Review

Thermophilic ethanologenesis: future prospects for second-generation bioethanol production

Mark P. Taylor[1,2], Kirsten L. Eley[1], Steve Martin[1], Marla I. Tuffin[2], Stephanie G. Burton[3] and Donald A. Cowan[2]

[1] TMO Renewables Ltd, Guildford, Surrey, GU2 7YF, UK
[2] Institute for Microbial Biotechnology and Metagenomics (IMBM), University of the Western Cape, Cape Town, 7535, South Africa
[3] Department of Chemical Engineering, University of Cape Town, Cape Town, 7011, South Africa

Strategies for improving fermentative ethanol production have focused almost exclusively on the development of processes based on the utilization of the carbohydrate fraction of lignocellulosic material. These so-called 'second-generation' technologies require metabolically engineered production strains that possess a high degree of catabolic versatility and are homoethanologenic. It has been suggested that the production of ethanol at higher temperatures would facilitate process design, and as a result the engineered progeny of *Geobacillus thermoglucosidasius*, *Thermoanerobacterium saccharolyticum* and *Thermoanerobacter mathranii* now form the platform technology of several new biotechnology companies. This review highlights the milestones in the development of these production strains, with particular focus on the development of reliable methods for cell competency, gene deletion or upregulation.

Introduction

Climate change, dramatic fluctuations in the cost of oil and price increases for basic foodstuffs have together focused political and scientific attention on the pursuit of renewable alternatives to oil-based fuels [1]. There is particular interest in biofuels that are derived from renewable materials, typically grains and sugar cane for bioethanol and plant-derived oils for biodiesel. These products are either blended with, or replace, conventional fuels such as gasoline and diesel and present an opportunity to tackle key environmental issues, such as the high levels of greenhouse gas (GHG) emissions from transport. These emissions have recently been estimated by the Intergovernmental Panel on Climate Change (IPCC) to constitute 13% of all GHG emissions and more than 50% of all CO_2 emissions (IPCC Climate Change Report 2007, http://www.ipcc.ch/pdf/assessment-report/ar4/syr/ar4_syr_spm.pdf).

According to the Renewable Fuels Association (RFA), in 2007 the global production of ethanol was 13 billion US gallons (2007 World Fuel Ethanol Production, http://www.ethanolrfa.org/industry/statistics/), 85% of which was produced in the USA and Brazil. The technology used to produce this ethanol, referred to as 'first-generation bioethanol', is relatively mature and based upon traditional brewing techniques involving the fermentation of sugars, such as sucrose and glucose, by variants of the yeast *Saccharomyces cerevisiae*. In this organism, the key ethanol production steps involve the enzymes pyruvate decarboxylase and alcohol dehydrogenase, which are encoded by the *pdc* and *adh* genes, respectively. In the USA, the primary feedstock for ethanol production has been the processed starch fraction of yellow corn (maize), whereas sucrose derived from sugar cane is used in Brazil.

First-generation bioethanol is well established; recent 'green' legislation in both the USA and EU (see below) suggests that it will play a significant part in reducing gasoline use in transport fuels for the foreseeable future. It is clear from a historic perspective in the USA that fuel ethanol production is increasing exponentially (Figure 1) and that in recent years ethanol imports have increased to fill production deficiencies. In 2005, the Energy Policy Act was signed into law in the USA, establishing the Renewable Fuels Standard (RFS), which proposed the use of 7.5 billion gallons of ethanol by 2012 [2]. This target was revised in December 2007 by the Energy Independence and Security Act (EISA), which sets the new target of 36 billion gallons of biofuels by 2025, whereby the contribution from corn ethanol is to be capped at 15 billion gallons. The remaining 21 billion gallons will come from advanced, so-called 'second-generation' technologies, such as cellulosic ethanol, that exhibit significant GHG savings and improvements in other sustainability criteria. Within the European Union, Directive 2003/30/EC specifies that 5.75% of overall transport fuel (by energy content) is to come from renewable sources by 2010, rising to 10% by 2020 [3]. Similar initiatives have been implemented in Canada, Thailand, Argentina, India and South Africa [4].

Although there is no doubt that biofuels are 'renewable', in that the feedstock can be re-grown, their potential to reduce GHG emissions, to provide energy security by reducing dependence upon foreign oil and to help rural development is dependent upon the type of biofuel being produced and the carbohydrate source. Criticisms have been raised concerning the marginal net energy values and GHG savings of corn ethanol [5], the potential diversion of food and fodder-grade feed stocks from the food chain into fuel production, also referred to as the 'food versus fuel' debate [6,7], and the indirect effects of biofuel

Corresponding author: Cowan, D.A. (dcowan@uwc.ac.za).

0167-7799/$ – see front matter doi:10.1016/j.tibtech.2009.03.006 Available online 28 May 2009

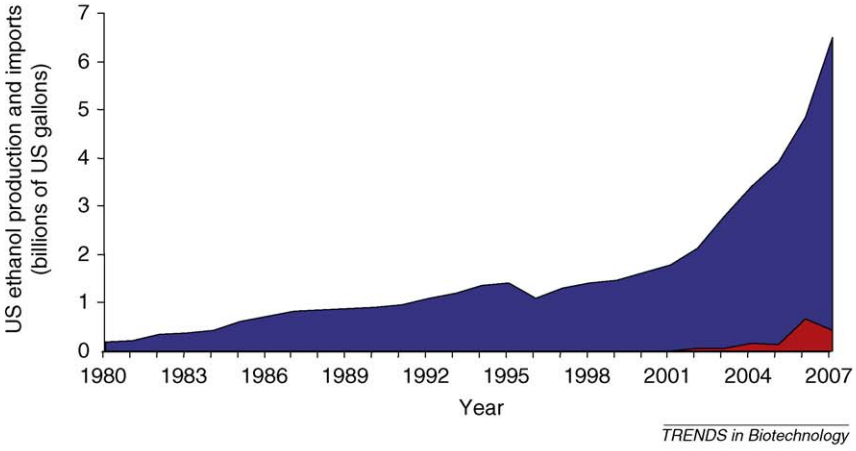

Figure 1. The production of fuel ethanol in the USA between 2002 and 2007. The area shown in blue is the bioethanol production by the US, whereas the area in red indicates recent imports. Data from http://www.ethanolrfa.org/industry/statistics/.

production on land use [8]. It is clear that if biofuels are to deliver on their promise of environmental benefits, they must meet rigorous sustainability criteria and utilize best practice, taking both direct and indirect environmental and socio-political effects into account.

