Engineered microorganisms for sustainable production of diesel fuel and other oleochemicals from renewable plant biomass

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Abstract

The looming exhaustion of fossil resources requires new processes for the use of renewable materials and regenerative energies to meet the demand for substrates by the industry and to accommodate our energy needs. Biotechnological processes can make pivotal contributions to this. Processes employing microorganisms for production of bioenergies such as bioethanol, biogas and hydrogen are already well established. This article provides a short survey over a novel process termed Microdiesel. Microdiesel technology employs genetically modified microorganisms for the production of biodiesel-equivalent fatty acid ethyl esters, which are a fully-fledged substitute for petroleum-based diesel fuel, completely derived from renewable plant biomass. By combining the enzymatic potential of an unspecific bacterial acyltransferase with our concept of modular biotechnology, engineered microorganisms also show great promise for the biosynthesis of a broad range of other tailored oleochemicals from bulk plant material.

Microorganismos "diseñados" para la producción sustentable de combustible diesel y otros productos oleoquímicos a partir de biomasa vegetal

El agotamiento de los recursos fósiles, que se vislumbra, demanda procesos nuevos en el uso de materiales renovables y energía regenerativas para poder responder a la exigencia de la industria por sustratos y para satisfacer nuestros requerimientos energéticos. Para esto, los procesos biotecnológicos pueden ofrecer una contribución significativa y fundamental. Los procesos que emplean microorganismos para la producción de bioenergías tales como el bioetanol, biogás e hidrógeno, ya se encuentran bien establecidos. Este artículo ofrece una reseña breve de un proceso novedoso denominado Microdiesel. La tecnología Microdiesel emplea microorganismos modificados genéticamente para la producción de ésteres etílicos de ácidos grasos, equivalentes en el biodiesel, que son un sustituto completo para el diesel combustible derivado del petróleo, pero totalmente derivados de biomasa vegetal. El resultado de la combinación del potencial enzimático de la aciltransferasa bacteriana no específica con nuestro concepto de biotecnología modular, hace que los microorganismos modificados puedan también mostrar grandes promesas para la biosíntesis de una amplia variedad de otros oleoquímicos diseñados específicamente a partir de biomasa vegetal.

Maßgeschneiderte Mikroorganismen für eine nachhaltige Produktion von Dieselkraftstoff und anderen Oleochemikalien aus Pflanzenbiomasse

Der sich abzeichnende Verbrauch fossiler Rohstoffe erfordert neue Prozesse zur Nutzung nachwachsender Rohstoffe und regenerativer Energien, um den Bedarf der chemischen Industrie für Rohstoffe und unseren Energiebedarf auch in der Zukunft befriedigen zu können. Biotechnologische Verfahren können dabei entscheidende Beiträge leisten. Mikroorganismen werden bereits zur Produktion von Bioethanol, Biogas und Wasserstoff eingesetzt. Dieser Aufsatz gibt einen kurzen Überblick über ein neues als Mikrodiesel bezeichnetes Verfahren. Beim Mikrodiesel-Verfahren werden gentechnisch veränderte Mikroorganismen zur Produktion von Biodiesel-äquivalenten Fettsäureethylestern eingesetzt, die dem Öl-basierten Dieseltreibstoff gleichwertig sind. Durch Kombination einer unspezifischen bakteriellen Acyltransferase mit unserem Konzept der modularen Biotechnologie, sind darüber hinaus entsprechend veränderte Mikroorganismen auch viel versprechende Kandidaten zur Erzeugung einer breiten Palette von maßgeschneiderten Lipiden und anderen chemischen Verbindungen ausgehend von Biomasse aus Pflanzen, die im Überschuss zur Verfügung steht.

Introduction

In the past the thriving and prospering economy and wealth of the industrial nations have been fueled by energy obtained from fossil resources like natural gas, coal and oil. However, recent years have witnessed a dramatic and steady increase in the price of these fossil fuels drastically reminding us that the supplies of those fossil energy resources are finite and will become successively exhausted within the next few decades. Furthermore, the lavish use of fossil energy material as heating and transportation fuel is driving the worsening global warming process by causing massive emissions of the greenhouse gas carbon dioxide. Therefore, the development of sustainable, economically viable, and ecologically friendly alternative energy sources is urgently required.

