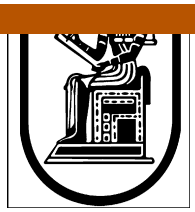
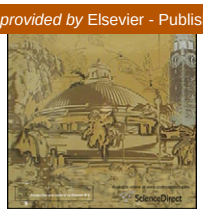
Journal of Advanced Research (2010) **1**, 103–111



Cairo University

Journal of Advanced Research



REVIEW

Microbiological aspects of biofuel production: Current status and future directions

Mostafa S. Elshahed *

Department of Microbiology and Molecular Genetics, 1110 S. Innovation Way, Room 226, Oklahoma State University, Stillwater, OK 74074, USA

Available online 6 March 2010

KEYWORDS

Biofuels;
Microbial fermentations;
Ethanol;
Biodiesel

Abstract Biofuel research is currently an area of immense interest due to the increase in global energy demand by emerging economies and the recent increases in global oil prices. Multiple approaches are currently being researched for the use of microorganisms in the production of various biofuel (e.g. alcohols, hydrogen, biodiesel, and biogas) from multiple starting materials. This review provides a brief overview on the research currently underway on laboratory and industrial scales in the area of biofuels, with specific emphasis on the economic viability of various approaches currently being utilized.

Introduction

Biofuel research aims at producing energy products such as alcohols (mainly ethanol, but also propanols and butanols, as well as propane and butane diols), diesel, hydrogen, and biogas from biological (mainly plant) sources. Research on the production of ethanol from plant materials started by German scientists as early as 1898, and continued in the United States during World War I. These processes involved the use of acidification to produce glucose from woods and subsequent fermentation by anaerobic microorganisms. During the same era, the ability of anaerobic microorganisms to ferment sugars to alcohols and ketones was documented and used not only in biofuel research, but also for the production of explosives during World War I. Research during the mid-twentieth century convincingly demonstrated the ability of various fungi and bacteria to degrade cellulose and other plant polymers. Such research was mainly of academic interest due to the presence of an abundant, secure, and inexpensive supplies of fossil fuels. The oil shock of 1973–1974, where dramatic increase in oil prices occurred, resulted in the intensification of the research in this area, and the exploration various avenues for its commercialization. It is currently an area of immense interest for scientists and policy makers due to the anticipated increase in global oil demand by the emerging economies of China and India, and the recent increase in global oil prices during the last two years. In addition, biofuels are also viewed as more environmentally friendly sources of energy since burning of biofuels (alcohols, hydrogen) produce much lower (if any) carbon emission to the atmosphere compared to burning of fossil fuels. Finally, the starting materials for biofuels (crops, perennial plant materials) are abundant in the United Sates and other industrially developed, oil-importing economies, and thus biofuel research is a politically correct issue in these societies and is seen as a way to minimize or eliminate the dependence of these countries on foreign oil.

This review provides a brief overview on the research currently underway on laboratory and industrial scales in the area of biofuels, with specific emphasis on the economic viability of various approaches currently being utilized. The subject is immense, rapidly evolving, and new discoveries are being reported on a daily basis. A collection of web-based biofuel databases has recently been com-

* Tel.: +1 405 744 3005; fax: +1 405 744 1112.
E-mail address: Mostafa@okstate.edu

doi:10.1016/j.jare.2010.03.001

piled in Wackett [1]. Also, a great, authoritative book that has recently been published by American Society for Microbiology is a must for biofuel researchers [2].

Alcohols as biofuels

Ethanol

In general, two main approaches are currently used in biofuel research aiming at alcohol production: direct fermentation and indirect fermentation. Direct fermentation depends on the conversion of various plant materials to biofuels, mainly ethanol. In principle, two processes are involved: the degradation of starting plant material into fermentable sugars, and the conversion of sugar to alcohol. Indirect fermentation is less commonly used, and depends on pyrolysis (burning) of the starting plant material, followed by the conversion of the produced gas (Syngas, a mixture consisting mainly of carbon monoxide, hydrogen, and carbon dioxide) to ethanol using acetogenic bacteria [3].

Direct fermentations

As mentioned earlier, direct fermentation starts with plant materials and converts it to ethanol. The process involves identification of starting plant material, isolation and development of bacterial and fungal strains, and design of appropriate protocols for efficient conversion of plant material to sugar monomers. Sugars are then converted to ethanol by yeasts or genetically engineered bacterial strains (see below). The first step, converting plant material to sugar is the most important and most active part of biofuel research. Various starting plant materials are available and each has a different composition, ranging from molasses from sugar cane, starch in corn kernels, as well as various forms and quantities of cellulose, hemicellulose, and lignin polymers in plant tissues. Therefore, different kinds of microorganisms, enzymes, incubation conditions, and engineering schemes are required for efficient depolymerization. In general, crop materials that are homogenous in nature are easily metabolized to sugars (e.g. molasses from sugar cane, starch from corn kernels). On the other hand, less expensive materials, e.g. crop residue, grasses, weeds, and other non-crop plants (collectively called lignocellulolytic material) are less expensive, but due to their heterogeneous nature (mixture of cellulose, hemicellulose, and lignin), are harder to degrade [4].

Depending on the starting material converted to sugars, biofuel research could be divided into two different generations. The first-generation biofuels use agricultural crops to produce simple sugars, which are subsequently converted to ethanol. The second-generation biofuels use specific native, perennially growing plants that require no cultivation, or entire prairie system flora for sugar and eventually ethanol production. The use of photosynthetic algae to produce biodiesel (see below) has often been referred to as the third-generation biofuels.

First-generation biofuels

Brazil uses sugar cane as an energy crop, and is currently the only country producing ethanol in a massive, economically competitive scale. In 2005, Brazil produced 3.8 billion gallons of ethanol, representing 40% of the country's fuel consumption in that year [5]. This is due to multiple reasons that are unique to this country: (1) early investments in this area starting in the 1970s have led to accumulation of immense research and industrial expertise. (2) The unique nature of sugar cane in which the product (sucrose) is not a polysaccharide but rather a disaccharide and so it does not require processing of complex polymeric plant molecules. (3) The availability of vast areas of extremely fertile land with ample rain that was initially part of the Amazon forest and has been cleared for huge sugar cane plantations. (4) The availability of cheap labor and close proximity of production sites to processing sites.