In response, principally to these concerns, current research has sought to develop novel ethanologenic strains with broader catabolic properties. It is anticipated that these strains will ferment sugars derived from the hydrolysis of the hemicellulosic fraction of biomass, thus conserving food-grade carbohydrate supplies for human and animal consumption [9]. In this review, we briefly discuss the concept of second-generation ethanol (Box 1) and current mesophilic-based technologies and then highlight the advances in the development of thermophilic genetic systems, mainly in the catabolically versatile hosts *Geobacillus thermoglucosidasius*, *Thermoanerobacter mathranii* and *Thermoanerobacterium saccarolyticum*, which are considered as viable candidates for second-generation processes.

Box 1. What is second-generation bioethanol?

Although conventional ethanol production relies on food crops, such as maize and sugar cane, fermentable carbohydrates are also found in more recalcitrant organic materials, such as bagasse, straw, corn stover, wood waste and municipal and agricultural waste. The production of ethanol from these materials forms the basis of what are commonly known as second-generation biofuel technologies [75]. Although first-generation bioethanol probably will continue to dominate the biofuels markets in the short and medium term, it is clear that various government mandates are driving the development and commercialization of second-generation ethanol, anticipating that these processes will initially complement existing technologies and then perhaps become dominant over the next 10 to 20 years. A recent joint study by the US Department of Agriculture and the US Department of Energy has shown that more than 1.3 billion tons of agricultural and forestry waste biomass could be made available annually in the USA for conversion into biofuels, which would be enough to displace more than 30% of US transportation fuel requirements [76].

A particular challenge in the development of a viable second generation process is the natural resistance of biomass to degradation, the so-called biomass recalcitrance. Effective processing to yield a fermentable carbohydrate stream will undoubtedly generate a high proportion of pentose sugar polymers that cannot be fermented by conventional ethanolgenic yeasts. This, in part, has been the motivation for engineering catabolic versatility in conventional strains and improving ethanol yields from unconventional strains, such as the thermophiles discussed in this review. A more detailed description of the current barriers to commercialization, as well as a proposed research strategy to overcome these, have been described in detail in a report by the US Department of Energy [77].

Life at high temperatures can lead to process advantages

Several diverse organisms are capable of converting sugars to ethanol, the most widely known being *Saccharomyces cerevisiae* and *Zymomonas mobilis*, both of which lack the metabolic capacity to ferment non-C6 sugars. However, the catabolic versatility of *S. cerevisiae* and *Z. mobilis* could be improved by the functional expression of a variety of foreign genes that are associated with D-xylose and L-arabinose assimilation and catabolism [10–12]. A different approach in hosts with a broad catabolic phenotype has been to express of the *pdc* and *adh* genes from *Z. mobilis*, essentially creating an Entner–Doudoroff type metabolism. Host strains that were engineered in this manner include *Escherichia coli* and *Klebsiella oxytoca* [13], and significant success has been reported in diverting carbon flux from glucose, through glycolysis and towards ethanol synthesis (reviewed in Ref. [9]). However, an industrially viable engineered mesophilic microbial strain, which would be suitable for large-scale ethanol production from hydrolysates as part of a second-generation process, has yet to be developed. Possibly the most interesting alternative approach to generating a mesophilic host would be to use the three thermophilic bacteria that are amenable to metabolic engineering, namely *G. thermoglucosidasius*, *T. mathranii* and *T. saccharolyticum*, because they possess catabolic flexibility, enhanced ethanol synthesis capacity and other physiological characteristics that together would benefit an industrial bioprocess.

In general, an ethanologenic process based on thermophiles has several distinct advantages over a mesophilic process. Thermophiles are commonly able to readily ferment not only the pentose and hexose sugar fraction of biomass but also hydrolysate materials [14] and even, in some cases, polymeric precursors or structurally complex polycarbohydrates, such as cellulose [15]. In addition, their

remarkable ability to tolerate fluctuations in pH, temperature and environmental change [16–18] is a clear advantage because these criteria are often cited as crucial in the development of a commercially viable process [9,14]. The use of high temperatures will also facilitate downstream product recovery, because aqueous ethanol will readily vaporize at temperatures over 50°C [16], potentially allowing ethanol removal and recovery to be achieved by applying only a mild vacuum, which will facilitate continuous distillation or 'stripping' of ethanol as opposed to conventional distillation. Steam stripping in this manner could be coupled to vapour compression and permeation membrane separation of ethanol, which could provide further savings in the energy required for product recovery [19].

Currently, microbial contamination is a significant problem in yeast-based bioethanol production because it reduces ethanol yields and potentially compromises the viability of the ethanologen [20]. The main contaminating species are lactobacilli, which compete for available carbohydrates and require the addition of antibiotics to control their growth [21]. This situation is far from ideal from a cost perspective [22] and could be avoided with the use of a thermophilic production strain that is grown at a temperature higher than the maximum permissible growth temperature of the common feedstock-derived contaminants. In addition, gas solubility is significantly lower at 65°C compared with 37°C, which not only helps to maintain the near anaerobic environment, favouring a fermentative process, but also minimizes the growth of obligatory aerobic contaminants. Thermophilic strains can furthermore improve process economics by helping to reduce energy input, which is required to cool mesophilic fermentations between the pre-treatment of feedstock and the post-fermentative distillation process. A thermophilic-based process could maintain temperatures above 50°C throughout the fermentation phase.

However, the production of high ethanol yields (>90% theoretical) and high ethanol tolerance (>40 gL^{-1}) are typically lacking in thermophilic ethanologens [9,14]. In addition, low product yield is often a result of mixed acid fermentation, which reduces the yield of the metabolite of interest (illustrated in Figures 2 and 3). Solvent tolerance to ethanol is typically low in thermophilic ethanologens [23–25] but can be enhanced through genetic modification. For example, engineered strains of *Thermoanaerobacter ethanolicus* that are able to survive and replicate in up to 8% v/v ethanol have been developed [26]. Ethanol tolerance (but presumably not active growth) as high as 10% v/v has been reported in *Geobacillus thermoglucosidasius* M10EXG [27] and 15% v/v in *Anoxybacillus* sp. WP06 [28]. At the time of writing, we are unaware of any reports of thermophilic ethanologens capable of active growth in 12–14% v/v ethanol, which is the 'normal' range for the mesophilic ethanologens *Z. mobilis* and *S. cerevisiae*. For more detailed information on the mechanisms of ethanol tolerance, see [29] and [30].

