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Sustainable production of microbial oleo-chemicals for high value applications in the pharmaceutical and chemical industry

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I Abstracts

Chapter 1

In the first chapter, the successful alteration of the fatty acid profile of *Escherichia coli* (*E. coli*), towards the generation of longer chain fatty acids (FAs), through the recombinant expression of the *Arabidopsis thaliana* chloroplastic β-ketoacyl-[acyl carrier protein] synthase I and II (KAS I and KAS II) was demonstrated. The recombinant expression of KAS I in *E. coli* resulted in an increase of total FA amount and a shift in the FA profile towards unsaturated fatty acids. Alternatively, the recombinant expression of KASII yielded a targeted increase in stearic and vaccenic acid, resulting in a shift in the fatty acid profile towards longer chain FAs, without affecting final yields. Remarkably, the tightly regulated FA biosynthesis pathway of *E. coli* could functionally accommodate the *A. thaliana* KAS enzymes. More interestingly, the *A. thaliana* KAS II enzyme could complement the loss of function of the native *E. coli* β-ketoacyl-[acyl carrier protein] synthase II (FabF). The utilization of the chloroplastic *A. thaliana* KAS enzymes in *E. coli* allows the generation of long chain FAs, suitable for further processing into high-end oleo-chemicals.

Chapter 2

The second chapter addressed, the production of very long chain fatty acids (VLCFAs) in *Escherichia coli* (*E. coli*). Therefore, a polycistronic expression cassette was designed and constructed, harboring the *Arabidopsis thaliana* (*A. thaliana*) elongase multi-enzyme-complex. The four enzymes building the complex are: 3-ketoacyl-CoA synthase 18 (*KCS18),* catalyzing the rate limiting step in VLCFA biosynthesis*,* Very-long-chain 3-oxoacyl-CoA reductase 1 (*KCR1*), Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase (*PAS2*), and Very-long-chain enoyl-CoA reductase (*CER10*). Consequently, we were able to successfully express the four elongase enzymes in combination with a self-assembly GFP system, to maximize solubility and enzyme interaction. We successfully demonstrated, for the first time, the generation of arachidic acid (C20:0, elongated stearic acid), cis-13-eicosenoic acid (C20:1, elongated vaccenic acid), cis-11-eicosenoic acid (C20:1, elongated oleic acid) and erucamide (C22:1, amide of erucic acid) in *E. coli*, via the *A. thaliana* elongase complex, after supplementation with oleic acid and cerulenin. Although further optimization of the VLCFA complex in *E. coli* is required to enhance product yield, this work paves the way for the future production of novel, high value very long chain oleo-chemicals in *E. coli*.

II Zusammenfassungen

Kapitel I

Im ersten Kapitel konnte, durch die rekombinante Expression der aus *Arabidopsis thaliana* (*A. thaliana*) stammenden, Chloroplasten-assoziierten β-ketoacyl-[acyl carrier protein] Synthasen I und II (KAS I & KAS II), eine Verschiebung des Fettsäureprofils von *Escherichia coli* (*E. coli*), in Richtung langkettiger Fettsäuren, demonstriert werden. Die heterologe Expression von KAS I in *E. coli* resultierte in einer erhöhten Fettsäuremenge und einer Verschiebung des Fettsäureprofils, hin zu mehr ungesättigten Fettsäuren. Die rekombinante Expression von KAS II hingegen, führte zur angestrebten Erhöhung der Ausbeute an Stearin- und Vaccensäure und sorgte damit gleichzeitig für ein verändertes Fettsäureprofil, mit höheren Anteilen an ungesättigten Fettsäuren, ohne die totale Fettsäureausbeute zu beeinflussen. Es konnten also die aus *A. thaliana* stammenden KAS-Enzyme erfolgreich in die in hohem Maße regulierte, wirtseigene Fettsäurebiosynthese eingebettet werden. Hierbei ist besonders hervorzuheben, dass durch die Expression von KAS II aus *A. thaliana* der Funktionsverlust der nativen β-ketoacyl-[acyl carrier protein] Synthase II (FabF), in einer entsprechenden *E. coli*-Mutante, komplementiert werden konnte. Die Verwendung der Chloroplasten-assoziierten KAS-Enzyme aus *A. thaliana* ermöglichen damit die Produktion von langkettigen Fettsäuren in *E. coli*, die sich zur weiteren Verarbeitung zu hochwertigen Oleochemikalien eignen.

Kapitel II

Das zweite Kapitel befasste sich mit der Produktion von „very long chain fatty acids (VLCFAs)“ (Fettsäuren mit mehr als 22 C-Atomen) in *Escherichia coli* (*E. coli*). Hierzu wurde eine polycistronische Expressionskassette ausgearbeitet und konstruiert, welche den aus *Arabidopsis thaliana* (*A. thaliana*) stammenden Elongase-Multienzymkomplex abbildet. Der Komplex besteht aus den folgenden Enzymen: der 3-ketoacyl-CoA Synthase 18 (*KCS18),* welche den ratenlimitierenden Schritt der VLCFA-Biosynthese katalysiert, der Very-long-chain 3-oxoacyl-CoA Reduktase 1 (*KCR1*), der Very-long-chain (3R)-3-hydroxyacyl-CoA Dehydratase (*PAS2*), sowie der Very-long-chain enoyl-CoA Reduktase (*CER10*). Der Multienzymkomplex konnte erfolgreich in Kombination mit einem „self-assembly“ GFP-System expremiert werden, wodurch eine maximale Löslichkeit, sowie eine verbesserte Interaktion der Enzyme erreicht werden konnten. Durch die Expression des *A. thaliana* Enzymkomplexes, sowie die Zugabe von Ölsäure und Cerulenin, konnten erstmalig Arachinsäure (C20:0, verlängerte Stearinsäure), Paullinsäure

(C20:1 cis-13, verlängerte Vaccensäure), Gondonsäure (C20:1 cis-11, verlängerte Ölsäure) und Erucasäure-Amid (C22:1 Amid) erfolgreich in *E. coli* hergestellt werden. Obwohl zur Steigerung der entsprechenden Fettsäureausbeuten noch Optimierungspotential für die Verwendung des VLCFA-Komplexes in *E. coli* besteht, konnte durch diese Arbeit der Grundstein für die Produktion von neuen, hochwertigen, auf VLCFAs basierenden Oleochemikalien in *E. coli* gelegt werden.

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V List of Abbreviations

°C	degree Celsius
µM	micro molar
AccABCD	acetyl coenzyme A carboxylase
ACP	acyl carrier protein
ALK	alkane
ATP	adenosine triphosphate
cDNA	complementary DNA
CER10	very-long-chain enoyl-CoA reductase
CoA	coenzyme A
ddH_2O	double distilled water
FabA	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase
FabB	3-oxoacyl-[acyl-carrier-protein] synthase 1
FabD	malonyl-CoA:ACP transacylase
FabF	3-oxoacyl-[acyl-carrier-protein] synthase 2
FabG	3-oxoacyl-[acyl-carrier-protein] reductase
FabH	3-oxoacyl-[acyl-carrier-protein] synthase 3
FabI	enoyl-[acyl-carrier-protein] reductase
FabZ	3-hydroxyacyl-[acyl-carrier-protein] dehydratase
FadD	fatty acyl-CoA ligase
FadE	acyl-coenzyme A dehydrogenase
FadL	long-chain fatty acid transport protein
FAEE	fatty acid ethyl ester
FAMEs	fatty acids methyl esters
FAOH	fatty alcohol
FATB	palmitoyl-acyl carrier protein thioesterase
FFA	free fatty acids
FAS I	fatty acid synthase I

FAS II	fatty acid synthase II
GFP	green fluorescent protein
HCl	hydrochloric acid
IPTG	isopropyl β-d-1-thiogalactopyranoside
KASI	β-ketoacyl-[acyl carrier protein] synthase I
KASII	β-ketoacyl-[acyl carrier protein] synthase II
KCS18	3-ketoacyl-CoA synthase 18
KCR1	very-long-chain 3-oxoacyl-CoA reductase 1
KO	knock-out
MK	methyl ketone
MMT	million metric tons
mM	millimolar
PAS2	3-hydroxyacyl-CoA dehydratase
PCR	polymerase chain reaction
RBS	ribosomal binding site
TAE	tris base, acetic acid and ethylenediaminetetraacetic acid buffer
TesA	thioesterase A
vvm	volume of medium per min
w/v	weight per volume

1. Introduction

1.1 Oleo-chemicals

Oleo-chemicals are a group of chemical products derived from animal or vegetable oil and fat feedstock. Oleo-chemicals are used for various applications in the food, cosmetic, pharmaceutical and chemical industry. The basic oleo-chemical substances are fatty acids, fatty acid derivatives, and glycerol. The synthetic alternatives to oleo-chemicals, being petrochemicals, are derived from petroleum feedstock [1-5]. Oils and fats of vegetable and animal origin currently represent the most important renewable feedstock for the chemical industry, required for the production of various oleo-chemicals. Common examples of vegetable oil feedstock include olive oil, rapeseed oil, sunflower oil, soybean, palm and palm kernel oil. Fatty acid composition varies between different plant oils in terms of chain length and level of saturation, ultimately dictating the properties of the final product [6]. The major constituents of animal and vegetable oils or fats are triglycerides (TAGs), which are composed of three fatty acids bound to a glycerol backbone. [1-6].

Various established chemical and enzymatic reactions are implemented for the generation of basic oleo-chemical substances from triglycerides, found in animal and vegetable oils and fats [1-8]. The hydrolysis of triglycerides, the chemical breakdown of triglycerides into glycerol and their respective fatty acids under the influence of water, pressure and temperature, is one of the chemical processes used for transforming oils and fats into oleo-chemicals [9,10]. The generated free fatty acids can be further modified by reactions at the carboxyl group to yield fatty alcohols [11-13], fatty amides and amines and fatty esters [2,3,14]. Methanol transesterification of triglycerides, on the other hand, is used to yield glycerol and fatty acids methyl esters (FAMEs) [2]. The hydrogenation of both, free fatty acids and fatty acid methyl esters, is one of the processes used for the production of fatty alcohols [1,7]. Currently, oleo-chemical industry utilizes plant extracts as starting material for the chemical generation of the desired products. An alternative to the common, chemical production route could be offered by the conversion of biological waste streams to fatty acid products, via microbial fermentation techniques [8] (Figure 1).

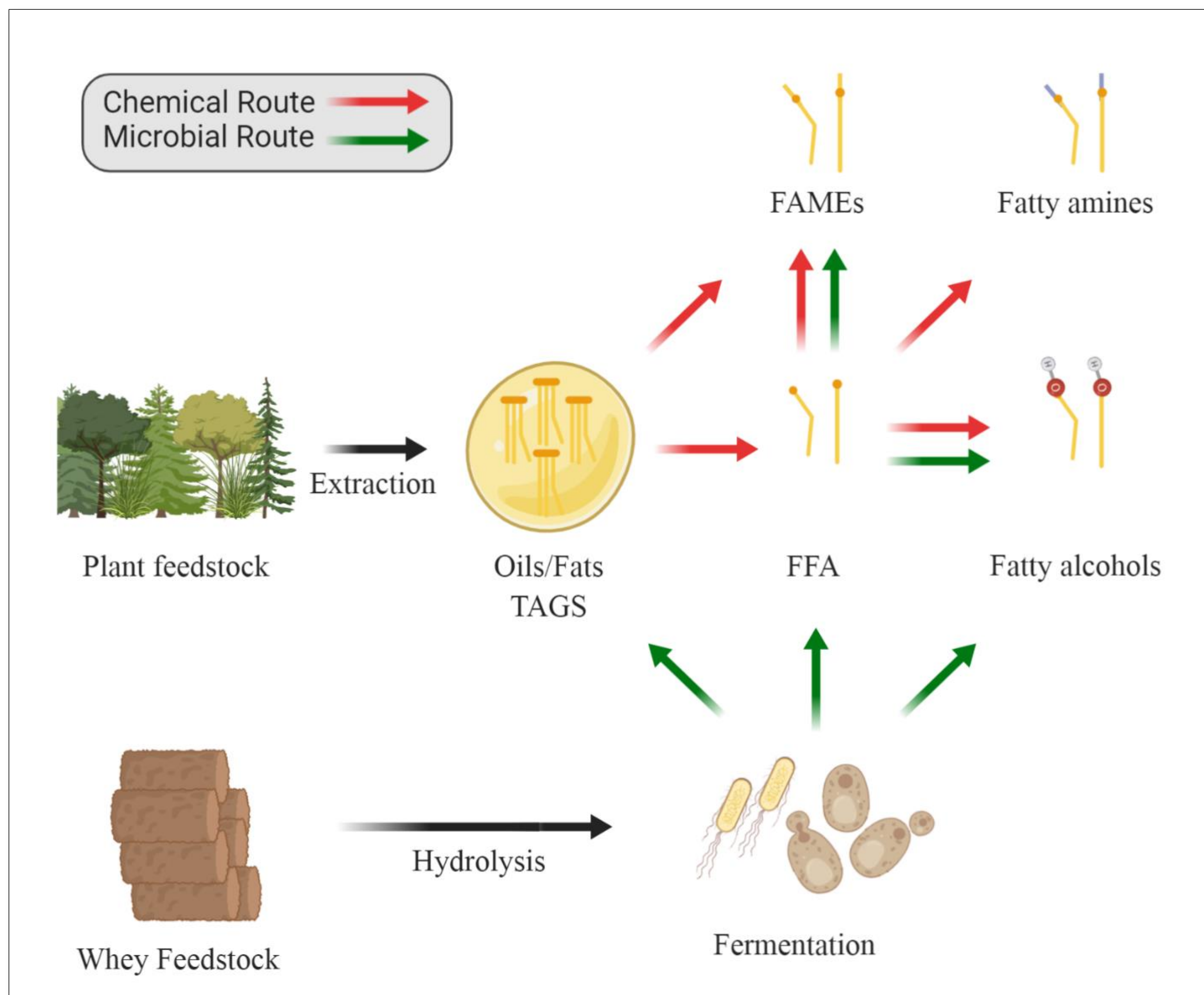

Figure 1. Typical chemical routes for basic oleo-chemicals production from plant extracts and alternative microbial routes [3,13].

The basic oleo-chemicals (free fatty acids, fatty methyl esters, fatty alcohols and fatty amines) can be thereafter converted into more advanced chemicals with a wide range of applications in various industries. Nowadays, oleo-chemicals are utilized in the production of soaps and detergents, cosmetics and personal care products, lubricants and surfactants, paint and surface coatings, rubber and plastic polymers, biofuels and biodiesel [1,3-5,8]. A short summary of basic oleo-chemicals, their derived chemicals and industrial applications is presented in Table 1.

Table 1: Oleo-chemicals, derivatives and applications [1,3,5]

Basic Oleochemicals	**Free Fatty Acids**	**Fatty Acids Methyl Esters**	**Fatty alcohols**
Derivatives	Fatty acid esters Conjugated fatty acids Alkyl epoxyesters Fatty acid ethoxysulfates	Sulfo fatty acid esters	Fatty alcohol sulfates Fatty alcohol ethoxylates and ethoxysulfates
Applications	Soaps and Detergents Rubber and plastics Paper Food and feed Personal care and cosmetics Paints and coatings lubricants	Biofuels Biodiesel Surfactants Plasticizers	Personal care Cosmetics Pharmaceuticals Detergents Textiles Agriculture

Currently, plant oils represent the major feedstock for renewable ole-chemical production. The world production volume of vegetable oils increased in the last decade from 149 million metric tons (MMT) in 2011, to 170 MMT in 2014, reaching 207 MMT in 2019 [3,15]. Palm and soybean together account for 65% of the global production of plant oils [4,16]. More specifically, 77 % of the 157 MMT of vegetable oils produced in 2013, were utilized in food industry, while the remaining 23% were used for the production of oleo-chemicals and biodiesel [3].

Although the use of vegetable-oils for oleo-chemicals production offers significant advantages (biodegradability, lower CO_2 footprint) over petroleum-derived chemicals, it is still associated with negative environmental and economic impacts [17]. To meet the rising annual global-demand, more than double of the currently arable land would be required, to cultivate crops that are explicitly grown for oleo-chemicals production [18]. This would lead to deforestation, destruction of ecosystems and deterioration of soils [19]. Furthermore, the use of edible crops for generation of oleo-chemicals jeopardizes food security and drives food prices up [20]. The use of plant-based oleo-chemicals is by far not the optimal solution to achieve a fossil-fuel independent society [18-20].

By contrast, a sustainable and eco-friendly production of oleo-chemicals requires the implementation of bioprocesses based on microbial cell-factory platforms. In fact, the use of

microorganisms as a production platform offers various advantages over vegetable oils such as: a) renewability and sustainability, b) faster growth rates, c) no requirement for arable land and precious agricultural resources, d) no requirement for fresh water irrigation, e) utilize waste biomass as feedstock, f) product yields and quality derived from microbial platforms are not affected by season, climate or geographic location, g) have a small carbon footprint, h) do not jeopardize food security, and i) are easily modifiable to generate custom tailored fatty acid profiles [8,18,21-23].

1.2 Established microbial platforms

Microorganisms enjoy a long history as workhorses in the food industry, dating back to the dawn of civilization. Baker's yeast (*Saccharomyces cerevisiae*) is amongst the most exploited for baking and the production of fermented beverages [24]. In the last century, the advent of synthetic biology expanded the application range of microorganisms. Single-cell-factory platforms are utilized in the production of oleo-chemicals, biofuels, proteins, bio-actives and pharmaceuticals. The use of microorganisms for the production of industrially relevant compounds from renewable or waste feedstock has gained substantial interest from companies, governments, and the general public [25-27].

In 1884, German scientist Theodor Escherich discovered what is now known as the Rock Star of biology: *Escherichia coli* [28,29]. It is a model organism, where most strains are non-pathogenic, easy to handle, and can grow quickly on many different nutrients. Groundbreaking studies on the basic aspects of life, such as decrypting the genetic code, transcription, translation and replication, were first worked out with *E. coli* [30,31]. Contributions to the fields of biology (restriction enzymes, life cycle of viruses), medicine (in vivo synthesis of insulin and human growth hormone) and energy (biofuels) were made with this microorganism [32-34]. *E. coli* exhibits particularly useful properties, such as high transformation efficiency, the ability to take up and maintain large plasmids and the ease of screening for successful transformants [35].

The genome of *E. coli* encodes for more than 4000 genes involved in hundreds of well chartered metabolic pathways, which are responsible for producing thousands of metabolites [36]. A selection of molecules that are either naturally occurring in *E. coli*, or produced by genetically modified strains is shown in Figure 2 [33].

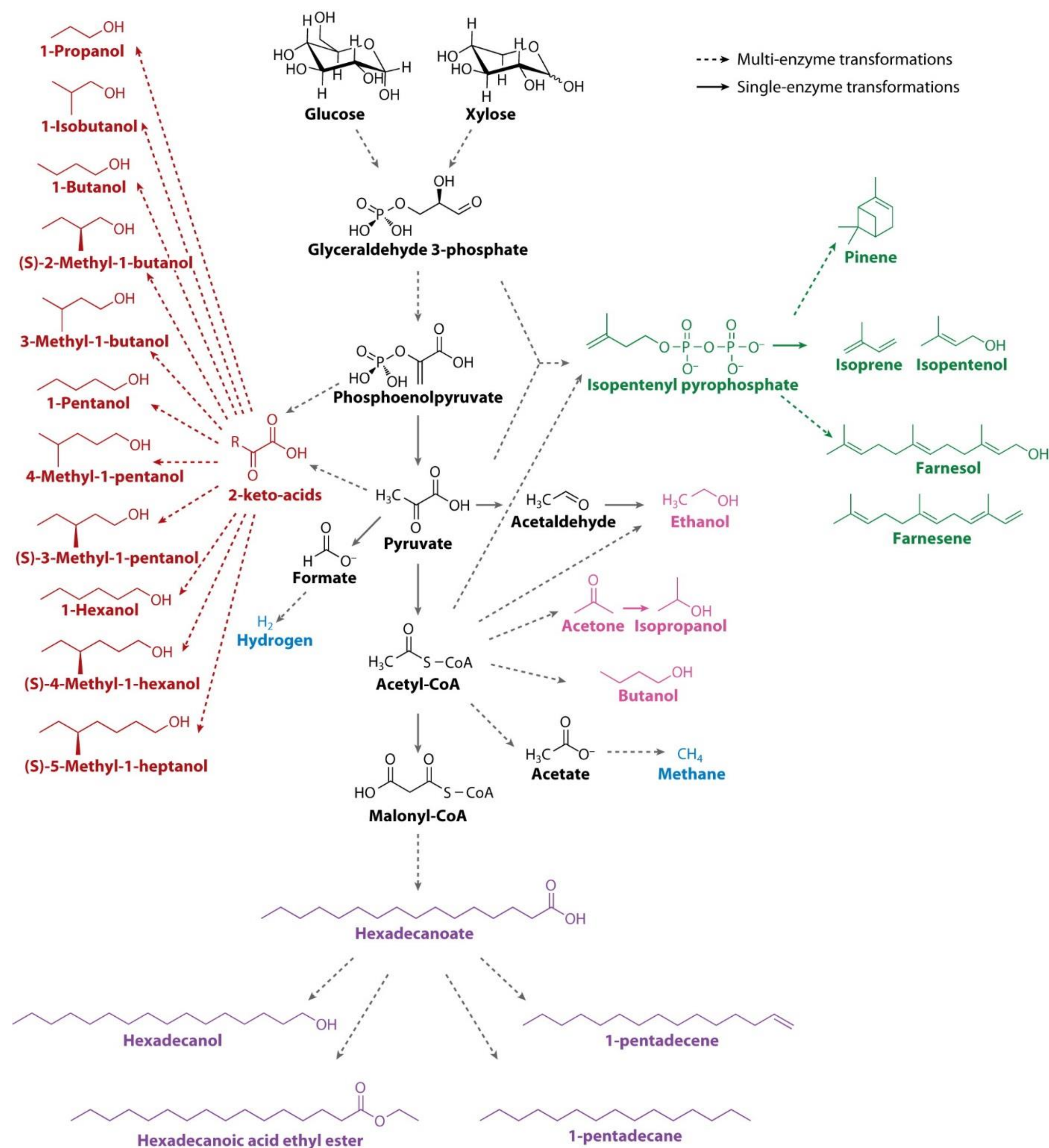

Figure 2. Illustration of different classes of fuel-like biomolecules in *E. coli*. Solid arrows indicate reactions catalyzed by a single enzyme. Dashed arrows represent pathways or multi-enzyme reactions [33].

The rapid development of this "*E. coli* toolbox", regarding gene regulation, protein engineering, pathway manipulation, synthetic biology and metabolic engineering, provides new biotechnological capacities, to address a sustainable, microbial production of high-value oleochemical [3,14,33,37-39].

