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[ChTitle] Biobased production of chemicals through metabolic engineering

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Abstract: Energy resources are one of the most central topics of our century. Even though fossil fuels met the needs for many years, reserves diminish faster than they regenerate. Therefore, alternatives have been found with biofuel production using microorganisms. High efficiency processes have been developed, particularly for bioethanol with productivity up to 125 g/L; however, this chapter focused on drop-in fuel that can be used directly in current engines and obtained through metabolic engineered microorganisms. Many efforts have been made and research teams were capable to produce bio-hydrocarbons with concentrations up to $\square$ 500 mg/L. Yet, these accumulation rates are still insufficient for a profitable process. This chapter also discussed the bottlenecks and solutions that would allow solving current limitations.

Key Words: Hydrocarbons, Drop-in fuels, Microorganisms, Metabolic engineering, Sustainable, Bio-refinery.

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1. Introduction:

In a context of increasing scarcity and price of fossil resources, petro-based chemicals' sourcing is a central concern among the scientific community. Nowadays, 70% of crude oil is used as fuel and 30% by chemical industries.¹ The demand keeps growing from year to year, while the oil reserve is irremediably diminishing. This can result in excessively expensive crude oil and can

provoke both an energetic and economic crisis, bringing unprecedented consequences to our planet and to our society.

In order to avoid these outcomes, the use of available biomasses as carbon sources has been considered. Although it is very attractive, this approach raises multiple questions. Is there enough feedstock? Is it sustainable? Are biomass prices going to increase because of the increasing consumption of this resource? The fact is: biomass is limited and most of the industrialized countries, such as USA and China, or regions such as Europe, North Africa and Middle East, have already been considered as "biocapacity debtors" in 2014. This means that their footprints are larger than their biocapacities. Moreover, these countries represent less than 50% of Earth's land surface. In other words, the world's ecological footprint is higher than its biocapacity. Under such considerations, the conclusion that can be drawn is that the surface of a 1.7 Earth would be necessary to meet the needs of humanity. It is also clear that all energy usages cannot be compensated with the available biomass (Figure 1).²

Fig. 1. An overview of human activities global footprint²

The fuel transition from fossil-resources to biomass has already started with first-generation bioethanol that consists in fermenting sugars contained in starch, found in feedstocks such as cereals (e.g., wheat, barley, corn and sorghum) and sugarcane. A massive drawback of this approach is that it uses the grain of the feedstock, resulting in either the overexploitation of the land to produce them or in the potential destabilization of the existing food/feed sectors. Therefore, second-generation bioethanol has been developed where the sugar sources are cellulose and hemicelluloses, two non-food resources. These polysaccharides are found in feedstocks such as wheat straw, corn stover, wood or agricultural residues, by-products that otherwise would be of low value. As a continuation of those efforts, the possibility of replacing petro-based chemicals (e.g., alcohols, dicarboxylic acids, diols, alkanes, alkenes, aromatics and thermoplastics) was explored. This transition must, however, meet industrial requirements: (1) feedstocks must be available and at a low price, (2) it is imperative that

their developed processes are at high yields, (3) their systems are robust and that they are inserted into an existing industrial ecosystem.

Along with the need to substitute petro-based chemicals on account of their decreasingly availability, industries are also highly motivated to realize this transition, influenced by the growing demand for natural or biobased products, especially for the pharmaceutical, cosmetic and food industries. As a consequence, bio-refinery that converts biomass into fuels, power, heat, and value-added chemicals has emerged. In synergy with existing industries, these bio-refineries participate to a circular economy, also called "blue economy", in which the by-product of one process becomes the starting material of another one.

While this bio-refinery approach is very promising, there is still plenty of room for improvement. For instance, the scale-up is a real issue. As for chemical transformations, it is a difficult and long process to go from the laboratory to the industrial reactor. However, when it comes to fermentation, new constraints must be taken into consideration during the development of the process. Microorganisms must be robust, with a low contamination risk, in order to resist to harsh reactor conditions (e.g. temperature, pH, pressure). Fermentation conditions also need to be anticipated (aerobic, anaerobic) depending on the specifications of the industry involved. To address all these potential constraints, metabolic engineering has been developed. Such genetic and regulatory processes optimizations within microorganisms to increase the production of a specific chemical are already an industrial reality.

2. Metabolic engineering:

The capacity of a microorganism to produce a specific molecule is defined by its genomic DNA. However, the corresponding metabolic pathways do not lead to all known petro-based chemicals and the natural productivity does not necessarily meet the industrial needs. Metabolic engineering is a scientific field that aims to overcome these limitations. It was defined by Bailey in 1981 as "the improvement of cellular activities by manipulating enzymatic, regulatory, and transport functions of the cell".³ Until nowadays, it relies on the principle of fermentation. A source of carbon, typically glucose, or another sugar, is made available to a particular microorganism to be catabolized into pyruvate. The latter will be converted into acetyl-CoA which "feeds" the Krebs cycle and the respiratory chain, allowing the production of energy in the form of adenosine triphosphate (ATP). The metabolites produced by these pathways are used for the biosynthesis of the necessary building blocks (e.g., amino acids, ribonucleotides, deoxyribonucleotides, fatty acids, vitamins) needed to synthesize macromolecules or biopolymers (e.g., proteins, lipids, nucleic acids, polysaccharides).

Metabolic engineering consists in making cells overproduce a metabolite or biosynthetic product of interest. The first technic to improve strains consisted in inducing mutations in genomic DNA using carcinogen chemicals. However, the randomness of this method makes it time consuming and the target phenotype is not necessarily reached. The progress that was made in recombinant DNA and gene knockout technology provided a powerful toolbox. It became possible to improve directly specific properties through the modification of particular biochemical reactions.

Metabolic engineering was brought to a new level with the advent of synthetic biology. This strategy consists in using computer-assisted biological engineering to (re)design and build biological components and systems that do not exist in Nature. It differs from classical technics by bringing an "engineering design and development" component (primarily computation modeling) to the thinking process. Using this strategy, a significant number of molecules have been produced by academic

laboratories for a wide variety of industrial sectors, essentially agricultural, pharmaceutical, food or energy industries.

3. Different carbon sources for the microorganisms:

When producing high added value chemicals *via* metabolic engineering, a carbon source is required to feed the micro-organisms of interest. The first generation of bioprocesses used sugars, mainly glucose, or molasses that had been fermented to give the target molecule. This is the case for first-generation bioethanol produced through natural strains (*S. cerevisiae* or *Z. mobilis*). While this approach is perfectly valid for small and medium scale production, it is not applicable for a global scale. Indeed, several times the production capacity of land-based agriculture would be necessary to completely substitute the quantity of fossil hydrocarbons that is currently extracted. Knowing that 80% of crude oil is used for fuels, first generation bioethanol cannot meet this demand. Moreover, the overexploitation of the land with soybean, cereal, cane and sugar beet crops, leads to depletion of soils and water resources. At the same time, the use of pesticides leads to the disappearance of insects and increases pollution. For that reason, significant efforts needed to be done in order to find an environmentally friendly alternative solution.

A second generation of carbon was found in biomasses. These plant and agricultural residues and other organic waste are highly abundant on the planet and very little valued. They are composed for the most part of cellulose and hemi-cellulose (~75%); polymers structured around six different building blocks: glucose, xylose, galactose, mannose, arabinose and rhamnose. It is a massive reserve of carbon that is only waiting to be used. However, most microorganisms cannot use biomass directly because of the complexity of the structures. It must therefore be decomposed beforehand to release the sugars that compose it.

There are two major approaches with the intent on deconstructing biomass. The first one is thermochemical degradations, such as gasification or liquefaction processes. These methods consist in roasting and gasification. The first one stabilizes the biomass, and the second gasifies the substrate under a controlled atmosphere, as a way to obtain a gas composed of simple organic molecules (carbon monoxide and hydrogen). Those can be then treated by conventional chemical catalysts or digested by microorganisms. Yet, this technology is energy intensive and produces substantial carbon dioxide. It can thus only be considered in the case of a superabundant and low-cost biomass.

The second approach consists in decomposing the biomass without affecting the integrity of the building blocks. This approach is particularly relevant for the production of chemicals through metabolic engineering in which microorganisms use glucose, arabinose or xylose as carbon source. This technic is based on a steam explosion pretreatment followed by an enzymatic hydrolysis. The sugars that are produced can be used for fermentation. The next step in this strategy is to artificially manufacture a strain or a consortium of microbial strains capable of digesting complex carbon matrices and form a finished compound in a single step.

Also, encouraging works are currently performed towards a third generation of carbon, the carbon dioxide present in our atmosphere. There are two different strategies developed around this research topic: (1) micro-algae that utilize light and photosynthesis as an energy source, and (2) natural or genetically modified microorganisms that use hydrogen as energy source.

4. Success in producing chemicals through metabolic engineering:

Nowadays, microorganisms can produce a wide range of biobased chemicals that are useful for various industries. These chemicals can have very different applications going from fuel and commodity plastics to pharmaceutical and agricultural products.

4.1. Biofuels:

Historically, lower alcohols, such as ethanol and butanol, were the first chemicals produced by fermentation. There are two principal reasons for this particular interest. Firstly, studies on several microorganisms (*E. coli*, *S. cerevisiae*, *C. acetobutylicum* or *Z. mobilis*) showed that natural metabolic pathways were leading to these chemicals. Secondly, and most importantly, these lower alcohols can be used as fuels.

Considerable amount of work was done on the production of bio-ethanol. Many microorganisms were studied, going from Gram-negative bacterium to yeast. It is probably, along with *n*-butanol, the molecule that gathers the most data when it comes to fermentation. The original metabolic engineering work that led to production of ethanol was realized in 1987 where Ingram *et al.* inserted the genes encoding essential enzymes of the fermentative pathway for ethanol production in *Z. mobilis* into *E. coli*.⁴ This modified strain, as well as the fermentation conditions, were deeply studied and optimized to obtain an efficient microorganism capable of producing ethanol from glucose and xylose at a titer of 48 and 40 g/L, respectively.⁵⁻⁷ Further work was realized to enhance ethanol tolerance in *Escherichia coli*,⁸ ameliorate the productivity⁹ or substitute the carbon source toward glycerol.¹⁰ Other bacteria strains (*C. phytofermentas*, *C. thermocellum*, *C. glutamicum*, *C. cellulovorans* DSM 743B and *C. beijerinckii* NCIMB 8052 consortium and *Z. mobilis*)¹¹⁻¹⁶ were also studied. While none of them reach the productivity level of *E. coli*, some proved to be interesting on account of their abilities to digest directly cellulosic biomass into ethanol. Finally, some yeast strains (*P. stipitis* and *S. cerevisiae*)¹⁷⁻²² also showed very good productivity (~125g/L). The utilization of yeast for industrial purposes is particularly interesting as these robust microorganisms are capable of working in acidic conditions (pH 4), where risks of contamination is low.

