Metabolic Engineering 13 (2011) 713–722

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Efficient free fatty acid production in *Escherichia coli* using plant acyl-ACP thioesterases

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ARTICLE INFO

Article history:
Received 15 March 2011
Received in revised form
20 September 2011
Accepted 22 September 2011
Available online 6 October 2011

Keywords:
Fatty acid production
Acyl-ACP thioesterases
Fatty acid composition
E. coli

ABSTRACT

Microbial biosynthesis of fatty acid-like chemicals from renewable carbon sources has attracted significant attention in recent years. Free fatty acids can be used as precursors for the production of fuels or chemicals. Free fatty acids can be produced by introducing an acyl–acyl carrier protein thioesterase gene into *Escherichia coli*. The presence of the acyl-ACP thioesterase will break the fatty acid elongation cycle and release free fatty acid. Depending on their sequence similarity and substrate specificity, class FatA thioesterase is active on unsaturated acyl-ACPs and class FatB prefers saturated acyl group. Different acyl-ACP thioesterases have different degrees of chain length specificity. Although some of these enzymes have been characterized from a number of sources, information on their ability to produce free fatty acid in microbial cells has not been extensively examined until recently. In this study, we examined the effect of the overexpression of acyl-ACP thioesterase genes from *Diploknema butyracea*, *Gossypium hirsutum*, *Ricinus communis* and *Jatropha curcas* on free fatty acid production. In particular, we are interested in studying the effect of different acyl-ACP thioesterase on the quantities and compositions of free fatty acid produced by an *E. coli* strain ML103 carrying these constructs. It is shown that the accumulation of free fatty acid depends on the acyl-ACP thioesterase used. The strain carrying the acyl-ACP thioesterase gene from *D. butyracea* produced approximately 0.2 g/L of free fatty acid while the strains carrying the acyl-ACP thioesterase genes from *R. communis* and *J. curcas* produced the most free fatty acid at a high level of more than 2.0 g/L at 48 h. These two strains accumulated three major straight chain free fatty acids, C14, C16:1 and C16 at levels about 40%, 35% and 20%, respectively.

1. Introduction

Microbial biosynthesis of fatty acid-like chemicals from renewable carbon sources as well as metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants has attracted significant attention in recent years (Alonso et al., 2010; Lu et al., 2008; Radakovits et al., 2011; Petrie et al., 2010; Paul et al., 2011). Free fatty acids can be used as precursors for the production of fuels or chemicals (Nikolau et al., 2008; Lennen et al., 2010; Liu et al., 2010; Steen et al., 2010; Handke et al., 2011).

Under normal conditions, the biosynthesis of fatty acid is highly regulated (Fujita et al., 2007; Cronan and Thomas, 2009). Unlike most oleaginous microorganisms or plants, which accumulate significant quantity of free fatty acids naturally, *Escherichia coli* normally does not accumulate free fatty acids as intermediates in lipid biosynthesis (Voelker and Davies, 1994). Wild type *E. coli* strains produce fatty acids mainly for the biosynthesis of lipids and cell membranes. Fig. 1A shows the fatty acid biosynthesis pathways together with a simplified central aerobic metabolic pathway of *E. coli* using glucose as a carbon source. Free fatty acids can be produced by introducing an acyl–acyl carrier protein (ACP) thioesterase (TE) gene (see Fig. 1A, dotted box) into *E. coli*. The presence of the acyl-ACP thioesterase will break the fatty acid elongation cycle and release free fatty acids (Jiang and Cronan, 1994; Cho and Cronan, 1995; Davies et al., 1993; Lu et al., 2008; Cantu et al., 2010). Furthermore, it has been reported that fatty acid overproduction induces the fatty acid degradative pathways (Voelker and Davies, 1994).

An acyl-ACP thioesterase terminates fatty acyl group extension by hydrolyzing the acyl moiety from the acyl-ACP at the appropriate chain length, releasing free fatty acids (Shine et al., 1976; Salas and Ohlrogge, 2002; Thelen and Ohlrogge, 2002). Depending on their sequence similarity and substrate specificity, class FatA

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doi:10.1016/j.ymben.2011.09.007