1.3 Metabolic Engineering

A metabolic pathway is a series of chemical and enzymatic reactions, which occur within a cell. Metabolic pathways exist for the conversion of raw materials into substances essential for the cell's survival [40]. Metabolic engineering is principally altering and enhancing metabolic pathways and regulatory processes within a cell, to achieve a specific phenotype [40,41]. It is a technique broadly used for the optimization of one or many metabolic pathways within a cell, in order to comprehend and utilize such pathways for scientific and industrial applications [41-43]. In industrial biotechnology, it is applied to achieve an enhanced cellular production of a certain substance on an industrial scale and in a cost effective manner [43]. Metabolically engineered organisms are successfully utilized for the production of high value chemicals for various commercial applications, such as specialty chemicals, pharmaceutical and medical compounds and biofuels [42,43].

Various genetic engineering tools are employed to modify a metabolic pathway within a cell. One of the conventional strategies in metabolic engineering is to determine the rate limiting reaction in the targeted metabolic process [40,41,43-45]. Based on these data, genetic techniques, such as the homologous expression of the host gene(s), responsible for the protein catalyzing the rate limiting step, are used to increase product titers [40,41,43]. In a similar manner, deleting or downregulating the expression level of gene(s), involved in competing metabolic pathways, that further utilize or modify the desired product in the cell, contribute towards increasing titers. Additionally, it is possible, that regulatory mechanisms, such as product inhibition can be alleviated from one enzyme or the specificity towards a specific substrate can be altered [40,43,45]. Another strategy in metabolic engineering involves the heterologous expression of a gene or gene clusters, isolated from a different organism, for the purpose of enhancing the production of an already available compound, or producing a new compound [42].

Most organisms have evolved strategies to combat genetic mutations and alterations through feedback loops and regulatory mechanisms that recruit and activate various metabolic pathways, to ensure survival of the host [40-42,44]. Therefore, genetic manipulations can lead to unwanted and unnecessary metabolic burdens on the host, which would hinder growth or cause cell death [40-43]. A clear understanding of the genetics of the host organism and its metabolic network is required, to expand the host cell's capability to generate the desired product and to minimize undesired metabolic stress on the host [40-42].

In that regard, *E. coli* has established itself as a model organism to study and tailor microbial fatty acid biosynthesis, using a concerted metabolic engineering approach [3,8,14,38,39,46]. While *E. coli* is not an oleaginous organism per se the option for extensive genetic alternation allowed the generation of diverse oleo-chemical products [3,8,14,33,38,39,46].

1.4 Fatty acids

Fatty acids are carboxylic acids with a straight or branched aliphatic carbon chain. Fatty acids are often classified into two groups; unsaturated fatty acids if one or more carbon to carbon double bonds are present within the carbon chain and saturated fatty acids when the carbon chain is devoid of carbon to carbon double bonds [47,48]. Unsaturated fatty acids are further characterized according to their geometrical isomerism; whether in *cis* or *trans* configuration[48]. Moreover, fatty acids are categorized according to the carbon chain length; short-chain fatty acids have aliphatic chains of five carbons or less, medium-chain fatty acids have aliphatic chains of six to twelve carbon atoms, long-chain fatty acids have aliphatic chains of thirteen to eighteen carbon atoms [49,50] and very long-chain fatty acids have aliphatic chains of twenty carbon atoms and more [46,51-53] (Table 2). The differences between the diverse types of fatty acids are crucial for the generation of a diverse group of oleo-chemicals with various applications [6,8,14,37,39].

Table 2. Overview of fatty acid classifications.

Fatty acids		
Saturated	Unsaturated (*cis*/*trans*)	
	Mono-unsaturated	Poly-unsaturated
short-chain fatty acids	max 5 carbon atoms	
medium-chain fatty acids	6 to 12 carbon atoms	
long-chain fatty acids	13 to 18 carbon atoms	
very long-chain fatty acids	more than 18 carbon atoms	

In organisms, synthesized fatty acids are further processed for the biosynthesis of energy-rich storage molecules, such as wax esters, diacylglycerols and triacylglycerols [8,33,37,50,54-56]. Furthermore, fatty acids are essential for a cell's membrane integrity and homeostasis. In eukaryotes, fatty acids are vital precursors for the biosynthesis of phospholipids, sphingolipids and sterols [50,51,53,56-62].

1.5 Fatty acid synthase

Fatty acids are synthesized *in vivo* through the catalytic action of the Fatty Acid Synthase (FAS). In nature, two types of fatty acid synthases exist, type I and type II [63]. The Fatty Acid synthase type I system (FAS I) is predominantly found in animals and fungi, but also in certain groups of bacteria, including corynebacteria and mycobacteria [63-65]. The FAS I system is composed of one large multifunctional protein complex, that contains all the catalytic centers and catalyzes all the steps of fatty acid biosynthesis [64]. In animals FAS I is encoded by a single gene and the resulting polypeptide undergoes homo-dimerization to become catalytically active [62]. While in yeast, FAS I is encoded by two genes and their polypeptide products are required to complex into a heterododecamer to be catalytically active [64]. On the other hand, bacteria, archaea and plants harbor the Fatty Acid synthase type II system (FAS II), which is composed of a group of mono-functional enzymes, with each enzyme catalyzing a single step of fatty acid biosynthesis [66]. Consequently, the enzymes comprising the FAS II system in bacteria and plants are encoded by several genes. The main difference between the FAS I and FAS II systems is, that the set of enzymes that form the FAS II system are neither encoded by a single gene nor organized into an operon [63-66]. Even though, two different systems have evolved for the *de novo* biosynthesis of fatty acids, both systems perform the same catalytic reactions and the pathway itself remains highly conserved [8,66]. The advantage of the dissociated FAS II multi-enzyme nature, allows the prospect to fine tune selectively and customize the process towards desired product profiles [3,14].

1.6 De novo fatty acid biosynthesis (FAS II)

Fatty acids synthesized via the FAS II system are built in three main phases: initiation, elongation and termination. Each phase gives rise to the opportunity to manipulate, modulate or engineer the enzymes in order to selectively produce various products (Figure 3) [3,8,14,38,66].

Initiation of *de novo* fatty acid biosynthesis starts with diverting acetyl-CoA towards the production of malonyl-CoA, which is a key intermediate in fatty acid biosynthesis. In *E. coli*, malonyl-CoA is only consumed by the FAS complex for the biosynthesis of fatty acids [66]. The acetyl coenzyme A carboxylase (ACC) is the first set of enzymes, which initiate fatty acid biosynthesis through the carboxylation of acetyl-CoA to malonyl-CoA, with hydrogencarbonate at the expense of one ATP molecule [67-70]. The pathway proceeds by the transfer of the malonyl group of malonyl-CoA to an acyl carrier protein (ACP) moiety through the action of the malonyl-CoA:ACP transacylase (FabD), producing malonyl-ACP. The transfer of the malonyl group by FabD is a reversible reaction, where the production of the CoA ester is favored over the production of malonyl-ACP [71].

The complete inhibition or deletion of *fabD* in *E. coli* inhibits the production of malonyl-ACP, which in turn would inhibit fatty acid biosynthesis and leads to cell death [72]. 3-ketoacyl-synthase III (FabH) catalyzes the final step in the initiation phase of fatty acid biosynthesis producing acetoacetyl-ACP by a condensation reaction of acetyl-CoA and Malonyl-ACP [73]. The acetoacetyl-ACP produced then enters the next phase of fatty acid biosynthesis, the elongation phase.

The elongation cycle consists of four enzyme activities, that catalyze the sequential addition of a two-carbon moiety from a malonyl-ACP to a growing acyl-ACP chain (after each round), in the presence of NADH and NADPH. The 3-Ketoacyl-ACP reductase, FabG, is the first enzyme in the cycle that accepts the generated acetoacetyl-ACP and is subsequently reduced to a β-hydroxyacyl-ACP in the presence of NADPH [74]. The intermediate is rapidly dehydrated by the catalytic action of the 3-hydroxyacyl-ACP dehydratases (FabA or FabZ) generating an enoyl-ACP [75]. The cycle is completed when the enoyl-ACP is reduced to a 3-ketoacyl-ACP, by the enoyl-ACP reductase, FabI, in the presence of NADH [76]. The first round of elongation is initiated through the formation of acetoacetyl-ACP by FabH, which eventually generated a butyryl-ACP. Further rounds of elongation are initiated by the 3-Ketoacyl-ACP synthases 1 and 2 (FabB and FabF) [66]. Elongation is inhibited by the length of the growing acyl-ACP chain, where FabB cannot accept acyl-ACP chains of 16 carbon atoms or longer, while FabF only catalyzes the elongation from a 16 carbon acyl-ACP to an 18 carbon acyl-ACP. Consequently, the *E. coli* FAS II system is not capable of generating fatty acids greater than 18 carbon atoms [8,38,46,66].

Fatty acid biosynthesis is terminated when the fatty acyl chain is cleaved from the ACP molecule. Termination, in *E. coli,* is usually performed by an ACP-thioesterase that cleaves the ester bond, releasing a free fatty acid and an ACP molecule that is reused in fatty acid synthesis [3,8,66].

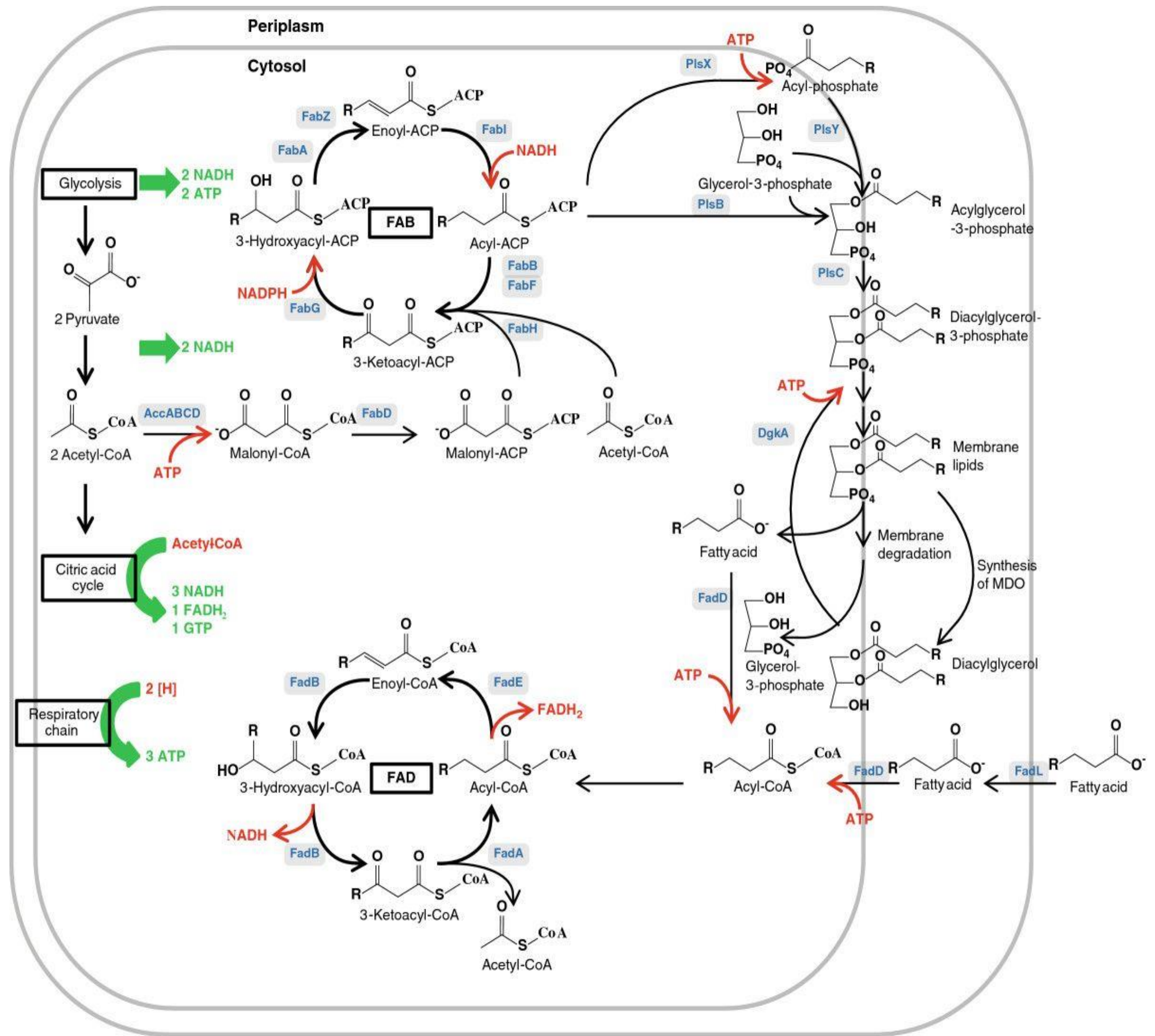

Figure 3. Schematic of the pathways involved in fatty acid biosynthesis and fatty acid degradation [8].

2. Methods

The following section represents a summary and overview of the most important materials, methods and procedures used in this thesis. The genetic engineering strategies employed to generate the respective mutant strains are described, in addition to details of the basic process steps and data analysis.. Further detailed information is represented within the individual materials and methods section and supplementary data sections of the respective published manuscripts.

Chemicals and reagents

All chemicals and reagents used in the described studies were purchased from standard sources that adhere to the highest quality standards. Components, that comprise the cultivation media, were purchased from Applichem GmbH, Roth chemicals and Merck. Toluene, hexane, ethanol and methanol were obtained from Roth chemicals. Methano/HCl solution was purchased from. All enzymes used for polymerase chain reactions, DNA restriction, digestion and ligation were purchased from Themo Fischer Scientific. DNA preparation kits used for mini and midi-preps were purchased from Thermo Fischer Scientific.

Media composition

Luria-Bertani Broth (pH 7)

Peptone	10 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	5 g L^{-1}
ddH_2O	up to 1 L

Minimal M9 media stock solutions

10X M9 salts solution (pH=6.9)			
	Na2HPO4	60 g L^{-1}	33.7 mM
	KH2PO4	30 g L^{-1}	22.0 mM
	NaCl	5 g L^{-1}	8.55 mM
	NH4Cl	10 g L^{-1}	9.35 mM
1 M $MgSO_4$			
1 M $CaCl_2$			

0.1M Fe(III)-Citrate
1 M Thiamine HCl
40% (w/v) Glucose (400 g L^{-1})

100 X trace element solution

EDTA	5.0 g L^{-1}	13.4 mM
ZnCl2	84 mg L^{-1}	0.62 mM
CuCl2-2H2O	13 mg L^{-1}	76 µM
CoCl2-2H2O	10 mg L^{-1}	42 µM
H3BO3	10 mg L^{-1}	162 µM
MnCl2-4H2O	1.6 mg L^{-1}	8.1 µM

Minimal M9 Broth

10X M9 salts solution	100 ml L^{-1}
1 M $MgSO_4$	4.88 ml L^{-1}
1 M $CaCl_2$	0.1 ml L^{-1}
0.1M Fe(III)-Citrate	2.45 ml L^{-1}
1 M Thiamine HCl	0.0134 ml L^{-1}
100x Trace element solution	1 ml L^{-1}
40% (w/v) Glucose (400 g L^{-1})	10 ml L^{-1}
1000x Antibiotics	1 ml L^{-1}
ddH_2O	up to 1 L

Complete modified M9 medium for fermentation

Batch Medium	940 ml L^{-1}
NaH_2PO_4 4 g L^{-1}	
K_2HPO_4 13.3 g L^{-1}	
NH_4Cl 8 g L^{-1}	
1 M $MgSO_4$	4.88 ml L^{-1}
1 M $CaCl_2$	0.1 ml L^{-1}
0.1M Fe(III)-Citrate	2.45 ml L^{-1}
1 M Thiamine HCl	0.0134 ml L^{-1}

100x Trace element solution	1 ml L^{-1}
40% (w/v) Glucose (400 g L^{-1})	50 ml L^{-1}
1000x Antibiotics	1 ml L^{-1}

Feeding solution

Glucose	500 g L^{-1}
MgSO4·7H2O	20 g L^{-1}
100× trace elements solution	16 ml L^{-1}
0.1M Fe(III)-Citrate	5 ml L^{-1}

Cloning, plasmid construction and culture conditions

All bacterial strains used in this thesis were obtained from Merck Millipore. *E. coli DH5 alpha* strain was used for cloning and plasmid amplification. Cultures were grown at 37°C in Luria-Bertani broth with the appropriate antibiotics (Kanamycin 50 µg/mL and/or Chloramphenicol 34 µg/mL). All PCR primers, genes and gene fragments were synthesized by Eurofins Scientific. All plasmid backbones were obtained from Novagen/Merck Millipore.

Standard protocols were followed for polymerase chain reaction (PCR), DNA restriction, digestion and ligation. The standard 1 % (v/m) agarose gel in TAE buffer was used for PCR product separation and purification. The appropriate gene bands were isolated and extracted using the innuPREP DOUBLEpure Kit (Analytik Jena) according to manufacturer's protocol. Standard protocols for heat shock transformation in chemical competent cells and electroshock transformation in electro competent cells were performed when necessary. Plasmid isolation and purification was conducted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's protocol. Plasmid constructs were validated by sequencing (Eurofins Scientific).

Gene and protein sequences

The gene sequences used in this thesis were codon-optimized for expression in *E. coli* and gene fragments were chemically synthesized by Eurofins Scientific. Cloned genes were confirmed by sequencing (Eurofins Scientific). The following gene sequences were used in this thesis:

Long chain fatty acid synthesis genes
3-ketoacyl-[acyl carrier protein] synthase I (*KASI*) from *Arabidopsis thaliana.* (Gene ID: 834671)
3-ketoacyl-[acyl carrier protein] synthase II (*KASII*) from *Arabidopsis thaliana.* (Gene ID: 843835)
Very long chain fatty acid synthesis genes
3-ketoacyl-CoA synthase 18 (*KCS18*) from *Arabidopsis thaliana.* (Gene ID: 829603)
3-ketoacyl-CoA synthase 1 (*KCS1*) from *Arabidopsis thaliana.* (Gene ID: 839395)
3-ketoacyl-CoA synthase 6 (*KCS6*) from *Arabidopsis thaliana.* (Gene ID: 843182)
3-oxoacyl-CoA reductase 1 (*KCR1*) from *Arabidopsis thaliana.* (Gene ID: 843098)
3-hydroxacyl-CoA dehydratase 2 (*PAS2*) from *Arabidopsis thaliana.* (Gene ID: 830912)
Trans-2,3-enoyl-CoA reductase 10 (*CER10*) from *Arabidopsis thaliana.* (Gene ID: 824702)
Self-assembly GFP protein sequences
Self-assembly GFP 1-10 protein sequence obtained from [77,78].
Self-assembly GFP 11 protein sequence obtained from [77,78].

VLCFA and sa-GFP expression cassette design

The four genes involved in very long chain fatty acid synthesis complex and interact together, therefore an expression cassette was designed for optimal expression in *E. coli.* In order to control translation initiation and protein expression rates of each gene in the expression cassette, the ribosomal binding site of each gene was predicted using the RBS Calculator software [79,80]. A minimum target translation initiation rate was set for the in-silico prediction of the synthetic ribosomal binding site of each gene. The predicted RBS sequences should, with high fidelity, allow a minimum expression rate of each protein. The genes with the respective RBS sequences were then assembled in-silico into an expression cassette that was further evaluated for protein expression using the Operon Calculator [81,82].

Plasmid construction

Long chain fatty acid genes

The chloropalstic transit peptide of both *KASI* and *KASII* was predicted using *SignalP 4.1 Server* and removed from the DNA sequence. 5' *BamH1* and a 3' *Not1* restriction sites were used to clone the genes into an empty pET28a vector.

Very long chain fatty acid genes

The 3-ketoacyl-CoA synthase KCS1, KCS6 and KCS18 genes were first obtained by PCR from a cDNA library of *Arabidopsis thaliana.* The PCR products were then inserted into an empty pET28a vector via enzymatic restriction digestion and ligation. Subsequently, the codon optimized sequences of *KCS18*, *KCR1, PAS2, CER10, GFP 1-10* and *GFP 11* were chemically synthesized. The synthesized *GFP 1-10* sequence was inserted into an empty pET28a vector via enzymatic restriction digestion and ligation.

Very long chain fatty acid and sa-GFP expression cassette

Notably, due to the expression cassette design, that includes repetitive sequences, where each gene has an *11-GFP* sequence attached to its N-terminus, the expression cassette could not be chemically synthesized. Therefore, different strategies were employed, in order to construct the vector with the expression cassette. The very long chain fatty acid expression cassette was divided into five separate fragments (F1, F2, F3, F4 and F5) and chemically synthesized. Seamless or scar-free cloning strategies, such as overlap-PCR and Gibson assembly, were followed according to standard procedures and manufacturer's protocol. However, the presence of the repetitive sequences of the *11-GFP* inhibited the complete assembly of the cassette and yielded incomplete or false constructs. Only fragments F1 and F2 were successfully joined, via overlap PCR. *SapI* (*LguI*) restriction enzyme, with the recognition sequence 5'GCTCTTC(N1) and its complement 3'GAAGAGC(N4) (the enzyme cuts at the first nucleotide "N1" after the recognition sequence in the 5' orientation and at the fourth nucleotide "N4" after the recognition sequence in the 3' orientation), similarly allows for seamless or scar-free cloning, where the recognition sequence (GCTCTTC) is cleaved off during restriction digestion. The following strategy was employed for the very long chain fatty acid vector construction: the first two fragments (F1 and F2) were joined, via overlap PCR, and inserted, by enzymatic restriction digestion and ligation, into an empty pACYC vector. The generated pACYC vector was then fully amplified using a forward primer containing a *5'-SapI* restriction site and a reverse primer containing a *3'-SpeI* restriction site. Additionally, a *5'-SapI* and a *3'-SpeI* restriction site sequence

were inserted via PCR at the 5' and 3' ends of fragments F3, F4 and F5 respectively. Subsequently, the restriction digested PCR product of the pACYC plasmid, containing the first two fragments (F1 and F2), was ligated with the restriction digested product of fragment F3, hence yielding the pACYC vector, with fragment F3 inserted sequentially after fragments F1 and F2. The same procedure was repeated until each remaining fragment (F4 and F5) was sequentially inserted into the pACYC vector, containing the previous fragments. The final construct, comprising the very long chain fatty acid cassette, was verified by sequencing. The use of *SapI* and *SpeI* restriction enzymes in that order allowed for the cloning of the 5 synthesized fragments in a predetermined order into the pACYC vector, with no sequence restrictions or scars within the expression cassette (Figure 4.).

