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Enhanced Fatty Acid Production in *Escherichia coli* by Over-Expression of NADPH Generating Enzymes

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Abstract: NADPH is an essential factor for Fatty Acid (FA) biosynthesis. The effect of NADPH generating enzymes [$NADP^+$-dependent malic enzyme (NADP-ME), $NADP^+$-dependent isocitrate dehydrogenase (NADP-IDH), Glucose 6-Phosphate Dehydrogenase (GPD) and 6-Phosphogluconate Dehydrogenase (PGD)] on FA biosynthesis was investigated in an engineered *Escherichia coli* BL21 ΔfadE/pTE. Among NADPH-generating enzymes, GPD and ICD increased total FA production by 60 and 16% respectively. Especially medium-chain FA production was increased up to 4.2 and 2.5 folds respectively. In addition, over-expression of the endogenous Thioesterase (TE) reduced Cyclopropane Fatty Acid (CFA) production by 40% and over-expressing of GPD and ICD further decreased CFA production by 23 and 30%, respectively compared to the TE over-expressed strain. Over-expression of TE appeared to be a good strategy to produce high quality biodiesel with medium-chain FAs as major Component (62%) and with Minimal Amount of CFA (4%). This study reveals that NADPH-generating enzyme GPD is associated with FA biosynthesis in *E. coli*, but not all reducing power generating enzymes, are involved in FA biosynthesis in bacteria.

Keywords: *Escherichia coli*, Fatty Acid, Malic Enzyme, Reducing Power

Introduction

To meet the increasing fuel demand and to provide an alternative source to the limited fossil fuel supply, the development of renewable energy has become increasingly urgent. Biodiesel such as Fatty Acid Ethyl Ester (FAEE) and Fatty Acid Methyl Ester (FAME), which has high energy density and low water solubility (Bharathiraja *et al*., 2017), is rapidly moving toward the mainstream of alternative energy sources of fossil diesel. However, biodiesel is usually derived from animal fats or plant oils (triacylglycerides, TAGs), which is accompanied by high cost and inadequate supply. This then limited the development of biodiesel production. Microbial oil production by fermentation using a cheap carbon source offers a promising approach for the cost-effective production of biodiesel. Oleaginous microorganisms can accumulate a great deal of lipids, but slow growth rate and complicated regulation mechanisms of these organisms limit their use in biodiesel production (Meng *et al*., 2011). *Escherichia coli* has become an alternative Fatty Acid (FA) producer because it possesses several attractive advantages such as a known genomic sequence, available genetic tools and a rapid growth rate.

Nevertheless, in wild-type *E. coli*, both FA biosynthesis and degradation are regulated by transcriptional and post-transcriptional controls (Magnuson *et al*., 1993). In addition, its strong product feedback inhibition also works to maintain membrane lipid homeostasis (Fujita *et al*., 2007). Extensive genetic studies have been carried out to increase FA production in *E. coli*. So far, the major reported genetic strategies include four aspects: (1) expressing endogenous or exogenous thioesterase genes in *E. coli* to provide the cell with a free FA sink (Cho and Cronan, 1995; Steen *et al*., 2010), (2) blocking FA β-oxidation pathway by knocking out *fadD* or *fadE* (Duan *et al*., 2011; Lu *et al*., 2008; Steen *et al*., 2010), (3) enhancing the availability of the precursor malonyl-CoA by over expressing the Acetyl-CoA Carboxylase (ACC) (Davis *et al*., 2000; Lee *et al*., 2013) and (4) manipulation of the multi gene FA biosynthesis pathway and its global regulator (Xu *et al*., 2013; Zhang *et al*., 2012). However, only a few researches have been paid to the effect of NADPH generating enzymes on FA synthesis in *E. coli*, which provide the reducing power in the chain elongation pathway.

