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Review

Fueling the future with biomass: Processes and pathways for a sustainable supply of hydrocarbon fuels and biogas

Global economic growth, wealth and security rely upon the availability of cheap, mostly fossil-derived energy and chemical compounds. The replacement by sustainable resources is widely discussed. However, the current state of biotechnological processes usually restricts them to be used as a true alternative in terms of economic feasibility and even sustainability. Among the rare examples of bioprocesses applied for the energetic use of biomass are biogas and bioethanol production. Usually, these processes lack in efficiency and they cannot be operated without the support of legislation. Although they represent a first step towards a greater share of bio-based processes for energy provision, there is no doubt that tremendous improvements in strain and process development, feedstock and process flexibility as well as in the integration of these processes into broader supply and production networks, in this review called smart bioproduction grids, are required to make them economically attractive, robust enough, and wider acceptance by society. All this requires an interdisciplinary approach, which includes the use of residues in closed carbon cycles and issues concerning the process safety. This short review aims to depict some of the promising strategies to achieve an improved process performance as a basis for future application.

Keywords: Biogas / Biomass / Drop-in biofuels / Renewable energy / Smart bioproduction grids

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1 Introduction

The increased awareness of the man-made climate change and the dependency of many economies on crude oil and natural gas imports underline perspectives of using bioenergy as renewable alternatives for liquid and gaseous fuels. In 2014, 6.6 billion liter of bioethanol were produced in the EU, which represented an increase of 13% compared with 2013 [1]. In Europe, more than 14 000 biogas plants with a total capacity of 7900 MWel power were installed in 2014 [2]. Both processes are conducted quite differently as the latter one is applied with an undefined composition of microbes, poor characterization of the feedstock composition and great variation of process and reactor design. Biofuels, namely bioethanol and fatty-acid methyl esters (FAME), also referred to as biodiesel, represent so-called first generation biofuels. The production of bioethanol is solely based on the naturally high production titers of the yeast *Saccharomyces cerevisiae* and its ability to ferment sugars to ethanol. Biodiesel is produced by the transesterification of plant and animal fat derived triacylglycerides with methanol.

Biogas and liquid biofuels have technical, commercial and ecological drawbacks in comparison to the currently used hydrocarbon compounds derived from natural gas and fossil fuels. Biogas is not pure enough to release it to the gas grid, purification to biomethane is costly and usually not applicable at most plants due to their size, which is restricted by the availability of feedstock nearby. Sustainability of purification is doubtful in comparison

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Abbreviations: **ACR**, fatty acyl-coenzyme A reductase; **ANN**, artificial neural network; **BFABCEs**, branched fatty acid branched-chain esters; **CAR**, fatty acyl-CoA reductase; **CoA**, coenzyme A; **DMAPP**, dimethylallyl diphosphate; **DOX**, fatty acid α-dioxygenase; **FABCEs**, fatty acid branched-chain esters; **FAEE**, fatty acid ethyl esters; **FAME**, fatty acid methyl esters; **FAR**, fatty acyl-carrier protein reductase; **FAS**, fatty acid synthase; **GMO**, genetically modified organisms; **IPP**, isopentenyl diphosphate; **IspS**, isoprene synthase; **MDD**, mevalonate diphosphate decarboxylase; **MSW**, municipal solid waste; **NIR**, near-infrared spectroscopy; **PKS**, polyketide synthase; **TAG**, triacylglycerides; **TENIRS**, transflexible embedded near-infrared sensor

to flexible operation [3]. Nevertheless, a flexible feedstock load is often not applied as it leads to increased risks in operation and might require sophisticated pre-treatment. Monitoring tools are not adequate to counteract these increased risks. Together with some other factors, the current situation leads to an inflexible, but rather stable operation as preferred by plant operators. The advantage a complex culture might have, namely to cope with substrate alterations, is not applied. Hence, although in many countries industrially operated biogas plants are installed, the potential is neither used for an advanced integration in an energy supply chain to balance other fluctuating renewable resources like wind and solar power, nor for a suitable integration in regional carbon cycles. For a wider substrate flexibility, attempts have been made to improve substrate pre-treatment and conduct hydrolysis separately from the anaerobic digestion stage, but practical installation requires research as there is little standardization: every plant is operated with feedstock of different quality and composition. On the research level, the utilization of pure cultures have been forced to gain a better control of the process, however, the lack of suitable organisms that can convert a variety of substrates and produce methane with compatible yields like in a complex culture restricts any application so far. The potential of the biogas process to convert a variety of substrate even of poor quality was recently proposed for the design of biorefineries, in which the biogas production is the last step to convert residues of other processes. This links biogas production to other bulk chemical production processes. The concept was also evaluated for bioethanol production, if the remaining culture broth was used as a feedstock for biogas production [4]. The amount of energy required from external sources, as well as time and efforts for feedstock pre-treatment needed for the biogas process were reduced. Also in this case, an improved control strategy in order to achieve a stable and safe process with an optimized yield remain a challenging task, although operational flexibility is restricted in such an integrated concept.

Many studies have been performed in the last ten years, which explored the biogas yield of various biogenic resources, like maize and corn crops, animal manure, sewage sludge and municipal solid waste (MSW) [5]. Based on the Shell 2050 studies' energy scenarios [6], the primary energy production based on biomass is foreseen to increase 3three-fold by 2050. Thus, biogas production still represents an important process for the future biomass-based energy provision.

Similar challenges exist in case of process control and product quality for the production of liquid biofuels. Due to the contained hydroxyl moieties in ethanol and FAME, they are polar and thus, by attracting water, can act corrosive on engines and the fuel supply infrastructure originally designed for hydrophobic, apolar hydrocarbons. Both biofuels have a lower energy content [7], FAME (~9%) and ethanol (~30%), in comparison to fossil oil derived fuels. Furthermore, biodiesel is less suitable for the use at cold temperatures, since it has a higher cloud point (the temperature, at which wax crystals start to form) than conventional Diesel fuel, resulting in the plugging of engine filters.