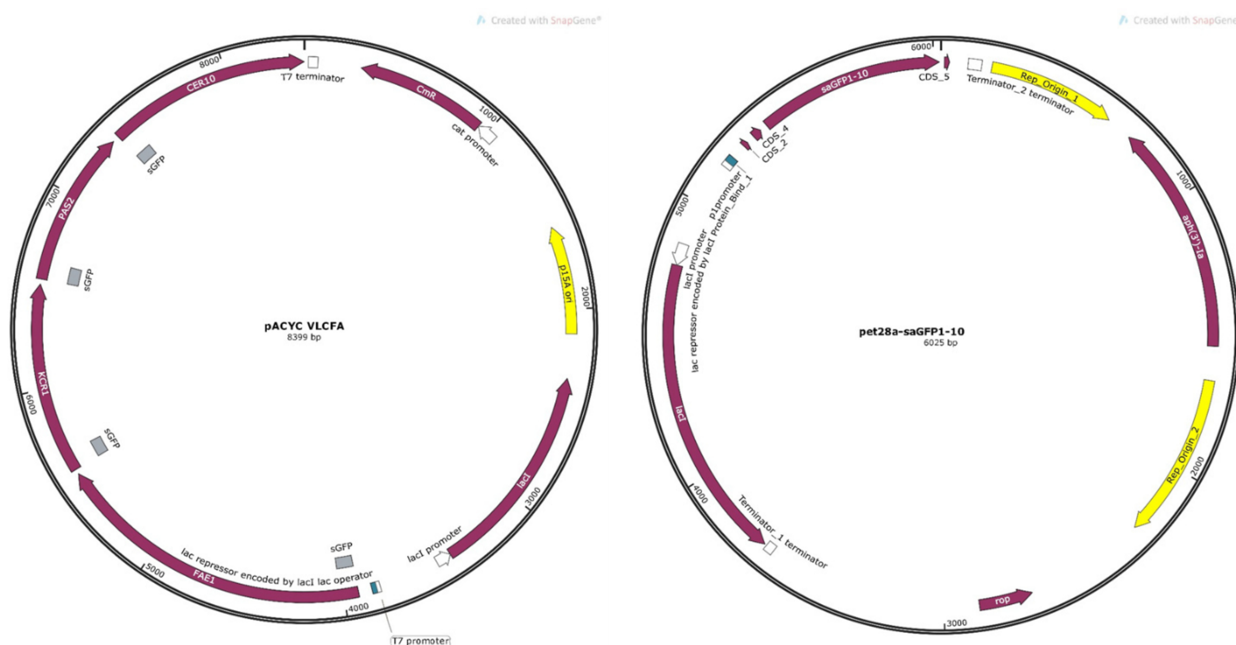

Figure 4. Plasmid map of the pACYC plasmid harboring the VLCFA polycistronic cassette and the pET28a.

Genomic engineering

The generation of gene knock outs in *E. coli BL21 (DE3)* was performed using the pRED/ET kit, following the protocols set by the manufacturer (Gene Bridges GmbH). In summary, a functional cassette, carrying the selectable marker of choice, was used as a "landing pad", to specifically integrate at, or knock out the targeted locus, via homologous recombination. To target the genome at the site of choice, it is necessary to incorporate short homology regions into the functional cassette carrying the selectable marker. The marker cassette was amplified beforehand, using two oligonucleotide primers (forward and reverse), each flanked by 50 base pairs corresponding to the homology regions directly adjacent or within the targeted locus. The pRED/ET plasmid was

transformed into electro-competent *E. coli BL21 (DE3)* cells. The clone harboring the pRED/ET plasmid was cultured overnight with shaking at 30°C in YLG media. The culture was subsequently induced with 0.2% L-arabinose at an OD_{600} of 0.2, to allow the expression of the Red/ET recombinant proteins. Directly after induction, the cultures were incubated at 37°C until OD_{600} reached 0.6. The induced cells were then collected and prepared for electroporation, following standard protocols for the generation of electro-competent *E. coli* cells. Consequently, the landing pad or marker cassette, with the flanked homology regions, was transformed into the electro-competent *E. coli BL21 (DE3) pRED/ET* cells, via electroporation. The transformed cells were then plated out on selective media and incubated overnight at 37°C. Single colonies were picked and the genomic knock out was further validated through a PCR of the targeted locus and sequencing.

Knocked out genes and homology sequences

The following gene sequences were used as references for the generation of the homology regions, which were incorporated at both ends of the marker cassette.

Gene sequences
fabB: beta-ketoacyl-[acyl carrier protein] synthase I (*Escherichia coli str. K-12 substr. MG1655*) Gene ID: 946799
fabF: beta-ketoacyl-[acyl carrier protein] synthase II *(Escherichia coli str. K-12 substr. MG1655)* Gene ID: 946665
fadD: fatty acyl-CoA synthetase *(Escherichia coli str. K-12 substr. MG1655)* Gene ID: 946327

Fatty acid production

Shake flask

Shake flask studies were conducted in samples of 50 ml, supplemented with the proper antibiotic (100 µg/mL ampicillin; 50 µg/mL kanamycin; 34 µg/mL chloramphenicol), in 250 ml baffled shake flasks for the functional screening and production of fatty acids. Baffled shake flasks were used to improve oxygen and gas transfer to the cultures. Screening was conducted in both, LB and minimal M9 media. However, Minimal M9 media was preferred for the production of fatty acids,

due to its defined basic constituents and lack of lipids or fatty acids that can be up taken by the cells. Samples, cultured in Minimal M9 media, were limited by the nitrogen source to induce *de novo* fatty acid biosynthesis. Only single colonies of *E. coli*, harboring the appropriate plasmid, were picked for overnight cultures. Shake flask samples were inoculated with 1ml of the overnight culture. Cultivations were carried out at 37°C in a shaking incubator at 170 rpm for 48 hours. At an OD_{600} of 0.6, 0.05 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the cultures to induce protein expression. For Shake flask studies, conducted for the functional screening of very long chain fatty acids, 0.5% oleic acid (5 g L-1) was further supplemented to the Minimal M9 media. 24 hours after the IPTG induction, 5 µM cerulenin (Cayman chemicals, USA) was added to the cultures. Samples for fatty acid analysis were collected before induction, 24 hours and 48 hours after induction with IPTG.

Bioreactor

Technical scale fermentations for the clones, generated in this thesis, were performed in a DASGIP® 1.3 L parallel reactor system (Eppendorf AG). A modified M9 media [8 g L^{-1} NH_4Cl, 13.3 g L^{-1} KH_2PO_4, 1.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.42 g L^{-1} $FeCl_3$- $6 \cdot H_2O$, 20 g L^{-1} glucose] was used as batch media, supplemented with 1 mL 100× trace elements solution and the proper antibiotics. Fermenters were inoculated from an overnight pre-culture (with a starting inoculation OD_{600} of 0.1). The cultivation temperature was kept constant at 37 °C for the technical scale production of long chain fatty acids and at 30 °C for the technical scale production of very long chain fatty acids. Initial stirring velocity and airflow was set to 200 rpm and 0.2 volumes of air per volume of medium per min (vvm), respectively. Dissolved oxygen was kept at 30% by successive increases of the stirrer velocity, the oxygen proportion, and eventually the airflow. A pH value of 7.00 was controlled by the addition of 6 M aqueous NaOH. A pH value shift above 7.05 initiated a feed shot of 40 mL. The feed solution consisted of 500 g L^{-1} glucose, 5 g L^{-1} oleic aicd, 20 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 mg L^{-1} thiamine–HCl, 16 mL 100× trace elements solution (pH = 7.00). Samples were taken at different time points to determine the OD_{600}. Once the clones reached limitation, due to the depletion of the nitrogen source, they were induced with 0.05 mM IPTG. For the technical scale production of very long chain fatty acids, 0.5% oleic acid (5 g L^{-1}) was supplemented to the feed solution and 5 µM cerulenin (Cayman chemicals, USA) was added to the fermenters 24 hours after induction with IPTG.

Fatty acid extraction and methylation

Before analysis could be conducted, lipids were extracted from dry cell biomass and converted to fatty acid methyl esters (FAMEs) by methanol transesterification [83]. Samples, collected from shake flask and technical scale fermentation studies, were centrifuged and subsequently washed twice with ddH_2O, to remove any excess salt from the biomass. The collected pellets were then lyophilized to remove water. Equal amounts of dry biomass were weighed in glass tubes with a silicon/PTFE-sealed caps, to inhibit solvent evaporation. 450 µl toluene was added to the collected samples for total lipid extraction. Then, 50 µl of a 2 mg/ml glyceryl trinonadecanoate (C19:0 triglyceride) in toluene solution was added as an internal standard to determine esterification efficiency. 100 µl of 2,2-dimethoxypropane, a water scavenger, was then added to remove any remaining excess water that can interfere with the transesterification reaction. Subsequently, 1 ml of 0.5 M sodium-methoxide in methanol, which is an alkaline catalyst required for the transesterification of complex lipids, was added. The samples were then directly incubated in a shaking heat block at 80°C with vigorous shaking at 1000 rpm for 20 min. Samples were cooled on ice for 5 min and 1 ml of 5% v/v HCl (37%) in methanol was added before repeating the same incubation procedure. The 5% v/v HCl (37%) in methanol solution is an acid catalyst used for the transesterification of complex lipids as well as free fatty acids. After samples were cooled on ice for 5 min following the second incubation, 400 µl of ddH_2O was added followed by 360 µl Hexane and 40 µl of a 2 mg/ml methyl decanoate (C10:0 fatty acid methyl ester) in hexane solution. Methyl decanoate was used to determine fatty acid methyl ester extraction efficiency. Samples were then mixed by vortexing and then centrifuged for 1 min at 4000 rpm. 200 µl of the upper Hexane-Toluene layer was taken for analysis.

Fatty acid analysis

Shimadzu GC-2010 Plus gas chromatograph with flame ionization detector (FID) was used for fatty acid analysis. 1 µL sample was injected via an AOC-20i auto injector (Shimadzu) on to a Phenomenex ZB-WAX column (length 30 m, 0.32 mm ID, 0.25 µm df). The column was heated up with 5°C min-1 to 240°C maintained for 5 min. Hydrogen was used as carrier gas with a flow rate of 3 mL min-1 and constant flow compensation. Additionally, 1 µl of each sample was injected into a Thermo Scientific™ TRACE™ Ultra Gas Chromatograph instrument (GC-FID) for separation and quantification of fatty acid methyl esters. GC–MS was performed with the Thermo Scientific™ TRACE™ Ultra Gas Chromatograph instrument coupled to a Thermo DSQ™ II mass spectrometer and the Triplus™ Autosampler injector. MS was performed in positive ion mode.

The analysis was carried out using a Stabilwax® fused silica capillary column (30 m x 0,25mm, with a film thickness of 0.25 µm). The run was under an optimized temperature as follows: initial column temperature 50°C, programmed to increase at a rate of 4 °C/min up to a final temperature of 250°C. Hydrogen was used as the carrier gas at a flow rate of 35mL/min with constant flow compensation.

Marine Oil FAME Mix (RESTEK USA), containing 20 individual fatty acid methyl ester components, was used as standard for GC-FID and GC-MS analysis. The esterification efficiency of each sample analyzed was determined according to the peak area and concentration of the internal standard Glyceryl trinonadecanoate (C19:0 TAG) (Sigma, Germany). Fatty acid methyl ester extraction efficiency was calculated relative to the Methyl decanoate (C10:0 FAME) (Sigma, Germany) standard used. Individual FAME concentrations were based on peak areas relative to a standard concentration curve derived from the individual components of the Marine oil Mix standard used [83].

3. Publication summaries

3.1 Summaries of Included Publications

Engineering *Escherichia coli* FAB system using synthetic plant genes for the production of long chain fatty acids

The article "Engineering Escherichia coli FAB system using synthetic plant genes for the production of long chain fatty acids" has been published in Microbial Cell Factories in October 2019 https://microbialcellfactories.biomedcentral.com/articles/10.1186/s12934-019-1217-7. The author of this thesis, Elias Kassab, developed the concept of the publication, designed the methodological approach and performed the experiments, evaluated and validated the experimental data and prepared the original manuscript.

The aim of the publication entitled "Engineering *Escherichia coli* FAB system using synthetic plant genes for the production of long chain fatty acids" was to assess the physiological impact of the *Arabidopsis thaliana* derived beta-keto-acyl synthases KASI and KASII on the fatty acid biosynthesis system and the fatty acid profile of *Escherichia coli* (*E. coli*). Driven by demands of the food, chemical- and pharmaceutical industry production of plant and animal based lipids increased by approximately 65% in the last decade. Although the food industry accounts for the majority of market consumption, the spike in demand is mainly attributed to the expanding applications of natural oils in biofuels, oleo-chemicals and bioactive substances. To that end, microbial oils have been designated as a potentially sustainable alternative to plant and animal based lipids.

In this study, we demonstrated that the supplementation of the native *E. coli* fatty acid biosynthesis pathway with the plant derived keto-acyl-synthases (KAS I and II) resulted in an increase in the fatty acid concentration in addition to a shift in the fatty acid profile towards the production of plant like C16-C18 type fatty acids. More specifically, KASI resulted in an overall increase in fatty acid concentration, with a concurrent increase in unsaturated C16-C18 fatty acids. By contrast, the expression of KAS II did not alter the overall fatty acid concentration, but selectively shifted the native fatty acid profile towards the generation of vaccenic acid (trans-C18:1Δ11). While KAS II in plants will generate oleic acid, we observed the production of vaccenic acid in *E. coli*, due to the different position of double bonds present in fatty acid synthase priming molecules. The overexpression and the deletion of the native *E. coli* beta-keto-acyl synthases fabB and fabF has been previously reported. As opposed to the KAS enzymes, the overexpression of fabB does not

result in a detectable, physiological change and the overexpression of fabF leads to cell death. On the other hand, the deletion of the *fabB* gene was lethal to the cell and the deletion of the *fabF* gene inhibited the production of stearic (C18:0) and vaccenic acid. The overexpression of the KASII enzyme in the *fabF* knock out strain restored the production of stearic and vaccenic acid, and further lead to an increase in palmitoleic and vaccenic acid amounts, relative to the wild type *E. coli*.

In summary, this study demonstrates that the *Arabidopsis thaliana* chloroplastic KAS enzymes can supplement the native *E. coli* fatty acid biosynthesis pathway, allowing for an increase in the fatty acid pool, while driving the fatty acid biosynthesis towards longer chain, plant like fatty acids. Hence, this system can be applied as a new platform for generating plant like fatty acids, without competing with agricultural activity or aggravating land use, which results in a negative environmental impact.

GFP Scaffold-Based Engineering for the Production of Unbranched Very Long Chain Fatty Acids in Escherichia coli with Oleic Acid and Cerulenin Supplementation

The article "GFP Scaffold-Based Engineering for the Production of Unbranched Very Long Chain Fatty Acids in Escherichia coli With Oleic Acid and Cerulenin Supplementation" has been published in Frontiers in Bioengineering and Biotechnology - Synthetic Biology in December 2019 https://www.frontiersin.org/articles/10.3389/fbioe.2019.00408/full. *The author of this thesis, Elias Kassab, developed the concept of the publication, designed and performed the experiments, evaluated the experimental data and wrote the manuscript.*

The aim of the publication was to demonstrate that *Escherichia coli* could serve as a sustainable production host for pharmaceutically and cosmetically relevant high value very long chain fatty acids, thereby presenting an alternative source of very long chain fatty acids with a reduced environmental impact, compared to conventional sources.

The very long chain fatty acid synthase complex, termed "elongase", is a membrane bound enzyme complex, located in the endoplasmic reticulum of plant cells. The complex is composed of four distinct enzymes that catalyze the elongation of long acyl-CoAs by the addition of a two carbon moiety, malonyl-CoA, through a four-step cycle. The rate limiting step, the first reaction of the elongation cycle, is catalyzed by a 3-ketoacyl-CoA synthase (KCS). At least 21 different gene homologues have been reported in the *A. thaliana* genome encoding a KCS. The homologues differ in their biological function, tissue expression patterns, substrate specificity and affinity to saturated and unsaturated long acyl-CoAs of varying chain length. Consequently, the KCS substrate specificity and expression level determines the final product's chain length, degree of desaturation, and yield. The three other steps of the elongation cycle are catalyzed by a 3-oxoacyl-CoA reductase (KCR), a 3-hydroxacyl-CoA dehydratase (HCD), and a trans-2,3-enoyl-CoA reductase (CER), respectively. In order to successfully solubilize and guide the plant cytosolic membrane bound complex in *Escherichia coli*, an innovative approach, using a self-assembly GFP system, was applied as a scaffold. A two plasmid system was used to implement that approach. The first plasmid was constructed to include a polycistronic expression cassette of the four elongase enzymes, where each enzyme sequence was tagged with the GFP11 sequence at the N-terminus. The RBS sequence, superseding each gene, was calculated to yield specific translation initiation and expression rates. The second plasmid included only the GFP1-10 sequence. Oleic acid and cerulenin were supplemented to cultures in order to initiate very long chain fatty acid biosynthesis. In that regard, we successfully report for the first time, the production of arachidic acid (C20:0) and cis-13-eicoseboic acid (C20:1), which are the two carbon elongated

versions of stearic acid (C18:0) and vaccenic acid (C18:1 Δ11) respectively. Both stearic acid and vaccenic acid are natively produced in *E. coli*. In addition, we can report the elongation of exogenous oleic acid (C18:1 Δ9) to cis-11-eicosenoic acid (C20:1) and erucic amide. The latter detection of erucic amide is very surprising and might indicate a direct cross- talk of the endogenous amino acid biosynthesis of *E.coli* with the heterologous VLCFA enzyme cluster. The production of total VLCFAs was calculated to be 6 mg per liter culture with an estimated productivity of 0.25 mg/l/h.

3.2 Full Length Publications

Engineering *Escherichia coli* FAB system using synthetic plant genes for the production of long chain fatty acids

Kassab *et al. Microb Cell Fact* (2019) 18:163
https://doi.org/10.1186/s12934-019-1217-7

Microbial Cell Factories

RESEARCH

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Engineering *Escherichia coli* FAB system using synthetic plant genes for the production of long chain fatty acids

Elias Kassab, Monika Fuchs, Martina Haack, Norbert Mehlmer and Thomas B. Brueck*

Abstract

Background: Sustainable production of microbial fatty acids derivatives has the potential to replace petroleum based equivalents in the chemical, cosmetic and pharmaceutical industry. Most fatty acid sources for production oleochemicals are currently plant derived. However, utilization of these crops are associated with land use change and food competition. Microbial oils could be an alternative source of fatty acids, which circumvents the issue with agricultural competition.

Results: In this study, we generated a chimeric microbial production system that features aspects of both prokaryotic and eukaryotic fatty acid biosynthetic pathways targeted towards the generation of long chain fatty acids. We redirected the type-II fatty acid biosynthetic pathway of *Escherichia coli* BL21 (DE3) strain by incorporating two homologues of the beta-ketoacyl-[acyl carrier protein] synthase I and II from the chloroplastic fatty acid biosynthetic pathway of *Arabidopsis thaliana*. The microbial clones harboring the heterologous pathway yielded 292 mg/g and 220 mg/g DCW for KAS I and KAS II harboring plasmids respectively. Surprisingly, beta-ketoacyl synthases KASI/II isolated from *A. thaliana* showed compatibility with the FAB pathway in *E. coli*.

Conclusion: The efficiency of the heterologous plant enzymes supersedes the overexpression of the native enzyme in the *E. coli* production system, which leads to cell death in *fabF* overexpression and *fabB* deletion mutants. The utilization of our plasmid based system would allow generation of plant like fatty acids in *E. coli* and their subsequent chemical or enzymatic conversion to high end oleochemical products.

Keywords: Type-II fatty acid synthase, Fatty acid biosynthesis, Long chain fatty acids, Heterologous expression, Chloroplast, β-Ketoacyl-[acyl carrier protein] synthase I and II, *Escherichia coli*, *Arabidopsis thaliana*

Background

To circumvent the ecological impact of plant oil production for generation of oleochemical building blocks, there is an increasing industrial demand for microbial generated fatty acids derived from bacteria, yeast or algae [1, 2] The chain length and degree of saturation of renewable fatty acids channels their use into either the biofuel, pharmaceutical or chemical industry. Wilde type *E. coli* produces a small range of saturated and unsaturated fatty acids with a chain length ranging from C12 to C18. Compared to other organisms it is neither a lipid accumulating organism nor a direct producer of high value fatty acid derivatives. However, due to its ease of genetic manipulation, *E. coli* has been successfully modified for the production for high value lipids and has been established as a model for generation of tailor made fatty acids [1, 3–7].

The sustainable production of microbial fatty acids and their derivatives as a renewable alternative to petroleum and other natural hydrocarbons has been the research focus over the past decades [1, 3, 5–10]. *Escherichia coli*'s fatty acid biosynthesis pathway has been thoroughly investigated [3, 11, 12]. A profound understanding of the *E. coli* fatty acid biosynthesis pathway and the enzymes

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involved is the basis for the remodeling its biosynthetic capacity to generate tailor made fatty acid profiles [1, 13–17].

