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Review Article

Making Biofuels Competitive: The Limitations of Biology for Fuel Production

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Abstract

Our main energy consumption is based on the use of non-sustainable sources (fossil fuels). The use of biological entities utilizing renewable (biomass) is one way to provide energy rich compounds and molecules for fuels, with emphasis on liquid fuels. Their utilization could substitute for a part of our present use of fossil fuels with the added attractiveness that they have the potential to be sustainable. Bio based fuels have been classified as first, second and third generation fuels. First generation biofuels are already produced on a large scale, mainly bioethanol and biodiesel. The present manufacture of both compounds is regulation driven and subsidized. Second generation biofuels are based on cellulose or waste and are still mainly in research and development phases. The first large scale facilities were launched in 2014. Pretreatment of the lignocellulose and the complexity of waste as a substrate are the economic impediment for these raw materials. Third generation biofuels are higher alcohols (butanol), hydrogen, other drop in fuels (hydrocarbons, isoprenoids) and the use of algae with CO_2 as carbon and light as energy source. The compounds produced by algae that are of interest as biofuels are fatty acids for biodiesel production or ethanol. Except for bioethanol produced in Brazil, which is based on sugar cane as substrate, and during periods of positive economics, these processes do not allow free competition with non-sustainable energy sources. It is important to have a holistic view at the whole life cycle of the processes in order to develop a competitive alternative fuel. The benchmark is economic competiveness compared with conventional fuels. There are many intrinsic limitations for implementation of economical biofuel production one of which is thermodynamics. However, with these intrinsic limitations continued research should be able to give novel and creative solutions to solve the problem of economical competitiveness. The evaluation of the thermodynamical feasibility of the possible pathways is a first requirement. Novel pathways of cellular energetics, related to what is thermodynamically possible need to be developed. The recent decrease in price for fossil fuels, the development of novel sources (i.e. shale gas) has had a very negative effect on the progress in the biofuels manufacture and marketing. Recent standards for volumes to be reached in the coming years are decreasing both in Europe and in the USA.

ABBREVIATIONS

LCA: Life Cycle Analysis

INTRODUCTION

The main drivers for development of alternative energy sources are climate change, the finite amount of fossil fuels and the desire to be energy independent. One of the major forms of energy is in its liquid form. Liquid energy is the most versatile and useful kind of energy which is transportable, with the potential for high energy density and flexibility in use. The need for liquid fuels is especially evident for aviation purposes. One approach to make substitutes for conventional liquid fuels is the use of biomass as the source for liquid energy manufacture. It is the solar energy as fixed by the primary producers (plants and algae) by photosynthesis that is utilized. The choice of the photosynthetic entity to be the source for energy production as well as method and product to be generated varies. This may depend on geographical location and conditions that allow for "economical" manufacture of a specific energy rich compound.

Until today the main manufacture of alternative liquid fuels are **first generation biofuels**, such as ethanol and biodiesel, produced at large volumes. Global production of ethanol for 2014 was estimated at 14-340 million gallons [1] and for biodiesel the projection for 2012 was 5,670 million gallons [2]. These production volumes are a result of regulations and subsidies adopted by various governments. The products would not be able to compete with conventional fuels on an equal basis on the free market. The energy balance for their production, which should be positive (i.e. get more energy out of the fuel than is invested to produce it) is dependent on the substrate, method of production, recovery pathway and eventual further chemical modification required to obtain the desired product.

The **second-generation biofuels** are still in development, even though some industries have gone online with large-scale facilities during 2014. In the report of the National Research Council of National Academies [3] it is stated that "calculations showed that the prices that suppliers are willing to accept to break even exceed the prices that biofuel processors are willing to pay". The utilization of lignocelluloses, the main substrate for second generation biofuels, is technologically possible, but economically unfeasible; with the result that lignocellulose as a source for biofuels is still not a major contributor to the biofuels market.

Third generation biofuels are still in the research stage, and comprise higher alcohols and other more novel energy rich compounds (hydrocarbons, isoprenoids etc.) as well as the algal products.

Criticism of biofuels usually emphasizes land use change, virtual water use, balances for carbon and energy, competition with food supplies, however, it is also important to include the intrinsic limitations of the biological processes to be used for the manufacture of biofuels.

