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Extremophiles in biofuel synthesis

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The current global energy situation has demonstrated an urgent need for the development of alternative fuel sources to the continually diminishing fossil fuel reserves. Much research to address this issue focuses on the development of financially viable technologies for the production of biofuels. The current market for biofuels, defined as fuel products obtained from organic substrates, is dominated by bioethanol, biodiesel, biobutanol and biogas, relying on the use of substrates such as sugars, starch and oil crops, agricultural and animal wastes, and lignocellulosic biomass. This conversion from biomass to biofuel through microbial catalysis has gained much momentum as biotechnology has evolved to its current status. Extremophiles are a robust group of organisms producing stable enzymes, which are often capable of tolerating changes in environmental conditions such as pH and temperature. The potential application of such organisms and their enzymes in biotechnology is enormous, and a particular application is in biofuel production. In this review an overview of the different biofuels is given, covering those already produced commercially as well as those under development. The past and present trends in biofuel production are discussed, and future prospects for the industry are highlighted. The focus is on the current and future application of extremophilic organisms and enzymes in technologies to develop and improve the biotechnological production of biofuels.

Keywords: biofuel; extremophiles; biocatalyst

1. Introduction

By definition, biofuels are the fuel products obtained from biomass (including sugar cane, corn, beets, wheat, sorghum, rapeseed, sunflower, soybean, palm, coconut and Jatropha) as well as the biodegradable component of industrial and municipal wastes [1]. The earliest attempts to produce biofuels on a commercial scale date back to 1975, when a programme called PROALCOOL was launched in Brazil [2]. However, fossil fuels remained a cheaper option at the time, and it is only in the last five years that there has been a renewed interest in biofuels.

Between 2006 and 2030, 80% of the predicted increase in global liquid fuel consumption is attributed to transportation [3]. The rising price of crude oil, the diminishing supply of fossil fuels and the global warming observed as a consequence of increasing greenhouse gas emissions have all stimulated interest in developing biofuels as an alternative fuel source [1,4,5]. Progress in this regard is reflected in the increasing global biofuel production and consumption figures, with total biofuel production having increased from 5639 million tons of oil equivalent (Mtoe) to 8165 Mtoe and an increase in total biofuel consumption from 5625 kilotons of oil equivalent (ktoe) to 10064 ktoe for the period between 2006 and 2008 in the EU27 countries alone [6].

Although chemical and thermo-chemical processes are current technologies for biofuel production [5], the biological conversion of biomass to biofuel by microorganisms is more cost-effective and has gained great momentum over the last several years. Furthermore, the potential application of extremophiles and their robust enzymes in this process has recently been explored. Extremophiles refer to organisms, mostly prokaryotic, which thrive in environmental conditions considered to be hostile to humans [7]. The parameters which contribute towards the extreme conditions include temperature, pH salinity, pressure, radiation, light and water content.

This review gives a brief introduction to the different biofuels and a summary of the research conducted on their development as a commercial product, covering both the current applications and the future potential, focussing particularly on extremophiles and/or their enzyme products in these production practices.

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2. Types of biofuels

First generation biofuels can be described as those produced from readily available crops containing sugar, starch and oil and using conventional technologies, and they include bioethanol, biodiesel and biobutanol [5] (Figure 1). Second generation biofuels are produced from raw materials which are not as easily hydrolysed, such as lignocellulosic material. The main biofuels currently in production on an industrial scale are biodiesel and bioethanol, comprising more than 90% of the total biofuel market [4]. Their success within this market is determined by various prerequisites defined by both chemical and physical properties.

2.1. Bioethanol

With its largest impact in the transportation industry, bioethanol has applications as blends or in its pure form as a substitute for petroleum-based systems. Tremendous progress in the field of biotechnology has led to the development of established bioethanol production practices. Fermentation based on *Saccharomyces cerevisiae*, using predominantly sugar cane molasses or enzymatically hydrolysed starch (obtained for example from wheat, corn, cassava, rye, barley and triticale), has been well developed for the production of ethanol [8–11]. This process has traditionally been a sequential procedure whereby the cellulosic hydrolysis and subsequent fermentation are performed separately. A simultaneous saccharification and fermentation approach can also be applied, in which the cellulases and the fermentative microorganism are added concomitantly [9,12–14]. Optimization of *S. cerevisiae* as well as the fermentative process has resulted in the increase of ethanol yields of up to 10% v/v in large-scale starch-derived production facilities [4,8]. Despite these improvements, *S. cerevisiae* lacks the ability to ferment many pentose sugars, one of the major constituents in enzyme hydrolysates from lignocellulosic biomass, reducing the efficiency of the conversion process. Although this is currently being