Prokaryotes like *E. coli* and prokaryote derived plant plastids both harbor the type-II fatty acid synthase (FAS), which is responsible for de-novo fatty acids biosynthesis. In *E. coli*, the FAS enzyme complex is made up of the FAB cluster, comprising the enzymes *fabA*, *fabB*, *fabD*, *fabF*, *fabG*, *fabH*, *fabI* and *fabZ*. The complete FAB complex is responsible for fatty acid production from Acetyl-CoA and the subsequent elongation using Malonyl-ACP (Acyl carrier protein) (Fig. 1). The natural fatty acid distribution ranges from C12 to C18 saturated and unsaturated fatty acids, with low amounts of vaccinate (C18:1). The formation of 3-Ketoacyl-ACP is catalyzed by the 3-Ketoacyl-ACP synthases (FabH, FabB and FabF)

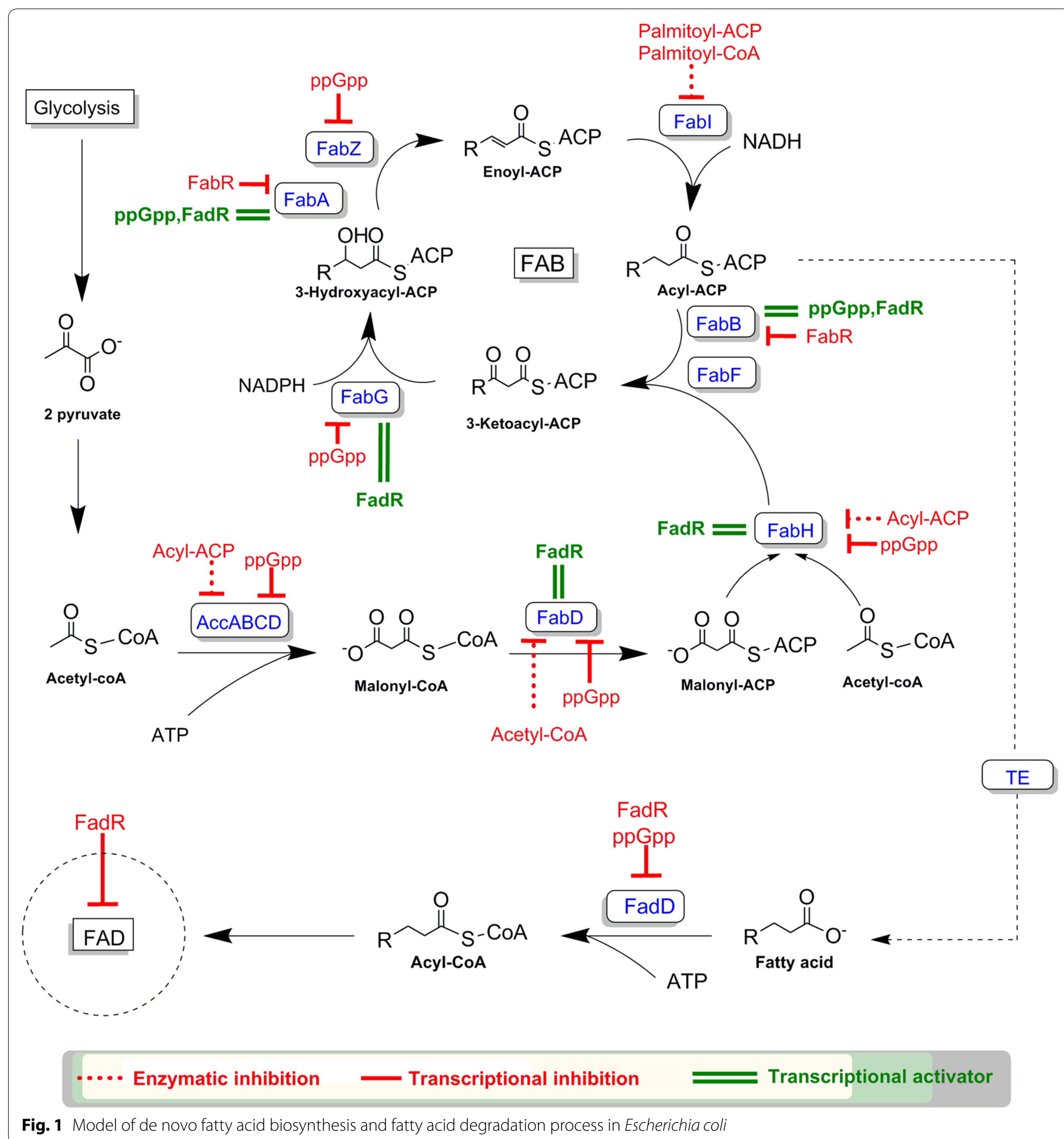

Fig. 1 Model of de novo fatty acid biosynthesis and fatty acid degradation process in *Escherichia coli*

through the condensation of an acyl-ACP with malonyl-ACP. The first condensation reaction of acetyl-CoA and Malonyl-ACP is performed by FabH, initiating the first cycle of elongation. The proceeding two carbon elongation steps are performed solely by FabB and FabF (Fig. 1) [1, 14, 18, 19]. Unlike plants, wild type *E. coli* does not possess a native desaturase [12], therefore the synthesis of unsaturated fatty acids is catalyzed anaerobically by FabA and FabB [1, 12]. FabA is specific towards the 10-carbon acyl chain intermediate (3-hydroxydecanoyl-ACP) and introduces a double bond at the 10th carbon yielding trans-2-decenoyl-ACP which is then further isomerized by FabA to cis-3-decenoyl-ACP. FabB subsequently catalyzes the elongation of cis-3-decenoyl-ACP, which is the rate limiting step in unsaturated fatty acid synthesis. The overexpression of either FabA or FabB alone does not improve unsaturated fatty acid concentrations [1, 12].

Both FabB and FabF show activity towards saturated fatty acyl-ACPs up to C14, however FabB has low activity towards C14:0, while FabF only shows low activity towards C16:0 [1, 12, 19]. Regarding the synthesis of unsaturated fatty acids, FabB performs the elongation steps up to C16:1, while FabF only catalyzes the last elongation step leading to cis-vaccenic acid (C18:1). Due to the temperature sensitive nature of the FabF protein, which is expressed and active at low temperatures [20], FabB is more active and abundant [1, 12, 19]. The overexpression of FabB alone does not improve fatty acid titers and its deletion causes auxotrophy for unsaturated fatty acids. The overexpression of FabF is lethal to *E. coli* probably due to the high level of the enzyme both binding and inhibiting the function of FabD thus blocking the FAB cycle and increasing malonyl-CoA levels. The deletion of *fabF* inhibits C18:1 and C18:0 fatty acid production [1, 12, 19, 21, 22].

The fatty acid biosynthesis process in *E. coli* has been extensively investigated and modified in order to increase the total fatty acid titers, shifting the fatty acid production towards a specific chain length or altering the degree of saturation of the cellular fatty acid pool. Such metabolically engineered *E. coli* strains have made use of different ACP-Thioesterases, for example, the leaderless version of the native *E. coli* TesA (the leaderless version possesses a broader substrate preference) or the *Arabidopsis thaliana* AtFatA [10, 23]. Most thioesterases have a wide range of activity towards ACP-fatty acid chain length with a preferred specificity towards a specific chain length. Thioesterases provide a metabolic sink by deregulating the negative feedback mechanism of ACP bound fatty acids rather than specifically altering the FAB pathway [24]. Expression levels of each thioesterase must be carefully optimized since a slight increase above the optimal concentration leads to the inhibition of FFA production [23, 25]. Other engineered strains have deleted genes of the FAD pathway in order to prevent fatty acid degradation. Specifically, the deletion of *fadD* (fatty-acyl-CoA ligase) or *fadE* the first enzyme in the fatty acid oxidation cycle inhibit fatty acid catabolism, thereby leading to intracellular fatty acid accumulation. However, the fragile balance of the FAB pathway and its strict regulation have left little or no option for manipulation.

*KAS*I and *KASII* (β-ketoacyl-[acyl carrier protein] synthase I and II) are the respective homologues of *fabB* and *fabF* in plant chloroplasts [26–28]. Both *A. thaliana* KAS enzymes share a mere 36% identity to their *E. coli* homologues, with the active site and catalytic triad being conserved in both species. Furthermore, KASII is not temperature sensitive as its counterpart, FabF, in *E. coli* [26].

Escherichia coli has an enormous in vitro potential for fatty acid synthesis, however several bottlenecks limit its in vivo production capacity [29, 30]. In this study, we address one of the bottlenecks and report the successful manipulation of the very heavily modulated FAB elongation system in *E. coli* driving the already high flux pathway towards the production of longer chain fatty acid. We successfully implemented the ability of chloroplastic *A. thaliana* enzymes to complement and synergistically function with the *E. coli* FAB system in the selective production of long chain fatty acids.

Results

Expression of KASI and KASII in wildtype BL21DE3

Expression of both plant enzymes in wild type *E. coli* BL21 (DE3) resulted in an increase in the intracellular fatty acid pool. Moreover, we observed a shift in the fatty acid distribution relative to the wild type BL21 (DE3) strain harboring an empty pet28a-plasmid.

In the KASII clones, we detected an increase in unsaturated fatty acids, particularly long chain fatty acids (i.e. C16:1 and C18:1) with regard to the wildtype BL21 (DE3) strain.

By comparison, KASI clones have shown improved fatty acids concentrations with specific increases in C14:0, C16:0, C16:1, C18:0 and C18:1 fatty acids. Saturated fatty acids constituted 52% (mol/mol) of the total fatty acid pool in our KASI clone versus 56% (mol/mol) in the control (Fig. 2). Amounts of C18:1 and its cyclic propane derivative C19:0c constitute 36% (mol/mol) of total fatty acids in our KASI clone versus 38% (mol/mol) in the control. However, we have noticed a decrease in cyclopropane conversion in our KASI strain, from 13% (mol/mol) in the control down to 3% (mol/mol) in our clone. Moreover, palmitoleic acid constitutes up to 12% (mol/mol) of the total fatty acid pool in our KASI clone versus 6% (mol/mol) in the control. Notably, unsaturated

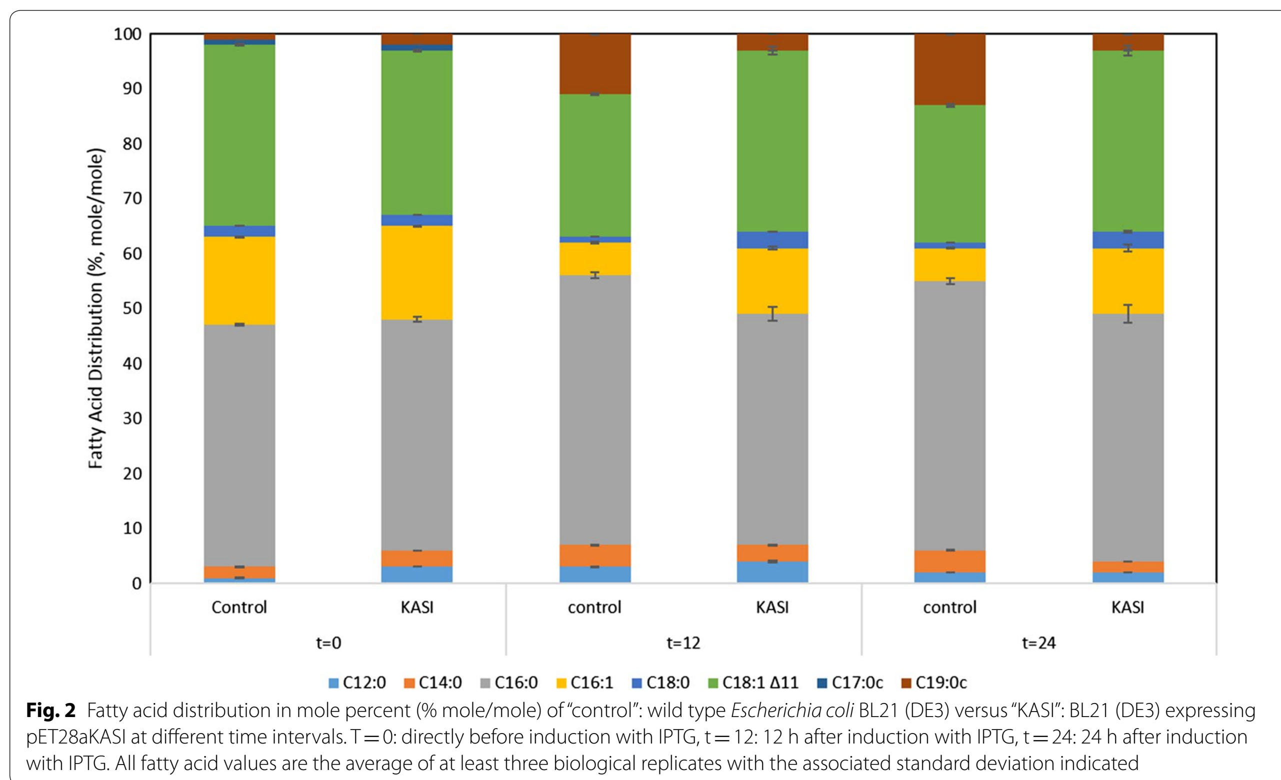

Fig. 2 Fatty acid distribution in mole percent (% mole/mole) of "control": wild type *Escherichia coli* BL21 (DE3) versus "KASI": BL21 (DE3) expressing pET28aKASI at different time intervals. T = 0: directly before induction with IPTG, t = 12: 12 h after induction with IPTG, t = 24: 24 h after induction with IPTG. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated

fatty acids constitute 44% (mol/mol) of total fatty acids in the wild type strain versus 48% (mol/mol) in the KASI clone.

KASI and KASII in *fadD* knockouts

In the ΔfadD strain a different fatty acid distribution was observed with a slight increase in fatty acid concentrations relative to the control strain, which is in accordance with previous literature [31, 32]. Our ΔfadD KASI clone showed a significant increase in overall fatty acid accumulation (see Additional file 1: Table S3). Interestingly, cis-vaccenic acid did comprise 4% (mol/mol) of the total fatty acids in the ΔfadD strain compared to 21% (mol/mol) in our ΔfadD KASI clone. The total amount of generated cis-vaccenic acid including the proportion converted to C19:0c, in the ΔfadD strain amount to 17% (mol/mol) of the total fatty acids produced versus 24% (mol/mol) in our ΔfadD KASI clone (Fig. 3). In our control strain, we observed an almost complete conversion of palmitoleic acid (1% mol/mol) to its cyclopropane fatty acid form (26% mol/mol). The ΔfadD KASI clone retains 18% (mol/mol) of the total fatty acids in the palmitoleic acid form versus 7% in the cyclopropane fatty acid form.

The ΔfadD KASII clone retains 16% of the total fatty acids in palmitoleic form versus 4% in cyclopropane form. Moreover, the fatty acid distribution in the ΔfadD KASII clone shows 30% of its total fatty acids are C18 versus 23% in the ΔfadD KASI clone and 18% in the ΔfadD strain. We observe an increase in the total amounts of unsaturated fatty acids from 44% in the ΔfadD strain, to 47% in the ΔfadD KASI strain to 49% in the ΔfadD KASII strain (Fig. 3).

KASII in *fabF* knockouts

The generated *Δ*fabF strain, due to the loss of the FabF protein responsible for the elongation of 16 carbon atom acyl ACPs, showed a significant decrease in C18 fatty acid species (see Additional file 1: Table S4), which is in accordance with previous literature [22]. The deletion also resulted in a shift in fatty acid distribution where palmitic and palmitoleic fatty acids accumulated making up more than 70% of the total fatty acids. After the cloning of the *Arabidopsis thaliana* beta ketoacyl acp synthase II (KASII) gene, a significant increase of C18 fatty acid levels were observed, 3.5-fold increase compared to the *Δ*fabF strain, in addition to a 2.6-fold increase in palmitoleic acid. The fatty acid distribution of our *Δ*fabF KASII clone highly varies from that of the *Δ*fabF strain as seen in Fig. 4. It is noteworthy to state that C19:0c fatty acids were nonexistent in both strains, and a significant reduction in C17:0c fatty acids, from 34% in the ΔfabF strain to 9% in the ΔfabF KASII clone, has been observed. No significant change in fatty acid concentration could be detected.

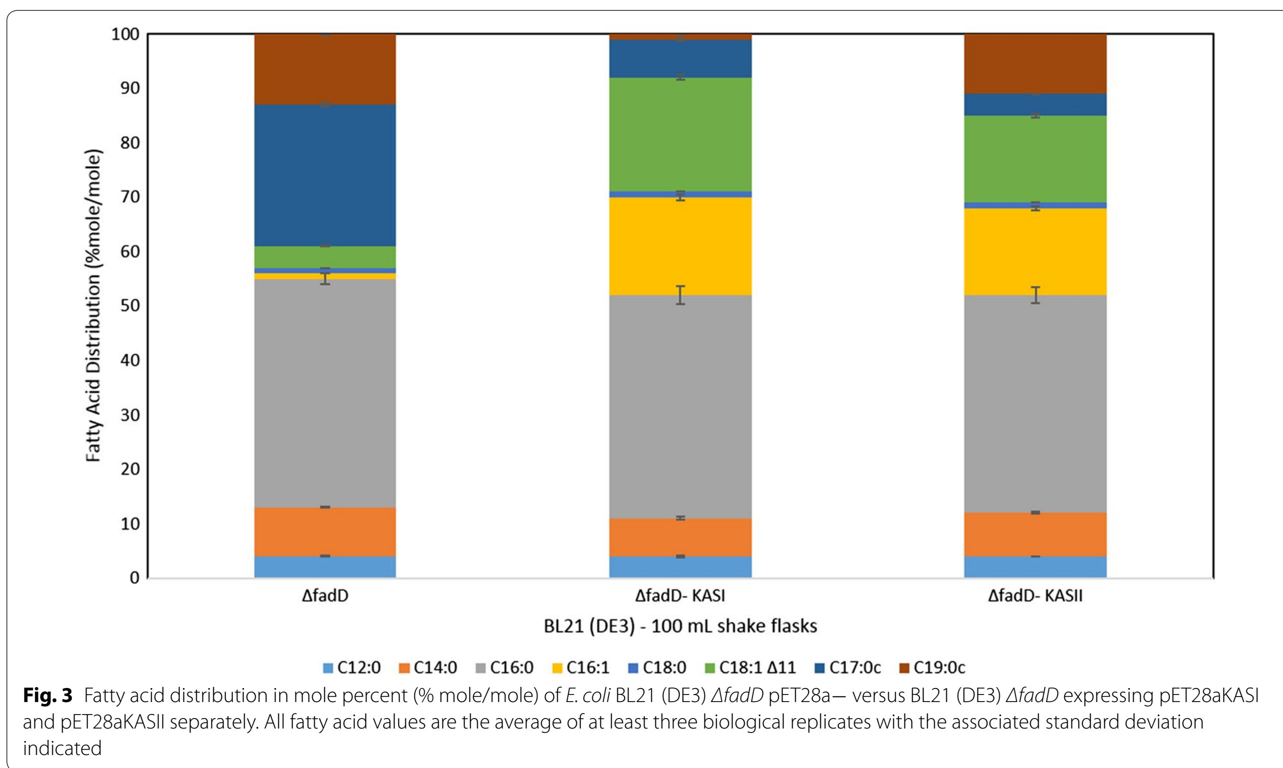

Fig. 3 Fatty acid distribution in mole percent (% mole/mole) of *E. coli* BL21 (DE3) *ΔfadD* pET28a— versus BL21 (DE3) *ΔfadD* expressing pET28aKASI and pET28aKASII separately. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated

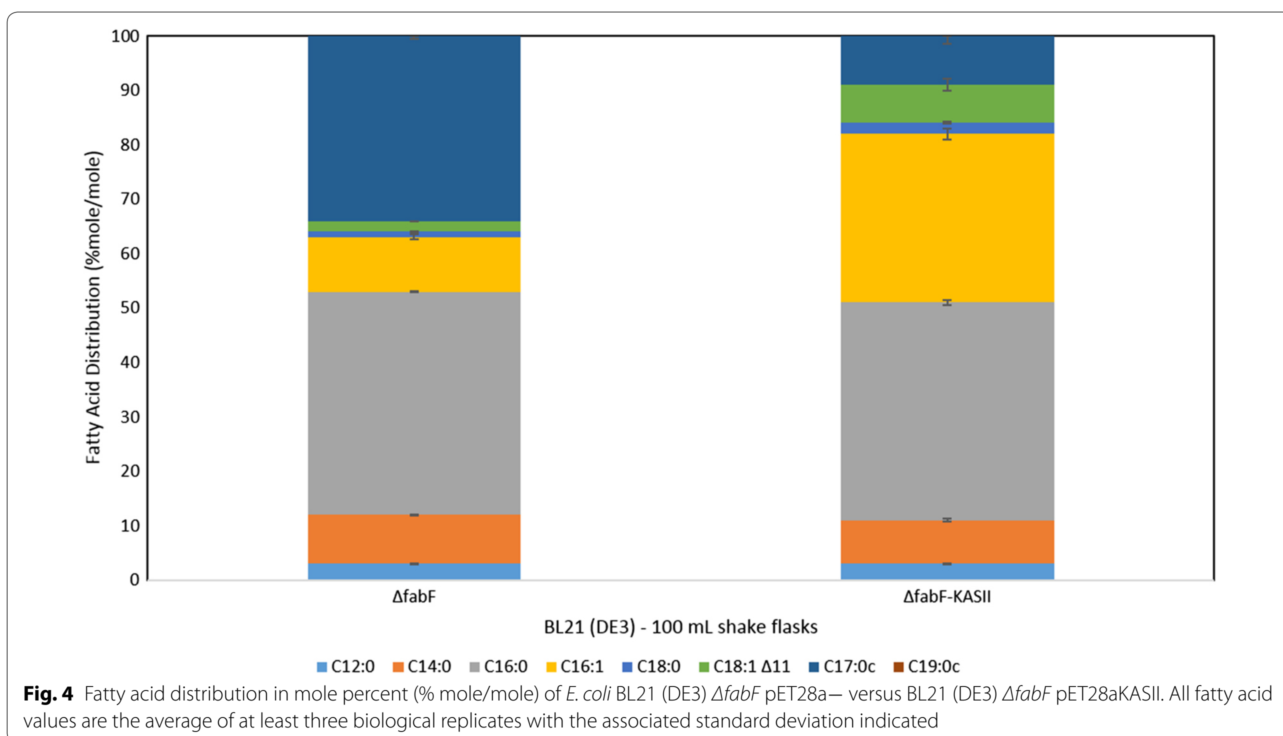

Fig. 4 Fatty acid distribution in mole percent (% mole/mole) of *E. coli* BL21 (DE3) *ΔfabF* pET28a— versus BL21 (DE3) *ΔfabF* pET28aKASII. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated

KASI in *fabB* knockouts

According to literature, the deletion of *fabB* causes auxotrophy to saturated fatty acids and inhibits growth [20]. We attempted to knock out *fabB* in our BL21 (DE3) strain using the same procedure as the *fabF* knock out. However, we were unable to retrieve viable colonies that had the gene knocked out. We assume that *fabB* is crucial for survival in BL21 (DE3) even in the presence of solid media supplemented with saturated fatty acids including oleic acid.

KASI and KASII fermentation

The shake-flasks experiments described above clearly indicate that successful cloning of plant KAS enzymes and their incorporation within the FASII system is feasible in recombinant *E. coli*. To evaluate the performance of these enhanced strains at an industrial-process, fed-batch fermentations were performed in a bioreactor with defined media.

All clones were induced under nitrogen limiting conditions to enhance lipogenesis. Samples were collected after 48 h of induction with IPTG. Total fatty acid concentration of the KASI and KASII clone in ΔfadD strain reached 292 mg/g And 220 mg/g DCW (see Additional file 1: Table S5 and S6). In particular, ΔfadD KASI showed an 80% increase in fatty acid concentrations, while ΔfadD KASII also showed a 40% increase compared to ΔfadD strain. The total amount of unsaturated fatty acids was higher in the clones expressing the KASII protein compared to all other clones (Fig. 5b).

Our fermentation results demonstrate that the ΔfabF strain's C18 fatty acids constitute only 4–5% of the total fatty acids. Moreover, the KASII complemented strain shows an increase of C18 fatty acid species to by about 13% with regard to the total fatty acid pool (Fig. 5a). By contrast, complementation with KASI resulted in 10% of total fatty acid species being C18 fatty acids. It is noteworthy that the increase of C18 fatty acids in the KASI strain was stearic acid, whereas the increase in the KASII strain was cis-vaccenic acid. This has been also observed in the ΔfadD strain containing KASI and KASII. Most notably, neither the increase in fatty acid concentrations nor the shift in the fatty acid profile was lethal to the cell. This is based on the fact that control and mutant strains have all exhibited almost the same growth rate with almost the same final OD_{600}).

Discussion

Decades of work have been dedicated to the biochemical optimization and metabolic engineering of *E. coli* aiming to increase fatty acids yield with tailored profiles [1, 3, 4, 33]. A common approach in early metabolic engineering studies involved addressing the bottlenecks observed in the native *E. coli* FAS system and its tight regulation, optimizing precursor supply, redirecting the carbon flux into a product of interest and downregulating or eliminating competing pathways [1, 3–6, 29, 34]. Despite this body of work, additional improvement in fatty acid production yield and profile specificity still requires further examination of pathway limitations and alleviation of the tight feedback regulations. However, these strategies are time-consuming and necessitate fine-tuning individual pathway components and targeting each rate-limiting factor until the desired goal is reached [3, 5, 6, 8, 11, 16].