The major obstacle to addressing these issues has historically been the paucity of suitable genetic systems

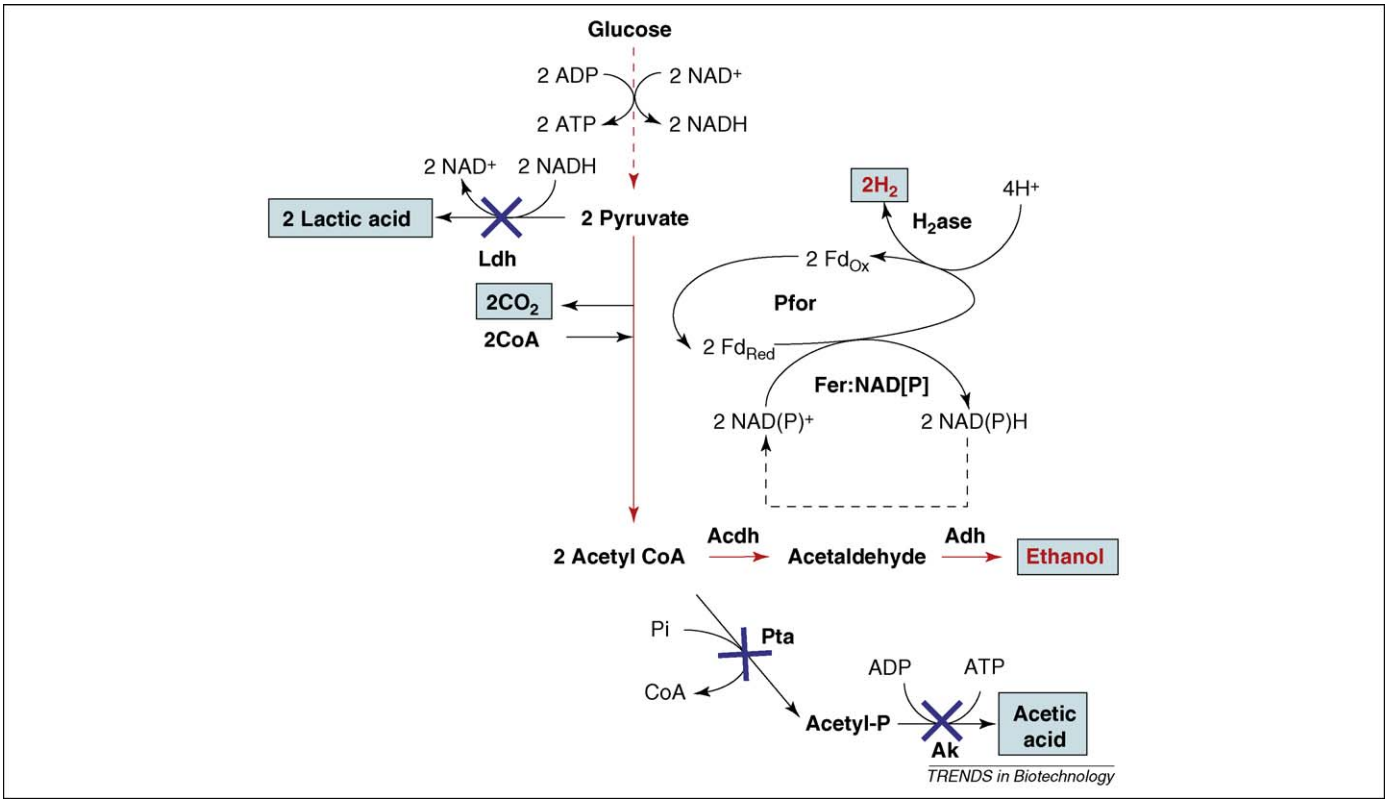

Figure 2. A summary of fermentative metabolism in *Thermoanaerobacterium* spp. Enzyme abbreviations: Acdh, acetaldehyde dehydrogenase; Adh, alcohol dehydrogenase; Ak, acetate kinase; Fer:NAD[P], ferredoxin:NAD(P) oxidoreductase; H_2ase, hydrogenase; Ldh, lactate dehydrogenase; Pfor, pyruvate:ferredoxin oxidoreductase; Pta, phosphotransacetylase. Blue crosses indicate metabolic steps that have been targeted by gene knockout. Red arrows highlight the route of increased metabolic flux as a consequence of these knockouts. The fermentative end products of the mutant strains are shown in red. The dashed red arrow summarizes several metabolic steps of glycolysis not shown in detail in this schematic. The reaction indicated by the dashed black arrow denotes NAD(P)H recycling to NAD(P). Reproduced, with permission, from Ref. [54]: Copyright (2008), National Academy of Sciences, USA.