Fossil oil derived fuels are a complex mixture of hydrocarbons containing nitrogen and sulfur compounds, which upon combustion are released as oxides of nitrogen (NO_x) and sulfur (SO_x). They in turn have adverse health effects. Additionally, these components lead to the condensation of air plane exhausts forming so-called contrails [8], which are considered to be major contributors to global warming caused by plane traffic [9].

The importance of renewable fuels is well recognized by authorities, as they are addressing future topics like securing the energy supply, fostering rural economic development, and reducing emissions from the transportation sector. The EU administration considers these issues within the Renewable Energy Directive (2009/28/EC), the Fuel Quality Directive (2009/30/EC) and in the US with the Energy Independence and Security Act (110th Congress Public Law 140). Thus, there is a need to develop a new generation of biofuels based on non-food biomass. One such fuel could be based on hydrocarbons produced by microorganisms, as it falls into the category of so-called drop-in biofuels. These are substantially similar to current fossil-derived fuels and are compatible to combustion engines and transportation infrastructure. In recent years, several microbial pathways for the direct biosynthesis of hydrocarbons have been discovered and heterologously expressed in different host bacteria, algae and yeast.

In conclusion, the major challenges for the energetic use of biomass are the utilization of alternative substrates, as well as the increase of efficiency and product quality. In order to avoid transportation costs and to increase sustainability, while reducing effects on market prices and a competition with food supply, these processes should be designed so that they can be integrated in closed carbon cycles. The feedstock used should depend on the availability and adjusted to other processes, including farming. Analogous to smart grids, in which energy generation and consumption are adjusted to each other in order to save resources, the implementation of, as what the authors call smart bioproduction grids, should transfer this idea to mass bioproduction, which relies on renewable resources and biogenic waste. Nevertheless, these processes have to be conducted very cost-efficient in comparison to many other, so far industrially applied biotechnological processes. An overview of these challenges and strategies to overcome them as they are addressed in the following parts of this review is summarized in Fig. 1.

2 Biofuel and biogas processes—Current technologies

2.1 Liquid biofuels

Major biofuels sold on the market are ethanol and FAME, commonly referred to as biodiesel. Bioethanol production is based on the high production titers of naturally occurring pathways, namely alcoholic fermentation of yeast. Biodiesel is produced by the chemical transesterification with methanol of plant oils and animal fats. These, often described as first generation biofuels, are based on sugars from corn, sugar cane and sugar beet or oils from canola or oil palm, and therefore compete with arable land and products, which are required for food production ("food or fuel"). Second generation biofuels aim at relying these well-established fuels on non-food substrates, mainly hydrolysates of lignocellulosic biomass. A variety of techniques have been developed for this hydrolysis, which have been

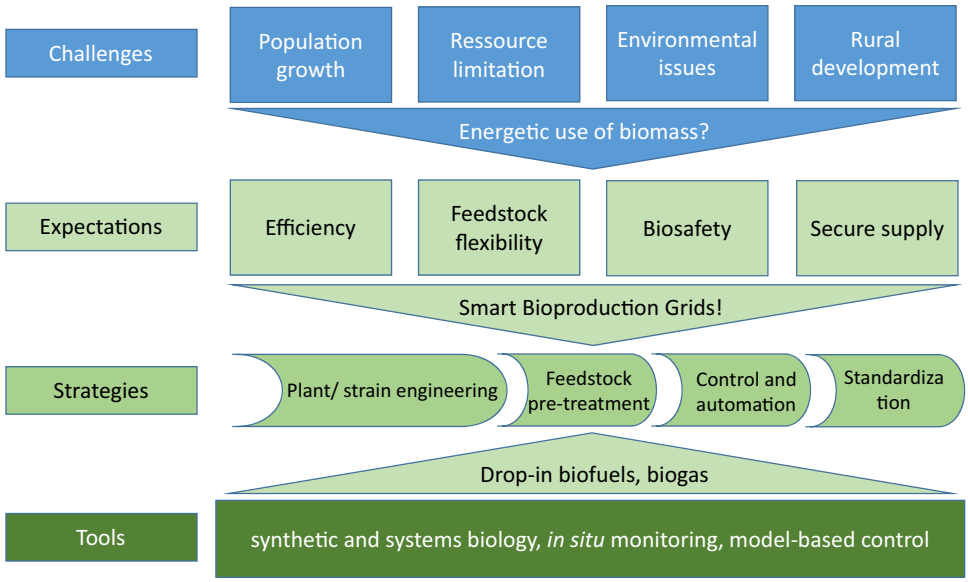

Figure 1. Overview of challenges and tools for achieving cost-reduction and an improved feedstock flexibility of biotechnological processes for the energetic use of biomass.

reviewed elsewhere [10]. The main production host applied for ethanol synthesis, the yeast *S. cerevisiae,* is not able to use the pentose sugar D-xylose, the main component of lignocellulose beside glucose. Therefore, so-called C5-yeasts have been developed, which are not only able to efficiently use lignocellulose hydrolysates, but as well cope with the inhibitors present [11]. Additionally, hexose transporters have been engineered to allow a simultaneous utilization of glucose and xylose [12], thus avoiding a diauxic phase and allowing shorter process times. Research was also intensified for other biofuel processes like the ABE-process for microbial butanol production [13], a model example for the application in biorefinery processes, in which the organic acids from an ABE-process are used as a C-source for bio-lipid production (see below) [14].