In the fatty acid biosynthetic pathway of *E. coli*, both FabB and FabF proteins function in the condensation reaction and are the first enzymes in the fatty acid elongation system [1, 14]. Their expression level and

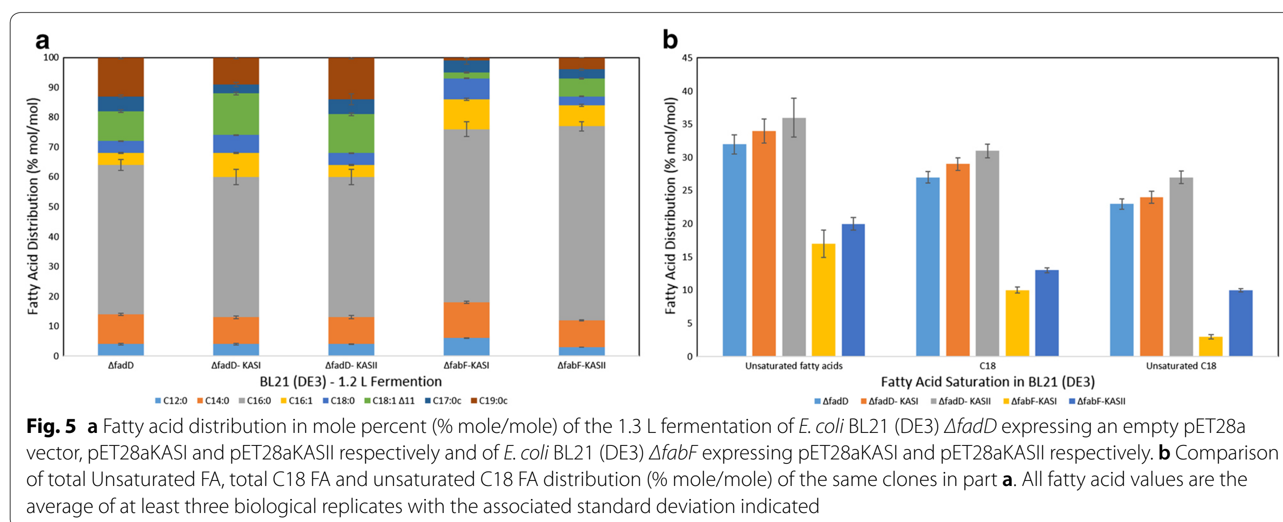

Fig. 5 **a** Fatty acid distribution in mole percent (% mole/mole) of the 1.3 L fermentation of *E. coli* BL21 (DE3) *ΔfadD* expressing an empty pET28a vector, pET28aKASI and pET28aKASII respectively and of *E. coli* BL21 (DE3) *ΔfabF* expressing pET28aKASI and pET28aKASII respectively. **b** Comparison of total Unsaturated FA, total C18 FA and unsaturated C18 FA distribution (% mole/mole) of the same clones in part **a**. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated

specificity towards the substrate greatly affects the fatty acid profile of the organism. The FadD protein is responsible for the initial step of fatty acid degradation by adding a CoA group to a free fatty acid. In comparison, in plant chloroplasts, three isoforms of ketoacyl-ACP synthase exist, encoded by the *KASI*, *KASII* and *KASIII* genes [26, 27]. The KASIII enzyme is responsible for the initial condensation reaction and accepts only acetyl-CoA as the priming unit. In contract, KASI and KASII use beta-ketoacyl-[acp] as the priming unit and are crucial for further elongation of the carbon chain from C4 to C16 for KASI and from C16 to C18 for KASII [26, 27].

In the present study, wild type *E. coli* Bl21 (DE3) strain was genetically modified for the purpose of enhancing the strictly modulated FASII system for the production of long chain fatty acids. *KASI* and *KASII* genes were synthesized following codon optimization for optimal expression and the endogenous *E. coli fadD* was knocked out to inhibit the degradation of free fatty acids [1, 3, 4]. We demonstrate for the first time that eukaryotic, plant KAS enzymes are functional within the native FASII system of *E. coli*, allowing for a definitive shift in the fatty acid profile.

Previous studies showed that the overexpression of FabB alone or in combination with other enzymes of the FAS system in *E. coli* did not improve fatty acid concentrations [1, 12]. In this study, we demonstrate that the overexpression of the *A. thaliana KASI* gene, the homologue of FabB, in wild type *E. coli* shows an improvement in the overall production of saturated fatty acids with increases in myristic, palmitic acid, and stearic acid in addition to cis-vaccenic acid. We were unable to knock out the *fabB* gene at its locus. However, at present there is only one literature precedence that reports a *fabB* deletion mutant [35], which may be an indication of the difficulties to generate a knock-out at this particular locus. It is noteworthy that the lack of the *fabB* deletion mutant does not limit the conclusions drawn from our data.

Overexpressing the native *E. coli* FabF was previously reported to inhibit the FASII system and therefore fatty acid production. This lead to an intracellular accumulation of malonyl-CoA, which induced cell death [22]. FabF is reported to complex with FabD. Overexpression of FabF leads quantitative elimination of free FabD proteins in the cell, which stalls fatty acid synthesis. This hold true since FabD requires sequential complexation with FabH and FabB in order to activate the FAS system [22]. In turn, this drives malonyl-CoA accumulation, which in turn also inhibits fatty acid production and is lethal to the cell [22].

The overexpression of FabF has been widely applied to increase malonyl-CoA pools for subsequent production of polyketides and flavonoids [21]. Cloning of the *A. thaliana KASII* gene—the homologue of *fabF*—in *E. coli* was not lethal to the cell and did not inhibit the endogenous fatty acid synthase. Hence KAS II overexpression allowed fatty acid synthesis to commence without accumulation of manolyl CoA. Overexpression of KASII additionally provided an improvement in the overall fatty acid concentrations, particularly driving up the palmitoleic and cis-vaccenic acid pool. In contrast to its *E. coli* equivalent FabF [20], the *A. thaliana* KASII is active over the broad temperature range (25–37 °C) assayed in this study (data not shown).

In accordance with the literature, knocking out the *fabF* gene severely inhibited the production of C18 species [1]. The expression of the KASII protein in the *fabF* knock out strain not only complemented the loss of function of the endogenous protein, but it also leads to a significant increase in both palmitoleic and cis-vaccenic acids relative to the wild type *E. coli*. The expression of *KASI* did not complement the loss of the native *fabF* gene, however, it confirmed the pattern of *KASI* expression in other clones where significant increases in palmitoleic and stearic acid were measured.

The complementation of ΔfabF strain with KASII, suggests that chloroplast enzymes, while only sharing 33% identity with the native *E. coli* enzymes, function efficiently in the prokaryotic system. Moreover, our cumulative data suggests that the overexpression of both KASI and KASII did not induce a transcriptional inhibition by FabR.

Several studies have extensively targeted the upstream processes of the fatty acid synthase system [8]. Whether, to increase malonyl-CoA levels, the main precursor for the FAS, by targeting the *AccABC* and *AccD* genes or by overexpressing or cloning a recombinant KASIII enzyme—the enzyme responsible for the initial precursor condensation in plants [8, 25]. However, these systems were limited at the second condensation reaction coordinated by the FabB and FabF enzymes. The second condensation reaction is a very rapid step, where elongation is fast and reaches its limit according to what substrate each enzyme can accept. In that regard, FabB was limited at C14, while FabF is only limited to accept C16 fatty acid species as substrates. The use of *A. thaliana* KASI and KASII enzymes drove the elongation step beyond the acceptance of C16 fatty acids as FAS substrates. This KAS dependent feature results in a significant change in the fatty acid distribution. KAS enzyme overexpression alone is not sufficient to drastically increase fatty acid concentrations. Surprisingly; together our synergistic plant and bacterial fatty acid biosynthesis system can improve fatty acid concentrations and distributions by cumulatively enhancing both upstream and downstream fatty acid biosynthesis processes. Most notably, both beta-ketoacyl

synthases KASI/II isolated from *A. thaliana* are completely compatible with the *E. coli* FAB pathway. It is imperative to state that both enzymes have shown to be more effective than the overexpression of the native enzyme.

Conclusion

Escherichia coli has become the model organism to study microbial fatty acid biosynthesis. However, while *E. coli* itself is not a dedicated oleaginous organism, extensive genetic engineering allowed for generation of respectable product titers. However, increasing the total fatty acid concentration and shifting the native fatty acid profile towards longer chain fatty acids with industrial application in the food and oleochemical industry remains a scientific challenge. This study has examined the effects of the plant derived, chloroplastic β-ketoacyl synthases (KAS) on the fatty acid biosynthesis of *E. coli*. We could demonstrate that KAS I and KAS II can complement the native fatty acid biosynthesis in *E. coli* and shift product profiles towards longer chain (C16–C18) type fatty acids. Interestingly, we these plant enzymes exert equivalent effects in *E. coli* as observed in their native plant source. In that respect, KAS I increases relative fatty acid concentrations of the cell, particularly promoting formation of C16–C18 type fatty acids. By contrast, KASII did not lead to a significant increase in the total fatty acid concentration but lead to a targeted relative increase of the C18:1, cis-vaccenic acid. Since, plant derived triglycerides mainly feature C16–C18 fatty acids, the integration of the plant derived KASI and II can mimic this fatty acid profile in *E. coli*. Therefore, utilization of our plasmid based system would allow generation of plant like fatty acids in *E. coli* and their subsequent chemical or enzymatic conversion to high end oleochemical products. One such example could be the conversion of cis-vaccenic acid to 10-hydroxy stearic acid using a dedicated vaccinate hydratase enzyme [36]. We are continuously probing and expanding our portfolio of *E. coli* variants for tailored fatty acid biosynthesis. In that regard, we focus on further extending the fatty acid chain length towards very long chain fatty acids, such as Eicosanoic acid (C20:0), Eicosenoic acid (C20:1) and Erucic acid (C22:1), which are in demand and associated with high pricing regimes in the production of cosmetics, lubricants and polymer industry [37, 38].

Methods

Genes and plasmids

cDNA sequences of both beta-ketoacyl-[acyl carrier protein] synthase I and II (*KASI* Gene ID: AT5G46290.3 and *KASII* Gene ID: AT1G74960.2) were obtained from The Arabidopsis Information Resource (TAIR), on www.arabidopsis.org. The chloropalstic transit peptide of both were predicted using *SignalP 4.1 Server* and removed from the mRNA sequence. The mature sequences were codon optimized for expression in *E. coli* and chemically synthesized by Eurofins Scientific. The genes were synthesized to include a 5′ *Bam*HI and a 3′ *Not*I restriction site that were later used to be cloned into the MCS of an empty pET28a vector. The cloned genes were confirmed by sequencing (Eurofins Scientific). All primers were synthesized by Eurofins Scientific and all plasmids were obtained from Novagen/Merk Millipore.

Bacterial strains and growth conditions

All bacterial strains used were obtained from Merk Millipore. *E. coli* DH5 alpha strain grown at 37 °C in Luria–Bertani medium was used for cloning and plasmid amplification. *E. coli* BL21 (DE3) strain was used for expression and fatty acid production. Minimal M9 media supplemented with 0.4% glucose and a pH = 6.9 was used for the shake flask studies. For shake flask studies, all clones were grown at 37 °C with the appropriate antibiotics (Kanamycin 50 μg/mL) and induced at an OD600 of 0.6 with 0.05 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Fatty acid methylation and analysis

Samples taken from shake flask and fermentation studies were centrifuged and subsequently washed twice with ddH_2O. The samples were then lyophilized and equal amounts of dry cell weight were taken for analysis. Methanol transesterification according to the protocol of Griffiths et al. was used to directly convert dry cell biomass to fatty acid methyl esters (FAMES) 0.1 μL of each sample was injected into gas chromatograph–flame ionization detector (GC–FID) for separation and quantification. GC–MS was performed with the Thermo Scientific™ TRACE™ Ultra Gas Chromatograph instrument coupled to a Thermo DSQ™ II mass spectrometer and the Triplus™ Autosampler injector. The analysis was carried out using a Stabilwax® fused silica capillary column (30 m × 0.25 mm, with a film thickness of 0.25 μm). The analysis was performed using the following temperatures: initial column temperature 50 °C, programmed to increase at a rate of 4 °C/min up to a final temperature of 250 °C. The carrier gas used was hydrogen at a constant flow rate of 35 mL/min. FAMEs Marine Oil Standard was used as a standard reference, containing 20 components from C14:0 until C24:1. At least three biological replicates were used for fatty acid analysis. The statistical differences and percent change presented in this manuscript are only shown for differences that were statistically significant ($P < 0.05$).

Fermentation

The DASGIP® 1.3 L parallel reactor system (Eppendorf AG) was used to perform parallel fermentations. A modified M9 media consisting of 8 g L^{-1} NH4Cl, 13.3 g L^{-1} KH2PO4, 1.24 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.42 g L^{-1} $FeCl^{3-}{\cdot}6H_2O$, 40 g L^{-1} Glucose was used as batch media. Fermenters were inoculated with an overnight pre-culture with a starting OD600 of 0.1. The cultivation temperature was kept constant at 37 °C. Initial stirring velocity and airflow was set to 200 rpm and to 0.2 volumes of air per volume of medium per min (vvm), respectively. Dissolved oxygen was maintained at 30% by successive increases of the stirrer velocity, the oxygen concentration, and eventually the airflow. A pH value of 7.00 was controlled by the addition of 6 M aqueous NaOH. A pH value shift above 7.05 initiated a feed shot of 40 mL. The feed solution consisted of 500 g L^{-1} glucose, 20 g L^{-1} $MgSO4{\cdot}7H_2O$, 2 mg L^{-1} thiamine–HCl, 16 mL 100× trace elements solution (5 g L^{-1} EDTA; 0.83 g L^{-1} $FeCl^{3-}{\cdot}6H_2O$; 84 mg L^{-1} ZnCl2, 13 mg L^{-1} $CuCl^{2-}{\cdot}2H_2O$, 10 mg L^{-1} $CoCl^{2-}{\cdot}2H_2O$, 10 mg L^{-1} H_3BO_3, and 1.6 mg L^{-1} $MnCl^{2-}{\cdot}4H_2O$) (pH = 7.00). Samples were taken at different time points to determine the OD600. Once the clones reached limitation due to the depletion of the nitrogen source, they were induced with 0.05 mM IPTG. Samples for fatty acid analysis were collected at different intervals.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-019-1217-7.

Additional file 1: Table S1. Fatty acid distribution in µg/g DCW of wild type *Escherichia coli* BL21 (DE3) at different time intervals. T = 0: directly before induction with IPTG, t = 12: 12 h after induction with IPTG, t = 24: 24 h after induction with IPTG. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated. **Table S2.** Fatty acid distribution in µg/g DCW of *Escherichia coli* BL21 (DE3) expressing pET28aKASI at different time intervals. T = 0: directly before induction with IPTG, t = 12: 12 h after induction with IPTG, t = 24: 24 h after induction with IPTG. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated. **Table S3.** Fatty acid distribution in µg/g DCW of *Escherichia coli* BL21 (DE3) ΔfadD pET28a− versus BL21 (DE3) ΔfadD expressing pET28aKASI and pET28aKASII separately. Samples were collected 48 h after induction with IPTG. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated. **Table S4.** Fatty acid distribution in µg/g DCW of *Escherichia coli* BL21 (DE3) ΔfadF pET28a− versus BL21 (DE3) ΔfadD expressing pET28aKASII. Samples were collected 48 h after induction with IPTG. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated. **Table S5.** Fatty acid distribution in µg/mg DCW of the 1.3 L fermentation of *Escherichia coli* BL21 (DE3) ΔfadD expressing an empty pET28a vector, pET28aKASI and pET28aKASII respectively. Samples were collected 48 h after induction with IPTG. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated. **Table S6.** Fatty acid distribution in µg/mg DCW of the 1.3 L fermentation of *Escherichia coli* BL21 (DE3) ΔfabF expressing pET28aKASI and pET28aKASII respectively. Samples were collected 48 h after induction with IPTG. All fatty acid values are the average of at least three biological replicates with the associated standard deviation indicated. **Table S7.** Growth analysis of M9 minimal media shake flask studies of wild type *Escherichia coli* BL21 (DE3) expressing pET28a−, pET28aKASI and pET28aKASII respectively. Absorbance values (OD600) are the average of at least three biological replicates. **Figure S1.** Growth curve of M9 minimal media shake flask studies of wild type *Escherichia coli* BL21 (DE3) expressing pET28a−, pET28aKASI and pET28aKASII respectively. Absorbance values (OD600) are the average of at least three biological replicates with the associated standard deviation indicated.

Abbreviations

FA: fatty acid; FAB: fatty acid biosynthesis; FAS: fatty acid synthase; DCW: dry cell weight; ACP: acyl carrier protein; CoA: coenzyme A; KASI: β-ketoacyl-[acyl carrier protein] synthase I; KASII: β-ketoacyl-[acyl carrier protein] synthase II; FabB: 3-oxoacyl-[acyl-carrier-protein] synthase 1; FabF: 3-oxoacyl-[acyl-carrier-protein] synthase 2; FadD: fatty acyl-CoA ligase.

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Authors' contributions

Conceived the project: EK and TB. Designed and performed the experiments: EK. Analyzed the data: EK and MH. Prepared the manuscript: EK, MF, NM and TB. Supervised the whole work: TB, MF, and NM. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional file. Additional data required is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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GFP Scaffold-Based Engineering for the Production of Unbranched Very Long Chain Fatty Acids in *Escherichia coli* With Oleic Acid and Cerulenin Supplementation

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Currently, very long chain fatty acids (VLCFAs) for oleochemical, pharmaceutical, cosmetic, or food applications are extracted from plant or marine organism resources, which is associated with a negative environmental impact. Therefore, there is an industrial demand to develop sustainable, microbial resources. Due to its ease of genetic modification and well-characterized metabolism, *Escherichia coli* has established itself as a model organism to study and tailor microbial fatty acid biosynthesis using a concerted genetic engineering approach. In this study, we systematically implemented a plant-derived (*Arabidopsis thaliana*) enzymatic cascade in *Escherichia coli* to enable unbranched VLCFA biosynthesis. The four *Arabidopsis thaliana* membrane-bound VLCFA enzymes were expressed using a synthetic expression cassette. To facilitate enzyme solubilization and interaction of the synthetic VLCFA synthase complex, we applied a self-assembly GFP scaffold. In order to initiate VLCFA biosynthesis, external oleic acid and cerulenin were supplemented to cultures. In this context, we detected the generation of arachidic (20:0), cis-11-eicosenoic (20:1) and cis-13-eicosenoic acid (20:1).

Keywords: VLCFA, *Escherichia coli*, *Arabidopsis thaliana*, self-assembly GFP, fatty acid biosynthesis

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INTRODUCTION

Microbial oils have been recently designated as a sustainable alternative to plant- and animal-based lipids. In that regard, *E. coli* has established itself as a model organism to study and tailor microbial fatty acid biosynthesis using a concerted genetic engineering approach (Janssen and Steinbuchel, 2014). While *E. coli* is not an oleaginous organism *per se* the option for extensive genetic alternation has allowed the generation of respectable product titers (Janssen and Steinbuchel, 2014). However, despite giant strides in increasing the intracellular fatty acid pool and diversifying the profile of natural fatty acids by genetic engineering, currently the ability to generate plant-like fatty acids with industrial demand is very limited (Handke et al., 2011; Janssen and Steinbuchel, 2014; Pfleger et al., 2015). While *E. coli* naturally generates medium chain fatty acids (C6–C12), most genetic engineering efforts have focused either on increasing the natural fatty acid pool or extending the natural profile toward generation of long-chain fatty acids (C13–C19). However, the optimized production of multi-methyl-branched VLCFAs has been successfully reported in *E. coli* by coupling the heterologous pathway for mycocerosic acid production of *M. tuberculosis* with the fatty acid biosynthetic pathway of *E. coli* (Menendez-Bravo et al., 2014, 2016). Currently, there are a

few studies that describe the generation of unbranched VLCFAs (C20–C28) that could be applied as food additives or in specialized high value chemical applications, such as performance lubricants (Handke et al., 2011; Pfleger et al., 2015).

More generally, fatty acids (FAs) are carboxylic acids with an aliphatic tail. They are naturally produced by both prokaryotes and eukaryotes and are precursors for the biosynthesis of essential building blocks such as sterols, phospholipids and sphingolipids. Fatty acids are often classified according to their chain length and degree of saturation. In eukaryotic and prokaryotic organisms the chain length can range from short chain fatty acids with aliphatic tails >4 carbons, to VLCFAs with aliphatic tails of >20 carbons. Independent of the chain length, FAs with a single double bond or more are classified as unsaturated FAs, whereas FAs that lack a double bond are classified as saturated FAs (Janssen and Steinbuchel, 2014; Beld et al., 2015, 2016).

VLCFAs are predominantly found in eukaryotic cells. Here they are the precursors for sphingolipid biosynthesis, which is essential for growth (Dickson et al., 2006). In yeast, VLCFAs are reported to be involved in the transport of proteins across membranes by assembling into lipid-protein complexes and play an essential role in the synthesis of the glycosylphosphatidylinositol lipid anchor (Gaigg et al., 2006). In mammals, VLCFAs perform a wide array of physiological functions and are abundant in the myelin sheath of the brain and the lipid barrier of the skin (Jakobsson et al., 2006). In plants, VLCFAs are components neutral lipids, such as triacylglycerol and wax esters, that are used for energy storage in plant seeds and as hydrophobic polymers located on leaf surfaces to prevent water loss and provide resistance to temperature changes (Cassagne et al., 1994; Trenkamp et al., 2004; Dickson et al., 2006; Joubes et al., 2008).

VLCFAs are synthesized in the endoplasmic reticulum of cells by a membrane-bound enzyme complex that catalyzes the sequential addition of a two-carbon moiety from a malonyl-CoA to a long chain acyl-CoA in the presence of NADH and NADPH (Jakobsson et al., 2006). The complex constitutes four enzymes that perform four distinct reactions that form the elongation cycle (Domergue et al., 2000). 3-ketoacyl-CoA synthases or KCSs catalyze the first step of the cycle through the condensation of a long-chain fatty acyl-CoA with a malonyl-CoA forming a very long chain 3-oxoacyl-CoA (Ghanevati and Jaworski, 2001). The condensing reaction is the rate limiting reaction, therefore the expression level of the condensing enzyme (KCS) highly affects the rate of the overall cycle. The second step of the biosynthetic cycle is the reduction of the very long chain oxoacyl-CoA to (*3R*)-*3*-hydroxyacyl-CoA by a 3-oxoacyl-CoA reductase or KCR. The third step is the dehydration of the (*3R*)-*3*-hydroxyacyl-CoA into a *trans*-2,3-enoyl-CoA by a 3-hydroxacyl-CoA dehydratase or HCD. The final reduction step is catalyzed by a trans-2,3-enoyl-CoA reductase or CER, yielding a two carbon longer acyl-CoA (Zheng et al., 2005; Jakobsson et al., 2006; Joubes et al., 2008; **Figure 1**).