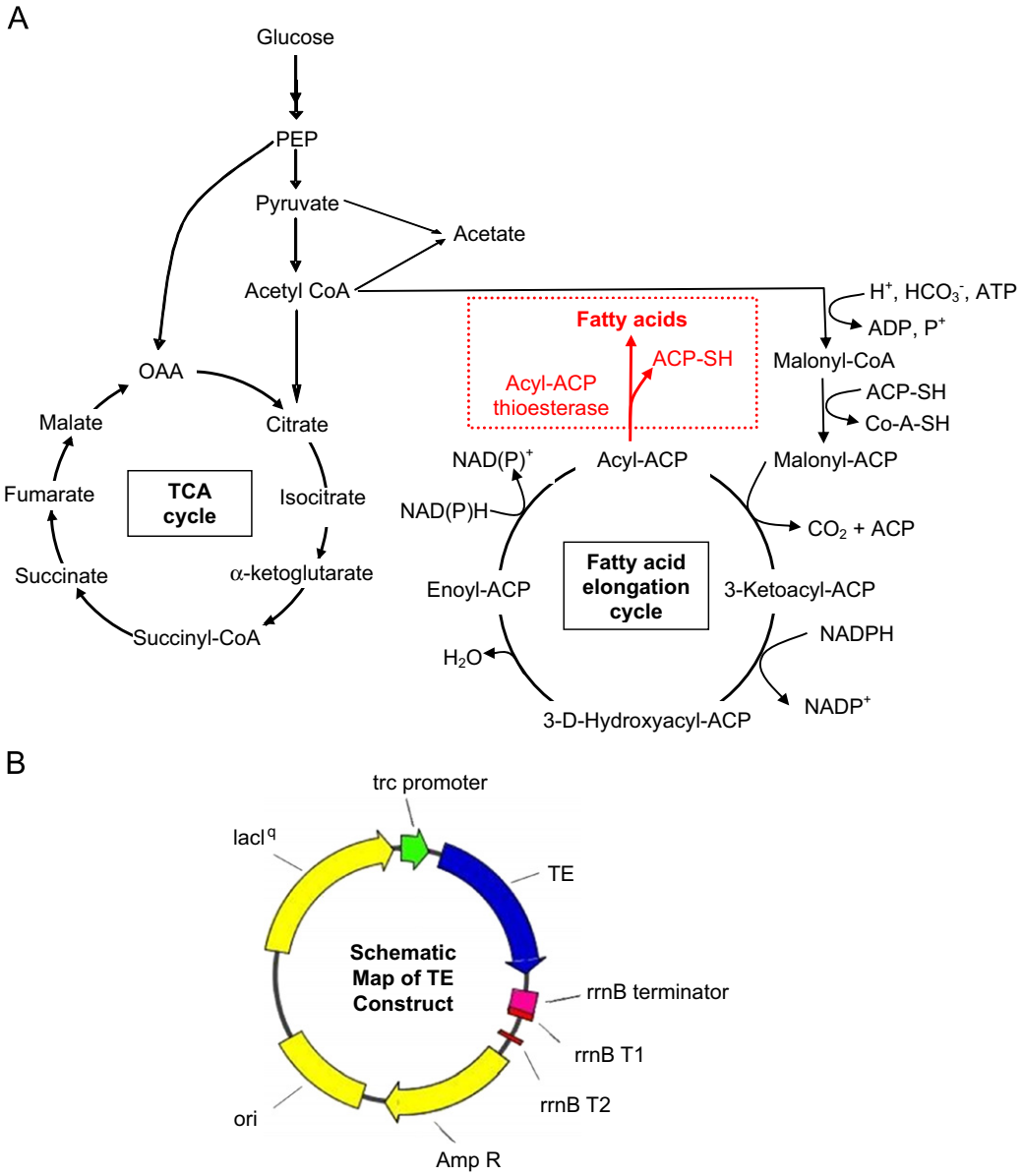

Fig. 1. (A) Simplified central aerobic metabolic pathway of *Escherichia coli*, including the free fatty acid production pathway. (B) Schematic of the plasmid construct.

thioesterase is active on unsaturated acyl-ACPs and class FatB prefers saturated acyl groups (Salas and Ohlrogge, 2002). Different acyl-ACP thioesterases have different degrees of chain length specificity (de Renobales et al., 1980). Although some of these enzymes have been characterized from a number of sources, information on their ability to produce free fatty acids in microbial cells has not been extensively examined until recently. In this study, we examined the effect of the overexpression of acyl-ACP thioesterase genes from *Diploknema butyracea*, *Gossypium hirsutum*, *Ricinus communis* and *Jatropha curcas* on free fatty acid production. In particular, we are interested in studying the effect of different acyl-ACP thioesterase on the quantities and compositions of free fatty acid produced by an *E. coli* strain ML103 carrying these constructs. It is shown that the amount of free fatty acid accumulated depends on the acyl-ACP thioesterase used. The strain carrying the acyl-ACP thioesterase genes from *D. butyracea* produced the quantity of free fatty acid (>0.2 g/L) while the strains carrying the acyl-ACP thioesterase genes from *R. communis* and *J. curcas* produced the most free fatty acid, more than 2.0 g/L at 48 h.

2. Materials and methods

2.1. Strains

An *E. coli* strain ML103 was used as the host strain in this study.

2.2. Plasmid construction

Four acyl-ACP thioesterase genes from *Diploknema butyracea* (GenBank accession number AAX51636), *G. hirsutum* (GenBank accession number Q9SQI3), *R. communis* (GenBank accession number XM002515518) and *J. curcas* (GenBank accession number ABU96744) were codon optimized, and chemically synthesized by Epoch Life Sciences (Sugarland, TX). Acyl-ACP thioesterase genes

from these plants were chosen mainly based on efficient oil accumulation in the plants seeds or related literature (Jha et al., 2006; Yoder et al., 1999; Devendra and Raghavan, 1978; Wu et al., 2009). The synthesized genes were subcloned into the cloning vector pTrc99a. The expression of the thioesterases gene is under the control of a strong inducible *trc* promoter system. The fidelity of the inserts was reconfirmed based on DNA sequencing results. The schematic of the plasmid construct is shown in Fig. 1B and strain and plasmid constructs used in this study are listed in Table 1.

2.3. Inoculum preparation

A single colony from a freshly transformed plate was grown for 10 h in 2 mL of LB medium supplemented with 100 mg/L ampicillin

Table 1
List of strains and plasmids used.

Strain name	Relevant genotype	Source or reference
MG 1655	*F⁻ lambda⁻ ilvG⁻ rfb⁻ rph⁻*	ATCC 47076
ML 103	MG1655(*fadD⁻*)	Li et al. (Submitted for publication)
Plasmid Name		
pTrc99a	pTrc99a, cloning vector	Amersham Pharmacia
pXZ16	pTrc99a carries an acyl-ACP thioesterase from *Diploknema butyracea* (GenBank accession number: AAX51636)	This work
pXZCO16	pTrc99a carries an acyl-ACP thioesterase from *Gossypium hirsutum* (GenBank accession number: Q9SQI3)	This work
pXZ18	pTrc99a carries an acyl-ACP thioesterase from *Ricinus communis* (GenBank accession number: XM002515518)	This work
pXZJ18	pTrc99a carries an acyl-ACP thioesterase from *Jatropha curcas* (GenBank accession number: ABU96744)	This work

