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Recent advances in the microbial production and recovery of apolar molecules

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Several apolar molecules of interest for the production of fuels and chemicals can nowadays be produced by fermentation. Those secreted from the microbial cell are of particular interest for large scale bioprocessing, since they allow for cell reuse, *in situ* product recovery and competitive production levels. So far, however, bioprocess strategies for fermentation and product recovery have been developed for addressing needs at the laboratory scale, rather than the process scale. Most commonly used strategies include extractive fermentations, product stripping in the gas phase, and off-line de-emulsification followed by intensive centrifugation. At the same time, current techno-economic studies at process scale have demonstrated the absolute need for significant improvements in both microorganism *and* process technology, for these processes to become competitive.

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Introduction

Industrial biotechnology is playing a significant role in the production of fuels, chemicals and materials from renewable resources. A broad range of molecules can be synthesised from biomass by fermentation or enzymatic conversion, as recently reviewed by Straathof [1]. The last years have seen important advances in industrial implementation such as DuPont's 1,3-propanediol, Genomatica's 1,4-butanediol, several succinic acid initiatives, and the recent opening of the first POET-DSM commercial-scale plant for cellulosic ethanol in the U.S. (http://poetdsm.com/pr/first-commercial-scale-cellulosic-plant).

Apolar and other hydrophobic molecules produced by fermentation encounter applications in energy (gas and liquid biofuels), materials (bioplastics) and platform chemicals (lipids, fatty acids, isoprenoids). Apolar molecules, also referred to as non-polar molecules, are typically hydrophobic. Molecules such as fatty acids consist of an apolar hydrocarbon chain and a polar carboxyl group and hence, their hydrophobicity depends on the length and structure of the hydrocarbon chain. All these molecules, when secreted from the microbial cell, lead to potential advantages for large scale bioprocessing such as cell reuse, *in situ* product recovery, less complex by-product profile and less complex unit operations for product recovery, as discussed in [2]. In this review, we discuss the recent advances in the microbial production and recovery of apolar molecules with an emphasis on liquid biofuels for diesel and jet fuel applications. Table 1 provides an overview of currently produced molecules, their relevant properties for product recovery, and the challenges and opportunities they represent for large scale bioprocessing. A summary of the microbial platforms used is shown in Box 1.

Advances in the production of apolar molecules by fermentation

Fatty acids and fatty-acid derivatives

Fatty acid biosynthesis (FAB) is the preferred pathway to accumulate energy storage compounds in many organisms [3]. It has been well-studied in bacteria and yeast, the focus being in the production of free fatty acids (FFAs) and fatty-acid derivatives such as fatty-acid alkyl esters (FAAEs) and alkanes.

Fatty acids are synthesised via an iterative reduction cycle, also known as fatty acid elongation pathway, adding two carbons from malonyl-ACP to a growing acyl-chain until long-chain acyl-ACPs are synthesised. When present as sole carbon source, FFAs can be utilised by generating acetyl-CoA via the β-oxidation pathway. The first step is the activation of FFAs to acyl-CoAs which, in the same way as the acyl-ACPs generated from FAB, are the starting point for synthesising relevant derivatives such as FAAEs and alkanes. A broad range of studies have looked at the engineering of these pathways, the best results so far being obtained through strategies focusing on: first, improving thioesterase expression, the catalytic link between FAB and a product sink; and second, reversing the β-oxidation pathway. Lennen and Pfleger [4] normalised the wealth of studies available by looking at the percentage of theoretical yield achieved. The theoretical yield was calculated from metabolic network modelling in *Escherichia coli*, and for

Table 1

Summary of extracellular apolar or hydrophobic molecules currently produced by fermentation and the technical challenges and opportunities they represent for large scale bioprocessing

Molecules	Relevant properties	Challenges and opportunities
Fatty acids and derivatives: Free fatty acids (FFAs) Fatty acid alkyl esters (FAAEs) Alkanes Isoprenoids: Isoprene Monoterpenes Sesquiterpenes	Phase forming of the (by-)products: Volatility (vapour pressure, boiling point) Melting point Solubility Toxicity to the microorganism Chemical stability Medium and broth properties: Viscosity, density Ionic strength, pH Impurity profile For liquid products or solvents: Droplet size under bioreactor operation Presence of surface active components	Challenges: Emulsion formation (for liquid products or *in situ* solvent extraction) Mass transfer limitations (for *in situ* solvent extraction) Product-cells separation (for solid products or in case of emulsion formation) Opportunities: Product recovery from the gas phase (i.e. stripping, for vapour products) *In situ* product formulation (i.e. using a liquid product as solvent) Direct product recovery during fermentation Cell reuse

C_{12} to C_{16} FFAs was shown to range from 0.3 to 0.4 g FFA/g carbon source. Improvement of thioesterase expression has led up to 56% of the theoretical yield; in contrast, reversal of the β-oxidation pathway has led to 85% of the theoretical yield and extracellular titres up to 7 g/L under minimal oxygen [5]. Recently, the production of shorter chains — allegedly more suitable for direct use as petrol replacement — was demonstrated in *E. coli* through engineering of FAB and degradation pathways [6]. The authors reported titres up to 313 mg/L FFAs (ranging from C_8 to C_{16}), and further modifications led to 580.8 mg/L of short chain alkanes (mostly nonane and dodecane).

FAAEs are not known to be generated naturally by any microorganism [7]. Microorganisms are being engineered to directly produce these compounds, in particular fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs), which can be used in the same way as biodiesel. A combined strategy involving improved FFA availability from FAB and controlled expression of downstream enzymes led to titres up to 427 mg/L FAEEs from aerobic growth solely on glucose, and up to 674 mg/L when a dodecane overlay was added [8] (see also next section).

There have been a few reports on the microbial production of alkanes and alkenes. Through decarbonylation of fatty aldehydes directly generated from fatty acyl-ACPs, Schirmer *et al.* [9] obtained a mixture of uneven C_{13} to C_{17} alkanes and alkenes, mostly extracellular. Following the same decarbonylation route, but altering the enzyme specificity at the start of the fatty acid elongation pathway, Harger *et al.* [10] obtained alkanes in the same range of change length, but with larger fractions of even alkanes (mostly C_{14} and C_{16}). Recently, Howard *et al.* [11] implemented a pathway in which fatty acids, instead of fatty acid thioesters, are the starting point of alkane synthesis. In this way, by modifying the FFA pool — either by further engineering or media supplementation — they broadened the product spectrum to include linear and branched C_{13} to C_{17} alkanes and alkenes. Although titres are still in the order of a few mg/L, these studies show the potential of synthetic biology for tailoring the product composition so that it is closer to current liquid fuels.

Isoprenoids

Isoprenoids or terpenoids are a highly diverse set of compounds that are all built from at least one C_5 isoprene unit via head-to-tail addition of the key intermediate isopentenyl diphosphate (IPP) [7]. Metabolic engineering of this pathway originated in medicine applications, in particular through the development of a microbial route for the production of the antimalarial drug precursor artemisinic acid [12]. Since then, the pathway has received enormous attention for its potential for generating replacements for diesel and jet fuel. The focus has been mainly on isoprene (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}) and a few higher terpenoids ($>C_{20}$). Metabolic engineering strategies for improved isoprenoid biosynthesis have been extensively reviewed throughout the last decade [13–15].

Microbial isoprene production has been driven by the role of this molecule in the petrochemical industry, in particular for the manufacture of tires. A research collaboration