The cofactor NADPH has generally been considered as exclusive reducing power for FA biosynthesis (Liu *et al*., 2010). There are several potential NADPH sources for FA biosynthesis (Fig. 1), such as $NADP^+$-dependent malic enzyme (NADP-ME, EC 1.1.1.40), Glucose 6-Phosphate Dehydrogenase (GPD, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44) and $NADP^+$-dependent isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42). The role of NADP-ME in filamentous fungi has been extensively studied (Ratledge and Wynn, 2002) and cytosolic NADP-ME appears to be the major NADPH provider for lipid accumulation. Also over-expression of NADP-ME leads to enhanced lipid accumulation in several fungi (Hao *et al*., 2014; Zhang *et al*., 2007). However, some oleaginous yeast such as *Yarrowia lipolytica* (Zhang *et al*., 2013a) and *Lipomyces starkeyl* (Tang *et al*., 2010) lack cytosolic NADP-ME, so most of the NADPH for FA biosynthesis is from Pentose Phosphate Pathway (PPP). Similarly, oleaginous microalga *Chlorella protothecoides* does not have a cytosolic NADP-ME activity and the PPP provided about 60% of NADPH for lipid biosynthesis, the rest of NADPH may be provided by NADP-ICDH (Xiong *et al*., 2010). Though, it is not clear whether this enzyme is in the cytoplasm or mitochondria. Cytosolic NADP-ICDH may be vital in FA biosynthesis when glucose is depleted from the culture (Varecza *et al*., 2006) or when the organism is grown on a non-fermentable carbon source (Minard and McAlister-Henn, 2005). However no clear evidence has been reported to link NADP-ICDH with FA biosynthesis in microorganisms, so far it is clear that NADP-ICDH plays a critical role in animal lipid metabolism (Koh *et al*., 2004).

The role of NADPH-generating enzymes on FA accumulation has barely been studied in bacteria. Questions such as, whether NADPH is a limiting factor for FA biosynthesis and whether some or all of the reducing power generating enzymes are involved in FA biosynthesis, remain elusive. It had reported in previous study (Meng *et al*., 2011) that over expression of NADP-ME and adding malate to the medium significantly enhanced the FA yield in *E. coli* BL21, which is likely to be an artifact as the bacterium cannot accumulate FAs without a free FA metabolic sink. In this study, we over-expressed NADP-ME, NADP-IDH, GPD and PGD in an engineered *E. coli* strain BL21 ΔfadE/pTE with a FA metabolic sink and investigated the effects of these reducing power generating enzymes on FA production.

Materials and Methods

Strains and Culture Medium

E. coli TOP10 was used for the plasmid manipulations and *E. coli* BL21 (DE3) ΔfadE, donated by Lu *et al*. (2008), was used for protein expression. *E. coli* strains were grown in Luria Broth (LB) medium at 37°C during strains construction and M9 minimal medium (per liter: 20 g glucose, 6 g Na_2HPO_4, 1 g NH_4Cl, 0.5 g NaCl and 0.24 g $MgSO_4$) for FA production. Antibiotics (50 mg L^{-1} kanamycin and 34 mg L^{-1} chloramphenicol) were used for screening.

Plasmids and Strains Construction

Standard protocols were followed for the DNA manipulations (Sambrook and Russell, 2001). All of the primers in this study are showed in Table 1. Genomic DNA was isolated from *E. coli* BL21 (DE3) and the *maeB* gene encoding NADP-ME was amplified from genomic DNA using NADPME-F and NADPME-R primers, digested by *Hin*d III and *Bam*H I and cloned into pET28a (+), resulting in pME. Genes *icd*, *zwf* and *gnd* (encoding NADP-IDH, NADP-GPD and NADP-PGD) were amplified from genomic DNA. PCR products were then cloned into pET28a (+) resulting in pIDH, pGPD and pPGD plasmids (Table 2).

A pTE plasmid was also developed to provide *E. coli* with a FA metabolic sink by expressing a cytosolic thioesterase (TesA'). The *tesA*' gene with deleted signal peptide coding sequence was amplified from genomic DNA using the TE-F and TE-R primers and inserted behind the T7lac promoter of pACYCDuet-1 between *Bam*H I and *Hin*d III restriction enzyme cutting sites to obtain the pTE plasmid pTE was co-transformed with each of the plasmids (pET28a, pME, pIDH, pGPD and pPGD) into *E. coli* BL21 ΔfadE to construct the recombinant strains, designated YS1-YS5 (Table 2).