Due to the high percent of sucrose in sugar cane syrup, extraction of sucrose from sugar cane is a relatively simple process that requires no microbial or enzymatic treatment. In this process, sugar cane is chopped and milling is used to extract the sucrose-rich juice from sugar cane and the resulting juice is concentrated by evaporation and subjected to subsequent fermentation [6].

These ideal conditions for sugar production in Brazil are not available in the United States, Japan or other industrial countries. Availability of cheap labor and fertile land is a major problem. More importantly, sugar cane could not be cultivated in colder climates. For these reasons, the United States relies on corn, rather than sugar cane, and uses starch in corn kernels as a starting point for ethanol production. In this approach, corn kernels are separated from the chaff and milled to coarse flour. Production of sugars from this starch-rich flour is achieved using either a dry milling or a wet milling procedure. The technical details of these procedures are described elsewhere [6,7], but both involve the use of a glucoamylase enzyme to cleave starches and dextrins α-1,4-glucosidic linkages, which releases glucose and maltose for fermentation.

Few commercial ethanol-from corn plants are already starting to spring up in the United States. However, it has clearly been shown that ethanol produced from this route will always be a much more expensive alternative to oil. The only reason these commercial ethanol production plants are being built is the massive government subsidies. Moreover, a vast amount of land, more than the entire continent of North America, is needed to provide the ethanol the United State needs to substitute for oil. As such, ethanol from corn is more of a feel-good approach to stimulate local economies but provides very little practical alternative.

Second-generation biofuels

A huge backlash against using crops for energy has developed in 2008. The price of many food commodities has increased and it was blamed on farmers growing energy crops instead of food or animal feed crops. In addition the extensive use of fertilizers in the United States in 2008 to grow energy crops resulted in a huge environmental impact, e.g. the increase in fertilizer concentrations in natural streams, and increase in the dead zone in the Gulf of Mexico where the Mississippi river flows.

As a result, scientists now are looking to harvest energy from weeds and other plants that grow naturally on marginal, non-agricultural lands (A process that has been termed cellulosic ethanol production). Currently, there is a lot of emphasis on using crop residues, e.g. stover, straws, hulls, stems, and stalks [8] as well as common weeds present in the southern part of the United States as energy crops. For example the state of Oklahoma, USA launched a major initiative towards using switch grass, a common weed within the state, for ethanol production. Other US states (e.g. Minnesota) are looking into collectively using multiple plants within their tall grass prairie ecosystems as the feedstock for energy production (an approach that has been called low-impact high diversity or LIHD approach) [9]. The composition of these materials varies but, in general, the major polymers within lignocellulosic biomass is cellulose (35–50%), followed by hemicellulose (20–35%), and lignin (10–25%) [10].

Although more appealing than using crops for biofuel production, second-generation biofuels have their own drawbacks. Switch grass and other non-crop plants planned to be used are usually not native plants, but rather invasive species, or weeds. These plants, if cultivated or encouraged to spread, could conceivably destroy the entire ecosystem. Further, using these plants for ethanol production is much more difficult since they are more complex to degrade than better-studied energy crops [11].

Since the entire plant material is used, degradation of cellulose, hemicellulose, and lignin is required. Lignin (10–25% of plant biomass) has not yet been convincingly shown to be degraded anaerobically (ethanol is produced by fermentative or facultative bacteria only in the absence of oxygen) and is thus removed in pretreatment. Pretreatment is also required to increase the surface area of exposed cellulose and hemicellulose for microbial and/or enzymatic degradation. Various pretreatment approaches are used and include the use of alkaline peroxidases, concentrated acids, dilute acids, alkali, alkali peroxidases, wet oxidation, steam explosion, ammonia fiber explosion, liquid hot water, or organic solvent treatments. Interested readers should consult the excellent review by Wyman [12] on that subject.

Cellulose is a linear homopolymer of D-glucose units linked by 1,4-β-glucosidic bonds. The length of the chain usually ranges between 4000 and 8000 monomers. Efficient cellulose hydrolysis to glucose requires the concerted action of endo1,4-β-gluconase, exo-1,4-β-gluconase, and β-galactosidase. The first enzyme randomly attacks internal β-glucosidic bonds within the chain. The second enzyme removes cellobiose units from the non-reducing ends of the chain, and the third enzyme converts cellobiose to glucose. The presence of active cellulase systems (of all three enzymes) is widespread within the fungi, aerobic, and anaerobic bacteria [13]. Cellulase enzymes are either produced extracellularly, mainly in aerobic fungi, or produced as a complex structure called the cellulosome that is bound to the cell membrane in anaerobic bacteria (e.g. *Clostridia*), as well as members of the *Neocallimastigales*, anaerobic fungi present in the gut of rumens and other herbivores [14]. Currently, enzymes derived from the aerobic fungal genera *Trichoderma* and *Aspergillus* are most widely used in industrial settings [6].

Hemicellulose is a heteropolymer of pentoses, hexoses, and sugar acids. Xylans are the most common form of hemicellulose and are heteropolysaccharide with a backbone consisting of a relatively short chain (around 200 units) of 1,4-linked β-D-xylopyranose units. In addition, minor quantities of arabinose, glucuronic acid, and acetic, ferulic, and *p*-coumaric acids might be present in xylan. The exact composition of hemicellulose depends on its source. Enzymes required for the depolymerization of hemicellulose are collectively known as hemicellulases. The total degradation of xylan requires endo-β-1,4-xylanase, which attacks the main chain of xylans. Subsequently, β-xylodase degrades the produced xylooligosaccaharides produced to xylose. In addition, various accessory enzymes are required for the degradation of various additional components and substitutions within the xylan polymer.

The presence of the entire suite of enzymes capable of hemicellulose degradation within a single microorganism is less common than the presence of complete cellulase machinery. Nevertheless, several microorganisms are known to completely depolymerize hemicellulases (mainly xylans) to xylose. These include the fungi *Penicillum capsulatum* and *Talaromyces emersonii* [15], the thermophilic actinomycete *Thermomonospor fusca* [16], the hyperthermophile *Caldicellulosiruptor saccharolyticus* [17], and several other microorganisms (Uffen [18] provides a detailed review on that subject).