The traditional source of triacylglycerides (TAG) required for the transesterification to produce FAME are plant oils and animal fats. A current development is to produce the TAG with so-called oleaginous microorganisms. These yeast, bacteria and algae can accumulate over 20% of their cell dry weight (cdw) as so-called oil bodies [15]. One of the most advanced organisms is the yeast *Yarrowia lipolytica,* which has been engineered to produce over 80% fatty acids of its cdw [16]. It has been enabled to use glycerol, the main side stream of chemical transesterification of TAG to FAME, as feedstock for lipid production [17]. Other oleaginous yeasts like *Cutaneotrichosporon curvatus* [18] or *Trichosporon oleaginosus* [19] are able to grow on a variety of biomass hydrolysates and are currently developed to serve as future production hosts for TAG and other fatty-acid derived products. Phototrophic microalgae are as well able to accumulate TAG, while at the same time CO_2 can be used, for example from steel mill off-gas. Zhao and co-authors composed a thorough review about the production of hydrocarbons from microalgae oil, including the decarboxylation by means of different metal-catalysts [20].

Research is also conducted to produce FAME in a one-pot reaction by applying recombinant microorganisms, which produce the required alcohol (e.g. ethanol), TAG and perform an enzymatic transesterification. Production titers of fatty-acid ethyl esters (FAEE) of 34 mg/L in *S. cerevisiae* [21] and 1.5 g/L in *E. coli* were reached [22]. Using microorganisms that are able to produce higher and branched alcohols (e.g. isobutanol), microbial production of fatty acid branched-chain esters (FABCEs) and branched fatty acid branched-chain esters (BFABCEs) with current titers of 273 mg/L in *E. coli* was achieved [23]. For a very thorough overview, which covers a wide area of microbial biofuel pathways, the reader is referred to a recently published review [24].

A next generation of biofuels, which aims at being drop-in replacements and second generation in respect to non-food feedstocks, is currently being developed and will be described in several of the following sections.

2.2 Biogas

In practice, biogas is produced by anaerobic digestion of mixed cultures, including a hydrolysis, acido- and acetogenesis, in which short-chain fatty acids and alcohols, CO_2 and H_2 are formed, and finally the methanogenesis, in which these intermediate products are converted into methane. The biogas usually consists of 50–60 % (v/v) methane, the residual gas mainly consists of CO_2. The consortium of microbes varies between production plants and can even vary over time in a single process, although operated continuously in a pseudo-steady state. The dry mass content differs between 5 to 6% (w/w) in wet fermentation and more than 15% (w/w) in dry fermentation. Most processes are conducted under mesophilic conditions (about 40°C) and some under thermophilic conditions (above 50°C) in a continuous mode. Biogas production offers a possibility to convert many kinds of feedstock that could otherwise not be used for other biotechnological processes. Moreover, landfilling of biologically degradable material instead of using it for any bioprocess could cause natural problems like groundwater pollution by nitrate leaching [25] and methane release. Biogas is

primarily used for electricity and heat generation and after upgrading and purification to bio-methane and as gaseous fuel. The application of biogas production-based digestate contributes to closed carbon cycles, since phosphate and other compounds are redistributed to the arable land [26, 27].

From a life cycle assessment perspective, several studies have reported benefits for several feedstock applications due to the reduction of greenhouse gas emissions, air pollution, acidification, or eutrophication, see, e.g. [28]. However, the effect of mitigation might not sufficient due to the amount of energy and material used for cultivation, as well as the transport and pre-treatment requirements of feedstock (for reviews of pre-treatment methods see, for example, [29, 30]). Environmental impacts strongly depend on the crop production [31–33]. Surplus emissions occur from the biogas plant operation, biogas utilization, and the use of digestate, respectively. All these steps are subject to optimization by ongoing research and development.

3 Next generation technologies

3.1 Microbial pathways to hydrocarbons as optimization target

Liquid hydrocarbon based fuels are the current choice of energy carrier for the transportation of humans and goods. Currently, two routes are focused in research for the production of hydrocarbons in microorganisms: the decarboxylation of carboxylic acids, especially fatty acids, by a variety of different reactions (see Fig. 2), mainly resulting in unbranched, medium to long chain hydrocarbons (C11 to C17) as well as isoprenoid-derived molecules resulting in branched aliphatic and aromatic hydrocarbons from short to long length. Short terminal olefins, which are platform molecules that can be polymerized to a variety of renewable alternatives to current petrochemical products, can be derived from isoprenoids, fatty acids and additionally from amino acid synthesis pathways as well.

3.1.1 Decarboxylation of carboxylic acids

Fatty acid synthesis is part of the primary metabolism. An acetyl-CoA as a primer is extended with a malonyl-CoA followed by reactions, which result in a saturated acyl chain that can be elongated by an additional malonyl-CoA molecule. This reaction is catalyzed by a multi-domain enzyme complex, namely the fatty acid synthase (FAS). The conversion requires two NADPH per elongation cycle, in turn resulting in a molecule, which stores a large amount of energy. Another secondary metabolism pathway resulting in a very large variety of complex carboxylic molecules are polyketide synthases (PKS). These multienzyme complexes share similarity to the FAS as they contain multiple domains, which elongate a starter molecule with acetyl- and malonyl-CoA. PKS can be subdivided into modular and iterative synthases. The former usually leads to larger structures of a higher degree of complexity, while the latter results in smaller molecules [34]. Both fatty acid and PKS-pathways are being explored for the production of hydrocarbons by decarboxylation.