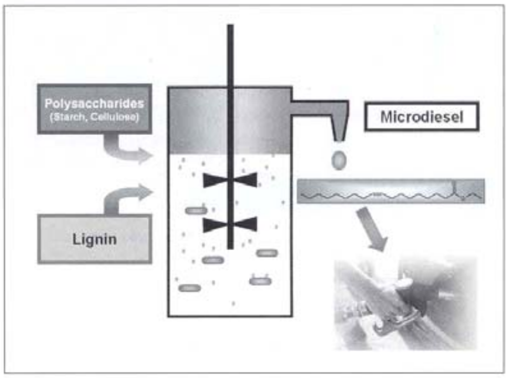
Benefits and drawbacks of biodiesel

Our energy supply in the future will probably consist of a cocktail of various alternative regenerative energy resources among which also so-called bioenergies or biofuels will surely play an important role. Bioenergies or biofuels are a variety of alternative energy resources made from renewable plant biomass that can contribute to a sustainable energy supply as well as to a reduction of carbon dioxide emission since they are based on almost completely closed carbon cycles. Bioethanol, biogas and molecular hydrogen are biofuels that are already produced by microorganisms by fermentation processes. Biodiesel, in contrast, which is in Europe the most important bioenergy by production volume, could so far be synthesized only chemically. Biodiesel is produced from triacylglycerols derived from plant oils like soybean, palm oil and in Europe mainly rapeseed which are transesterified employing a chemical catalyst in a highly energy-consuming process in presence of methanol yielding fatty acid methyl esters (FAMEs), the main constituents of biodiesel. Aside from its environmental benefits, biodiesel is particularly popular since it is a fully-fledged substitute for fossil diesel fuel that can be used in conventional diesel engines requiring no or only marginal modifications. Furthermore, it can be distributed using the existent infrastructure. Is biodiesel therefore the key technology allowing a gradual and seamless transition to alternative biofuels to achieve independence from fossil energy resources in the near future? Unfortunately not, because biodiesel, in the way it is currently produced at a technical scale, has also numerous drawbacks and limitations seriously hampering a more widespread use in the future. The vegetable oils needed for biodiesel production can only be obtained from the seeds of oilseed crops, but not from the other, by far more abundant parts of plant biomass. Thus, this results in a very low yield and in an excessive demand of acreage for production of the required oil seed feedstock. This acreage is then not available for food production anymore, and this competition between food and fuel is not least also ethically questionable regarding the many starving people in the developing countries. The large demand of acreage is intensified by the fact that oilseed crops like rapeseed and soybean are not self-compatible and that their cultivation requires a frequent crop-rotation regime. Furthermore, the chemical transesterification process and the subsequent purification steps are costly and energy consuming, thereby greatly diminishing the possible energy yield and increasing the price. Finally, the methanol used for biodiesel synthesis is primarily derived from natural gas. Thus, FAME-based biodiesel is ultimately not a true natural product due to its partial fossil origin. In consequence, a substantial increase of biodiesel production and a more significant substitution of petroleum-based diesel fuel in the future will only be feasible when processes could be developed enabling biodiesel synthesis not only from plant oils but also from more readily available bulk plant materials such as sugars and starch, and in particular cellulose, hemicellulose and lignin.

The Microdiesel process

Towards this end, we generated in our laboratory a genetically modified strain of the well-characterized gut bacterium *Escherichia coli*, a frequently used workhorse in molecular biological and biotechnological processes. By combining genes from three different bacteria based on the concept of "modular biotechnology" we created a novel, non-naturally biosynthetic pathway enabling the engineered *E. coli* strain to produce large amounts of fatty acid ethyl esters (FAEEs). These FAEEs are not only chemically very similar to the FAME constituents of "classical" biodiesel, they also have virtually identical fuel properties and are an equally appropriate substitute for petroleum-based diesel fuel. The peculiarity of bacterial FAEE biosynthesis, for which we coined the term "Microdiesel", and its advantage over classical biodiesel production is that it is synthesized utilizing fatty acids and glucose as substrates. Therefore, it is completely made from renewable plant materials without requiring toxic methanol of fossil origin (4). How does the Microdiesel process works in detail? The basis is a novel enzyme, WS/DGAT, from *Acinetobacter baylyi* involved in storage lipid biosynthesis in this bacterium (1). WS/DGAT is an acyltransferase, an enzyme transferring fatty acid moieties to compounds containing hydroxyl groups. Biochemical characterization revealed that this acyltransferase has an extraordinary low substrate specificity (2, 3, 6, 7). For Microdiesel production, we exploited this promiscuity and extended the genetic repertoire of *E. coli* by two additional metabolic modules. First, we cloned two genes from the ethanol-producing bacterium *Zymomonas mobilis* (pyruvate decarboxylase and alcohol dehydrogenase) which enables *E. coli* to synthesize ethanol from simple sugars like glucose. To this ethanol production module we introduced in the second step the WS/DGAT from *A. baylyi* which then allowed subsequent esterification of the synthesized ethanol with fatty acid moieties resulting in FAEE formation. This engineered designer *E. coli* strain is already capable to produce more than 1 g l^{-1} Microdiesel during fermentation in a bioreactor (4). Although this clearly demonstrates that the concept principally works, the current yields are still significantly below the needs of a viable industrial process. What has to be done in the future for further improvement of the Microdiesel technology and development of a profitable biotechnological production process (see Fig. 1)? At present Microdiesel production relies on glu-