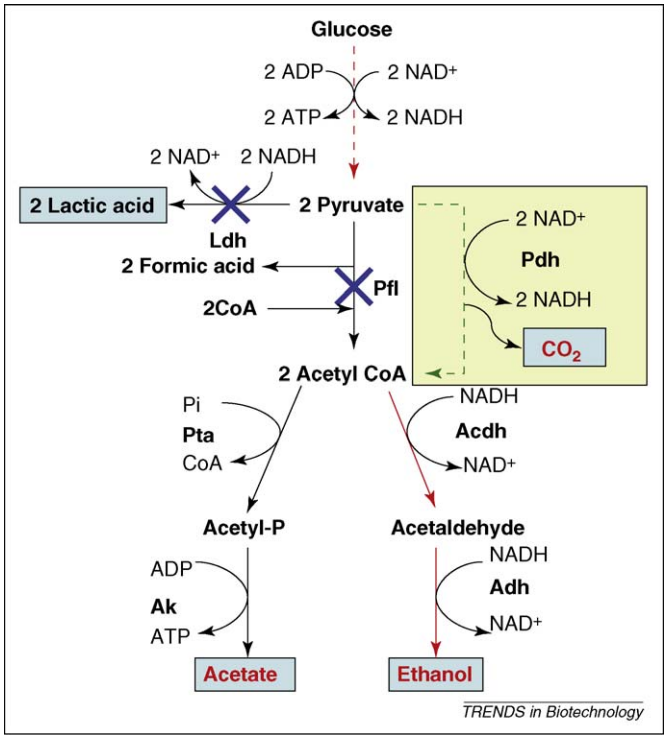

Figure 3. A summary of fermentative metabolism typical of *Geobacillus* spp.. Enzyme abbreviations: Acdh, acetaldehyde dehydrogenase; Adh, alcohol dehydrogenase; Ak, acetate kinase; Ldh, lactate dehydrogenase; Pdh, pyruvate dehydrogenase; Pfl, pyruvate-formate lyase; Pta, phosphotransacetylase. Blue crosses indicate genes that have been knocked out to increase ethanol yields. Red arrows highlight the route of increased metabolic flux as a consequence of these knockouts. The fermentative end products of the mutant strains are shown in red. The dashed red arrow summarizes several metabolic steps of glycolysis not shown in this schematic. The dashed green arrow illustrates the metabolic activity of the native Pdh, which is usually only active aerobically. The *pdh* promoter has been replaced with an *ldh* promoter from a *Geobacillus* sp., inducing activity of Pdh fermentatively.

and molecular tools that would assist and enable a thermophilic strain development programme. However, several recent novel techniques and tools have resulted in the development of engineered thermophilic ethanologenic strains, the best yields of which, on a variety of sugars, are summarized and compared in Table 1, and in turn this has led to the establishment of several new biotechnology companies exploiting thermophiles.

Thermophilic *Clostridia*

Thermophilic *Clostridia*, fermentative anaerobes growing optimally at 60–65°C, have been of interest as potential producers of ethanol (and other solvents) for several decades [25,31–33]. Their principal advantage is their ability to degrade and ferment crystalline cellulose via the utilization of several endo-β-glucanases, exoglucanases, cellobiose phosphorylases, cellodextrin phosphorylases and β-glucosidases [24]. These enzymes are often found in the cell in a structure termed the cellulosome, a multienzyme cellulose-degrading complex located and embedded on the external surface of the cell membrane. Two species of *Clostridia*, *Clostridia thermocellum* and *Clostridia thermohydrosulfuricum,* have attracted most attention, and the cellulosome of *C. thermocellum* has been extensively characterized (reviewed in [24]).

A wide substrate range is a distinct advantage for any second-generation bioethanol process, and thus considerable research has focused on investigating and improving the substrate profiles of thermophilic *Clostridia*. For example, *C. thermocellum* ATCC 27405 and a strain of *C. thermohydrosulfuricum* have been reported to be able to ferment cellulose, cellobiose and a range of other carbohydrates, but the extent of substrate consumption and specificity varied considerably between different strains and in different culture media [33,34]. *C. thermocellum* (JW20) also has a broad substrate specificity and was able to grow on cellulose, cellobiose and xylooligomers, as well as, after adaptation, on glucose, fructose and xylose [35].

In an attempt to maximize the utilization of carbon biomass, *C. thermocellum* has been co-cultured with other thermophilic strains, including *Clostridia thermosaccharolyticum* [24], *C. thermohydrosulfuricum* [36], *T. ethanolicus* [24], *Geobacillus stearothermophilus* [37] and *Thermoanaerobacter brockii* [24,32]. In all instances, co-cultures showed

Table 1. Ethanol yields of thermophilic strains compared with current best strains of *S. cerevisiae*

Production strain	Genotype	Fermentation substrate	Yield of ethanol (gg^{-1})[a]	Refs
Thermoanaerobacter mathranii BG1L1 (Biogasol)	Δ*ldh*	Mixed sugars	0.39–0.42[b]	[59,60]
T. mathranii BG1L1	Δ*ldh*	Mixed sugars (predominately D-xylose)	0.39–0.42[c]	[59,60]
Thermoanaerobacterium saccharolyticum JW/SL-YS485 (Mascoma)	Δ*ldh* Δ*ak* Δ*pta*	Mixed sugars (glucose, xylose, galactose and mannose)	0.38[d]	[54]
Geobacillus thermoglucosidasius TM242 (TMO Renewables Ltd)	Δ*ldh* Δ*pfl* $pdh_{upregulated}$	D-glucose	0.41–0.44[e]	[f]
G. thermoglucosidasius TM242	Δ*ldh* $pdh_{upregulated}$	D-xylose and D-glucose	0.44[e]	[f]
G. thermoglucosidasius TM242	Δ*ldh* $pdh_{upregulated}$	D-xylose, D-glucose and L-arabinose	0.41[e]	[f]
Saccharomyces cerevisiae strain IMS0010	Expression of xylose isomerase (XI) and L-arabinose assimilation genes.	D-glucose, D-xylose and L-arabinose	0.43	[73]
S. cerevisiae	L-arabinose assimilation genes from *Lactobacillus plantarum*	L-arabinose	0.43	[74]

[a]Grams of ethanol produced per gram of sugar consumed.
[b]Yield on mixed sugars from a wheat straw hydrolysate. Media supplemented with yeast extract, vitamins and trace elements.
[c]Yield on mixed sugars from a corn stover hydrolysate. Media supplemented with yeast extract, vitamins and trace elements.
[d]Maximum yield on mixed pure sugars from a fed batch fermentation (50 gL^{-1} total sugars loaded and 3 gL^{-1} of sugars fed over 16 h). Media supplemented with yeast extract.
[e]Yield data from batch fermentation in yeast extract supplemented medium.
[f]Eley, K. *et al.* (2008) The development of TM242: a novel thermophilic Bacillus capable of high yield ethanol production. Presented at *Symposium on Biotechnology for Fuels and Chemicals, Society for Industrial Microbiology: 2008 May 4–7; New Orleans*. Abstract found at http://sim.confex.com/sim/30th/techprogram/P5090.HTM.

higher ethanol yields than either strain grown independently, with yields as high as 1.8 mol of ethanol per mol of anhydrous glucose unit of cellulose reported in a co-fermentation of *C. thermocellum* strain LQRI and *T. ethanolicus* 39E [32]. *C. thermocellum* has been reported to be able to degrade both acid pre-treated hardwood and Avicel© (crystalline cellulose) [38], and a 90% conversion of substrate to ethanol was achieved in continuous culture.