Figure 2 summarizes engineered pathways from fatty-acyls to hydrocarbons. A major achievement for these pathways was the identification of two enzymes from cyanobacteria [35]: the fatty acyl-ACP reductase (FAR, cyanobacteria) catalyzing the formation of a fatty aldehyde from fatty acids bound to an acyl carrier protein and a fatty-aldehyde deformylating oxygenase (ADO, cyanobacteria), which decarboxylates the fatty aldehyde to the corresponding alkan/en. While the FAR prefers ACP bound fatty acids, the fatty acyl-coenzyme A reductase (ACR, *Acinetobacter calcoaceticus*) forms fatty aldehydes from coenzyme A (CoA) bound fatty acids [36]. Using either a fatty acyl-CoA reductase (CAR, *Mycobatcerium marinum*) [37] or a fatty acid α-dioxygenase (DOX, *Oryza sativa*) [38], long chain free fatty acids can be converted to fatty aldehydes and to alkan/ens by ADO. All these enzymes prefer different chain length and saturation levels of the substrate fatty acyl and require different co-factors, a fact that has to be considered for pathway engineering. Additionally, the fatty aldehyde is prone to site reactions like fatty-alcohol formation [39]. A major bottleneck remains fast decarboxylating enzymes. The most prominently used enzyme, despite its slow turnover numbers ($k$cat = 0.36 min^{-1}, Km (stearoyl-CoA) = 31.9 μM) [40], is the ADO. The lack of alternative enzymes makes it one of the rate limiting steps in the hydrocarbon formation from fatty-aldehydes. However, the availability of a protein structure [41] will doubtlessly yield variants improved by rational protein engineering in due time.

Polyketide synthases are explored for the production of aromatic and complex hydrocarbon molecules, but as well for linear, medium chain hydrocarbons. All these molecules originate from secondary metabolism and are therefore mostly produced in miniscule amounts. The endophytic fungi *Nigrograna mackinnonii* E5202H is able to produce a volatile nonatetraene while growing on wood debris [42]. The discovery of terminal alkene formation by application of a decarboxylating thioesterase [43] will allow us to form hydrocarbons from heterologously expressed and modified PKS. Heterologously expressed, iterative PKS were already applied to produce pentadecane (140 mg/L) in *E. coli* [44]. The company RHO Renewables is applying PKS to produce cyclic aromates as fuels and fuel additives (3-methylanisole).

3.1.2 Isoprenoid derived hydrocarbons

Isoprenoid synthesis is based on the condensation of the C_5-phosphate esters isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Two different pathways exist for the synthesis of IPP and DMAPP, namely the mevalonate (MEP, dominant in eubacteria and plants) and the methylerythritol (MEV, dominant in eukaryotes and archae) pathways. The number of condensed isoprene molecules determines their different classes: monoterpenes (C_{10}, IPP+DMAPP), sesquiterpenes (C_{15}, 2IPP+DMAPP) and diterpenes (C_{20}, 3IPP,+DMAPP). From these basic structures, over 50 000 different compounds have been shown to be synthesized in nature making isoprenoids one of the most diverse groups of natural products and creating a huge potential for applications.

Being pure hydrocarbons, which are formed in natural pathways, the sesquiterpenes farnesene and bisabolene are promising candidates as drop-in fuel replacements. Isoprenoids with their branched and aromatic structures have desirable fuel properties resulting in higher octane ratings. By expressing plant derived

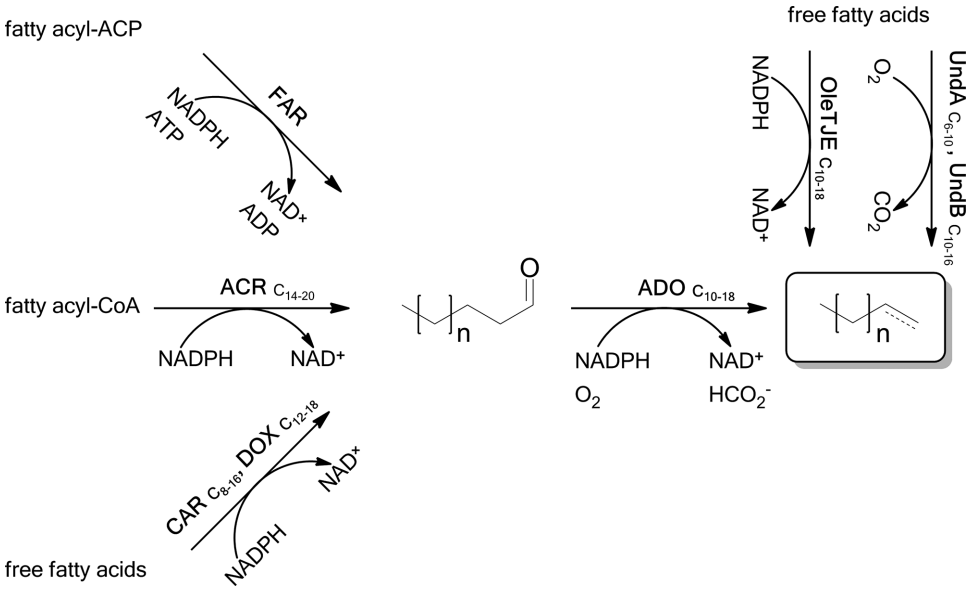

Figure 2. Microbial pathways from fatty acids to alkans/ens. Preferred carbon chain length is indicated in subscript next to the enzymes. CAR: carboxylic acid reductase from *Mycobacterium marinum* [37]; DOX: fatty acid a-dioxygenase from *Oryza sativa* [38]; ADO: fatty aldehyde deformylating oxygenase from Cyanobacteria [35]; FAR: fatty acyl-CoA/ACP reductase from Cyanobacteria [35]; ACR: fatty acyl coenzyme A reductase from *Acinetobacter calcoaceticus* [36]; UndA: from *Pseudomonas* species [118]; UndB: from *Pseudomonas* species [119]; OleTJE: P450 fatty acid decarboxylase from *Jeotgalicoccus* sp. [120].

farnesene synthases in *S. cerevisiae,* the biotech company Amyris was able to produce 104.3 g/L farnesene which is marketed as Biofene. The metabolism of the yeast strain was previously optimized for the anti-malaria drug pre-cursor artemisininic acid boosting the initial yield of 100 mg/L to 25 g/L applying systems and synthetic biology [45].