In this review intrinsic biological limitations are scrutinized, to allow for better control of which process to develop for large-scale production. This is valid for all three generations of biofuels. The limitations of biology must take into account the economic, environmental and social soundness for production of very high volumes of low value compounds. Biology (Biotechnology) is the basis for a large industry for production of both high volume-low value commodities and more specialty compounds (low volume – high value), all economically viable. The main issue is, if we really can make large volumes of biofuel (or energy rich biofuels) energy, utilizing biology and do it environmentally and socially sound and also economical? In this contribution liquid fuel for transport is the main issue to be analyzed. The volumes of energy used globally on a daily basis are enormous, in 2013 90.35 million barrels were consumed per day (a barrel being 159 liters) and it is forecast to reach over 94 million barrels per day for 2016 [4].

There is great awareness of the necessity to develop alternative fuels [5]. Many countries have stipulated goals for the volumes of alternative energy of the total to be produced in the future relying on the industry to advance towards these production goals. However, the industry relies on the stability in the forecasts to enable them to invest in a costly infrastructure to allow them to reach the regulatory authorities preset goals. The possibility to maximize the outcome of biological processes with novel solutions is an important parameter to allow for the stability of these processes, independent of which generation of biofuels is considered. The fluctuations in the conventional energy sources and the recent expanded capabilities in volumes of natural gas and shale gas has radically changed the energy market.

The limitations of biology for metabolite production

The use of biological entities for production of metabolites as consumer products or included in consumer products is well established. The existence of a biological industry is based on the capability to make processes profitable, however, in the biofuels field; this has not always been the case. The regulations and laws adopted by various authorities have given the possibility for development of processes that are not profitable in themselves. The promise of biology for the production of energy rich compounds has often overlooked the intrinsic limitations of biology including the substrates, the organisms, the biochemistries involved, the purification, the delivery and the end use. The biology has no influence of the fluctuations on the market of fossil fuels, and at each point in time will have to compete with the current price levels, which are impossible to predict. These can be influenced by many factors, including political ones, and the task to compete on an equal basis becomes unfeasible. The biology is limited by what is thermodynamically possible, even though there is still room for improvement, but we should always be aware of the theoretical limitations on any one process. Three indices are used for determination of feasibility. The first is the yield, one of the main challenges in biology today is to better utilize the carbon given to the cell and its conversion to the end product as close to the maximal theoretical yield as possible. The final concentration is directly coupled to the downstream processes and the productivity, as measured by gram of product per volume and time is crucial for industrial realization of a process. The current aerobic processes must be considered as wasteful since a large part of the carbon is lost as CO_2 and the theoretical yields are therefore such that large part of the substrate never ends up in the product. There are recent efforts to use and engineer pathways for more efficient use of carbon [6].

The present production processes

Biofuels presently produced at large volumes are bioethanol and biodiesel, so called first generation biofuels, which are mainly based on the utilization of starch or sugar rich crops for bioethanol.

The processes in use for bioethanol formation are yeast fermentations with amylase hydrolyzed starch (mainly from corn and cassava) and sucrose from sugar cane or in temperate climates sugar beet. The process in Brazil, which is made at non sterile conditions, has recently been described in detail in the literature. The ethanol is manufactured for 6 to 8 months, when sugar cane is available. The production is uninterrupted using acid to wash the recycled yeast between batches, to lower the bacterial count [7-9]. The yeasts have been characterized and are often polyploidy and very different from the *S. cerevisiae* researched in the lab [8].

Substrates

The substrates in use for first generation bioethanol are limited to compounds available in large quantities allowing for a steady supply of raw material ideally throughout the year. Even in Brazil, with a tropical climate, the growth periods for sugar cane are seasonal and the fermentations are not performed over the whole year. Sugarcane is a prime substrate for the fermentation of ethanol, with the highest decrease in greenhouse gas discharges comparing first generation substrates. Life cycle analysis shows that greenhouse gases are reduced between 19-48 % for corn and 40-62 % for sugarcane [10]. Sugarcane also has a high energy balance and high yield per hectare [11] compared to other first generation biofuel crops used in temperate climates.

