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Microbial production of 1-octanol: A naturally excreted biofuel with diesel-like properties

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ABSTRACT

The development of sustainable, bio-based technologies to convert solar energy and carbon dioxide into fuels is a grand challenge. A core part of this challenge is to produce a fuel that is compatible with the existing transportation infrastructure. This task is further compounded by the commercial desire to separate the fuel from the biotechnological host. Based on its fuel characteristics, 1-octanol was identified as an attractive metabolic target with diesel-like properties. We therefore engineered a synthetic pathway specifically for the biosynthesis of 1-octanol in *Escherichia coli* BL21(DE3) by over-expression of three enzymes (thioesterase, carboxylic acid reductase and aldehyde reductase) and one maturation factor (phosphopantetheinyl transferase). Induction of this pathway in a shake flask resulted in 4.4 mg 1-octanol L^{-1} h^{-1} which exceeded the productivity of previously engineered strains. Furthermore, the majority (73%) of the fatty alcohol was localised within the media without the addition of detergent or solvent overlay. The deletion of *acrA* reduced the production and excretion of 1-octanol by 3-fold relative to the wild-type, suggesting that the AcrAB–TolC complex may be responsible for the majority of product efflux. This study presents 1-octanol as a potential fuel target that can be synthesised and naturally accumulated within the media using engineered microbes.

1. Introduction

The demand for diesel fuel continues to increase (Cames and Helmers, 2013). In response to these demands, and amid concerns over the environmental impact of fossil fuels, intensive research efforts have been made in developing renewable and sustainable methods for the production of diesel substitutes (Peralta-Yahya et al., 2011; Steen et al., 2010). To date, biodiesel is the most extensively researched diesel fuel replacement (Atabani et al., 2012). Its synthesis traditionally involves chemical or mechanical extraction of oils from plant or algal sources followed by trans-esterification to yield either fatty acid methyl esters or ethyl esters. Innovations in microbial engineering have led to bioprocesses which obviate the need for a separate esterification step and potentially allow multiple industrial waste streams to be utilised (Kalscheuer et al., 2006; Steen et al., 2010). The major downside to biodiesel or even precursor fuels such as the recently reported bisabolane (Peralta-Yahya et al., 2011) is that they require energy intensive extraction procedures (*e.g.* usage of organic solvents for end-product isolation, high centrifugal forces for biomass recovery, physical methods for disruption of biomass) and/or further chemical modifications, and this can present a major economic barrier for the purpose of fuel commercialisation (Chisti, 2013).

Alcohols and alkanes are both highly attractive biofuel candidates as they do not require further chemical modification. In diesel engines, one particularly notorious issue with the use of alkanes is the formation of particulate matter. These carbonaceous particles, also known as soot, are attributed to incomplete combustion and have been shown in several studies to exacerbate respiratory illnesses and contribute to global warming (Bond et al., 2013; D'Amato et al., 2013). In this regard, alcohols have generated a considerable amount of interest since their increased oxygenated content can significantly stimulate the completion of combustion and thereby lower the production of particulate matter.

Herein, we evaluate the fuel and physicochemical characteristics of saturated fatty alcohols and conclude that the C8 fatty alcohol, 1-octanol, is a highly attractive biofuel with diesel-like properties. Previously, 1-octanol had been synthesised by (i) the reversal of beta-oxidation (Dellomonaco et al., 2011), (ii) rerouting

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branched-chain amino acid biosynthesis (Marcheschi et al., 2012) and (iii) extending the 1-butanol pathway (Machado et al., 2012). In this study, we engineer a novel metabolic route for the production of 1-octanol in *Escherichia coli* and furthermore show that it can be naturally excreted into the media.

2. Materials and methods

2.1. Strains and plasmids

E. coli BL21(DE3) was purchased from Novagen. The *E. coli* BL21 (DE3)Δ*acrA* strain was constructed by P1 phage transduction, described previously (Datsenko and Wanner), using the BW25113Δ*acrA* strain (The Coli Genetic Stock Center, Yale, USA) as the donor strain. These two strains were engineered using genes encoding for *Mycobacterium marinum* carboxylic acid reductase (CAR), Sfp from *Bacillus subtilis*, *tes3* (thioesterase from *Anaerococcus tetradius* with accession no. EEI82564) and Ahr (an aldehyde reductase previously known as *yjgB*) from *E. coli*. The construct cloning approach was described previously (Akhtar et al., 2013; Kallio et al., 2014). The plasmids pET-TPC3 (encoding Tes3, Sfp and CAR (TPC3, Akhtar et al., 2013; Kallio et al., 2014), assembled as a synthetic operon (Akhtar and Jones, 2009), and pCDF-Ahr$_{his}$ (encoding Ahr with an N-terminal 6xHis-tag) were transformed into chemically competent *E. coli* BL21 (DE3) to generate the TPC3 Ahr strain.