Although metabolic engineering succeeded in building microorganisms capable of producing bioethanol at an industrial level, using it directly as fuel poses some issues. First, its efficiency is one third lower than a traditional fuel. Secondly, its high hygroscopic properties can damage the engine. Therefore, it is highly recommended to use it in a mix with traditional fuel.

In order to overcome the issues cited above, the scientific community got interested in the production of *n*-butanol, isobutanol, propanol and isopropanol due to their better efficiency and lower hygroscopic properties. The first reported work was done by Charles Weizmann that discovered a microorganism capable of producing acetone and *n*-butanol from maize or other grain starch.²³ Unfortunately, the name of the microorganism and the productivity data does not appear in his patent document. As for ethanol, *E. coli* was largely studied and it resulted in engineered strains that produce *n*-butanol with relatively high productivity (~30g/L).²⁴⁻²⁹ Other studied microorganisms provided lower accumulation levels but used different carbon source such as mannitol or xylose.^{14, 30, 31} Though the accumulation levels cannot be compared, interesting work was done on cyanobacteria that were capable of producing *n*-butanol using CO₂ as carbon source.³² However, it has to be noted that, in the case of *n*-butanol, metabolic engineering did not succeed in producing the best strain in term of productivity. Still nowadays, the natural strain *C. acetobutylicum* is capable of reaching accumulation levels higher than 100 g/L.³³ Unfortunately, metabolic engineered strains are far from this level of productivity.

Other alcohols, such as 1-propanol, *iso*-butanol and *iso*-propanol, have been considered as biofuels and strains were engineered to give relatively good productivity levels. The metabolic engineered strains were especially efficient in the case of *iso*-butanol and *iso*-propanol, reaching productivity levels of 50 and 140 g/L, respectively.³⁴⁻³⁶ Furthermore, other defined biofuels were studied (e.g., hydrogen, iso-butyraldehyde and triacylglycerol) but showed relatively low productivity levels.³⁷⁻³⁹

Another approach for the production of biofuel is to focus on a mixture of combustible compounds rather than on a particular molecule. Biodiesel is such a mixture and it can be obtained through the transesterification of vegetable oil (e.g., soybean, rapeseed and palm) with methanol or ethanol. However, the production of biodiesel from these oils raises the same issues than the ones raised by first generation bioethanol. Therefore, research has been carried out to produce biodiesel of second generation. Different microorganisms were identified (bacteria, yeasts and micro-algae). However, unlike second generation bioalcohol production, the accumulation levels reached with engineered microorganisms were not high enough to consider industrialization. This is mostly due to the fact that lipases involved in the formation of the biodiesels require chaperones and/or post-translational modifications for proper refolding.⁴⁰ The lack of knowledge on these mechanisms greatly hampered the development of efficient engineered strains. Nonetheless, the capacity of modified yeasts (*Y. lipolytica* and *S. cerevisiae*) and microalgae (*P. tricornutum*) to produce biodiesels at relatively interesting concentration levels have been shown with accumulation levels of 3 000, 400 and 150 mg/L, respectively.⁴¹⁻⁴³

Table 1. List of biofuels produced by metabolic engineered strains.

Product	Organism	Carbon source	Maximum Productivity (mg/L)
Ethanol	<i>E. coli</i>	Glucose	48 000
		Glycerol	810
	<i>C. phytofermentas</i>	Cellulose	1 150
	<i>C. thermocellum</i>	Cellulose biomass	3 210
	<i>C. glutamicum</i>	Glucose	9 200
	<i>C. cellulovorans</i> DSM 743B and <i>C. beijerinckii</i> NCIMB 8052	Deshelled corncobs	6 370
	<i>Z. mobilis</i>	Glucose	25 550
	<i>P. stipitis</i>	Xylose	36 000
	<i>S. cerevisiae</i>	Glucose	125 000
<i>n</i> -Butanol	<i>E. coli</i>	Glucose	30 000
	<i>C. tyrobutyricum</i>	Mannitol	16 000
	<i>C. beijerinckii</i>	Xylose	16 910
	<i>C. cellulovorans</i> DSM 743B and <i>C. beijerinckii</i> NCIMB 8052	Deshelled corncobs	11 500
	Cyanobacteria	Carbon dioxide	14
1-Propanol	<i>E. coli</i>	Glucose	7 000
<i>iso</i> -Butanol	<i>E. coli</i>	Glucose	50 000

<i>iso</i> -Propanol	<i>E. coli</i>	Glucose	140 000
Biodiesel	<i>Y. lipolytica</i>	Glycerol	3 000
	<i>P. tricornutum</i>	Carbon dioxide	400
	<i>S. cerevisiae</i>	Glucose	150

4.2. Polymers:

The second largest industry depending on fossil resources, after the fuel industry, is probably the plastic industry that manufactures polymer materials. This industry is very much criticized because of the ecological concerns it raises (e.g., pollution, toxicity). It is subjected to fossil resources that are not renewable and their disposals and recyclability are expensive and quite complicated. Furthermore, some additives that confer specific properties to polymers are known to be toxic. Polyvinyl chloride (PVC), the third-most widely produced polymer after polyethylene and polypropylene,⁴⁴ contains phthalates that have neurotoxic and carcinogenic potentials. Other plastics contain Bisphenol-A (BPA), a chemical compound that has been identified as endocrine disruptor. Since plastics are known to release their monomers and additives over time because of different factors (e.g., temperature, light), the presence of these molecules in their contents falls under a public health problem.

Therefore, there is an urgent need to substitute these petrosourced polymers with non-toxic alternatives that are recyclable and biodegradable. Nowadays, there are strains capable of producing monomers identical to those of petrochemicals (e.g., 5-aminovaleric acid, ethylene, styrene, isobutene).⁴⁵⁻⁴⁹ Although the bioproduction of these compounds through microorganisms is encouraging, it does not solve the problems of biodegradability of their respective polymers. Fortunately, there are strains capable of producing new materials with interesting properties. Some of them are still under development, such as 3-hydroxybutyric acid,⁵⁰⁻⁵² but others are already an industrial reality. The most studied monomers are probably the lactic and succinic acids that can be accumulated by modified microorganisms at levels of 122 and 146 g/L, respectively (Table 2).⁵³⁻⁶¹

Table 2. Bioproduction of lactic and succinic acids by metabolic engineered strains.

Product	Organism	Carbon source	Maximum Productivity (mg/L)
Lactic acid	<i>E. coli</i>	Glucose	2 000
	<i>K. oxytoca</i>	Glucose	24 000
		Maltodextrin	34 000
	<i>L. citreum</i>	Glucose	61 000
	<i>S. cerevisiae</i>	Cane juice-based medium	122 000
Succinic acid	<i>E. coli</i>	Glucose	7 000
	<i>E. coli</i> consortium	Glucose and Xylose	40 000
	<i>S. cerevisiae</i>	Glucose	3 620
	<i>C. glutamicum</i>	Glucose	146 000

Other monomers of interest can be produced by microorganisms, and more specifically by recombinant *E. coli*. 3-hydroxypropionic acid, listed in the top 10 most wanted building blocks, can be obtained with high concentration level (185 g/L).⁶² Production of muconic acid can reach accumulation of 37 g/L.⁶³ Glucaric acid can be produced at concentration up to 4.85 g/L.⁶⁴

Chitinbiose, a precursor for chitinoligosaccharides, can be accumulated at 4 g/L.⁶⁵ These new materials are usually co-polymerized with diols which are also produced *via* fermentation. Among them, we can mention the four most used: 1,2-propanediol, 1,3-propanediol, 1,4-butanediol and 2,3-butanediol.⁶⁶⁻⁶⁹

Considerable work has been done to obtain the monomers cited above. However, another strategy can be used in order to produce directly the corresponding polymers. Indeed, the progress of metabolic engineering gave access to strains capable of handling these relatively complex materials. Nowadays, modified microorganisms can efficiently produce unnatural polymer such as poly(lactic acid) and poly(3-hydroxypropionic acid) using *E. coli* or poly(hydroxyalkanoic acid) using *P. putida*.⁷⁰⁻⁷²

4.3. Therapeutic molecules:

Most of therapeutic molecules that are used in the pharmaceutical industry are either natural products or one of their analogs. It is very likely that microorganisms, having metabolic pathways to produce them, exist in Nature. However, these microorganisms can be difficult to handle or the metabolic pathways associated to the molecule simply does not exist; this is mostly true for natural compound analogs. In any case, synthetic biology is particularly relevant for this industry. Firstly, the need in term of quantity is way smaller compared to other industries (e.g., biofuels or plastics). Secondly, the added value to the final target allows more flexibility in terms of cost of the production process. Therefore, although a high productivity process will be preferred, relatively average accumulation levels do not hinder a production.

There are many therapeutic molecules produced through synthetic biology.⁷³ Although not all these molecules are at the same level of development, few of them can be mentioned to illustrate the importance of metabolic engineering in this field of research. Penicillin (Figure 2), the best known antibiotic, can be efficiently excreted by modified yeast, *H. polymorpha*, enabling exploration of yeast-based cell factories for antibiotic production.⁷⁴ Artemisinin (Figure 2), an antimalarial drug, can be obtained by a tedious and costly total chemical synthesis.⁷⁵ However, its production is highly simplified when artemisinic acid (Figure 2) is used as precursor.^{76, 77} The latter can be produced using a modified strain of *S. cerevisiae* with accumulation levels of 100mg/L, contributing to a reliable and cost-effective source of Artemisinin.⁷⁸⁻⁸⁰ Hydrocortisone (Figure 2) can be produced by recombinant *S. cerevisiae* strains with accumulation levels ~10 mg/L.⁸¹ These examples show perfectly how a strain that would not be selected in fuel industry because of its relatively low productivity would be a hit in the pharmaceutical industry.

Fig. 2. Therapeutic molecules produced by engineered microorganisms

4.4. Food additives and supplements:

Food additives and supplements are ubiquitous molecules in the food industry. Therefore, their production represents a major challenge. Chemical processes have been developed in order to meet the demand. However, the current trend among consumers is a return to "healthy eating" and there is a

growing concern for the environment, which translates into a demand for natural products, locally produced or in short circuits and in the logic of sustainable development. The biosourced character of food additives and supplements has, therefore, become an essential marketing argument for the food industry.