However, it should be remembered that sugar cane is a tropical plant with limited geographical spread. One parameter in use for comparison is the net energy value per liter of product. The net energy value includes the water and land requirement, the CO_2 emission and surface run off of nitrogen and phosphate as a measure of fertilization. For corn ethanol it varies from 462 to 1757 kJ l^{-1} and for sugarcane it is between 16 057 and 17 092 kJ l^{-1} [10]. No other plant will be able to compete with the use of sugar cane grown in Brazil, a result of a combination of factors, including land area that is in use and potential novel areas that can be added for use, the relatively cheap manpower and many years of experience (since 1975).

Biodiesel manufacture is based on natural sources or wastes containing a high percentage of fatty acids and other lipid compounds. The main supply of fatty acids is mainly from oil palm and mustard plants. There are also attempts to use lipid containing wastes, but this is on a much smaller scale compared to the use of plants. Using plants, the volumes of biodiesel that can be produced are limited, because areas for growth of the oleaginous crops compete with other uses (mainly food). The biology involved in their manufacture is mainly through improving the lipid amount and fatty acid composition of the target plants. The trans esterification to produce the functional biodiesel is a chemical reaction of the fatty acid with an alcohol catalyzed by base or acid, even though there are biological processes for this that are being developed [12].

Second generation substrates have better environmental characteristics compared to first generation biofuels, with the reservations that even though large scale facilities have been commissioned, the economics of the use of corn stover, switchgrass or miscanthus is still disputed [10]. The pretreatment costs are the main unsolved problem [13,14] and the price of the hydrolysis needed to obtain a mixture of the hexose and pentose sugars low in inhibitors. The use of both sugars is crucial for the economics of the process and it can still not be fully exploited effectively for ethanol formation [15,16].

For third generation biofuels in development (butanol, isoprenoids, hydrocarbons etc), as far as is known, the common sugar (mainly glucose) is the substrate of choice for heterotrophic processes. The intentions are to use cheaper substrate sources (lignocellulose) with similar unsolved issues as for second generation biofuels.

The use of organic wastes, from a plethora of sources, as substrate has many advantages. The main difficulty with wastes is their mixed composition and high moisture content. One method is the gasification and production of syngas as the substrate for the fermentations [17]. However, there are still limiting factors such as low volumetric product concentrations, feedback inhibition and low rates of mass transfer of the gas to the liquid. One alternative, already in use is anaerobic digestion with the formation of methane [18], however, it is not economic as a primary product, and usually is a byproduct with energy value (mainly in sewage treatment). The use of organic and food wastes are minor compared to substrates such as manure and energy crop wastes [19].

The carbon should ideally be obtained from the atmosphere by photosynthesis allowing for cycling of the CO_2 between fuel and air, thus also solving the problem of accumulating green house gases. The efficiency of photosynthesis is low both for plants and for algae, and less than 10 % (in the best case) of the energy is absorbed and used to form potential organic molecules to be used either directly as fuel or by fermentation. One of the great challenges has been to improve the efficiency of photosynthesis which at maximum short term can reach 4.3% for C4 plants [20] and up to 7 % in bubbled bioreactors with algae as the organism [21].

The substrate choice is still the main issue in research for efficient, voluminous and steady formation of liquid biofuels.

The Microorganisms

Yeast - Most of the industrial production of bioethanol is based on the fermentation by the conventional yeast *Saccharomyces cerevisiae* on sugars that are naturally utilized (see above). The yeast has been suggested as one of the platform organisms for the cost-effective production of drop in fuels [22]. The conventional yeast is naturally limited by its restricted capabilities in substrate use and many naturally occurring and abundant carbon sources such as the carbohydrate polymers of lignocellulose cannot be utilized directly [23]. The yeast is also being engineered to produce energy rich compounds other than ethanol, such as fatty acids, ethyl esters, fatty alcohols, and alkanes [24].

