 87]. There is ample energy in lignin to power the fermentation process and produce power for sale.

Currently, the pulp and paper industry in the USA processes 108 million tons of wood. Pre-extraction of wood chips could provide 14 million tons of hemicellulosics to the bioethanol refineries [90], simultaneously enhancing production of kraft pulps. The major constituents of wood are 41–51% cellulose, 23–38% hemicellulose, and 19–33% lignin.

It is important to appreciate that, in contrast to corn to ethanol, biomass feedstocks require more advanced pretreatment to unlock the fermentable sugars from the cellulose, hemicellulose and lignin components [1, 34, 40, 116].

Difficulties with hydrolysis of cellulose to unlock fermentable sugars

Lignocellulose is difficult to hydrolyze because it is (1) associated with hemicellulose, (2) surrounded by a lignin seal which has a limited covalent association with hemicellulose, and (3) much of it has a crystalline structure with a potential formation of six hydrogen bonds, four intramolecular and two intermolecular, giving it a highly ordered, tightly packed structure [109]. An important requirement for the cost effective production of liquid biofuels is to unlock the fermentable sugars present in cellulosic and hemicellulosic polysaccharides which are associated with and surrounded by the lignin seal. In order to deconstruct highly ordered, tightly packed crystalline and amorphous cellulose regions, pretreatment technologies are necessary. This helps to disrupt crystallinity, remove the lignin seal, increase pore volume, and solubilize cellulose and hemicellulose, thus making target polymers susceptible to enzymatic attack [116]. Continuous technology optimization through coordinated development efforts on biomass pretreatment technologies is a very active field dedicated to enhance the total yield of fermentable sugars prior to the microbial fermentation stage [74].

Bioethanol production

Yeasts

Ethanol is a primary metabolite produced by fermentation of sugar, or of a polysaccharide that can be depolymerized to a fermentable sugar. S*accharomyces cerevisiae* is used for the fermentation of hexoses, whereas *Pichia stipitis* or *Candida* species is employed for pentose utilization.

Under optimum conditions, approximately 10–12% ethanol by volume can be obtained from sugar by *S. cerevisiae* within 5 days. Such a high concentration slows down growth and the fermentation ceases. With special yeasts, the fermentation can be continued to produce alcohol concentrations of 20% by volume, but these concentrations are attained only after months or years of fermentation. At present, all beverage alcohol is made by fermentation. Industrial ethanol is mainly manufactured by fermentation, but some is still produced from ethylene by the petrochemical industry.

A review [70] on ethanol fermentation lists *S. cerevisiae* processes yielding 97 g/l in 96 h on sucrose, 70 g/l on sugar cane molasses in 30 h and 53 g/l on beet molasses in 192 h.

A number of genetic manipulations have been carried out to increase ethanol production by yeasts. For example, eliminating the glycerol-3-phosphate dehydrogenase gene *gdp1* and over-expressing the glutamate synthase gene *glt-1* increased ethanol production by *S. cerevisiae* by 11% to 80 g/l while decreasing formation of glycerol, acetate and pyruvate [62]. The *glt-1* over-expression worked by reducing NADH and making a more acceptable NAD^+/NADH ratio. In another study, over-expression of *glt-1* and deletion of glycerol export gene *fps1* in *S. cerevisiae*, resulted in the improvement of ethanol production on glucose from 108 to 123 g/l [61].

Hahn-Hägerdal et al. [46] have reviewed the production of ethanol by pentose-fermenting yeasts. The genome of the lignocellulose-degrading xylose fermenter, *P. stipitis*, has been sequenced [54]. Fed-batch culture of *P. stipitis* can produce 50 g/l ethanol from xylose with yields of 0.35–0.44 g/g xylose and ferment hydrolysates at 80% of theoretical yield [47, 84]. *P. stipitis* contains many genes encoding endoglucanases, β-glucosidases, xylanases, mannanase, chitinase, and xylosidases and can use cellobiose and other oligomers.

Fusants of *S. cerevisiae* with the pentose-fermenting *Candida shehatae* are capable of converting wood biomass to ethanol after dilute acid pre-treatmemt and addition of *Aspergillus niger* cellulase [86]. From 150 g/l of substrate, 84 g/l of sugars were produced (equivalent to 89% hydrolysis of carbohydrates in lignocellulose) which were converted to 32 g/l of ethanol with 90% fermentation efficiency.

Gram-negative bacteria

Escherichia coli and *Klebsiella oxytoca* have been genetically converted from producers of mixed acids into ethanol producers by recombinant DNA techniques. By cloning and expressing the alcohol dehydrogenase and pyruvate decarboxylase genes from *Zymomonas mobilis* in *K. oxytoca*, the recombinant strain is able to convert crystalline cellulose to ethanol in high yield when fungal cellulase is added [28]. The percent of maximum theoretical yield was 81–86% and titers as high as 47 g/l ethanol were produced from 100 g/l cellulose. Other genetically engineered strains of *E. coli* can produce 60 g/l of ethanol [117]. Ethanol production has been increased further by metabolic engineering techniques [85].

Recombinant *E. coli* strains produced 35 g/l ethanol from corn fiber hydrolysate [24]. The time was 55 h and yield was 0.46 g ethanol per g of available sugar which is 90% of maximum attainable. Corn fiber contains 70% by weight of carbohydrate, being made up of cellulose and hemicellulose. Corn fiber is produced at 3.4 million dry tons per year which could yield up to 4 billion gallons of ethanol, assuming an 80% conversion. The best pretreatment of corn fiber appears to be dilute acid, which avoids production of inhibitory compounds such as furfural or 5-hydroxymethyl furfural acid from lignin. Addition of cellulase and β-glucosidase yielded 85–100% of theoretical yield of monomeric sugars [94]. Fermentation of corn fiber pre-treated by ammonia fiber explosion yielded 27 g/l ethanol by recombinant *E. coli* [80].

Z. mobilis is also used for ethanol production. Most recombinant strains of *E. coli*, *Zymomonas*, and *Saccharomyces* convert corn fiber hydrolysate to 21–34 g/l with yields of 0.41–0.50 ethanol per g of sugar consumed [14]. The best was *E. coli* strain KO11 [119].

The need for fungal cellulase

The yeast processes and the Gram-negative bacterial processes discussed above all require the addition of fungal cellulase (cellobiohydrolase I) which is made by the mold *Trichoderma reesei.* This has resulted in a process known as simultaneous saccharification and fermentation (SSF) [89]. Only the Gram-positive anaerobic processes ("Gram-positive bacterial anaerobes") do not need the enzyme because the cellulase is normally made by the fermenting bacterium.

Cellulase is induced in *T. reesei* by cellulose. The low constitutive level of cellulase appears to hydrolyze some of the cellulose to small soluble inducers such as sophorose which then induces the formation of high concentrations of the enzyme [29]. In the past, the cost of this enzyme was prohibitively high but in the last few years, a greater than tenfold reduction in cost of *T. reesei* cellulase was accomplished by enzyme engineering and fermentation process development at Genencor International and Novozymes with DOE grants (totaling $30 million). This is claimed to yield an enzyme cost of only 10–20 cents per gallon of ethanol produced compared to the original price of $5/gallon [42]. However, further reductions in enzyme price are needed.

