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Microdiesel: *Escherichia coli* engineered for fuel production

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Biodiesel is an alternative energy source and a substitute for petroleum-based diesel fuel. It is produced from renewable biomass by transesterification of triacylglycerols from plant oils, yielding monoalkyl esters of long-chain fatty acids with short-chain alcohols such as fatty acid methyl esters and fatty acid ethyl esters (FAEEs). Despite numerous environmental benefits, a broader use of biodiesel is hampered by the extensive acreage required for sufficient production of oilseed crops. Therefore, processes are urgently needed to enable biodiesel production from more readily available bulk plant materials like sugars or cellulose. Toward this goal, the authors established biosynthesis of biodiesel-adequate FAEEs, referred to as Microdiesel, in metabolically engineered *Escherichia coli*. This was achieved by heterologous expression in *E. coli* of the *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase and the unspecific acyltransferase from *Acinetobacter baylyi* strain ADP1. By this approach, ethanol formation was combined with subsequent esterification of the ethanol with the acyl moieties of coenzyme A thioesters of fatty acids if the cells were cultivated under aerobic conditions in the presence of glucose and oleic acid. Ethyl oleate was the major constituent of these FAEEs, with minor amounts of ethyl palmitate and ethyl palmitoleate. FAEE concentrations of 1·28 g l^{-1} and a FAEE content of the cells of 26 % of the cellular dry mass were achieved by fed-batch fermentation using renewable carbon sources. This novel approach might pave the way for industrial production of biodiesel equivalents from renewable resources by employing engineered micro-organisms, enabling a broader use of biodiesel-like fuels in the future.

INTRODUCTION

A major challenge mankind is facing in this century is the gradual and inescapable exhaustion of the earth's fossil energy resources. The combustion of those fossil energy materials lavishly used as heating or transportation fuel is one of the key factors responsible for global warming due to large-scale carbon dioxide emissions. In addition, local environmental pollution is caused. Thus, alternative energy sources based on sustainable, regenerative and ecologically friendly processes are urgently needed.

One of the most prominent alternative energy resources, attracting more and more interest in recent years with the price for crude oil reaching record heights, is biodiesel, which is a possible substitute for petroleum-based diesel fuel. Biodiesel is made from renewable biomass mainly by alkali-catalysed transesterification of triacylglycerols (TAGs) from plant oils (Ma & Hanna, 1999). It consists of monoalkyl esters of long-chain fatty acids with short-chain alcohols, primarily methanol and ethanol, resulting in fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). Biodiesel offers a number of interesting and attractive beneficial properties compared to conventional petroleum-based diesel (for an overview see Krawczyk, 1996). Most important, the use of biodiesel maintains a balanced carbon dioxide cycle since it is based on renewable biological materials. Additional environmental benefits are reduced emissions (carbon monoxide, sulphur, aromatic hydrocarbons, soot particles) during combustion. Biodiesel is non-toxic and completely biodegradable. Due to its high flash point, it is of low flammability and thus its use is very safe and non-hazardous. Furthermore, it provides good lubrication properties, thereby reducing wear and tear on engines. Pure biodiesel or biodiesel mixed in any ratio with petroleum-based diesel can be used in conventional diesel engines with no or only marginal modifications, and it can be distributed using the existing infrastructure. Biodiesel is already produced in a growing number of countries on a large scale (e.g. 1 080 000 t biodiesel was produced in Germany in 2004: Bockey & von Schenck, 2005).

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Abbreviations: FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester; TAG, triacylglycerol; WS/DGAT, wax ester synthase/acyl-coenzyme A : diacylglycerol acyltransferase.

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Despite these positive ecological aspects, however, biodiesel, as currently produced on a technical scale, has also numerous drawbacks and limitations. (1) Production is dependent on the availability of sufficient vegetable oil feedstocks, mainly rapeseed in Continental Europe, soybean in North America and palm oil in South East Asia. Therefore, industrial-scale biodiesel production will remain geographically and seasonally restricted to oilseed-producing areas. (2) Vegetable oils predominantly consisting of TAGs can not be used directly as diesel fuel substitute, mainly because of viscosity problems. Additional problems are the reliability of product quality in bulk quantities and filter plugging at low temperatures due to crystallization. Therefore, plant oils must be transesterified with short-chain alcohols like methanol or ethanol to yield the FAME and FAEE constituents of biodiesel. This transesterification process and the subsequent purification steps are cost intensive and energy consuming, thereby reducing the possible energy yield and increasing the price. (3) FAMEs and FAEEs have comparable chemical and physical fuel properties and engine performances (Peterson *et al.*, 1995), but for economic reasons, only FAMEs are currently produced on an industrial scale due to the much lower price of methanol compared to ethanol. Methanol, however, is currently mainly produced from natural gas. Thus, FAME-based biodiesel is not a truly renewable product since the alcohol component is of fossil origin. Furthermore, methanol is highly toxic and hazardous, and its use requires special precautions. Use of bioethanol for production of FAEE-based biodiesel would result in a fully sustainable fuel, but only at the expense of much higher production costs. (4) The major limitation impeding a more widespread use of biodiesel is the extensive acreage needed for production of oilseed crops. The yield of biodiesel from rapeseed is only 1300 l ha^{-1}, since only the seed oil is used for biodiesel production, whereas the other, major part of the plant biomass is not used for this purpose. Furthermore, oilseed crops like rapeseed and soybean are not self-compatible; therefore, their cultivation requires a frequent crop-rotation regime. In consequence, biodiesel based on oilseed crops will probably not be able to substitute more than 5–15 % of petroleum-based diesel in the future.