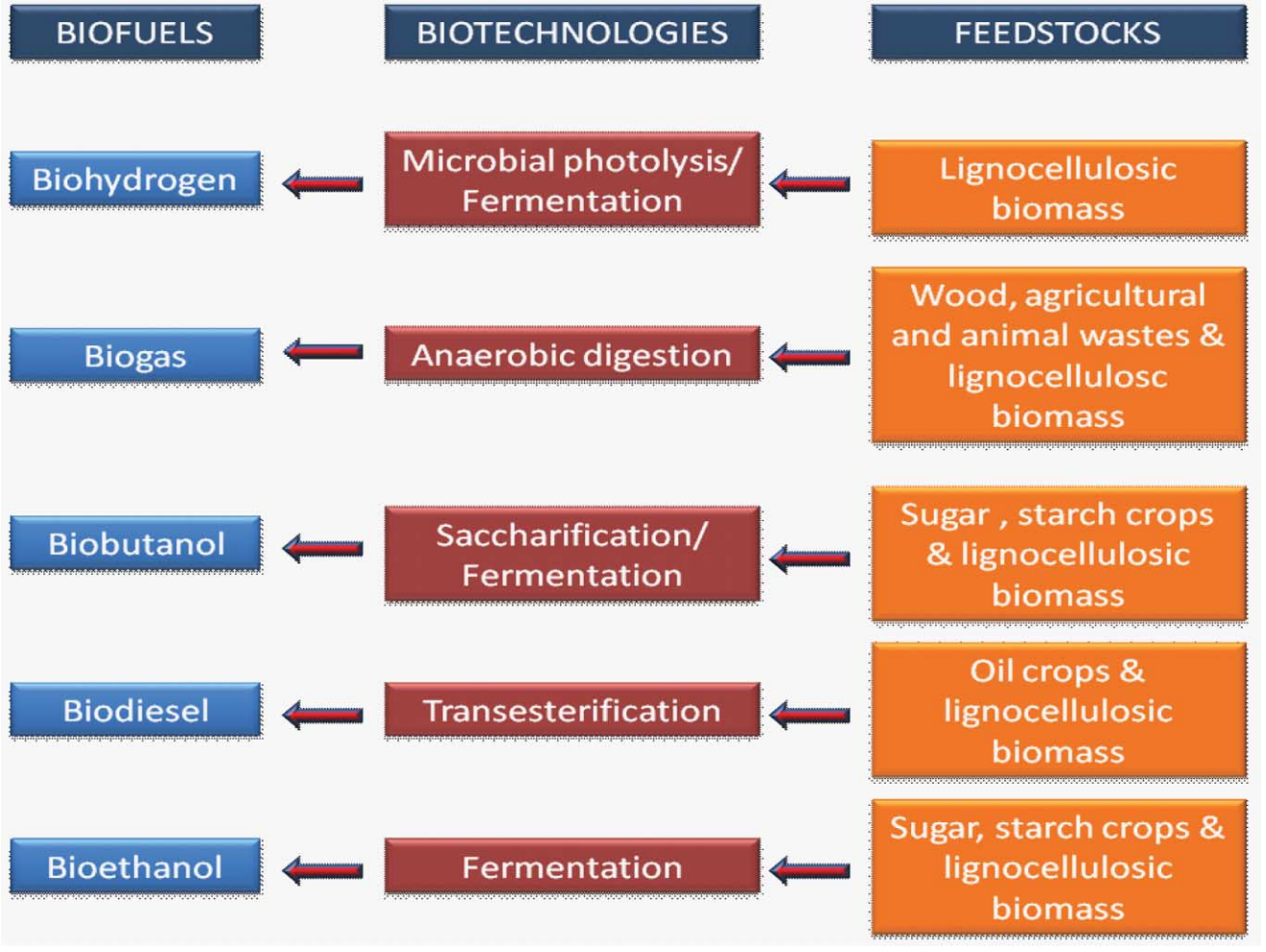

Figure 1. Various methodologies for the production of biofuels.

addressed through genetic engineering [12,15,16], alternative solutions are also being considered. Many bacterial organisms are capable of utilizing the pentose sugars as a carbon source during fermentation, including *Mucor indicus*, *Chalara parvispora*, *Pachysolen tannophilus*, *Zymomonas mobilis*, *Streptococcus fragilis*, *Kluyveromyces fragilis*, *Clostridium thermocellum*, *Clostridium ljungdahlii*, *Escherichia coli*, *Pichia stipitis*, *Klebsiella oxytoca*, *Moorella* ssp. and *Carboxydocella* spp. [4,8,9,17–23]. However, the ethanol yields tend to be markedly lower than with yeast fermentations because of competing reactions, resulting in other by-products [4], and these organisms typically display poor ethanol tolerance [8,14]. As a result, the development of a suitable fermentative microorganism remains one of the major bottlenecks in advancing bioethanol commercialization from biomass.

2.2. Biodiesel

Biodiesel displays physical and energetic properties similar to those of petroleum diesel, enabling it to serve efficiently, either pure or as a blend, in unmodified diesel engines [24,25]. The advantageous aspects of biodiesel include its non-toxic, sulphur-free and biodegradable nature. It also displays desirable flash point and aromatic content properties and is known to extend engine life and reduce maintenance costs as a consequence of its desirable lubricity [5]. Most importantly, the use of biodiesel results in less combustion emission, carbon monoxide and unburned hydrocarbons compared with regular petrol-based diesel [26]. By the end of 2007, 165 companies in the USA alone were already producing biodiesel, with a further 85 plants under construction [8].

The production of biodiesel relies on a catalyst-driven chemical reaction (transesterification) between a vegetable oil (including peanut, soybean, babassu, palm, sunflower, rapeseed, rice bran oil, olive oil, canola oil and used corn oil) and an alcohol (methanol, ethanol, propanol, isopropanol, butanol and pentanol) [27–40]. This process of transesterification is influenced by a number of factors, including the type of catalyst employed, alcohol/oil ratios, temperature and water content. The catalyst is usually a strong base, such as sodium or potassium hydroxide, and the resulting products are methyl esters, alternatively known as biodiesel. The major disadvantages of the process are the need to remove the alkaline catalyst and the glycerol from the final product and the treatment of the resulting alkaline wastewater [41]. However, improvements in microbial biotechnology have provided alternative methods to overcome these obstacles. In particular, the use of biocatalysts, such as lipases, for enzymatic transesterification holds several advantages. Employing biocatalysts for large-scale production, however, presents other drawbacks, including the high costs involved as well as methanol and/or glycerol inhibition [42]. Well-optimized reactions that consider parameters such as substrate molar ratio, solvent, temperature, water and free fatty acid content are being investigated, which could lead to both lower costs and higher yields [43].