Gram-positive bacterial anaerobes

Bacteria such as clostridia are now being seriously considered for ethanol production [18, 76]. They are of interest since they can utilize lignocellulosic waste and generate ethanol, a rare combination among living organisms. Indeed, their cellulase systems mediate hydrolysis of lignin-containing materials such as hardwood pretreated with dilute acid as well as model substrates not containing lignin. In principle, the concept of an anaerobic ethanol

fermentation is a very simple one, i.e., a single fermentation with five biologically mediated events involved in ethanol production: (1) cellulase and hemicellulase formation, (2) cellulose and hemicellulose hydrolysis, (3) uptake of sugars and oligosaccharides, (4) hexose fermentation, and (5) pentose fermentation, all consolidated in a single process step. An advantage of such an anaerobic process is the reduced need for power-consuming agitation/aeration of reaction vessels for making biomass-degrading enzymes. Also, anaerobes generally have a low cellular growth yield, hence more of the substrate can be converted to ethanol, and in situ cellulase production is much more economical than addition of cellulase. Although mesophilic anaerobes might be usable, anaerobic thermophiles are favored as "ethanologens" for the following reasons: (1) single step nature of enzyme production, saccharification and ethanol production thus eliminating the cost of fungal enzyme(s), (2) thermophiles appear to be robust and contain stable enzymes, (3) thermophilic fermentations run at 60°C are less prone to detrimental effects of contamination, (4) growth at higher temperatures may facilitate the removal and recovery of volatile products such as ethanol, (5) high rates of growth and metabolism of cellulose and hemicellulose, (6) low cell yield allowing more substrate to be converted to ethanol, and (7) reduced need for power involved in oxygen transfer, agitation and cooling of fermentors. Anaerobiosis is an advantage because one of the most expensive steps in industrial fermentations is that of providing adequate oxygen transfer; with these anaerobes, this is not required.

Integration of cellulase production, saccharification and fermentation into the microbial cell involves the use of thermophilic, anaerobic, ethanologenic bacteria such as *Clostridium thermocellum* [8, 19, 74]. *C. thermocellum* can convert both waste cellulose (i.e. biomass) and crystalline cellulose directly to ethanol [21]. The cellulase system in *C. thermocellum* is comprised of multiple enzyme complexes [9, 15, 45]. It contains multiple endo-β-glucanases, four exoglucanases, two β-glucosidases, one cellodextrin phosphorylase, one cellobiose phosphorylase, six xylanases, minor β-xylosidase, minor β-galactosidase; minor β-mannosidase, two lichenases, two laminarinases, pectin lyase, polygalacturonate hydrolase, and pectin methylesterase.

C. thermocellum breaks down cellulose with the formation of cellodextrins and cellobiose as main products. These products, containing glucose moieties held together by beta-1,4-linkages, can be taken into the cells and further utilized; the final end products are ethanol, acetic acid, lactic acid, hydrogen and carbon dioxide [67]. This organism has the highest rate of cellulose utilization and the highest growth rate on crystalline cellulose among all known bacteria [39, 74]. Conversion of mixed hardwood flour to ethanol in a continuous fermentation was 2.5 times greater using *C. thermocellum* than with the SSF process using *S. cerevisiae* and the addition of both fungal cellulase and β-glucosidase and [100]; furthermore, the rate of conversion was four times higher.

Since clostridia such as *C. thermocellum* do not have the ability to convert pentose sugars derived from hemicellulose to ethanol, the concept of mixed cultures ("co-cultures") was born. Mixed cultures of anaerobic thermophiles offer the potential of decreasing production costs of lignocellulosic biomass to ethanol by twofold [72]. With resources dedicated to the exploration of these combinations of bacteria, the conversion of agricultural, forest, and urban resources into ethanol could become an economic substitute for petroleum fuels. This modern technology named "consolidated bio-processing (CBP)" [74] could become an important means of producing ethanol commercially. The advantages of an anaerobic coculture system includes elimination of capital or operating costs for enzyme production, greatly reduced diversion of substrate for enzyme production, and compatible enzyme and fermentation systems.

The coculture process maximizes the hydrolysis and fermentation of lignocellulosic/hemicellulosic biomass after mild acid treatment [21]. *C. thermocellum* has the capacity to produce cellobiose and cellodextrins from cellulose which are transformed into ethanol, lactic acid and acetic acid. In the case of hemicellulose, enzymatic saccharification by *C. thermocellum* occurs generating xylose and xylobiose; both are utilized by *Thermoanaerobacterium saccharolyticum* for production of ethanol, lactic acid and acetic acid. Steps of CBP include (1) cellulase and hemicellulase production by *C. thermocellum*, (2) cellulose hydrolysis to cellooligomers and cellobiose, (3) conversion of hemicellulose including arabinoxylans to monomeric xylans, xylobiose, xylooligosaccharides and pentose sugars, (4) uptake of oligosaccharides and sugars, (5) hexose fermentation to ethanol by *C. thermocellum*, and (6) pentose fermentation to ethanol by *T. saccharolyticum.*

The uptake of oligosaccharides and sugars from cellulose by the coculture occurs via phosphorolytic cleavage using cellodextrin phosphorylase and cellobiose phosphorylase. The phosphorylative cleavage by cellular phosphorylases seems to be much more active than the exogenous hydrolysis of cellulose to cellobiose due to lower K_m values; thus the cellodextrins are better utilized than is cellobiose [120]. Another key item for cell metabolism is the positive ATP balance generated during phosphorylation [74]. In addition to all the beneficial properties of the anaerobic thermophilic system listed above, the coculture system has the ability to use cellulose, hemicellulose, glucose, starch, xylan, xylose, mannose, galactose and arabinose [23, 74, 103, 104].

Although the coculture system is very promising, several barriers exist such as end product inhibition by the

produced ethanol [48]. However, this disadvantage can almost be counterbalanced by the process of ethanol distillation from dilute broths. Continuous ethanol removal gives a twofold increase in ethanol yield when only 37% of the ethanol has been removed. Furthermore, a mutant of *C. thermocellum* has been obtained that is tolerant to 60 g/l of ethanol and is capable of producing 26 g/l ethanol [76]. Also, a strain of *Thermoanaerobacter* has been isolated (see below) that is resistant to ethanol up to 8.3% (equivalent to 65 g/l of ethanol) [36].

