Microbial Synthesis of Wax Esters

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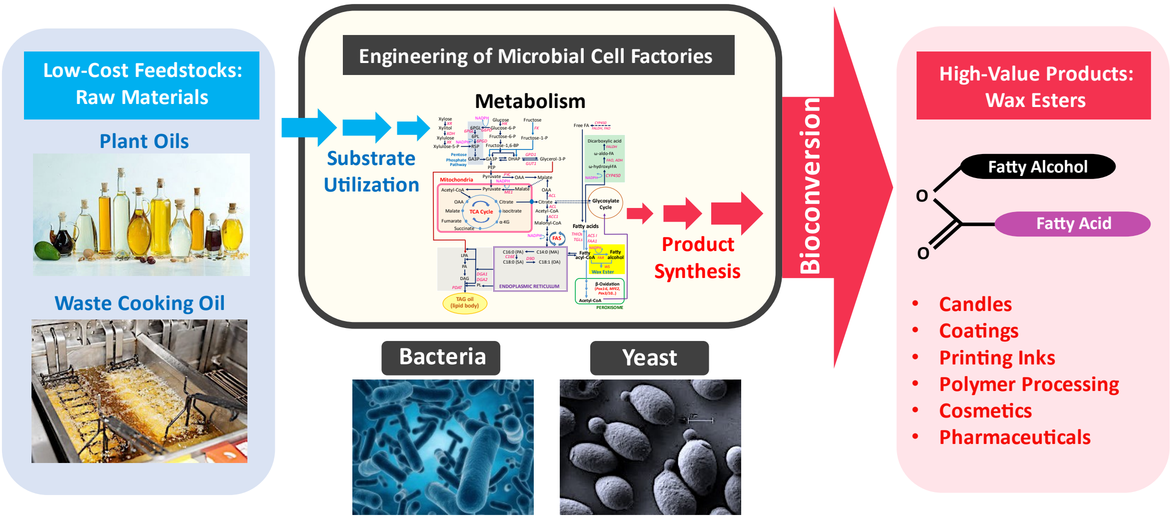
Highlights

- Both *Y. lipolytica* and *E. coli* were engineered to produce wax esters at the highest level up to date.
- *MhFAR* and *AbWS* genes were heterologously expressed and the metabolic pathways were optimized.
- Using a lipid substrate to replace glucose led to increase in wax ester production by up to 60-fold.
- The engineered *Y. lipolytica* produced 7.6 g/L wax esters, which contributed to 57% of the yeast DCW.
- Wax esters produced from FFA or TAG oils had longer chain length than those produced from glucose.



Graphical Abstract

Abstract

This paper summarizes our recent research effort in microbial synthesis of a class of very important molecules, wax esters (WE), which are widely used for applications in chemical, pharmaceutical, and food industries. Currently the nature sources of WE, jojoba plant and sperm whale, are very limited due to the harsh requirement of agriculture system and hunting ban. Here we report to engineer the yeast *Yarrowia lipolytica* and bacterium *Escherichia coli* to produce WE at the highest level up to date. First, the key genes encoding fatty acyl-CoA reductases (FAR) and wax ester synthase (WS) from different sources were investigated, and the expression system for two different *Y. lipolytica* hosts were compared and optimized for enhanced WE production and the strain stability. To improve the metabolic pathway efficiency, different carbon sources including glucose, free fatty acid (FFA), soybean oil, and waste cooking oil (WCO) were compared, and the corresponding pathway engineering strategies were optimized. It was found that using a lipid substrate such as WCO to replace glucose led to a 60-fold increase in WE production. The engineered yeast was able to produce 7.6 g/L WE with a yield of 0.31 (g/g) from WCO within 120 hr and the produced WE contributed to 57% of the yeast DCW. After that, *E. coli* BL21(DE3), with faster growth rates than the yeast, was engineered to significantly improve the WE production rate. Optimization of the expression system and the substrate feeding strategies led to production of 3.7~4.0 g/L WE within 40 hr in a 1-L bioreactor. GC analysis showed that the intracellular WE produced by both *Y. lipolytica* and *E. coli* were C_{36}, C_{34} and C_{32}, respectively, in order of decreasing abundance and with a large proportion being unsaturated. This work paved the way for biomanufacturing of WE at large scale.

Keywords: Wax Esters, fatty alcohol reductase (FAR), wax ester synthase (WS), *Yarrowia lipolytica*, *Escherichia coli*, metabolic engineering

Abbreviations: WE, wax esters; FAR, fatty acyl-CoA reductase; WS, wax ester synthase; WCO, waste cooking oil; FFA, free fatty acids; OLA, oleic acid; Glc, glucose; TAG, triacylglycerols; DCW, dry cell weight

1. Introduction

Wax esters (WE) are neutral lipid compounds composed of fatty acids esterified to the long-chain fatty alcohols. Physicochemical properties and applications of WE vary due to the number of the carbon atoms of the fatty acid and the fatty alcohol moieties as well as the degree of the unsaturation that affect melting temperature, oxidation, and pressure stability. The global WE market size was valued at $9.9 billion in 2019 and is expected to register a compound annual growth rate of 2.8% from 2020 to 2027 (Size, 2020). Therefore, WE as valuable chemicals are derived from the petroleum, chemical synthesis, or the natural sources. However, WE made from different sources have different application. Petroleum and chemical synthesis-derived WE are mainly applied to the candles, lubricants, coatings, printing inks, rubber and plastic processing, whereas the bio-based WE are used in the pharmaceuticals and personal care products such as cosmetics, hair care, skin care, and other allied products. In addition, WE with longer carbon chain lengths (C_{32}~C_{36}) will be more valuable in the market (Doan et al., 2017; Fiume et al., 2015; Petersson et al., 2005).

WE are widely distributed in living species from microbes, plants to animals, and provide a variety of biological functions. Similar to triacylglycerols (TAG), WE facilitate carbon and energy storage, and provide the protective coating resistant to dehydration, ultraviolet rays, and pathogens (Jetter and Kunst, 2008; Wältermann et al., 2005). However, the natural sources of liquid WE are still rare. Only few organisms such as desert shrub jojoba (*Simmondsia chinensis*) and sperm whale (*Physeter macrocephalus*) are capable of accumulating high amount of intracellular waxes (predominately C_{28} to C_{36} and C_{38} to C_{44}, respectively) (Metz et al., 2000; Voelker and Kinney, 2001; Wenning et al., 2017). The high price of spermaceti oils and the jojoba oil have limited their use to be an ingredient in cosmetics and pharmaceutical excipient as a basis for ointments, creams, and other personal care products (Kalscheuer et al., 2006b). In fact, WE are in a short supply due to the global ban on whale hunting and the strict requirement of modern agriculture system for jojoba cultivation (Miwa, 1971). Currently, WE are mainly produced via chemical routes or biotechnological process employing immobilized lipases (Hills, 2003; Li et al., 2011a). The drawback of chemical synthesis process includes the corrosive acids required, high energy consumption, degradation of synthesized esters and inconsistent performance. Even in the lipase-catalytic esterification process, the chemically synthesized fatty alcohols are required as substrates for WE production. In contrast to the conventional methods, microbial production of WE presents a more promising and environmentally sustainable alternative approach worth exploring.