A recent study assessing the use of bioethanol for fuel came to the conclusion that large-scale use will require a cellulose-based technology (Farrell *et al.*, 2006). A substantial increase of biodiesel production and a more significant substitution of petroleum-based diesel fuel in the future will probably only be feasible when processes are developed enabling biodiesel synthesis from bulk plant materials such as sugars and starch, and in particular cellulose and hemicellulose.

Intracytoplasmic storage lipid accumulation in the Gram-negative bacterium *Acinetobacter baylyi* strain ADP1 (formerly *Acinetobacter* sp. strain ADP1: Vaneechoutte *et al.*, 2006) is mediated by the wax ester synthase/acyl-coenzyme A : diacylglycerol acyltransferase (WS/DGAT; the *atfA* gene product). This unspecific acyltransferase simultaneously synthesizes wax esters and TAGs by utilizing long-chain fatty alcohols or diacylglycerols and fatty acid coenzyme A thioesters (acyl-CoA) as substrates (Kalscheuer & Steinbüchel, 2003). Biochemical characterization of WS/DGAT revealed that this acyltransferase exhibits an extremely low acyl acceptor molecule specificity *in vitro*. The remarkably broad substrate range of WS/DGAT comprises short chain-length up to very long chain-length linear primary alkyl alcohols; cyclic, phenolic and secondary alkyl alcohols; diols and dithiols; mono- and diacylglycerols as well as sterols (Kalscheuer *et al.*, 2003, 2004; Stöveken *et al.*, 2005; Uthoff *et al.*, 2005). By expression of WS/DGAT in different recombinant hosts, this substrate promiscuity has already been exploited to synthesize various fatty acid ester molecules *in vivo*. The type of fatty acid ester synthesized by WS/DGAT was determined by the physiological background of the expression host regarding the provision of substrates accomplished by natural metabolism, medium supplementation or genetic engineering. Examples of those recombinantly synthesized fatty acid ester derivatives are wax esters in recombinant *Pseudomonas citronellolis* (Kalscheuer & Steinbüchel, 2003), wax esters and fatty acid butyl esters (FABEs) in recombinant *Escherichia coli* (Kalscheuer *et al.*, 2006), wax diesters and wax thioesters in the mutant *A. baylyi* strain ADP1*acr1*ΩKm (Kalscheuer *et al.*, 2003; Uthoff *et al.*, 2005), and TAGs, FAEEs and fatty acid isoamyl esters (FAIEs) in recombinant *Saccharomyces cerevisiae* (Kalscheuer *et al.*, 2004). Although only trace amounts were produced, recombinant biosynthesis of FAEEs and FAIEs in yeast as well as FABEs in *E. coli* indicated that production of biodiesel-appropriate fatty acid monoalkyl esters might in principle be feasible by using recombinant WS/DGAT-expressing micro-organisms. The objective of our present study was thus the development of a microbial process for the production of FAEEs for use as biodiesel from simple and renewable carbon sources. For this approach, the natural WS/DGAT host *A. baylyi* strain ADP1 was not a suitable candidate since it is a strictly aerobic bacterium not able to form ethanol. We therefore established FAEE biosynthesis in recombinant *E. coli* by coexpression of the ethanol production genes from the ethanol-producing fermentative bacterium *Zymomonas mobilis* in combination with the WS/DGAT gene from *A. baylyi* strain ADP1.

METHODS

Strains, plasmids and cultivation conditions. *Escherichia coli* TOP10 (Invitrogen) was used in this study. The plasmids used are pLOI297 harbouring the *Zymomonas mobilis* genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) cloned in pUC18 collinear to the *lacZ* promoter (Alterthum & Ingram, 1989), and pKS : : *atfA* and pBBR1MCS-2 : : *atfA* harbouring the WS/DGAT gene from *A. baylyi* strain ADP1 collinear to the *lacZ* promoter in pBluescript KS^{-} or pBBR1MCS-2, respectively (Kalscheuer & Steinbüchel, 2003). The construction of plasmid pMicrodiesel is described in Results.

Recombinant strains of *E. coli* were cultivated in LB medium (0·5 %, w/v, yeast extract, 1 %, w/v, tryptone and 1 %, w/v, NaCl) containing 1 mM IPTG and 2 % (w/v) glucose at 37 °C in the presence of ampicillin

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(75 mg l^{-1}) and kanamycin (50 mg l^{-1}) for selection of pLOI297, pKS::*atfA* and pMicrodiesel or pBBR1MCS-2::*atfA*, respectively. Where indicated, sodium oleate was added from a 10 % (w/v) stock solution in H_2O to a final concentration of 0·1 or 0·2 % (w/v). Cells were grown aerobically in 300 ml baffled Erlenmeyer flasks containing 50 ml medium on an orbital shaker (130 r.p.m.).