Another disadvantage has been a low yield due to the production of side-products lactate and acetate. One solution is the elimination of metabolic branches which result in ethanol formation being the sole means for the cell to get rid of excess reducing equivalents. In doing so, factors had to be investigated which were likely to impact gene transfer in the two organisms of the coculture. For a number of years, this was hampered by the paucity of information on the molecular genetics of these cultures. One obvious approach was to knock out the genes encoding acetate kinase and lactate dehydrogenase, which were responsible for the branched metabolic pathways. A prerequisite for such manipulations was the ability to introduce foreign DNA into these bacteria. Some progress was made in a joint Dartmouth College-M.I.T. investigation of *C. thermocellum* and coculture partner *Clostridium thermosaccharolyticum* dealing with restriction endonuclease systems [59]. *C. thermosaccharolyticum* was successfully transformed by electrotransformation and the foreign erythromycin resistance character was expressed [22, 60]. More recently, an efficient high frequency gene transfer system was developed by the Lynd laboratory at Dartmouth College. Electrotransformation of *C. thermocellum* was achieved using plasmid p1Km1 with selection based on resistance to erythromycin and lincomycin [103, 104]. Genes *ldh* and *ack* are involved in side product production in both members of the coculture. In the case of *T. saccharolyticum* [23], single mutant knockouts for lactate and acetate were obtained first and then a double knockout mutant was generated which presented a substantial reduction of lactate and acetate production and four times higher ethanol production than the wild strain. In addition, the double knockout mutant was capable of utilizing xylose more efficiently [76, 104].

In recent years, more work is being done on the pentose-utilizing member of the coculture system. Thermophilic anaerobe *Thermoanaerobacter* BG1L1 is capable of converting dilute sulfuric acid pre-treated corn stover to ethanol without detoxification of the corn stover hydrolysate [37]. The system used was a continuous immobilized fermentation and up to 15% total solids were tolerated, yielding ethanol at 0.39–0.42 g/g sugar consumed. The system was run for 135 days with no contamination. The culture does not produce lactate due to elimination of lactate dehydrogenase. It was resistant to ethanol up to 8.3% [36].

T. saccharolyticum, an anaerobic non-cellulolytic thermophile which can use xylan and sugars from biomass, was genetically engineered to produce ethanol in high yield [97]. The new pathway uses pyruvate:ferredoxin oxidoreductase (POR) and electron transfer from ferredoxin to NAD. The original strain produced acetic acid from pyruvate via POR, phosphate acetyl transferase (PTA) and acetate kinase (ACK). It also produced lactic acid from pyruvate via lactate dehydrogenase (LDH). In new strain ALK1, genes L-*ldh* and *ack/pta* were knocked out, eliminating production of lactic and acetic acids. When grown in continuous culture with xylose, the culture produced 33 g/l ethanol at a volumetric productivity of 2.2 g/lh and was stable over hundreds of generations. In a fed-batch fermentation with a mixture of glucose, xylose, galactose and mannose, 37 g/l ethanol was produced with a maximal productivity of 2.7 g/lh. The titer of 37 g/l is higher than ever achieved by a thermophilic bacterium [74].

The workhorse of the cellulolytic anaerobes is the cellulosome [6, 105]. In 1983, important events occurred in this regard. Eric Johnson in his Ph.D. work at M.I.T. noted that the cellulase activity of *C. thermocellum* was part of a large structure with a molecular weight of over 1.5×10^6 [55]. In the same year, Lamed, Bayer and coworkers [4, 69] in Israel purified a multi-subunit complex from the culture supernatant which they named the cellulosome [66]. Cellulosomes are crucially important for the efficient breakdown and utilization of crystalline cellulose. The cellulosome is a macromolecular machine (multienzyme complex) which, like a ribosome, is dedicated to organized, concerted, synergistic, and efficient catalysis of cellular activities [7]. Cellulosomes are unique in that no other extracellular protein complexes with the size and complexity of cellulosomes have been reported. They have molecular masses of 2×10^6–6×10^6, diameters of about 18 nm and contain 14–50 polypeptides ranging in size from 37 to 210 kDa. Over 95% of the endoglucanase activity of *C. thermocellum* is associated with the cellulosome.

The highly ordered arrangement of the cellulosome gives it stability but makes purification of individual proteins extremely difficult. However, purification of this complex aggregate of cellulolytic proteins was accomplished [112, 113] Using Avicel (crystalline cellulose) breakdown as a turbidimetric assay for "true cellulase" activity and carboxymethyl cellulose (CMC) hydrolysis as an assay for endoglucanases, the aggregate was found to contain at least four endoglucanases of different molecular weights accompanying true cellulase activity. It was dissociated by mild sodium dodecylsulfate (SDS) treatment plus EDTA and DTT but the resulting individual fractions exhibited only endoglucanase activity, the true cellulase activity being

lost. Reconstitution of true cellulase activity was accomplished by combining two of the major components which were called S_S (M_r = 82,000) and S_L (M_r = 250,000). They were purified by gel filtration chromatography and by elution from an SDS-polyacrylamide gel, respectively. S_S alone acted on CMC but S_L alone had little to no enzymatic activity [68, 69, 113]. The enzymatic activity of S_S on CMC was not enhanced by S_L, but its adsorption to crystalline cellulose was improved [113]. It was hypothesized that the cooperative degradation of crystalline cellulose involved an interaction between S_S (and presumably other cellulases), S_L and the insoluble substrate. S_L (an anchorage subunit) would function to bind S_S (and other catalytic proteins of the complex) to the cellulose surface in a manner optimal for hydrolysis [111, 112], consistent with the "anchor-enzyme" hypothesis [38]. The anchor-enzyme model was further confirmed using recombinant forms of S_S and S_L [63]. The anchorage function of protein S_L is the basis of the current understanding of cellulosome structure. S_L, which is equivalent to component S1 of Lamed et al. [68], is now called the cellulosome-integrating protein (CipA), or the scaffolding protein, or 'scaffoldin' [5]. It contains approximately 1,850 amino acid residues and is the most important protein of the cellulosome. In addition to its function of binding other members of the cellulose complex to itself, it also binds to cellulose [10]. Its nucleotide sequence revealed a deduced protein size of 196,800 Da, a cellulose binding domain (CBD) [8] and nine enzyme receptor domains of about 150–166 amino acid residues each. The nine repeated sequences, called 'cohesins' by Bayer et al. [5], are quite similar to each other, i.e., exhibiting between 60 and 100% identity, with six of the nine domains being at least 90% identical. They are the receptors that bind the individual cellulases, xylanases and other enzymes to CipA. The work by Bayer and Lamed [3] on the structure of the cellulosome was very important for understanding the detailed interactions between the organism and its enzymes, and the binding affinity to cellulose through the CBD.

Protein S_S, the exoglucanase which is also called CelS [108], is the major catalytic subunit of the cellulosome, and is equivalent to component S8 of the Israeli group [81]. Of great interest was the sequencing of the *celS* gene encoding S_S [108] which revealed an open reading frame of 2,241 bp encoding 741 amino acid residues with a predicted molecular weight of 80,670 [107, 108]. The sequence indicated that CelS belonged to a new cellulase family [108] and was later classified as a member of family 48 glycosyl hydrolases. Although it is the most abundant catalytic subunit of the cellulosome, its low or complete lack of activities on CMC and other synthetic substrates explains why it had been difficult to clone its gene. Family 48 enzymes are found mostly in bacterial cellulase systems and are now considered key components in the bacterial scheme for breaking down cellulosic materials [52]. CelS contains a duplicated 24 amino residue dockerin, the site of binding to scaffoldin. Like the cellulosome itself, recombinant CelS is competitively inhibited by cellobiose and only marginally so by glucose [64]. Thus, an association is formed by a synergistic cassette of catalytic proteins, which is optimal for hydrolysis of insoluble polymers to the level of soluble oligosaccharides.