The biosynthesis and accumulation of TAG and WE has been reported in some soil and marine bacteria including *Acinetobactor* (Ishige et al., 2002; Santala et al., 2014)*, Marinobactor* (Willis et al., 2011)*, Mycobacterium* (Sirakova et al., 2012)*, Streptomyces* (Röttig et al., 2016), *Euglena* (Tomiyama et al., 2017), and *Rhodococcus* (Round et al., 2019) genera under nitrogen-limited conditions. The synthesis of WE in prokaryotes proceeds via two sequential reactions: the formation of fatty alcohols from fatty acyl-CoAs or fatty acyl-ACPs, followed by the esterification of the resulting fatty alcohols with fatty acyl-CoAs to form WE. The first step can be catalyzed by two enzymes, fatty acyl-CoA reductase (FAR) and fatty aldehyde reductase (Alvarez, 2016; Mcdaniel et al., 2011), to first reduce fatty acyl-CoA or fatty acyl-ACP into fatty aldehyde, and then further reduce it to fatty alcohol. Interestingly, a novel FAR enzyme (Maqu_2220 and Maqu_2507; termed FarA or MhFAR and AcrB) derived from *Marinobacter hydrocarbonoclasticus* strain (also known as *M. aquaeolei* VT8) was found to be able to directly convert a fatty acyl-CoA substrate ranging from $C_{8:0}$ to $C_{20:4}$ to the corresponding fatty alcohol via a four-electron reduction without a need of an aldehyde reductase (Hofvander et al., 2011; Liu et al., 2016; Wahlen et al., 2009; Willis et al., 2011). The second step is catalyzed by a CoA-dependent acyltransferase enzyme known as wax ester synthase/diacylglycerol acyltransferase (WS/DGAT) (Arabolaza et al., 2008; Daniel et al., 2004; Hernández et al., 2013; Holtzapple and Schmidt-Dannert, 2007). WS/DGAT (or AbWS) from *Acinetobactor baylyi* ADP1 exhibits a low specificity regarding the chain length of the substrates, which indicates that acyl-CoA substrates can broadly esterify either diacylglycerols (DAG) or long-chain fatty alcohols to synthesize TAG or WE, respectively, with a diversity of chain lengths (Kalscheuer et al., 2004; Kalscheuer and Steinbüchel, 2003; Wältermann et al., 2007; Wenning et al., 2017). Additionally, previous studies have demonstrated that WS/DGAT is involved in the lipid droplet (also referred to as lipid bodies) formation through the hydrophobic interactions with the synthesized lipids (Reiser and Somerville, 1997). The hydrophobic domain of *AbW*S facilitates the agglomeration of phospholipid-coated lipid droplet as an oleaginous layer parallel to the plasma membrane and acquisition of TAG and WE as a hydrophobic core of the lipid droplet (Alvarez and Steinbüchel, 2002; Garay et al., 2014; Heilmann et al., 2012; Wältermann et al., 2005). Though people have gained much understanding of the WE biosynthesis pathways and the available genes for the key biochemical steps, only very poor titer and yield of WE production have been reported. Further metabolic engineering studies including choosing fast-growing new host strains, optimization of WE synthesis pathway, improving the expression of key gene, and selection of economical substrates are needed to meet the need for large scale production.

The selection of microbial production host for an industrial biotechnology process is primarily determined by its potential to efficiently produce the product of interest. Over the past decade, *Escherichia coli* has become one of the most well-known and intensively studied prokaryotic model organism. Characteristics features such as a fast specific growth rate, favorable growth condition, easier and versatile genetic manipulation tools and high level of recombinant proteins synthesis rate make *E. coli* an ideal manufacturing platform for the development of industrially viable productions (Baeshen et al., 2015; Pontrelli et al., 2018). Great advances have been made in producing fatty alcohols from fatty acyl-CoA, fatty acyl-ACP, and fatty acids through the catalysis of FAR in *E. coli* (Akhtar et al., 2013; Guo et al., 2015). Moreover, the WS/DGAT from *A. baylyi* ADP1 has also been adopted in *E. coli* for in vivo ester production (Duan et al., 2011; Elbahloul and Steinbüchel, 2010; Kalscheuer et al., 2006a). However, the up-to-date titer and yield are still too low to be commercially attractive. Compared to *E. coli*, yeast strains have also been widely used as metabolic engineering hosts for production of various bioprivileged molecules due to their advantages in resistance to phage contamination, tolerance to high levels of products and byproducts, and unique pathways for some specific products, especially the ones whose synthesis requires cytochorome P450 enzyems. For WE synthesis, the nonconventional yeast *Y. lipolytica* is of our particular interest due to its high fatty acyl-CoA metabolic flux and excellent lipid accumulating capability, which provide enough precursors for WE synthesis (Abdel-Mawgoud et al., 2018; Beopoulos et al., 2010; Ledesma-Amaro and Nicaud, 2016; Markham and Alper, 2018; Soong et al., 2019). For example, heterologous expression of FAR from *M. hydrocarbonoclasticus* strain VT8 enabled to produce 5.75 g/L fatty alcohols in *Y. lipolytica* when grown on modified YPD medium (Zhang et al., 2019; Zhao, 2017). Similar research result was also recently reported (Cordova et al., 2020). In addition, *Y. lipolytica* was engineered to produce fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) by introducing wax ester synthase (Gao et al., 2018; Xu et al., 2016). Further engineering of the *Y. lipolytica* cytosolic redox metabolism converting glycolytic NADH into the lipid biosynthetic precursors NADPH and acetyl-CoA has been proved to further improve FAME production (Qiao et al., 2017; Xu et al., 2017). However, there has not been research on co-expression of the FAR and WS/DGA1 enzymes in *Y. lipolytica* for producing long chain WE of a higher value.

In addition to host selection, choosing an economical substrate is not only critical for reducing the material cost of biomanufacturing, but also for metabolic pathway design and optimization. For some commodity fermentation products, the raw material cost may contribute to 50~80% of the total manufacturing cost. Sugars, especially glucose, are the dominant substrates that are used for making various fermentation products. However, many other renewable agriculture feedstocks such as plant oils and rendered animal fats can serve as the carbon and energy source for manufacturing of fermentation products (Enshaeieh et al., 2012; Fontanille et al., 2012; Makkar et al., 2011; Spier et al., 2015; Uprety et al., 2017). The global production of plant oils is approximately 206 million tons in total, whereas sugar is estimated around 174 million tons in the 2019 to 2020 crop year according to United States Department of Agriculture-Foreign Agriculture Service (USDA-FAS, 2020). Although sugars are widely used in biotechnology industry to make fuels, chemicals, and valuable products, plant oils are primarily used in food and feed applications generating low or limited economic values. Moreover, substantial quantities of waste cooking oil (WCO) generated during the food application is becoming a worldwide concern due to improper disposal to the environment. There is an urgent need of developing practical ways for reutilization of WCO (Lam et al., 2010). WCO mainly contains TAG oil, but can be contaminated by some derivatives generated during the frying process, such as free fatty acids (FFA), heterocycles and so on (Mannu et al., 2020). The degree of such degradation depends on the number of frying cycles, frying time, temperature, and the specific vegetable oil (Awogbemi et al., 2019). During deep frying, many volatile compounds are generated as a consequence of a combination between high temperature and oxygen, which promotes oxidation processes and other transformations (e.g., the Maillard process) (Saguy and Dana, 2003; Ziaiifar et al., 2008). In addition, food and tool exposure during frying process promote leaching and enrich the oil composition with metal traces, spices, and other organic molecules (Pokorny, 1989). Therefore, the composition of WCO is quite complicated and varies from batch to batch. In the past decades, an enormous amount of research efforts have been focused on the production of biodiesel from WCO, but the biodiesel industry is facing lots of uncertainties due to market changes (Chrysikou et al., 2019; Phan and Phan, 2008; Zhang et al., 2003) and the technical challenges in eliminating undesired byproducts such as FFA and glycerol (Salzano et al., 2010).

In this paper, a novel approach of microbial synthesis of WE from various feedstocks is presented. First, the nonconventional yeast *Y. lipolytica* was used as the host strain to produce WE from glucose, FFA, soybean oil, or WCO. The key genes required for WE biosynthesis pathway were determined by comparing the performance (in terms of WE production levels) of several candidate genes in the *Y. lipolytica* Po1f strain. After that, the selected *FAR* and *WS* genes were further integrated into the chromosome of a faster-growing, wild type *Y. lipolytica* (ATCC20362). The medium and process conditions were optimized to achieve high titer and yield. In parallel, the bacterium *E. coli* BL21(DE3) was also tested to express *FAR* and *WS* genes for WE production. The expression system in the *E. coli* strain was also optimized to produce WE from a medium containing glucose, FFA, soybean oil, or WCO. Finally, the WE production was demonstrated with high titer, rate, and yield under both shake flask and fed-batch bioreactor conditions.