The chain length and yield of the final product is highly dependent on the KCS expression level and substrate specificity. In *A. thaliana* 20 different genes have been identified encoding a KCS, and are expressed in different tissues and life cycles of the plant depending on the tissue specificity for VLCFAs (Blacklock and Jaworski, 2002; Joubes et al., 2008). Each KCS has a different substrate affinity for saturated and unsaturated long acyl-CoAs of different chain length (Millar and Kunst, 1997). The three other enzymes involved in the elongation process have a broad range of substrate specificity (Fehling and Mukherjee, 1991; Millar and Kunst, 1997).

The four reactions in the VLCFA elongation cycle are similar to that of *de novo* fatty acid biosynthesis, found in prokaryotes and eukaryotes (Jakobsson et al., 2006; **Figure 2**). However, in *de novo* fatty acid biosynthesis malonyl-ACP is used as a substrate in the condensing reaction.

In *E. coli,* malonyl-CoA synthesized by the acetyl-CoA carboxylase is directly converted to malonyl-ACP by the malonyl-CoA:ACP transacylase (FabD) and directed toward fatty acid biosynthesis (Joshi and Wakil, 1971; Cronan and Waldrop, 2002). The amount of malonyl-CoA synthesized is tightly, transcriptionally and translationally, regulated in *E. coli* (Cronan and Waldrop, 2002). The fatty acid profile ranges from lauric acid (C12:0) up to stearic acid (C18:0), and low amounts of vaccenic acid (C18:1 Δ11). The most abundant fatty acid is palmitic acid (C16:0). Unsaturated fatty acids, mainly composed of palmitoleic and vaccenic acids, constitute 35% of total membrane lipids. Free fatty acids cleaved from the membrane of *E. coli* or taken up from the environment are activated by FadD to Acyl-CoAs and consumed via the beta oxidation pathway (Janssen and Steinbuchel, 2014).

In this study, we have systematically extended our toolbox for engineering of *E. coli* toward the generation of VLCFA's including eicosenoic acid (20:1) and arachidic acid (20:0). We focus on the expression of four enzymes involved in the plant-based VLCFA biosynthesis of *A. thaliana* using a synthetic polycistronic expression cassette in combination with a self-assembly GFP system. The application of the self-assembly GFP system is a highly innovative method to enable simultaneous solubilization and guided interaction of the plant-derived fatty acid biosynthesis enzyme systems (Cabantous et al., 2005; Cabantous and Waldo, 2006; Venning-Slater et al., 2014; Xie et al., 2017). Furthermore, oleic acid and cerulenin were required to initiate VLCFA biosynthesis.

MATERIALS AND METHODS

Genes and Plasmids

Nucleotide sequences of *KCS18* (Gene ID: 829603), *KCR1* (Gene ID: 843098), *PAS2* (Gene ID: 830912), and *CER10* (Gene ID: 824702) were obtained from The Arabidopsis Information Resource (TAIR), on www.arabidopsis.org database. Nucleotide sequences for the self-assembly GFP were obtained from the published sequences of Cabantous et al. (2005). The putative transmembrane domain of only *KCR1* (NP_564905.1) was predicted using the TMHMM Server v. 2.0 (Krogh et al., 2001) and removed from the mature DNA sequence. The mature sequences were codon-optimized for expression in *E. coli* and chemically synthesized by Eurofins Scientific. The RBS calculator software tool was used to design and evaluate the

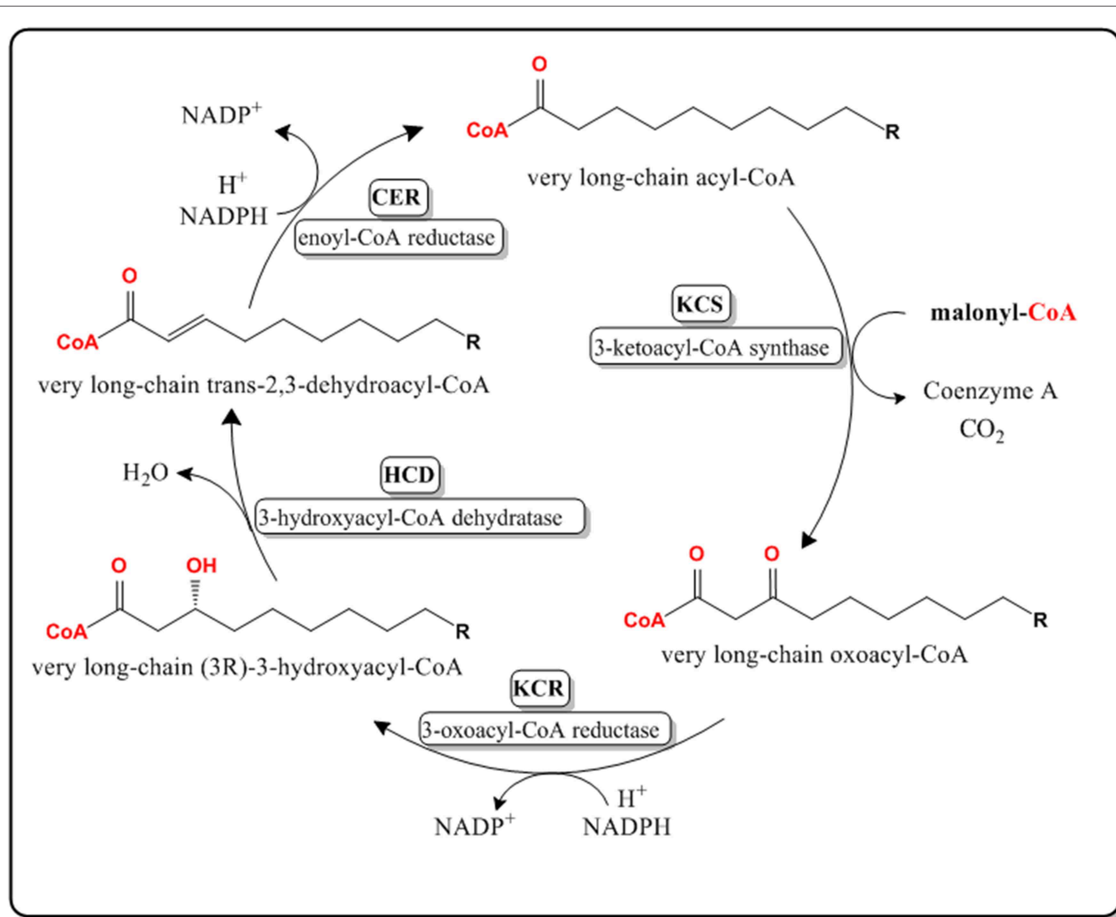

FIGURE 1 | Pathway for the biosynthesis of VLCFAs in the endoplasmic reticulum of *Arabidopsis thaliana* cells. Malonyl-CoA is used as the 2-Carbon elongation unit. *Very long chain* refers to a chain length of 20 carbon atoms or higher.

relevant ribosomal binding sites (RBS) (Salis et al., 2009; Espah Borujeni et al., 2014). The cloned genes were confirmed by sequencing (Eurofins Scientific). All primers were synthesized by Eurofins Scientific and all plasmids were obtained from Novagen/Merk Millipore.

Bacterial Strains and Growth Conditions

All bacterial strains used were obtained from Merk Millipore. For cloning and plasmid amplification, *E. coli* DH5 alpha strain was used. *E. coli* BL21 (DE3) strain was used for expression and fatty acid production. Minimal M9 media [1 g L^{-1} NH_4Cl, 0.5 g L^{-1} NaCl, 3 g L^{-1} KH_2PO_4, 6 g L^{-1} Na_2HPO_4, 0.493 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.011 g L^{-1} $CaCl_2$, 0.42 g L^{-1} $FeCl_3$-$6{\cdot}H_2O$] supplemented with 0.4% glucose and a pH of 6.9 was used for the shake flask studies, clones were grown at 37°C with the appropriate antibiotics (Kanamycin 50 μg/mL and Chloramphenicol 34 μg/mL) and induced at an OD_{600} of 0.6 with 0.05 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Fluorescence Microscopy

For microscopy, cells were washed and resuspended in ddH_2O. Microscope photographs were acquired on an Axio Lab. A1, fluorescence microscope equipped with an Axio Cam ICm1 (Zeiss, Oberkochen, Germany).

Fatty Acid Methylation

Samples taken from shake flask and fermentation studies were centrifuged and subsequently washed twice with ddH_2O. Subsequently, the pellets were then lyophilized and equal amounts of dry cell weight were weighed and taken for analysis. Methanol transesterification according to the protocol of Griffiths et al. (2010) was used to directly convert dry cell biomass to fatty acid methyl esters (FAMES).

Fatty Acid Analysis

One microliter of each sample was injected into Gas Chromatograph-Flame Ionization Detector (GC-FID) for separation and quantification of the FAMEs. GC–MS was performed with the Thermo Scientific™ TRACE™ Ultra Gas Chromatograph instrument coupled to a Thermo DSQ™ II mass spectrometer and the Triplus™ Autosampler injector. MS was performed in positive ion mode. The analysis was carried out using a Stabilwax® fused silica capillary column (30 m × 0.25 mm, with a film thickness of 0.25 μm). The

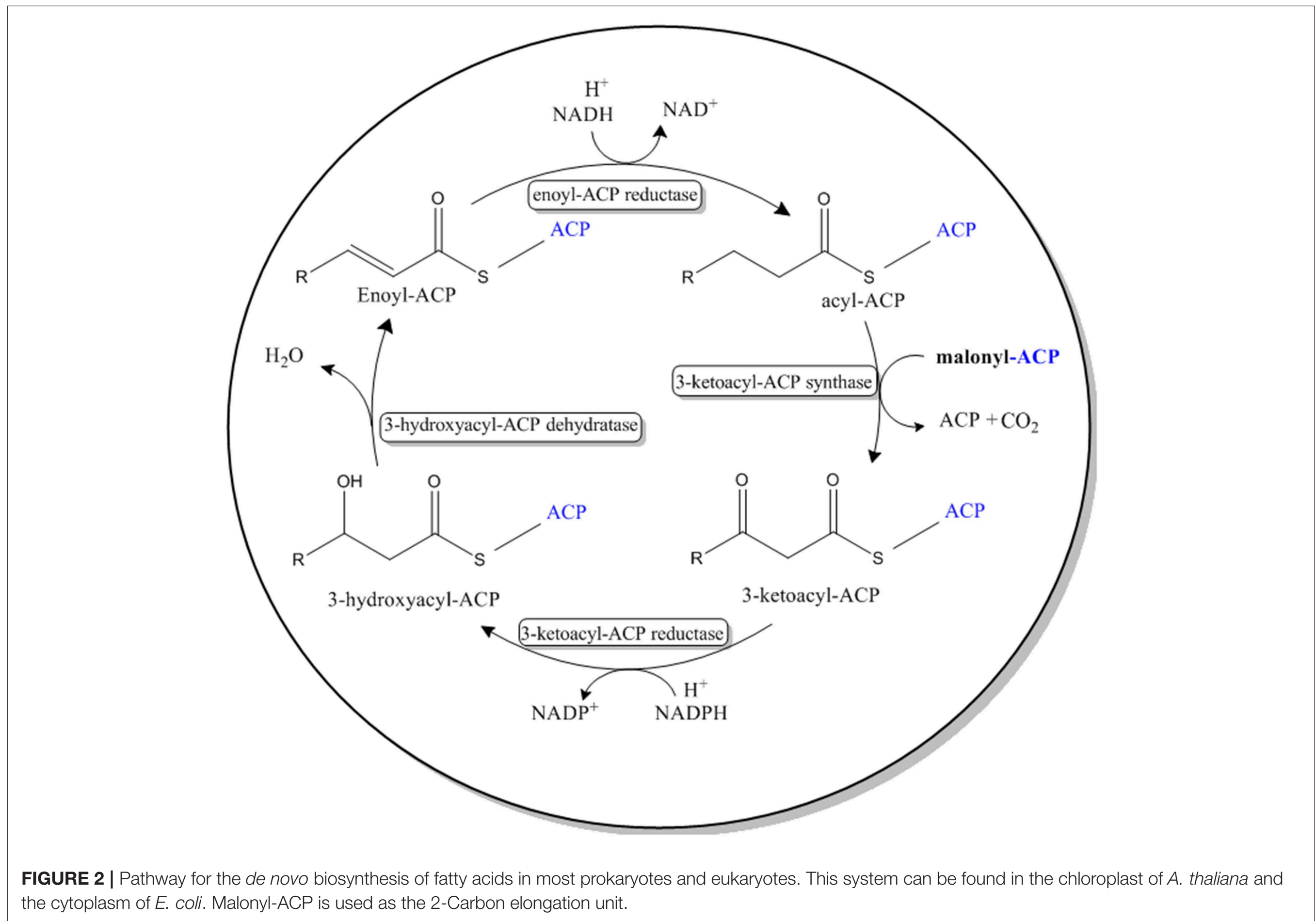

FIGURE 2 | Pathway for the *de novo* biosynthesis of fatty acids in most prokaryotes and eukaryotes. This system can be found in the chloroplast of *A. thaliana* and the cytoplasm of *E. coli*. Malonyl-ACP is used as the 2-Carbon elongation unit.

run was under an optimized temperature as follows: initial column temperature 50°C, programmed to increase at a rate of 4 °C/min up to a final temperature of 250°C. Hydrogen was used as the carrier gas at a flow rate of 35 mL/min with constant flow compensation. Additionally, Shimadzu GC-2010 Plus gas chromatograph with flame ionization detector (FID) was also used for fatty acid analysis. One microliter sample was injected via an AOC-20i auto injector (Shimadzu) on to a Phenomenex ZB-WAX column (length 30 m, 0.32 mm ID, 0.25 µm df). The column was heated up with 5°C min-1 to 240°C maintained for 5 min. Hydrogen was used as carrier gas with a flow rate of 3 mL min-1 and constant flow compensation. FAMEs Marine Oil Standard (Marine Oil FAME Mix, RESTEK USA) was used as a standard reference, containing 20 components from C14:0 until C24:1. Glyceryl trinonadecanoate (C19:0 TAG) (Sigma, Germany) was used as internal standard to determine esterification efficiency. Individual FAME concentrations were based on peak areas relative to methyl non-adecanoate (C19:0) (Griffiths et al., 2010).

Fermentation

The DASGIP® 1.3 L parallel reactor system (Eppendorf AG) was used to perform parallel fermentations. A modified M9 media [8 g L^{-1} NH_4Cl, 13.3 g L^{-1} KH_2PO_4, 1.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.42 g L^{-1} $FeCl_3$-$6{\cdot}H_2O$, 20 g L^{-1} glucose] was used as batch media and supplemented with 1 mL 100× trace elements solution (5 g L^{-1} EDTA; 0.83 g L^{-1} $FeCl_3$- $6{\cdot}H_2O$; 84 mg L^{-1} $ZnCl_2$, 13 mg L^{-1} $CuCl_2$-$2{\cdot}H_2O$, 10 mg L^{-1} $CoCl_2$-$2{\cdot}H_2O$, 10 mg L^{-1} H_3BO_3, and 1.6 mg L^{-1} $MnCl_2$-$4{\cdot}H_2O$) and the proper antibiotics. Fermenters were inoculated with an overnight pre-culture with a starting OD_{600} of 0.1. The cultivation temperature was kept constant at 30 °C. Initial stirring velocity and airflow was set to 200 rpm and to 0.2 volumes of air per volume of medium per min (vvm), respectively. Dissolved oxygen was kept at 30% of the maximum dissolved oxygen concentration (mg/L) by successive increases of the stirrer velocity, the oxygen proportion, and eventually the airflow. A pH value of 7.00 was controlled by the addition of 6 M aqueous NaOH. A pH value shift above 7.05 initiated a feed shot of 40 mL. The feed solution consisted of 500 g L^{-1} glucose, 5 g L^{-1} oleic acid, 20 g L^{-1} $MgSO_4{\cdot}7H_2O$, 2 mg L^{-1} thiamine–HCl, 16 mL 100 × trace elements solution (pH = 7.00). Samples were taken at different time points to determine the OD_{600}. Once the clones reached the stationary phase of growth, they were induced with 0.05 mM IPTG. Five micromolar Cerulenin (Cayman chemicals, USA) was added 24 h after induction with IPTG.

RESULTS AND DISCUSSION

KCSs in *E. coli* System

In order to enable the recombinant production of VLCFA in *E. coli* BL21 (DE3), we constructed and expressed the characterized *Arabidopsis thaliana* VLCFA multi-enzyme elongase system using a synthetic expression cassette. The first step of fatty acid elongation is the condensation of a fatty-acyl-CoA with a malonyl-CoA catalyzed by 3-ketoacyl-CoA synthase (Ghanevati and Jaworski, 2001).Twenty-one gene-homologs of the 3-ketoacyl-CoA synthase have been identified in *A. thaliana* (Costaglioli et al., 2005). The different KCS homologs were classified into 8 subclasses according to their phylogeny, duplication history, genomic organization, protein topology and 3D modeling (Joubes et al., 2008). Of the 21 KCS homologs, only eight (*KCS1, KCS5, KCS9, KCS11, KCS14, KCS17, KCS18,* and *KCS20*) have been tested and demonstrated to encode proteins able to catalyze VLCFA production (Trenkamp et al., 2004; Blacklock and Jaworski, 2006; Paul et al., 2006). Furthermore, *KCS1, KCS6* and *KCS10* were reported to be the most expressed of all the homologs, suggesting that the other homologs are functionally redundant (Joubes et al., 2008). Three genes, *KCS1, KCS6,* and *KCS18* each falling within a different subclass, were individually expressed in *E. coli* BL21 (DE3). Out of the 21 homologs, *KCS1, KCS6, and KCS18* were chosen based on the differences in substrate specificity, biological function, and expression patterns in *A. thaliana* (Blacklock and Jaworski, 2006; Joubes et al., 2008; **Table 1**). Respectively, we have performed a fatty acid analysis in order to determine any changes in the fatty acid profile of *E. coli.* Three pET28a vectors with T7 promoters, each harboring one of the KCS homologs, were used for expression in BL21 (DE3). Clones were cultivated at 37°C and 30°C in both LB and M9 minimal media respectively. After induction with 0.05 mM IPTG, we performed a lipid analysis where we found that the individual expression of three 3-ketoacyl-CoA synthases (*KCS1, KCS6,* and *KCS18*) in *E. coli* BL21 (DE3) did not result in detectable amounts of VLCFA (**Supplementary Material**). Since wild type *E. coli* is endogenously not capable of generating VLCFAs, the entire *A. thaliana* elongase multi-enzyme complex was cloned to enable VLCFA biosynthesis independent of the *E. coli* native fatty acid synthase.

Synthetic Design of an Elongase Expression Cassette Using RBS Prediction

Since we could not detect a change in fatty acid distribution by the individual expression of the three KCSs homologs, we referred back to literature. Based on the collected information *KCS18* was chosen as the first enzyme in the complex due to its broader range of substrate specificity compared to equivalent homologs (Ghanevati and Jaworski, 2001; Blacklock and Jaworski, 2002; Paul et al., 2006; Joubes et al., 2008). The additional three enzymes (KCR1, PAS2, and CER10) involved in the elongase complex have a broad range of specificity and are expressed in all tissues exhibiting VLCFA (Fehling and Mukherjee, 1991; Millar and Kunst, 1997). Two of these enzymes (KCR1, CER10) involved in the complex are well characterized in A. *thaliana*: KCR1, 3-ketoacyl-CoA reductase, performs the second step of the elongation process (Xu et al., 1997, 2002; Beaudoin et al., 2002), PAS2, 3-hydroxacyl-CoA dehydratase, the least characterized of the four, catalyzes the third step (Bach et al., 2008; Roudier et al., 2010) and CER10, trans-2,3-enoyl-CoA reductase, performs the last step of the elongation process. PAS2 is known to interact with CER10 in the ER of *A. thaliana* (Zheng et al., 2005). The ribosomal binding site of each gene on the VLCFA expression cassette was predicted using the RBS Calculator software (**Supplementary Material**) to control translation initiation and protein expression rates (Salis et al., 2009; Espah Borujeni et al., 2014). Since KCS18 catalyzes the rate limiting step in VLCFA biosynthesis, the synthetic RBS sequence of *KCS18* was calculated to yield a double translation initiation rate and double protein expression level to that of *KCR1, PAS2,* and *CER10*. The synthetic RBS sequences of *KCR1, PAS2,* and *CER10* were calculated to yield equimolar expression levels of each protein. The expression cassette was further evaluated for protein expression using operon expression calculator (Salis et al., 2009; Espah Borujeni et al., 2014).

TABLE 1 | Properties of A. *thaliana* ketoacyl-CoA synthases used in this study.

KCS	Biological function	Expression patterns	Substrate specificity	Product	References
KCS1	Required for cuticular wax production	Expressed in all tissues; Highest expression in siliques, flowers and stems	C16:0, C16:1, C18:0, C18:1 (very low activity) and C20:1	C20:0, C20:1, C22:0, C22:1, C24:0 and C26:0	Todd et al., 1999; Joubes et al., 2008; Haslam and Kunst, 2013
KCS6	Required for cuticular wax production	Expressed in all tissues; specialized expression in epidermis	C22:0, C24:0 and C26:0	C24:0, C26:0 and C28:0	Hooker et al., 2002; Haslam and Kunst, 2013; Janssen and Steinbuchel, 2014
KCS18 (FAE1)	Required for the production of VLCFA for TAG storage in seeds	Highest in seeds; Found in carpels and siliques	C16:0, C16:1, C18:0, C18:1; lower activity with 20:0 and 20:1	C18:1; C20:0; C20:1; C22:0; C22:1; Low amounts of C24 and C26	James et al., 1995; Joubes et al., 2008; Haslam and Kunst, 2013

Design of GFP-Based Scaffold Elongase Expression Cassette

Green Fluorescent Protein (GFP) is a barrel-shaped protein consisting of 11 Betta-sheets. Previous studies have developed a mutated version of GFP, termed split-GFP or self-assembly GFP, where the gene encoding the first 10 beta sheets (GFP1-10) and the gene encoding the last beta sheet (GFP11) are expressed separately but are able to self-assemble and fluoresce (Cabantous et al., 2005; Cabantous and Waldo, 2006). Self-assembly GFP has been previously reported to enhance protein solubility, reduce formation of bacterial inclusion bodies and for the immobilization of various enzymes in recombinant *E. coli* BL21 (DE3) (Cabantous and Waldo, 2006; Venning-Slater et al., 2014). It has also been applied for topology analyses of membrane-bound enzymes in N. *benthamiana*, without affecting their biological functionality (Xie et al., 2017). In order to enhance solubilization and immobilize the VLCFA elongase system in *E. coli,* each gene in the VLCFA expression cassette was genetically tagged with the *GFP11* sequence at its N-terminus. Concomitantly, the *GFP1-10 was* cloned and co-expressed on a separate *pET28a* vector (**Figure 3**). Notably, due to the several repetitive regions implemented on each gene, cloning was very challenging. Therefore, the expression cassette was split into five fragments and chemically synthesized. The first two fragments were joined via overlap PCR and cloned into an empty pACYC vector. The remaining fragments were added sequentially to the pACYC vector containing the first two fragments using a series of restriction and ligations with *SapI* and *SpeI* restriction enzymes respectively.