At least 71 open reading frames coding for proteins containing dockerins have been found by sequencing and annotating the genome of *C. thermocellum* [121, 122]. They include cellulases, xylanases/xyloglucanases, a chitinase, mannanases, lichenases, carbohydrate esterases, pectinases, pectic lyase, glycosidases, a mixed-linkage β-glucanase, putative proteases and protease inhibitors. Thirty-three of these contain a CBM.

Expression of scaffoldin-related proteins is coordinately regulated by catabolite repression as shown by a quantitative proteomic analysis [39], confirming the hypothesis first proposed in 1980 [35].

The cohesins bind the dockerin subunits (type 1 dockerins) of the relevant enzymes. The scaffoldin also contains dockerins (type II) that anchor the cellulosomes to the cell surface. Much information on the scaffoldin structure was revealed by the genetic sequencing of the gene by Gerngross et al. [38]. Cellulosomes are bound to the cell surface during early log phase of growth and are released into the medium during late log phase. During the stationary phase, the cellulosome complex remains attached to cellulose in the medium [7]. Extensive work has been dedicated to the understanding of each component, including the purification, cloning, expression and sequencing of each relevant gene. Besides *C. thermocellum*, there are other microorganisms which contain cellulosome type structures such as *Clostridium cellolovorans*, *Clostridium cellulolyticum*, *Clostridium josui*, *Clostridium papyrosolvens*, *Clostridium acetobutylicum*, *Acetovibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Vibrio* sp., and some species of the fungal genera *Neocallimastix*, *Piromyces* and *Orpinomyces* [98].

A gene, *glyR3*, was found to be co-transcribed in an an operon with cellulase/hemicellulase genes *celC* and *licA* [83]. Gene *glyR3* bound specifically to the *celC* promoter region. The binding was inhibited by laminaribiose. Protein GlyR3 is a negative regulator of the operon. Laminaribiose is the inducer of the operon. It is a β-1-3 linked glucose dimer produced by breakdown of cellulose.

Researchers at the University of Georgia [32, 51] found even larger aggregates, ca. 108×10^6 Da which they called "polycellulosomes". Protuberances covering the surface of the cell are packed with polycellulosomes, each protuberance containing several hundred cellulosomes [65]. When

cells are grown on cellobiose, cellulosome complexes are packed into such discrete exocellular structures. When grown on cellulose, these polycellulosome-containing organelles (protuberances of diameter 60–200 nm) undergo extensive structural modification [3]. After attachment to the insoluble substrate, the protuberances rapidly aggregate into "contact corridors" which physically mediate between the cellulosome, which is attached to the cellulose, and the bacterial cell surface. The proteins of the cellulosome are arranged in a highly ordered chain-like array [78]. The cellulose-bound cellulosome clusters appear to be the sites of active cellulolysis and the products may be channeled down the fibrous structures to the cell.

Biobutanol

Other clostridia produce acetate, lactate, acetone, ethanol and butanol. Butanol has 1/3 higher energy density (36 vs. 27 kJ/g) than ethanol. Also, butanol use in cars does not require engine modification until it reaches 40% of total fuel; ethanol requires it at concentrations of over 15%. At one time, the acetone–butanol–ethanol (ABE) fermentation was used commercially to produce the solvents acetone and butanol, but the fermentation was replaced by less expensive chemical procedures. Today, there is renewed interest in this fermentation to produce biobutanol.

Application of gas stripping during fed batch fermentation of liquefied corn starch allowed higher production of acetone, butanol and ethanol (ABE) by *C. beijerinkii* [30]. In a batch process, only 18 g/l ABE was produced. With gas stripping and fed batch fermentation, 81 g/l was produced from 225 g/l sugar in the substrate. Of this 81 g/l, butanol was at 56 g/l, acetone at 24 g/l and ethanol at 1 g/l.

Biodiesel

Whereas gasoline contains hydrocarbons of 4–12 carbon atoms, diesel (=petrodiesel) is made of alkanes and aromatic hydrocarbons, C_{10}–C_{15} in length (some sources say C_9–C_{23}) whereas biodiesel contains alkyl fatty esters (fatty acids with alkyl chains of 16–24 carbon atoms). Biodiesel is made by chemically *trans*-esterifying vegetable oils or animal fats from excess soybean oil or restaurant grease with an alcohol, usually methanol, to make monoalkyl (ethyl or methyl) esters [77]. The process is known as alcoholysis. Worldwide biodiesel production between 2004 and 2007 quintupled to 2.4 billion gallons [102]. It can be used as a fuel for vehicles in its pure form, but it is generally used as a petroleum diesel additive to reduce levels of particulates, carbon monoxide, hydrocarbons and air toxics from diesel-powered vehicles. It can be blended with diesel up to 20% ("B20"). It is about 90% as effective as diesel. Like diesel, it can be used in unmodified engines but only 200 million gallons were made in the USA in 2006 which is only a minor amount as compared to the 65 billion gallons of diesel used in 2007 in the USA. However, this can be compared to only 30 million gallons made in 2004 and 75 million gallons in 2005. 300 million gallons were expected to be made in 2007. There is not enough soy oil around to make diesel at high levels. Thus, biodiesel using microbiologically produced fats and oils will be important. Similar to bioethanol, biodiesel can help reduce greenhouse gas emissions since the organisms that produce it remove CO_2 from the atmosphere as a part of their natural metabolism.

Glycerol is a waste product of the biodiesel process, which if converted into a useful product, would make the biodiesel process much more economical [118]. Such products might be PDO (1,3-propane-diol), butanol, ethanol, propionic acid (42 g/l) and succinic acid (19 g/l).

Biodiesel from the photosynthetic microalgae and cyanobacteria which grow on CO_2 has great potential as a biofuel [16]. Some microalgae have as much as 80% of their dry weight as oils. In contrast, current crops used for biodiesel, e.g., soy bean and oil palm, possess only 5% of their biomass as oils. Thus, these organisms are being seriously considered as a substitute for plant oils to make biodiesel. Such biomass can have a very high (30–80%) lipid content. They grow rapidly and can produce 7,000 gallons of biodiesel per acre per year. Growth will be in small photobioreactor ponds allowing light penetration and temperature control. Land area requirements will be lowered if algae substitute for vegetable oils. Fermentation alcohols (ethanol, propanol or butanol) can be used instead of methanol. *Chlorella pyrenoidosa* is known to produce up to 70% of its biomass as extractable lipid [17]. Other algae produce from 25 to 54% lipid.