Besides farnesenes, bisabolene and dimerized pinene have been shown to possess favorable fuel properties and are suitable replacements for Diesel and jet fuel [46]. Heterologous production was achieved in *E. coli* (pinene: 32 mg/L [47] and bisabolene: 900 mg/L [46]) and *S. cerevisiae* (bisabolene: 900 mg/L [46]), and more recently in *Streptomyces venezuelae*, an actinobacterium able to utilize glucose polymers for growth, resulted in the production of 10.52 mg/L bisabolene [48].

3.1.3 Short hydrocarbons

Besides these long chain liquid hydrocarbons, pathways for short hydrocarbons like propane, isobutene, pentane and isoprene have been developed. Since these molecules are volatile in typical cultivation conditions (e.g. 30–37°C), they can be recovered from the off-gas stream. This avoids energy intensive downstream processing. Applying the above described ADO with an engineered substrate-access channel has improved activity towards short chain aldehydes, which results in an improved production of propane (3.4 mg/L) using *E. coli* [49]. Short hydrocarbons containing a terminal double-bond are desirable platform chemicals. Isobutene, for example, can be processed to a variety of fuel additives (MTBE, ETBE, isooctane) or polymers like butyl rubber [50]. The company Global Bioenergies is applying a mevalonate diphosphate decarboxylase (MDD) to decarboxylate and dehydrate 3-hydroxyisovalerate to isobutene in a commercial process. An optimized *E. coli* strain is used to heterologously express an MDD from the archaeon *Picrophilus torridus.* Titers are not being disclosed by Global Bioenergies, but a second pilot plant with a capacity of 100 t/a of polymer-grade isobutene will start production in 2016 (http://www.global-bioenergies.com/, as of 15th of April 2016). A primary feedstock of the process will be sugar beet as underlined by a joint venture of Cristal Union (one of the largest sugar producers of France) and Global Bioenergies named IBN-One with a license for a 50 000 tons capacity plant to be located in France (information obtained from Global Bioenergies' webpage).

Isoprene is a short hydrocarbon by-product of the above described isoprenoid pathways. An isoprene synthase (IspS) has been identified in plants, in which the production of isoprene constitutes a plant protecting agent against abiotic stress conditions [51]. Isoprene production was also detected in bacteria [52], but no responsible enzyme could be identified. One of the early examples of a heterologous expression of a plant IspS was performed in the cyanobacterium *Synechocystis* sp. PCC 6803, which yielded approx. 50 μg isoprene per g dry cell weight per day during a CO_2 accumulating, photosynthetic process [53]. Highest production titers of 60 g/L were described in a patent application of Cervin et al. (US patent no.: US20090203102 A1, patent owner: Danisco and Goodyear) by the heterologous expression of isoprene synthase (*Populus alba*) and MVA pathway genes from several organisms in *E. coli.* Yang and co-authors recently achieved a yield of 620 mg/L isoprene in an *E. coli* fed-batch cultivation [54]. The strain was created by assembling a hybrid pathway consisting of the upper MVA pathway from *Enterococcus faecalis*, OleTJE (see Fig. 2) from *Jeotgalicoccus* sp. ATCC 8456 and an oleate hydratase from *Elizabethkingia meningoseptica.* Using the oleaginous yeast *Y. lipolytica* in order to heterologously express a lipoxygenase, Blazeck and co-authors were able to produce 4.98 mg/L of pentane [55].

3.1.4 Natural producers of hydrocarbon fuels

Besides these already applied synthetic pathways, promising new natural pathways are discovered and are further elucidated. A strain of the yeast *Aureobasidium pullulans* [56] was reported to secrete amounts of 32 g/L heavy oil within 168 h and several endophytic fungi have been reported to produce a variety of

Table 1. Selection of drop-in hydrocarbons producing companies

Company	Feedstock	Organism	Products	Stage	Pathway	Partner
Amyris	Sugar	*S. cerevisiae*	Diesel fuel, terpenes, fine chemicals	Commercial (Biofene®)	Terpenoid pathway	Total
Genomatica	Sugar	*E. coli*	Butadiene	Pilot	Aminoacid biosynthesis	Braskem, BASF
Global Bioenergies	Sugar	*E. coli*	Isobuten	Commercial demonstration	Aminoacid biosynthesis	Audi
Joule	Light, CO_2	Cyanobacteria	Gasoline, diesel jet fuel	Commercial demonstration	Decarboxylation of fatty acids	Audi
RHO Renewables	Sugar	*S. cerevisiae*	Monocyclic aromatic hydrocarbons as fuel additives	Pre-pilot	Polyketide-synthases	Sasol
REG Life Science (LS9)	Sugar	*E. coli*, *S. cerevisiae*	Renewable chemicals and fuels	Research	Decarboxylation of fatty acids	REG
Sapphire Energy	Light, CO_2	Undisclosed algae and cyanobacteria	Gasoline, Diesel and jet fuel	Commercial demonstration	Terpenoid pathway	Phillips 66

hydrocarbons during growth on decaying wood [42, 57]. These phenotypes were shown to be linked to polyketide synthases. As highlighted in a recent review by Stroebel [58], great potential for the formation of a multitude of hydrocarbons are encoded in fungal genomes, which could be transferred to production strains. The review describes the importance of testing novel compounds (e.g. 1,8-cineole, a Diesel fuel additive) for their performance in combustion engines.

3.2 Companies producing hydrocarbon fuels

Table 1 contains a selection of companies, which produce hydrocarbon-based biofuels via microbial synthesis. All companies aim at creating "drop-in" replacements for the well-established infrastructure for fossil fuel supply. Due to the volatile oil price, these companies diversified their portfolios to higher-value products like platform molecules, pharmaceutical precursors, lubricants and flavors.