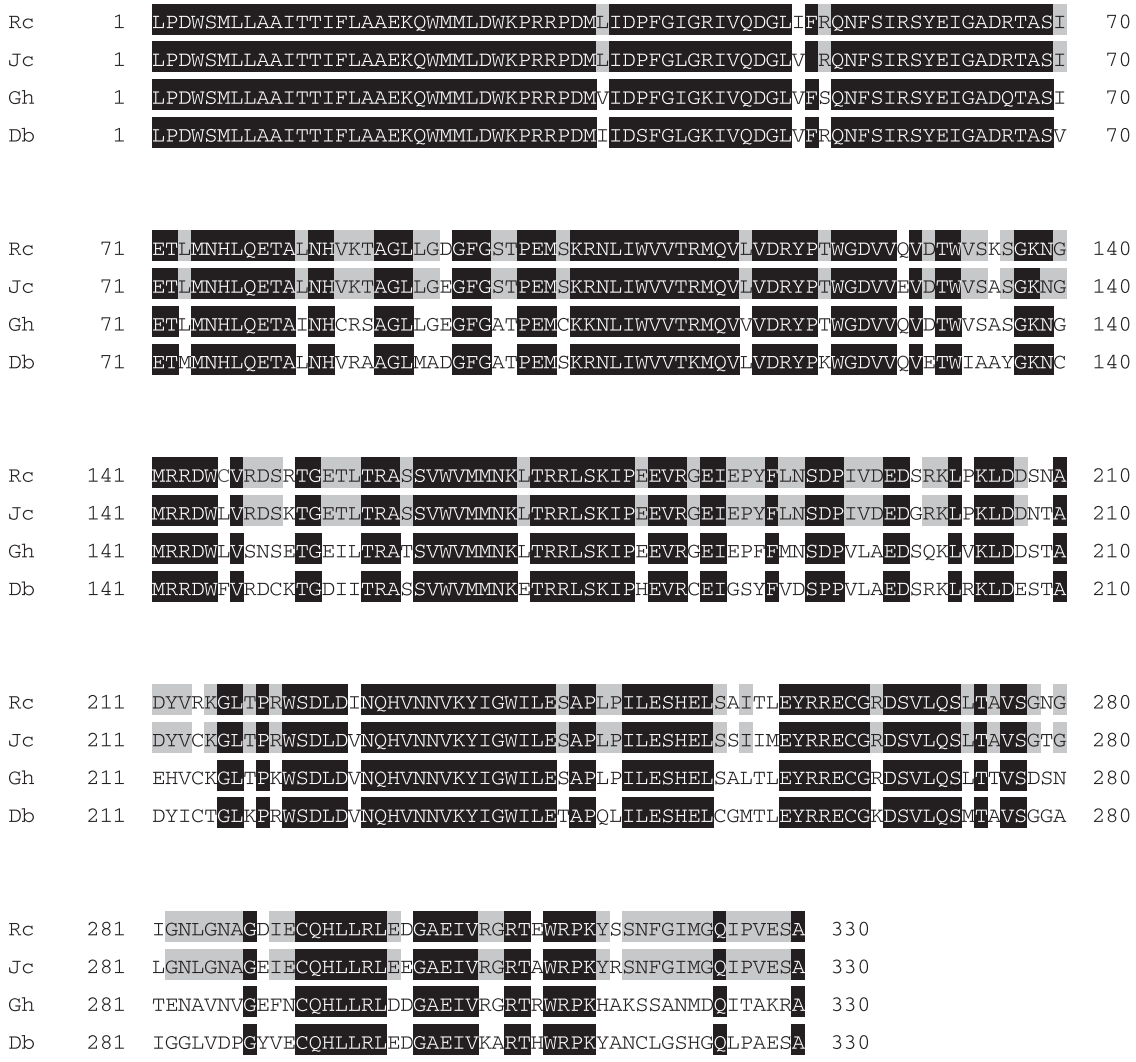

Fig. 2. Amino acid sequence alignment of *Ricinus communis* (Rc), *Jatropha curcas* (Jc), *Gossypium hirsutum* (Gh), and *Diploknema butyracea* (Db). Identical amino acids are shaded in black with additional identical amino acids between *R. communis* and *J. curcas* shaded in gray.

(Fisher BioTech) in an orbital shaker (New Brunswick Scientific, NJ) operated at 250 rpm and 37 °C.

2.4. Shake flask cultures

Luria–Bertani (LB) broth medium supplemented with 15 g/L of glucose and an appropriate quantity of ampicillin was used for aerobic cultivations. The expression of the acyl-ACP thioesterase was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was inoculated into a 250 flask containing 40 mL of the culture medium at a final OD600 of 0.01. The cells were then cultivated in an orbital shaker (New Brunswick Scientific, NJ) operated at 250 rpm and 30 °C. This temperature was chosen because we observed strains carrying the plant acyl-ACP thioesterase are not stable at 37 °C. This observation is consistent with an early study by Voelker and Davies (1994). Samples were taken at four time points (16, 24, 36, and 48 h) for fatty acid and extracellular metabolite analysis.

2.5. Analytical techniques

Optical density was measured at 600 nm with a spectrophotometer (Bausch & Lomb Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl. Previously established HPLC methodology was used to analyze the extracellular metabolites (Dittrich et al., 2005; Lin et al., 2005). Briefly, 1 mL of culture was centrifuged and the supernatant was then filtered through a 0.45-μm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems, Shimadzu, Colombia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, CA), a UV detector (Shimadzu SPD-10A) and a differential refractive index (RI) detector (Waters 2410, Waters, Milford, MA). A mobile phase of 2.5 mM H_2SO_4 solution at a 0.6 mL/min flow rate was used and the column operated at 55 °C. Standards were prepared for glucose, succinate, acetate, ethanol, lactate and pyruvate for both the RI detector and UV detector, and calibration curves were created. Metabolites such as glucose, acetate, ethanol and lactate were measured by the RI detector and pyruvate was measured by the UV detector at 210 nm.