2. Materials and Methods

2.1. Strains, growth and culture conditions

Escherichia coli NEB5α was primarily used for plasmid construction and propagation. *E. coli* BL21(DE3) was used as the host strain for wax ester production. The *Y. lipolytica* stains used in this study were derived from Po1f (Yeastern Biotech Company) ~~(show the phenoptype of po1f)~~ or ATCC20362 (American Type Culture Collection). The *Y. lipolytica* VSYU1, the uracil auxotrophic variant of ATCC20362, serves as the host strain for yeast plasmid construction. All strains used in this study are listed in **Table 1**.

Media and growth conditions for *E. coli* have been previously described by Sambrook and Russell (Sambrook et al., 1989), and those for *Y. lipolytica* have been described by Barth and Gaillardin (Barth and Gaillardin, 1997). *E. coli* strains are grown at 37°C with constant shaking in Luria–Bertani Broth supplemented with 100 mg/L ampicillin or 50 mg/L kanamycin. Rich medium (YPD) for *Y. lipolytica* strain were prepared with 5 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, 1 mg/L thiamine hydrochloride, 6 g/L KH_2PO_4, and 2 g/L Na_2HPO_4. Minimal medium (YNB) was made with 6.7 g/L yeast nitrogen base with ammonium sulfate and 20 g/L glucose. Selection for *Y. lipolytica* strains with URA3 selection marker was done in minimal medium with 1.92 g/L yeast synthetic uracil drop-out supplement (Sigma-Aldrich). For URA3 counter-selection, 0.5 g/L 5-fluoroorotic acid (Zymo Research) and 12 mg/L uracil were additionally supplemented.

Shake flask experiments for *E. coli* strains. The fatty acyl-CoA reductase and wax ester synthase were expressed in ***E. coli*** BL21(DE3) grown in the ZYM-5052 auto-induction medium, which contained 1% tryptone, 0.5% yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.2X trace elements, 0.5% glycerol, 0.05% glucose and 0.2% α-lactose. A single transformed colony was selected from an LB plate with an appropriate antibiotic for selection and used to inoculate a starter culture in LB medium. The starter culture was grown overnight at 37°C and was freshly used at a dilution of 1:50 to inoculate expression cultures in ZYM-5052 auto-induction medium. For some wax ester production experiments, glycerol in the ZYM-5052 medium was replaced by 5% (W/V) oleic acid, soybean oil or waste cooking oil (canola oil) as specified in the section of results. The fatty acid composition in soybean oil and WCO were described in **Table S1**. Lipase was supplemented to the medium containing oil substrate. The expression cultures were grown for 30 hr at 37°C for maximum WE production.

Shake flask experiments for *Y. lipolytica* strains. Enzyme expression and wax ester production in *Y. lipolytica* was performed in the nitrogen-limited medium, which contained 2.5 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$, 6 g/L KH_2PO_4, 2 g/L K_2HPO_4, 1 mM $MgSO_4$, 0.05X trace metal, and was supplemented with different carbon sources, including 40 g/L glucose (YD), 2.48% (w/v) oleic acid (YFA), 2.48% (w/v) soybean oil (YO) or 2.48% (w/v) waste cooking oil (french-fried canola oil obtained from a local restaurant) (YWCO). A single transformed colony was selected from a selective plate and used to inoculate a starter culture in the minimal medium. The starter culture was grown for 24-48 hr at 30°C, 250 rpm and was freshly used at a dilution of 1:50 to inoculate the expression cultures (i.e., YD, YFA, YO or YWCO medium) for wax ester production. Carbon source (glucose, FFA, soybean oil, or WCO) was added 3 times at 24 hr, 48 hr and 96 hr time-points. Phosphate and carbonate supplements (6 g/L KH_2PO_4, 2 g/L K_2HPO_4, and 14 g/L $NaHCO_3$) were also supplemented to the expression medium for adjusting the pH value to near neutral. The expression cultures were grown for 5 additional days at 30°C for WE production.

Protocols for bioreactor scale (1-L) fermentation experiments for both *E. coli* and *Y. lipolytica* strains are described in the Supplementary Materials.

2.2. Construction of *E. coli* and *Y. lipolytica* Production Strains

General molecular biology methods. Restriction enzymes and DNA polymerases were procured from New England Biolabs. PCR amplifications were conducted with Q5 High-Fidelity DNA Polymerase or Taq DNA polymerase at recommended conditions. PCR products and DNA fragments were purified using Monarch® PCR & DNA Cleanup Kit (New England Biolabs). The resultant PCR amplicons were electrophoresed through a 1-2% agarose gel. Gel extraction was performed using the QiAquick Gel Extraction kit (Qiagen). DNA oligonucleotides used for PCR are listed in **Table S3**. Plasmid constructs used in this study are listed in **Table 2**. Plasmid containing the desired genes were constructed using NEBuilder HiFi DNA Assembly cloning kit (New England Biolabs) and the pUC19, pET21b(+) or pET28a(+) plasmid was used as backbone.

Plasmid construction for *E. coli* strains. To construct the plasmids expressing the WE pathway genes in *E. coli*, genes *MhFAR* (accession number: WP_011785687.1) from *M. hydrocarbonoclasticus* strain VT8 and codon-optimized *AbWS* (accession number CAG67733.1) from *A. baylyi* ADP1 were synthesized. PCR was performed using primers NdeI_MhFAR_FP/ tT7_XhoI_MhFAR_RP and pT7_NdeI_WS_FP/ tT7_XhoI_WS_RP and the productct was cloned between NdeI and XhoI sites of pET21b(+) and pET28a(+) for construction of pFAR and pWS, respectively (**Fig. S5**). pFAR-WS was constructed by amplifying the *MhFAR* and *AbWS* gene coding FAR and WS and ligated into KpnI, AvrII and NheI restriction sites of a starting vector pET21b(+). To increase the efficiency of WE production, the fusion enzyme was constructed by ligating the coding sequence of *MhFAR* (with the stop codon removed?) and *AbWS* without a linker and introduced into the pET21b(+) plasmid. pET-based plasmids contained an insert for incorporation of an 6X His-tag following the in-frame insertion.

Plasmid and linearized gene cassette construction for *Y. lipolytica* strains. To construct the WE biosynthesis pathway in *Y. lipolytica* Po1f or Le3 (Po1f-ΔPEX10) strain (Gao et al., 2016), *ScFAR, MmFAR* or *MhFAR* together with the codon-optimized *AbWS* was amplified and cloned into a pUC-derived plasmid (**Fig. 1A**). The promoter of translation elongation factor-1α (TEF1) was amplified from ATCC20362 to control the expression of *ScFAR, MmFAR, MhFAR* and *AbWS*. The codon-optimized hygromycin phosphotransferase (*HPT*) and *URA3* genes were used as selection markers for random integration into the *Y. lipolytica* Po1f strain genome. Each of the selection markers was placed in between the *Leu2* minimal promoter and a short synthetic terminator T_{Synth8} (Alper and Curran, 2016; Curran et al., 2015). The MhFAR-HPT and WS-URA3 linear cassettes were assembled by overlap extension PCR (**Fig. 1B**) and integrated into the Po1f chromosome to generate the SI and DI strains. The gene expression cassettes of WE (*pTEF1-MhFAR-tCYC1-pTEF1-WS-tCYC1-YlURA3*) were linearized from pUC19-FAR-WS-URA3 plasmid (**Fig. 3A**). Transformaion of *Y. lipolytica* was described in supplementary information. Linear WE gene cassettes was subsequently transformed into VSYU1 and the yeast transformants were selected by uracil auxototroph. The WE-producing *Y. lipolytica* strains, VSWE1-VSWE4, were generated using non-homologous random integration. To increase the expression of WS, a second copy of *AbWS* gene was introduced into the chromosome of VSWE1. The *AbWS* under the control of *TEF1* promoter with *YlURA3*-flanked fragments was cloned into the pUC plasmid to yield pUC19-5'URA3-WS-3'URA3 (**Fig. S2**). *YlURA3* gene deletion cassette was amplified from this construct and transformed to *Y. lipolytica* VSWE1 strain to replace the URA3 marker by WS, generating a WS-overexpression strain, VSWE5.