It is likely that the success of these companies is related to a common issue in the development of a bio-economy: during a period of elevated oil prices, public and private financial efforts support research, which results in the foundation founding of start-ups that acquire capital to improve and scale-up their processes. When the oil price drops, making the optimistic economic targets unachievable within the expected time frame, investments and research are suddenly focused on alternative products. This scenario is repeated within a couple of years.

3.3 Process monitoring

Both, biofuel and biogas processes, are currently not equipped with much of monitoring devices. In contrast to pharmaceutical processes, low requirement of process documentation is needed for biofuel production. If the future processes should be characterized by a high tolerance against alterations of substrate quality and loading rates at biogas production, the monitoring equipment has to be improved in order to reduce the risks of process failures, especially during adoption times. Numerous sensor technologies have been developed and tested in a lab environment, a very comprehensive review was recently published [59]. However, many of these methods were not commercialized yet. This has mainly two reasons: (i) the sensor probes were just too costly or (ii) the methods and technologies were firstly developed particularly for aerobic processes, in which sensitive parameters and requirements of the probes differ from those in anaerobic processes and they still lack reliability in industrial scale. Beside the gas phase composition, which is usually monitored in biogas plants and liquid biofuel processes, the pH-value is the only one frequently measured parameter in the liquid phase. Nevertheless, process disturbances are originated in the cell or its environment. Thus, an improved monitoring in this phase is mandatory, e.g. an increased level of short chain fatty acids would already indicate a process disturbance in anaerobic digestion, while the methane production might not be effected until a certain level is reached. In order to identify zones in the liquid phase, which are representative and suitable for the installation of probes, multiposition monitoring devices for the simultaneous measurement of several parameters have been developed [60,61]. Probes that have been commercialized recently or are promising to broaden the application for monitoring the liquid phase, have been adopted and characterized for the use in anaerobic processes. Near-infrared spectroscopy (NIR) was applied by Jacobi and co-authors in order to evaluate the feasibility to monitor relevant process parameters, e.g. total volatile fatty acids, acetic acid and propionic acid in anaerobic digestion [62]. A further development for *on line* and *at line* NIR analysis is the Transflexive Embedded Near-Infra Red Sensor (TENIRS) technology. The TENIRS system is based on transflexion, a combination of transmission and reflectance, and requires the utilization of a transmission vial for the scanning process. Transmission is usually used for analysis of liquids, whereas solids are scanned by their reflectance. Hence, both phases, e.g. in manure and slurries

can be analyzed with the same device [63]. The TENIRS technology was used for monitoring acetic acid and propionic acid in the substrate at two different temperatures in a biogas digester of 400 L [64]. Based on a calibration model, process instabilities were rapidly detected. The authors also applied NIRS-technology at a range between 960 and 1700 nm for the *on line* determination of organic acid concentrations in anaerobic digestion for the classification of the process state [65, 66]. Raju and co-authors developed a diffuse reflectance probe, which can be attached to a NIR spectrometer to monitor the total ammonia nitrogen (TAN) content in an anaerobic digester [63]. Raman technology is also promising for future application, as probes are now much more convenient for biotechnological application. Ávila et al. coupled multivariate analysis to Raman spectroscopy for the *on line* determination of glucose, ethanol, and glycerol during a fermentation with *S. cerevisiae* [67]. The simultaneous saccharification and fermentation of corn mash by *S. cerevisiae* was monitored in situ by determining saccharides and ethanol with detection limits of 0.19% (Ethanol) and 0.25% (Glucose) as well as quantitation limits of 0.61% (Ethanol) and 0.82% (Glucose) [68]. Further studies report the utilization of Raman spectroscopy for monitoring ethanol production in yeast fermentation based on lignocellulosic biomass and under high hydrostatic pressure [69, 70]. Beside yeast fermentation, it has been shown that Raman spectroscopy was able to determine glucose and ethanol concentration also after enzymatic hydrolysis and fermentation of biomass [71]. The progression of pre-treatment, in particular hydrolysis prior to ethanol production, was monitored [72], even in the presence of an elevated background caused by lignin-derived compounds, if lignocellulose was used as feedstock. A recent publication describes the use of an amperometric microbial electrochemical sensor for acetate measurement using *Geobacter sp.* that oxidize acetate. A real-time measurement of this electroactive biofilm yielded a resolution of 0.25–1 mmol/L of acetate [73]. Dissolved methane and sulfide concentration determination was conducted by membrane extraction [74].

Calorimetric and acoustic methods for the analysis of anaerobic bioprocesses regarding biological parameters are subject to optimization in research projects, which try to use the potential of these methods for monitoring feedstock composition. The determination of in situ particle distribution is a promising method to evaluate pre-treatment methods. Own experimental work indicates the laser-light back reflection to be a suitable method for the detection of the particle size distribution in anaerobic digestion broths. In this case, the change of the particle size distribution after filtration and centrifugation steps of biogas culture broth samples could be determined in situ. Current investigations try to separate living cells from other carbon material based on the optical reflectance profile. By this, also agglomeration of cells, which might support the intercellular exchange of compounds, e.g. between acidogenic and methanogenic microorganisms, might become tractable.