2.3. Biobutanol

Although butanol is synthesized chemically, biobutanol was one of the first industrial-scale fermentation technologies, developed in the 1960s [44,45]. Predominantly used as an industrial solvent, biobutanol is a colourless liquid with a distinct odour. As a biofuel, biobutanol shows even greater potential than bioethanol in the transportation industry as it contains 25% more energy than bioethanol (per volume) [45]. Additionally, its use as a blend with gasoline and diesel does not require any modification to existing vehicles [8]. The current production practices, however, render it the more costly option of the two. This is largely as a consequence of product inhibition during fermentation, brought on at concentrations of 1–2% butanol. The production of biobutanol involves a two-step bioconversion process carried out by *Clostridium* spp., and is referred to as the ABE fermentation, yielding acetone:butanol:ethanol at a ratio of 3:6:1 [44,46]. Raw biomass (e.g. corn, sugar beet, sorghum) is partially converted to hydrogen and butyric acid, which is subsequently converted to butanol [47]. Current research in this field is directed at genetically altering *Clostridium* spp. to enhance both its fermentative performance and final butanol yield [44,48]. Additionally, research has been focused on ways to circumvent product inhibition by removal of butanol as fermentation progresses [44,45,48–50].

2.4. Methane/biogas

Biogas is a product of anaerobic degradation of organic substrates, and can be relatively easily produced in small-scale industrial units. Its applications vary from the production of electrical power or heat to its use in combustion engines. The greatest appeal of biogas lies in its environmentally friendly status, potentially providing an opportunity for complete recycling of minerals and nutrients from the soil. Substrates for the microbial production of biogas can be obtained from a diverse source, including manure (pigs, horses, cows or chickens), used frying oil, organic household and garden waste, and municipal refuse. Often, energy crops such as grass, poplar, maize and willow are also harvested as feedstock for biogas production [4].

Generating methane from plant material through microbial technologies usually occurs in a three-step process. Firstly, the polysaccharides in the organic substrates are hydrolysed to fermentable sugars, which, subsequent to fermentation, yield acetic, propionic and butyric acid, alcohols, carbon dioxide, hydrogen and several other by-products. The second and most limiting step of the process (as a consequence of extended generation times of the bacteria involved) is known as acetogenesis, of which acetic acid and carbon dioxide are the main products. The third and final step is known as methanogenesis, performed by slow-growing, fastidious archaea, yielding up to 70% CH_4 and CO_2, NH_3 and H_2S as by-products [4]. The exact microbial communities involved in each of the three steps is variably dependent on the process (mesophilic or thermophilic) as well as the type of substrate and fermenter employed [51–54]. In large-scale industrial biogas plants, the two-stage process is gaining preference, separated into hydrolysis and methanogenesis/acetogenesis, which allows for the optimization of each process individually (pH and temperature). More recently, the use of thermophilic organisms for these purposes has gained momentum, based largely on their ability to speed up the process [4].

2.5. Hydrogen

Hydrogen has gained much interest as a viable biofuel because of its ability to be converted to electrical energy in fuel cells [55]. Generating hydrogen from microbial origins certainly meets the requirements of a viable biofuel prospect, providing a cost-effective, pollution-free and energy-saving alternative to current production practices [4,56,57]. Although microbial production of H_2 has not yet been developed to an economically viable status, research into its production from renewable biomass is ongoing [58]. Several options for the biological production of H_2 are being investigated, including biophotolysis of water through algae and cyanobacteria, the use of photosynthetic bacteria for the photofermentation of organic substances, and 'dark' fermentation of organic substances by anaerobic organisms [59]. In particular, the use of thermophilic microorganisms such as *Caldicellulosiruptor saccharolyticus* and *Thermotogo elfii* has shown promising results [4,60–62].

3. Extremophiles used in the production of biofuels

There are a number of advantages to using extremophiles in industrial applications, particularly in the production of biofuels. Extremophiles are robust organisms producing stable enzymes, and are often able to tolerate changes in environmental conditions, such as pH and temperature. In reviewing the information available on the use of extremophiles in biofuel production, it became apparent that the majority are of thermophilic source. This is not surprising since thermophiles have a remarkable ability to tolerate fluctuations in pH, temperature and environmental change [7], an attribute which offers a clear advantage in the development of a commercially viable process [63,64]. Thermophiles readily ferment pentose and/or hexose sugars from biomass and, in some cases, even structurally complex carbohydrates, a quality which is particularly important for production of second-generation biofuels [64,65]. Furthermore, thermophilic industrial fermentations are less prone to microbial contamination and require lower energy inputs as a result of the reduced cooling steps needed between the fermentation steps. Also, the removal of any volatile products, which in turn minimizes the problem of product inhibition, is facilitated. Despite the dominance of thermophiles in biofuels, other extremophile groups have also been applied in this field, including methanogens (typically thermophilic, anaerobic archaea) and psychrophiles. Methanogens play a crucial role in the production of biogas, whereas psychrophiles are being exploited for their cold-adapted lipases for use in biodiesel. The application of these extremophilic organisms and their enzymes in the production of biofuels, particularly for bioethanol and to a lesser extent in the production of other biofuels, will be discussed below.

3.1. Bioethanol

Ideal microbiological strains for bioethanol production should produce high yields of ethanol, with few side products, and have low inhibitor sensitivity and high ethanol tolerance. Industrial production of bioethanol, using improved strains of *Saccharomyces cerevisiae* from sugar cane molasses or enzymatically hydrolysed starch, yields as much as 20% (v/v) of ethanol [4]. The bacterium *Zymomonas mobilis* has also been used for bioethanol production as it possesses an ethanol fermentation pathway, resulting in a higher ethanol yield than *S. cerevisiae*, and it can tolerate up to 120 g/L of the product [66]. However, for bioethanol to become economically viable, the use of lignocellulosic material as a source of bioethanol production is a requirement. This process requires the hydrolysis of cellulose, which is catabolised into hexose sugars and hemicellulose, consisting mostly of pentose sugars [67]. Unlike *S. cerevisiae* and *Z. mobilis*, which can utilize only hexose sugars, a large number of thermophiles are able to ferment both hexose and pentose sugars derived from biomass and hydrolysates [68]. This allows for high growth and

metabolic rates of organisms growing on both cellulose and hemicellulose.