Some oleaginous molds, yeasts and bacteria produce a high content of lipids as storage compounds [93]. The molds include *Mucor circinelloides* (27% of dry weight), *Crythecodinium cohnii* (30%), *Mortierella alpina* (50%) and *Thraustchytrium aureum* (80%). *M. alpina* is used commercially to produce the polyunsaturated fatty acid arachidonic acid. The procedure used allows the organisms to run out of nitrogen in the medium by using a high C:N ratio. A crucial enzyme for lipid accumulation is ATP:citrate lyase (ACL) but it is difficult to clone this enzyme into fungi. Cloning it into plants increased lipid accumulation but only by 16%. Also important appears to be malic enzyme (ME) which supplies NADPH for acetate condensation into the growing fatty acyl chain. Other important molds are *Mortierella vinacea* (66% of dry weight), *Mucor circinelloides* (65%), *Penicillium spinulosum* (64%), *Aspergillus terreus* (57%), *Penicillium lilacinum* (56%), *Aspergillus nidulans* (51%), *Chaetomium globosum* (54%),

Fusarium sp. (52%), *Fusarium bulbigenum* (50%), *Mucor mucido* (51%), *Aspergillus ochraceus* (48%) and *Pythium ultimum* (48%) [91]. Over 75 species of molds producing from 23 to 66% lipid are listed by Ratledge [92].

The yeast *Lipomyces starkeyi* is also important in lipid accumulation. In a fed-batch process, after ammonium, zinc or ferrous ion became deficient, it produced 83 g/l of lipids in 153 g/l cells. Other yeasts which are good lipid producers are *Rhodotorula gracilis* (74% of dry weight), *Cryptococcus terricolus* (renamed *Cryptococcus albidus* var. *albidus*) (68%), *Lipomyces tetrasporus* (64%), *Candida curvata* (60%), *Endomyces vernalis* (renamed *Trichsporon pullulans*) (57%), *Rhodotorula glutinus* var. *glutinus* (58%), *Geotrichum candidum* (50%), *Lipomyces lipofer* (49%), and *Candida* 107 (possibly *Candida humicola*) (44%) [91, 92].

The bacterium *Arthrobacter* sp. produces up to 80% of its dry weight as lipid [92]. Genetically modified bacteria can produce biodiesel from plant materials. Kalscheuer et al. [57] modified *E. coli* by incorporating genes from other bacteria, two from *Z. mobilis* allowing alcohol production from glucose, and a third gene from *Acinetobacter bavlvi* to combine the alcohol with the plant oils (from olive oil). What was made was a fatty acid diesel substitute which they called microdiesel.

Other biofuels

Some scientists are developing *E. coli* strains which produce short chain alcohols from intermediates of amino acid biosynthesis [2] via 2-keto acid decarboxylases and alcohol dehydrogenases. These include isobutanol (from 2-ketoisovalerate in valine biosynthesis), 3-methyl-1-butanol (from 2-keto 4-methyl pentanoate in leucine biosynthesis), 1-butanol (from 2-ketovalerate in norvaline biosunthesis), 1-propanol and 2-methyl-1-butanol (from 2-ketobutyrate and from 2-keto-3-methyl valerate respectively in isoleucine biosynthesis), and 2-phenylethanol (from phenylpyruvate in phenylalanine biosynthesis [58]. The benefit of these branched chain higher alcohols is their higher energy density and lower hygroscopicity as compared to ethanol. They are also less volatile. The LS9 company aims to transform *E. coli* fatty acids into specific hydrocarbon fuels [102].

Commercial developments

Since 2006, many biotechnology companies have been established in the area of biofuels either alone or with companies of the petroleum and chemical industries. Even before 2004, Iogen (a Canadian enzyme company) had built a demonstration plant in Ottawa in 2004 with backing from Shell and Petro-Canada. A new plant in Germany was planned by Iogen, Shell and VW. New partnerships in the bioethanol field include (1) Cellunol and Diversa, (2) BP, UC Berkeley, University of Illinois and Lawrence Berkeley National Laboratory, (3) Mascoma and Genencor, (4) Codexis and Shell, (5) Iowa State and Broin, (6) DuPont and Genecor International, (7) Dupont and BP, (8) Chevron and Weyerheuser, and others. At present, there are 137 US plants with capacity to convert over 2 million bushels of corn into 7.6 billion gallons of ethanol. Corn to ethanol efforts have received a tax credit of $0.54 per gallon. In addition, there are 62 plants being built and another eight under expansion.

In late 2007, 165 companies were producing biodiesel in the USA and 85 plants were under construction. ADM is the leading producer of biodiesel. Dow Haltermann Custom Processing (DHCP; part of Dow Chemical) is also a producer. UOP and ENI (Rome) use hydrogen and vegetable oil to produce a high cetane biodiesel fuel.

US government initiatives

In 2005, the US Congress mandated that 7.5 billion gallons of ethanol and biodiesel be produced per year by 2012. Also, the US Department of Agriculture offered $188 million in loan guarantees and grants for renewable energy and energy efficiency efforts [82]. Congress in 2007 mandated that by 2022 there will be 36 billion gallons of ethanol produced with 44% of it from cellulosic biomass. In 2007, DOE allocated over $1 billion to biofuels as follows: six commercial scale biorefineries ($385 million), development of pilot scale tests of cellulosic refining processes ($200 million), construction of three bioenergy R&D centers ($400 million), research to develop efficient microbes to convert biomass to ethanol ($23 million), plus four projects on gasification processes to convert grasses, corn stover and other plant materials to biofuels. Industrial contributions were expected to bring the total to over $2 billion. The three research centers are to be established at Oak Ridge National Laboratory, University of Wisconsin, and Lawrence Berkeley National Laboratory in California. The commercial plants will be constructed in Kansas by Abengoa, in Idaho by Iogen, in Iowa by Poet, in Georgia by Range Fuels, in Florida by Alica, and in Southern California by BlueFire Ethanol.

Final comments

We have been in a troubled energy situation since the 1970s. About the only positive events that occurred soon thereafter was the production of bioethanol from corn in the

USA and from sugar cane in Brazil in the 1980s. However, at least in the USA, we have essentially "run out" of corn and something new has to be done. A tremendous amount of lignocellulosic biomass is available here and elsewhere on farms and in the forests. Later, even municipal waste can come into the picture as a source of feedstock for biofuels production. The biological products of the future include bioethanol, biobutanol, biodiesel, other short chain alcohols, and biohydrocarbons. Additional biological possibilities include biomethane, biomethanol, biohydrogen, and microbial fuel cells, as described in a new book on bioenergy [106]. These potential solutions will aid our drive to energy independence, homeland security, reduction in greenhouse gas emissions and resultant environmental improvement, as well as providing a boost to our troubled economy. Much of the basic microbiological and genetic work has been done on a variety of microorganisms. What remains is a major effort and challenge to biochemical engineering at the many new plants being built for biofuel production. The new processes have to be scaled up and carried out in a cost effective way. The future of biofuels looks very bright. It is obvious that the best is yet to come.

Acknowledgment I thank Professor Lee R. Lynd of Dartmouth College for much advice over the years on this subject.