Conversion of sugars to alcohols

Regardless of the starting plant material, the degradation of starch, cellulose, or hemicellulose yields hexoses and pentoses that need to be fermented to ethanol. Multiple fermentation schemes are known to produce ethanol as one of the end products in the process, e.g. mixed acid fermentation by enteric bacteria, hetereolactic acid fermentation by some lactic acid bacteria, e.g. various *Leuconostoc* spp. However, for industrial purposes, ethanol needs to be the major end product. Two groups of microorganisms naturally produce 2 moles of ethanol per mole of hexose during fermentation. The yeast *Saccharomyces cerevisiae,* and members of the genus *Zymomonas*, e.g. *Z. mobilis*. In both microorganisms, pyruvate produced by the Embden–Meyerhoff (glycolytic) pathway in *S. cerevisiae* or Entner–Doudoroff pathway in *Z. mobilis* is converted to alcohol via pyruvate decarboxylase/alcohol dehydrogenase enzymes.

Conversion of hexoses to ethanol using *S. cerevisiae* is one of the best-studied and perfected industrial processes in ethanol production. The use of strains capable of efficient simultaneous uptake of multiple sugars (through genetic manipulation of sugar transporters), and directed laboratory evolution results in near stoichiometric production of ethanol from glucose [10]. The process could occur at high substrate levels, high turnover rate, and industrial strains can withstand relatively high levels of ethanol [19]. Further, strains growing in the presence of naturally occurring plant compounds that inhibit sugar fermentation, e.g. furfural and 5-hydroxyfurfural were obtained through engineering [20]. The availability of the genome sequences of *S. cerevisiae,* and the presence of a reliable genetic system for this microorganism allows for continuous genetic manipulations and strain improvements [21].

Xylose, a C5 sugar is eventually metabolized to pyruvate using the pentose phosphate pathway. Multiple microorganisms are naturally capable of xylose metabolism, including the yeast *Pichia stapis*, anaerobic fungi, and multiple groups of mesophilic and thermophilic anaerobic bacteria (e.g. several members of the order *Thermoanaerobacteriales*) [18]. However, since hemicellulose, the precursor of xylose is always present in plant material with cellulose (the precursor of glucose), a microorganism capable of efficiently and simultaneously metabolizing both sugars is needed. Due to the industrial strength and background knowledge working with *S. cerevisiae*, efforts were focused on introducing this ability into *Saccharomyces* strains. Through genetic engineering, strains that efficiently degrade xylose were obtained and shown to work well with pure substrates as well as sugars released by enzymatic treatment of plant materials [22–25].

Another approach, pioneered by Lonnie Ingram group at the University of Florida is to use genetically engineered *Escherichia coli* (and closely related enteric strains belonging to the genera *Klebsiella* and *Erwinia*) for alcohol production from hexose, pentoses, and enzymatically treated lingocellulosic materials. This research started in the 1980s with *Zymomonas* as a model microorganism. However, due to difficulties, e.g. temperature dependence of alcohol tolerance coupled to the ease of genetically manipulating *E. coli*, and the fact that *E. coli* could metabolize pentose sugars, the research shifted to inserting *Zymomonas* genes encoding alcohol production enzymes into *E. coli*. In a landmark paper, the Alcohol dehydrogenase and acetaldehyde decarboxylase enzymes were expressed in *E. coli* strain TC4 on PUC18 plasmid under the control of a *lac* promoter, and the resulting strain produced ethanol as the

principal fermentation product from glucose [26]. Multiple strains with varying degrees of environmental hardiness and ethanologenic capabilities have been developed since, and are being commercially tested for their abilities to metabolize pretreated lignocellulosic material for the production of ethanol, e.g. from sugarcane bagasse in southern Louisiana [27], and corn stover [8].

Consolidated bioprocessing

Within the biofuels industry, approaches to lower costs are highly desirable, since most of the cost is in the production, rather than the starting material stages. Consolidated biological processing refers to attempts for one step conversion of plant materials to biofuels using microbial agents, with no need of saccharolytic enzyme treatments. Such approach has long been recognized as the most promising way for making biofuel production more cost effective compared to first-generation biofuel schemes that are currently used commercially [28].

As recently stated: "Realization of the potential of ethanol production via CBP requires a microbe, or combination of microbes, able to rapidly utilize cellulose and other components of pretreated biomass while at the same time producing ethanol at high yield and titer" [29]. Aerobic fungi are capable of plant material degradation by a one step process. However, aerobic microorganisms produce CO_2 as the final end product rather than ethanol. This is because electrons produced are shuttled to the respiratory chain for oxidative phosphorylation rather than ethanol production via substrate level phosphorylation involved in fermentative pathways. Members of the *Neocallimastigales* (anaerobic fungi) represent a great yet untapped resource due to their combination of invasiveness, and ability to degrade plant materials fermentatively to various fermentation end products, including ethanol [30]. However, they are hard to grow and maintain, no genetic system for manipulation is yet available, and so far, all isolates produce ethanol only as a minor fermentation end product.

Most of the recent research on consolidated biological processing (CBP) has been pioneered by Professor Lee Lynd group at Dartmouth college. The group utilizes thermophilic gram-positive Firmicutes belonging to the orders *Clostridiales* and *Thermoanaerobiales.* The rate of cellulose metabolism is known to increase with temperature and thermophilic Clostridia (e.g. *Clostridium thermocellum*) has some of the highest cellulose degradation rates known [29]. In addition, many clostridia produce ethanol from the sugar produced from cellulose degradation. This dual polymer to sugar and sugar to ethanol ability within members of these two orders makes them ideal candidates for CBP. While promising, the amount of ethanol produced (on a w/v scale) rarely exceeds 5% in such schemes and an advanced, streamlined, economically sound CBP using thermophilic anaerobes have not yet been realized on a commercial scale.