Bioreactor cultivation. Fermentation experiments were done in a 2 litre stirred bioreactor (B. Braun Biotech International) with an initial volume of 1·5 l LB medium containing 0·2 % (w/v) sodium oleate, 2 % (w/v) glucose, 1 mM IPTG and appropriate antibiotics for plasmid selection (see above). Cultivations were done at 37 °C and at a stirring rate of 200 r.p.m. If not stated otherwise, the pH was controlled at 7·0 by automated addition of 4 M HCl or NaOH. Cells were cultivated either aerobically (aeration rate 3 vvm), under restricted oxygen conditions (aeration rate 0·75 vvm), or anaerobically. Inoculum was 5 % (v/v) of saturated overnight cultures.

Thin-layer chromatography. TLC analysis of lipid extracts from whole cells was done as described previously (Kalscheuer & Steinbüchel, 2003) using the solvent system hexane/diethyl ether/acetic acid (90 : 7·5 : 1, by vol.). Lipids were visualized by spraying with 40 % (v/v) sulfuric acid and charring. Ethyl oleate was purchased from Sigma-Aldrich Chemie and used as reference substance for FAEEs.

GC and GC/MS analysis of FAEEs. For quantification of FAEEs, 5 ml culture broth was extracted with 5 ml chloroform/methanol (2 : 1, v/v) by vigorous vortexing for 5 min. After phase separation, the organic phase was withdrawn, evaporated to dryness, and redissolved in 1 ml chloroform/methanol (2 : 1, v/v). FAEEs were analysed by GC on an Agilent 6850 GC (Agilent Technologies) equipped with a BP21 capillary column (50 m × 0·22 mm, film thickness 250 nm; SGE) and a flame-ionization detector (Agilent Technologies). A 2 µl portion of the organic phase was analysed after split injection (1 : 20); hydrogen (constant flow 0·6 ml min^{-1}) was used as carrier gas. The temperatures of the injector and detector were 250 and 275 °C, respectively. The following temperature programme was applied: 120 °C for 5 min, increase of 3 °C min^{-1} to 180 °C, increase of 10 °C min^{-1} to 220 °C, 220 °C for 31 min. Identification and quantification were done by using authentic FAEE standards.

For coupled GC/MS analysis, FAEEs were purified by preparative TLC. GC/MS analysis of FAEEs dissolved in chloroform was done on a Series 6890 GC system equipped with a Series 5973 EI MSD mass-selective detector (Hewlett Packard). A 3 µl portion of the organic phase was analysed after splitless injection on a BP21 capillary column (50 m × 0·22 mm, film thickness 250 nm; SGE). Helium (constant flow 0·6 ml min^{-1}) was used as carrier gas. The temperatures of the injector and detector were 250 °C and 240 °C, respectively. The same temperature programme as described for GC analysis was applied. Data were evaluated by using the NIST-Mass Spectral Search Program (Stein *et al.*, 1998).

Ethanol quantification. Ethanol in cell-free aqueous culture supernatants was determined by GC essentially as described above for FAEE quantification, but applying a modified temperature programme: 70 °C for 20 min, increase of 10 °C min^{-1} to 180 °C, increase of 10 °C min^{-1} to 220 °C, 220 °C for 25 min.

General molecular biological techniques. Standard molecular biological techniques were applied according to Sambrook *et al.* (1989).

RESULTS

Establishment of FAEE biosynthesis in recombinant *E. coli* TOP10 by metabolic engineering

The unspecific acyltransferase WS/DGAT from *A. baylyi* strain ADP1 has been shown to be capable of utilizing ethanol to some extent as an acyl acceptor substrate (Kalscheuer *et al.*, 2004; Stöveken *et al.*, 2005). However, heterologous expression of the WS/DGAT-encoding *atfA* gene alone from pBBR1MCS-2::*atfA* did not result in FAEE formation in *E. coli* TOP10 during cultivation in LB medium containing 2 % (w/v) glucose, 1 mM IPTG and 0·1 % (w/v) sodium oleate under either aerobic or anaerobic conditions (data not shown). Although *E. coli* is known to form ethanol during mixed acid fermentation, obviously ethanol synthesis and/or uptake of oleic acid from the medium and activation to the acyl-CoA thioester were too inefficient to support detectable FAEE formation under anaerobic conditions. However, increased ethanol production has been achieved in *E. coli* upon heterologous expression of pyruvate decarboxylase (the *pdc* gene product) and alcohol dehydrogenase (the *adhB* gene product) from the strictly anaerobic ethanologenic Gram-negative bacterium *Zymomonas mobilis*. Using this system, efficient ethanol biosynthesis was achieved from glucose via the glycolysis product pyruvate even under aerobic conditions (Ingram *et al.*, 1987; Alterthum & Ingram, 1989).