Bacteria - Other platform organisms that have been suggested are *E.coli* [25- 27] and *Corynebacteriumglutamicum* [28]. *E.coli* based on the well developed metabolic engineering possibilities for this bacterium and *C.glutamicum* as a well established producer of high concentrations of metabolites. The conventional yeast and the two bacteria are considered to be organisms with robust assets such as tolerance towards stress, good fermentation performance and capability of using a great variety of abundantly occurring substrates. Many other microbes are also described in the literature such as *Zymomonasmobilis, Clostridia* spp. with good characteristics but when looking at the overall (holistic) performance that are still not advantageous for large scale production of any of the energy rich candidate compounds. The main reasons being limited substrate utilization, low resistance to high product concentrations and low productivities [29]. The choice of organism to use as the large scale producer will of course depend on the product aimed for production. The limitations of the organism as to its efficiency is limited to what is thermodynamically possible and the inherent constraints have been elegantly analyzed recently [30]. The main argument is the relation of the obtained change in Gibbs energy that will decide the ratio of reverse and forward fluxes and gives a way to compare pathway chemistries leading to the identical compound. For biofuels, as low value – high volume compounds, it is preferred that the most efficient pathway in conserving both material and energy is chosen. However, it often is not obvious that the conventionally used heterotrophic pathways are fulfilling these criteria. A novel look at other possible routes for formation is necessary.

Our own experience with a sequenced *Micrococcus* strain [31] illustrates some of these points. In attempts to optimize growth, the continuous culture technique was utilized to reach high-density populations [32]. Many different media compositions were tested (Battat& Goldberg, unpublished results) to obtain a high cell concentration of the *Micrococcus luteus* (Fleming strain). Very high optical densities (OD_{600} ~ 80) were obtained augmenting two conventional laboratory media LB and Nutrient Broth where the first is known to be imbalanced both in carbon source and magnesium concentration [33]. In order to get good growth, sucrose was added as carbon source, since *M.luteus* is unable to utilize glucose [31] and it also became evident that the amino acid glutamic acid is required for the buildup of a high cell concentration. The yield of fatty acids is at its best 35% for ethyl hexadecanoate [34], and decreases for other fatty acids. This means a loss of over 60 % of the expensive carbon sources that will not end up in the expected end product, and for alkenes it is probably lower. The comparatively high cost of the medium ingredients for production of alkenes, using the quite fastidious *M. luteus* led us to the conclusion that with all the other expenses involved in making a high volume low value compound it would never enable its competitiveness with conventional fuels.

Biochemistry of formation

The main heterotrophic growth used in the conventional large scale biotechnological production of biochemicals is routine in the fermentation industry, using conventional carbon sources with, in most cases, aerobic growth of the producer organism. This involves the loss of a large percent of the carbon, and depending on the product, the theoretical yields vary.

The non fermentative pathways are not present in nature, but utilizing the decarboxylation of amino acid intermediates it has been possible to obtain alcohols [36]. Enzymes from *L.lactis*(decarboxylase) and *S. cerevisiae* (alcohol dehydrogenase) both have wide substrate specificity that allow for conversion of different 2-keto acids resulting in high concentrations of isobutanol (22 g/L) with engineered *E.coli* [37].

Novel biochemical routes for energy rich compounds

The production of biofuels from plant material is inefficient since CO_2 is first reduced to complex organics (sugar) requiring hydrolysis (lignocellulose) followed by growth sugar based fermentations in which around 50 % of the carbon is lost as CO_2 and is not found in the final product. In that respect in the syngas fermentation, where a chemical conversion of the biomass is initially performed there is better utilization of the total lignocellulose, not only the 2 sugar polymers (cellulose and hemicellulose). CO is the product of the pyrolysis at limiting oxygen that is fermented to CO_2 and acetyl CoA. Use of photosynthetic organisms are limited by the cell concentration obtainable since at a comparatively low cell concentration they will cause shade to the underlying cells (an intrinsic limitation that cannot be engineered away), and even the optimization of photosynthesis will not lead to the orders of magnitude increase in order to make these production processes competitive. The heterotrophic growth results in much higher cell concentrations and also product concentrations. Better use of CO_2 has been a target for improvement of biological means to decrease loss of product formation. Carbon fixation by primary producers is the main supplier for carbon in all living cells.

Novel pathways are described where the stoichiometric conversion of 1mol of glucose to 3 moles of acetate is possible [38]. In the normal glycolysis decarboxylation of pyruvate leads to the loss of a carbon equivalent. It was shown that it is possible to construct a synthetic non oxidative glycolysis pathway, where no carbon is lost, by rearrangements with different configurations with fructose 6-phosphate as the starting material. Both *in vitro* and *in vivo* experiments were performed with xylose as the carbon source resulting in the formation of 2.2 moles of acetate (2.5 theoretical) and above the 1.67 moles formed by the oxidative Embden Meyerhof Parnaspathway which exists in nature [39].