Indirect fermentation approaches

A promising approach for the production of ethanol is indirect fermentation. In this approach, starting plant material is pyrolyzed (burned) to produce Syngas. Syngas, which consists primarily of CO, CO_2, and hydrogen, is converted to ethanol by acetogenic bacteria. Acetogens are strict anaerobic microorganisms that use C1 compounds in the Wood–Ljungdahl pathway to produce C_2 products, mainly acetate (hence the name acetogens) [31]. These strict anaerobic microorganisms are usually gram positive sporulating bacteria belonging to the class *Clostridia* within the phylum *Firmicutes*, although acetogenesis has been proven to occur in members of other phyla, e.g. the anaerobic *Spirochetes* [32]. In addition, many of the acetogenic Clostridia are also capable of anaerobic fermentation when grown on hexose sugars, producing various fermentation end products, e.g. acetate, butyrate, and ethanol.

The exact biochemical pathways and regulatory mechanisms involved in producing ethanol from Syngas are not completely understood. Presumably, ethanol production occurs as part of the Wood-Ljungdahl pathway where ethanol is produced instead of acetate [33]. A pH drop in the media usually results in shifting acetogenic fermentation from acetate to ethanol. Also, the higher alcohol:acid ratio observed in the presence of CO in the headspace suggests that CO results in the ability of acetogens to reduce acids to alcohols, coupled with the oxidation of CO to CO_2.

Using these approaches, scientists at the University of Oklahoma have been working on isolating acetogenic bacteria that are capable of producing high yields of alcohol from Syngas [34]. The process starts by isolation of acetogens from various sources (either on CO:H_2 headspace or using fermentable sugars). Isolates are then evaluated for their ability to produce ethanol and promising strains are continuously subcultured. Using directed laboratory evolution and careful assessment of the metabolic needs (cofactors, vitamins, and minerals), alcohol production could be increased, as well as the cell's tolerance to higher levels of alcohols produced in the medium, and relative tolerance to oxygen exposure. Such approaches resulted in the isolation of various *Clostridia* and *Moorela* strains with high ethanol production and tolerance that attracted commercial interest [34].

In principle, this indirect fermentation approach has several advantages. Any plant material, or even non-plant wastes that could be pyrolized could theoretically be used in such approach, since pyrolysis produces the same product (Syngas) (7). The approach makes use of all plant components, including lignin, which is generally not utilized in direct fermentation approaches, and can use mixed plant flora within a batch (e.g. using entire flora of an ecosystem in a LIHD approach [9]).

However, key technical difficulties still exist. These include relatively low growth rates and low product concentration in aqueous phase when compared to yeast fermentation [35]. In addition, the anaerobic *Clostridia* and *Moorela* sp. used in the process are very oxygen sensitive, metabolically fastidious, and, inspite of considerable improvements, still produce considerable amounts of acetate together with ethanol from Syngas. Such considerations, as well as the fact that only a fraction of the energy in plant materials is captured in the pyrolysis process, renders this approach as it stands today economically unattractive. However, it is assumed that continuous steady improvements in the properties of the microbial agents utilized as well as in the engineering process will bring the cost of such process down to economic feasibility, without the need for any new breakthrough discoveries. Indeed, an American company (Coskata Corp.) has already committed to building an ethanol from Syngas plant in the United States.

Longer chain alcohols as biofuels

Historically, ethanol has been the biofuel product of choice. This is mainly due to the accumulated wealth of knowledge regarding the biochemistry, physiology, and industrial aspects of its production, mainly from the food and beverage industries. However, it could technically be argued that ethanol is not the best compound to be used for biofuel. For example, the water solubility of ethanol makes it less suited for pipeline transport, and easier to be watered down. In

addition, the energy content of ethanol is approximately two-thirds that of an equivolume of a standard petroleum mix, as opposed to 86% for longer chain alcohols [36].

For these reasons, multiple researchers and start up companies are now eying C3–C5 normal and branched alcohols as alternative biofuel molecules. They are less water soluble, with higher energy contents and are clean burning molecules. Several anaerobic microorganisms, e.g. *Clostridium acetobutylicum* have long been known for their ability to produce butanol, isobutanol, and propanols as products of sugar metabolism [37–39]. However, these products, usually produced during sporulation phase, constitute a minor fraction of the substrate utilized, and blocking of multiple pathways for production of other enzymes are often required to enhance the yield of these microorganisms.

In a recent breakthrough, researchers at University of California, Los Angeles (UCLA) used several modifications in the amino acid production pathways in *E. coli* to produce alcohols (n-propanol, n-butanol, isopropanol, 2-methyl-1-butanol, and 3-methyl-1-butanol) from *E. coli* [40–43]. These exploitations of non fermentable pathways for the production of C3–C5 alcohols represent a major discovery, since 86% of the theoretical alcohol yields from glucose has already been reported, far better than those reported by natural fermentative pathways. The approach has already attracted funding from multiple start up companies.

In addition to direct fermentation, C3 and C4 alcohols could theoretically be produced via indirect fermentation, and there is an increasing interest in exploring the possibility of producing butanol from Syngas. However, so far, the amount produced appears to be a minor product, compared to ethanol and acetate produced by such fermentations, and selection of microorganisms capable of higher levels of butanol production is underway [35].

Biodiesel as biofuel

Biodiesel is defined as non-petroleum-based diesel fuel consisting of alkyl esters (mainly methyl, but also ethyl, and propyl) of long chain fatty acids. Biodiesel could be produced from various animal and plant sources by esterification of triglycerides with methanol [44]. In addition, biodiesel could be produced from various species of microalgae [45]. Research on biodiesel from algae has been funded in US national laboratories through the aquatic species program, launched in 1978 and sponsored by the department of energy. The production of biodiesel from microalgae has multiple advantages and has been termed the third-generation biofuels [36]. Unlike other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. Microalgae commonly double their biomass within 24 h, and biomass doubling times during exponential growth are commonly as short as 3.5 h. Oil content in microalgae can exceed 80% by weight of dry biomass [46,47], and oil levels of 20–50% are quite common. An excellent review on this topic has recently been published [45].

Most importantly, due to their photosynthetic nature, autotrophic algae do not compete with starting plant materials for biofuel production. On the contrary, algae fix and thus reduce the amount of CO_2 in the atmosphere, a gas that contributes to the process of global warming. In fact, few start up companies are now experimenting with the idea of harvesting carbon dioxide streams emitted from coal plants for the autotrophic, photosynthetic growth of microalgae [36].