The most representative example of this category is vanillin, one of the most widely used flavoring agents. Because of its success in food industry, the demand became rapidly greater than the offer. Finding an alternative to natural vanillin (Figure 3) being a major stake, processes to obtain synthetic vanillin were developed. However, the growing will for natural products forced the food industry to imagine new production ways. Metabolic engineering seemed to be the best solution to overcome the latter issues. Several modified microorganisms were thus designed to meet industry expectations. Among them, three microorganisms stood out. Hansen et al. modified a *S. cerevisiae* and *S. pombe* strains and were able to report the first relatively high vanillin productivity with maximum concentration of 45 and 65 mg/L, respectively.⁸² A year later, Brochado *et al.* improved the productivity of *S. cerevisiae* with a different approach. They targeted vanillin- β -D-glucoside and used a gene deletion strategy. Doing so, concentration up to 500 mg/L were reached.⁸³ Interesting works have been reported by Ruzzi *et al.* that engineered a *P. fluorescens* strain capable of producing vanillin from ferulic acid at titer of 1.3 g/L.⁸⁴ However, modified *E. coli* outdid all other microorganisms that allowed the production of vanillin from isoeugenol with concentration up to 28.3 g/L.⁸⁵ Other major food additives and supplements, such as malic acid (34 g/L) acetic acid (78 g/L) or citric acid (87 g/L) (Figure 3) can be produced by metabolic engineering⁸⁶⁻⁸⁸

Fig. 3. Food additives and supplements produced by engineered microorganisms

5. Biobased production of alkanes and alkenes through metabolic engineering:

While significant progress has been done for alcoholic biofuels, as we previously have seen in this chapter, advances made in renewable hydrocarbon biofuels (aka "green" hydrocarbons, biohydrocarbons, "drop-in" biofuels, and sustainable or advanced hydrocarbon biofuels) are still limited. This is all the more unfortunate because these "drop-in" biofuels are similar to petroleum gasoline, diesel, or jet fuel in chemical makeup and are therefore considered infrastructure-compatible fuels. Faced with the challenges of their bioproduction, the metabolic pathways involved in their biosynthesis were studied in order to design an efficient microorganism applying metabolic engineering strategies.

5.1. Biosynthesis of hydrocarbons:

The presence of alkanes and alkenes (e.g., hydrocarbons) was observed in Nature in different types of organisms. For instance, these chemicals are used by plants as cuticular waxes, by insects as pheromones, and were found in a series of microorganisms such as *E. coli*, *R. rubrum* or *Saccharomyces sp.*⁸⁹⁻⁹¹ Most of the pathways described to date for the biosynthesis of hydrocarbons in these organisms use fatty acids as intermediary metabolites. These molecules are obtained from acetyl and malonyl CoA being the sources of acyl groups for the fatty acid synthase complex. Using acetyl and malonyl CoA-ACP transacylase, AT and MT, respectively, the acetyl group is added to the

cysteine –SH group of the β -ketoacyl-ACP synthase (KS), and the malonyl group is added to the –SH group of 4'-phosphopantetheine of the ACP. The orientation of the fatty acid synthase places the malonyl and acetyl groups in close proximity. The following four steps take place iteratively to yield the saturated carbon chain of the fatty acid molecule: condensation by β -ketoacyl-ACP synthase (KS), reduction of the ketone by β -ketoacyl-ACP reductase (KR), dehydration by β -hydroxyacyl-ACP dehydratase (HD) and reduction of the double bond by enoyl-ACP reductase (ER) (Figure 4).⁹² These steps are repeated until the carbon chain reaches the wanted size (usually 16 carbons).

Fig. 4. Biosynthesis of fatty acids

Four metabolic pathways that convert fatty acids, or their derivatives, into straight-chain hydrocarbons have been identified and previously described by Wang *et al.*⁹³ The "elongation-decarboxylation" pathway (blue pathway in Figure 5) is probably the most widely discussed. It consists in a classical *de novo* synthesis of fatty acids (Figure 4) followed by a decarboxylation step. This route was first discovered in the 60's while studying the metabolism of plants.⁹⁴ It was later on found that *E. coli* would use this pathway as well.⁹⁵ A big concern raised by this pathway was the high energy-demanding decarboxylation step that needs to be activated by a β -substituent. This question was solved in 2011 by Rude *et al.* who highlighted that the decarboxylation of fatty acids was catalyzed by OleT_{JE}, a P450 peroxygenase from the cyp152 family.⁹⁶

The "head-to-head condensation" pathway (green pathway in Figure 5) is the second most widely discussed. It was first reported by Albro and Dittmer in 1969.⁹⁷ It is only 30 years later than the genes involved in this transformation were characterized. Bioinformatics and biochemical analysis allowed the identification of a three-gene cluster from *M. luteus*.⁹⁸ The head-to-head condensation reaction requires four protein families (OleABCD). The first step, namely the OleA-catalyzed Claisen condensation, generates a β -ketoacid capable to spontaneously decarboxylate to yield the corresponding ketone.⁹⁹ OleC and OleD then convert this product to olefins.¹⁰⁰ It is still not clear what the role of OleB is, but its presence in the cluster suggests that it is necessary for olefins biosynthesis.

The third route, also called PKS pathway (orange pathway in Figure 5), highly similar to the "elongation-decarboxylation" pathway, was elucidated thanks to Gu *et al.* who were interested in the enzyme *curM* responsible for the formation of the terminal double bond of Curacin A.¹⁰¹ In parallel, it has been shown that a marine cyanobacterium, *Synechococcus sp.* PCC 7002, was capable of producing 1-nonadecene and 1,14-nonadecadiene. Therefore, BLAST of *curM* sequence against the genome of *Synechococcus sp.* PCC 7002 was performed which resulted in the discovery of an open reading frame (ORF) encoding a protein sharing 45% homology with *curM*. This ORF was named olefin synthase (Ols). Ols is a part of a multi-domain type I polyketide synthases (PKS). The fatty acid

moiety of the acyl-ACP is transferred to the ACP1 of the PKS domain *via* by the loading domain (LD). A condensation of the acyl-ACP1 with malonyl-CoA catalyzed by the central extension module (ketosynthase (KS), acyltransferase (AT), ketoreductase (KR)) form a β -hydroxy group that is activated *via* sulfation using the sulfotransferase (ST) domain. It has been hypothesized that subsequent dehydration and decarboxylation reactions could be catalyzed by the C-terminal thioesterase (TE) domain.¹⁰²

A fourth route (red pathway in Figure 5), called AAR/ADO pathway, was identified when studying cyanobacteria. It has been established that Acyl-acyl carrier protein (ACP) can be reduced to the corresponding aldehyde by an acyl-ACP reductase (AAR).¹⁰³ The latter can be converted to hydrocarbons by an aldehyde-deformylating oxygenase (ADO).¹⁰⁴ The formation of formate alongside hydrocarbons is done *via* a redox oxygenation process. ADO was characterized as being a non-heme di-iron oxygenase, which requires a reducing system (NADPH) and O₂ to catalyze the reaction. It is noteworthy to mention that ADO activity is reversibly inhibited by H₂O₂. This inhibition can be solved by the use of a fusion catalase.¹⁰⁵

Fig. 5. Straight-chain hydrocarbons biosynthesis pathways

5.2. Production of Hydrocarbons by engineered microorganisms:

Attempts to improve the productivity of the natural pathways have been investigated. Most of the work has been done on long and very-long chain hydrocarbons, C9 to C31, and on two different host microorganisms, *E. coli* and *S. cerevisiae*. Two other microorganisms, *Synechocystis* sp. PCC6803 and *S. globisporus*, presented encouraging data as well.

"Elongation-decarboxylation" pathway:

Few studies have been realized on this pathway in order to obtain good production of hydrocarbons. Among them, one engineered microorganism reached relatively high concentrations. In 2014, Li *et al.* reported the *in vitro* decarboxylation activity of OleT_{JE} from *Jeotgalicoccus* sp. ATCC 8456 on fatty acids in presence of H₂O₂. Furthermore, RhFRED reductase domain from *Rhodococcus* sp. was fused to the C-terminus of OleT_{JE} to see if it could act as a monooxygenase. Authors reported that the OleT_{JE}-RhFRED converted lauric acid to 1-undecene in presence of NADPH with half the efficiency of OleT_{JE} in presence of O₂. Their insertion in different *E. coli* strains overproducing free fatty acids, and more specifically in the XL-100 strain (an *E. coli* BL21 fadD deletion mutant strain), resulted in a overproduction of hydrocarbons (C11 to C17) with a maximum concentration at 97.6 mg/L.¹⁰⁶ A year later, despite the significant lower productivity, interesting work has been realized on *S. cerevisiae* by Wook Chang *et al.* By inserting OleT_{JE} gene from *Jeotgalicoccus* sp. ATCC 8456 into the yeast, deleting genes using H₂O₂ as cofactor (catalase A (CTA1), cytochrome C peroxidase (CCP1) and catalase T (CTT1)) to redirect the flux towards OleT_{JE} and deleting the genes coding for

enzymes converting free fatty acids to fatty acyl-CoAs (fatty acyl-CoA synthetases (FAA1 and FAA4)), the authors were able to reach accumulation of terminal alkenes up to 3.7 mg/L.¹⁰⁷

"Head-to-head condensation" pathway:

Due to the relative complexity of this pathway involving four different enzymes, and because their involvement in hydrocarbons biosynthesis is not completely understood yet, the "head-to-head condensation" pathway has been very little studied. Nevertheless, Beller *et al.* attempted to engineer an *E. coli* strain overproducing long chain hydrocarbons *via* this route. The heterologous expression of a three-gene cluster from *M. luteus* (Mlut 13230, 13240 and 13250), coding for OleA, a fusion of OleB and OleC, and OleD, in a fatty acid-overproducing *E. coli* strain allowed the formation of long-chain alkenes (C27 and C29). Unfortunately, authors reported very low productivity with concentrations up to 40 µg/L.⁹⁸

PKS pathway:

Very encouraging work has been done on the PKS pathway. Contrarily to the "head-to-head condensation" pathway, competitive productivity has been reported. In 2011, Shen *et al.* engineered a heptaene-overproducing *S. globisporus* strain while attempting to ameliorate the production of C-1027, a relatively complicated chromophore. In a previous study, authors identified three regulatory enzymes (SgcR1, SgcR2, and SgcR3), part of the Ols family, as being involved in C-1027 biosynthesis. The overexpression of SgcR1 in the *S. globisporus* wild-type strain resulted in a overexpression of C-1027 as well as 1,3,5,7,9,11,13-pentadecaheptaene (reaching concentration of 66 mg/L for the latter).¹⁰⁸ Nonetheless, Shen *et al.* took a step further by studying the influence of two additional C-1027 regulatory enzymes, sgcE1 and sgcR. It resulted in a heptaene-overproducing *S. globisporus* strain which was obtained by deletion of sgcE1 and sgcR genes, and overexpression of sgcR1. By doing so, authors increased 2 fold the production of heptaene reaching concentration up to 129.3 mg/L.¹⁰⁹ Few years later, Liu *et al.* implanted this pathway into *E. coli* in order to access pentadecaheptaene (PDH). Their initial *in vitro* results suggested that alkene production was linked to the SgcE10: SgcE1 ratio. Therefore, authors developed an *E. coli* strain with fine tuning of sgcE10 expression by using synthetic promoters in order to control the quantity of targeted enzymes in the microorganism. Through fed-batch fermentation, accumulation of PDH reached concentration up to 140 mg/L. A flow chemical hydrogenation was used to get to the corresponding alkane.¹¹⁰