2.6. Total fatty acid analysis

Cell cultures were harvested and prepared for fatty acid analysis, as described earlier (Voelker and Davies, 1994). Authentic standards were used to quantify individual fatty acids, and a pentadecanoic fatty acid (C15) was added as an internal standard in all samples. The fatty acid content of each sample was quantified by GC–FID/MS system (GC/MS QP 2010 from Shimadzu

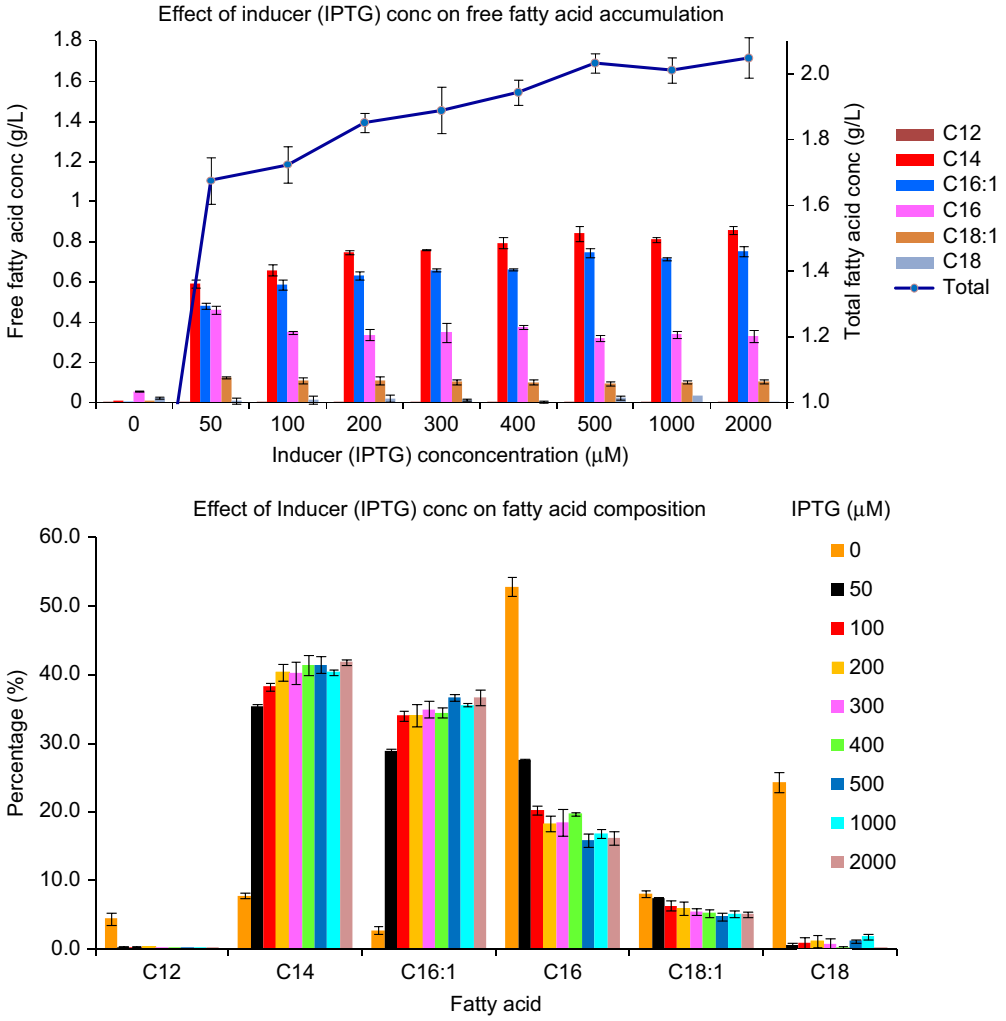

Fig. 3. Accumulation and compositions of fatty acids by the strain ML103 (pXZ18) at different IPTG concentrations. The strains were grown in 250 mL flasks, with 40 mL Luria-Bertani (LB) broth medium supplemented with approximately 15 g/L of glucose, varied IPTG concentrations, and appropriate amount of ampicillin. The cultures were grown in a rotary shaker at 250 rpm and 30 °C. Samples of the media were taken 48 h after inoculation. The data shown are means ± standard deviation for triplicate experiments.

Scientific, MD) using a single quadrupole mass spectrometer with an electron impact ionization source and a FID detector. The GC–FID/MS system was also equipment with two auto-injectors. Two identical DB-5MS columns (30 m × 0.25 mm × 0.25 μm, Agilent Co., USA) were directly connected to either the flame ionization detector (FID) or the mass spectrometer (MS). During analysis, two identical samples were injected to each column individually and sequentially. We found out that this configuration yielded better data quality than the single column split detector configuration. Helium was used as the carrier gas and the flow rate was set at 1 mL/min. The oven temperature was initially held at 50 °C for 2 min. Thereafter, the temperature was raised with a gradient of 4 °C/min until the temperature reached to 220 °C. This temperature was then held for 10 min. Other system settings were as follows: 280 °C interface temperature, 250 °C ion source temperature, and electron impact ionization (EI) was set 0 kV relative to the tuning result. Mass spectra were analyzed by full scan mode. Raw mass spectrometry data were processed using the program GCMS post run analysis to obtain a spectrum and to identify a peak together with running the standards.

3. Results and discussions

3.1. Effect of IPTG concentrations

The effect of the inducer (IPTG) concentrations on the free fatty acid accumulation was examined using the strain ML103(pXZ18) in shake flasks with 40 mL LB broth supplemented with 15 g/L of glucose and varied concentrations of IPTG at 30 °C. The uninduced culture produced very little of free fatty acid, about 0.1 g/L. Upon induction, the total free fatty acid accumulated exhibited a dosage response behavior up to 500 μM of IPTG. Beyond 500 μM, the cultures accumulated similar quantity of free fatty acid at approximately 2.0 g/L (Fig. 3). The observation that the free fatty acid accumulation with increasing IPTG concentration suggested that the production of fatty acid is not limited by precursor availability but rather the acyl-ACP thioesterase activities. The low free fatty acid reported in the literature (Jha et al., 2006; Steen et al., 2010) might be due to a less active acyl-ACP thioesterase. The free fatty acid composition changes drastically between the uninduced and the induced cultures. The percentages of C14 and C16:1 straight chain fatty acids increase significantly. The percentage of the C14 straight chain fatty acid increased from less than 10% to more than 35% while the C16:1 increased from less than 5% to about 30% (Fig. 3). At the same time, the percentage of C16 and C18 dropped drastically from more than 50% to about 30% and from more than 20% to less than 1%, respectively. Further increase in the IPTG concentrations resulted in further increase in the percentage of C14 and C16:1 components and further decrease in the C16 percentage. The composition of the free fatty acid stabilized to steady levels for IPTG above 200 μM.