Cultivation of E. coli Strains for FA Production

E. coli strains were cultured in 300 ml M9 medium (1 L flasks) with appropriate antibiotics at 37°C. The induction of recombinant proteins was achieved by adding 0.5 mM IPTG when the culture OD_{600} reached at 0.6. After induction, cell cultures were grown by shaking for 20 h. Cells were then harvested by centrifugation (6,000 g for 5 min) and washed twice with distilled water for further analysis.

Protein Determination and Enzyme Activity Assays in vitro

The harvested cells were re-suspended with buffer a (Song *et al*., 2001) and disrupted by sonication. Cell debris was removed by centrifugation and the supernatant served as a crude extract. Protein concentration was determined by the Bradford method with BSA as a standard and the supernatant was also separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The enzyme activity *in vitro* was determined spectrophotometrically by measuring the absorbance at 340 nm and 30°C. The specific methods used were NADP-ME (Hsu and Lardy, 1969), NADP-IDH (Kornberg, 1955), GPD (Kuby and Noltmann, 1966) and PGD (Ruda *et al*., 2010). At least three measurements for each enzyme activity were carried out to assess reproducibility.

Table 1. Primers used in this study

Genes	Primers	Sequences (5'-3')	Annealing temperature (°C)
maeB	NADPME-F	CGCGGATCCGATGACCAGTTAAAACAAAG	62.53
	NADPME-R	AGGCCCAAGCTTTTACAGCGGTTGGGTTTGCG	69.76
icd	IDH-F	CGCGGATCCGAAAGTAAAGTAGTTGTTCC	61.91
	IDH-R	AGGCCCAAGCTTTTACATGTTCTTGATGATCG	63.08
zwf	GPD-F	CGCGGATCCGCGGTAACGCAAACAGCCCA	71.89
	GPD-R	AGGCCCAAGCTTTTACTCAAACTCATTCCAGG	64.15
gnd	PGD-F	CGCGGATCCTCAAAGCAACAGATCGGCGTC	68.43
	PGD-R	AGGCCCAAGCTTTTAATCCAGCCATTCGGTATG	65.68
'*tesA*	TE-F	CGCGGATCCGGCGGACACGTTATTGATT	66.95
	TE-R	CCCAAGCTTTTATGAGTCATGATTTACT	56.23

The underlined portions indicate the restriction enzyme cutting sites

Table 2. Strains and plasmids used in this study

Plasmids	Characteristics	Sources
pET28a(+)	*ColE1(pBR322), Kmr, T7lac promoter*	Novagen
pME	pET28a(+) carrying *maeB*	This study
pIDH	pET28a(+) carrying *icd*	This study
pGPD	pET28a(+) carrying *zwf*	This study
pPGD	pET28a(+) carrying *gnd*	This study
pACYCDuet-1	P15A(pACYC184), *Camr,T7lac promoter*	Novagen
pTE	pACYCDuet-1 carrying '*tesA*	This study
E. coli BL21 (DE3)	*F-ompT, hsdSB(rB-mB-), gal(λcI857, ind1, Sam7, nin5, lacUV5-T7gene1) , dcm(DE3)*	Novagen
*E. coli TOP*10	*F- mcrA∆ (mrr-hsdRMS-mcrBC) Φ80 lacZ∆ M15 ∆ lacX74 recA1 ara∆ 139 ∆ (ara-leu) 7697 galUgalKrpsL(Strr) endA1 nupG*	Novagen
E. coli BL21(DE3)∆fadE	*E. coli* BL21 (DE3) lacking *fadE*	(Duan *et al*., 2011)
YS1	*E. coli* BL21 (DE3) ∆fadE/pTE/pET28a	This study
YS2	*E. coli* BL21 (DE3) ∆fadE/pTE/pME	This study
YS3	*E. coli* BL21 (DE3) ∆fadE/pTE/pIDH	This study
YS4	*E. coli* BL21 (DE3) ∆fadE/pTE/pGPD	This study
YS5	*E. coli* BL21 (DE3) ∆fadE/pTE/pPGD	This study