Within the thermophilic *Clostridia*, central carbon metabolism typically consists of glycolysis and the pyruvate-ferredoxin oxidoreductase-mediated conversion of pyruvate to acetyl coenzyme A (CoA) (similar to the scheme shown in Figure 2). Fermentative end products include lactate, acetate and ethanol [24]. Many mesophilic *Clostridium* species are known to produce acetone, butanol, ethanol (so-called ABE fermentation) and, under some conditions, hydrogen, but we are not aware of any references to thermophilic *Clostridia* producing butanol or acetone. Fermentative thermophiles often produce a range of 'by-products', of which lactate is frequently the most significant. This has prompted a range of mutational studies aimed at eliminating lactate production to increase carbon flux to ethanol. Novel mutants of *C. thermocellum* have been developed using UV mutation and post-mutational selection of fluoropyruvate-resistant colonies as an indicator for the disruption of lactate dehydrogenase (Ldh), as this enzyme converts fluropyruvate to the cytotoxic compound fluorolactate. The resulting mutant *C. thermocellum* strains were capable of producing ~12.5 gL^{-1} ethanol at pH 6.5 compared with only 5 gL^{-1} produced by the parent strain [39]. The same mutants also demonstrated increased ethanol tolerance of up to 4% v/v [40]. Lactate production could also be reduced by medium development [41].

Several recent reports have described the development of new genetic tools for engineering thermophilic *Clostridium* species. The successful electrotransformation (10^5 transformants per μg of plasmid DNA) of *C. thermocellum* [24] suggests that this approach could form the basis of a gene transfer method suitable for gene knockout or gene expression studies [24,42]. Recently, a reliable gene transfer system for *C. thermocellum* [43] has been used to delete the *ldh* and acetate kinase/phosphotransacetylase genes (*ak*/*pta*) in a related strain, *Thermoanerobacterium saccharolyticum*, resulting in reduced production of lactate and acetate [43]. The reader is referred to Ref. [29] for a comprehensive review of the work undertaken in the characterization and development of *Clostridium* species for bioethanol production.

Two significant hurdles to the development of industrially viable *Clostridium* species currently remain: (i) the inability of *C. thermocellum* to consistently ferment pentose sugars [24,33] and (ii) the inhibition of cell growth by relatively low concentrations of ethanol [34,35,44]. An ethanol-tolerant variant of *C. thermocellum* (strain C9) able to grow in ethanol concentrations up to 2.5% v/v ethanol [44] was a marked improvement over the wild type, which could only tolerate 0.5% v/v. It has been suggested that ethanol tolerance in strain C9 was linked to increases in normal and anteisobranched fatty acids in the cell membrane (at the expense of isobranched fatty acids), giving the cell membrane a lower T_m and increased fluidity [45]. It has also been suggested that ethanol intolerance might be a growth response aimed at moderating ethanol production [46,47].

Thermoanaerobacter

Species of the genus *Thermoanaerobacter,* once classified as members of the genus *Clostridium*, are physiologically similar to the thermophilic Clostridia. They have been reported to possess an Embden–Meyerhof glycolytic metabolism with measurable activities of lactate dehydrogenase, acetaldehyde dehydrogenase, hydrogenase, alcohol dehydrogenase, acetate kinase, ferredoxin-linked pyruvate dehydrogenase and pyridine nucleotide oxidoreductases [31,48]. The pathways that are associated with ethanol and fermentative end product formation are shown in Figure 2. Metabolic analysis of *Thermoanaerobacter* fermentations suggests that they are mainly ethanol and lactate producers [31] and have a moderately broad substrate range; for example, *T. ethanolicus* is able to ferment both D-glucose and D-xylose to ethanol [49].

Both ethanol tolerance and production in *T. ethanolicus* have been shown to be closely linked to the presence and function of alcohol dehydrogenase [26,50,51]. The primary (*adhA)* and secondary (*adhB*) alcohol dehydrogenase genes have been cloned, expressed and characterized, and studies have suggested that AdhA functions primarily in ethanol consumption, whereas AdhB mediates ethanol production. AdhA has also been implicated in ethanol tolerance; an Δ*adhA* strain was isolated that showed markedly improved tolerance compared with that of the wild type [50,52].

An early example of successful site-directed gene disruption in *T. saccharolyticum* resulted in the diversion of metabolic carbon flux from lactate to ethanol [53]. The Δ*ldh* mutation was reported to be stable and the mutant strain produced no lactic acid but showed only marginally increased ethanol production compared with the wild-type strain [53]. With further genetic engineering approaches, the same research group has reported a variety of other mutant progeny with combinatorial disruptions in the loci of the acetate kinase (*ak*) and *ldh* genes (these knockouts are highlighted in Figure 2). A double mutant strain, ALK2, (Δ*ak*, Δ*ldh* and adapted to growth on D-xylose) possessed a stable homoethanologenic phenotype (>150 generations in continuous culture) and catabolic repression was apparently absent, enabling the strain to co-utilize D-xylose, D-glucose, mannose and L-arabinose, with maximum ethanol titres of 37 gL^{-1} [54]. The commercialization and development of this research is currently promulgated by the US-based company Mascoma (http://www.mascoma.com) [55].

Thermoanaerobacter mathranii BG1L1, isolated from a hot spring in Iceland, is currently being developed for application in biofuel production by the Danish company Biogasol (http://www.biogasol.com) [56]. A variant, *Thermoanaerobacter* A10, grows optimally at 70°C and tolerates ethanol concentrations of up to 4.7% v/v [57]. *Thermoanaerobacter* BG1L1 (a Δ*ldh* mutant) has been shown to be capable of consuming 42% of the xylose component of a lignocellulosic hydrolysate and has shown acquired adaptive alcohol tolerance after continuous exposure to ethanol [58]. BG1L1 has been reported to digest untreated corn stover hydrolysate (with a maximum

of 15% total solids) in a laboratory-scale fluidized bed reactor with optimum D-xylose conversion occurring at a 10% total solids load [59]. Under these conditions, between 0.39 and 0.42 g of ethanol were produced per gram of the total sugars consumed [59]. Similar ethanol yields have also been reported for the strain from the fermentation of a wheat straw hydrolysate containing predominately glucose and xylose [60].

Geobacillus spp

The genus Geobacillus, first proposed by Nazina *et al.* in 2001 [61], includes a wide range of thermophilic Bacilli with different physiologies. Some, such as *Geobacillus thermodenitrificans,* are dentrifiers, whereas others, such as *G. stearothermophilus,* have been shown to be mixed acid producers. *G. stearothermophilus* has been studied in some detail with respect to ethanol production, and initial reports predicted that this organism is capable of generating ethanol from sucrose at 70°C and producing yields comparable to those of yeast [62].