Expression in BL21 (DE3)

The synthetic *VLCFA* expression cassette and in combination with the self-assembly GFP constructs were co-expressed in *E. coli* BL21 (DE3). The cultivation was conducted with minimal M9 media supplemented with 0.5% glucose at 30°C. Cultures were induced with 0.05 mM IPTG at an OD_{600} of 0.6 (**Figure 4**). Samples were collected 24 h after induction, the lipid fraction was extracted and methylated. After analysis, we could not detect VLCFA production (**Supplementary Material**).

KCS18 is reported to have high affinity for oleyl-CoA (James et al., 1995; Paul et al., 2006; Sun et al., 2013). Additionally, *E. coli* is reported to be able to grow on oleic acid as sole carbon source (Janssen and Steinbuchel, 2014). Hence, external oleic acid is transported from the media into the cell via the membrane transport protein (FadL), which is then activated into oleoyl-CoA by FadD (Campbell and Cronan, 2002; Lepore et al., 2011). In order to increase the amount acyl-CoAs, M9 media was supplemented with 0.5 % oleic acid. Cultures were induced with 0.05 mM IPTG when OD_{600} of 0.6 was reached. In order to increase malonyl-CoA concentrations as the second substrate required for fatty acid elongation, 5 μM cerulenin was added to the cultures at the stationary phase (van Summeren-Wesenhagen and Marienhagen, 2015). Cerulenin binds irreversibly to the native *E. coli* beta-ketoacyl-ACP synthases (FabB and FabF) leading to the accumulation of malonyl-CoA (Janssen and Steinbuchel, 2014). Subsequently, cerulenin also irreversibly binds to KCS18, however the concentration of cerulenin used should not completely inhibit the function of KCS18 (Schneider et al., 1993). After 24 h of induction with IPTG, samples were collected and analyzed for their lipid content by GC-FID. In samples expressing the VLCFA expression cassette, we detected minor peaks corresponding to arachidic acid (20:0) methyl ester and eicosanoic acid (20:1) methyl ester. Quantitative analysis revealed a change in the fatty acid distribution of the clone expressing the VLCFA cassette (**Figure 5**).

Fermentation and Scale Up

Fed batch fermentation experiments were carried out in a 1.3 L parallel fermenter in order to investigate the full potential of the recombinant VLCFA system in an optimized microbial system. The feed was supplemented with 0.5 % oleic acid and 0.05 mM IPTG was used for induction. Five micromolar

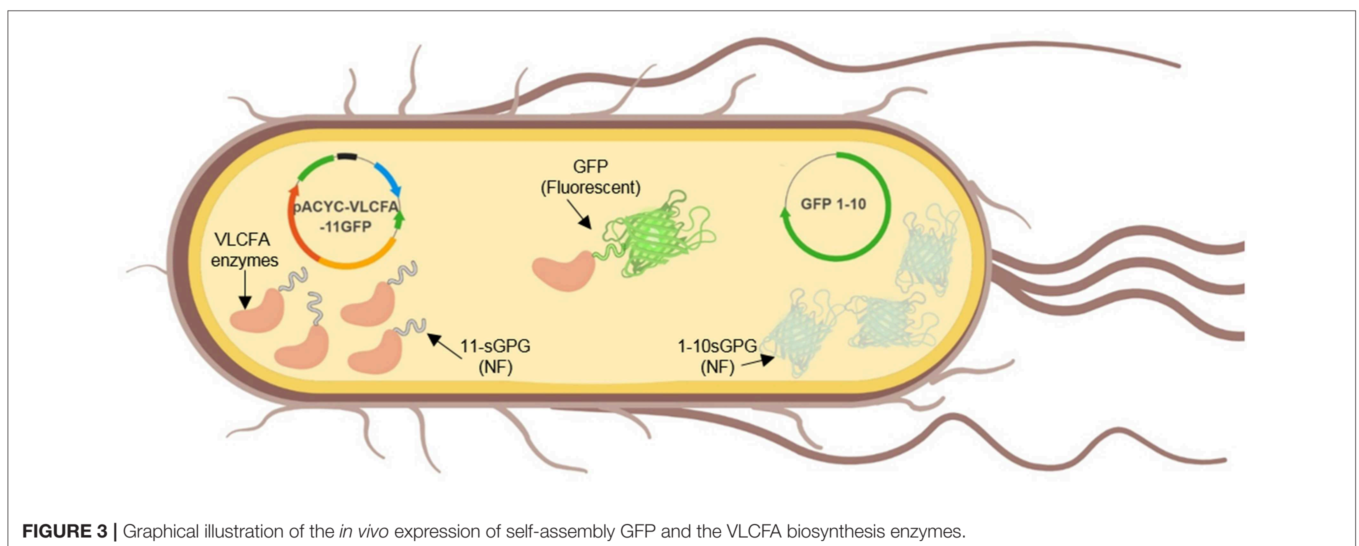

FIGURE 3 | Graphical illustration of the *in vivo* expression of self-assembly GFP and the VLCFA biosynthesis enzymes.

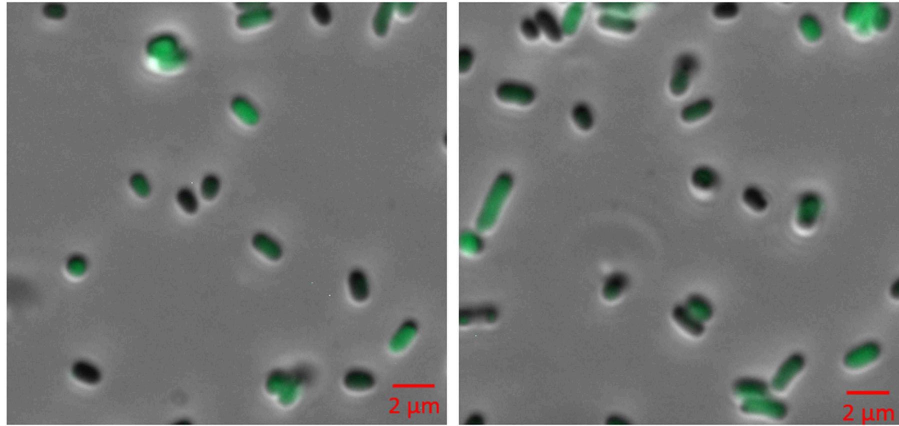

FIGURE 4 | Fluorescence microscopy of BL21 (DE3) cells expressing pACYC-VLCFA-11GFP and pET28a-1-10GFP. Cells were cultured in LB media and induced with 0.05 mM IPTG for 6 h. For microscopy, cells were washed and re-suspended in ddH_2O.

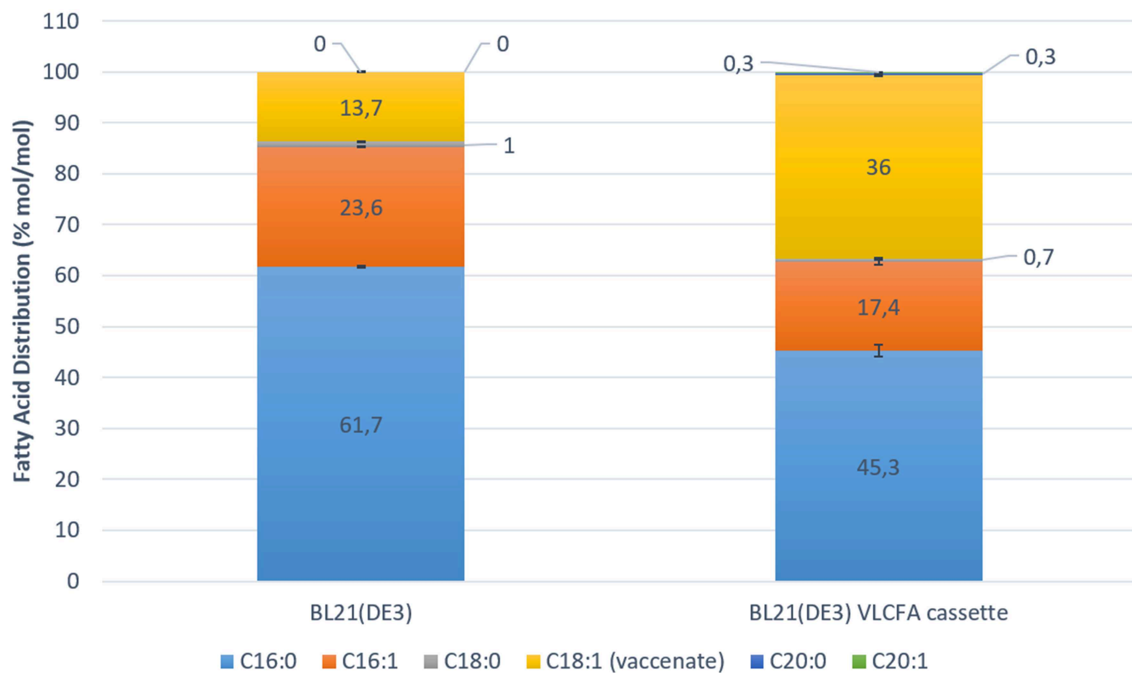

FIGURE 5 | Fatty acid distribution mol/mol of control *Escherichia coli* BL21 (DE3) expressing pACYC-11-saGFP and pET28a-1-10saGFP vs. BL21 (DE3) expressing pACYC-VLCFA-11GFP and pET28a-1-10GFP in 100 ml shake flask studies. The overall increase in C18:1 fatty acids is highly noticeable. The BL21 (DE3) VLCFA cassette profile clearly shows the production of longer than C18 fatty acids (C20:0 and C20:1) which are absent in the control.

cerulenin was added to the cultures 24 h after induction with IPTG. Quantitative analyses using GC-FID revealed a change in the fatty acid distribution of the clone harboring the VLCFA cassette. Interestingly, stearic acid (18:0) contributed 11.4% mol/mol of the total fatty acid pool, while vaccenic acid (18:1) made up 25.3% mol/mol. The total C18 fatty acid fraction was therefore 36.7% mol/mol of the total fatty acid pool. Arachidic acid (20:0) comprised 1% mol/mol of the total fatty acid pool and eicosenoic acid (20:1) comprised 2% mol/mol of the total fatty acid pool (**Figure 6**). We estimate that we obtained 6 mg of total VLCFAs per liter of culture with an estimated productivity of 0.25 mg/l/h.

GC-MS could confirm the presence of arachidic acid methyl ester, eicosenoic acid methyl ester and surprisingly erucamide, an amide of erucic acid (C22:1), in the lipid fraction of the VLCFA clone (**Figure 7**). Two separate signals referring to cis-11-eicosenoic acid methyl ester and cis-13-eicosenoic acid methyl ester were also identified (**Supplementary Material**).

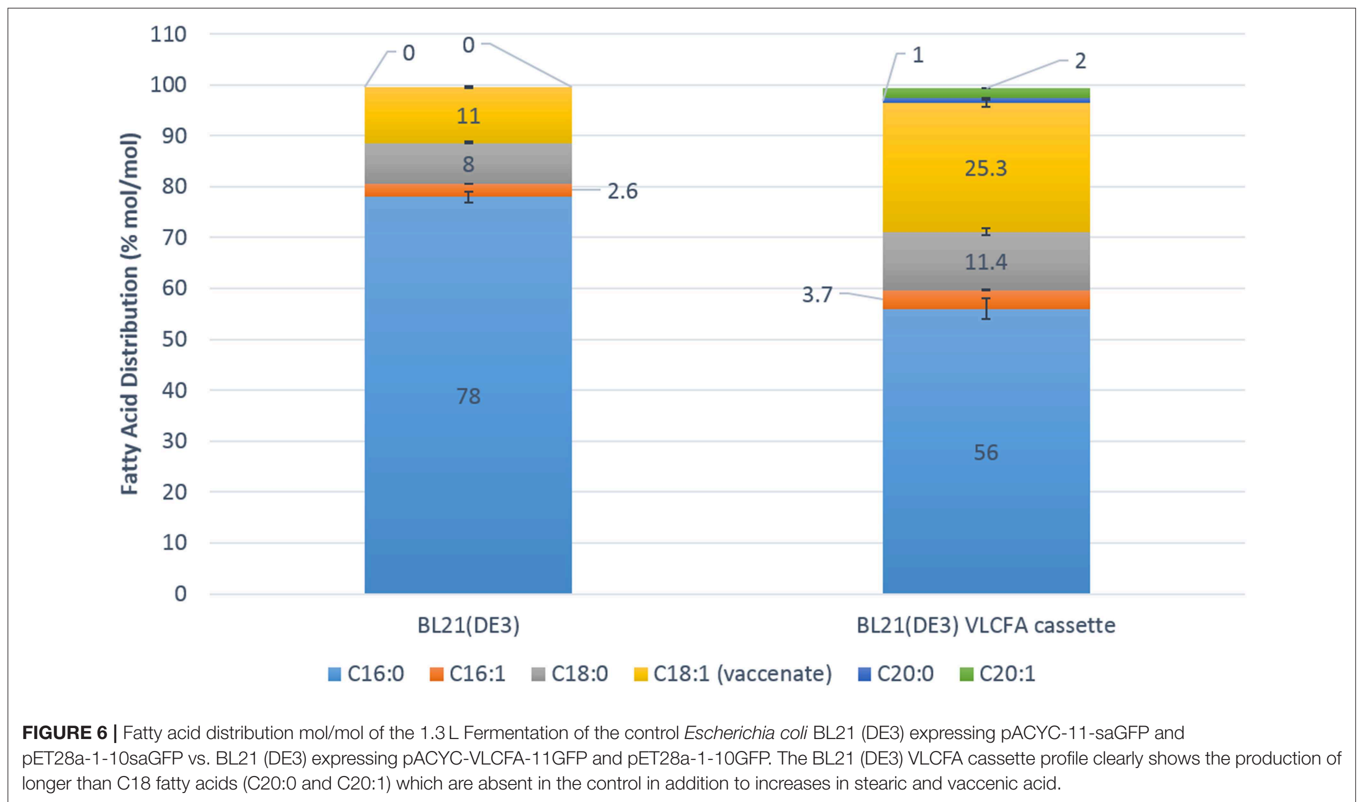

FIGURE 6 | Fatty acid distribution mol/mol of the 1.3 L Fermentation of the control *Escherichia coli* BL21 (DE3) expressing pACYC-11-saGFP and pET28a-1-10saGFP vs. BL21 (DE3) expressing pACYC-VLCFA-11GFP and pET28a-1-10GFP. The BL21 (DE3) VLCFA cassette profile clearly shows the production of longer than C18 fatty acids (C20:0 and C20:1) which are absent in the control in addition to increases in stearic and vaccenic acid.

Cis-11-eicosenoic acid (20:1) is the two carbon elongated version of the cis-9-octadecenoic acid (oleic acid 18:1), whereas cis-13-eicosenoic acid (20:1) is the derivative of the cis-11-octadecenoic acid (vaccenic acid 18:1), which is natively produced *de novo* in *E.* coli. We also observed and confirmed the presence of erucamide in the lipid fraction of our clones. Erucic acid (C22:1) is the 4 carbon elongated derivative of oleic acid. While we currently cannot explain the presence of the amidated version of erucic acid, we are conducting a detailed metabolomic study to elucidate its potential biosynthetic origin.

Further optimization of upstream and downstream processes is required to efficiently produce and increase VLCFA titers in *E. coli*. As *E. coli* is not a lipid-accumulating organism *per se*, the biosynthetically generated fatty acids are commonly transported and stored in the membrane as diacylglycerols (DAGs). It is currently uncertain whether *E. coli* can incorporate VLCFAs in its membrane or activate VLCFAs to be degraded via the beta oxidation pathway.

CONCLUSION

VLCFAs are essential building blocks in most eukaryotic and multicellular organisms. VLCFAs and their derivatives are crucial for plant survival and are important for the development and maintenance of nervous and cardio-vascular system in mammals. The high value applications of VLCFA and their derivatives has been reported across the chemical, pharmaceutical, food and cosmetic industries. The ease and the extensive genetic manipulation of *E. coli* for the production of high value chemicals have been shown to be a sustainable alternative to plant-based platforms. In this study, we cloned and expressed the *A. thaliana* VLCFA synthase complex in *E. coli* for the generation of arachidic and eicosenoic acid by supplementing oleic acid and cerulenin. The solubilization of the membrane associated, plant-derived enzyme cascade that enabled VLCFA production in *E. coli* was facilitated by application of an innovative self-assembly GFP system. In that context, the fusion of individual enzyme activities that are part of the heterologous VLCFA biosynthesis complex, to the engineered GFP system also facilitated the interaction of relevant enzyme systems within the *E. coli* cell.

Since wild type *E. coli* is endogenously not capable of generating VLCFAs, their metabolism, which may include further enzymatically mediated functionalization, is hitherto not established. Further systems biology analysis is required in order to assess the impact of VLCFAs production on *E. coli* metabolism. In this context, the detection of erucamide in this study, suggested that there is a cross talk between, the endogenous, primary amino acid biosynthesis and the heterologous VLCFA biosynthetic cascade. While the biosynthesis of primary amides is not well-characterized, their natural occurrence in fungi, plants and mammals has been well-documented (Sun et al., 2016; Li et al., 2017; Kim et al., 2018). While, a discrete investigation of metabolic effects is beyond the scope of this study, we are currently addressing the observed metabolic effects by a concerted systems biology approach. Furthermore, additional genetic strategies are

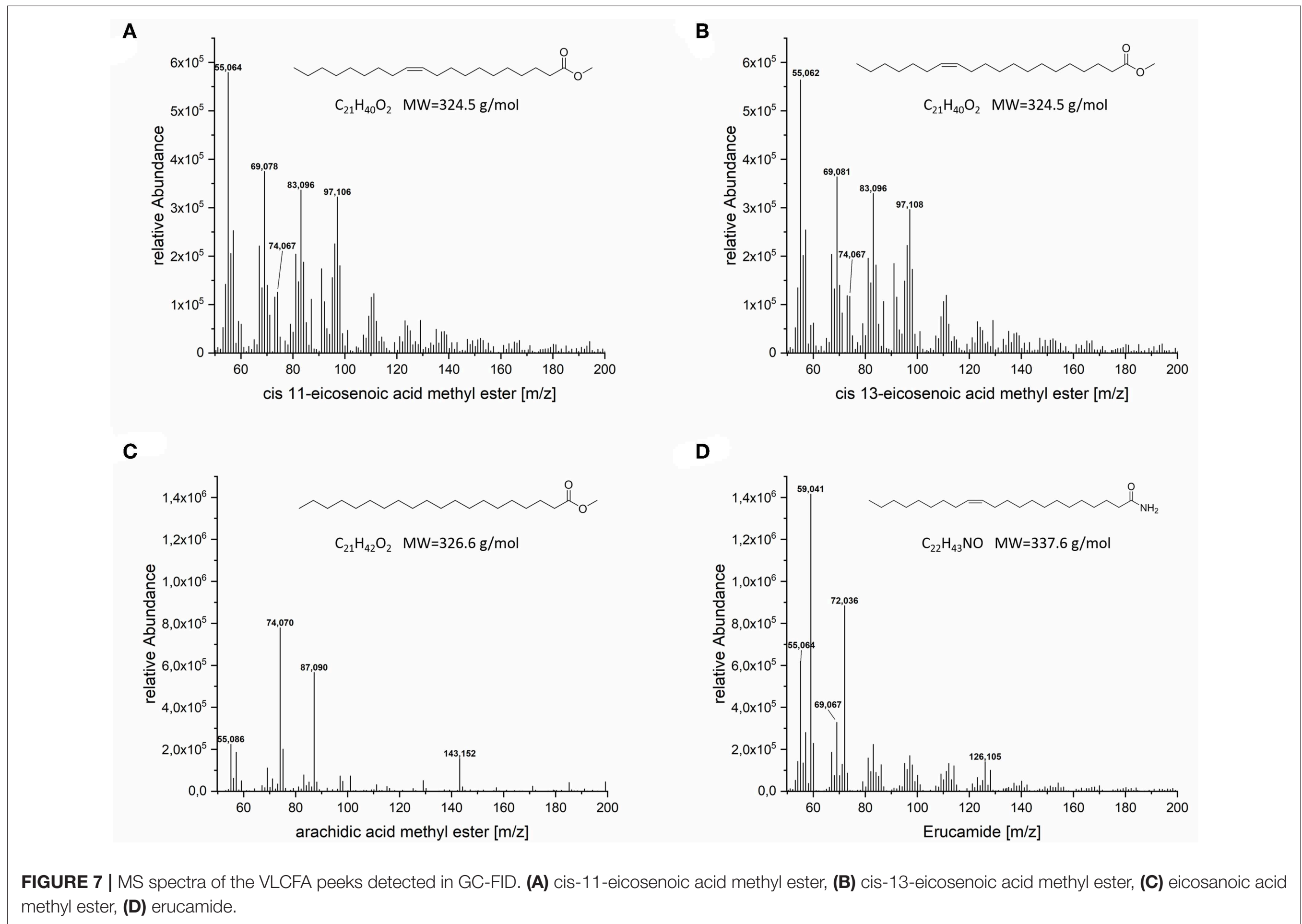

FIGURE 7 | MS spectra of the VLCFA peeks detected in GC-FID. **(A)** cis-11-eicosenoic acid methyl ester, **(B)** cis-13-eicosenoic acid methyl ester, **(C)** eicosanoic acid methyl ester, **(D)** erucamide.

required to further increase malonyl-coA levels and to create a metabolic sink for recombinantly generated fatty acids, thereby increasing space-time and total concentrations of tailored fatty acid products.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**. Additional data required is available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

EK and TB conceived the project. EK designed and performed the experiments. EK and NM analyzed the data. EK, NM, and TB prepared the manuscript. TB and NM supervised the whole work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2019.00408/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4. Discussion and Outlook

Arabidopsis thaliana (*A. thaliana*) is a weed plant that belongs to the *Brassicaceae* family of medium-sized crop flowering plants. Contrary to economically important members of the *Brassicaceae* family, which include cabbage, broccoli, mustard and turnip, *A. thaliana* is not considered to have agronomic significance. However, studies on *A. thaliana* as a model organism have had high impacts on advances in agro science, medical research, improving food security and plant biology. Several advantages have propelled *A. thaliana* to become a model organism for the study of the cellular, genetic and molecular biology of flowering plants. The genome of *A. thaliana*, consisting of 5 chromosomes, has been fully sequenced and extensively annotated in 2000. Compared to other plants, *A. thaliana* has a relatively short / fast life cycle that yields numerous self-progeny (seeds), requires minimal space and resources for cultivation, in addition to a relatively small genome. Consequently, *A. thaliana* is more easily and rapidly manipulated through concerted metabolic engineering approaches, including established genetic transformation methods, by utilizing *Agrobacterium tumefaciens*. Overall, *A. thaliana* has been designated as a reference for the study of fatty acid biosynthesis in plants [84].