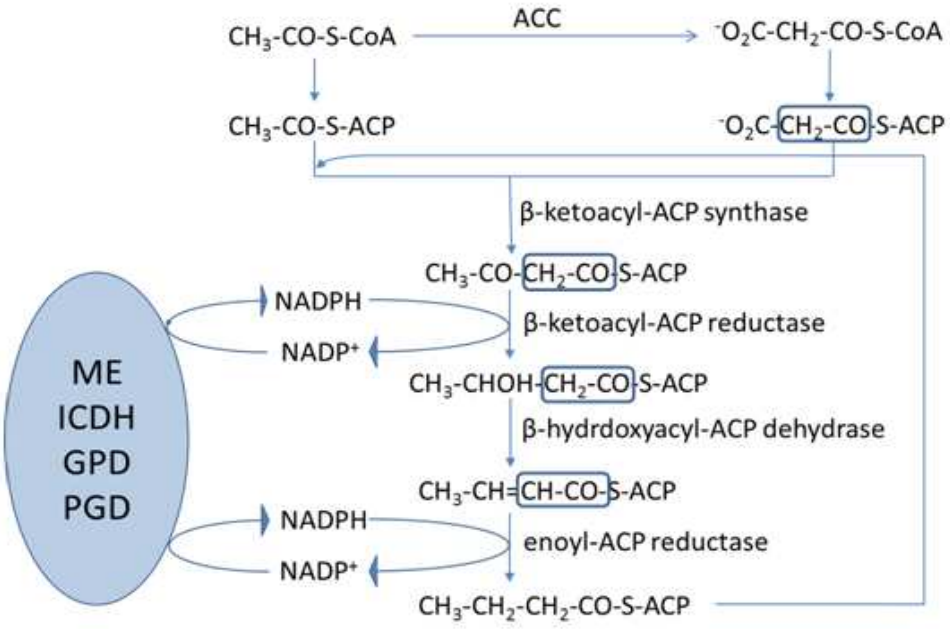

Fig. 1. FA biosynthesis pathway in *E. coli*. Reducing power NADPH, provided by ME, ICDH, GPD and PGD, is necessary for two reductive steps of FA biosynthesis pathway

Lipid isolation and FA analysis

The harvested cells after freeze-drying (100 mg) were subjected to lipids extraction by directly transmethylated according to Sakuradani and Shimizu (2003) with Pentadecanoic acid (C15:0, Sigma) as internal standard. FA Methyl Esters (FAMEs) were then analyzed by GC and GC/MS-MS according precious method (Hao *et al*., 2014).

Results

Recombinant Protein Expression in E. coli BL21 ΔfadE

To construct the strains with over expressed reducing power generating enzyme and a FA metabolic sink, the *E. coli* BL21 ΔfadE competent cells were co-transformed by pTE with each of the plasmids (pME, pIDH, pGPD and pPGD) designated YS2-YS5 respectively (Table 2). The pET28a and pTE plasmids were co-transformed into *E. coli* BL21 ΔfadE competent cells and the resulting strain was used as a control (YS1). The thioesterase (TesA') and reducing power generating enzymes were all induced by adding IPTG.

The expression level of these proteins was analyzed by SDS-PAGE (Fig. 2). All four proteins of NADP-ME, NADP-IDH, GPD and PGD were overproduced and the sizes of the induced proteins were in accordance with their estimated molecular weights 82, 46, 56 and 51 kDa, respectively (Fig. 2). A band of approximately 20 kDa was present in all strains which was consistent with the estimated size of TesA' according to its gene sequence (Fig. 2).

The *in vitro* enzyme activities were also assayed and compared with the control strain YS1 (Table 3). Over-expression of NADP-ME, IDH, G6D and PGD in the recombinant strains led to about 826, 11, 96 and 14 folds increase in the corresponding enzyme activity, respectively.

Over-Expression of NADPH Generating Enzymes Changed the FA Production

To investigate the effects of over expressing enzymes on FA profile, we determined the FA yields of the reconstructed strains after IPTG induction for 20 h (Fig. 3). In the control strain YS1, the total FA production was 212.3 mg/g Dry Cell Weight (DCW). Among the other four strains, Over-expression of GPD in strain YS4 improved the FA yield by 1.6 fold. Strain YS3 (ICD over-expressing strain) showed a slight increase (16%) in FA production, while YS5 strain had no significant difference with the control strain YS1. Surprisingly, over-expression of NADP-ME in strain YS2 reduced the FA production by 15% compared with the control strain.