References

1. Allen SG, Schulman D, Lichwa J, Antal MJ, Jennings E, Elander R (2001) A comparison of aqueous and dilute-acid single-temperature pretreatment of yellow poplar sawdust. Ind Eng Chem Res 40:2352–2361. doi:10.1021/ie000579+
2. Atsumi S, Hanai T, Liao JC (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as fuels. Nature 451:86–89. doi:10.1038/nature06450
3. Bayer EA, Lamed R (1986) Ultrastructure of the cell surface cellulosome of *Clostridium thermocellum* and its interaction with cellulose. J Bacteriol 167:828–836
4. Bayer EA, Kenig R, Lamed R (1983) Adherence of *Clostridium thermocellum* to cellulose. J Bacteriol 156:818–827
5. Bayer EA, Morag E, Lamed R (1994) The cellulosome—a treasure-trove for biotechnology. Trends Biotechnol 12:379–386. doi:10.1016/0167-7799(94)90039-6
6. Bayer EA, Setter E, Lamed R (1985) Organization and distribution of the cellulosome in *Clostridium thermocellum*. J Bacteriol 163:552–559
7. Bayer EA, Shimon LJW, Shoham Y, Lamed R (1998) Cellulosomes—structure and ultrastructure. J Struct Biol 124:221–234. doi:10.1006/jsbi.1998.4065
8. Beguin P, Aubert JP (1994) The biological degradation of cellulose. FEMS Microbiol Rev 13:25–58. doi:10.1111/j.1574-6976.1994.tb00033.x
9. Beguin P, Lemaire M (1996) The cellulosome: an exocellular, mutiprotein complex specialized in cellulose degradation. Crit Rev Biochem Mol Biol 31:201–236. doi:10.3109/10409239609106584
10. Beguin P, Millet J, Aubert J-P (1992) Cellulose degradation by *Clostridium thermocellum*: from manure to molecular biology. FEMS Microbiol Lett 100:523–528. doi:10.1111/j.1574-6968.1992.tb05750.x
11. BioCycle eNews Bulletin (2005) Creating cellulosic ethanol: spinning straw into fuel by Diane Greer. (http//:www.harvestcleanenergy.org/enews/enew_0505/enews_0505_Cellulosic_Ethanol.htm)
12. Blanch HW, Adams PD, Andrews-Cramer KM, Frommer WB, Simmons BA, Keasling JD (2008) Addressing the need for alternative transportation fuels: the joint bioenergy institute. ACS Chem Biol 3:17–20. doi:10.1021/cb700267s
13. Bothast R, Schlicher MA (2005) Biotechnological processes for conversion of corn into ethanol. Appl Microbiol Biotechnol 67:19–25. doi:10.1007/s00253-004-1819-8
14. Bothast R, Nichols NN, Dien BS (1999) Fermentations with new recombinant organisms. Biotechnol Prog 75:867–875. doi:10.1021/bp990087w
15. Bumazkin BK, Velikodsorsakaya GA, Tuka K, Mogutov MA, Strongin A (1990) Cloning of *Clostridium thermocellum* endoglucanase genes in *Escherichia coli*. Biochem Biophys Res Commun 168:1326–1327
16. Chisti Y (2008) Biodiesel from micralgae beats bioethanol. Trends Biotechnol 26:126–131. doi:10.1016/j.tibtech.2007.12.002
17. Collyer DM, Fogg GE (1955) Studies on fat accumulation by algae. J Exp Bot 6:227–256. doi:10.1093/jxb/6.2.256
18. Demain AL (2006) The crucial importance of bioethanol and the contribution of anaerobic bacteria and cellulosomes. In: Uversky V, Kataeva IA (eds) Cellulosome. Nova Science Publishers Incorporation, New York, pp 1–9
19. Demain AL, Wu JHD (1989) The cellulase complex of *Clostridium thermocellum*. In: Ghose TK (ed) First generation of bioprocess engineering. Ellis Horwood, Chichester, pp 68–86
20. Demain AL, Lynd LR (1994) Turning garbage into motor fuel: fanciful dream or feasible scheme? In: Shimada K, Hoshino S, Ohmiya K, Sakka K, Kobayashi Y, Karita S (eds) Genetics, biochemistry and ecology of lignocellulose degradation. Uni Publ Co Ltd, Tokyo, pp 573–583
21. Demain AL, Newcomb M, Wu JHD (2005) Cellulase, clostridia and ethanol. Microbiol Mol Biol Rev 69:124–154. doi:10.1128/MMBR.69.1.124-154.2005
22. Demain A, Klapatch TR, Jung KH, Lynd LR (1996) Recombinant DNA technology in development of an economical conversion of waste to liquid fuel. Ann N Y Acad Sci 782:402–412. doi:10.1111/j.1749-6632.1996.tb40578.x
23. Desai SG, Guerinot ML, Lynd LR (2004) Cloning of the L-lactate dehydrogenase gene and elimination of lactic acid production via gene knockout in *Thermoanerobacterium saccharolyticum* JW/SL-YS485. Appl Microbiol Biotechnol 65:600–605. doi:10.1007/s00253-004-1575-9
24. Dien BS, Nichols NN, O'Bryan PJ, Bothast RJ (2000) Development of new ethanologenic *Escherichia coli* strains for fermentation of lignocellulosic biomass. Appl Biochem Biotechnol 84–86:181–196. doi:10.1385/ABAB:84-86:1-9:181
25. DOE report (2005) Genomics: GTL roadmap: systems biology for energy and environment. US Department of Energy Office of Science, p 298. (http://genomicsgtl.energy.gov/roadmap/)
26. DOE and Argon Labs report (2005) Ethanol study. DOE office of energy efficiency and renewable energy. (http://www.ncga.com/public_policy/PDF/03_26_Hahn05ArgonneNatlLabEthanolStydy.pdf)
27. DOE report (2006) Breaking the biological barriers to cellulosic ethanol: a joint research agenda. A research roadmap resulting from the biomass to biofuel workshop, 7–9 December 2005 at Rockville. US Department of Energy. Office of science and office of energy efficiency and renewable energy. (http://genomicsgtl.energy.gov/biofuels/b2bworkshop.shtml)