2.2. In vivo production of fatty alcohols

Strains were cultivated based on a slight modification of the method described previously (Akhtar et al., 2013). LB, containing a 2% (v/v) overnight, LB-based inoculum (30 °C/150 rpm/~18 h), was incubated at 30 °C/150 rpm until it had reached an OD600 of 0.5–0.7. Cells were harvested (17, 000g/1 min), washed twice with modified minimal medium (Akhtar et al., 2013) prior to resuspension in the same medium. After addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 20 μM, cells were incubated at 30 °C for 14 h. The cellular density was determined from absorbance measurements (attributed to light scattering) at 600 nm ($OD_{600\ nm}$) and glucose levels were quantified at 340 nm, based on the reduction of NAD^+ by glucose-6-phosphate dehydrogenase. Yields were determined by expressing the total production of fatty alcohol (described in detail below) per gram of consumed glucose.

2.3. Fatty alcohol quantification

For total fatty alcohol quantification, a 100 μl cell culture was mixed with 200 μl acetone (containing 0.2 mM 1-heptanol as the internal standard). For cellular and extracellular quantification of fatty alcohols, cell cultures were first separated into cellular and media fractions by centrifugation. The cellular fraction was resuspended in 100 μl of water prior to mixing with 200 μl acetone, while the media fraction was directly mixed with 200 μl acetone. All samples were centrifuged and the supernatant transferred to GC vials. Metabolite analysis was performed with an Agilent 7890A gas chromatograph equipped with a 5975 mass spectrometry detector, as described previously (Akhtar et al., 2013). For 1-octanol and 1-hexanol identification, fragmentation patterns and retention times of the analytes were compared with the NIST mass spectral library and commercially available fatty alcohol standards. A standard curve for quantification was prepared with commercially sourced fatty alcohols. Data was normalised with respect to the internal standard and optical density at 600 nm. All given values are an average mean of measurements obtained from at least 6 independent cultures with error bars representing standard error.

3. Results

3.1. A comparison of the fuel characteristics between fossil-derived diesel and saturated alcohols

Having recently demonstrated the *in vitro* production of a broad range of fatty alcohols based on the activities of CAR and AHR enzymes (Akhtar et al., 2013), we surveyed the literature and compared the fuel characteristics of fossil-derived diesel and saturated fatty alcohols ranging in chain length from 2 to 12 carbons.

Table 1
Fuel and physicochemical characteristics of petroleum-derived fuels and its potential substitutes.

	Energy content (MJ/L)	Solubility[a] (g/L)	Cetane number[b]	Lubricity[c] (μm corrected wear scar)	Viscosity[d] cST	Density[a]	Auto ignition temperature[b] (°C)	Boiling point[a] (°C)	Flash point[a] (°C)	Vapour pressure[a] (mmHg)	Freezing point[a] (°C)
Methanol	16	Miscible	2	1100	0.6@40 °C	0.79	463	65	11	127	−98
Ethanol	19.6	Miscible	11	603	1.1@40 °C	0.79	420	78	17	55	−114
1-Butanol	29.2	77	17	623	1.7@40 °C	0.81	343	117	29	7	−90
1-Hexanol	31.7	7.9	23	534	2.9@40 °C	0.81	285	158	59	1	−45
1-Octanol	33.7	0.59	39	404	4.4@40 °C	0.83	270	195	81	0.08	−16
1-Decanol	34.6	~0.04	50	406	6.5@40 °C	0.83	255	233	108	<0.1	6
1-Dodecanol	35.3	~0.004	64	345	9.0@40 °C	0.83	275	261	119	<0.1	24
Hydrogenated bisabolene[e]	~37	Immiscible	42	Unknown	2.91	0.82	Unknown	267	111	<0.01	<−78
Biodiesel	32.1	Immiscible	60	314	4–6@40 °C	0.87 (avg)	177–330	315–350	100–170	<1	−3 to −5
Petrodiesel[f]	40.3	Immiscible	45–50	315	1.8–5.8@40 °C	0.84 (avg)	210	150–350	52–96	0.4	−12
Petroleum[g]	32.1	Immiscible	13–17	711–1064	0.4–0.8@20 °C	0.82 (avg)	246–280	27–225	−40	275–475	−60

[a] Linstrom and Mallard, 2014.
[b] Harnisch et al. (2013).
[c] Weinebeck and Murrenhoff (2013).
[d] Viswanath et al. (2007).
[e] Peralta-Yahya et al. (2011).
[f] NREL (2009).
[g] Louis and Arkoudeas (2012).