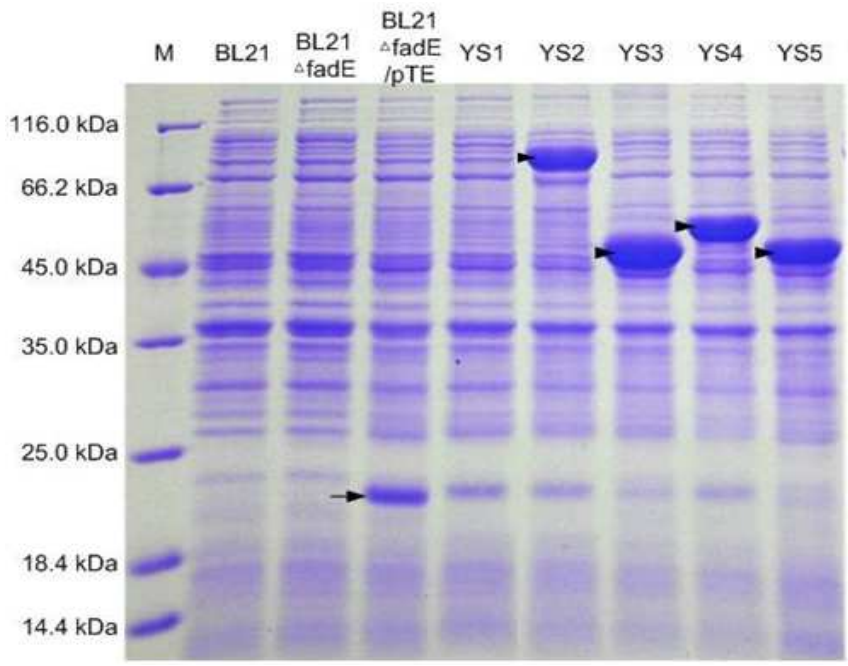

Fig. 2. SDS-PAGE analysis of overexpressed reducing power generating enzymes and 'TesA. The cells were cultivated in M9 culture medium for 20 h after induction and then harvested and broken for SDS-PAGE testing. The tags on the lanes stand for the different strains. The tailless arrows mark the overproduced reducing power generating enzymes and the normal arrow marks the 'TesA protein

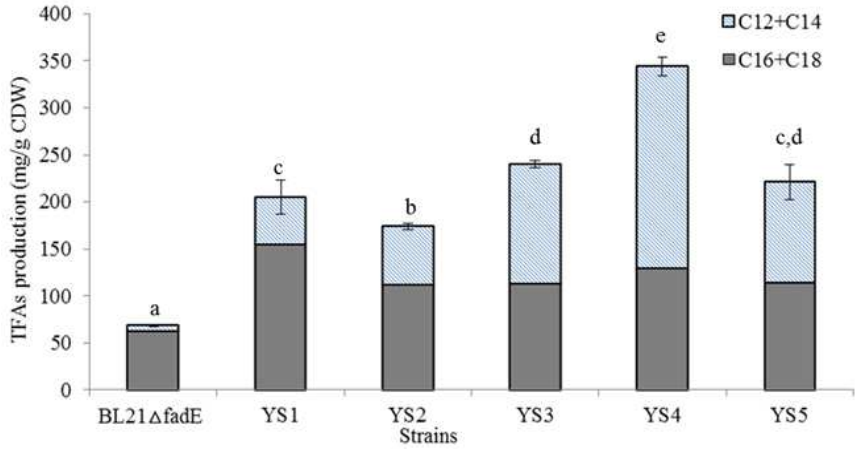

Fig. 3. Total Fatty Acids (TFAs) production in the control strain and five reducing power generating enzyme overexpressing strains. When OD_{600} reached 0.6, 0.5 mM IPTG was added to each strain culture. The cells were cultivated for 20 h after induction and harvested to analyze their FA production. The experiments were conducted in triplicate. The letters (a-e) on the columns denote significantly different (Duncan's multiple range test, $p<0.05$) FA productions