2.3. Wax Ester Extraction, Detection and Quantification

Gas Chromatography. The organic fraction of WE extracted from the *E. coli* and *Y. lipolytica* cell pellets was used for GC analysis by using the method described by Inui *et al* (Inui et al., 1982) with slight modifications. In brief, cell pellets were harvested from 500 µL of culture and washed with 500 µL deionized water. The sample was centrifuged for 3 min at the maximal speed to remove the supernatant. To identify each WE product, a list of authentic WE standards purchased from NU-CHEK Prep, Inc. were used in GC (Table S2). To quantify the produced WEs, only 25 µL of 5 g/L stearyl palmitate ($C_{16:0}$-fatty acid/$C_{18:0}$-fatty alcohol; $C_{34}H_{68}O_2$) as an internal wax ester standard was added. The total wax esters were extracted from cells with 500 µL of a mixture containing methanol, hexane, deionized water at a ratio of 5:1:16 (v/v/v). In each organic solvent adding interval, the cell mixture was homogenized by sonication or vigorous shaking for 30 min. The mixture was centrifuged at 5,000 rpm for 30 sec after agitation. The hexane phase was transferred to a chromatography vial. The extraction was repeated by adding hexane, and the combined supernatant were collected for wax ester quantification via gas chromatography.

The separation of individual WE was carried out on a GC-2014 gas chromatograph (Shimadzu) equipped with a flame ionization detector. A DB-1HT fused-silica capillary column (15 m X 0.25

mm, film thickness of 0.10 μm; Agilent Technologies) was used. The sample (3 μL) was injected at 35°C. Chromatographic separation was initially set at 35°C for 2 min. The oven was programmed from 35°C to 240°C at a rate of 20°C /min and maintained at 240°C for 6 min, then the temperature was increased from 240°C to 310°C at a rate of 20°C /min and maintained for 2 min, and then the temperature was elevated from 310°C to 360°C at a rate of 8°C/min. The finial temperature was held for 2 min. Helium was used as a carrier gas at a constant flow rate of 2.0 mL/min. Each individual WE was determined by GC-MS via comparing with the authentic starndards. All data acquisition and processing were performed with the GC-FID solution software (Shimadzu).

3. Results

3.1. Engineering *Y. lipolytica* Po1f for biosynthesis of WE

Construction of Po1f-based strains for WE production. Synthesis of fatty alcohols as intermediates is a bottleneck that limits the WE production and thus FAR is the critical catalytic factor to drive the metabolic flux from fatty acyl-CoA to fatty alcohols. To select the best combination of *FAR* and *WS* for WE production, three *FAR* gene candidates including *ScFAR* from jojoba plant (*Simmondsia chinensis*), *MmFAR* from house mouse (*Mus musculus*), and *MhFAR* from *M. hydrocarbonoclasticus* strain VT8 were first co-expressed with the *WS* gene from *A. baylyi* ADP1 (*AbWS*) in the *Y. lipolytica* Po1f strain. Since WE synthesis requires fatty acyl-CoA as precursors and inactivation of the β-oxidation can significantly improves lipid accumulation (Xue et al., 2013; Xie et al., 2015), strain Le3 was generated from Po1f by deletion of the *pex10* gene involved in peroxisome biogenesis to examine the effect of β-oxidation on WE production. The *FAR* and *AbWS* genes were cloned into two separate plasmids, which were co-transformed into strain Po1f and Le3 for WE production evaluation. All the strains and plasmid information are shown in **Table 1** and **2**. A schematic diagram of the strain construction process is shown in **Fig. 1**. The WE production results from all the strains tested are shown in **Fig. 2**.

Determination of the best pair of *FAR* and *WS* genes for WE production. As results shown in **Fig. 2A**, among the three FARs, the MhFAR led to production of most fatty alcohols from glucose. Major types of fatty acids produced by *Y. lipolytica* are ~~saturated $C_{16:0}$ and $C_{18:0}$ and~~ unsaturated

$C_{16:1}$, $C_{18:1}$, and $C_{18:2}$ (Xue et al., 2013). FARs have distinct preferences on fatty acid substrates of different chain lengths. ScFAR mainly catalyzes $C_{18:0}$, $C_{20:1}$ and $C_{22:1}$ acyl-CoAs (Miklaszewska and Banaś, 2016), while MmFAR utilizes $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ acyl-CoAs (Cheng and Russell, 2004b) and MhFAR prefers $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{20:0}$ acyl-CoAs (Hofvander et al., 2011). Thus, MmFAR and MhFAR enzymes are considered to be favored by the dominant C_{16}-C_{18} acyl-CoA pool in *Y. lipolytica*. Our experiments showed that MhFAR gave the highest fatty alcohol production. In addition to the formation of fatty alcohols, **Fig. 2B** shows that co-expression of *MhFAR* with *AbWS* gave much higher WE production than co-expressions of *ScFAR* or *MmFAR* with *AbWS*. The possible reason is that both MhFAR and AbWS are cytosolic enzymes considering that they originate from prokaryotes while ScFAR is a membrane-associated enzyme involved in lipid metabolism in endoplasmic reticulum (Metz et al., 2000) and MmFAR is a transmembrane protein residing in the peroxisome (Cheng and Russell, 2004a; Heilmann et al., 2012) in their original hosts. Therefore, *MhFAR* with *AbWS* should be the pair of genes chosen for further studies on WE synthesis.

Effect of β-oxidation on WE synthesis. It has been well studied that lipid accumulation in *Y. lipolytica* can be significantly improved by repressing or deletion of β-oxidation genes including *PEX10*, *MFE1*, and *POX1-6* to prevent the accumulated lipids from degradation (Dulermo and Nicaud, 2011; Tai and Stephanopoulos, 2013; Xie et al., 2015; Xue et al., 2013). Surprisingly, it was found the three Le3 production strains with peroxisome biogenesis disrupted (Sc-Le3, Mm-Le3 and Mh-Le3) produced less fatty alcohols and WE compared to the Po1f production strains (Sc-Po1f, Mm-Po1f and Mh-Po1f) (**Fig. 2A** and **2B**). It seemed that higher carbon flux flew to the fatty acid or TAG synthesis rather than to the WE branch when β-oxidation was inactivated.

Comparison of gene expression via plasmids and integration. In general, ~~using plasmid should introduce multiple copies of the target genes than integrating a single copy of the gene in the chromosome, and thus may lead to significantly higher WE production. On the other hand,~~ an industrial production strain prefers to have all foreign genes integrated into its chromosome to maintain its genetic stability after several dozens of generations of cell growth, especially in the commercial-scale fermentation process. In *Yarrowia*, all of the currently available plasmids are known to have constrained genetic stability, which caused significantly varied WE production when cultures were initiated from different colonies on a single transformation plate. To improve the strain's genetic instability, strain SI was generated by integrating one-copy *MhFAR* gene into the chromosome of Po1f by random insertion while *AbWS* was still expressed by the plasmid pLZ2-AbWS. For the SI strain, 48 colonies were randomly picked and cultured in Sc-Leu medium containing 200 μg/mL hygromycin B for three days, then the lipids from cell samples were extracted and the total lipid analysis were conducted by thin layer chromatography (TLC). Among all 48 colonies, only 11 of them exhibited relatively large and bright WE spots (**Fig. S1A and S1B**). Colony #35 of the SI strain produced the highest amount of WE (**Fig. S1D**). At the same time, strain DI was generated by randomly integrating one copy of both *MhFAR* and *AbWS* genes (**Fig. 1B**). Different from the SI strain, almost all 20 colonies produced WE from the DI strain (**Fig. S1C**). Only 6 colonies were picked, cultured in Sc-Ura medium, and analyzed for WE production by GC-MS (**Fig. S1D**).