We therefore attempted to establish FAEE biosynthesis in a recombinant *E. coli* by combining expression of the *Z. mobilis* genes *pdc* and *adhB* and of the *atfA* gene from *A. baylyi* strain ADP (Fig. 1) using plasmids pLOI297 (*pdc* and *adhB*) and pBBR1MCS-2::*atfA*. Recombinant strains carrying either plasmid alone did not exhibit FAEE levels detectable by TLC (Fig. 2a, lanes 1 and 2). However, coexpression of all three relevant genes in a strain carrying both plasmids resulted in significant FAEE formation (Fig. 2a, lane 3). FAEE biosynthesis was strictly dependent on the presence of sodium oleate in the medium (data not shown). Growth of strains harbouring plasmid pLOI297 was very poor in LB medium without glucose addition, and FAEE synthesis was not observable in *E. coli* TOP10 harbouring both plasmids under these conditions (data not shown). The FAEEs formed were accumulated intracellularly, and no significant extracellular lipids were found in cell-free culture supernatants (data not shown).

GC/MS analysis of FAEE isolated from *E. coli* TOP10(pBBR1MCS-2::*atfA* + pLOI297) cultivated in medium supplemented with sodium oleate revealed a mixture of esters mainly consisting of ethyl oleate plus minor amounts of ethyl palmitate and ethyl palmitoleate (Fig. 2b). The presence of ethyl palmitate indicated that also some fatty acids derived from *de novo* fatty acid biosynthesis were channelled into FAEE production. When technical-grade sodium oleate (content ~80 %) was used for cultivations at a larger scale, low amounts of ethyl

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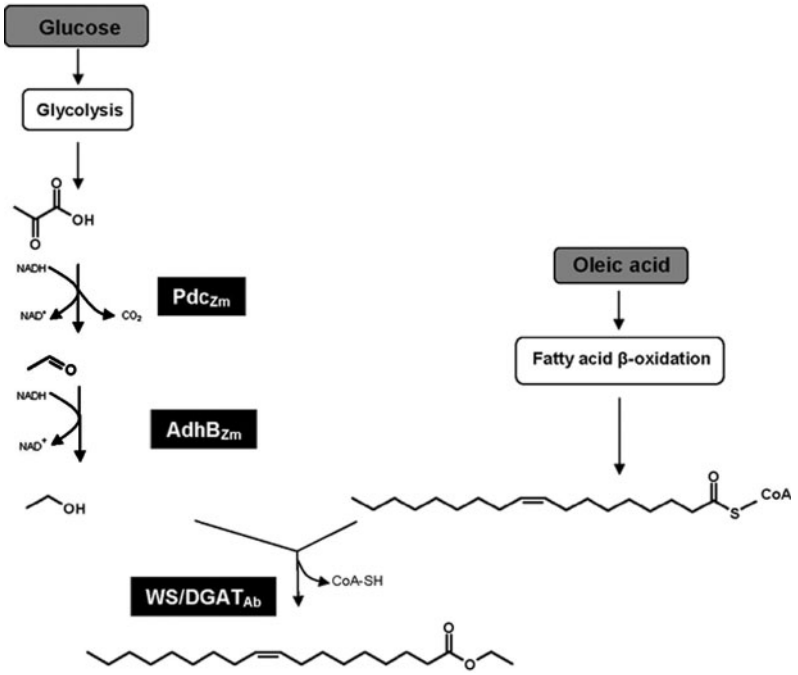

Fig. 1. Pathway of FAEE biosynthesis in recombinant *E. coli*. FAEE formation was achieved by coexpression of the ethanolic enzymes pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (AdhB) from *Z. mobilis* and the unspecific acyltransferase WS/DGAT from *A. baylyi* strain ADP1.

myristate (C14 : 0-ethyl ester, $m/z=256$ $[C_{16}H_{32}O_2]^+$), ethyl myristoleate (C14 : 1-ethyl ester, $m/z=254$ $[C_{16}H_{30}O_2]^+$) and ethyl linoleate (C18 : 2-ethyl ester, $m/z=308$ $[C_{20}H_{36}O_2]^+$) were also observed due to the presence of the corresponding fatty acid impurities (data not shown).

Batch fermentations of *E. coli* TOP10(pBBR1MCS-2 : : *atfA*+pLOI297) for FAEE production