Table 3. Enzyme activity

	Enzyme activity (nmol.min^{-1}.mg protein^{-1})	
Enzyme	Control strain	Over-expressing strain
NADP-ME	12.5±0.8	9,915.6±231.5
IDH	750.6±23.3	8,468.3±354.4
GPD	247.2±16.1	23,628.6±869.3
PGD	127.1±2.6	1,805.6±106.0

Data are expressed as the means±SD of the three replicates

Table 4. Fatty acid composition of each *E. coli* strain

	Fatty acid composition (w/w, %)										
Strains	C12:0	C12:1	C14:0	C14:1	C16:0	C16:1	C17:0$^{\Delta a}$	C18:0	C18:1	C19:0$^{\Delta b}$	UFA/SFAc
BL21 ΔfadE	2.52±0.04	N.D.	6.98±0.02	0.15±0.01	44.52±0.06	0.64±0.02	25.48±0.12	0.49±0.05	1.11±0.01	18.15±0.07	0.02(0.84)
YS1	24.35±0.60	4.19±0.17	35.13±0.37	6.73±0.07	8.08±0.20	13.86±0.20	1.66±0.40	0.04±0.003	5.63±0.06	0.33±0.06	0.45(0.48)
YS2	7.08±0.44	0.41±0.05	26.47±0.68	1.87±0.05	25.98±0.39	12.86±0.23	6.20±1.33	0.21±0.05	16.50±0.55	2.43±0.38	0.46(0.67)
YS3	13.41±0.11	1.22±0.01	33.75±0.43	2.92±0.01	18.85±0.25	13.16±0.23	4.01±0.90	0.14±0.01	11.33±0.36	1.20±0.20	0.40(0.51)
YS4	18.77±0.30	1.31±0.24	37.52±1.02	4.81±0.05	12.23±0.39	13.63±0.47	2.90±0.70	0.08±0.01	7.55±0.27	1.12±0.38	0.38(0.46)
YS5	12.56±0.20	0.96±0.02	32.40±0.41	2.67±0.08	20.62±0.90	12.50±0.32	4.57±0.90	0.15±0.01	11.87±0.59	1.71±0.25	0.39(0.52)

a, C17:0$^{\Delta}$, cis-9,10-methylenehexadecanoic acid. b, C19:0$^{\Delta}$, cis-9,10-methyleneoctadecanoic acid. c, the ratio of total Unsaturated FA (UFA) to Saturated FA (SFA), the No. in the bracket showed the ratio when C17:0 and C19:0 cyclopropane FAs were treated as unsaturated FAs. N.D.= not detectable

Over-Expression of NADPH Generating Enzymes Changed the FA Composition

The FA compositions of the reconstructed strains were also analyzed by GC-MS and were shown in Table 4. In the *fadE*-knockout strain, C16:0 was the most abundant FAs (44.52%), followed by two CFA, cis-9,10-methylenehexadecanoic acid (C17:0$^{\Delta}$) and cis-9,10-methyleneoctadecanoic acid (C19:0$^{\Delta}$). Over-expression of thioesterase (control strain YS1) reduced the production of C17:0$^{\Delta}$ and C19:0$^{\Delta}$ greatly and as a result, increased C16:1 and C18:1 concentration, furthermore it also increased medium chain FAs (C12-C14) production. Over-expression of reducing power generating enzymes in TE genetic background leads to the further decrease of CFA production and massive increase of the medium FA production, especially GPD increased the medium FA production for about 300%. However the overall ratio of unsaturated FAs verses saturated FAs (UFA/SFA) was slightly decreased. The FA profile of NADP-ME over-expressing strain, which decreased the total FA production slightly, was similar to the control strain YS1.