Beside metabolite monitoring in the liquid phase, a method is still lacking that enables the monitoring of cell physiology *on line* or *at line* in anaerobic digestion. Flow cytometry, a rather established method for cell culture and also widely applied for microbal cultivation, faces several problems when used in these processes, as the composition of the culture broth leads to high background signals. Additionally, the diverse composition of the microbial community causes difficulties in finding suitable staining methods, which provide comparable results. Suitable measurements on the single-cell level are rather hard to obtain. We have recently adopted another method, in which cells are opposed to an electrical field. The time of reorientation is measured as inducible polarizability of cells. Although this method is not able to quantify the polarizability on a single-cell level, but rather as a mean of the whole culture, it was shown that this parameter can be related to the methane production and overall metabolic activity of the culture as previously shown [75]. Thus, this electrooptical measurement, which has been applied for pure anaerobic cultures successfully before when quantifying acid and solvent fluxes in *C. acetobutylicum* batch cultivations [76], could be well-adopted to the complex culture. The method allows a semi-automated sample preparation, which leads to reproducible results. When this method is applied for screening, e.g. for the evaluation of different feedstock composition, the costly and sophisticated measurement of the gas phase composition or single metabolite concentrations in parallel cultivation experiments can be avoided. This allows the application of a parallel cultivation methodology also for anaerobic digestion, which is up to now often restricted due to the lack of rapidly conductible analysis tools.

The above-mentioned devices and some other promising techniques support the application of an improved monitoring for process development and optimization for both, anaerobic pure and mixed culture processes. Nevertheless, a next step is the combination of data with model-based approaches like they have been introduced in the last decade for accelerated process development of aerobic processes, e.g. recombinant protein production.

3.4 Model-based approaches

Monitoring and control of complex cultures like in anaerobic digestion remains still a challenge, because many parameters can't be determined as easily as in pure cultures. Temporary process instabilities that might arise from alternating process conditions or in general a poor process performance can typically be solved by re-adjusting the feedstock load and composition in biogas processes [77], if the problem was diagnosed correctly and promptly. In this case, a process simulation gain real benefits, as usually an accurate prediction of the process performance allows for a risk reduction. Furthermore, a model-based control increases the probability that a process disturbance is interpreted correctly based on a certain, simultaneous change of process parameters, instead of relying only on a few measurements itself.

Suitable models have been developed for anaerobic digestion, which consider different microbial populations, predicting the change of VFA, pH-value and biogas production [78–81]. Substrate conversion is a central aspect in the modeling of the biogas process [82], especially under transient or generally dynamic conditions. There are four models, which incorporated more detailed kinetics about this process step [81, 83, 84], the IWA AD model No1 – ADM1 [85], and its subsequent implementations [86], respectively. Although a detailed model-based description about the complex processes enables the

simulation of transient conditions, the various data required usually exceeds by far what can be measured in industrial plants. The AM2 (or AMOCO) model [87] can serve as a kind of reduced model for anaerobic digestion. Only two populations are considered, namely for the acidogenic and methanogenic phase. The model performs well when compared with the ADM1 as reference model [88, 89]. A simulated data set was used to describe acidification with the AM2 model for the purpose of parameter identification. So far, the simulation of data of a lab-scale biogas process pulse-wise fed with maize silage was performed [90]. In case anaerobic digestion is split in several process stages, the evolvement of these in the separate biotechnological conversions has to be controlled as well. One advanced model contributes to this challenge, while it simulates either a two-stage biogas production process, and after adaptation, the distribution in a leach-bed bioreactor. The model includes a coupling of chemical reactions with transport of process liquid variation of microbial population [91, 92]. The link from a model to control a plant at industrial scale is described for a dynamic plant operation using non-linear predictive control [93].

The use of artificial neural network (ANN) models in simulation and optimization of biogas processes might have some advantages over non-linear estimation approaches. These models do not require former data for the identification of interconnections between variables [80]. Both ANN and hybrid modeling represent an option to conventional theoretical models, particularly when detailed knowledge of the metabolic paths, of the real kinetic mechanisms, and of transport phenomena are hardly achievable [94]. ANN models have been applied to simulate biogas production using one or two hidden layers and fourfold cross-validation for reliability [95], or in a full-scale plant from organic waste using two hidden layers [96]. A non-linear autoregressive exogenous neural network model was applied to predict daily biogas production from cattle manure in a waste to energy plant for 15 days [97]. The results show a +/-8% deviation between model and real data from digesters.

The coupling of such models to control strategies in industrial scale remain a challenge and will be an important step towards improved process risk reduction under unstable operation conditions towards an increased substrate flexibility on the process level and the integration of such plants into smart bioproduction grids. This was also already conducted at dynamic model-based approaches for fed-batch fermentation of hydrolysate sugars for bioethanol production with *S. cerevisiae* [98].

3.5 Increasing substrate flexibility

As mentioned in the introduction, substrates for the production of biofuels have to be gained regionally, and concomitantly, processes have to be adopted to the corresponding requirements of the available feedstock. It is neither economically nor ecologically responsible to use fuel to transport substrates in order to produce fuel. Air traffic is a prime example for the need to achieve similar product qualities with different feedstock: planes require the same fuel quality at all destinations, e.g. between Central Europe and South Africa. This fuel would be produced under completely different climatic conditions and different available side-streams (e.g. plant residues from fall harvest in Central Europe and algal-biomass and -oil produced in the South African summer).

The currently applied model organisms for heterologous pathway expression in research and industry are the gram-negative bacterium *E. coli* and the yeast *S. cerevisiae* (see Table 1). Both organisms are ubiquitous in research labs, very well characterized and a multitude of molecular techniques are available for their manipulation, making them the convenient choice for developing processes. Nevertheless, both organisms are not the obvious choice with respect to their substrate flexibility. For example *S. cerevisiae* is natively unable to utilize pentose sugars like xylose, a major component of lignocellulose. *E. coli* is only moderately able to utilize oligosaccharides. Other well-characterized organisms like the gram-positive bacterium *Bacillus subtilis* are genetically amendable and have a substrate scope, which includes lignocellulosic biomass [99]. Especially for fatty acid derived products, the oleaginous yeast *Y. lipolytica* is starting to be explored in an increasingly intensified manner, including tools for improved genetic engineering [55] and the broadening of its substrate scope [100]. Oleaginous yeasts are suitable for the production of fatty acid based hydrocarbon fuels, since they exhibit a strong flux towards fatty acids. Accumulated oil bodies could be used as a large substrate reservoir for decarboxylation. Metabolic engineering attempts of *S. cerevisiae* have been successful at transfer some of the TAG formation capabilities from oleaginous yeast [101]. Substrate flexibility requires the development of robust transcription factors, which allow a consistent expression of all genes involved in a substrate independent manner. A promising development to address this problem could be the application of orthogonal transcription factors based on TALE or CRISPR/Cas [102]. These artificial transcription factors would allow controlled gene expression without any regulation by the metabolic state of the organism.