The shake-flask experiments under aerobic conditions described above clearly proved the concept that FAEE biosynthesis is feasible in recombinant *E. coli*. Oxygen availability might have a great influence on the ethanol synthesis rate in this recombinant system, with low-oxygen conditions supposed to favour ethanol formation, and thus might also have a profound impact on the FAEE biosynthesis rate. We therefore cultivated *E. coli* TOP10(pBBR1MCS-2 : : *atfA* + pLOI297) under conditions permissive for FAEE formation with different controlled oxygen conditions (Fig. 3). Although ethanol production was slightly higher under anaerobic conditions (maximal 4·39 g l^{-1} after 17 h), only a very low FAEE content was observed, plateauing already after 18 h at a concentration of 0·05–0·07 g l^{-1} (Fig. 3b). In contrast, FAEE biosynthesis was significantly higher under aerobic conditions (aeration rate 3 vvm). FAEE formation was not restricted to a certain growth phase but continued throughout the cultivation period, finally reaching 0·26 g l^{-1} after 48 h (Fig. 3a). With a final cellular dry biomass of 4·3 g l^{-1} obtained by aerobic cultivation this corresponds to a cellular FAEE content of 6·1 % (w/w). When the cells were cultivated under oxygen-restricted conditions (aeration rate 0·75 vvm) a final FAEE concentration of 0·16 g l^{-1} was obtained after 48 h (data not shown). Under all three cultivation conditions ethanol concentration reached a maximum after 15–20 h cultivation, after which a rapid decrease was unexpectedly observed (Fig. 3a, b), which has not to our knowledge been described before for ethanologenic *E. coli* strains employing the *Z. mobilis pdc* and *adhB* genes for recombinant ethanol synthesis.

Construction of plasmid pMicrodiesel

To simplify the process by reducing the number of antibiotics required for plasmid stabilization and to potentially increase FAEE yield by providing all three relevant genes on a high-copy-number vector, plasmid pMicrodiesel was constructed. For this, a 3·2 kbp DNA fragment was amplified from plasmid pLOI297 by tailored PCR using the oligonucleotides 5′-AAAGGATCCGCGCA-ACGTAATTAATGTGAGTT-3′ (forward primer) and 5′-TTTGGATCCCCAAATGGCAAATTATT-3′ (reverse primer) introducing *Bam*HI restriction sites (underlined). This 3·2 kbp *Bam*HI fragment, which comprised the *Z. mobilis* genes *pdc* and *adhB* and the upstream *lacZ* promoter region, was cloned into *Bam*HI-linearized pKS : : *atfA*, a derivative of the high-copy-number plasmid pBluescript KS$^-$ (Kalscheuer & Steinbüchel, 2003), yielding pMicrodiesel (Fig. 4). The orientation of *atfA*, *pdc* and *adhB* was determined by *Eco*RI restriction and DNA sequence analysis. Plasmid pMicrodiesel carried all three genes relevant for FAEE synthesis in a collinear orientation, with *atfA* driven by a *lacZ* promoter and with *pdc* and *adhB* controlled by a second *lacZ* promoter, thereby ensuring effective transcription of all three genes.

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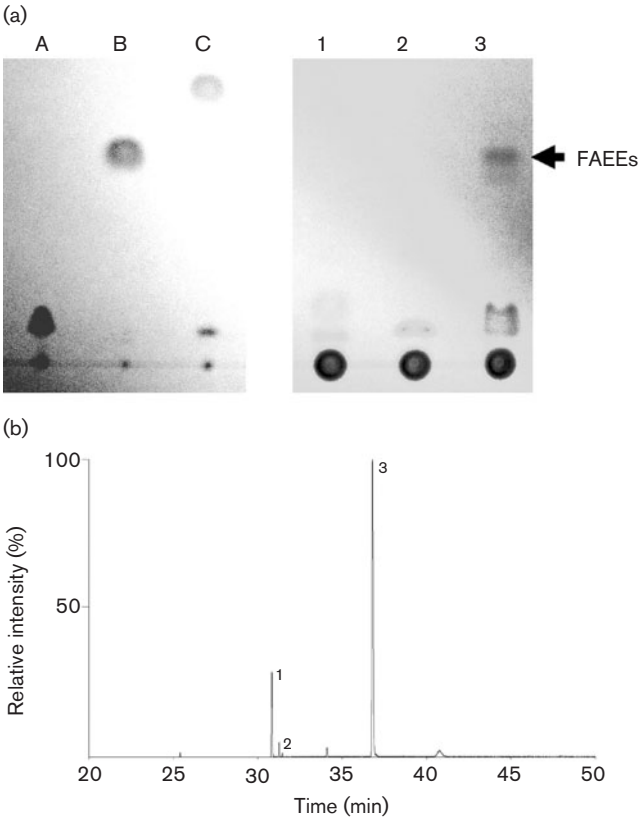

Fig. 2. Chemical analysis of FAEEs produced by recombinant *E. coli* TOP10. (a) TLC analysis of intracellular lipids accumulated by recombinant *E. coli* TOP10. Cells were cultivated aerobically in shake flasks for 24 h at 37 °C in LB medium containing 2 % (w/v) glucose, 0·1 % (w/v) sodium oleate, 1 mM IPTG and appropriate antibiotics as described in Methods. A, oleic acid; B, ethyl oleate; C, oleyl oleate; 1, *E. coli* TOP10(pLOI297); 2, *E. coli* TOP10(pBBR1MCS-2 : : *atfA*); 3, *E. coli* TOP10(pBBR1MCS-2 : : *atfA* + pLOI297). Total lipid extracts each obtained from 1·5 mg lyophilized cells were applied in lanes 1–3. (b) Total ion profile of GC/MS analysis of FAEEs isolated from *E. coli* TOP10(pBBR1MCS-2 : : *atfA* + pLOI297). Cells were cultivated as described above. FAEEs were purified by preparative TLC. Identified substances: 1, ethyl palmitate (C16 : 0-ethyl ester, $m/z = 284$ $[C_{18}H_{36}O_2]^+$); 2, ethyl palmitoleate (C16 : 1-ethyl ester, $m/z = 282$ $[C_{18}H_{34}O_2]^+$); 3, ethyl oleate (C18 : 1-ethyl ester, $m/z = 310$ $[C_{20}H_{38}O_2]^+$).