Discussion

Genetic engineering of *E. coli* for the production of FAs or biodiesel has been studied extensively in the past decade and the yield of FA production in *E. coli* has been increased up to 8.6 g L^{-1} (Xu *et al.*, 2013), however, lipid content per cell dry weight is only about 21%, there is still much room to be improved. The main

genetic work has focused on reconstructing the metabolic pathway for FA production but not much works had been done to investigate the effect of NADPH generating enzymes on FA production. Although one report showed that over-expression of NADP-ME increased FA production in *E. coli* BL21 significantly (Meng *et al*., 2011), but this result was irreproducible in our study. We conducted a preliminary experiment with *E. coli* BL21 (DE3) as a host strain to over express NADP-ME and other reducing power generating enzymes to compare the FA production. In comparison with the control strain *E. coli* BL21/pET28a, the FA production of the over-expressing strains did not increase but rather presented modest decreases. Although the corresponding substrate of the enzymes was added to the culture medium, the FA production stayed the same as that of the cells without exogenous substrates (data not shown). In fact, genetically non-engineered *E. coli* does not accumulate FAs and FA metabolic pathways must be regulated tightly to maintain membrane lipid homeostasis. So what Meng *et al*. (2011) found is very likely an artifact. Liu *et al*. (2010) also suggested that under the physiological condition of *E. coli* XL100 lacking the *fadD* gene, the NADPH supply is unlikely to be the limiting factor in FA production. Therefore, only when *E. coli* was engineered to have a FA metabolic sink by genetic modifications such as over-expressing a TesA', then its FA production capacity can be manipulated by over-expression of NADPH-generating enzymes or other enzymes that regulate FA production.

In oleaginous eukaryotic microorganisms, only cytosolic NADPH-generating enzymes, such as ME, GPD and PGD, are involved in FA biosynthesis. However in prokaryotic microorganisms, as mitochondrion is absent, all NADPH-generating enzymes may be involved in anabolic pathways. In our work, among the NADPH-generating enzymes, GPD of PPP has most effectively increased the FA production (60%), ICD of TCA cycle slightly increased the FA production (16%). These results are consistent with the findings regarding genetic engineering of NADPH regenerators for enhanced production of GDP-L-fucose in *E. coli* as over-expression of GPD and ICD increased GDP-L-fucose production by 46, 31%, respectively (Lee *et al*., 2011). Also these findings appear to be true for other non-FA production pathways that need NADPH as reviewed by (Lennen *et al*., 2010). Solid evidences of the contributions of individual NADPH generating enzymes for anabolic metabolism has been found by metabolic flux analysis. Earlier work did by Sauer *et al*. (2004) showed that PPP, ICD and transhydrogenase provided 35-35, 20-25 and 35-45% NADPH respectively in *E. coli* MG 1655. A previous work conducted by He *et al*. (2014) showed that PPP, ICD and transhydrogenase provided 32, 8 and 60% NADPH respectively for biosynthetic pathway in a FA-over-production engineered strain (*E. coli* DH1).

Although it has generally been assumed that NADP-ME provides NADPH for the biosynthesis of FA in various eukaryotic microorganisms (Ratledge and Wynn, 2002), over expression of NADP-ME did not lead to increased FA production in this study. On the contrary, it decreased FA production for 15%. Similarly, a previous work showed that over-expression of NADP-ME in *E. coli* BL21 reduced GDP-L-fucose production by 24% metabolic flux analysis by He *et al*. (2014) has clearly showed that flux ratio through NADP-ME is negligible in both control and engineered *E. coli*, this suggests that NADP-ME is not involved in providing NADPH for FA biosynthesis. Potential role of NADP-ME has been suggested to provide NADPH for bacterial growth on two carbon compounds, such as acetate (Wang *et al*., 2011). Therefore, the extremely high expression of a "non-functional" NADP-ME in our experiment (the *in vitro* NADP-ME activity in strain YS2 was 826 times that in the control strain YS1) either burdened the host or caused an imbalanced metabolism in the FA pathway (Yu *et al*., 2011), which lead to the decreased production of FA.