The SI strain accumulated more fatty alcohols (80 mg/L) than Mh-Po1f (14.5 mg/L), but the WE production decreased to 276 mg/L, presumably due to the instability of LZ2-AbWS plasmid during the cultivation in shaking flask (**Fig. 2B**). For strain DI, both fatty alcohol (15 mg/L) and WE (84 mg/L) production significantly dropped when compared to the SI and Po1f strains. The copy number of *AbWS* gene was measured in both strain SI and DI at three time-points (24 hr, 48 hr, and 72 hr). It was found that the copy number of *AbWS* gene randomly integrated into the genome was one in strain DI, while the copy number dropped from 2.1 at 24 hr to 1.5 at 72 hr in strain SI (**Fig. S1E**). Nonetheless, the copy number of *AbWS* gene in SI strain was greater than one as obtained in the DI strain, which could explain why strain SI produced more WE than strain DI. The decrease of the *AbWS* copy number in the SI strain indicated the plasmid instability issue.

Fed batch fermentation of strain SI. To further evaluate the strain's performance, strain SI was further tested in 1-liter fed-batch bioreactor (**Fig. 2C**). Although the highest titer of fatty alcohols (590 mg/L) was achieved at 106 hr, very poor WE production (~90 mg/L) was achieved, and the titer stopped increasing after 54 hr. As mentioned above, the plasmid instability issue may have caused the inefficient expression of *AbWS* under bioreactor conditions, which led to accumulation of fatty alcohols and decreased WE formation. In addition, a very-slow-growth phenotype (12.3 g/L DCW) was observed for strain SI even sufficient nutrients were provided and all the process parameters were controlled under typical fed-batch conditions for *Y. lipolytica* (Xie et al., 2017). This is presumably due to either the deficiencies of several amino acid synthesis genes in the original Po1f strain or the condition non-optimized for WE formation. Therefore, we decided to switch to a new wild type *Y. lipolytica* ATCC20362, which is able to grow more than 100 g/L dry cell weights (DCW) under fermentation conditions (Xie et al., 2015 and 2017), as a new production strain.

3.2. Engineering the wild type *Y. lipolytica* ATCC20362 for biosynthesis of WE

Construction of *Y. lipolytica* strain VSWE1~4. To avoid the growth defect of Po1f-based strains and improve the WE production, the wild type *Y. lipolytica* ATCC20362 strain was used as the host to build the WE biosynthesis pathway by integrating both *MhFAR* and *AbWS* into its chromosome via non-homologous recombination. A recombinant WE plasmid (pFAR-WS-URA3) was constructed, which included both *MhFAR* and *AbWS* regulated under the control of *TEF1* constitutive promoter and *CYC1* terminator. The *URA3* was used as the selection marker (**Fig. 3A**). The *FAR-WS-URA3* gene cassette was linearized from the recombinant plasmid via double digestion and then randomly inserted into the *Y. lipolytica* VSYU1 strain (i.e., ATCC20362 with *URA3* mutation). Among the 12 colonies selected, four of them were identified to have successful insertion of *MhFAR* and *AbWS*, which were named VSWE1~4 (**Fig. S2**). The lipid extracts from VSWE1~4 strains subjected to nitrogen-limited medium with glucose as carbon source were analyzed by TLC (**Fig. S2E**) and GC (**Fig. S3**) to identify the WE production. The titers of WE varied from 0.07 to 0.11 g/L among these four strains, with VSWE1 producing the highest amount of WE. The difference in production was caused by the different locus where *MhFAR-WS-URA3* was randomly inserted in the chromosome.

Construction of VSWE5 by adding a second copy of *AbWS* gene to VSWE1. In order to further increase the efficiency of WE bioconversion, a second copy of *AbWS* was introduced into the URA3 locus of the VSWE1 strain to generate a new strain VSWE5. This step was achieved by homologous recombination, and the homologous arms was designed based on the sequences flanking the URA3 gene (**Fig. S2B**). In the shaking flask experiments, the VSWE5 strain turned out to produce WE at a lower titer than strain VSWE1 (**Fig. S4**), but the specific WE titer (g WE titer/g dry cell weight) was very similar to that of VSWE1. A small defect in the cell growth was observed, which might account for the decrease in the overall WE production titer.

3.3. Modulation of fatty acyl-CoA substrate pool for enhanced biosynthesis of WE

As the direct substrate of FAR to make fatty alcohols, fatty acyl-CoA is speculated as a limiting factor for the synthesis of fatty alcohol and WE. We attempted to use alternative carbon sources, whose catabolism is different from glucose metabolism but can significantly increase the intracellular fatty acyl-CoA pool via either a de novo lipid synthesis pathway or an ex novo pathway (**Fig. 3B**). For this purpose, strain VSWE1 was selected to analyze its capability of producing WE in different culture media containing glucose, oleic acid ($C_{18:1}$), soybean oil, or WCO ~~(Fig. S3)~~.

Y. lipolytica strain was able to grow well in the medium with hydrophilic or hydrophobic substrates (**Fig. 4A**). The flask fermentations with any lipid substrates (oleic acid, soybean oil, and WCO) had significantly higher DCW (12~18 g/L) as compared to that with glucose (~7 g/L). This is mainly due to the significant increase in the intracellular lipid level when extracellular lipid substrate was used to grow the *Y. lipolytica* cells. Interestingly, we also observed 20- to 60-fold increases in WE production when the carbon source was changed from glucose to any types of lipid. While the run with glucose produced only 0.12 g/L WE, the runs with oleic acid, soybean oil, and WCO produced 3.4 g/L, 2.5 g/L, and 7.6 g/L WE, respectively (**Fig. 4B**). The total WE with WCO as a substrate reached 57% of the DCW, which was so far the highest level of WE produced by any microorganisms up to date. High lipid content contributed up to 70% of the DCW, which consisted of 81% WE and 14% TAG in total lipid content. Moreover, high levels of lipid accumulation were easily visualized by light microscopy, where significantly larger size of lipid bodies were observed in those cells grown in a medium with any of the hydrophobic substrates (**Fig. 4C**).

The product identity was highly dependent on the carbon source used in the flask fermentation medium. While the WE products from the glucose runs were mainly C_{28} and C_{30}, with small portions of C_{34} and C_{36}, the predominant WE in the runs with soybean oil and WCO were C_{36}, C_{34} and C_{32}, in an order of decreasing abundance, with a large proportion being unsaturated (**Fig. 4D and 4E**). Due to the high C_{18} content in vegetable oils, the fatty acid and fatty alcohol moieties of WE were also mainly C_{18}. GC-MS analysis confirmed the presence of $C_{18:2}/C_{18:2}$, $C_{18:1}/C_{18:2}$ and $C_{18:1}/C_{16:0}$ when the strain was grown on soybean oil or WCO.