The full genomes of two *Geobacillus* species, *Geobacillus kaustophilus* [18] and *G. thermodenitrificans* [63], are available, as well as the partial sequence of *G. stearothermophilus* from the *Bacillus (Geobacillus) stearothermophilus* Genome Sequencing Project (http://www.genome.ou.edu/bstearo.html). All of the genes responsible for the Embden–Meyerhof glycolytic metabolism have been identified, together with those encoding lactate dehydrogenase, acetaldehyde dehydrogenase, alcohol dehydrogenase, acetate kinase and pyruvate dehydrogenase. The presence of this gene complement is consistent with the mixed acid production seen in many *Geobacillus* strains, but *G. stearothermophilus* is known to produce formate under fermentative conditions [16], and although no pyruvate formate lyase (*pfl*) gene has been identified in its partial genome sequence, this gene was suggested to be present in *G. thermoglucosidasius*[*]. Pyruvate formate lyase is therefore included in the summary of the metabolic pathways of *Geobacillus* (Figure 3).

G. stearothermophilus produces lactate, formate, acetate and ethanol from glucose [16]. The initial results of strain development and characterization were mixed; the successful isolation of *ldh*-deficient variants was reported [16], but these were subsequently shown not to have originated from NCA1503 [64] and to be unstable in continuous culture at high growth rates [65]. Absolute nutritional requirements have been defined both aerobically and anaerobically [64,66], and the ability to ferment the carbohydrate fraction of various biomass hydrolysate materials has been demonstrated [65,67].

The functional expression of the *pdc* gene from *Z. mobilis* in *G. thermoglucosidasius* has been reported [68], and it was shown to retain activity up to 52°C but not beyond 54°C. A thermostable shuttle vector has been used for the functional expression of the *Zymomonas palmae* Pdc in a Δ*ldh* strain of *G. thermoglucosidasius* [69] and serves as a platform for the further forced evolution of thermostable Pdc variant that is functional at higher temperatures. Recently, significant progress in the commercial development of enhanced ethanologenic *Geobacillus* spp. has been made by the UK-based company TMO Renewables Ltd (http://www.tmo-group.com) with the generation of genetically altered ethanologenic progeny of *G. thermoglucosidasius*, along with the development of genetic engineering methods for several selected *Geobacillus* strains [70–72]. Using these approaches, several mutant strains, incorporating gene deletions or upregulation events, have been developed. One particular strain (Δ*ldh*, Δ*pfl*, $pdh_{upregulated}$) was able to produce ethanol yields that approached the theoretical maximum on a variety of C5 and C6 sugars[*]. The combinatorial effects of these gene manipulations are further illustrated in Figure 3.

Conclusion

It is anticipated that the transition from first- to second-generation biofuel processes must involve a move from conventional wild-type production strains towards genetically engineered variants with superior catabolic properties and homoethanologenic production phenotypes. In addition, it is widely accepted that the preferred substrate (especially in developing economies) should be the fermentable carbohydrate fraction of hydrolysed biomass. The development of mesophilic organisms such as *E. coli* as ethanologens has benefited from an established range of genetic tools and a detailed molecular understanding gained from years of scientific research. A parallel research strategy in thermophiles has been hindered by a lack of the same degree of knowledge, which is likely to be why the development of these organisms has never been a main focus of established companies active in the bioenergy and biofuels field. Crucially, transformation, gene transfer and genomic integration systems are now emerging that will allow the potential of thermophilic microorganisms to be developed. The potential commercial viability of engineered thermophilic bacterial strains has been demonstrated by the formation of several new bioenergy-focused biotechnology companies that specialize in high-temperature production of ethanol. Many relevant process issues remain, not least the selection or development of ethanol-tolerant variants of the currently available strains. In addition, the ability to reproduce ethanol yield results in the presence of pretreatment fermentation inhibitors such as acetate will also be crucial, as will addressing any catabolic repression issues that might hinder mixed sugar consumption. However, equipped with suitable genetic methods and an increasing understanding of the physiology of these strains, these problems can be tackled. The benefits associated with working at high temperatures, such as energetic advantages, reduction of contamination and high rates of production, coupled with the successful selection of organisms that are able to utilize a wide substrate range, suggest that thermophilic ethanologens have the potential to overcome the commercial barriers to second-generation biofuels and make lignocellulosic ethanol a reality in the near future.

Disclosure statement

M.P.T., K.L.E. and S.M. are involved in the commercialization of part of the technology described within the review.

[*] Cripps, R. and TMO Renewables Ltd (2006) Biofuel Production From Plant Biomass Derived Sugars, *Department of Trade and Industry* (http://www.berr.gov.uk/files/file37691.pdf).

Acknowledgements

We thank the South African National Research Foundation and the Universities of the Western Cape and Cape Town for financial support.