3.2. Effect of different thioesterases from different source on total fatty acid accumulations

Four acyl-ACP thioesterases from four different plants are selected for this study. Based on the reported amino acid sequence in GenBank, the acyl-ACP thioesterases of *R. communis* (Rc), *J. curcas* (Jc), *G. hirsutum* (Gh) and *D. butyracea* (Db) are codon optimized and chemically synthesized. The amino acid sequence alignment of these four acyl-ACP thioesterases is shown in Fig. 2 with identical amino acids are shaded in black with additional identical amino acids between *R. communis* and *J. curcas* shaded

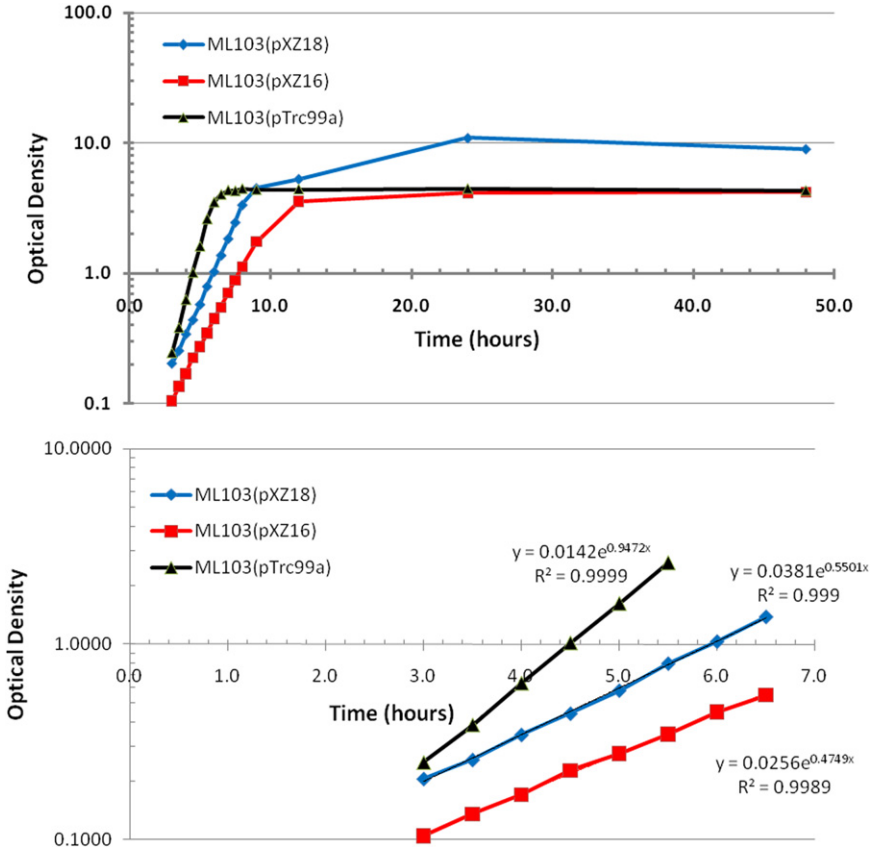

Fig. 4. Growth curve of ML103(pXZ18), ML103(pXZ16) and ML103(pTrc99a) strains. The strains were grown in 250 mL flasks, with 40 mL Luria-Bertani (LB) broth medium supplemented with approximately 15 g/L of glucose, 1 mM IPTG, and appropriate amount of ampicillin. The cultures were grown in a rotary shaker at 250 rpm 30 °C.

in gray. The four acyl-ACP thioesterases show high sequence similarity at the N-terminal domain which was reported to have effect on substrate specificity (Mayer and Shanklin, 2005).

The effect of acyl-ACP thioesterase overexpression on cell growth was examined. The specific growth rates of ML103(pXZ18) and ML103(pXZ16) during the exponential phase were much lower than that of the control strain, ML103(pTrc99a), Fig. 4. These results are expected as cells will have to divert resources to produce the thioesterase and to produce the free fatty acids. The final optical density of strain ML103(pXZ16) is similar to that of the control strain. The strain ML103(pXZ18), however, has a much higher final optical density than the control strain (Fig. 4).

The total free fatty acids produced by the four different acyl-ACP thioesterases together with the control (the host ML103 carrying the cloning vector pTrc99a) were shown in Fig. 5A. The four acyl-ACP thioesterases exhibited drastically different behaviors in terms of the quantities of free fatty acid produced over time. The control strain ML103(pTrc99a), which carries the cloning vector pTrc99a produced very little free fatty acids, less than 0.2 g/L (Fig. 5A). The ML103(pXZ16) strain which carries an acyl-ACP thioesterase from *D. butyracea* produced slightly more free fatty acids, about 0.5 g/L at around 24 h. This level is more than the reported free fatty acid production using the *E. coli* acyl-ACP thioesterases TesA reported by Steen et al. (2010) and others (Jiang and Cronan, 1994; Lu et al., 2008). The amount of free fatty