3.4. Engineering *E. coli* for biosynthesis of WE

Introducing WE biosynthesis pathway into *E. coli* BL21(DE3). In comparison with yeast *Y. lipolytica*, the *E. coli* bacterial expression system has the advantage of the rapid growth and production rates, easy gene manipulation, inexpensive culture and efficient production. To establish the inducible gene expression system, the *E. coli* BL21(DE3) as T7 RNA polymerase-based system was employed to overexpress the pET plasmids carrying the *MhFAR* and *AbWS* under the control of strong bacteriophage T7 transcription. Three different strategies were designed for WE biosynthesis in BL21(DE3), as shown in **Fig. 5A**. The presence of polyhistidine-tagged MhFAR and AbWS enzymes in the putative mutants were detected by immobilized metal affinity chromatography. SDS-PAGE analysis revealed the molecular weight of 57 kDa for MhFAR and 54 kDa for AbWS, and 110 kDa for the fusion enzyme (**Fig. S5B**).

WE production by the engineered *E. coli* strains. Engineered bacteria were cultured in the modified ZYM-5052 auto-induction medium, in which glycerol was replaced by oleic acid, soybean oil or WCO for wax ester production in shaking flask. Since *E. coli* cannot directly grow on fatty acids, glucose was still included in the modified ZYM-5052 medium. In shaking flask experiments, lactose was added to induce the MhFAR and AbWS expression after glucose was depleted, then the hydrophobic substrate (oleic acid or WCO) was used as the carbon source for maintenance and WE synthesis. As shown in **Fig. 5B** and **5C,** the *E. coli* strain with the fused *MhFAR-AbWS* expression produced WE at significantly higher titer than the strains with *MhFAR* and *AbWS* expressed separately in two different plasmids or in the same plasmid ~~(**Fig. 5B and 5C**)~~. While only 0.1 g/L WE were produced in the run with glucose and glycerol in the medium, replacing glycerol with oleic acid ($C_{18:1}$ free fatty acid) significantly increased the WE production, and the maximum WE titer was 0.74 g/L for strain BL21(DE3)/p(FAR-WS). However, all the engineered *E. coli* strains produced less than 0.2 g/L intracellular WE after 30 hr when medium used soybean oil or WCO to replace glycerol. This is because the vegetable oils are in the format of triglycerides (TAG), which require addition of lipase to decompose TAG into FFA before they can be utilized by the *E. coli* cells. Using WCO was slightly better than using soybean oil since WCO contains small portion of FFA. Similar to what we observed in the *Y. lipolytica* strains, the major chain length of WE produced from the oil substrate were C_{36}, C_{34} and C_{32} (**Fig. 5D and S6**), but the majority for those from the glucose and glycerol substrates were C_{34} and C_{32}. In addition, the majority of the produced WE were formed by $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ fatty acids and fatty alcohols.

Fed-batch fermentation of engineered *E. coli* strain. Strain *E. coli* BL21(DE3) transformed with p(FAR-WS) was further studied for WE production in a 1-liter fed-batch bioreactor, where the initial medium contained only glucose for cell growth. After the initial glucose was consumed, glucose was continuously fed to maintain at minimal level (< 0.1 g/L) to provide energy maintenance. IPTG was added in the mid-log phase (t = 15 hr and OD_{600} was around 55) to induce WE production. At the same time, oleic acid and lipase-hydrolyzed WCO were injected into the bioreactor to a final total concentration (20 mL/L) for production of WE. The growth phase of engineered *E. coli* strain was in first 18 hr and after that the cells entered to the production phase. As shown in **Fig. 6A**, approximately 20 g/L of DCW were achieved in glucose feeding period before the hydrophobic substrate was added. A specific titer of 0.08 mg/g DCW and a WE titer of 1.7 g/L were obtained at 40 hr when using glucose as sole carbon source. WE production titers and specific titers were significantly enhanced when the oleic acid or hydrolyzed WCO was fed during the production phase, which showed a specific titer of 0.2 mg/g DCW and a WE titer of 3.98 g/L for oleic acid and a specific titer of 0.19 mg/g DCW and a WE titer of 3.7 g/L for WCO, respectively (**Table 3**). The composition of the produced WE were analyzed by GC and shown in **Fig. 6B** and 6C. Again, the chain length of produced WE were mainly C_{32} and C_{30} when glucose was the only carbon source provided in the fermentation medium, while longer chain length of WE including C_{34} and C_{36} were observed when the medium was supplemented with oleic acid or WCO (**Fig. 6D**).

4. Discussion

The production of the intracellular neutral lipid wax esters is limited by enzyme efficiency, substrate specificity, lipid-storage capacity, and tolerance of potential toxicity of high levels of FFA. Various FAR and WS exhibit different preferences for fatty acyl-CoA substrates with varied chain length and specificity (Shi et al., 2012). Using the oleaginous yeast *Y. lipolytica* as a host can be advantageous due to its capability of producing high levels of intracellular lipids and acyl-

CoAs (Beopoulos et al., 2009; Papanikolaou et al., 2002), the latter of which can be directly converted to fatty alcohols and WE. Three *FAR* candidate genes coding for ScFAR from *S. chinensis*, MmFAR from *M. musculuc* and MhFAR from *M. hydrocarbonoclasticus* were investigated. Compared to ScFAR that catalyzes the acyl-CoAs with very long carbon chain lengths (> C_{18}) and MmFAR, a transmembrane protein resided in the peroxisome (Cheng and Russell, 2004a; Heilmann et al., 2012), MhFAR demonstrated the highest activity in production of fatty alcohols and WE from the C_{16} and C_{18} acyl-CoAs in the *Y. lipolytica* Po1f strain. In addition to the three FARs that were investigated in this paper, both MaFAR from *Marinobacter aquaeolei* and TAFAR from barn owl *T. alba* were also used for biosynthesis of very long-chain WEs (Gao et al., 2020). A co-expression of TaFAR and AbWS in a VLCFA (very long chain fatty acid) producing *Y. lipolytica* led to production of 0.65 g/L very long-chain WEs (C_{32}-C_{42}) from glucose in flask fermentation and 2 g/L in a 5-L bioreactor. AbWS derived from *A. baylyi* ADP1 showed a broad substrate specificity that naturally prefers acyl-groups with chain lengths of C_{16}-C_{18} and linear fatty alcohols with chain lengths ranging from C_{14} to C_{20}, which is consistent with the reported activities of WS/DGAT by other researchers (Cordova et al., 2020; Hofvander et al., 2011; Li et al., 2008). Presumably, both MhFAR and AbWS were distributed in the cytoplasm of *Y. lipolytica* considering both genes are originated from prokaryotes and no additional signal peptides were added, which facilitate the esterification reaction when compared to the endoplasmic reticulum-located ScFAR and peroxisomal transmembrane protein MmFAR (van der Zand et al., 2010; Wang et al., 2015).

Heterologous gene expression can be achieved via either a plasmid or integration into chromosome. Using plasmid to express a foreign gene may cause a genetic instability problem (e.g., inefficient plasmid segregation and inconsistent fermentation performance between different strain stocks) especially when cells grow through many generations or under stressful conditions (Fakruddin et al., 2012). Integrating the target gene into chromosome usually results in a single copy in each operation but the integrated gene will be associated with a higher genetic stability, which is desired especially if the strain will be potentially used for a large-scale production. When *MhFAR* and *AbWS* genes were expressed in the *Y. lipolytica* Po1f strains via the ARS18-based plasmids, we observed that WE production from different single colonies of the same strain varied from almost non-detectable to 0.6 g/L, which indicated serious genetic instability issue. However, strain VSWE1~4, which were generated by integrating only one copy of *MhFAR* and *AbWS* under the control of TEF1 strong constitutive promoter into the genome of *Y. lipolytica* ATCC20362, demonstrated high genetic stability and as a result, the highest levels of WE production.