References

1 Haber, W. (2007) Energy, food, and land: the ecological traps of humankind. *Environ. Sci. Pollut. Res. Int.* 14, 359–365
2 US Congress (2005) Energy Policy Act of 2005, 109th cong., 58th sess. (http://www.epa.gov/oust/fedlaws/publ_109-058.pdf)
3 The European Parliament and the Council of the European Union (2003) Directive 2003/30/EC of the European Parliament and of the Council of 8 May 2003 on the promotion of the use of biofuels or other renewable fuels for transport. In *Official Journal of the European Union*, L123/42, pp. 42–46 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:123:0042:0046:EN:PDF)
4 Otero, J.M. *et al.* (2007) Fueling industrial biotechnology growth with bioethanol. *Adv. Biochem. Eng. Biotechnol.* 108, 1–40
5 Farrell, A.E. *et al.* (2006) Ethanol can contribute to energy and environmental goals. *Science* 311, 506–508
6 Nordhoff, S. (2007) Editorial: food vs fuel – the role of biotechnology. *Biotechnol. J.* 2, 1451
7 Tenenbaum, D.J. (2008) Food vs. fuel: diversion of crops could cause more hunger. *Environ. Health Perspect.* 116, A254–A257
8 Metzger, J.O. and Huttermann, A. (2009) Sustainable global energy supply based on lignocellulosic biomass from afforestation of degraded areas. *Naturwissenschaften* 96, 279–288
9 Dien, B.S. *et al.* (2003) Bacteria engineered for fuel ethanol production: current status. *Appl. Microbiol. Biotechnol.* 63, 258–266
10 Jeffries, T.W. (2006) Engineering yeasts for xylose metabolism. *Curr. Opin. Biotechnol.* 17, 320–326
11 Pronk, J. *et al.* (2005) Engineering *Saccharomyces cerevisiae* for xylose utilization. *J. Biotechnol.* 118 (Suppl. 1), S86–S87
12 Yanase, H. *et al.* (2005) Ethanol production from cellulosic materials by genetically engineered *Zymomonas mobilis*. *Biotechnol. Lett.* 27, 259–263
13 Burchhardt, G. and Ingram, L.O. (1992) Conversion of xylan to ethanol by ethanologenic strains of *Escherichia coli* and *Klebsiella oxytoca*. *Appl. Environ. Microbiol.* 58, 1128–1133
14 Zaldivar, J. *et al.* (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl. Microbiol. Biotechnol.* 56, 17–34
15 Sommer, P. *et al.* (2004) Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. *Biochem. Soc. Trans.* 32, 283–289
16 Hartley, B.S. and Sharma, G. (1987) Novel ethanol fermentations from sugar cane and straw. *Philos. T. Roy. Soc. A* 321, 555–568
17 Hild, H.M. *et al.* (2003) Effect of nutrient limitation on product formation during continuous fermentation of xylose with *Thermoanaerobacter ethanolicus* JW200. *Appl. Microbiol. Biotechnol.* 60, 679–686
18 Takami, H. *et al.* (2004) Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. *Nucleic Acids Res.* 32, 6292–6303
19 Vane, L.M. and Alvarez, F.R. (2008) Membrane-assisted vapor stripping: energy efficient hybrid distillation–vapor permeation process for alcohol–water separation. *J. Chem. Technol. Biotechnol.* 83, 1275–1287
20 Skinner, K.A. and Leathers, T.D. (2004) Bacterial contaminants of fuel ethanol production. *J. Ind. Microbiol. Biotechnol.* 31, 401–408
21 Bischoff, K.M. *et al.* (2007) Antimicrobial susceptibility of *Lactobacillus* species isolated from commercial ethanol plants. *J. Ind. Microbiol. Biotechnol.* 34, 739–744
22 Schell, D.J. *et al.* (2007) Contaminant occurrence, identification and control in a pilot – scale corn fibre to ethanol conversion process. *Bioresour. Technol.* 98, 2942–2948
23 Ben-Bassat, A. *et al.* (1981) Ethanol production by thermophilic bacteria: metabolic control of end product formation in *Thermoanaerobium brockii*. *J. Bacteriol.* 146, 192–199
24 Demain, A.L. *et al.* (2005) Cellulase, clostridia, and ethanol. *Microbiol. Mol. Biol. Rev.* 69, 124–154
25 Zeikus, J.G. *et al.* (1981) Thermophilic ethanol fermentations. *Basic Life Sci.* 18, 441–461
26 Burdette, D.S. *et al.* (2002) Physiological function of alcohol dehydrogenases and long-chain (C-30) fatty acids in alcohol tolerance of *Thermoanaerobacter ethanolicus*. *Appl. Environ. Microbiol.* 68, 1914–1918
27 Fong, J.C. *et al.* (2006) Isolation and characterization of two novel ethanol-tolerant facultative – anaerobic thermophilic bacteria strains from waste compost. *Extremophiles* 10, 363–372
28 Peng, H. *et al.* (2008) The high ethanol tolerance in a thermophilic bacterium *Anoxybacillus* sp. WP06. *Sheng Wu Gong Cheng Xue Bao* 24, 1117–1120
29 Ramos, J.L. *et al.* (2002) Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.* 56, 743–768
30 Taylor, M. *et al.* (2008) Microbial responses to solvent and alcohol stress. *Biotechnol. J.* 3, 1388–1397
31 Lamed, R. and Zeikus, J.G. (1980) Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. *J. Bacteriol.* 144, 569–578
32 Ng, T.K. *et al.* (1981) Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. *Appl. Environ. Microbiol.* 41, 1337–1343
33 Weimer, P.J. and Zeikus, J.G. (1977) Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence of *Methanobacterium thermoautotrophicum*. *Appl. Environ. Microbiol.* 33, 289–297
34 Wiegel, J. *et al.* (1979) Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. *J. Bacteriol.* 139, 800–810
35 Freier, D. *et al.* (1988) Characterization of *Clostridium thermocellum* JW20. *Appl. Environ. Microbiol.* 54, 204–211
36 Mori, Y. (1990) Characterization of a symbiotic coculture of *Clostridium thermohydrosulfuricum* YM3 and *Clostridium thermocellum* YM4. *Appl. Environ. Microbiol.* 56, 37–42
37 Sharma, G. (1991) Prospects for ethanol production from cellulose with *Clostridium thermocellum–Bacillus stearothermophilus* co-cultures. *Biotechnol. Lett.* 13, 761–764
38 Lynd, L.R. *et al.* (1989) Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. *Appl. Environ. Microbiol.* 55, 3131–3139
39 Tailliez, P. *et al.* (1989) Enhanced cellulose fermentation by an asporogenous and ethanol-tolerant mutant of *Clostridium thermocellum*. *Appl. Environ. Microbiol.* 55, 207–211
40 Tailliez, P. *et al.* (1989) Cellulose fermentation by an asporogenous mutant and an ethanol-tolerant mutant of *Clostridium thermocellum*. *Appl. Environ. Microbiol.* 55, 203–206
41 Sato, K. *et al.* (1992) Effect of yeast extract and vitamin B(12) on ethanol production from cellulose by *Clostridium thermocellum* I-1-B. *Appl. Environ. Microbiol.* 58, 734–736
42 Tyurin, M.V. *et al.* (2004) Electrotransformation of *Clostridium thermocellum*. *Appl. Environ. Microbiol.* 70, 883–890
43 Tyurin, M.V. *et al.* (2006) Gene transfer systems for obligately anaerobic thermophilic bacteria. *Extremophiles* 35, 309–330
44 Herrero, A.A. and Gomez, R.F. (1980) Development of ethanol tolerance in *Clostridium thermocellum*: effect of growth temperature. *Appl. Environ. Microbiol.* 40, 571–577
45 Herrero, A.A. *et al.* (1982) Ethanol-induced changes in the membrane lipid composition of *Clostridium thermocellum*. *Biochim. Biophys. Acta* 693, 195–204
46 Lovitt, R.W. *et al.* (1984) Ethanol production by thermophilic bacteria: physiological comparison of solvent effects on parent and alcohol-tolerant strains of *Clostridium thermohydrosulfuricum*. *Appl. Environ. Microbiol.* 48, 171–177
47 Lovitt, R.W. *et al.* (1988) Ethanol production by thermophilic bacteria: biochemical basis for ethanol and hydrogen tolerance in *Clostridium thermohydrosulfuricum*. *J. Bacteriol.* 170, 2809–2815
48 Lamed, R. and Zeikus, J.G. (1980) Glucose fermentation pathway of *Thermoanaerobium brockii*. *J. Bacteriol.* 141, 1251–1257
49 Lacis, L.S. and Lawford, H.G. (1991) *Thermoanaerobacter ethanolicus* growth and product yield from elevated levels of xylose or glucose in continuous cultures. *Appl. Environ. Microbiol.* 57, 579–585