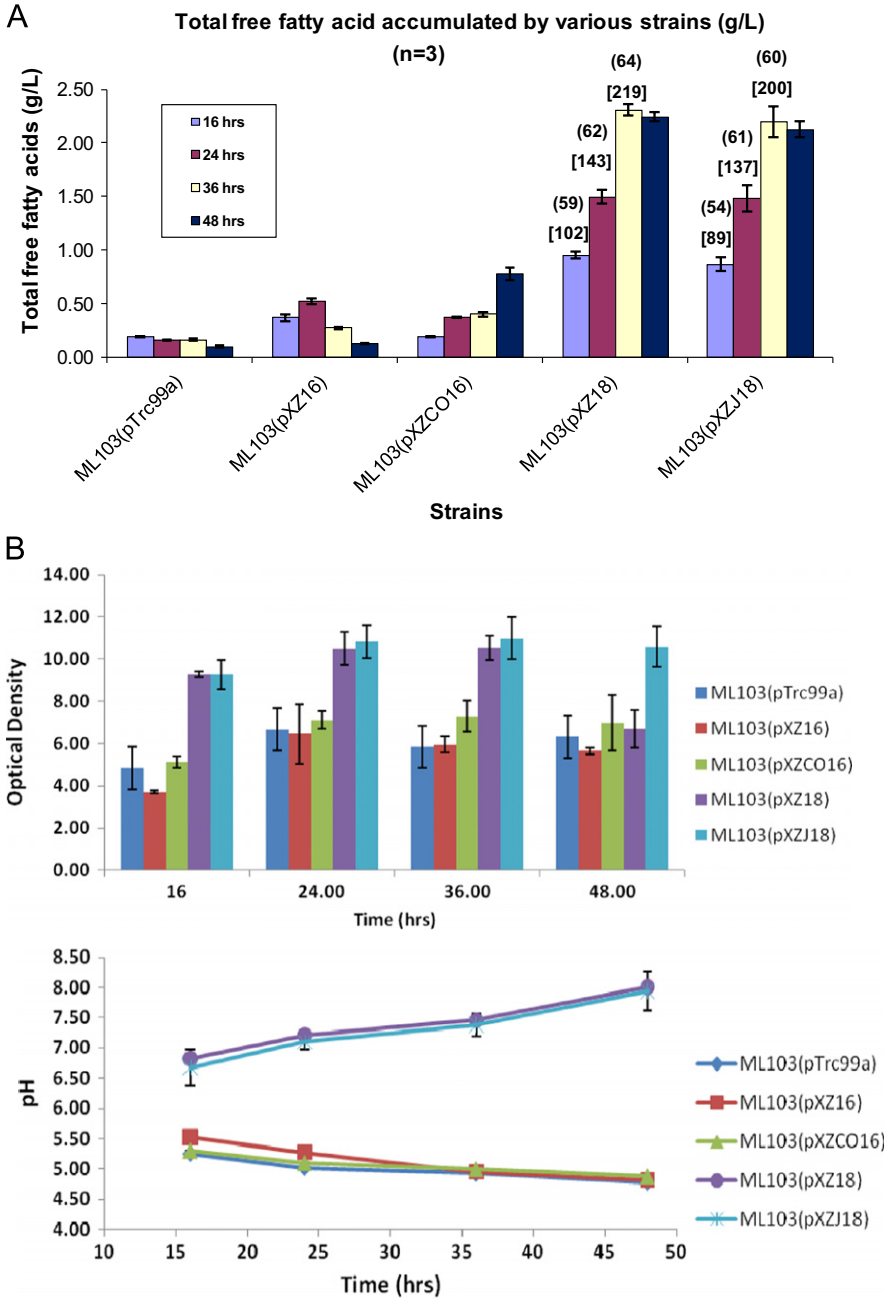

Fig. 5. (A) Accumulation of fatty acids by various strains carrying an acyl-ACP thioesterase. The strains were grown in 250 mL flasks, with 40 mL Luria–Bertani (LB) broth medium supplemented with approximately 15 g/L of glucose, 1 mM IPTG, and appropriate amount of ampicillin. The cultures were grown in a rotary shaker at 250 rpm 30 °C. Samples of the media were taken at 16, 24, 36 and 48 h after inoculation. The data shown are means ± standard deviation for triplicate experiments. Numbers in parenthesis are the average free fatty acid production rates in mg/L/h and numbers in square brackets are the average free fatty acid produced per OD in mg/L/OD at 16, 24, and 36 h for the two high producing strains. (B) Optical density and culture pH of various strains carrying an acyl-ACP thioesterase at 16, 24, 36 and 48 h.

acids dropped to less than 0.2 g/L at 48 h (Fig. 5A). The ML103(pXZCO16) strain carrying an acyl-ACP thioesterase from *G. hirsutum* produced slightly more free fatty acid at 48 h, approximately 0.8 g/L (Fig. 5A). The free fatty acid production by the strain ML103 (pXZ18) which carries an acyl-ACP thioesterases from *R. communis* is very similar to that of ML103 (pXZJ18), which carries an acyl-ACP thioesterases from *J. curcas*. Both strains produced extremely high level of free fatty acid about 1.5 g/L at 24 h and more than 2.1 g/L at 36 h. The free fatty acid concentrations for both strains dropped slightly at 48 h. The average free fatty acid production rates at 16, 24, and 36 h for the two high producing strains, ML103(pXZ18) and the ML103(pXZJ18), are very similar around 60 mg/L/h (Fig. 5A). The average free fatty acid produced per OD (mg/L/OD) at 16, 24 and 36 h for the two high producing strains however showed an increasing trend (Fig. 5A). The average free fatty acid produced per OD increased from about 100 mg/L/OD at 16 h to more than 200 mg/L/OD at 36 h. The increase in the free fatty acid produced per OD suggests the cells are producing fatty acid even at the stationary phase since the optical densities of these two strains did not change much (Fig. 5B). The pH profiles of the control and the low producing strains are very similar showing a decreasing trend from about 5.5 at 16 h to below 5.0 at 48 h. The decrease in pH is mainly due to acetic acid accumulations (Fig. 6). Interestingly, the pH profiles of the two high producing strains showed an opposite trend, increasing from 6.5 at 16 h to 7.5 at 48 h (Fig. 5B).

The protein sequence of the acyl-ACP thioesterases from *R. communis* showed high sequence similarity to the acyl-ACP