E. coli BL21(DE3) in combination with the T7 polymerase-based pET prokaryotic expression system has been frequently employed due to its advantage in lacking protease to keep high FAR and WS activities (Pan and Malcolm, 2000). The T7 promoter system present in the pET-based vector was used in this study for heterologous enzyme expression in *E. coli* BL21(DE3). The pET vector exists as a low copy number (15-20 copies per cell) and maintains a relatively higher plasmid stability (Birnbaum and Bailey, 1991). Although the protein of interest may be overexpressed in a short period of time and produced up to 50% of total protein (Baneyx, 1999; Graumann and Premstaller, 2006), this study showed that the MhFAR and AbWS enzyme expression was only close to 30% of the total cell protein in the *E. coli* BL21(DE3) strain. In general, peptides larger than 50 kDa with two or more domains are independently folded and difficult to express (Erickson, 2009). In our case, the reduced expression of MhFAR (57 kDa) and AbWS (54 kDa) protein did not significant affect WE production if sufficient acyl-COAs are provided.

Development of bifunctional fusion enzyme was inspired to promote active site coordination and substrate channeling (Lin et al., 2014; Quin et al., 2017). In the strain BL21(DE3) transformed with p(FAR-WS), the *MhFAR* and *AbWS* genes were fused and placed downstream of a shared same promoter. The catalytic activity of MhFAR-AbWS fusion enzyme was confirmed, which showed the highest efficiency for the WE production compared to the two-plasmid system (pFAR and pWS) and the one-plasmid system (pFAR-WS) with two gene expressed individually under their own promoters (**Fig. 5A**). Hence, the relay of the substrate and the intermediate from one enzyme to another prevented the accumulation of intermediates, minimized the undesired side reactions, and eventually improved the overall product formation (Bauler et al., 2010; Küchler et al., 2016; Wheeldon et al., 2016). The applicability of fusing MhFAR and AbWS should also be investigated in *Y. lipolytica* in the future.

Considering that *E. coli* is not naturally capable of producing lipase, both glucose and lipase-hydrolyzed oils (or oils treated with lipase) were co-fed into the fermentation medium to enhance WE production. In this study, high intracellular WE productions up to 0.74 g/L in shaking flask and 3.98 g/L in 1-liter fed-batch bioreactor were achieved by strain BL21(DE3) transformed with p(FAR-WS) when both glucose and oleic acid ($C_{18:1}$) were present as carbon sources. Though the titers achieved by the engineered *E. coli* were lower than the *Y. lipolytica* (**Table 3**), the production rate was much faster. A typical *E. coli* fermentation requires only 24-48 hr while a *Y. lipolytica* fermentation may take 5-7 days to reach the maximum production. Therefore, production of WE and other lipid-derived high-value products by engineered *E. coli* is still attractive for commercial application if the native fatty acid synthesis can be boosted or an efficient strategy of providing fatty acid-containing feedstock becomes feasible.

In general, the partial or complete shutdown of β-oxidation pathway by disrupting *POX*, *PEX*, and *MFE* genes should be efficient to improve intracellular lipids (Xue et al., 2013; Xie et al., 2015), which may benefit WE synthesis. However, our initial effort of *PEX10* disruption in Po1f (Le3 strain) with co-expression of MhFAR and AbWS failed to improve WE production as compared to the parent strain. It seemed that *PEX10* disruption was efficient in improving lipid accumulation in the format of TAG but not necessarily in providing more fatty acyl-CoAs to WE synthesis. In addition, some previous studies also exhibited contradictory results that disruption of β-oxidation by the knockout of *MFE1* encoding multifunctional β-oxidation protein in *Y. lipolytica* showed a deleterious effect on the accumulation of the lipid-derived products (Schwartz et al., 2017; Zhao, 2017). Disruption of *POX* or *MFE* may be helpful in further improving fatty acyl-CoA levels for WE synthesis, which needs to be further investigated in the future.

Plasmid instability presents another issue resulting in decreased production in *Y. lipolytica*. The growth deficiency of Po1f and its derived strains in the fermentation medium might further limit the strains' capability of producing high levels of WE. To address this issue, the fast-growing wild type *Y. lipolytica* ATCC20362 was engineered for WE production. In addition, our strategy of using a sugar-free medium containing waste oils/fats as alternative carbon source requires an active β-oxidation to provide both carbon and energy for cell growth and product formation during the fermentation process. Since *Y. lipolytica* can grow directly on oils and fats by secreting lipases to decompose TAG into FFA, which can be brought inside the cells for further WE synthesis, we optimized the culture medium by using the carbon sources other than glucose, such as oleic acid, soybean oil, and WCO. Our study showed that *Y. lipolytica* VSWE1 strain was able to assimilate both hydrophilic (e.g., glucose) and hydrophobic (e.g., FFA and TAG) substrates for WE production. Accelerated growth, larger lipid body accumulation, and higher WE titer have been found when VSWE1~4 were grown in a medium supplemented with oil-based substrates in the absence of glucose (**Table 3**). In regard to the composition profile of the produced WE, it was found that WE produced from the hydrophobic substrates had longer carbon chain length (C_{32}-C_{36}) as compared to the WE produced from glucose (C_{28}-C_{34}). This finding is in agreement with previous research that the composition of the fatty acids produced by *Y. lipolytica* depended on the carbon source chosen to use (Fontanille et al., 2012; Papanikolaou et al., 2009; Papanikolaou et al., 2006).

For the engineered *Y. lipolytica* strain VSWE1, using soybean oil to completely replace glucose in the flask fermentation increased WE production by 26-fold. Moreover, a 70-fold increase in WE titer was achieved when WCO was used, reaching a maximum 7.6 g/L or 57% of DCW after 120 hr of cultivation. This further increase is mainly due to the higher content of FFA in WCO, which reduces the requirement for the secreted lipase to hydrolyze TAG into FFA. This also suggests overexpression of lipase in the engineered strains should be explored in the future to further improve the WE production from a TAG oil-based substrate. Considering that WCO is a low-cost (due to the fluctuation and uncertainty of biodiesel market) or even free renewable feedstock, our study provides a novel and economically attractive route of biomanufacturing by turning a widely abundant waste into a high-value product with very minimal concerns of environmental contamination.

For the engineered *E. coli* strains, which do not have a capability of direct assimilation of TAG oils/fats due to the inability of producing lipase, co-feeding FFA or lipase-hydrolyzed TAG oil with glucose should be the fermentation strategy to improve WE production. It has been reported that a maximum 11 g/L fatty acid ethyl esters (FAEE) was produced by an engineered *E. coli* strain when glucose was co-fed with oleic acid (Elbahloul and Steinbüchel, 2010). High lycopene production (2.7g/L) was produced in engineered *E. coli* strain FA03-PM in fed-batch fermentation co-fed with glucose and oleic acid or hydrolyzed WCO (Liu et al., 2020). Interestingly, the fatty acyl-CoA synthases activity was improved in wild-type *E. coli* and *Salmonella enterica* with either decanoic acid ($C_{10:0}$) or oleic acid as a substrate (Iram and Cronan, 2006; Polacco and Cronan, 1977). Hence, co-feeding glucose with FFA or hydrolyzed TAG oils is able to provide a sufficient fatty acyl-CoA pool for high-yield WE production. Unlike the oleaginous yeast, *E. coli* only maintains a small amount of lipids in cell, impeding its utility to overproduce and accumulate WE (Meng et al., 2011). Introducing a lipase expression and further engineering the de novo fatty acid biosynthesis pathway in *E. coli* are the promising strategies to overcome this hurdle.

Raw material cost may contribute to more than half of the total biomanufacturing cost of WE, we have demonstrated in shaking flasks that using 24.8 g/L WCO to produce 13.3 g/L yeast biomass, which contained 7.6 g/L WE at 120 hr. To accelerate WE production in the future, protein engineering can be applied to improve MhFAR and AbWS activities; other genetic engineering strategies such as increasing genetic stability, fusing MhFAR and AbWS to facilitate substrate channeling, and implementing stronger promoters, could be attempted to increase the expression. In addition to waste oils/fats, lignocellulosic sugars may be another feedstock option. *Y. lipolytica* has been successfully engineered to use both C5 and C6 sugars derived from the cellulosic biomass (Ledesma-Amaro et al., 2016). Integrating the WE synthesis with those metabolic engineering efforts may provide another economically viable solution to large scale production of WE.