3.1.1. Thermophilic clostridia

Thermophilic clostridia are fermentative anaerobes with an optimal growth between 60 and 65 °C [69]. They are able to degrade lignin-containing materials, such as lignocellulosic waste, because of the presence of multiple cellulases and hemicellulases often contained within the cellulosome [70]. The cellulosome is a multi-enzyme complex located on the outside of the cell membrane and is involved in the enzymatic degradation of cellulosic substances, including crystalline cellulose [70]. The enzymes incorporated in this complex include endo-β-glucanases, exoglucanases, β-glucosidases, cellodextrin phosphorylases, cellobiose phosphorylases, xylanases, lichenases, laminarinases, pectin lyases, polygalacturonate hydrolases, pectin methylesterase, β-xylosidases, β−galacosidases and β−mannosidases [25,70]. The cellulosome of *Clostridium thermocellum* allows for the degradation of cellulose to cellobiose and cellodextrins, and hemicellulose to xylose, xylobiose and other pentose sugars. For an extensive review on cellulosomes, the reader is referred to Demain *et al*. [70]. Cellobiose and cellodextrins are taken into the cell, where *C. thermocellum* is able to ferment them to ethanol, acetate, lactate, H_2 and CO_2 [69]. *Clostridium thermocellum*, therefore, is a good candidate for ethanol fermentation from cellulosic biomass. There are, however, a number of disadvantages associated with its application in bioethanol production. One of these is that most strains are sensitive to high ethanol concentrations [4,25]. This could be overcome by the continuous removal of ethanol as it is being produced, facilitated by the high volatility of the product at higher temperatures [25,71], or by engineering a strain tolerant to higher ethanol concentrations. Another negative aspect is the low ethanol yields produced, due to the formation of by-products such as lactate and acetate [25,70]. This could be solved by the engineering of strains with knockouts of acetate kinase and lactate dehydrogenase. The tools needed for engineering thermophilic clostridia are becoming available [72–74], with a genetically engineered strain of *C. thermocellum* already having been successfully generated, resulting in a mutant capable of tolerating up to 60 g/L of ethanol and also able to produce as much as 26 g/L ethanol [75]. In another study, a *C. thermocellum* lactate dehydrogenase (*ldh*) mutant, with higher ethanol production and enhanced product tolerance, was constructed [76].

The biggest disadvantage in using *C. thermocellum* is that, despite its ability to degrade lignocellulosic waste to both hexose and pentose sugars, it is only able to utilize hexose sugars from cellulose and not the pentose sugars derived from hemicellulose [14,77]. This drawback could be solved by the use of mixed cultures for the degradation and fermentation of all sugars derived from lignocellulosic materials. The use of other thermophilic bacteria, able to ferment pentose sugars for the production of ethanol, together with *C. thermocellum* seems to be a possibility. The production cost of bioethanol from lignocellulose could be decreased two-fold when using thermophilic anaerobic mixed cultures [78]. The complete degradation and utilization of lignocellulose would involve five steps: first, the formation of cellulase and hemicellulase enzymes by *C. thermocellum*; second, the hydrolysis of cellulose to cello-oligomers and cellobiose, and hemicellulose to xylans, xylobiose and other pentose sugars; third, the uptake of these sugars; fourth, the fermentation of hexose sugars by *C. thermocellum* to produce ethanol; fifth, the fermentation of pentose sugars by another thermophilic organism able to produce ethanol. To this regard, *C. thermocellum* has been used in mixed fermentations with *Thermoanaerobacterium thermosaccharolyticum* [25,70], *Thermoanaerobacter thermohydrosulphuricum* [79], *Thermoanaerobacter ethanolicus* [70], *Geobacillus stearothermophilus* [80], *Thermoanaerobacter brockii* [70,81] and *Thermoanaerobacterium saccharolyticum* [25].

3.1.2. Thermoanaerobacterium saccharolyticum

Thermoanaerobacterium is a hemicellulolytic thermophilic anaerobe [82]. It is capable of utilizing pentose sugars such as xylose to produce ethanol, as well as organic acids (acetic acid is formed by pyruvate:ferredoxin oxidoreductase (POR), phosphate acetyltransferase (Pta) and acetate kinase (Ack), while lactic acid is formed by L-lactate dehydrogenase (Ldh) [83]). To increase ethanol yields, metabolic engineering of end-product metabolism has been carried out to generate a single knockout mutant for lactate dehydrogenase in *Thermoanaerobacterium saccharolyticum* [84]. This mutant had reduced levels of lactate production and a four-fold increase in ethanol yields. Furthermore, the new strain TD1 was able to utilize xylose more efficiently. Recently, a *T. saccharolyticum* strain, ALK1, was engineered to produce ethanol as the only organic product [85]. This was attained by knocking out the genes involved in lactate (*ldh*) and acetate (*ack/pta*) production. Ethanol was produced from pyruvate using POR, a pathway different from that in previously described microbes with a homo-ethanol fermentation. Strain ALK1 was cultivated in continuous culture with higher concentrations of xylose progressively generating *T. saccharolyticum* strain ALK2. This new strain was able to utilize xylose, glucose, mannose and

galactose, producing 33 g/L ethanol in continuous culture and 37 g/L in fed-batch cultures. These are the highest yields of ethanol production by thermophilic anaerobes reported to date.