Fed-batch fermentation of *E. coli* TOP10(pMicrodiesel) for FAEE production

Shake-flask experiments with *E. coli* TOP10 harbouring either pMicrodiesel alone or pLOI297 plus pBBR1MCS-2 : : *atfA* revealed a more than twofold higher FAEE production using the newly constructed plasmid pMicrodiesel (0·64 g l^{-1} compared to 0·26 g l^{-1}) whereas ethanol concentrations were similar. This indicated the positive influence of provision of all three relevant genes on a high copy-number vector and, as consequence, potentially higher expression rates on FAEE yield.

We then aspired to further optimize FAEE production by *E. coli* TOP10(pMicrodiesel), employing an aerobic fed-batch fermentation regime. Initial optimization experiments revealed that no regulation of medium pH during cultivation, resulting in a slightly acidic pH of 6·0–6·5 at the end, rather than a strict regulation at pH 7·0, might be favourable for FAEE biosynthesis (data not shown). Thus, the pH value was only roughly regulated automatically between 6·0 and 8·5 during the following fed-batch fermentation experiment (Fig. 5). To avoid carbon limitation, glucose was fed several times during the cultivation period. FAEE concentration continuously increased throughout the fermentation process, whereas its composition remained relatively constant (similar to the results shown in Fig. 2b). Employing this fed-batch strategy, a final FAEE content of 1·28 g l^{-1} was achieved after 72 h, which was about five times higher compared to aerobic batch fermentation of the *E. coli* TOP10 strain harbouring pLOI297 plus pBBR1MCS-2 : : *atfA* (Fig. 3a). With a final cellular dry biomass of 4·9 g l^{-1} this corresponds to an impressive cellular FAEE content of 26 % (w/w). Referred to the initial amount of 2 g l^{-1} present in the medium at the beginning of the cultivation, sodium oleate was converted to FAEEs with an efficiency of 62·7 % on a molar basis.

DISCUSSION

Biodiesel is an interesting alternative energy source and is used as substitute for petroleum-based diesel. Offering numerous environmental benefits, it has attracted broad public interest and is being produced in increasing amounts (see Introduction). However, a broader use of biodiesel and a more significant substitution of petroleum-based fuels in the future will only be possible if production processes are developed that are not solely based on oilseed crops but on more bulk plant materials like cellulose. Toward this goal, we report here on a novel approach to establish biotechnological production of biodiesel using metabolically engineered micro-organisms, which we refer to as Microdiesel. The early optimization studies described here revealed FAEE yields of up to 26 % of the bacterial dry biomass. Although these yields are still far below the needs for an industrial process, this study has clearly proved the feasibility, in principle, of this novel approach. Therefore, the present study might open new avenues potentially enabling microbial production of fuel equivalents from cheap and readily available renewable bulk plant materials like sugars, starch, cellulose or hemicellulose in the future.

Microbial FAEE biosynthesis for Microdiesel production is based on the exploitation of the extraordinarily low substrate specificity of the acyltransferase (WS/DGAT) of *A. baylyi* strain ADP1, which in its natural host mediates wax ester and TAG biosynthesis from acyl-CoA thioesters plus long chain-length fatty alcohols or diacylglycerols (Kalscheuer & Steinbüchel, 2003). *E. coli* does not produce such substances by its natural metabolism; however, recombinant strains enabled to produce large amounts of