AAR/ADO pathway:

Researches on the AAR/ADO pathway seemed to have given the best results. Firstly, proofs of concept have been validated using yeast and cyanobacteria: *S. cerevisiae* and *Synechocystis sp.* PCC6803. Nielsen *et al.* focused on the modifications of *S. cerevisiae*'s metabolism to access long chain hydrocarbons (C10 to C17). Fatty acyl-ACP/CoA reductase (AAR) and a fatty aldehyde deformylating oxygenase (*Se*ADO), encoded by *S. elongatus* orf1594 and *S. elongatus* orf1593 respectively, were introduced into *S. cerevisiae* using plasmids. However, no production of hydrocarbons was observed despite the inserted heterologous biosynthetic pathway. It has been hypothesized that this lack of activity was due to the presence of an aldehyde dehydrogenase naturally present in yeast's metabolism. Deletion of the gene encoding for hexadecenal dehydrogenase (Hfd1) resulted in the formation of alkane at concentration up to 22 µg/L.¹¹¹

Access to very-long chain alkanes (C27 to C31) was made possible by Joubes *et al.* that imported the alkane biosynthetic enzymes of *A. thaliana* into *S. cerevisiae*. It has been shown that *A. thaliana* Eceriferum 1 (CER1), from the family of fatty aldehyde deformylating oxygenase (ADO),

was an essential protein for wax alkane biosynthesis. However, its expression in heterologous systems did not result in the formation of alkanes.¹¹² CER1 was found to interact with *A. thaliana* Eceriferum 3 (CER3) and with the cytochrome b5 isoforms (CYTB5s). The co-expression of CER 1 and 3 allowed the conversion of fatty aldehydes into the corresponding alkanes, amplified in presence of CYTB5s. However, accumulation remained very low with a maximum concentration of 86 µg/g of dry weight.¹¹³

Interesting work has also been performed on a cyanobacteria strain, *Synechocystis sp.* PCC6803. It is, for modified AAR/ADO pathway, the second best microorganism in term of hydrocarbons productivity. It has been shown that the natural strain of *Synechocystis sp.* PCC6803 possesses the AAR/ADO pathway in their metabolism. The access to acyl-ACPs can proved difficult because of their incorporation to membrane lipids. However, the latter can release free fatty acids during its degradation that will be converted back to acyl-ACPs *via* an acyl-ACP synthetase (AAS). Recombinants plasmids were inserted into *Synechocystis sp.* PCC6803 in order to overexpress these key proteins (AAS, AAR and ADO). This strategy redirected the carbon flux to acyl-ACPs and favored the production of hydrocarbons, with reported accumulations up to 26 mg/L.¹¹⁴

Researches done on cyanobacteria allowed 1000 fold higher concentration of hydrocarbons, del Cardayre *et al.* greatly improved the productivity by using *E. coli* as host microorganism. Although the wild strain produced no detectable hydrocarbons, insertion of *S. elongatus* PCC7942_orf1593 and orf1594, coding for AAR and ADO respectively, allowed a titers of 300 mg/L (~80% of excreted hydrocarbons) with the use of a modified mineral medium.¹⁰³

Other engineered *E. coli* have been reported in the literature, unfortunately with lower accumulation. Zhao *et al.* attempted to increase the productivity with the co-expression of a catalase aimed at withdrawing toxic byproducts of the culture media. They also introduced a type-I fatty acid synthase (FAS) from *C. ammoniagenes* to bypass regulatory mechanism of fatty acids production in *E. coli*. By doing so, authors were capable of producing hydrocarbons with maximum concentrations up to 57 mg/L.¹¹⁵ Zhu *et al.* proposed a similar modified microorganism than the one constructed by del Cardayre *et al.* Overexpression of AAR and ADO from *S. elongatus* PCC7942 along with ACP allowed an accumulation of hydrocarbons from 3.1 to 24 mg/L, depending on the expression strategy that was used. Authors improved greatly their strain by the identification of two genes: yqhD, an inherent aldehyde reductase in *E. coli*, and fadR, an activator for fatty acid biosynthesis. The deletion of the first and the overexpression of the second allowed a tenfold increase in hydrocarbons titer reaching concentration up to 255.6 mg/L.¹¹⁶

New pathways:

Although very encouraging improvements have been obtained by using natural pathways, stimulating works have been developed around alternatives pathways. Most of them have been conducted by substituting AAR, from AAR/ADO pathway, by another enzyme. Foo *et al.* designed a modified *S. cerevisiae* strain that utilized a fatty acid- α -dioxxygenase (α DOX) from *O. sativa* coupled with a gene deletion strategy of FAA1 and FAA4 to avoid side consumption of FFAs. What differentiate this route from the AAR/AADO pathway is the precursor (FAAs) and the cofactors involved: AAR and α DOX go through a NADPH/NAPD⁺ system and an O₂/CO₂ system, respectively. Though α DOX is less energy intensive due to its catalytic system, the accumulation levels of hydrocarbons were very low with maximum concentration of 73.5 µg/L.¹¹⁷

While other studies focused primarily on hydrocarbons productivity, Akhtar *et al.* got interested in the substrate selectivity of these enzymes and identified a carboxylic acid reductase

(CAR) from *M. marinum* that converts fatty acids (C6–C18) into corresponding aldehydes. *In vitro* trials showed the catalytic conversion of fatty acids to fatty alkanes (C7–C15) in presence of CAR and ADO. Applying this strategy *in vivo* by inserting both genes encoding for CAR and a chain-length specific thioesterase into *E. coli* BL21(DE3) revealed the production of alkanes (C7–C15). Unfortunately, low alkane productivity was reported with accumulation of 2 mg/L.¹¹⁸ Howard *et al.* followed the same strategy using a fatty acid reductase (FAR) from *P. luminescens* and an aldehyde decarbonylase (ADO) from *N. punctiforme* inserted into *E. coli* and reported production of a wide range of alkanes, and more surprisingly alkenes, to concentration up to 5 mg/L.¹¹⁹

Nielsen *et al.*, inspired by the work of Akhtar *et al.*, aimed to improve the hydrocarbons productivity by using a modified *S. cerevisiae* strain through CAR/ADO pathway. It has been established that free fatty acids (FFAs) can be accumulated to much higher levels than fatty acyl-CoA (>200 fold higher).¹²⁰ Therefore, they first designed a platform strain of *S. cerevisiae* that redirects citrate to acetyl-CoA that is then converted in FFAs by endogenous or heterogeneous enzymes. Furthermore, reverse pathways were knocked down through the deletion of the corresponding genes (hFD1, FAA 1,4 and POX 1). Doing so, FFAs accumulated in the modified strain to a maximum concentration of 10.4 g/L. Authors used their previous AAR/FADO system to produce alkanes with this new strain but observed low productivity of 100 µg/L. To overcome this issue, an alternative has been found by using carboxylic acid reductase (MmCAR) expressed in *M. marinum* activated by 4'-phosphopantetheinyl transferase (NpgA) from *A. nidulans*.¹¹⁸ This pathway enabled a higher alkane production up to 480 µg/L. The identification and deletion of genes converting fatty aldehydes to fatty alcohols, aldehyde reductases (ALRs) and/or alcohol dehydrogenases (ADHs), coupled with the expression of *SeADO*, permitted the intensification of the process. Finally, the additional expression of *N. punctiforme* (*NpADO*) increased alkane production to 820 µg/L.¹²¹

In 2013, Lee and Choi used knock out gene strategy in order to minimize the potential intermediates loss. β -oxidation pathway was disabled by deleting the *fadE* gene thus avoiding the degradation of fatty acyl-CoAs. Then, the authors deleted the *fadR* gene in order to prevent upregulation of the *fabA* and *fabB* genes responsible for unsaturated fatty acids biosynthesis.¹²² The absence of unsaturated fatty acids lifted the inhibition on 3-oxoacyl-ACP synthase (*FabH*) that favored the accumulation of medium-chain acyl-ACPs. The latter were converted into FFAs using a modified thioesterase (*tesA*), that were then converted into the corresponding alkanes through a sequence composed of an *E. coli* fatty acyl-CoA synthetase (*fadD*), a *C. acetobutylicum* fatty acyl-CoA reductase (*ACR*) and an *A. thaliana* fatty aldehyde deformylating oxygenase (*CER1*). By using this *E. coli* strain platform, authors reported a maximum accumulation of alkanes of 580.8 mg/L, consisting of nonane (327.8 mg/L), dodecane (136.5 mg/L), tridecane (64.8 mg/L), 2-methyl-dodecane (42.8 mg/L) and tetradecane (8.9 mg/L).¹²³

These last decades, many strategies have been developed to improve hydrocarbons productivity through microorganisms. Although modification or substitution of the enzymes directly involved in alka(e)nes biosynthesis (figure 5) allowed significant amelioration, it is clear that controlling the side metabolic pathways is essential in order to offer a competitive engineered microorganism. Major progresses have been made with the deletion of specific metabolic pathways, such as β -oxidation or fatty alcohols biosynthesis, and redirection of the metabolic flux towards fatty acyl-ACPs through acetyl-CoA (figure 6).

Fig. 6. Metabolic flux optimization for hydrocarbons production

5.3. Bottlenecks and solutions

Despite the promising results obtained with engineered microorganisms, the best strains do not present a sufficient hydrocarbons productivity for potential industrialization. Different factors could be studied to achieve it. Firstly, elements directly linked to enzymes (mostly their activities) can have a negative effect on the alkanes/alkenes productivity. The most efficient pathway (AAR/ADO) is hindered by the low activity of ADO that results in the accumulation of fatty alcohols from fatty aldehydes. This issue can partially be overcome *via* the overexpression of ADO gene to boost the productivity. Another strategy consists in using directed or random mutagenesis in order to ameliorate the specific activity of the enzyme. This approach allowed Khara *et al.* to enhance the activity of ADO from *P. marinus* towards short chain aldehydes by twofold.¹²⁴ Metabolism of host microorganisms can also affect the productivity of the target molecules. Different studies, discussed previously, strongly suggest that a number of metabolic pathways lower the biosynthesis of hydrocarbons. Knock-out gene strategy allowed researchers to redirect the metabolic flux towards the targeted route; however this can be at the expense of the growth of the modified microorganisms and so of the productivity itself. Nevertheless, strains that were designed so far permitted to improve the accumulation of alkanes/alkenes.