Figure 1. Microdiesel biosynthesis in the future after amelioration of the current production strain by additional metabolic capabilities



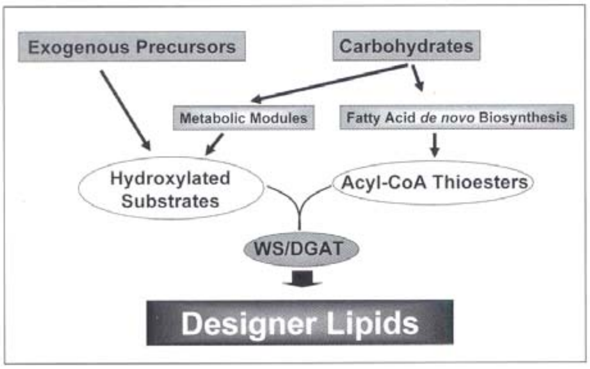
cose and fatty acids. The current *E. coli* strain shall get successively improved by addition of further metabolic modules allowing utilization of bulk plant polymers like starch, cellulose and lignin. By this, materials like straw and wood, but also recycling paper will become accessible substrates for Microdiesel synthesis. Furthermore, integration of a fatty acid biosynthesis module is aspired so that addition of fatty acids to the medium will become dispensable setting the whole process on a pure carbohydrate basis. Finally, addition of a secretion module is desirable so that synthesized Microdiesel is not accumulated intracellularly by the bacteria but secreted into the fermentation medium allowing a continuous production and siphoning of Microdiesel. A lot of development work is still needed, but Microdiesel has the perspective to become once part of the energy mix that keeps us mobile in the future avoiding the competition between food and fuel production since the waste products of food manufacturing can be the substrates for Microdiesel synthesis.

Harnessing WS/DGAT´s promiscuity for biosynthesis of other oleochemicals from renewables by recombinant microorganisms.

By virtue of the extremely broad substrate spectrum of WS/DGAT, it is easily conceivable to adapt our "modular biotechnology" concept also for the biosynthesis of other tailor-made oleochemicals from renewable plant biomass employing recombinant microorganisms. For this purpose other metabolic modules mediating formation of different hydroxylated compounds serving as alternative substrates have to be combined with WS/DGAT´s almost "unlimited" acyltransferase activity (Fig. 2). One such example that was implemented in our laboratory is the biosynthesis of jojoba oil-like wax esters in a genetically modified *E. coli* strain. Wax esters (oxo esters of long-chain fatty acids and long-chain fatty alcohols) are of considerable commercial importance and are today principally produced by chemical synthesis on a petrochemical basis. The oil from the jojoba plant is the main biological source of wax esters whose use however, despite of having multiple potential applications, is restricted due to its high price. By coexpression of a fatty alcohol-producing enzyme (a bifunctional acyl-coenzyme A reductase) from the jojoba plant recombinant, biosynthesis of jojoba oil-equivalent lipids from inexpensive plant fatty acid substrates was established in a WS/DGAT harbouring *E. coli* strain (5). Further strain improvement could also here enable the biotechnological production of such high-value lipids derived from inexpensive renewable plant carbohydrates.

In view of WS/DGAT´s huge enzymatic potential the range of tailored lipids synthesizable with engineered microorganisms from renewable plant materials is only limited by the availability of appropriate metabolic modules for provision of substrates for this enzyme. Alternatively, in cases where such metabolic modules are not yet available, designer lipids can also be made by medium supplementation with specific precursor substrates to WS/DGAT carrying microorganisms. This approach was already successfully applied in our laboratory for synthesis of unusual lipids such as wax diesters, thio wax esters and dithio wax esters (3, 7).

Figure 2. Strategies for the biosynthesis of tailored lipids in recombinant microorganisms



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