3.1.3. Thermoanaerobacter

Thermoanaerobacter species are thermophilic anaerobes which are very similar to thermophilic clostridia, and some were originally classified as *Clostridium* species [86,87]. The main products from *Thermoanaerobacter* fermentations are lactic acid and ethanol [69] via, among others, lactate dehydrogenase and alcohol dehydrogenase activities [88]. *Thermoanaerobacter ethanolicus* is able to ferment both D-glucose and D-xylose [89] to form ethanol; however, their ethanol tolerance is low [90], and a *T. ethanolicus* strain was adapted to tolerate up to only 4% (v/w) of ethanol [91]. Ethanol tolerance in *T. ethanolicus* seems to be linked to the function of alcohol dehydrogenase since a *T. ethanolicus* mutant in this gene was tolerant to 8% ETOH (ethanol) (v/v) [90]. *Thermoanaerobacter* BG1L1 is a lactate dehydrogenase mutant, deficient in lactic acid production [92], with resistance to 8.3% ethanol (equivalent to 65 g/L) after continuous exposure to the product. This strain is able to utilize xylose from lignocellulosic hyrolysates, and has been shown to digest corn stover pretreated with dilute acid [93] and wheat straw hydrolysate [94]. Other species which have been evaluated for the production of ethanol are *T. thermohydrosulphuricus* and *T. brockii* [69,95].

3.1.4. Geobacillus

Geobacillus are thermophilic bacilli with high catabolic flexibility and for which metabolic engineering is possible [96,97]. Certain species are able to ferment sugars such as D-glucose, D-xylose and L-arabinose at temperatures between 55 °C and 70 °C and to produce a mixture of lactate, formate, acetate and ethanol from glucose [98]. More complex carbohydrates such as xylan are also degraded by certain *Geobacillus* strains owing to the presence of xylanases [99]. *Geobacillus stearothermophilus* is able to produce ethanol at 70 °C at yields which are comparable to those of *S. cerevisiae* [100]. A *Geobacillus thermoglucosidasius* strain has been isolated which can tolerate ethanol as high as 10% (v/v), although without growth [101]. For these reasons, there is a great deal of interest in these organisms for industrial bioethanol production.

Saccharomyces cerevisiae and *Zymomonas mobilis* produce ethanol through the pyruvate decarboxylase (*pdc*) gene and the alcohol dehydrogenase (*adh*) gene [102,103]. These two enzymes are sufficient to convert intracellular pools of pyruvate and NADH to ethanol, where the acetaldehyde generated by the pyruvate decarboxylase from pyruvate is then converted to ethanol by alcohol dehydrogenase. Bacterial *pdc* genes are rare [104]; however, the few that have been identified have different kinetic properties and thermal stability [102,103,105–107]. These properties are likely to offer unique advantages for the development of desirable biocatalysts for use in the ethanol industry. Therefore, several attempts have been made to engineer this pathway in various organisms, including *Geobacillus* species [108–110]. Although functional expression was demonstrated in *G. glucosidasius*, the pyruvate decarboxylase activity was not stable above 54 °C [111]. However, this platform now provides the possibility to further develop the expression of *pdc* genes in this organism [75]. Further development of this organism for enhanced ethanologenic properties has been carried out by the company TMO Renewables Ltd. A mutant strain with knockouts in the lactate dehydogenase (*ldh*) and pyruvate formate lyase (*pfl*) genes as well as an upregulated pyruvate dehydrogenase (*pdh*) gene has been constructed [112]. This mutant has been shown to ferment both pentose and hexose sugars, producing ethanol at yields that approach the theoretical maximum at temperatures above 60 °C [112].

3.2. Biodiesel

Production of biodiesel is a mature technology for use in compression-ignition (diesel) engines. The cost of the plant raw materials averages 70% of the total production cost [113], which involves processing of the vegetable oils by transesterification into monoalkyl esters of the plant fatty acids. Unfortunately, these oleaginous plants produce fatty acids that account for around only 5% of their total biomass, providing small quantities of biodiesel to be used for blending with petroleum diesel. If biodiesel is to become an economically viable resource, more efficient novel sources of oil, such as microalgae as well as from extremophilic organisms, need to be researched.

3.2.1. Photosynthetic microalgae for production of biodiesel

Microalgae are eukaryotic photosynthetic microorganisms which convert sunlight, water and CO_2 to algal biomass. Under optimal growth conditions, these organisms produce fatty acids for esterification in to glycerol-based membrane lipids which can amount to 5–20% of their dry cell weight [114]. Under stressful environmental conditions some microalgae, such as *Botryococcus*

braunii, can produce very long chain hydrocarbons (C_{23} to C_{40}), similar to those in petroleum, which can exceed 80% of their dry cell weight [115,116]. There are several advantages to using microalgae for the production of lipids for conversion into biodiesel: (1) they accumulate lipids and oils in large amounts; (2) they grow rapidly, often doubling biomass within 24 hours [117]; (3) they are able to grow in saline waters and wastewaters without the need for fresh water [118]; (4) photobioreactors for growth of microalgae can be located in arid or semi-arid areas that are not suitable for agriculture; (5) the nutrients needed for growth can be provided from waste sources such as agricultural run-off, industrial or municipal wastewater, and animal feeds [114]; (6) they remove CO_2 emitted from burning fossil fuels; (7) unlike crops, their growth is not seasonal; (8) a large number of microalgae produce valuable by-products, such as biopolymers, pigments and polysaccharides, which can be harvested; (9) after lipid extraction, the algal biomass can be anaerobically converted into biogas, which can provide more energy than the energy produced from the lipids [119,120]. There are a large number of extremophilic microalgae, such as *Cyanidium caldarium* and *Galdieria sulphuraria*, which tolerate both high temperatures and low pH, having high growth rates at 50 °C and pH 1 [121,122]. The advantage of using extremophilic microalgae would be to minimize contamination within the photobioreactors, which tends to be problematic in outdoor cultures. Lipid content in certain microalgae, such as *Ochromonas danica* and *Nannocloropsis salina*, has been shown to increase with increasing temperature [114]. However, despite the potential for the use of microalgae in biodiesel production, the cost of algae oils is an impediment, due to the fact that the stressful conditions that high lipid production result in low growth rates [117,123]. Genetic engineering of microalgae will be required, therefore, to achieve enhanced lipid production under high growth rate conditions [124,125], and it is believed that this is the key for this technology to become commercially viable.