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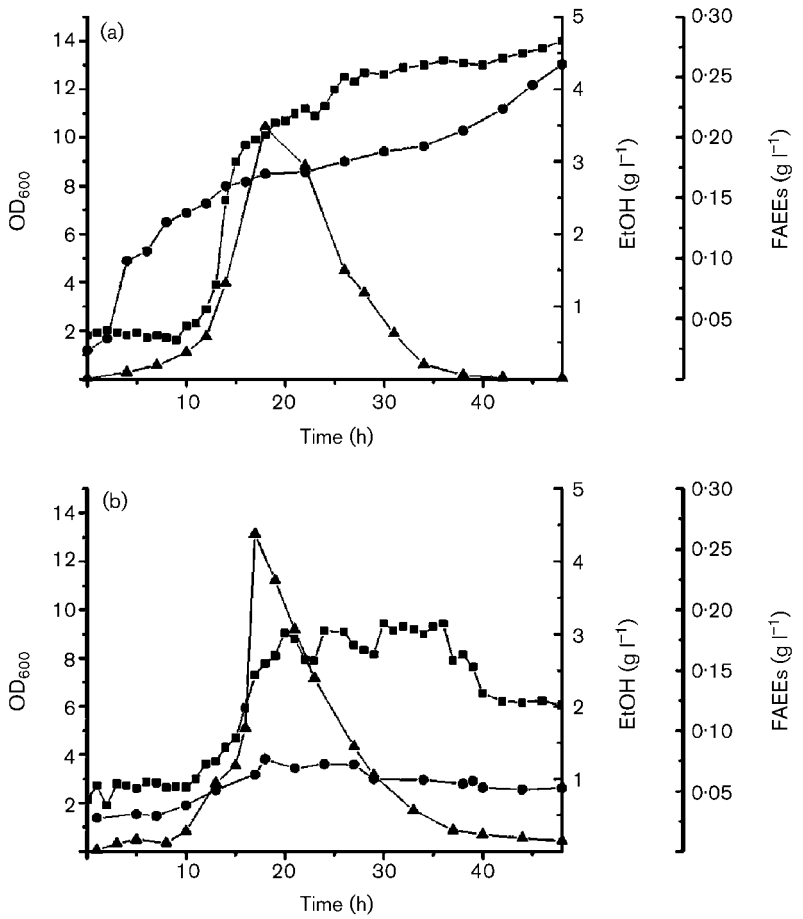

Fig. 3. FAEE production during batch fermentations of *E. coli* TOP10(pBBR1MCS-2::*atfA*+pLOI297). Cultivations were done in a 2 litre stirred bioreactor initially filled with 1·5 l LB medium containing 0·2 % (w/v) sodium oleate, 2 % (w/v) glucose, 1 mM IPTG, 75 mg ampicillin l^{-1} and 50 mg kanamycin l^{-1} as described in Methods. Sodium oleate causes turbidity of the medium, explaining the high initial optical densities. ■, OD_{600}; ▲, ethanol concentration; ●, FAEE concentration. (a) Cultivation under aerobic conditions (aeration rate 3 vvm). (b) Cultivation under anaerobic conditions.

ethanol and simultaneously expressing WS/DGAT provided an unusual, alternative substrate for this acyltranferase. This resulted in production of substantial amounts of FAEEs utilizing WS/DGAT's substrate promiscuity.

E. coli forms ethanol, among other fermentation products, during mixed acid fermentation under anaerobic conditions from acetyl-CoA via two sequential NADH-dependent reductions catalysed by a multifunctional alcohol dehydrogenase (the *adhE* gene product) (Goodlove *et al.*, 1989; Kessler *et al.*, 1992). However, ethanol levels naturally occurring in *E. coli* under anaerobic conditions are probably not sufficient to support formation of significant amounts of FAEE. In addition, several other fermentation products besides ethanol occur in substantial amounts. By using a recombinant system employing *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase, this limitation was circumvented, resulting in substantial amounts of ethanol under aerobic conditions, which is in accordance with previous reports (Ingram *et al.*, 1987; Alterthum & Ingram, 1989). In fed-batch fermentations conducted under controlled aeration rates, the highest FAEE levels were observed in recombinant *E. coli* under aerobic conditions (approximately five times higher compared to anaerobic conditions) although ethanol levels were similar. This indicates that uptake of exogenous fatty acids from the medium and their activation to the corresponding acyl-CoA thioesters is probably another factor limiting Microdiesel production in *E. coli* under anaerobic conditions.

Although an impressive FAEE content as high as 26 % of the cellular dry weight was finally obtained, *E. coli* is not ideal for Microdiesel production for various reasons. Although the occurrence of ethyl palmitate as a minor constituent indicated that fatty acids derived from *de novo* fatty acid biosynthesis were channelled into FAEE production, substantial FAEE biosynthesis was strictly dependent on supplementation of exogenous fatty acids. This indicates that *de novo* fatty acid biosynthesis, in contrast to fatty acid β-oxidation, can not provide sufficient intracellular acyl substrates for WS/DGAT-mediated FAEE synthesis. Therefore, it will be challenging to establish Microdiesel production solely from simple bulk plant materials like sugars, cellulose or hemicellulose in the future using *E. coli* as a production platform. As an alternative, storage-lipid-accumulating bacteria, in particular those of the actinomycete group, may be used; these bacteria are capable of synthesizing from simple carbon sources like glucose under growth-restricted conditions remarkably high amounts of fatty acids (up to ~70 % of the cellular dry weight) and

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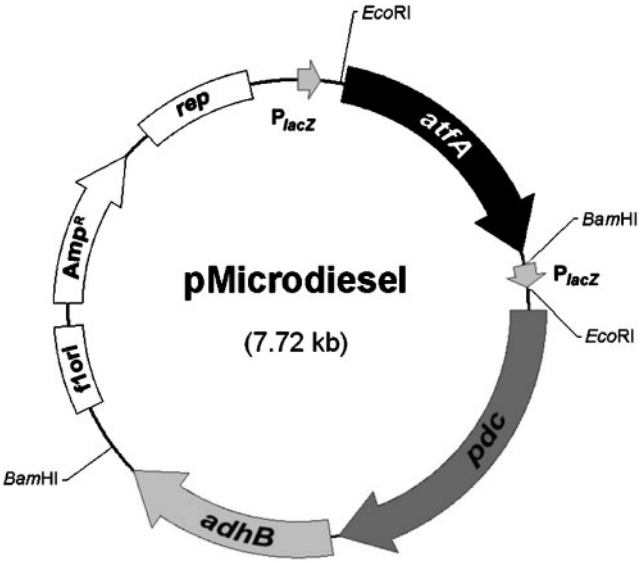