28. Doran JB, Ingram LO (1993) Fermentation of crystalline cellulose to ethanol by *Klebsiella oxytoca* containing chromosomaaly integrated *Zymomonas mobilis* genes. Biotechnol Prog 9:533–538
29. El-Gogary S, Leite A, Crivellaro O, Eveleigh DE, El-Dorry H (1989) Mechanism by which cellulose triggers cellobiohydrolase I gene expression in *Trichoderma reesei*. Proc Natl Acad Sci USA 86:6138–6141
30. Ezeji TC, Qureshi N, Blaschek HP (2007) Production of acetone butanol (AB) from liquefied corn starch, a commercial substrate, using *Clostridium beijerinckii* coupled with product recovery by gas stripping. J Ind Microbiol Biotechnol 34:771–777
31. Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM (2006) Ethanol can contribute to energy and environmental goals. Science 311:506–508
32. Felix CR, Ljungdahl LG (1993) The cellulosome: the extracellular organelle of *Clostridium*. Annu Rev Microbiol 47:791–819
33. Fox JL (2002) Legislation, technology boosting renewable fuel, materials uses. ASM News 68:480–481
34. Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618–628
35. Garcia-Martinez DV, Shinmyo A, Madia A, Demain AL (1980) Studies on cellulase production by *Clostridium thermocellum*. Eur J Appl Microbiol Biotechnol 9:189–197
36. Georgieva T, Mikkelsen M, Ahring B (2007) High ethanol tolerance of the thermophilic anaerobic ethanol producer *Thermoanaerobacter* BG1L1. Cent Eur J Biol 2:364–377
37. Georgieva TI, Ahring BK (2007) Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1. Appl Microbiol Biotechnol 77:61–68
38. Gerngross UT, Romaniec MPM, Huskisson NS, Demain AL (1993) Sequencing of a *Clostridium thermocellum* gene (*cipA*) encoding the cellulosomal S_L-protein reveals an unusual degree of internal homology. Mol Microbiol 8:325–334
39. Gold ND, Martin VJJ (2007) Global view of the *Clostridium thermocellum* cellulosome revealed by quantitative proteomic analysis. J Bacteriol 189:6787–6795
40. Gorhmann K, Torget R, Himmel M (1985) Optimization of dilute acid pretreatment of biomass. Biotechnol Bioeng Symp 15:59–80
41. Gray KA, Zhao L, Emptage M (2006) Bioethanol. Curr Opin Chem Biol 10:141–146
42. Green D (2005) Spinning straw into fuel. Biocycle 46:61–65
43. Greene N, Roth R (2006) Ethanol: energy well spent. A review of corn and cellulosic energy balances in the scientific literature to date. Ind Biotechnol 2:36–39
44. Greene N et al (2004) Growing energy. How biofuels can help end America's oil dependence. Natl Res Def Council Rept, New York, pp 1–94. (http://www.bio.org/ind/GrowingEnergy.pdf)
45. Guimaraes BG, Soucho H, Lytle BL, Wu JHD, Alzari PM (2002) The crystal structure and catalytic mechanism of the cellobiohydrolase CelS, the major enzymatic component of the *Clostridium thermocellum* cellulosome. J Mol Biol 320:578–596
46. Hahn-Hägerdahl B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund M (2007) Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol 74:937–953
47. Hahn-Hägerdahl B, Pamment N (2004) Special session A: microbial pentose metabolism. Appl Biochem Biotechnol 113–116:1207–1209
48. Herrero AA, Gomez RF, Roberts MF (1985) ^{35}P-NMR studies of *Clostridium thermocellum.* Mechanism of end product inhibition by ethanol. J Biol Chem 260:7442–7451
49. Hess G (2006) Bush promotes alternative fuel. Chem Eng News 84(10):50–56
50. Hess G (2006) Push for biofuels seen in farm bill. Chem Eng News 84(21):29–31
51. Hon-nami K, Coughlan MP, Hon-nami H, Ljungdahl LG (1986) Separation and characterization of the complexes constituting the cellulolytic enzyme system of *Clostridium themocellum.* Arch Microbiol 145:13–19
52. Irwin DC, Zhang S, Wilson DB (2000) Cloning, expression and characterization of a family 48 exocellulase, Cel48a, from *Thermobifida fusca*. Eur J Biochem 267:4988–4997
53. Jeffries TW (2005) Ethanol fermentation on the move. Nat Biotechnol 23:40–41
54. Jeffries TW et al (2007) Genome sequence of the lignocellulose-biocoverting and xylose-fermenting yeast *Pichia stipitis*. Nat Biotechnol 25:319–326
55. Johnson EA (1983) Regulation of cellulase activity and synthesis in *Clostridium thermocellum.* Ph.D. thesis, Massachusetts Institute of Technology, Cambridge
56. Johnson J (2006) Beyond corn: biomass to bioenergy. Chem Eng News 84(35):16
57. Kalscheuer R, Stölting T, Steinbüchel A (2006) Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152:2529–2536
58. Keasling JD, Chou H (2008) Metabolic engineering delivers next-generation biofuels. Nat Biotechnol 26:298–299
59. Klapatch TR, Demain AL, Lynd LR (1996) Restriction endonuclease activity in *Clostridium thermocellum* and *Clostridium thermosaccharoyuticum*. Appl Microbiol Biotechnol 45:127–131
60. Klapatch TR, Guerinot ML, Lynd LR (1996) Electrotransformation of *Clostridium thermosaccharoyuticum*. J Ind Microbiol 16:342–347
61. Kong Q-X, Gu J-G, Cao L-M, Zhang A-L, Chen X, Zhao X-M (2006) Improved production of ethanol by deleting *FPS1* and over-expressing *GLT1* in *Saccharomyces cerevisiae*. Biotechnol Lett 28:2033–2038
62. Kong Q-X, Cao L-M, Zhang A-L, Chen X (2007) Overexpressing *GLT1* in *gpd1* Δ mutant to improve the production of ethanol of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 73:1382–1386
63. Kruus K, Lua AC, Demain AL, Wu JHD (1995) The anchorage function of CipA (celL), a scaffolding protein of the *Clostridium thermocellum* cellulosome. Proc Natl Acad Sci USA 92:924–9258
64. Kruus K, Andreacchi A, Wang WK, Wu JHD (1995) Product inhibition of the recombinant CelS, an exoglucanase component of the *Clostridium thermocellum* cellulosome. Appl Microbiol Biotechnol 44:399–404
65. Lamed R, Bayer EA (1986) Contact and cellulolysis in *Clostridium themocellum* via extensile surface organelles. Experientia 42:72–73
66. Lamed R, Bayer EA (1988) The cellulosome of *Clostridium thermocellum*. Adv Appl Microbiol 33:1–46
67. Lamed R, Zeikus JG (1980) Ethanol production by thermophilic bacteria: relationship between fermentation product yields and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. J Bacteriol 144:569–578
68. Lamed R, Setter E, Bayer EA (1983) Characterization of a cellulose-binding, cellulase containing complex in *Clostridium thermocellum*. J Bacteriol 156:828–836
69. Lamed R, Setter E, Kenig R, Bayer AE (1983) The cellulosome—a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose binding and various cellulolytic activities. Biotechnol Bioeng Symp 13:163–181
70. Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642
71. Lynd LR (1996) Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment and policy. Annu Rev Energy Environ 21:403–465