Precursor sourcing can also be a problematic factor. Hydrocarbons biosynthesis being dependent on fatty acyl-ACPs and their derivatives (fatty acids and acyl-CoAs), any side reactions that consume them (fatty alcohols biosynthesis or β -oxidation) will directly affect the productivity. Deletion of the genes involved in these pathways seems to be mandatory to reach relatively good concentrations. In addition, it is also possible to intensify the production upstream. As seen previously in this chapter, acetyl- and malonyl-CoA are central intermediates in fatty acids biosynthesis; however they are also used in many other metabolic pathways. Therefore, intensifying their production would allow both supporting metabolism and boosting fatty acids production. Koffas *et al.* followed this strategy and designed an *E. coli* strain that overexpresses each enzyme involved in fatty acid biosynthesis. Combining it with deletion strategy on the precursor enzyme of β -oxidation (fadD), authors were able to reach high productivity with concentration up to 8.6 g/L.¹²⁵ It is easily conceivable to add the metabolic pathways yielding hydrocarbons to this microorganism and therefore improving the productivity.

While these approaches give positive preliminary results, it seems that environmental conditions greatly influence the hydrocarbons productivity as well. Temperature can play a crucial role in the process. Zhu *et al.* showed that growth of their modified strain was closely linked to this parameter. At 18 °C, cell mass reached an optical density at 600 nm (OD600) of 8.5 with accumulation of hydrocarbons up to 5.3 mg/L. At 30 °C, these values were quite similar with OD600 of 9.3 and an accumulation of 5.0 mg/L; however they dropped at 37 °C with an OD600 of 7.3 and accumulation of 4.2 mg/L. Surprisingly, the maximum titer was observed at 24 °C with an OD600 of 19 and accumulation of 26 mg/L. This observation shows the importance to bring the attention to the details with a 3.5 fold improvement in productivity when incubated the culture at an "unconventional" temperature. The composition of the culture medium can also improve the accumulation of hydrocarbons. By using a modified mineral medium, del Cardayre *et al.* developed a strain that is capable of excreting hydrocarbons up to 240 mg/L. This discovery opened the door to continuous extraction which makes industrialization more plausible.

Other major concerns are linked to the concept of fermentation in general. When it comes to incorporating a promising engineered strain in an industrialized process, several questions can be raised. The cost of the culture medium, the accumulation time of a wanted product and the robustness of the process are the main obstacles to scale up. The question of the carbon source, discussed in the introduction, is also a major issue. Use of first generation sugars has to be ruled out as it can destabilize an existing industry, especially the agricultural one. The valorization of biomasses allowed to take a step towards sustainability but is not sufficient when it comes to high productivity. Thus, the solution that offers metabolic engineering feeding from first and second generation sugars is only sustainable for low or medium production of molecules of specialty and fine chemicals. Using microorganisms that consume CO₂ as carbon source would allow considering large-scale fermentation, as for the production of biofuels. Moreover, industrial fermentation handles large amounts of water that must be recycled and reprocessed before being released. Therefore, the nature of the wanted product and the purification method to access it dictates the viability of a process. While approaches involving extraction with solvent have been developed, purification by distillation will be largely preferred when possible. Finally, parameters intrinsic to fermentation can hinder industrial viability such as contamination of cultures, formation of biofilms or oxygenation of the tank during use of aerobic processes.¹²⁶

Since production of "drop-in" biofuels revealed to be quite difficult, approaches have been developed in order to access small building blocks that can be later transform into hydrocarbons. These building blocks had to have reactive chemical functions, which precluded methane and small alkanes. However, molecules such as ethylene shown great interest. Three biosynthesis pathways have been reported in the literature. The first one, called the "ACC pathway", is mostly found in plants and uses methionine as precursor. The latter is transformed into *S*-adenosyl-methionine *via* SAM synthase. The intermediate is then converted into ACC and 5'-methylthioadenosine (MTA) *via* ACC synthase. Finally, ACC is converted into ethylene by an ACC oxidase. It has to be noted that MTA is regenerated into methionine by a sequence of 4 enzymes allowing the level of methionine to stay constant while ethylene accumulate.¹²⁷⁻¹²⁹ The second pathway, called the "KMBA pathway", can be found in microorganisms and uses as well methionine as precursor. The first step consists in a transamination involving a α -keto acid *via* a transaminase. The intermediate thus formed (KMBA) is oxidized *via* a peroxidase or an oxidoreductase resulting in the formation of ethylene.¹³⁰⁻¹³² This pathway has been found in various microorganisms such as *E. coli*, *S. albidus* or *B. cinerea*.¹³¹⁻¹³³ The third pathway uses 2-oxoglutaric acid as precursor which is converted into ethylene *via* an "ethylene-forming enzyme" (EFE). This pathway has been found mostly in fungi such as *P. cyclopium*, *P.*

digitatum or *F. oxisporum*.¹³⁴⁻¹³⁶ The comprehension and relative simplicity of these pathways allowed the development of modified microorganisms capable of good accumulation of ethylene. Therefore, Gill *et al.* performed metabolic engineering on an *E. coli* strain and reported accumulation of ethylene up to 5 g/L.¹³⁷

Another interesting building block for the production of "drop-in" biofuel is the isobutene. Its biosynthesis has been studied on a yeast strain called *R. minuta*.¹³⁸ Whereas the enzymes involved in this pathway are not identified, the different intermediates have been identified as being α -ketoisocaproic acid, *iso*-valeryl-CoA and 3-methylcrotonyl-CoA. Few published word is available on metabolic engineered strain capable of producing isobutene, however Marliere *et al.* published a patent that described the production of isobutene from 3-methylcrotonyl-CoA.¹³⁹ No data concerning concentration or productivity is revealed in this patent; however, it is the founding stone of the company called Global Bioenergies. They recently published a press release announcing the "First production of isobutene from wheat straw at the scale of a demonstrator" which leave no doubt on the efficiency of their process.¹⁴⁰

5.4. Conclusion

For many years, fossil resources have been overexploited to manufacture a wide variety of chemicals that are essential to everyday life. Ranging from polymers to therapeutic molecules or food additives, the applications are diverse. Nevertheless, the renewal of fossil resources is not fast enough for a global demand that keeps on growing exponentially. In order to overcome this major concern, bio-production of such chemicals has been developed through metabolic engineering. This approach led to industrial successes thanks to modified microorganisms capable of producing succinic acid, penicillin or vanillin at the industrial scale at relatively low cost. With regards to energy, it is also possible to produce biofuels, such as bioethanol or biodiesel, using this approach. However, as bioethanol cannot be used directly in classic engine, many efforts are being directed towards the production of "drop-in" biofuels.

Different host microorganisms have been studied such as yeast or cyanobacteria; however it seems that *E. coli* is the one which is the most suitable for hydrocarbons production. Although alka(e)nes accumulations up to 580.8 mg/L have been overserved, these concentrations are still way below the required rate. To compare, very efficient process for bioethanol production uses strains capable of accumulating ~125g/L; however a process can be profitable at lower concentration. In order to determine where the limit lies, costs of goods sold (COGS) values must be taken in consideration. COGS of 0.6 USD/L have been estimated for a microbial biofuel production to be viable. Afterwards, theoretical yields must be calculated to determine if the process could be profitable. These calculations have been realized on a particular biodiesel (oleyl ethyl ester) and can be compared to hydrocarbons since their energetic yields are quite similar (~0.82).¹⁴¹ Foremost, even if some metabolic pathways involving cofactors such as NADPH or ATP will reduce the yield on glucose, it has been calculated than the productivity would only be reduced by ~10% compare to ethanol. Then, it has been calculated that a productivity of 7.5 g/L per hour and a production scale of 50,000–75,000 tons/year with the sugar price not exceeding 0.07 USD/Kg would allow staying below COGS of 0.6 USD/L. Consequently, hydrocarbons productivity using modified *E. coli* must be improved by at least tenfold.

[Chapter References]

1. Nations, U., *Energy Statistics Yearbook 2016*. United Nations Publication 2018.
2. Lin, D.; Hanscom, L.; Murthy, A.; Galli, A.; Evans, M.; Neill, E.; Mancini, M. S.; Martindill, J.; Medouar, F.-Z.; Huang, S.; Wackernagel, M., Ecological Footprint Accounting for Countries: Updates and Results of the National Footprint Accounts, 2012–2018. *Resources* **2018**, *7*, 58-80.
3. Bailey, J. E., Toward a science of metabolic engineering. *Science* **1991**, *252*, 1668-75.
4. Ingram, L. O.; Conway, T.; Clark, D. P.; Sewell, G. W.; Preston, J. F., Genetic engineering of ethanol production in *Escherichia coli*. *Appl. Environ. Microbiol.* **1987**, *53*, 2420-5.
5. Neale, A. D.; Scopes, R. K.; Kelly, J. M., Alcohol production from glucose and xylose using *Escherichia coli* containing *Zymomonas mobilis* genes. *Appl. Microbiol. Biotechnol.* **1988**, *29*, 162-7.
6. Alterthum, F.; Ingram, L. O., Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. *Appl Environ Microbiol* **1989**, *55*, 1943-8.
7. Ohta, K.; Beall, D. S.; Mejia, J. P.; Shanmugam, K. T.; Ingram, L. O., Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. *Appl. Environ. Microbiol.* **1991**, *57*, 893-900.
8. Gonzalez, R.; Tao, H.; Purvis, J. E.; York, S. W.; Shanmugam, K. T.; Ingram, L. O., Gene array-based identification of changes that contribute to ethanol tolerance in ethanologenic *Escherichia coli*: comparison of KO11 (parent) to LY01 (resistant mutant). *Biotechnol. Prog.* **2003**, *19*, 612-623.
9. Trinh, C. T.; Unrean, P.; Srienc, F., Minimal *Escherichia coli* cell for the most efficient production of ethanol from hexoses and pentoses. *Appl. Environ. Microbiol.* **2008**, *74*, 3634-3643.
10. Trinh, C. T.; Srienc, F., Metabolic engineering of *Escherichia coli* for efficient conversion of glycerol to ethanol. *Appl. Environ. Microbiol.* **2009**, *75*, 6696-6705.
11. Tolonen, A. C.; Haas, W.; Chilaka, A. C.; Aach, J.; Gygi, S. P.; Church, G. M., Proteome-wide systems analysis of a cellulosic biofuel-producing microbe. *Mol. Syst. Biol.* **2011**, *7*, 461.
12. Balusu, R.; Paduru, R. M. R.; Seenayya, G.; Reddy, G., Production of ethanol from cellulosic biomass by *Clostridium thermocellum* SS19 in submerged fermentation: Screening of nutrients using Plackett-Burman design. *Appl. Biochem. Biotechnol.* **2004**, *117*, 133-141.
13. Inui, M.; Kawaguchi, H.; Murakami, S.; Vertes, A. A.; Yukawa, H., Metabolic Engineering of *Corynebacterium glutamicum* for Fuel Ethanol Production under Oxygen-Deprivation Conditions. *J. Mol. Microbiol. Biotechnol.* **2005**, *8*, 243-254.
14. Wen, Z.; Minton, N. P.; Zhang, Y.; Li, Q.; Liu, J.; Jiang, Y.; Yang, S., Enhanced solvent production by metabolic engineering of a twin-clostridial consortium. *Metab. Eng.* **2017**, *39*, 38-48.
15. Lee, K. Y.; Park, J. M.; Kim, T. Y.; Yun, H.; Lee, S. Y., The genome-scale metabolic network analysis of *Zymomonas mobilis* ZM4 explains physiological features and suggests ethanol and succinic acid production strategies. *Microb. Cell Fact.* **2010**, *9*, 94.
16. Rogers, P. L.; Jeon, Y. J.; Lee, K. J.; Lawford, H. G., *Zymomonas mobilis* for fuel ethanol and higher value products. *Adv. Biochem. Eng./Biotechnol.* **2007**, *108*, 263-288.
17. Jeffries, T. W.; Grigoriev, I. V.; Grimwood, J.; Laplaza, J. M.; Aerts, A.; Salamov, A.; Schmutz, J.; Lindquist, E.; Dehal, P.; Shapiro, H.; Jin, Y.-S.; Passoth, V.; Richardson, P. M., Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat. Biotechnol.* **2007**, *25*, 319-326.
18. Bai, F. W.; Anderson, W. A.; Moo-Young, M., Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.* **2008**, *26*, 89-105.
19. Bro, C.; Regenber, B.; Foerster, J.; Nielsen, J., In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. *Metab. Eng.* **2006**, *8*, 102-111.
20. Hjersted, J. L.; Henson, M. A.; Mahadevan, R., Genome-scale analysis of *Saccharomyces cerevisiae* metabolism and ethanol production in fed-batch culture. *Biotechnol. Bioeng.* **2007**, *97*, 1190-1204.