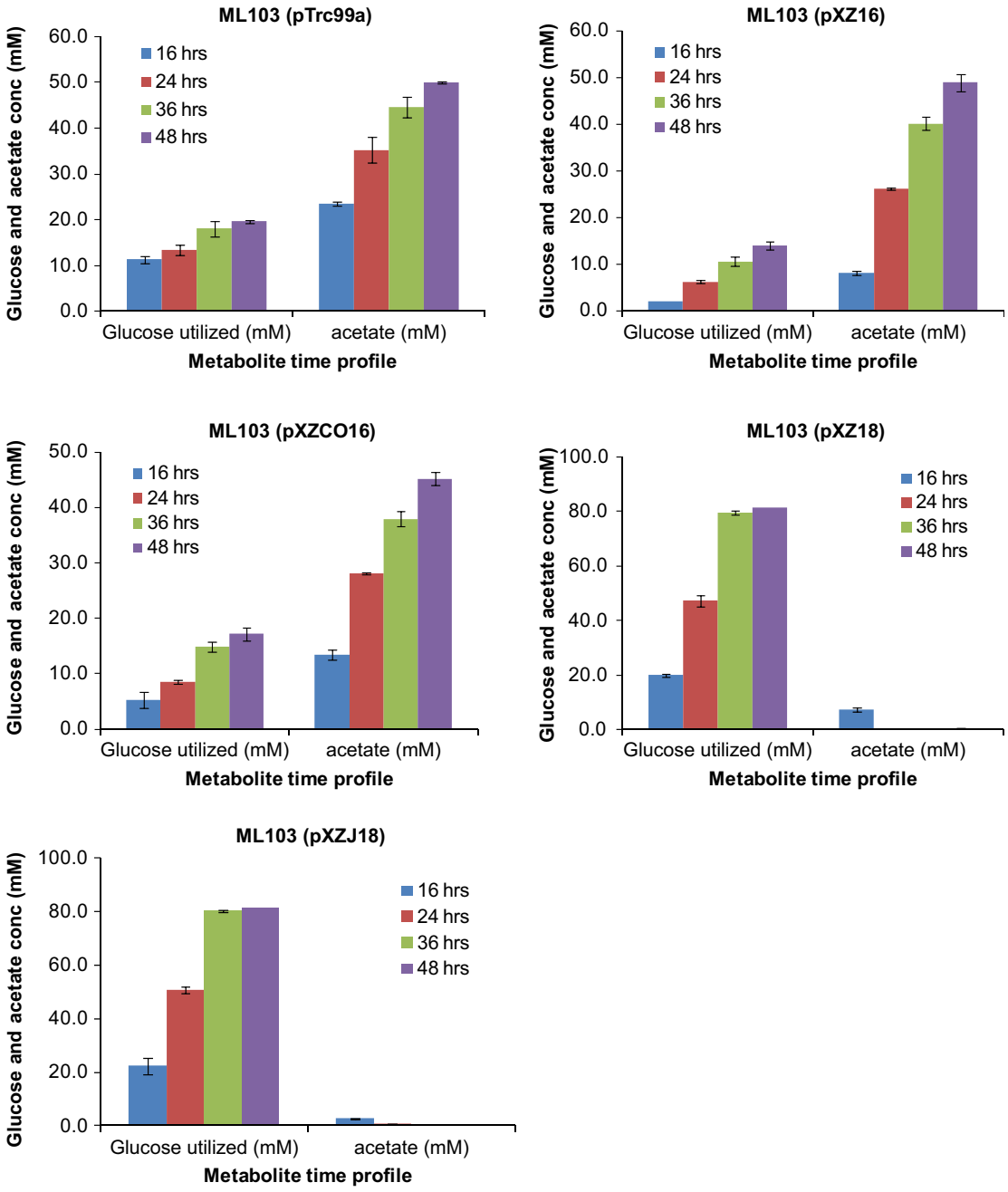

Fig. 6. Glucose utilization and acetate accumulations. Samples of the media were taken at 16, 24, 36 and 48 h after inoculation. The data shown are means ± standard deviation for triplicate experiments.

thioesterases from *J. curcas*. (Fig. 2). The relatively high free fatty acid production by *R. communis* and *J. curcas* acyl-ACP thioesterases appears to suggest the malonyl-CoA carboxylation activity, which is commonly accepted as the limiting step in fatty acid biosynthesis, is not the limiting when the free fatty production is at around 0.3 g/L level. Furthermore, this high sequence similarity might explain why both acyl-ACP thioesterases yielded very similar free fatty acid production results. The ability of *R. communis* and *J. curcas* acyl-ACP thioesterases to synthesize large quantities of free fatty acids may greatly facilitate the development of production processes of free fatty acid based biochemical products from renewable sources.