In addition, research is currently being conducted in using heterotrophic algae for biodiesel production using sugars as substrates [46,47]. Heterotrophic algae have the advantage of achieving much higher growth densities (and hence biodiesel concentrations) when compared to phototrophic algae. In addition, dark growth of heterotrophic algae poses no engineering challenge when compared to phototrophic algae. However, the process requires starting plant materials as substrates and the overall economic viability of the process is currently being researched.

It is envisioned that algae could be grown to generate biodiesel in dedicated artificial ponds. However, the economics of this process is still uncertain. While the microbiological aspects of the process are extremely promising, the engineering aspects pose the most challenge. The main engineering problem currently is the cost of collection and harvesting. Algae grow as a thin surface layer in ponds, so harvesting miles and miles of growth to get large amounts of biodiesel is needed. Huge ponds are required to grow microalgae in quantities that make the process commercially feasible. Growing of microalgae in natural lakes or ocean shores has been proposed. However the invasiveness of algae could present an environmental hazard, since the grown algae will destroy and overtake the ecosystem. Nevertheless, plenty of research funded by various US national agencies, as well as multinational oil companies and start up biotechnology companies is underway and aims at making algal biodiesel a significant fraction of the diesel used in the transportation in the next twenty years.

Biohydrogen as biofuel

Hydrogen is the cleanest of biofuels since it is oxidized to water, with no emission of carbon dioxide in the process. As such, hydrogen is a very popular biofuel with policy makers, and hydrogen-fueled concept cars are currently being produced and displayed by car companies to bolster their environmental credentials. Few hydrogen stations for refueling such cars are now present in large US cities. However, the bulk of hydrogen produced currently is derived from chemical modification of fossil fuels, e.g. oil and coal, rendering hydrogen-powered cars as responsible for carbon emissions as gasoline-powered cars, albeit in an indirect way.

Biohydrogen production offers an appealing alternative. Hydrogen has long been known to be produced as a final end product of fermentation or a side product in photosynthesis in multiple groups of microorganisms, and a vast body of literature is available regarding the properties, activities, structure, and kinetics of hydrogenase enzymes (enzymes that produce or consume hydrogen) in microorganisms [48,49]. Therefore, it is natural to envision exploiting this process for large-scale biohydrogen production. The US department of energy is currently funding a hydrogen initiative with the aim of developing processes to the point where they would be commercially feasible.

Three main processes are the focus of current biohydrogen production research. The most direct approach involves using photosynthetic microorganisms, e.g. *Cyanobacteria* and Green algae for biohydrogen production. Photosynthetic microorganisms have the ability to split water, i.e. produce electrons and oxygen from one molecule of water using sunlight as an energy source. The produced electrons are used for energy production through electron transport chain, as well as biomass production and sugar production using anabolic reactions (Calvin Benson cycle). However, they could also be converted to hydrogen by the action of hydrogenase enzymes. The appeal of this system is that it uses water as a substrate, and sunlight as an energy source, and for both of these precursors, a free, inexhaustible supply is present. Therefore, in principle, this

approach is extremely promising for low-cost hydrogen production [50].

However, a major problem is the extreme oxygen sensitivity of hydrogenases involved in hydrogen production. Therefore the two processes (photolysis and hydrogen production) need to be temporarily uncoupled. This crucial problem is not yet solved, and no commercial application of this approach has yet been announced. A proposed practical scheme to overcome this issue is to implement a two step process in which the microorganisms are incubated in aerobic conditions under light to stimulate oxygenic photosynthesis, then are transferred to oxygen limiting and/or dark conditions to induce hydrogenase activity and hydrogen production [50].

The second approach uses nitrogenase enzymes in anoxygenic photoheterotrophic microorganisms (the purple nonsulfur bacteria) for hydrogen production. The function of nitrogenase is to fix atmospheric N_2 gas to ammonia to be incorporated in cells biomass, thus enabling nitrogen-fixing microorganisms to grow in the absence of organic or inorganic nitrogen sources in growth media. However, nitrogenase enzymes are also capable of producing hydrogen from electrons and protons in the absence of oxygen and presence of light. When grown in the light and in absence of oxygen, purple non-sulfur bacteria can obtain adenosine triphosphate (ATP) and electrons through cyclic anoxygenic photosynthesis, and carbon from organic substrates. Electrons extracted from organic substrates could be used for hydrogen production using nitrogenase enzymes. This photoheterotrophic versatility of purple non-sulfur bacteria makes it theoretically possible to divert 100% of the electrons produced during carbon metabolism to hydrogen production, since electrons required for anabolic, biosynthetic reactions could be obtained via photosynthesis. Research on this approach has been conducted by Caroline Harwood group at the University of Washington using *Rhodopseudomonas palustris* as a model purple non-sulfur bacterium [51] and via additional genetic manipulations, a strain of *R. palustris* capable of producing 7.5 ml of hydrogen/liter of culture has been obtained, and initial engineering designs have been proposed [52].

The third approach is the production of hydrogen by fermentative bacteria. This approach uses organic substrates, e.g. sugar, lingocellulosic biomass, industrial, residential, and farming waste for anaerobic fermentation. Several groups of microorganisms are known to produce hydrogen as an end product of fermentation, e.g. *E. coli, Enterobacter aerogenes*, and *Clostridium butyricum*. In addition, mixed culture inocula, e.g. microorganisms in sludge have recently been utilized to produce hydrogen from waste materials. These "dark fermentation" reactions do not require light energy, so they are capable of constantly producing hydrogen from organic compounds throughout the day and night. However, production of hydrogen is only one of several electron sinks employed by fermentative microorganisms, since other fermentation end products are produced beside hydrogen [53]. It is estimated that only 15% could be diverted in anaerobic fermentations for hydrogen production [54].

Inspite-of the microbiological, engineering, and design improvements in all three areas of biohydrogen production [53], it does not appear that commercial, wide scale hydrogen use, especially in transportation is on the horizon. Due to its lower energy, large compressed tanks are needed for storage, which could be expensive and hazardous. A large infrastructure is also needed for supplying and adapting various energy-consuming economic activities to a hydrogen-based economy. This is a huge disadvantage when hydrogen is compared to alcohols and biodiesels, both of which could be transferred and utilized using existing infrastructure for fossil fuel products.