21. Mo, M. L.; Palsson, B. O.; Herrgard, M. J., Connecting extracellular metabolomic measurements to intracellular flux states in yeast. *BMC Syst. Biol.* **2009**, *3*, 37.
22. Pereira, F. B.; Guimaraes, P. M. R.; Teixeira, J. A.; Domingues, L., Optimization of low-cost medium for very high gravity ethanol fermentations by *Saccharomyces cerevisiae* using statistical experimental designs. *Bioresour. Technol.* **2010**, *101*, 7856-7863.
23. Weizmann, C. Production of Acetone and Alcohol by Bactebiological processes. US1315585A, 1919.
24. Shen, C. R.; Liao, J. C., Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. *Metab. Eng.* **2008**, *10*, 312-320.
25. Atsumi, S.; Cann, A. F.; Connor, M. R.; Shen, C. R.; Smith, K. M.; Brynildsen, M. P.; Chou, K. J. Y.; Hanai, T.; Liao, J. C., Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng.* **2008**, *10*, 305-311.
26. Inui, M.; Suda, M.; Kimura, S.; Yasuda, K.; Suzuki, H.; Toda, H.; Yamamoto, S.; Okino, S.; Suzuki, N.; Yukawa, H., Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2008**, *77*, 1305-1316.
27. Gulevich, A. Y.; Skorokhodova, A. Y.; Sukhozhenko, A. V.; Shakulov, R. S.; Debabov, V. G., Metabolic engineering of *Escherichia coli* for 1-butanol biosynthesis through the inverted aerobic fatty acid β -oxidation pathway. *Biotechnol. Lett.* **2012**, *34*, 463-469.
28. Saini, M.; Hong Chen, M.; Chiang, C.-J.; Chao, Y.-P., Potential production platform of n-butanol in *Escherichia coli*. *Metab. Eng.* **2015**, *27*, 76-82.
29. Shen, C. R.; Lan, E. I.; Dekishima, Y.; Baez, A.; Cho, K. M.; Liao, J. C., Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol.* **2011**, *77*, 2905-2915.
30. Yu, M.-R.; Zhang, Y.-L.; Tang, I. C.; Yang, S.-T., Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production. *Metab. Eng.* **2011**, *13*, 373-382.
31. Xiao, H.; Li, Z.; Jiang, Y.; Yang, Y.; Jiang, W.; Gu, Y.; Yang, S., Metabolic engineering of D-xylose pathway in *Clostridium beijerinckii* to optimize solvent production from xylose mother liquid. *Metab. Eng.* **2012**, *14*, 569-578.
32. Lan, E. I.; Liao, J. C., Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metab. Eng.* **2011**, *13*, 353-363.
33. Lee, J.; Jang, Y.-S.; Choi, S. J.; Im, J. A.; Song, H.; Cho, J. H.; Seung, D. Y.; Papoutsakis, E. T.; Bennett, G. N.; Lee, S. Y., Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. *Appl. Environ. Microbiol.* **2012**, *78*, 1416-1423.
34. Atsumi, S.; Hanai, T.; Liao, J. C., Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature (London, U. K.)* **2008**, *451*, 86-89.
35. Baez, A.; Cho, K.-M.; Liao, J. C., High-flux isobutanol production using engineered *Escherichia coli*: a bioreactor study with in situ product removal. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1681-1690.
36. Inokuma, K.; Liao, J. C.; Okamoto, M.; Hanai, T., Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping. *J. Biosci. Bioeng.* **2010**, *110*, 696-701.
37. Yoshida, A.; Nishimura, T.; Kawaguchi, H.; Inui, M.; Yukawa, H., Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. *Appl. Environ. Microbiol.* **2005**, *71*, 6762-6768.
38. Atsumi, S.; Higashide, W.; Liao, J. C., Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol.* **2009**, *27*, 1177-1180.
39. Santala, S.; Efimova, E.; Kivinen, V.; Larjo, A.; Aho, T.; Karp, M.; Santala, V., Improved triacylglycerol production in *Acinetobacter baylyi* ADP1 by metabolic engineering. *Microb. Cell Fact.* **2011**, *10*, 36.
40. Alnoch, R. C.; Stefanello, A. A.; Paula Martini, V.; Richter, J. L.; Mateo, C.; Souza, E. M. d.; Mitchell, D. A.; Muller-Santos, M.; Krieger, N., Co-expression, purification and characterization of the lipase and foldase of *Burkholderia contaminans* LTEB11. *Int. J. Biol. Macromol.* **2018**, *116*, 1222-1231.
41. Papanikolaou, S.; Aggelis, G., Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. *Bioresour. Technol.* **2002**, *82*, 43-49.

42. Radakovits, R.; Eduafo, P. M.; Posewitz, M. C., Genetic engineering of fatty acid chain length in *Phaeodactylum tricornutum*. *Metab. Eng.* **2011**, *13*, 89-95.
43. Tang, X.; Feng, H.; Chen, W. N., Metabolic engineering for enhanced fatty acids synthesis in *Saccharomyces cerevisiae*. *Metab. Eng.* **2013**, *16*, 95-102.
44. Allsopp, M. W.; Vianello, G., Poly(Vinyl Chloride). In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH, W., Ed.2012; Vol. 28, pp 441-468.
45. Park, S. J.; Kim, E. Y.; Noh, W.; Park, H. M.; Oh, Y. H.; Lee, S. H.; Song, B. K.; Jegal, J.; Lee, S. Y., Metabolic engineering of *Escherichia coli* for the production of 5-aminovalerate and glutarate as C5 platform chemicals. *Metab. Eng.* **2013**, *16*, 42-47.
46. Wang, J.-P.; Wu, L.-X.; Xu, F.; Lv, J.; Jin, H.-J.; Chen, S.-F., Metabolic engineering for ethylene production by inserting the ethylene-forming enzyme gene (efe) at the 16S rDNA sites of *Pseudomonas putida* KT2440. *Bioresour. Technol.* **2010**, *101*, 6404-6409.
47. Pirkov, I.; Albers, E.; Norbeck, J.; Larsson, C., Ethylene production by metabolic engineering of the yeast *Saccharomyces cerevisiae*. *Metab. Eng.* **2008**, *10*, 276-280.
48. McKenna, R.; Nielsen, D. R., Styrene biosynthesis from glucose by engineered *E. coli*. *Metab. Eng.* **2011**, *13*, 544-554.
49. Leeuwen, B. N. M.; Wulp, A. M.; Duijnste, I.; Maris, A. J. A.; Straathof, A. J. J., Fermentative production of isobutene. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 1377-1387.
50. Liu, F.; Jian, J.; Shen, X.; Chung, A.; Chen, J.; Chen, G.-Q., Metabolic engineering of *Aeromonas hydrophila* 4AK4 for production of copolymers of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoate. *Bioresour. Technol.* **2011**, *102*, 8123-8129.
51. Tseng, H.-C.; Martin, C. H.; Nielsen, D. R.; Jones-Prather, K. L., Metabolic engineering of *Escherichia coli* for enhanced production of (R)- and (S)-3-hydroxybutyrate. *Appl. Environ. Microbiol.* **2009**, *75*, 3137-3145.
52. Slater, S.; Mitsky, t. A.; Houmiel, K. L.; Hao, M.; Reiser, S.; Taylor, N. B.; Tran, M.; Valentin, H. E.; Rodriguez, D. J.; Stone, D. A.; Padgett, S. R.; Kishore, G.; Gruys, K. J., Metabolic engineering of *Arabidopsis* and *Brassica* for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production. *Nat. Biotechnol.* **1999**, *17*, 1011-1016.
53. Fong, S. S.; Burgard, A. P.; Herring, C. D.; Knight, E. M.; Blattner, F. R.; Maranas, C. D.; Palsson, B. O., In silico design and adaptive evolution of *Escherichia coli* for production of lactic acid. *Biotechnol. Bioeng.* **2005**, *91*, 643-648.
54. Sangproo, M.; Polyiam, P.; Jantama, S. S.; Kanchanatawee, S.; Jantama, K., Metabolic engineering of *Klebsiella oxytoca* M5a1 to produce optically pure D-lactate in mineral salts medium. *Bioresour. Technol.* **2012**, *119*, 191-198.
55. Ishida, N.; Suzuki, T.; Tokuhiko, K.; Nagamori, E.; Onishi, T.; Saitoh, S.; Kitamoto, K.; Takahashi, H., D-lactic acid production by metabolically engineered *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* **2006**, *101*, 172-177.
56. Chae, H. S.; Lee, S. H.; Lee, J.-H.; Park, S. J.; Lee, P. C., Use of a novel *Escherichia coli*-*Leuconostoc* shuttle vector for metabolic engineering of *Leuconostoc citreum* to overproduce D-lactate. *Appl. Environ. Microbiol.* **2013**, *79*, 1428-1435.
57. Ishida, N.; Saitoh, S.; Ohnishi, T.; Tokuhiko, K.; Nagamori, E.; Kitamoto, K.; Takahashi, H., Metabolic engineering of *Saccharomyces cerevisiae* for efficient production of pure L-(+)-lactic acid. *Appl. Biochem. Biotechnol.* **2006**, *129-132*, 795-807.
58. Lin, H.; Bennett, G. N.; San, K.-Y., Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. *Metab. Eng.* **2005**, *7*, 116-127.
59. Xia, T.; Altman, E.; Eiteman, M. A., Succinate production from xylose-glucose mixtures using a consortium of engineered *Escherichia coli*. *Eng. Life Sci.* **2015**, *15*, 65-72.
60. Okino, S.; Noburyu, R.; Suda, M.; Jojima, T.; Inui, M.; Yukawa, H., An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 459-464.