72. Lynd LR (1989) Production of ethanol from lignocellulosic material using thermophilic bacteria: critical evaluation of potential and review. Adv Biochem Eng Biotechnol 38:1–52
73. Lynd LR, Cushman JH, Nichols RJ, Wyman CE (1991) Fuel ethanol from cellulosic biomass. Science 251:1318–1323
74. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577
75. Lynd LR, Jin J, Michels JG, Wyman CE, Dale BE (2003) Bioenergy: background, potential, and policy. A policy briefing prepared for the Center for Strategic and International Studies Washington, DC. (http://i-farmtools.org/ref/Lynd_et_al_2002.pdf)
76. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583
77. Marchetti JM, Miguel VU, Errazu AF (2007) Possible methods for biodiesel production. Renew Sustain Energy Rev 11:1300–1311
78. Mayer F, Coughlan KP, Mori Y, Ljungdahl LG (1987) Macromolecular organization of the cellulolytic complex of *Clostridium thermocellum* as revealed by electron microscopy. Appl Environ Microbiol 53:2785–2792
79. Meilenz JR (2001) Ethanol production from biomass: technology and commercialization status. Curr Opin Microbiol 4:324–329
80. Mondruzzaman MM, Dien BS, Ferrer B, Hespell RB, Dale BE, Ingram LO, Bothast RJ (1996) Ethanol production from AFEX pretreated corn fiber by recombinant bacteria. Biotechnol Lett 18:985–990
81. Morag E, Bayer EA, Hazlewood GP, Gilbert HJ, Lamed R (1993) Cellulase Ss (Cel S) is synonymous with the major cellobiohydrolase (subunit S8) from the cellulosome of *Clostridium thermocellum*. Appl Biochem Biotechnol 43:147–151
82. Moriera N (2005) Growing expectations. New technology could turn fuel into a bumper crop. Sci News (Online) 168(14):216–218
83. Newcomb M, Wu JHD (2007) Induction of the *Clostridium thermocellum* CelC operon by laminaribiose. Proc Natl Acad Sci USA 104:3747–3752
84. Nigam JN (2001) Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. J Biotechnol 87:17–27
85. Nissen TL, Kielland-Brandt MC, Nielsen J, Villadsen J (2000) Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. Metab Eng 2:69–77
86. Pasha C, Kuhad RC, Rao CV (2007) Strain improvement of thermotolerant *Saccharomyces cerevisiae* VS3 strain for better utilization of lignocellulosic substrates. J Appl Microbiol 103:1480–1489
87. Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes J, Erbach DC (2005) Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. Oak Ridge Natl Lab, pp 1–78. (http://feedstockreview.ornl.gov/pdf/billion_ton_visio.pdf)
88. Pessoa A Jr, Roberto IC, Menossi M, Dos Santos RR, Filho SO, Penna TCV (2005) Perspectives on bioenergy and biotechnology in Brazil. Appl Biochem Biotechnol 121–124:59–70
89. Philippidis G, Smith TK, Wyman CE (1993) Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by simultaneous saccharification and fermentation process. Biotechnol Bioeng 41:846–853
90. Ragauskas AJ, Nagy M, Kim DH, Eckert CA, Hallett JP, Liotta CL (2006) From wood to fuels: integrating biofuels and pulp production. Ind Biotechnol 2:55–65
91. Ratledge C (1979) Resources conservation by novel biological processes I. Grow fats from wastes. Chem Soc Rev 8:283–296
92. Ratledge C (1982) Single cell oil. Enzyme Microb Technol 4:58–60
93. Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–51
94. Saha BC, Bothast RJ (1999) Pretreatment and enzymatic saccharification of corn fiber. Appl Biochem Biotechnol 76:65–77
95. Sanderson KW (2007) Are ethanol and other biofuels technologies part of the answer for energy independence? Cereal Food World 52:5–7
96. Schmer MR, Vogel KP, Mitchell RB, Perrin RK (2008) Net energy of cellulosic ethanol from switchgrass. Proc Natl Acad Sci USA 105:464–469
97. Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, Hogsett DA, Lynd LR (2008) Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc Natl Acad Sci USA 105:13769–13774
98. Shoham Y, Lamed R, Bayer EA (1999) The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol 7:275–281
99. Soetoert W, Vandamme E (2006) The impact of industrial biotechnology. Biotechnol J 1:756–769
100. South CR, Hogsett DA, Lynd LR (1993) Continuous fermentation of cellulosic biomass to ethanol. Appl Biochem Biotechnol 39–40:587–600
101. Sticklen M (2006) Plant genetic engineering to improve biomass characteristics for biofuels. Curr Opin Biotechnol 17:315–319
102. Tollefson J (2008) Energy: not your father's biofuels. Nature 45:880–883
103. Tyurin M, Desai SG, Lynd LR (2004) Electrotransformation studies in *Clostridium thermocellum*. Appl Environ Microbiol 70:883–890
104. Tyurin MV, Lynd LR, Weigel J (2006) Gene transfer systems for obligately anaerobic thermophilic bacteria. Methods Microbiol 35:309–330
105. Uversky V, Kataeva IA (eds) (2006) Cellulosome. Nova Sci Publ Inc., New York
106. Wall JD, Harwood CS, Demain A (eds) (2008) Bioenergy. ASM Press, Washington
107. Wang WK, Wu JHD (1993) Structural features of the *Clostridium thermocellum* cellulase Ss gene. Appl Biochem Biotechnol 39–40:149–158
108. Wang WK, Kruus K, Wu JHD (1993) Cloning and DNA sequence of the gene coding for *Clostridium thermocellum* cellulase Ss (CelS), a major cellulosome component. J Bacteriol 175:1293–1302
109. Weil J, Westgate P, Kohlmann K, Ladisch MR (1994) Cellulase pretreatments of lignocellulosic substrates. Enzyme Microb Technol 16:1002–1004
110. Wirth T, Gray CB, Podesta JD (2003) The future of energy policy. Foreign Aff 82:132–155
111. Wu JHD (1993) *Clostridium thermocellum* cellulosome: new mechanistic concept for cellulose degradation. In: Himmel M, Georgiou G (eds) Biocatalyst design for stability and specificity. American Chemical Society, Washington, DC, pp 251–264
112. Wu JHD, Demain AL (1988) Proteins of the *Clostridium thermocellum* complex responsible for degradation of crystalline cellulose. In: Aubert JP, Beguin P, Millet J (eds) Biochemistry and genetics of cellulose degradation. Academic Press, New York, pp 117–131
113. Wu JHD, Orme-Johnson WH, Demain AL (1988) Two components of an extracellular protein aggregate of *Clostridium thermocellum* together degrade crystalline cellulose. Biochemistry 27:1703–1709
114. Wyman CE (1994) Alternative fuels from biomass and their impact on carbon dioxide accumulation. Appl Biochem Biotechnol 45–46:897–915

115. Wyman CE, Hinman ND (1990) Ethanol. Fundamentals of production from renewable feedstocks and use as a transportation fuel. Appl Biochem Biotechnol 24–25:735–753
116. Wyman CE, Dale BD, Elander RT, Hotzapple M, Ladisch MR, Lee YY (2005) Coordinated development of leading biomass pretreatment technologies. Bioresour Technol 96:1959–1966
117. Yamano LP, York SW, Ingram LO (1998) Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J Ind Microbiol Biotechnol 20:132–138
118. Yazdani SS, Gonzalez R (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 18:213–219
119. Yomano LP, York SW, Zhou S, Shanmugam KT, Ingram LO (2008) Re-engineering *Escherichia coli* for ethanol production. Biotechnol Lett 30:2097–2103
120. Zhang Y-HP, Lynd LR (2004) Kinetics and relative importance of phosphorylytic and hydrolytic cleavage of cellodextrins and cellobiose in cell extracts of *Clostridium thermocellum*. Appl Environ Microbiol 70:1563–1569
121. Zverlov W, Kellermann J, Schwarz WH (2005) Functional subgenomics of *Clostridium thermocellum* cellulosomal genes: identification of the major catalytic components in the extracellular complex and detection of three new enzymes. Proteomics 5:3646–3653
122. Zverlov VV, Schantz N, Schmitt-Kopplin P, Schwarz WH (2005) Two new major subunits in the cellulosome of *Clostridium thermocellum*: xyloglucanase Xgh74A and endoxylanase Xyn10D. Microbiology 151:3395–3401