3.2.2. Fermentative use of microalgae for production of biodiesel

Solazyme, a company based in San Francisco, uses microalgae and standard commercial fermentation technologies to convert industrial and agricultural biomass directly into renewable oils (www.solazyme.com). The microalgae are grown in dark fermenters, where their photosynthetic apparatus is switched off, and they convert sugars to oil. This technology is well established, and Solazyme already produces and sells biodiesel, known as Soladiesel®. To date, this is the only oil-based fuel from microbes that is being produced in quantities of many thousands of gallons. Natural as well as engineered strains of microalgae are being used, and there is a potential for the use of extremophilic microalgae.

3.3. Biobutanol

There is a recent interest in biobutanol production. Various companies produce butanol via a microbial fermentative route. These include the French company Metabolic Explorer (www.metabolic-explorer.com), the US-based Cobalt Biofuels (www.cobaltbiofuels.com), Tetravitae Biosciences (www.tetravitae.com), the collaboration between BP and DuPont, known as Vivergo (www.vivergofuels.com), Environmental Energy, Inc. (www.butanol.com), Gevo Inc. (www.gevo.com), Green Biologics (www.greenbiologics.com), among others. *Clostridium* ABE fermentation is still the main process; however, the low butanol yield as well as the low product tolerance of the organisms is forcing researchers to look at alternative routes, including solvent-tolerant organisms.

3.3.1. Clostridium *ABE fermentation*

Certain clostridial species are well known for their ability to produce butanol via ABE fermentation [126]. The main disadvantages to this process are the low yield of butanol as well as the growth inhibition of the organisms by these solvents. Recent advances in metabolic engineering are concentrating on these two aspects. The engineering of *C. acetobutylicum* to provide hyperbutanol-producing strains is ongoing [127,128], while various other clostridial strains, able to produce higher butanol yields as well as being capable of hydrolyzing biomass to produce the solvent, have been isolated [129–131].

After decades of inactivity, a large number of biotechnological companies have recently started producing butanol by using clostridial fermentations. Tetravitae Biosciences (www.tetravitae.com) has improved the efficiency of the ABE process by using an extremely stable and robust mutant strain of *Clostridium beijerinckii*. This production strain has high selectivity to butanol production compared with the wildtype, has reduced product inhibition, and has the ability to effectively use low-cost cellulosic feedstocks. Tetravitae is continually developing enhanced strains with more efficient process engineering to significantly reduce production costs further. To date, ABE fermentation has not been shown in any thermophilic clostridia [77].

As mentioned above, the other aspect of the clostridial ABE fermentation that needs to be addressed for the process to become economically viable is the low butanol tolerance of the organisms. This can be circumvented by the engineering of solvent-tolerant

Clostridium. Overexpression of chaperones has been shown to increase the solvent tolerance of *C. acetobutylicum* [132].

3.3.2. The use of extremophile genes to engineer E. coli for the production of butanol

Escherichia coli, which is unable to produce butanol naturally, has been engineered with genes from other organisms, including extremophiles, for the production of higher chain alcohols. *Clostridium acetobutylicum* genes were overexpressed in *E. coli*, allowing this organism to produce butanol [133]. This strain was optimized for the production of 1-butanol by further deletion of certain *E. coli* pathways that compete with 1-butanol production. Additional engineering of *E. coli* strains has been done to produce 1-butanol, 1-propanol and other higher chain alcohols using the amino acid biosynthetic pathways of the host, diverting its 2-keto acid intermediates to alcohol synthesis, as opposed to the ABE fermentation [134–138]. Recently, the citramalate synthase (CimA) enzyme from the extremophile *Methanococcus jannaschii* was evolved for increased activity over a temperature range of 30 °C to 70 °C and overexpressed in *E. coli* for the production of both 1-propanol and 1-butanol [139]. The citramalate pathway directly converts pyruvate to 2-ketobutyrate bypassing threonine synthesis. This technology whereby *E. coli* is used for butanol production has been adopted by the biotechnology company Gevo™ (www.gevo.com).

3.3.3. Extremophiles in the production of butanol

Most microorganisms are unable to grow at butanol concentrations above 2% [140]. However, there are certain organisms, such as certain species of *Bacillus*, that are able to tolerate butanol concentrations as high as 2.5–7% [141]. Higher tolerance has been shown for organisms belonging to the *Pseudomonas* genus. *Pseudomonas* achieve high solvent tolerance by removal of solvent using efflux pumps and physico-chemical changes of their membrane lipids [142,143]. The *P. putida* S12 has inherent moderate tolerance to butanol [144], while other *P. putida* strains have been evolved to tolerate 6% w/v butanol [145]. This fact and the recent engineering of *P putida* to produce butanol [146] open up a new field of research for the production of butanol from solvent-tolerant organisms. The UK-based company Green Biologics (www.greenbiologics.com) uses a mixture of thermophiles as well as thermostable enzymes for the production of butanol from waste biomass. The thermophilic fermentations are conducted with genetically modified microbial strains optimized to produce butanol which is commercially sold as Butafuel™.