61. Raab, A. M.; Gebhardt, G.; Bolotina, N.; Weuster-Botz, D.; Lang, C., Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid. *Metab. Eng.* **2010**, *12*, 518-525.
62. Yu, S.; Yao, P.; Li, J.; Ren, J.; Yuan, J.; Feng, J.; Wang, M.; Wu, Q.; Zhu, D., Enzymatic synthesis of 3-hydroxypropionic acid at high productivity by using free or immobilized cells of recombinant *Escherichia coli*. *J. Mol. Catal. B: Enzym.* **2016**, *129*, 37-42.
63. Niu, W.; Draths, K. M.; Frost, J. W., Benzene-Free Synthesis of Adipic Acid. *Biotechnol. Prog.* **2002**, *18*, 201-211.
64. Shiue, E.; Prather, K. L. J., Improving D-glucaric acid production from myo-inositol in *E. coli* by increasing MIOX stability and myo-inositol transport. *Metab. Eng.* **2014**, *22*, 22-31.
65. Cottaz, S.; Samain, E., Genetic engineering of *Escherichia coli* for the production of NI,NII-diacetylchitobiose (chitinbiose) and its utilization as a primer for the synthesis of complex carbohydrates. *Metab. Eng.* **2005**, *7*, 311-317.
66. Jain, R.; Huang, J.; Yuan, Q.; Yan, Y., Engineering microaerobic metabolism of *E. coli* for 1,2-propanediol production. *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 1049-1055.
67. Lee, J. H.; Lama, S.; Kim, J. R.; Park, S. H., Production of 1,3-Propanediol from Glucose by Recombinant *Escherichia coli* BL21(DE3). *Biotechnol. Bioprocess Eng.* **2018**, *23*, 250-258.
68. Hwang, H. J.; Park, J. H.; Kim, J. H.; Kong, M. K.; Kim, J. W.; Park, J. W.; Cho, K. M.; Lee, P. C., Engineering of a butyraldehyde dehydrogenase of *Clostridium saccharoperbutylacetonicum* to fit an engineered 1,4-butanediol pathway in *Escherichia coli*. *Biotechnol. Bioeng.* **2014**, *111*, 1374-1384.
69. Erian, A. M.; Gibisch, M.; Pflugl, S., Engineered *E. coli* W enables efficient 2,3-butanediol production from glucose and sugar beet molasses using defined minimal medium as economic basis. *Microb Cell Fact* **2018**, *17*, 190.
70. Jung, Y. K.; Kim, T. Y.; Park, S. J.; Lee, S. Y., Metabolic engineering of *Escherichia coli* for the production of polylactic acid and its copolymers. *Biotechnol Bioeng* **2010**, *105*, 161-71.
71. Zhou, Q.; Shi, Z.-Y.; Meng, D.-C.; Wu, Q.; Chen, J.-C.; Chen, G.-Q., Production of 3-hydroxypropionate homopolymer and poly(3-hydroxypropionate-co-4-hydroxybutyrate) copolymer by recombinant *Escherichia coli*. *Metab. Eng.* **2011**, *13*, 777-785.
72. Poblete-Castro, I.; Binger, D.; Rodrigues, A.; Becker, J.; Martins dos Santos, V. A. P.; Wittmann, C., In-silico-driven metabolic engineering of *Pseudomonas putida* for enhanced production of poly-hydroxyalkanoates. *Metab. Eng.* **2013**, *15*, 113-123.
73. Pauthenier, C.; Faulon, J.-P., Composé produits par ingénierie métabolique. *Techniques de l'ingénieur* **2013**, BIO 801.
74. Gidijala, L.; Kiel, J. A. K. W.; Douma, R. D.; Seifar, R. M.; van Gulik, W. M.; Bovenberg, R. A. L.; Veenhuis, M.; van der Klei, I. J., An engineered yeast efficiently secreting penicillin. *PLoS One* **2009**, *4*, No pp. given.
75. Schmid, G.; Hofheinz, W., Total Synthesis of Qinghaosu. *J. Am. Chem. Soc.* **1983**, *105*, 624-625.
76. Roth, R. J.; Acton, N., A simple conversion of artemisinic acid into artemisinin. *J. Nat. Prod.* **1989**, *52*, 1183-5.
77. Haynes, R. K.; Vonwiller, S. C. Cyclic peroxyacetal lactone, lactol and ether compounds. . US 5310946 1994.
78. Liu, B.; Wang, H.; Du, Z.; Li, G.; Ye, H., Metabolic engineering of artemisinin biosynthesis in *Artemisia annua* L. *Plant Cell Rep.* **2011**, *30*, 689-694.
79. Yoon, Y. J.; Kim, E. S.; Hwang, Y. S.; Choi, C. Y., Avermectin: biochemical and molecular basis of its biosynthesis and regulation. *Appl Microbiol Biotechnol* **2004**, *63*, 626-34.
80. Ro, D.-K.; Paradise, E. M.; Ouellet, M.; Fisher, K. J.; Newman, K. L.; Ndungu, J. M.; Ho, K. A.; Eachus, R. A.; Ham, T. S.; Kirby, J.; Chang, M. C. Y.; Withers, S. T.; Shiba, Y.; Sarpong, R.; Keasling, J. D., Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **2006**, *440*, 940-3.
81. Szczepara, F. M.; Chandelier, C.; Villeret, C.; Masurel, A.; Bourrot, S.; Duport, C.; Blanchard, S.; Groisillier, A.; Testet, E.; Costaglioli, P.; Cauet, G.; Degryse, E.; Balbuena, D.; Winter, J.; Achstetter, T.;

- Spagnoli, R.; Pompon, D.; Dumas, B., Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat. Biotechnol.* **2003**, *21*, 143-149.
82. Hansen, E. H.; Moeller, B. L.; Kock, G. R.; Bunner, C. M.; Kristensen, C.; Jensen, O. R.; Okkels, F. T.; Olsen, C. E.; Motawia, M. S.; Hansen, J., De novo biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and Baker's yeast (*Saccharomyces cerevisiae*). *Appl. Environ. Microbiol.* **2009**, *75*, 2765-2774.
 83. Brochado, A. R.; Matos, C.; Moeller, B. L.; Hansen, J.; Mortensen, U. H.; Patil, K. R., Improved vanillin production in baker's yeast through in silico design. *Microb. Cell Fact.* **2010**, *9*, No pp. given.
 84. Di Gioia, D.; Luziatelli, F.; Negroni, A.; Ficca, A. G.; Fava, F.; Ruzzi, M., Metabolic engineering of *Pseudomonas fluorescens* for the production of vanillin from ferulic acid. *J. Biotechnol.* **2011**, *156*, 309-316.
 85. Yamada, M.; Okada, Y.; Yoshida, T.; Nagasawa, T., Vanillin production using *Escherichia coli* cells over-expressing isoeugenol monooxygenase of *Pseudomonas putida*. *Biotechnol. Lett.* **2008**, *30*, 665-670.
 86. Zhang, X.; Wang, X.; Shanmugam, K. T.; Ingram, L. O., L-Malate production by metabolically engineered *Escherichia coli*. *Appl. Environ. Microbiol.* **2011**, *77*, 427-434.
 87. Huang, Y. L.; Mann, K.; Novak, J. M.; Yang, S.-T., Acetic acid production from fructose by *Clostridium formicoaceticum* immobilized in a fibrous-Bed bioreactor. *Biotechnol. Prog.* **1998**, *14*, 800-806.
 88. Foerster, A.; Aurich, A.; Mauersberger, S.; Barth, G., Citric acid production from sucrose using a recombinant strain of the yeast *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 1409-1417.
 89. Samuels, L.; Kunst, L.; Jetter, R., Sealing plant surfaces: Cuticular wax formation by epidermal cells. *Annu. Rev. Plant Biol.* **2008**, *59*, 683-707.
 90. Tillman, J. A.; Seybold, S. J.; Jurenka, R. A.; Blomquist, G. J., Insect pheromones-an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol.* **1999**, *29*, 481-514.
 91. Ladygina, N.; Dedyukhina, E. G.; Vainshtein, M. B., A review on microbial synthesis of hydrocarbons. *Process Biochem. (Amsterdam, Neth.)* **2006**, *41*, 1001-1014.
 92. Hanada, K., Serine Palmitoyltransferase, a Key Enzyme of Sphingolipid Metabolism. *Biochim. Biophys. Acta* **2003**, *1632*, 16-30.
 93. Wang, W.; Lu, X., Microbial Synthesis of Alka(e)nes. *Front Bioeng Biotechnol* **2013**, *1*, 10.
 94. Kolattukudy, P. E., Biosynthesis of paraffins in *Brassica oleracea*: fatty acid elongation-decarboxylation as a plausible pathway. *Phytochemistry* **1967**, *6*, 963-75.
 95. Naccarato, W. F.; Gilbertson, J. R.; Gelman, R. A., Effects of different culture media and oxygen on lipids of *Escherichia coli* K-12. *Lipids* **1974**, *9*, 322-7.
 96. Rude, M. A.; Baron, T. S.; Brubaker, S.; Alibhai, M.; Del Cardayre, S. B.; Schirmer, A., Terminal olefin (1-alkene) biosynthesis by a novel P450 fatty acid decarboxylase from *Jeotgalicoccus* species. *Appl. Environ. Microbiol.* **2011**, *77*, 1718-1727.
 97. Albro, P. W.; Dittmer, J. C., The biochemistry of long-chain, nonisoprenoid hydrocarbons. 3. The metabolic relationship of long-chain fatty acids and hydrocarbons and other aspects of hydrocarbon metabolism in *Sarcina lutea*. *Biochemistry* **1969**, *8*, 1913-1918.
 98. Beller, H. R.; Goh, E.-B.; Keasling, J. D., Genes involved in long-chain alkene biosynthesis in *Micrococcus luteus*. *Appl. Environ. Microbiol.* **2010**, *76*, 1212-1223.
 99. Frias, J. A.; Richman, J. E.; Erickson, J. S.; Wackett, L. P., Purification and Characterization of OleA from *Xanthomonas campestris* and Demonstration of a Non-decarboxylative Claisen Condensation Reaction. *J. Biol. Chem.* **2011**, *286*, 10930-10938.
 100. Sukovich, D. J.; Seffernick, J. L.; Richman, J. E.; Hunt, K. A.; Gralnick, J. A.; Wackett, L. P., Structure, function, and insights into the biosynthesis of a head-to-head hydrocarbon in *Shewanella oneidensis* strain MR-1. *Appl Environ Microbiol* **2010**, *76*, 3842-9.
 101. Gu, L.; Wang, B.; Kulkarni, A.; Gehret, J. J.; Lloyd, K. R.; Gerwick, L.; Gerwick, W. H.; Wipf, P.; Hakansson, K.; Smith, J. L.; Sherman, D. H., Polyketide Decarboxylative Chain Termination Preceded by O-Sulfonation in Curacin A Biosynthesis. *J. Am. Chem. Soc.* **2009**, *131*, 16033-16035.