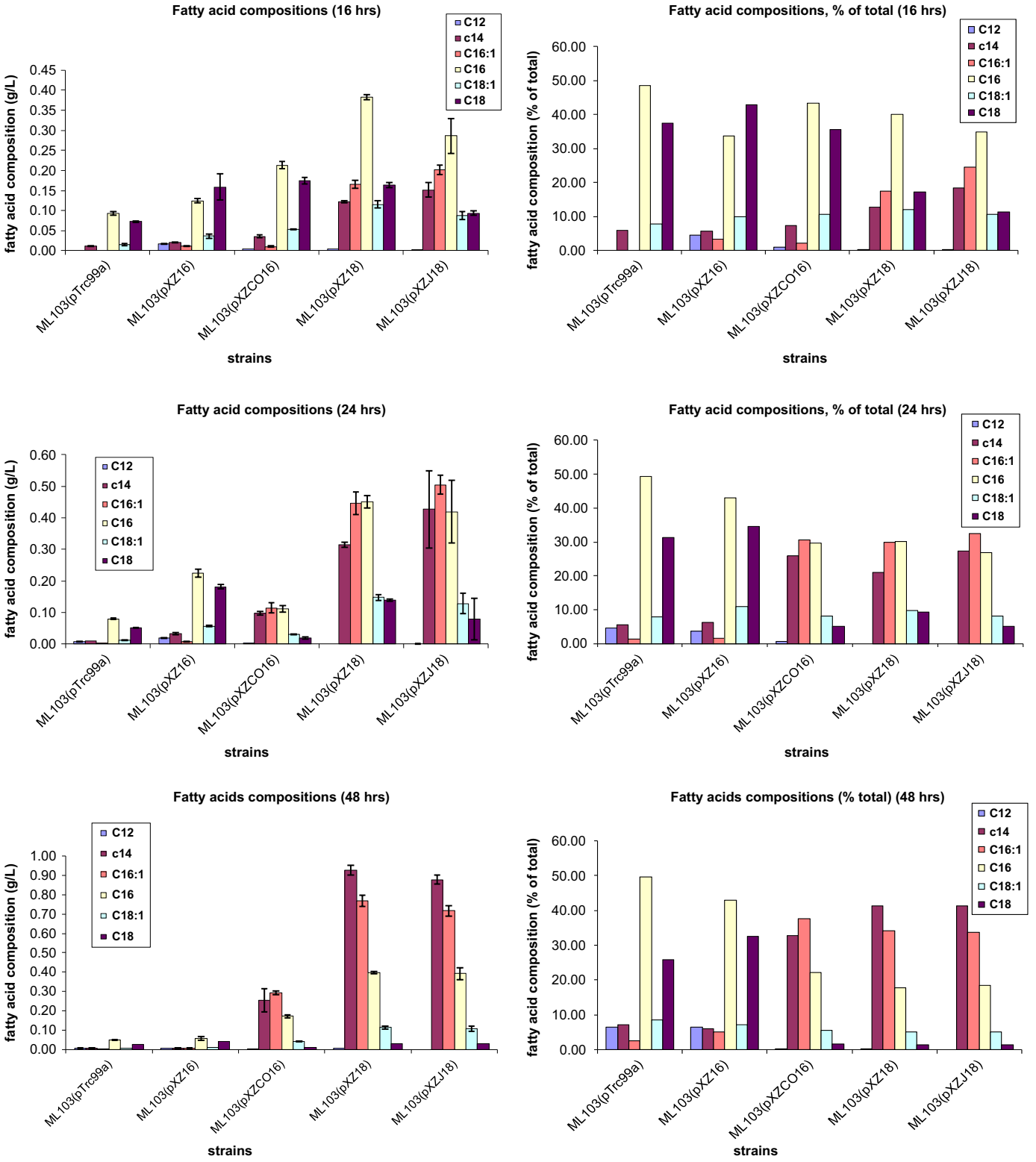

Fig. 7. Effect of acyl-ACP thioesterase on free fatty acid compositions at 16, 24 and 48 h.

3.3. Glucose utilization and acetate accumulations

The four acyl-ACP thioesterases also showed different glucose utilization and acetate accumulation patterns. The moderate free fatty acid producing strains ML103 (pXZ16) and ML103 (pXZCO16) as well as the control strain ML103 (pTrc99a) showed similar trends of moderate glucose consumption and high acetate accumulation (Fig. 6). The amount of glucose consumed by these moderate producing strains increased with time and consumed less than 20 mM of glucose at the end of 48 h. The acetate accumulation also simultaneously increased with time reaching a final level of around 50 mM at 48 h. On the other hand, the two high free fatty acid producing strains ML103 (pXZ18) and ML103 (pXZJ18) utilized glucose readily and consumed all the available glucose in the flask within 36 h. Interestingly, unlike the control strain and the moderate fatty acid producing strains, these two high producers did not accumulate detectable quantities of acetate except at the 24 h sampling point where a very low level of acetate, less than 10 mM, was recorded. One of the possible reasons for the no to low acetate accumulation observed in the high free fatty acid producing cultures may be due to a strong pull of acetyl-CoA pool to the fatty acid synthesis pathways through the formation of malonyl-CoA. Similar concept of diverting excessive precursors produced from the glycolysis pathways to other products in order to reduce acetate accumulation was demonstrated previously by Aristidou et al. (1994). In their study acetoin was produced instead of acetate through the overexpression of acetolactate synthase from *Bacillus subtilis*.

3.4. Free fatty acid compositions

The free fatty acid compositions (in g/L and in percentage of the total free fatty acid) of the five strains are shown in Fig. 7. The free fatty acid composition from the control strain ML103 (pTrc99a) mostly composed of C16 and C18 straight chain fatty acids. The pattern did not change much over time with C16 fatty acid being the most abundant followed by C18. The free fatty acids produced by the strain ML103 (pXZ16) are also mainly straight chain C16 and C18. However, C18 fatty acid is slightly more than that of the C16 at the earlier 16 h sample. At the 24 and 48 h samples, the free fatty acid composition patterns are very similar to that of the control strain with C16 slightly more than that of C18. The free fatty acid compositions by the strain ML103 (pXZCO16), however, change over time. At the earlier 16 h sampling, the free fatty acids are mainly composed of C16 and C18, with C16 slight more than the C18. At the 24 h time point, the composition of the free fatty acids changed significantly; the percentage of C18 dropped from more than 30% at 24 h to around only 5% while both C14 and C16:1 increased from less than 10% to around 30%. As a result, C14, C16:1 and C16 are the three major free fatty acids, which accounted for more than 85% of the total free fatty acid, produced at 24 h. At 48 h, both C14 and C16:1 increased slightly while C16 dropped to only around 20% (Fig. 7). The free fatty acid compositions of the two high producing strains ML103 (pXZ18) and ML103 (pXZJ18) showed similar pattern over time. The compositions of the high producing strains are quite different from the other strains. Even at the earlier 16 h sampling, while C16 was still the most abundant species, the percentage of C14 and C16:1 in the sample was already relatively high, around 10–20% each. At 48 h, the percentage of C14 further increased to about 40% of the total free fatty acids while C16:1 changed only slight but C16 dropped to less than 20%.

The changes in the compositions of the free fatty acid produced over time by the two high producing strains appear to follow a general trend in that the percentage of C14 increases with cultivation time while the percentage of C16 decreases with time. A possible explanation may be due to changes in cellular physiology at different growth phases. These changes in term might lead to variations in the compositions of the precursor pools presented to the acyl-ACP thioesterases. It suggests that further studies are needed to gain insight into the interplay between these composition changes and the growth phase and growth environment.

4. Summary

We have examined the effect of different acyl-ACP thioesterase on the quantities and compositions of free fatty acid produced by an *E. coli* strain ML103 carrying the thioesterase gene from four different plants. It is shown that the amount of free fatty acid accumulated depends on the acyl-ACP thioesterase used. The strain carrying the acyl-ACP thioesterase genes from *D. butyracea* produced the quantity of free fatty acid (>0.2 g/L) while the strains carrying the acyl-ACP thioesterase genes from *R. communis* and *J. curcas* produced the most free fatty acid, more than 2.0 g/L at 48 h. These two strains accumulated three major straight chain free fatty acids, C14, C16:1 and C16 at levels about 40%, 35% and 20%, respectively.

Acknowledgments

This work was supported in part by the National Science Foundation (BES-0420840; EEC-0813570).

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