3.4. Biohydrogen and biogas

3.4.1. Production of hydrogen by anaerobic fermentation

Thermophilic biohydrogen fermentations have higher H_2 yields than mesophilic ones because of the suppression of H_2-consuming bacteria, such as methanogens and sulphur-reducing bacteria [147,148]. Fermentations at 50–55 °C yield twice as much H_2 as those at 30–40 °C [149]. Several thermophiles are able to produce H_2 both from C5 and C6 sugars. The extreme thermophile *Caldicellulosiruptor saccharolyticus* produces H_2 from pentose sugars and contains a large number of genes involved in lignocellulosic degradation, as well as the presence of two distinct hydrogenases, as shown by its genome [150,151], while *Thermoanaerobacterium thermosaccharolyticum* W16 was shown to ferment a biomass hydrolysate containing a mixture of glucose and xylose for H_2 production and had high tolerance to inhibitors such as acetate and furfural found in the hydrolysate [152,153]. *Clostridium thermocellum*, which is able to utilize hexose sugars, has been shown to produce H_2 from delignified wood fibres [154]. However, higher H_2 yields seem to be produced from mixed cultures, as H_2 production in a co-culture of *C. thermocellum* and *T. thermosaccharolyticum* was increased two-fold [155]. Hydrogen yields were also shown to increase by the addition of *Caldicellulosiruptor saccharolyticus* to a biogas-producing microbial community [156]. This effect was likely due to the high cellulolytic activity of *C. saccharolyticus* as well as its H_2-producing capacity. *Thermoanaerobacterium* has similarly been identified to be a primary H_2-producer in mixed communities [157–159]. Production of hydrogen has been shown using carbohydrate-rich wastewaters and agricultural and food waste under thermophilic conditions [157,158,160,161], and this has been coupled to a fuel cell for energy production [162].

3.4.2. Possible use of bacterial hydrogenases in the production of H_2

Hydrogenases have been shown to produce nearly theoretical yields of H_2 from both glucose and starch in enzymatic reactions [163–165]. Hydrogenases are metalloenzymes which catalyse the reversible reduction of protons to hydrogen using an energy source derived from either organic matter or light [166]. There is a group of thermostable hydrogenases that are able to function at temperature ranges between 50 °C and 125 °C [167]. Most of these enzymes contain a nickel–iron (NiFe) active site, and they have been found in all thermophilic environments. One of the disadvantages of hydrogenases is their sensitivity to oxygen [168]. Various thermophilic hydrogenases have been studied, such as *Pyrococcus furiosus* membrane-bound hydrogenase, which retains a

fraction of its activity when exposed to oxygen [166]. *Aeropyrum camini* contains two hydrogenases, one of which has very high thermostability at 90 °C with a half-life of 48 hours and is very tolerant to oxygen, retaining 75% of its activity after exposure [169]. The thermostable hydrogenase of *Thermotoga maritima* has been recently shown to require both ferredoxin and NADH as electron carriers for production of H_2 [170]. This demonstrates that further research needs to be conducted in the use of hydrogenases for the production of biohydrogen.

3.4.3. Methane/biogas

Biomethane can be produced from a large number of substrates. These include manure from farm animals, fat from slaughter waste or even waste frying oil, organic household waste or municipal solid waste [25,171]. For optimal biomethane production, mixed bacterial communities need to be used, similar to those in cow's rumen [172] or wastewater treatment [173,174]. A number of organisms have been shown to be capable of methane production, including thermophilic *Methanobacterium* sp., *Methanosarcina thermophila* and *Methanothermococcus okinawensis* as well as psychrotolerant and psychrophilic *Methanosarcina lacustri*, *Methanolobus psychrophilus* and members of the genus *Methanosaeta* [172–180]. The industrial implications of these findings suggest that the anaerobic digestion process can be either thermophilic [54,171] or psychrophilic, potentially reducing operational costs [181]. Research is being conducted on the diversity of these mixed microbial communities, for the optimization of biomethane production.

4. Biocatalysts for biofuel production

Considering the amount of substrates available for biofuel production together with the limitations faced in current production practices, researchers are continually driven to improve the various aspects of such existing technologies. Countless studies have reported the application of enzymes derived from microorganisms, particularly those from extremophiles, each specific with regards to its intended purpose.

4.1. Lipases

Lipolytic enzymes have gained increasing attention owing to their potential in biotechnological applications, particularly with respect to biodiesel production. During the organic synthesis of biodiesel, most lipases used for transesterification originate from organisms such as *Candida rugosa*, *Pseudomonas fluorescens*, *Rhizopus oryzae*, *Burkholderia cepacia*, *Aspergillus niger*, *Thermomyces lanuginosa* and *Rhizomucor miehei* [27]. As with the conventional method of biodiesel production, several factors are known to influence lipase-mediated biodiesel production, including the type of lipase employed, water content, temperature, type of alcohol and the ratio of alcohol relative to the oil used.

Based on their successful application on an industrial scale, lipases from *R. delemar*, *R. miehei*, *C. rugosa*, *C. lypolytica*, *K. oxytoca*, *P. camembertii*, *P. fluorescens* and *P. cepacia* are summarized in Table 1, indicating the respective oil/alcohol combinations employed for biodiesel production [27]. However, the continued drive to identify novel lipases, particularly those capable of activity under extreme conditions, has led to the identification of several cold-active lipases. The application of such psychrophilic enzymes to catalyse reactions at low temperatures offers enormous industrial potential; however, the requirement for continued research in this area is reflected in the current industrial status of such cold-active lipases within the biodiesel production process. For a very extensive review on these cold-active lipases, the reader is referred to Joseph *et al*. [182]. For additional reviews regarding lipases, the reader is referred to Pandey *et al*. [183], Jaeger and Eggert [184], Vakhlu and Kour [185] and Levisson *et al*. [186].