102. Mendez-Perez, D.; Begemann, M. B.; Pflieger, B. F., Modular synthase-encoding gene involved in α -olefin biosynthesis in *Synechococcus* sp. strain PCC 7002. *Appl. Environ. Microbiol.* **2011**, *77*, 4264-4267.
103. Schirmer, A.; Rude, M. A.; Li, X.; Popova, E.; del Cardayre, S. B., Microbial Biosynthesis of Alkanes. *Science (Washington, DC, U. S.)* **2010**, *329*, 559-562.
104. Li, N.; Chang, W.-c.; Warui, D. M.; Booker, S. J.; Krebs, C.; Bollinger, J. M., Evidence for Only Oxygenative Cleavage of Aldehydes to Alk(a/e)nes and Formate by Cyanobacterial Aldehyde Decarbonylases. *Biochemistry* **2012**, *51*, 7908-7916.
105. Andre, C.; Kim, S. W.; Yu, X.-H.; Shanklin, J., Fusing catalase to an alkane-producing enzyme maintains enzymatic activity by converting the inhibitory byproduct H₂O₂ to the cosubstrate O₂. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 3191-3196.
106. Liu, Y.; Wang, C.; Yan, J.; Zhang, W.; Guan, W.; Lu, X.; Li, S., Hydrogen peroxide-independent production of α -alkenes by OleTJE P450 fatty acid decarboxylase. *Biotechnol. Biofuels* **2014**, *7*, 28/1-28/12, 12.
107. Chen, B.; Lee, D.-Y.; Chang, M. W., Combinatorial metabolic engineering of *Saccharomyces cerevisiae* for terminal alkene production. *bioRxiv, Microbiol.* **2015**, 22350/1-22350/34.
108. Chen, Y.; Yin, M.; Horsman, G. P.; Huang, S.; Shen, B., Manipulation of pathway regulation in *Streptomyces globisporus* for overproduction of the enediyne antitumor antibiotic C-1027. *J. Antibiot.* **2010**, *63*, 482-485.
109. Chen, Y.; Yin, M.; Horsman, G. P.; Shen, B., Improvement of the enediyne antitumor antibiotic C-1027 production by manipulating its biosynthetic pathway regulation in *Streptomyces globisporus*. *J. Nat. Prod.* **2011**, *74*, 420-424.
110. Liu, Q.; Wu, K.; Cheng, Y.; Lu, L.; Xiao, E.; Zhang, Y.; Deng, Z.; Liu, T., Engineering an iterative polyketide pathway in *Escherichia coli* results in single-form alkene and alkane overproduction. *Metab. Eng.* **2015**, *28*, 82-90.
111. Buijs, N. A.; Zhou, Y. J.; Siewers, V.; Nielsen, J., Long-chain alkane production by the yeast *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **2015**, *112*, 1275-1279.
112. Bourdenx, B.; Bernard, A.; Domergue, F.; Pascal, S.; Leger, A.; Roby, D.; Pervent, M.; Vile, D.; Haslam, R. P.; Napier, J. A.; Lessire, R.; Joubes, J., Overexpression of *Arabidopsis* ECERIFERUM1 promotes wax very-long-chain alkane biosynthesis and influences plant response to biotic and abiotic stresses. *Plant Physiol.* **2011**, *156*, 29-45.
113. Bernard, A.; Domergue, F.; Pascal, S.; Jetter, R.; Renne, C.; Faure, J.-D.; Haslam, R. P.; Napier, J. A.; Lessire, R.; Joubes, J., Reconstitution of plant alkane biosynthesis in yeast demonstrates that *Arabidopsis* ECERIFERUM1 and ECERIFERUM3 are core components of a very-long-chain alkane synthesis complex. *Plant Cell* **2012**, *24*, 3106-3118.
114. Wang, W.; Liu, X.; Lu, X., Engineering cyanobacteria to improve photosynthetic production of alka(e)nes. *Biotechnol. Biofuels* **2013**, *6*, 69.
115. Coursolle, D.; Lian, J.; Shanklin, J.; Zhao, H., Production of long chain alcohols and alkanes upon coexpression of an acyl-ACP reductase and aldehyde-deformylating oxygenase with a bacterial type-I fatty acid synthase in *E. coli*. *Mol. BioSyst.* **2015**, *11*, 2464-2472.
116. Song, X.; Yu, H.; Zhu, K., Improving alkane synthesis in *Escherichia coli* via metabolic engineering. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 757-767.
117. Foo, J. L.; Susanto, A. V.; Keasling, J. D.; Leong, S. S. J.; Chang, M. W., Whole-cell biocatalytic and de novo production of alkanes from free fatty acids in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **2017**, *114*, 232-237.
118. Akhtar, M. K.; Turner, N. J.; Jones, P. R., Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 87-92, S87/1-S87/38.
119. Howard, T. P.; Middelhaufe, S.; Moore, K.; Edner, C.; Kolak, D. M.; Taylor, G. N.; Parker, D. A.; Lee, R.; Smirnov, N.; Aves, S. J.; Love, J., Synthesis of customized petroleum-replica fuel molecules by targeted modification of free fatty acid pools in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 7636-7641, S7636/1-S7636/6.

120. Schjerling, C. K.; Hummel, R.; Hansen, J. K.; Boersting, C.; Mikkelsen, J. M.; Kristiansen, K.; Knudsen, J., Disruption of the gene encoding the acyl-CoA-binding protein (ACB1) perturbs acyl-CoA metabolism in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1996**, *271*, 22514-22521.
121. Zhou, Y. J.; Buijs, N. A.; Zhu, Z.; Qin, J.; Siewers, V.; Nielsen, J., Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. *Nat. Commun.* **2016**, *7*, 11709.
122. Nunn, W. D.; Giffin, K.; Clark, D.; Cronan, J. E., Jr., Role for fadR in unsaturated fatty acid biosynthesis in *Escherichia coli*. *J. Bacteriol.* **1983**, *154*, 554-60.
123. Choi, Y. J.; Lee, S. Y., Microbial production of short-chain alkanes. *Nature (London, U. K.)* **2013**, *502*, 571-574.
124. Khara, B.; Menon, N.; Levy, C.; Mansell, D.; Das, D.; Marsh, E. N. G.; Leys, D.; Scrutton, N. S., Production of Propane and Other Short-Chain Alkanes by Structure-Based Engineering of Ligand Specificity in Aldehyde-Deformylating Oxygenase. *ChemBioChem* **2013**, *14*, 1204-1208.
125. Xu, P.; Gu, Q.; Wang, W.; Wong, L.; Bower, A. G. W.; Collins, C. H.; Koffas, M. A. G., Modular optimization of multi-gene pathways for fatty acids production in *E. coli*. *Nat. Commun.* **2013**, *4*, ncomms2425, 8 pp.
126. Pauthenier, C.; Faulon, J. L., Ingénierie métabolique et biologie de synthèse pour la chimie verte. *Techniques de l'ingénieur* **2018**, BIO 800.
127. Cristescu, S. M.; De Martinis, D.; te Lintel Hekkert, S.; Parker, D. H.; Harren, F. J. M., Ethylene production by *Botrytis cinerea* in vitro and in tomatoes. *Appl. Environ. Microbiol.* **2002**, *68*, 5342-5350.
128. Stearns, J. C.; Glick, B. R., Transgenic plants with altered ethylene biosynthesis or perception. *Biotechnol. Adv.* **2003**, *21*, 193-210.
129. Chae, H. S.; Kieber, J. J., Eto Brute? Role of ACS turnover in regulating ethylene biosynthesis. *Trends Plant Sci.* **2005**, *10*, 291-296.
130. Graham, J. H.; Linderman, R. G., Ethylene production by ectomycorrhizal fungi, *Fusarium oxysporum* f. sp. *pini*, and by aseptically synthesized ectomycorrhizae and *Fusarium*-infected Douglas-fir roots. *Can. J. Microbiol.* **1980**, *26*, 1340-7.
131. Fukuda, H.; Takahashi, M.; Fujii, T.; Tazaki, M.; Ogawa, T., An NADH: Fe(III) EDTA oxidoreductase from *Cryptococcus albidus*: an enzyme involved in ethylene production in vivo? *FEMS Microbiol. Lett.* **1989**, *60*, 107-11.
132. Ince, J. E.; Knowles, C. J., Ethylene formation by cell-free extracts of *Escherichia coli*. *Arch. Microbiol.* **1986**, *146*, 151-8.
133. Chague, V.; Elad, Y.; Barakat, R.; Tudzynski, P.; Sharon, A., Ethylene biosynthesis in *Botrytis cinerea*. *FEMS Microbiol. Ecol.* **2002**, *40*, 143-149.
134. Pazout, J.; Pazoutova, S., Ethylene is synthesized by vegetative mycelium in surface cultures of *Penicillium cyclopium* Westling. *Can. J. Microbiol.* **1989**, *35*, 384-7.
135. Sprayberry, B. A.; Hall, W. C.; Miller, C. S., Biogenesis of ethylene in *Penicillium digitatum*. *Nature (London, U. K.)* **1965**, *208*, 1322-3.
136. Hottiger, T.; Boller, T., Ethylene biosynthesis in *Fusarium oxysporum* f. sp. *tulipae* proceeds from glutamate/2-oxoglutarate and requires oxygen and ferrous ions in vivo. *Arch. Microbiol.* **1991**, *157*, 18-22.
137. Lynch, S.; Eckert, C.; Yu, J.; Maness, P.-C.; Gill, R., Overcoming substrate limitations for improved production of ethylene in *E. coli*. *Biotechnol Biofuels* **2016**, *9*, 3.
138. Fujii, T.; Ogawa, T.; Fukuda, H., Isobutene production by *Rhodotorula minuta*. *Appl. Microbiol. Biotechnol.* **1987**, *25*, 430-3.
139. Allard, M.; Marliere, P. Metabolic pathways for isobutene production from 3-methylcrotonyl-CoA. WO2017162738A1, 2017.
140. <https://www.global-bioenergies.com/premiere-mise-en-production-disobutene-a-partir-de-paille-de-ble-a-lechelle-dun-demonstrateur/>
141. Caspeta, L.; Nielsen, J., Economic and environmental impacts of microbial biodiesel. *Nat. Biotechnol.* **2013**, *31*, 789-793.