As previously stated, fatty acid biosynthesis in both bacteria and plants occurs via the Fatty Acid Synthase type II (FAS II) system. Consequently, a multi-enzyme complex, encoded by several genes, performs the reactions required for the biosynthesis of fatty acids [66]. In the recent years, an increasing number of studies have aimed their work towards understanding and enhancing the FAS II system in *E. coli* and *A. thaliana* alike. Modulations in FAS II system of *A. thaliana* has led to the successful production of mutants [52,59,84-89]. The results of these studies were employed to enhance economically important crops, significantly impacting agro-science and agriculture [52,84,86,88]. The numerous advantages include: producing seeds with high oil content for the food, chemical, cosmetic and pharmaceutical industries, enhancing resistance to extreme weather conditions (fatty acid content, wax esters and TAG's), in addition to producing high value oleo-chemicals [52,84,86-89].

In *Escherichia coli,* various metabolic engineering strategies have been employed for the purpose of improving the rate of fatty acid biosynthesis, increasing the overall product yield, and enhancing the production of a specific chain length. Common approaches included the overexpression of the native *E. coli* FAS II enzymes or the deletion of genes in the beta-oxidation pathway, to prevent fatty acid degradation [3,8,14,33,38,39,46,69]. Other successful approaches included the expression of non-native FAS II genes from other prokaryotic and eukaryotic organisms, including *A. thaliana*

[38,46]. Deregulating the tight product inhibition of fatty acid biosynthesis, with the expression of an ACP-thioesterase, was reported to be the steppingstone for increasing fatty acid yields [90]. The expression of the leaderless version of the native *E. coli* thioesterase A (TesA) resulted in a 12- to 35-fold increase in free fatty acid yields, compared to wild type *E. coli* [91]. TesA has a broad substrate specificity towards fatty acids of 10 carbon chain length and higher [90]. Unlike TesA, the *A. thaliana* FATB ACP-thioesterase showed the highest specificity towards palmitoyl-ACP, and its expression in *E. coli* resulted in significant increase of palmitic acid in the fatty acid pool [91]. However, the expression of thioesterases must be finely tuned, as higher than optimal cytosolic levels of the enzyme has been shown to inhibit fatty acid biosynthesis in both *in vivo* and *in vitro* experiments [90,91].

Studies performed in *E. coli* strains, that lack the ability to degrade fatty acids, were achieved through the deletion of some of the genes responsible for or initiating the beta-oxidative degradation of fatty acids [91]. In *E. coli,* free fatty acids need to be esterified to a coenzyme-A before entering the beta-oxidative degradation pathway. The long-chain-fatty-acid—CoA ligase, FadD, is the only enzyme in *E. coli* that performs this function, hence initiating fatty acid degradation [92-94]. Subsequently, the generated fatty acyl-CoA undergoes a series of beta-oxidative reactions. The first is catalyzed by the Acyl-coenzyme A dehydrogenase, FadE [95]. Most studies report the use of an *E. coli* strain with a *fadD* deletion as a free fatty acid production strain, where free fatty acids accumulate in the cytosol. Further, *fadE* deletion strains were generated when the activation of free fatty acids to acyl-CoA esters was required for further product processing, such as the application of a delta-9-acyl-CoA desaturase for the production of delta-9 unsaturated fatty acids [91]. Other metabolically engineered approaches include the deletion of the gene coding for the long-chain fatty acid transport protein, *fadL*, to prevent the reuptake of excreted free fatty acids into the cell [95]. The expression of a thioesterase in *E. coli* strains with *fadD, fadE and fadL* deletions did not result in enhanced yields of free fatty acids [90,96].

Several studies have aimed their work towards the fine metabolic tuning of the *E. coli* FAS II system along the three phases of fatty acid biosynthesis: initiation, elongation and termination [3,14]. Such strategies were executed in combination with a thioesterase, or a strain lacking fatty acid degradation [8]. Some of the successful approaches included the finely tuned overexpression of the AccABCD genes, which was focused on increasing intracellular malonyl-CoA concentrations, to enhance the rate of fatty acid biosynthesis [97,98]. The equimolar expression of the ACC proteins with an improved acetate assimilation resulted in a 15-fold increase in malonyl-CoA levels, compared to wild type *E. coli* [99]. Another strategy, aiming at increasing malonyl-CoA levels,

involved the overexpression of the malonyl CoA-acyl carrier protein transacylase, FabD, along with *tesA*, yielding an 11% increase in total fatty acid concentrations compared to *tesA* overexpression alone [100]. Additionally, the overexpression of *fabD* alone did not yield a significant change in fatty acid titers, rather a slight shift in the fatty acid composition [101]. Furthermore, studies that report the overexpression of the native *E. coli* 3-oxoacyl-[acp] synthase 3, *fabH*, resulted in the cessation of fatty acid biosynthesis [102].

Extensive work has been done for modulating *E. coli* fatty acid pathway and improving total fatty acid titers. However, several bottlenecks in the FAS II system are yet to be addressed. In our study, we set out to examine the effect of the *A. thaliana* beta-ketoacyl-ACP I and II enzymes on the FAS II system in wild type *E. coli* BL21 (DE3) strain and in the *fadD* knock-out strain. We also demonstrated for the first time, that *A. thaliana* KAS II enzyme can complement the knocked-out homologue in *E. coli*, as restored levels of C18 were observed. In accordance with the literature, *fabF* knock-out strains were possible, by contrast *fabB* knock-out strains were non-viable. Interestingly, KAS II overexpression was not lethal and was able to complement the loss of endogenous FabF function and further enhance the titers of palmitoleic and cis-vaccenic acids. KAS I, however, resulted in fatty acid profile shifts towards palmitoleic and stearic acid respectively. Although plant-based/chloroplast KAS I and II share only one-third of its sequence identity with native *E. coli* FabB and FabF (Figure 5), this enduring functional complementation indicates strong conservation of the FAS II system across species. Figure 6 shows the crystal structures of the *E. coli* and *A. thaliana* proteins of interest respectively. Our approach in combining the strengths of that native *E. coli* FAS II enzymes with that of *A. thaliana* can be further modified with metabolic engineering efforts for the tailored production of high value oleo-chemicals at high titers, such as hydroxy-stearic acid, which is used a performance lubricant additive. Given the broad range of plant KASI and KAS II susceptibility, further studies should assess the host's resistance to low temperatures and the antimicrobial agent cerulenin [38].

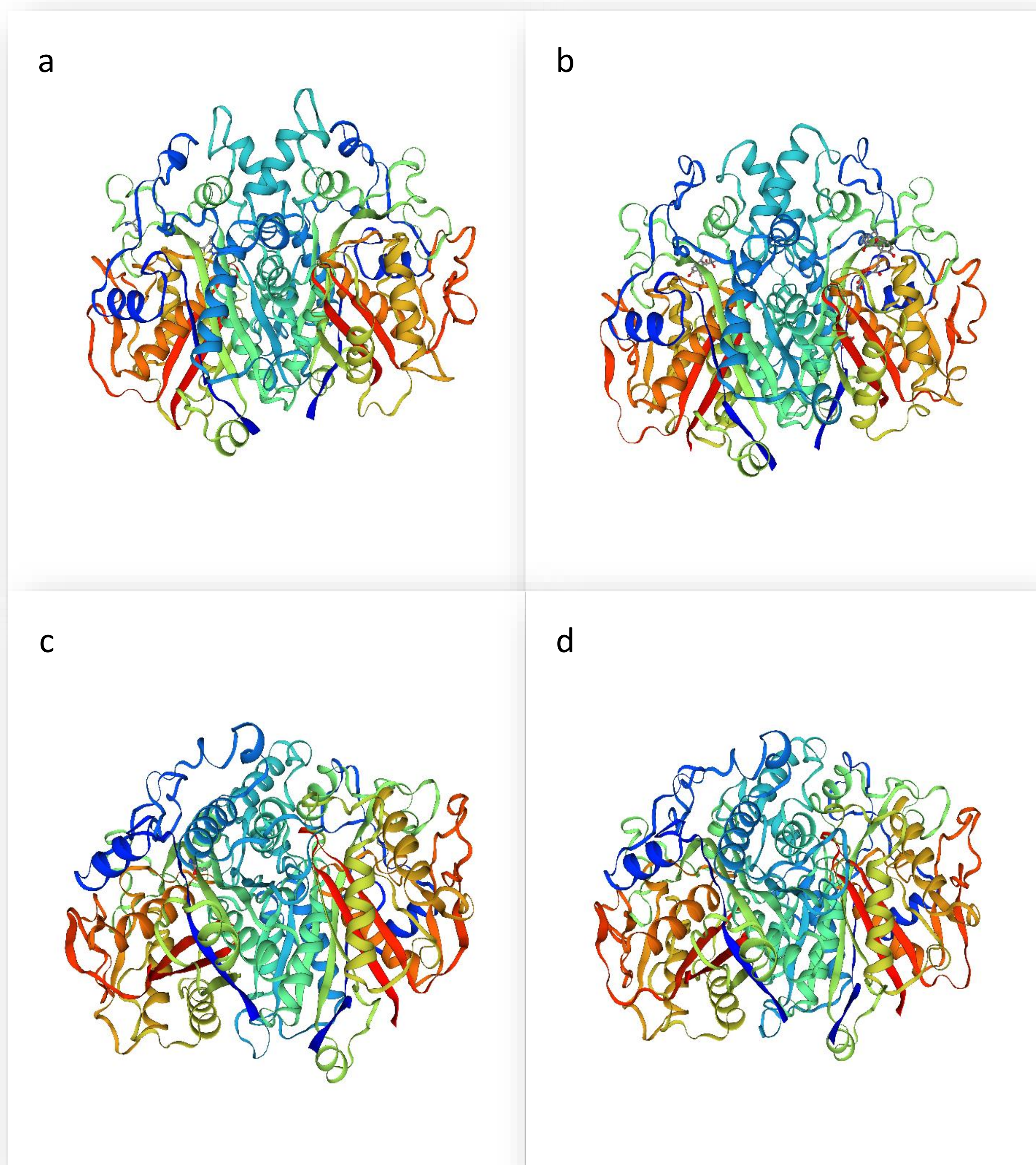

Figure 5: The crystal structures of *Escherichia coli* FabB (a), FabF (b), and *A. thaliana* KAS I (c) and KAS II (d) prepared via x-ray diffraction at 2.30, 2.40, 2.85 and 2.85 Å, respectively. The model of the structures was imported from Swissmodel.expasy.org [103-107].

Table 3. Summary of oleo-chemicals produced in *Escherichia coli* from different carbon sources [14].

	Chain-length	Yield (g/g)	Titer (g/L)	Carbons Source, Medium	Citations
MK	C_{11}-C_{17}	0.14 g/g fed glucose	3.4 g/L	20 g/L Glucose, M9-MOPS minimal medium	108
	C_{11}-C_{17}	0.054 g/g fed glucose	5.4 g/L	Glucose, M9-MOPS minimal medium, fed-batch	109
FAOH	C_6-C_{10}	0.084 g/g consumed glucose	0.42 g/L	20 g/L Glucose, minimal medium	110
	C_4-C_{16}	0.2 g/g consumed glucose	1.8 g/L	10 g/L Glucose, LB medium	111
FFA	C_{14}-C_{18}	0.28 g/g consumed glucose	~7 g.L	30 g/L Glucose, minimal medium	110
	C_8	0.17 g/g consumed glucose	1.7 g/L	4 g/L glycerol, LB medium	112
	C_6-C_{10}	0.0215 g/g fed glucose	0.43 g/L	20 g/L glycerol, modified MOPS minimal medium	113
	C_6-C_{10}	0.28 g/g consumed glucose	3.8 g/L	15 g/L glucose, modified MOPS minimal medium	114
	C_{10}	0.1 g/g fed glycerol	2.1 g/L	20 g/L glycerol, modified MOPS minimal medium	115
	C_{12}	0.1 g/g fed glycerol	0.4 g/L	4 g/L glycerol, LB medium	116
	C_{12}-C_{20}	0.26 g/g consumed glucose	5.2 g/L	20 g/L Glucose, M9 minimal medium	117
ALK	C_7	0.00035 g/g fed glucose	~0.0042 g/L	12 g/L Glucose, LB medium	118
	C_7-C_9	0.0001 g/g fed glucose	~0.0012 g/L	12 g/L Glucose, LB medium	118
	C_9-C_{13}	Not Reported	0.581 g/L	glucose, MR minimal medium, fed-batch	119
	C_{11}-C_{13}	0.000951 g/g fed galactose	0.0038 g/L	4 g/L arabinose, LB medium	120
	C_{13}-C_{17}	0.0213 g/g fed galactose	0.426 g/L	20 g/L glucose, M9 modified minimal medium	121
	C_{15}	0.00233 g/g consumed glucose	0.14 g/L	glucose, M9 minimal medium, fed-batch	122
FAEE	C_{10}-C_{14}	0.0159 g/g fed glucose	0.477 g/L	30 g/L glucose, MR minimal medium	119
	C_{12}-C_{18}	0.031 g/g fed sugar	0.682 g/L	20 g/L beechwood xylan and 2 g/L glucose, M9 minimal medium	123
	C_{12}-C_{18}	0.075 g/g fed glucose	1.5 g/L	20 g/L glucose, minimal medium	124
FAME	C_{12}-C_{14}	0.037 g/g fed glycerol	0.56 g/L	15 g/L glycerol, TB medium	125

Several studies carried the work further towards the modification and processing of free fatty acids in *E. coli*, to generate high value oleo-chemicals. In that respect, several plant and bacterial desaturases were successfully expressed in *E. coli*, for the production of unsaturated fatty acids [126,127]. These compounds have specific applications in the food, chemical and pharmaceutical industries. Other studies focused on the production of fatty acid ethyl esters and fatty acid methyl esters, as a potentially sustainable alternative sources for biofuel production [119,123,124]. Additionally, several fatty acyl-CoA reductases (in this case *A. thaliana*) were isolated and expressed in *E. coli* for the targeted production of fatty alcohols, which are main components in the production of detergents and surfactants [12]. Free fatty acids, generated in *E. coli*, are natively converted to diacylglycerols and stored in the membrane to maintain membrane homeostasis and survival [128,129]. The production of triacylglycerols in *E. coli* was also achieved with high specificity via the action of several diacylglycerol acyltransferase [130-132]. Other oleo-chemicals, biotechnologically produced in *E. coli*, such as alkanes and methyl ketones, are listed in Table 3.

Despite the broad range of oleo-chemicals produced in *E. coli*, the chain length of the end product determines its respective industrial application. For example, medium chain (C6-C12) fatty acid methyl esters (FAMEs) are favorable for the biofuel industry, whereas short chain (C1-C5) FAMEs are preferred for applications in the food and fragrance industries [3,8,14]. The scope of the above reported oleo-chemical production platforms has been confined to the chain length of the generated fatty acid profile of *E. coli.* In that regard, the FAS II system of *E. coli* is only capable of synthesizing fatty acids with a maximum chain length of 18 carbon atoms [8,66,133]. In our study, we focused on the elongation of free fatty acids towards the generation of very long chain fatty acids (VLCFAs; >C20), that make up precursors of high value oleo-chemicals. VLCFAs and their derivatives are crucial components of hydrophobic polymers, found on plant leaves surfaces, preventing water loss [46,134-137]. The industrial applications of very long chain fatty acids are diverse across several industries. Polyunsaturated very long chain fatty acids, such as omega-3- and omega-6-fatty acids, which constitute an essential supplement of the human diet, are sourced from marine crustaceans or fish oil for the food and pharma industry. However, alternative sustainable solutions for sourcing very long chain polyunsaturated fatty acids are required by industry to alleviate the environmental impact of conventional marine sources. Wax esters, derivatives of VLCFAs, serve several biological functions [138-141]. In humans, wax esters are the second major component of sebum (oil secreted by the human sebaceous gland). Such wax esters are essential components of cream and lotion formulations applied on the human skin, they help hydrate the human skin and enhance drug delivery to the epidermis [142]. Natural wax esters are commercially sourced from wax ester rich seed oils e.g. Jojoba or plant leaves e.g. carnuba.

Another industrially relevant VLCFA is erucic acid, which finds applications in the production of lubricants, detergents and coatings, while its amide derivative (erucamide) is used as an anti-block agent and in the production of plastic films [143-145]. High erucic acid rapeseed remain the major commercial source for erucic acid. Currently there are more than 1000 patented industrial applications for high erucic acid (C22:1) rapeseed oil, therefore alternative sources to crop sourced oils is required [140,141].

In that respect, several aspects still need to be addressed in the future for the optimal production of very long chain fatty acids and their derivatives in *E. coli.* One main aspect is to enhance the metabolic flux towards the production of malonyl-CoA, the key player in the *de novo* biosynthesis of fatty acids and their further elongation via the elongase complex [46]. Therefore, a successful strategy that can be employed would be the overexpression of the native *E. coli AccABCD* of *fabD* genes [97-99]. Additionally, enhancing the pool of long-acyl-CoA is also required to improve final yields [46]. In that regard, the expression of the *A. thaliana* KASI and KASII enzymes has resulted in a fatty acid profile shift towards long chain fatty acids [38]. At present, a combinatory approach employing both *A. thaliana* KAS I, KAS II and VLCFA elongase systems could be a promising strategy to increase final yields.

Although *E. coli* is not inherently an oleaginous organism that can accumulate fatty acids in high amounts, this study and others have demonstrated that it can be employed as a platform for the generation of high value substances. Very long chain fatty acids are mainly sourced from oil crop or petroleum resources. Because of their multiple applications across several industries, there is a high demand for these substances and their derivatives [46]. Additionally, the food, skin care and biofuel industries are in a rush to secure bio-based sources for these compounds, indirectly aggravating environmental and socioeconomic issues, such as increased land clearing and competition with food crops over arable land and valuable resources (freshwater irrigation) [17-20]. *E. coli* possesses several advantageous characteristics for oleo-chemicals production, including efficient growth at industrially relevant conditions, the ability to grow in minimal medium with inexpensive components, and the capability of utilizing a wide range of substrates including waste biomass. Furthermore, high productivities can also be achieved in *E. coli* due to its fast growth and tolerance to high concentrations of substrates and products [8,18,21-23]. So far, the utilization of *E. coli* as a microbial cell factory for the production of low volume high value fatty acids and their oleo-chemical derivatives could be a promising alternative for a sustainable future.

Oleaginous organisms including algae (i.e. *Microchloropsis salina*), yeast (e.g. *Yarrowia lipolytica* and *Cutaneotrichosporon oleaginosus*) and filamentous fungi (i.e. *Moritella alpine* and *Mucor*

circinelloides) are high volume producers that can accumulate lipids at more than 20% (w/w) of their total cellular weight [146-149]. One particular oleaginous yeast strain termed *C. oleaginosus* has developed into a workhorse for high titer microbial oil production due to its capacity to utilize multiple carbohydrate containing substrates including cheap and chemically complex biomass hydrolysates, including wheat straw, bran, wood and crustacean shell hydrolysates. Moreover, a recent publication demonstrated that under dual substrate cultivation *C. oleaginosus* generates oil yields in the order of 90% (w/w) of its dry cell weight. This high titer combined with improved oil recovery procedures allowed production of microbial lipids at the same cost as plant oil. In native *de-novo* oil producing organisms, such as oleaginous yeast like *C. oleaginosus* genetic engineering of tailored fatty acid profiles remains challenging due to the highly complex genome structure of these organisms and a lack of appropriate molecular biology tools [150]. To that end, this thesis demonstrated that the utilization and development of targeted molecular and biochemical tools for manipulation of fatty acid biosynthesis in non-native -oil generating, genomically well traceable organisms, such *E. coli*, are an efficient route for diversified production of low volume, high value oleo-chemical products.

5. List of Publications

Engineering Escherichia coli FAB system using synthetic plant genes for the production of long chain fatty acids.

Elias Kassab, Monika Fuchs, Martina Haack, Norbert Mehlmer and Thomas Brueck.

Microbial Cell Factories 18, 163 (2019).

https://doi.org/10.1186/s12934-019-1217-7

GFP Scaffold-Based Engineering for the Production of Unbranched Very Long Chain Fatty Acids in Escherichia coli with Oleic Acid and Cerulenin Supplementation.

Elias Kassab, Norbert Mehlmer and Thomas Brueck.

Frontiers in Bioengineering and Biotechnology 7 (408).

https://doi.org/10.1186/s12934-019-1217-7

Characterization of a new, recombinant thermo-active subtilisin-like serine protease derived from Shewanella arctica.

Farah Qoura, **Elias Kassab,** Steven Reisse, Garabed Antarikian and Thomas Brueck.

Journal of Molecular Catalysis B: Enzymatic 116:16

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6. Reprint Permission

1. **Engineering Escherichia coli FAB system using synthetic plant genes for the production of long chain fatty acids.**

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Engineering Escherichia coli FAB system using synthetic plant genes for the production of long chain fatty acids

Author: Elias Kassab et al

Publication: Microbial Cell Factories

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2. GFP Scaffold-Based Engineering for the Production of Unbranched Very Long Chain Fatty Acids in Escherichia coli with Oleic Acid and Cerulenin Supplementation.

Keywords: VLCFA, *Escherichia coli*, *Arabidopsis thaliana*, self-assembly GFP, fatty acid biosynthesis

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1- **Figure 3.** Illustration of different classes of fuel-like biomolecules in *E. coli*.

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2- **Table 3.** Summary of oleochemicals produced in *Escherichia coli* from different carbon sources

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7. Figures & Tables

Figure 1. Typical chemical routes for basic oleo-chemicals production from plant extracts and alternative microbial routes.

Figure 2. Illustration of different classes of fuel-like biomolecules in *E. coli*. Solid arrows indicate reactions catalyzed by a single enzyme. Dashed arrows represent pathways or multi-enzyme reactions.

Figure 3. Schematic of the pathways involved in fatty acid biosynthesis and fatty acid degradation.

Figure 4. Plasmid map of the pACYC plasmid harboring the VLCFA polycistronic cassette and the pET28a.

Figure 5: The crystal structures of *Escherichia coli* FabB (a), FabF (b), and *A. thaliana* KAS I (c) and KAS II (d) prepared via x-ray diffraction at 2.30, 2.40, 2.85 and 2.85 Å, respectively. The model of the structures was imported from Swissmodel.expasy.org.

Table 1: Oleo-chemicals, derivatives and applications.

Table 2. Overview of fatty acid classifications.

Table 3. Summary of oleo-chemicals produced in *Escherichia coli* from different carbon sources.

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