Promising results have been gained recently about a flexible operation of biogas plants at changing feeding regimes [103], although some challenges remain. From an engineering point of view, flexibility in plant operation for anaerobic digestion can be improved by separating the process in various steps, e.g. by coupling and de-coupling additional acetogenic or acidogenic processes to the methanogenic stage. These concepts have been examined with the aim to increase a demand-oriented provision of biogas supply [104]. They can be coupled with a supply-oriented feedstock use, e.g. for reducing the yield when the product demand is low and feedstock of poor quality needs to be converted. Beside the aforementioned enzymatic (pre-) treatment [105] and mechanical disruption, thermal hydrolysis offers a possibility to prepare different kinds of feedstock for anaerobic digestion [106]. The addition of enriched cultures for boosted cellulose degradation was proven recently as a feasible method to optimize hydrolysis and increase process robustness [107].

3.6 Process safety and "genetic firewalls"

Tight regulations exist in many countries for the application of genetically modified organisms (GMO). These stipulate biocontainment of GMO, which is a major cost factor in any GMO-bioprocess, since closed systems, decontamination procedures and highly trained personnel is required. In order to establish

a bio-economy for low-cost products like fuels and fostering rural economic development, public and political acceptance for GMO under certain conditions will be essential. Additionally, research funding for the development of new methods and containment strategies, which allow the optimization of production strains without the above-mentioned costly requirements, is needed. Current practice is the use of so-called self-cloned strains, which—in case of Germany—are defined by German legislation (§3 Nr. 3cc GenTG). Currently, bacterial strains can be considered self-cloned if any, or only small scars of foreign DNA remain in the host. This can be achieved, for example, with the Cre/lox-system, which is based on a site-specific recombinase that removes selection markers after deletion of unwanted regions, leaving a 34-basepair scar [108]. Scarless systems have been developed as well allowing for manipulations without any remaining DNA in *B. subtilis* and *E. coli* [109,110]. Modern genome editing tools such as zinc-finger nuleases, TALEN or CRISPR/Cas allow multiplex scarless deletion in eukaryotes [111].

Self-cloned strains only include the removal of unwanted traits or the re-regulation of pathways already present in the host, but not the integration of foreign DNA. In order to resolve this issue, special containment strains, which possess, for example, auxotrophies that inhibit growth outside a controlled environment, or killer-switch-systems like the induction of a toxin in absence of an artificial inducer not present in nature, can be applied and further developed [112, 113].

4 Concluding remarks

In this review, several promising, recently developed techniques were addressed to achieve a better integration of—in this case—biogas and liquid drop-in biofuels into regional carbon cycles and supply infrastructure, and thus support the idea of smart bioproduction grids. Such an idea is supported by systematic optimal design of biorefinery production chains and schedules [114, 115]. However, many efforts remain to be achieved. Even while the oil price reached above 100 US-$/barrel in some of the recent years, most of the commercial ventures were and are focusing on higher value bioproducts. Sapphire Energy is marketing high-value omega-3 oils like DHA and EPA, Amyris is developing its portfolio towards specialty fatty acids/hydrocarbons for fine chemistry and cosmetics, while other companies like Genomatica started off with smaller platform molecules that can be turned into fuels or fine chemicals. Nevertheless, in the long term, the developed pathways and processes might be adapted towards a low-cost product process. Neil Reninger, co-founder of Amyris, puts this vision for biofuels into more drastic words: "we need a product so cheap we can burn it". Albeit that previous public and private funding for biofuel research during peaks of the oil price (e.g. before the financial crisis of 2008) has not yet led to carbon neutral transportation, it has resulted in the development of novel concepts, methods and technologies to produce more and more fossil oil derived compounds in a renewable manner. With rapid and cheap genome sequencing enabling us to unlock nature's reservoir for biochemical pathways to hydrocarbons, the current trend of transferring these to microbial cell factories and optimizing them towards economically feasible production titers will continue. In Europe exists a strong public skepticism against genetically modified organisms, which are a cornerstone of a low-cost production. In other research fields, research groups have recently genetically modified the genome of human embryos, for example, to establish resistance against HIV infection [116]. Other scientists review the application of synthetic microbes as smart and adaptive drug delivery systems in the human body [117], indicating that globalized scientific progress has long surpassed public awareness and perception in some countries. An open discussion of risks, but also opportunities, is also mandatory in case of biofuel production. Besides, the large amount of substrate required usually attracts criticism as it is the case for biogas production in Germany, in which the renewable energy act in its first versions have boosted plant installation. Similar criticism has arisen for bioethanol production. What can be learned from this is the requirement of a better integration of these processes into infrastructure and feedstock supply chains at maximum efficiency, which anyway is the basis for a cost-efficient production. Therefore, all possibilities provided by technological development, have to be considered and evaluated for eventual benefits and risks without prejudices. As a final and closing remark, it must be stated that the presented scenarios and technologies are not likely to allow us to continue living on the high level of energy and resource consumption as is today, but that they can be part of a "big picture", which will include a variety of renewable energy sources, and most of all a responsible utilization of the available resources by everyone.

Practical application

Recently developed tools for strain and pathway engineering, feedstock flexibility, process monitoring, control and integration into broader concepts of biomass utilization for drop-in biofuel and biogas production are summarized.

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