50 Burdette, D.S. *et al.* (1997) Biophysical and mutagenic analysis of *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase activity and specificity. *Biochem. J.* 326, 717–724

51 Peng, H. *et al.* (2008) The aldehyde/alcohol dehydrogenase (AdhE) in relation to the ethanol formation in *Thermoanaerobacter ethanolicus* JW200. *Anaerobe* 14, 125–127

52 Arni, R.K. *et al.* (1996) Crystallization of the secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* 39E. *Protein Pept. Lett.* 3, 423–426

53 Desai, S.G. *et al.* (2004) Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Appl. Microbiol. Biotechnol.* 65, 600–605

54 Shaw, A.J. *et al.* (2008) Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13769–13774

55 Shaw, A.J. *et al* (2007) Mascoma and Dartmouth College. Thermophilic organisms for conversion of lignocellulosic biomass to ethanol, WO/2007/130984

56 Mikkelsen, M.J. and Ahring, B.K. (2007) Biogasol. *Thermoanaerobacter mathranii* strain BGl, WO/2007/134607

57 Georgieva, T.I. *et al.* (2007) Effect of temperature on ethanol tolerance of a thermophilic anaerobic ethanol producer *Thermoanaerobacter* A10: modeling and simulation. *Biotechnol. Bioeng.* 98, 1161–1170

58 Georgieva, T.I. *et al.* (2007) High ethanol tolerance of the thermophilic anaerobic ethanol producer *Thermoanaerobacter* BG1L1. *Cent. Eur. J. Biol.* 2, 364–377

59 Georgieva, T.I. and Ahring, B.K. (2007) Evaluation of continuous ethanol fermentation of dilute-acid corn stover hydrolysate using thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1. *Appl. Microbiol. Biotechnol.* 77, 61–68

60 Georgieva, T.I. *et al.* (2008) Ethanol production from wet-exploded wheat straw hydrolysate by thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. *Appl. Biochem. Biotechnol.* 145, 99–110

61 Nazina, T.N. *et al.* (2001) Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp nov and *Geobacillus uzenensis* sp nov from petroleum reservoirs and transfer of *Bacillus stearothermophilus Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans*. *Int. J. Syst. Evol. Microbiol.* 51, 433–446

62 Hartley, B.S. and Payton, M.A. (1983) Industrial prospects for thermophiles and thermophilic enzymes. *Biochem. Soc. Symp.* 48, 133–146

63 Feng, L. *et al.* (2007) Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc. Natl. Acad. Sci. U. S. A.* 104, 5602–5607

64 Amartey, S.A. *et al.* (1991) Development and optimisation of a defined medium for aerobic growth of *Bacillus stearothermophilus* LLD-15. *Biotechnol. Lett.* 13, 621–626

65 Amartey, S.A. *et al.* (1999) Fermentation of a wheat straw acid hydrolysate by *Bacillus stearothermophilus* T-13 in continuous culture with partial cell recycle. *Process Biochem.* 34, 289–294

66 San Martin, R. *et al.* (1992) Development of a synthetic medium for continuous anaerobic growth and ethanol production with a lactate dehydrogenase mutant of *Bacillus stearothermophilus*. *J. Gen. Microbiol.* 138, 987–996

67 Amartey, S.A. and Leung, P.C.J. (2000) Corn steep liquor as a source of nutrients for ethanologenic fermentation by *Bacillus stearothermophilus* T-13. *Bull. Chem. Technol. Macedonia.* 19, 65–71

68 Thompson, A.H. *et al.* (2008) Heterologous expression of pyruvate decarboxylase in *Geobacillus thermoglucosidasius*. *Biotechnol. Lett.* 30, 1359–1365

69 Taylor, M.P. *et al.* (2008) Development of a versatile shuttle vector for gene expression in *Geobacillus* spp. *Plasmid* 60, 45–52

70 Atkinson, A. *et al.* (2006). TMO Renewables Ltd. Thermophilic microorganisms with inactivated lactate dehydrogenase gene (*ldh*) for ethanol production, WO/2006/117536

71 Atkinson, A. *et al.* (2006). TMO Renewables Ltd. Modified microorganisms with inactivated lactate dehydrogenase gene, WO/2006/131734

72 Atkinson, A. *et al.* (2008). TMO Renewables Ltd. Thermophilic microorganisms for ethanol production, WO/2008/038019

73 Wisselink, H.W. *et al.* (2009) Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Appl. Environ. Microbiol.* 75, 907–914

74 Wisselink, H.W. *et al.* (2007) Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. *Appl. Environ. Microbiol.* 73, 4881–4891

75 Hahn-Hägerdal, B. *et al.* (2006) Bio-ethanol – the fuel of tomorrow from the residues of today. *Trends Biotechnol.* 24, 549–556

76 Perlack, R.D. *et al.* (2005) Biomass as Feedstock for a Bioenergy and Bioproducts Industry: The Technical Feasibility of a Billion-Ton Annual Supply, *US Department of Agriculture and US Department of Energy* (http://feedstockreview.ornl.gov/pdf/billion_ton_vision.pdf)

77 US Department of Energy (2006) Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda, *US Department of Energy* (http://genomicsgtl.energy.gov/biofuels/2005workshop/b2blowres63006.pdf)