Fig. 4. Map of plasmid pMicrodiesel. Relevant characteristics: *rep*, origin of replication; Amp^R, ampicillin-resistance gene; P_{lacZ}, *lacZ* promoter; *pdc*, pyruvate decarboxylase gene from *Z. mobilis*; *adhB*, alcohol dehydrogenase gene from *Z. mobilis*; *atfA*, WS/DGAT gene from *A. baylyi* strain ADP1.

accumulate them intracellularly as TAGs (Alvarez & Steinbüchel, 2002). If the flux of fatty acids could be directed from TAG towards FAEE biosynthesis by genetic manipulation, storage-lipid-accumulating bacteria might be promising candidates for more simplified Microdiesel production processes in the future. Establishment of recombinant ethanol biosynthesis in these aerobic, non-fermentative bacteria would be a prerequisite for this purpose. In this regard, a recently developed heterologous ethanol production system for Gram-positive bacteria could become of great value and utility (Talarico *et al.*, 2005). Future optimization of biotechnological Microdiesel production will also benefit from the progress made in recent years in lignocellulose utilization as feedstock for bioethanol production by recombinant micro-organisms (Dien *et al.*, 2003; Zaldivar *et al.*, 2001).

A further bottleneck in the path towards optimized FAEE levels is the relatively low reaction rate of WS/DGAT with ethanol in comparison with longer chain-length fatty alcohols (C_{10}–C_{18}) (Kalscheuer *et al.*, 2004; Stöveken *et al.*, 2005). Numerous genes encoding WS/DGAT homologues have been identified in several other bacteria (Kalscheuer & Steinbüchel, 2003). One of those acyltransferases might be more suitable for FAEE production since it may exhibit a higher specificity for ethanol. Alternatively, increase of the reaction rate of WS/DGATs with ethanol may be achieved by enzyme engineering.

Optimized Microdiesel production by engineered micro-organisms could finally offer some major advantages over established conventional production processes. Biotechnological Microdiesel production could be significantly less expensive than conventional biodiesel production if plant products like starch or lignocellulose are used for its production. These plant polymers are not only much cheaper than plant oils, but are also much more abundant, and Microdiesel production will not be restricted to oilseed-producing regions of the world. In contrast to conventional FAME-based biodiesel, Microdiesel is a fully sustainable biofuel completely derived from renewable materials, also avoiding the use of highly toxic methanol. In conclusion, this study provides a basis to achieve more competitive production costs, and therefore a more substantial substitution of petroleum-derived fuels by biofuels in the future.

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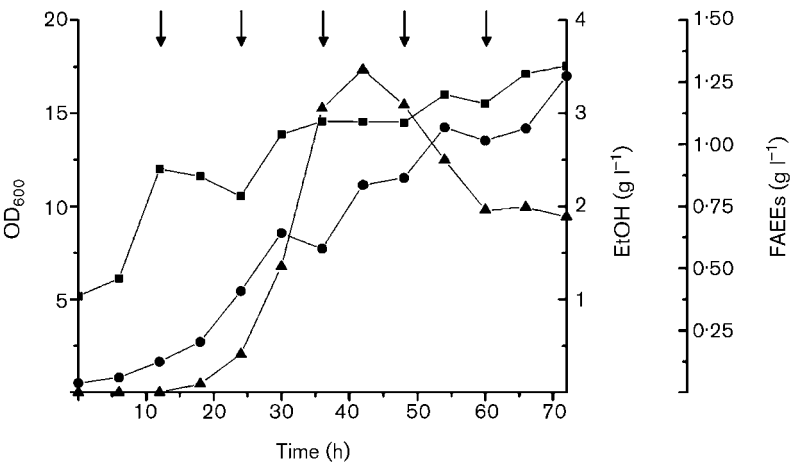

Fig. 5. FAEE production during fed-batch fermentation of *E. coli* TOP10(pMicrodiesel). Cultivation was done in a 2 litre stirred bioreactor initially filled with 1·5 l LB medium containing 0·2 % (w/v) sodium oleate, 2 % (w/v) glucose, 1 mM IPTG and 75 mg ampicillin l^{-1} under aerobic conditions (aeration rate 3 vvm) as described in Methods. The pH was kept between 6·0 and 8·5 by automated addition of 4 M HCl or NaOH. To prevent carbon limitation, 1 g glucose l^{-1} was fed several times during cultivation (indicated by arrows). Sodium oleate causes turbidity of the medium, explaining the high initial optical density. ■, OD_{600}; ▲, ethanol concentration; ●, FAEE concentration.

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