Similar to previous reports (Cao *et al*., 2010; Voelker and Davies, 1994), a large amount of CFA was produced in fadE-knockout strain of *E. coli* BL21 cultivated for 20 h, which is commonly produced by methylenation of the unsaturated FA of membrane phospholipid in bacteria grown at late exponential to stationary phase. CFA inhibits the activity of glycerol 3-phosphate acyltransferase (Kito *et al*., 1972) and works as a double switch to control phospholipid biosynthesis at stationary phase when cell proliferation is arrested, which resulted in the accumulation of acyl-ACPs and leads to the inhibition of FA synthesis. However, when an endogenous Thioesterase (TE) was expressed in the cytoplasm of *E. coli*, the synthesized acyl-ACPs can be hydrolyzed to release free FAs and thus feed-back inhibition on FAS was removed, which lead to the great increase of FA production (about 200% increase). Furthermore, CFA production was greatly inhibited (40% decrease), while unsaturated C16:1 and C18:1 production was massively increased (55 fold) in TE over expressing *E. coli*. Similar results has been reported by Voelker and Davies (1994) who observed that CFA (C17:0) is greatly reduced, while unsaturated FAs in phopholipid fraction is largely increased in *E. coli* strain over expressing a plant acyl-ACP thioesterase. This is likely due to the redirection of acyl-ACPS flux to free FA synthesis and limited the production of phospholipids, the substrate for CFA production. However if we consider CFAs as unsaturated FAs (as CFAs are produced from membrane phopholipid carried unsaturated FAs), the ratio of UFA/SFA was even slightly decreased rather than increased.

The effect of TEs on FA production and FA composition has been extensively studied in *E. coli* (Fan *et al*., 2013; Jing *et al*., 2011; Zhang *et al*., 2013b) and medium chain acyl-ACP TEs appear to be efficient for the production of medium chain FAs. In this study, over-expression of endogenous TE in *E. coli* increased the production of medium-chain FAs (C12-C14) and long chain FAs (C16-C18) by 8 and 2 folds respectively. This is not in accordance with the nature of endogenous TE of *E. coli*, as it prefers long chain FAs (C16-C18) to medium-chain FAs (C12-14) as substrates (Spencer *et al*., 1978). This is likely due to the fact that TE hydrolyze the intermediate product of long chain acyl-ACPs and released the feed-back inhibition of ketoacyl-ACP synthase III and enoyl-ACP reductase in the elongation cycle (initiation) of FA biosynthesis (Richard and Rock, 1996). This lead to the activated initiation of FA synthesis, resulted in a deficiency of malonyl-CoA for the terminal elongation step, thus more medium-chain FAs were produced.

Over-expression of GPD in TE-over expressed *E. coli*, resulted in massive increase of medium chain FAs (3.2 fold increase), while the long chain FAs (C16-C18) production is slightly decreased (Fig. 3). This suggests that overproduced NADPH, a substrate for enoyl-ACP reductase, further activate FA biosynthetic pathway, especially the initiation step, which leads to a deficiency of malonyl-CoA for the terminal elongation step (Richard and Rock, 1996; Magnuson *et al*., 1993). Therefore long chain FA production is restricted and more medium chain FA production is increased. In addition, CFA production was further reduced by 23% in G8PD over expressed strains respectively. Thus, engineering *E. coli* by co-expression of TE and NADPH-generating enzymes can provide a good cell factory to produce FAs with medium chain FAs as major component (62%, of TFA) and minimal amount of CFA (4%, of TFA), which is excellent source for biodiesel production.

Free FAs have been detected in the culture medium in *E. coli* with expressing the TesA' enzyme (Davis *et al*., 2000). However, we did not detect any FA in the culture medium (cells were excluded), suggesting that overproduced FAs might be intracellular, which is not too high to be secreted to the culture medium. To maximize the FA yields of these strains, culture medium and cultivation conditions are necessary to be optimized.

Conclusion

Four strains were constructed with over expressed four reducing power generating enzymes in *E. coli* BL21 ΔfadE/pTE. Among the NADPH generating enzyme GPD of PPP increased FA production (60%) more than ICD of TCA cycle (16%). Over-expression of endogenous TE in *E. coli*, not only increased the total FA production, but also increased unsaturated FA content and medium-chain FA (C12-C14) production. In addition, it greatly decreased CFA production at stationary phase. Over-expressing of NADPH-generating enzymes, especially GPD decreased CFA production. More importantly, it extraordinarily increased the production of medium-chain FAs, possibly because of activation of FA initiation by its synergistic action with TE.

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Author's Contributions

Huaiyuan Zhang: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Yongshuang Zhou: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Haiqin Chen: Designed the research plan, organized the study.

Yong Q. Chen: Designed the research plan, organized the study.

Yuanda Song: Designed the research plan, organized the study and amended the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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