Biogas as biofuels

Biogas, a mixture of methane and carbon dioxide, is produced from the methanogenic decomposition of organic waste under anaerobic conditions [55]. Biogas production could be achieved by a defined culture of a fermentor and/or syntroph in association with an aceticlastic (acetate degrading) and hydrogenotrophic (hydrogen-consuming) methanogen. In addition, undefined cultures (e.g. microorganisms in cow dung or waste water sludge) could be used as an inoculum for biogas production [56–58]. The thermodynamics, kinetics, and nature of syntrophic cooperation of these processes have extensively been investigated, as well as the various biochemical pathways for fermentation of fatty acids and methane production. The work by Schink [59] provides a comprehensive/review of the topic.

Currently, cost efficiency renders wide scale usage of biomass unfeasible. Natural gas, the fossil fuel competitor of biogas is currently very cheap ($3.60/MMBTU, March 2009), even at its highest level (13.5 MMBTu, July 2008) when compared to biogas (1 MMBtu = 28.263682 m^3 of natural gas at defined temperature and pressure). Also, natural gas is a relatively clean burning fuel. The United States have large reserves of natural gas, and other developed countries have developed pipelines and agreements for purchasing natural gas (e.g. Western European countries from Russia). As such, the need for biogas on a large scale is minimal.

However, on a local level, biogas could be and is currently used and exploited. For example, biogas-producing facilities, e.g. waste water treatment plants and landfills can use biogas produced during operation for running the plant, thus becoming energy neutral. The use of biogas on a local, residential scale could be exploited in the countryside of developing countries. India had great success in using biogas produced in pits associated with rural homes with no utilities connected for generation of biogas for cooking and electricity [56]. Cow dung was used as an inoculum in this effort. Such approach is currently being considered in Egypt for the treatment of rice straw and other low-nutrient agricultural waste that could not be fed to feedstock and is currently burned. Such burning practice is partly responsible for the "black cloud" phenomenon that has been periodically observed in Egypt in the past few years.

Use of microorganisms and microbial products for more efficient recovery of fossil fuel from existing oil and natural gas formations

All of the approaches described above produce fuels using biological agents (microorganisms), and mostly from biological sources (plant materials). Another potential use of microorganisms is to enhance the production of fossil fuels in existing oil and natural gas formations. As such, the product is not truly a biofuel since it is not produced from biological matters, but rather a biologically based approach for extracting conventional fossil fuels.

The economics of such approach is straightforward and appealing. Simply, if the cost of implementing a specific process is lower than the revenue obtained from selling the additionally recovered product, then the process is deemed economically sound. Therefore, the appeal of such processes is very dependent on changes in oil prices. These processes are usually used in oil wells where production is declining, or only recently ceased to occur. As such, all the infrastructure, transport, and marketing issues are usually in place for selling the additionally produced fossil fuel.

It is important here to differentiate between two interrelated approaches are described here: microbially enhanced oil recovery (MEOR), and microbially enhanced energy recovery (MEER). In MEOR, microorganisms and/or their products are introduced into oil formation to increase the production in oil wells that are in the tertiary stage of production and where level of oil production has decreased to a level that render the extraction process economically unattractive. Examples include injection of biosurfactant and/or biosurfactant producing bacteria into the formation to decrease oil water interfacial tension and improve oil recovery [55], as well as injection of acid- and gas-producing microorganisms to recover oil entrapped in carbonate formations. The reader is referred to a recently published comprehensive review on this subject [60].

In MEER, microorganisms capable of a specific transformation process are injected into the formation, to bring change in the fuel chemistry in situ, allowing more efficient energy recovery. Examples include injection of methanogenic consortia capable of anaerobic biodegradation of various hydrocarbons into oil or natural gas reservoirs to recover unrecoverable and/or recalcitrant compounds as methane [61], and exploring mechanisms of stimulating microorganisms originally present in petroleum formation to produce methane from natural gas [62]. A holy grail of the MEER research is developing a mechanism to stimulate methanogenesis in the vast coal formations in the United States, where many of the coal is unrecoverable or too dirty to be utilized under current environmental regulations. Extensive amounts of private money and leading world scientists are working on this issue. Although encouraging reports on the issue has recently been published [63,64], no known microorganism or consortia that could convincingly and reproducibly transform coal anaerobically to methane has yet been obtained.

Concluding remarks

The current research thrust in biofuel research appears to be a long-term sustainable effort and is conducted on previously unprecedented levels. In addition to governmental financial backing, funding from huge multinational oil companies, e.g. British Petroleum (BP) [65] and multiple venture capitalists around the world will sustain the efforts for future times to come [36]. The current level is a reflection of the recent realization that sooner or later the world will run out of fossil fuels and this will coincide with an explosion in the demand for energy due to dramatic increase in standards of living in the world two most populous countries: China and India. As such, research advances and discoveries will continue regardless of temporal fluctuations in oil prices.

Most probably, a single solution, approach, or standardized procedure for bioenergy production will not be the outcome of such research effort. Rather, a slow step-by-step advances on multiple fronts will occur, and the final scheme for biofuel production will be a combination of approaches. Currently, biodiesel production from algae seems to be the closest technology to economic viability, with the hurdles still to overcome being engineering, rather than biological hurdles [45]. A lot of progress is also being made in the production of ethanol, propanol, and butanols from lignocellulosic materials both in the polymer to sugar [66], and sugar to alcohol [40–42] phases, as well as in consolidated biological processing schemes [8]. On the other hand, commercial production of biohydrogen is not foreseeable within the next decade.

The choice of the bioenergy approach to use in a specific country/community will eventually depend on the energy needs (electricity, transportation fuel, and heating gas), flora (agricultural, grass, and forest), and political considerations. No doubt, the total global annual production of biofuels will continue to steadily climb in the near future, but these increases will be uneven, and yearly changes will still be correlated to fluctuations in oil prices, as well as political considerations and election results in developed countries. Nevertheless, the global energy landscape will be significantly different within the next two decades.