The acetogens, anaerobic bacteria, assimilating CO2 by the Wood-Ljungdahl pathway with two branches, the methyl and carbonyl branch, whereby single carbon molecules forms acetate (acetyl CoA) ending up either as biomass or acetate [40]. The capability under heterotrophic conditions to acquire electrons from a wide variety of substrates such as alcohols, organic acids and sugar allow for almost stoichiometric conversion of C_6 sugars to acetate, with an overall conversion of C_6 sugar to three moles of acetate and 4 moles of ATP (by substrate phosphorylation). The question is how effective, i.e. the required time frame to obtain these end products [41].

CO_2 capture is one of the solutions to achieve product formation with efficiencies that might enable commercialization. Non –natural routes where importance of ATP and cofactor driving forces allow for more effective utilization of the substrate. They should reach near to theoretical yields with and minimal energy going to maintenance. The growth rates obtained for the novel CO_2 assimilating non-natural pathways are far from what is a competitive compared to present pyruvate route with the loss of carbon. The redirection of the carbon fluxes is possible with the novel synthetic biology techniques that might bypass the inherent regulation. An example is the introduction of a bacterial pyruvate dehydrogenase complex, which is ATP independent [42]. For autotrophic growth there are several alternatives and relatively novel carbon fixation pathways have been described [43], two of them utilize reduced C-1 compounds (formate) attached to C-1 carrier compounds (tetrahydrofolate, methanopterin or methanofuran) and the reductive TCA cycle where CO_2 is fixed to other metabolites. Even though these pathways allow for the full utilization of the carbon sources, there are limitations to their competitiveness with more conventional biotechnological methods used in large scale operations. The gas liquid mass transfer limitations of sparingly soluble gases results in low cell concentrations and slow reactions and there are many issues that have to be overcome by creative research to make these processes efficient. Is this achievable? Novel enzymes have been described that allow for energy formation in unfavorable conditions overcoming thermodynamic limitations when a proton is used as electron acceptor [44]

Anaerobic culture conditions are appealing since costs for maintaining the high oxygen concentrations and optimization of reducing equivalents formed leads to high proficiency on a large scale. The capability to engineer pathways regenerating NADPH would be beneficial and increase productivity.

Toxicity and tolerance issues

The formation of high concentrations of organic chemicals, solvents such as ethanol, butanol or isobutanol all have detrimental effects on both growth and product accumulation. The accumulation results in multiple cellular changes such as slower growth rates, formation of undesired by-products and low productivities. The main target in the cell of the biofuel molecules is the membrane, sensitive to the accumulating products. Attempts to increase tolerance have been done for natural producers (*S.cerevisiae*, *C.acetobutylicum*) with limited success. The use of better understood microorganisms such as *E.coli* or *lactobacilli* has shown that both amino acid metabolism and osmoregulation are key protective mechanisms. Very recently GC/MS metabolomics of establish metabolite changes over time [45] showed that specific modules are present in the cell and it could be determined which module was specific for the accumulation of either ethanol, butanol or isobutanol.

FUTURE PROSPECTS

The use of LCA to determine the most sustainable way is used for manufacturing industries, and for biofuels. It is then possible to compare different processes leading to the same product and also comparison of products with analogous uses [46]. The efficiency of ethanol from corn is 0.03 % and from sugar cane 0.14% [47] showing the low efficiency of photosynthesis in combination with the formation of an oxidized chemical. LCA with the relevant boundaries for estimation, take into account the total substrate cost, energy input, wastes, land use and other relevant information and should allow for a “real” evaluation of the “costs” for biofuel production. Robust properties are taking into account such as stress tolerance, fermentation performance and substrate utilization. The establishment of a process with highest possible efficiency (yield) with no loss of carbon, minimal waste of the resources utilized and with productivities allowing for a competitive price with other fuels are the objectives. Before putting too much effort into engineering a specific pathway that leads to a biofuel that might seem worthwhile, it is important to determine the theoretical yield of the planned product since many of the currently investigated biofuels have an intrinsic low theoretical yield and therefore neither practical yield concentration nor productivity will be competitive.

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Conflict of Interest

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