4.2. Lignocellulose-degrading enzymes

The pretreatment of lignocellulosic feedstocks includes several options including physical, physical-chemical and chemical treatment methods. However, the development of biological treatments is currently at the forefront of biofuel biotechnologies. Lignocellulose-degrading enzymes (including cellulases, xylanases, lignases, lignin peroxidases and manganese peroxidases) have been identified in a number of thermophilic organisms, including *Geobacillus* sp. R7, *Phanerochaete chrysosporium*, *Sporotrichum thermophile*, *Thermoascus thermophile* var. coprophile, *Chaetomium thermophile*, *Coniochaeta ligniaria*, *Clostridium thermocellum*, *C. stercorarium*, *C. thermolacticum*, *C. thermocopriae*, *C. thermopapyrolyticum* and *Thermotoga* spp. [9,187–195]. Several thermophilic fungi producing high levels of cellulase activities (endoglucanases, exoglucanases, cellobiohydrolase and β-glucosidases) include *Chaetomium thermophilum* [196], *Thermoascus aurantiacus* [197,198], *Talaromyces emersonii* [199–201], *Humicola insolens* [202,203], *Melanocarpus albomyces* [204] and *Humicola grisea* var. thermoidea [205–207]. Nevertheless, the golden standard as an industrial choice is the cellulases obtained from *Trichoderma reesi*, a consequence of its capacity to secrete significant amounts of cellulases and hemicellulases [12,208]. Despite this predominant choice, as research continues to identify novel thermophilic counterparts, these alternatives continue to show

Table 1. Bacterial lipases used for biodiesel production.

Lipase	Oil	Alcohol	Reference
Novozyme 435[1]	Soybean	Methanol	[221,35,222]
	Soybean	Methyl acetate	[28]
	Canola	Methanol	[223]
	Rice bran	Methanol	[32]
	Olive	Methanol	[36]
	Vegetable	Methanol	[224]
	Waste ABE	Methanol, ethanol, 1-propanol, 1-butanol, iso-butanol, iso-amylalcohol and n-octanol	[33]
R. delemar	Vegetable	Methanol	[224]
R. miehei	Vegetable	Methanol	[224]
	Palm	Methanol	[225]
C. rugosa	Waste ABE	Methanol, ethanol, 1-propanol, 1-butanol, iso-butanol, iso-amylalcohol and n-octanol	[33]
	Jatropha	Ethanol	[226]
C. lypolytica	Soybean	Methanol	[221]
K. oxytoca	Soybean	Methanol	[221]
P. camembertii	Soybean	Methanol	[221]
P. fluorescens	Soybean	Methanol	[221]
	Triolein	1-Propanol	[227]
	Jatropha	Ethanol	[226]
P. cepacia	Soybean	Methanol and ethanol	[221,33]
	Jatropha		[226]

[1]Novozyme 435 contains a *C. antarctica* B lipase immobilized on acrylic resin.

promise in their application on an industrial scenario. For extensive reviews on xylanases and cellulases, the reader is referred to Kulkarni *et al.* [209], Gilbert and Hazelwood [210], Beg *et al.* [211], Subramaniyan and Prima [212] and Collins *et al.* [213].

4.3. α-Amylases

Traditionally, starches were acid-hydrolysed to yield starch-derived glucose, largely utilized by industry for ethanol production through fermentation. However, the preference in modern-day practices leans toward the use of microbial enzymes. A leading choice in this regard is α-amylase, such as that obtained from thermotolerant bacteria like *Bacillus licheniformis.* This enzymatic degradation of starch occurs optimally at high temperatures (90–110 °C) [8,9], making thermophilic organisms prime targets for novel α-amylase identification and isolation. Subsequent to this initial step of starch degradation referred to as liquification, the resulting dextrine and glucose solution is subjected to glucoamylase (generally obtained from *Aspergillus niger* or *Rhizopus* spp.) at lower temperatures (60–70 °C), in a process known as saccharification, which is followed by fermentation (by *Z. mobilis* or *S. cerevisiae* at 30–32 °C) [8,9]. Other extremophilic organisms investigated for the ability to produce α-amylase include *Pyrococcus furiosus* [214], *Bacillus stereothermophilus* [215], *Bacillus acidoaldarius* [216] and *Alteromonas* spp. [217]. For an extensive review on amylases, the reader is referred to Pandey *et al*. [218], Gupta *et al.* [219] and Sivaramakrishnan *et al.* [220].

5. Conclusion

We have been in a troubled energy situation for the last few decades, but it is only recently that urgency in the development of commercially viable technologies for biofuel production has been realized. Most of these, however, have depended on food-grade or expensive raw materials. Economically, biofuels will not be able to replace the demand for fossil fuels unless lignocellulosic biomass and wastewater are used in the fermentation processes. The most important biological fuel products are bioethanol, biodiesel, biobutanol and biogas. Until recently, mesophilic engineered organisms or enzymes have been the preferred choices for the production of biofuels. This has mainly been due to a deep knowledge of metabolic pathways in the organisms as well as established genetic tools for engineering. However, in the last few years, alternative approaches have arisen in the use of extremophilic organisms and their enzyme products, owing to their robustness and versatility. A major obstacle in this approach is the lack of understanding of the

physiology and metabolic pathways of these extremophiles, as well as the lack of genetic tools for enzyme/organism enhancement by genetic engineering. With the development of genomics and other molecular biology tools, improved extremophilic-derived products are envisaged. One of the biggest challenges in the future will be the scaling-up process in a cost-effective manner. There is little doubt, however, that microbial biofuels will be successfully brought in to commercial production in the future, and that extremophiles will play a significant role in this.

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