Will biofuel production completely replace oil and natural gas, become the main source of energy, and bankrupt oil and natural gas-producing countries? The answer is most certainly not. Oil and natural gas production costs continue to be exceptionally low compared to biofuel production, which continue to rely on government subsidies. Inspite of the fact that many countries, e.g. Iran, Venezuela, and Egypt have passed their peak fossil fuel production point [67], the gloom about lack of newer oil discoveries, and the increasing cost of production of oil from mature uneasily accessed reservoirs, oil production prices continue to be extremely inexpensive in many areas. An unofficial estimate puts the cost of producing one barrel of oil from the oil fields of Saudi Arabia at $2/barrel (159 liters), and around $28 from the vast Canadian tar sands reserves. Similarly, costs of natural gas production in Russia, or even within the United States (e.g. within the Barnett shale in north central Texas) is still low, and emerging engineering technologies (e.g. horizontal well drilling) continue to drive the cost lower or make it feasible in previously inaccessible formations. Therefore, biofuels will be an important future supplement for fossil fuel energy rather than the sole source of energy within the near and intermediate future.

Acknowledgments

Funding for biofuel research in my laboratory has been funded by the Oklahoma Bioenergy Center, and NSF EPSCoR award EPS 0814361. I thank Dr. Noha Youssef for helpful scientific input and for editing this manuscript.

References

[1] Wackett LP. Microbial ethanol for fuel and food. Environ Microbiol 2008;10(1):278–9.
[2] Wall JD, Harwood CS, Demain AL. Bioenergy. Washington DC: ASM Press; 2008.
[3] Klasson KT, Ackerson MD, Clausen EC, Gaddy JL. Bioconversion of synthesis gas into liquid or gaseous fuels. Enzyme Microb Technol 1992;14(8):602–8.
[4] Outlaw JL, Collins KJ, Duffield JA. Agriculture as a Producer and Consumer of Energy. Oxford, UK: CABI Publishing; 2005.
[5] Baez Vasquez MA, Demain AL. Ethanol, biomass and clostridia. In: Harwood CS, Demain AL, Wall JD, editors. Bioenergy. Washington, DC: ASM Press; 2008.
[6] Nichols NN, Dien BS, Monceaux DA, Bothast RJ. Production of ethanol from corn and sugarcane. In: Harwood CS, Demain AL, Wall JD, editors. Bioenergy. Washington, DC: ASM Press; 2008.
[7] Gulati M, Kohlmann K, Ladisch MR, Hespell R, Bothast RJ. Assessment of ethanol production options for corn products. Bioresour Technol 1996;58(3):253–64.
[8] Lau MW, Dale BE. Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A(LNH-ST). Proc Nat Acad Sci USA 2009;106(5):1368–73.
[9] Tilman D, Hill J, Lehman C. Carbon-negative biofuels from low-input high-diversity grassland biomass. Science 2006;314(5805):1598–600.

[10] Liu ZL, Saha BC, Slininger PJ. Lignocellulosic biomass conversion to ethanol by Saccharomyces. In: Harwood CS, Demain AL, Wall JD, editors. Bioenergy. Washington, DC: ASM Press; 2008.
[11] Raghu S, Anderson RC, Daehler CC, Davis AS, Wiedenmann RN, Simberloff D, et al. Adding biofuels to the invasive species fire? Science 2006;313(5794):1742.
[12] Wyman CE. Ethanol from lignocellulosic biomass: technology, economics and opportunities. Bioresour Technol 1994;50(1):3–15.
[13] Wilson DB. Three microbial strategies for plant cell wall degradation. Ann NY Acad Sci 2008;1125:289–97.
[14] Doi RH. Cellulases of mesophilic microorganisms: cellulosome and non-cellulosome producers. Ann NY Acad Sci 2008;1125:267–79.
[15] Filho EXF, Tuohy MG, Puls J, Coughlan MP. The xylan-degrading enzyme systems of *Penicillium capsulatum* and *Talaromyces emersonii*. Biochem Soc Trans 1991;19(1):25S.
[16] Bachmann SL, McCarthy AJ. Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Appl Environ Microbiol 1991;57(8):2121–30.
[17] Van De Werken HJG, Verhaart MRA, VanFossen AL, Willquist K, Lewis DL, Nichols JD, et al. Hydrogenomics of the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus*. Appl Environ Microbiol 2008;74(21):6720–9.
[18] Uffen RL. Xylan degradation: a glimpse at microbial diversity. J Indust Microbiol Biotechnol 1997;19(1):1–6.
[19] Walker GM. Yeast growth. In: Walker GM, editor. Yeast: Physiology and Biotechnology. New York: Wiley; 1998.
[20] Klinke HB, Thomsen AB, Ahring BK. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pretreatment of biomass. Appl Microbiol Biotechnol 2004;66(1):10–26.
[21] Goffeau A, Barrell G, Bussey H, Davis RW, Dujon B, Feldmann H, et al. Life with 6000 genes. Science 1996;274(5287):546–67.
[22] Ho NWY, Chen Z, Brainard AP. Genetically engineered Saccharomyces yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 1998;64(5):1852–9.
[23] Sedlak M, Ho NWY. Production of ethanol from cellulosic biomass hydrolysates using genetically engineered Saccharomyces yeast capable of cofermenting glucose and xylose. Appl Biochem Biotechnol 2004;114(1–3):403–16.
[24] Wouter Wisselink H, Toirkens MJ, Wu Q, Pronk JT, Van Maris AJA. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Appl Environ Microbiol 2009;75(4):907–14.
[25] Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, Van Dijken JP, Pronk JT. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 2005;5(10):925–34.
[26] Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF. Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 1987;53(10):2420–5.
[27] Luli GW, Jarboe L, Ingram LO. The development of ethanologenic bacteria for fuel production. In: Wall JD, Harwood CS, Demain AL, editors. Bioenergy. Washington, DC: ASM Press; 2008.
[28] Lynd LR, Van Zyl WH, McBride JE, Laser M. Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 2005;16(5):577–83.
[29] Lynd LR, Currie D, Ciazza N, Herring C, Orem N. Consolidated bioprocessing of cellulosic biomass to ethanol using thermophilic bacteria. In: Wall JD, Harwood CS, Demain AL, editors. Bioenergy. Washington, DC: ASM Press; 2008.
[30] Bauchop T. Biology of gut anaerobic fungi. Biosystems 1989;23:53–64.
[31] Müller V. Energy conservation in acetogenic bacteria. Appl Environ Microbiol 2003;69(11):6345–53.
[32] Leadbetter JR, Schmidt TM, Graber JR, Breznak JA. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 1999;283(5402):686–9.
[33] Drake HL. Acetogenesis. New York: Chapman and Hall; 1994.
[34] Liou JSC, Balkwill DL, Drake GR, Tanner RS. *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon and reclassification of the acetogen *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. nov. Int J System Evolut Microbiol 2005;55(5):2085–91.
[35] Tanner RS. Production of ethanol from synthesis gas. In: Wall JD, Harwood CS, Demain AL, editors. Bioenergy. Washington, DC: ASM Press; 2008.
[36] Tollefson J. Energy: not your father's biofuels. Nature 2008;451:880–3.
[37] Formanek J, Mackie R, Blaschek HP. Enhanced butanol production by *Clostridium beijerinckii* BA101 grown in semidefined P2 medium containing 6 percent maltodextrin or glucose. Appl Environ Microbiol 1997;63(6):2306–10.
[38] Jones DT, Woods DR. Acetone-butanol fermentation revisited. Microbiol Rev 1996;50(4):484–524.
[39] Lin YL, Blaschek HP. Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Appl Environ Microbiol 1983;45(3):966–73.
[40] Atsumi S, Hanai T, Liao JC. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 2008;451(7174):86–9.
[41] Hanai T, Atsumi S, Liao JC. Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl Environ Microbiol 2007;73(24):7814–8.
[42] Shen CR, Liao JC. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab Eng 2008;10(6):312–20.
[43] Atsumi S, Liao JC. Directed evolution of Methanococcus jannaschii citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. Appl Environ Microbiol 2008;74(24):7802–8.
[44] Fukuda H, Kondo A, Noda H. Biodiesel fuel production by transesterification of oils. J Biosci Bioeng 2001;92(5):405–16.
[45] Chisti Y. Biodiesel from microalgae. Biotechnol Adv 2007;25(3):294–306.
[46] Metting Jr FB. Biodiversity and application of microalgae. J Indust Microbiol Biotechnol 1996;17(5–6):477–89.
[47] Spolaore P, Joannis Cassan C, Duran E, Isambert A. Commercial applications of microalgae. J Biosci Bioeng 2006;101(2):87–96.
[48] Vignais PM, Billoud B. Occurrence, classification and biological function of hydrogenases: an overview. Chem Rev 2007;107(10):4206–72.
[49] Vignais PM, Colbeau A. Molecular biology of microbial hydrogenases. Curr Issues Mol Biol 2004;6(2):159–88.
[50] Prince RC, Kheshgi HS. The photobiological production of hydrogen: potential efficiency and effectiveness as a renewable fuel. Crit Rev Microbiol 2005;31(1):19–31.
[51] Rey FE, Heiniger EK, Harwood CS. Redirection of metabolism for biological hydrogen production. Appl Environ Microbiol 2007;73(5):1665–71.
[52] Gosse JL, Engel BJ, Rey FE, Harwood CS, Scriven LE, Flickinger MC. Hydrogen production by photoreactive nanoporous latex coatings of nongrowing *Rhodopseudomonas palustris* CGA009. Biotechnol Prog 2007;23(1):124–30.
[53] Hallenbeck PC, Ghosh D. Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol 2009;27(5):287–97.
[54] Angenent LT, Karim K, Al Dahhan MH, Wrenn BA, Domíguez Espinosa R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 2004;22(9):477–85.
[55] Youssef N, Simpson DR, Duncan KE, McInerney MJ, Folmsbee M, Fincher T, et al. In situ biosurfactant production by Bacillus strains injected into a limestone petroleum reservoir. Appl Environ Microbiol 2007;73(4):1239–47.
[56] Singh BP, Panigrahi MR, Ray HS. Review of biomass as a source of energy for India. Energy Sources 2000;22(7):649–58.
[57] Somayaji D, Khanna S. Biomethanation of rice and wheat straw. World J Microbiol Biotechnol 1994;10(5):521–3.
[58] Taniguchi M, Tanaka M, Matsuno R, Kamikubo T. Evaluation of chemical pretreatment for enzymatic solubilization of rice straw. Eur J Appl Microbiol Biotechnol 1982;14(1):35–9.
[59] Schink B. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 1997;61(2):262–80.

[60] Youssef N, El Shahed MS, McInerney MJ. Microbial processes in oil fields: culprits, problems and opportunities. Adv Appl Microbiol 2009;66:141–251.
[61] Gieg LM, Duncan KE, Suflita JM. Bioenergy production via microbial conversion of residual oil to natural gas. Appl Environ Microbiol 2008;74(10):3022–9.
[62] Grigoryan A, Voordouw G. Microbiology to help solve our energy needs: methanogenesis from oil and the impact of nitrate on the oil-field sulfur cycle. Ann NY Acad Sci 2008;1125:345–52.
[63] Strapoć D, Picardal FW, Turich C, Schaperdoth I, Macalady JL, Lipp JS, et al. Methane-producing microbial community in a coal bed of the Illinois Basin. Appl Environ Microbiol 2008;74(8):2424–32.
[64] Volkwein JC, Schoeneman AL, Clausen EG, Gaddy JL, Johnson ER, Basu R, et al. Biological production of methane from bituminous coal. Fuel Process Technol 1994;40(2–3):339–45.
[65] Kintisch E. BP bets big on UC Berkeley for novel biofuels center. Science 2007;315(5813):747.
[66] Alriksson B, Rose SH, Van Zyl WH, Sjöde A, Nilvebrant NO, Jönsson LJ. Cellulase production from spent lignocellulose hydrolysates by recombinant *Aspergillus niger*. Appl Environ Microbiol 2009;75(8):2366–74.
[67] Hirsch RL, Bezdek RH, Wendling RM. Peaking oil production: sooner rather than later? Issues Sci Technol 2005;21(3): 25–30.