Continuous biomanufacturing could also be considered to further improve productivity and reduce both operating cost and capital investment. Though conventional batch or fed-batch processes are still adopted in most fermentation facilities due to the operation flexibility and easy scale-up, more and more studies have shown great benefits from continuous fermentation in high productivities, simple operation, and stable quality of fermentation products (Ethier et al., 2011; Li et al., 2011b; Taylor et al., 1995; Xie et al., 2017). Recently, a unique two-stage continuous fermentation was developed to decouple *Y. lipolytica* cell growth and product formation in two separate fermenters operating under their own optimal conditions, which led to 120% increase in productivity and 70% improvement in product titer over the fed-batch process (Xie et al., 2017). We expect that applying the continuous biomanufacturing to WE production will significantly reduce the manufacturing cost by enhancing the production titer and rate while maintaining a similar yield.

5. Conclusion

Metabolic engineering of the oleaginous yeast *Y. lipolytica* and bacterium *E. coli* BL21(DE3) has been explored for biosynthesis of WE at high titer and yield. The MhFAR was selected from three candidate FARs to produce the highest levels of fatty alcohols. Co-expression of MhFAR and AbWS in both engineered *Y. lipolytica* and *E. coli* strains led to production of 0.1~0.57 g/L WE from glucose. Increasing lipid synthesis by inactivation of β-oxidation failed to improve WE synthesis. Genetic instability was observed in the *Y. lipolytica* Po1f strain when plasmid expression system was employed, and it was resolved by redesigning the genomic integration of the *MhFAR* and *AbWS* in the wild type *Y. lipolytica* ATCC20362. Using a lipid substrate such as FFA, soybean oil, and WCO to replace glucose led to a 60-fold increase in WE production. The new *Y. lipolytica* strain VSWE1 was able to produce 7.6 g/L WE from WCO within 120 hr and the intracellular WE contributed to 57% of yeast biomass, which is the highest levels reported so far. By further expression of the fused genes of *MhFAR* and *AbWS*, the *E. coli* BL21(DE3) produced nearly 4.0 g/L WE within 40 hr from a feed containing both glucose a fatty-acid substrate. In addition, WE produced from FFA or TAG oils had longer chain length than those products converted from sugars. The research results not only paved the way for large-scale biomanufacturing of WE in future, but also lay a solid foundation for producing a series of other lipid-derived or -assisted high-value molecules from low-cost agriculture feedstocks such as plant oils and animal fats.

CRediT authorship contribution statement

Y. H. V. Soong: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **L. Zhao:** Conceptualization, Methodology, Investigation. **N. Liu:** Investigation, Methodology, Writing - review & editing. **P. Yu:** Investigation. **C. Lopez:** Investigation. **A. Olsen:** Investigation, Writing - review & editing. **H.-W. Wang:** Methodology, Investigation. **Z. Shao:** Funding acquisition, Project administration, Supervision, Writing - review & editing. **D. Xie:** Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

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Conflicts of interest

The authors declare no other financial or commercial conflict of interest.

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Table 1. Strains used in this Study.

STRAIN	DESCRIPTION	PHENOTYPE	REFERENCE
E. coli			
NEB5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*		NEB
BL21(DE3)	*fhuA2 [, #1034] ompT gal (λ DE3) [Li, #568] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*		NEB
Y. lipolytica			
ATCC20362	Wild-Type	Ura$^+$	ATCC
Po1f	*MatA, leu2-270, ura3-302, xpr2-322, axp-2*	Leu$^-$, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	CLIB 724 (Madzak et al., 2000)
Le3	Derived from Po1f, *ΔPEX10*	Leu$^-$, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	Gift from Yu Jiang's Lab (Gao et al., 2016)
Sc-Po1f	Po1f, pLZ1-ScFAR, pLZ2-AbWS	Leu$^+$, Ura$^+$, ΔAEP, ΔAXP, Suc$^+$	This work
Sc-Le3	Le3, pLZ1-ScFAR, pLZ2-AbWS	Leu$^+$, Ura$^+$, ΔAEP, ΔAXP, Suc$^+$	This work
Mm-Po1f	Po1f, pLZ1-MmFAR, pLZ2-AbWS	Leu$^+$, Ura$^+$, ΔAEP, ΔAXP, Suc$^+$	This work
Mm-Le3	Le3, pLZ1-MmFAR, pLZ2-AbWS	Leu$^+$, Ura$^+$, ΔAEP, ΔAXP, Suc$^+$	This work
Mh-Po1f	Po1f, pLZ1-MhFAR, pLZ2-AbWS	Leu$^+$, Ura$^+$, ΔAEP, ΔAXP, Suc$^+$	This work
Mh-Le3	Le3, pLZ1-MhFAR, pLZ2-AbWS	Leu$^+$, Ura$^+$, ΔAEP, ΔAXP, Suc$^+$	This work
SI	Po1f, MhFAR-HPT (Integration), pLZ2-AbWS	Leu$^+$, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$	This work
DI	Po1f, MhFAR-HPT (Integration), AbWS-URA3 (Integration)	Leu$^-$, Ura$^+$, ΔAEP, ΔAXP, Suc$^+$	This work
VSYU1	ATCC20362, URA3Δ	Ura$^-$	This work
VSWE1	VSYU1, MhFAR-AbWS-URA3	Ura$^+$	This work
VSWE2	VSYU1, MhFAR-AbWS-URA3	Ura$^+$	This work
VSWE3	VSYU1, MhFAR-AbWS-URA3	Ura$^+$	This work
VSWE4	VSYU1, MhFAR-AbWS-URA3	Ura$^+$	This work
VSWE5	VSWE1, pTEF1-AbWS-tCYC1	Ura$^-$	This work

Table 2. Plasmids used in this study.

PLASMID	DESCRIPTION	REFERENCE
pLZ1-ScFAR	*Amp*^{R}, pUC57 derivative that contained *ARS18* and expressed *ScFAR* from the *TEF1* promoter and *CYC1* terminator, and *YlURA3* complete sequence	This work
pLZ1-MmFAR	*Amp*^{R}, pUC57 derivative that contained *ARS18* and expressed *MmFAR* from the *TEF1* promoter and *CYC1* terminator, and *YlURA3* complete sequence	This work
pLZ1-MhFAR	*Amp*^{R}, pUC57 derivative that contained *ARS18* and expressed *MhFAR* from the *TEF1* promoter and *CYC1* terminator, and *YlURA3* complete sequence	This work
pLZ2-AbWS	*Amp*^{R}, pUC57 derivative that contained *ARS18* and expressed codon-optimized *AbWS* from the *TEF1* promoter and *CYC1* terminator, and *YlLEU2* complete sequence	This work
pFAR	*Amp*^{R}, pET21b(+) derivative that expressed *MhFAR* from the *T7* promoter and terminator.	This work
pWS	*Kan*^{R}, pET28a(+) derivative that expressed codon-optimized *AbWS* from the *T7* promoter and terminator.	This work
pFAR-WS	*Amp*^{R}, pET21b(+) derivative that expressed *MhFAR* from the *T7* promoter and terminator, and codon-optimized *AbWS* from the *T7* promoter and terminator.	This work
p(FAR-WS)	*Amp*^{R}, pET21b(+) derivative that expressed recombinant *MhFAR* and codon-optimized *AbWS* fusion gene from the *T7* promoter and terminator.	This work
pFAR-WS-URA3	*Amp*^{R}, pUC19 derivative that expressed *MhFAR* from the *TEF1* promoter and *CYC1* terminator, the codon-optimized *AbWS* from the *TEF1* promoter and *CYC1* terminator, and *YlURA3* complete sequence.	This work
pUC19-5'URA3-WS-3'URA3	*Amp*^{R}, pUC19 derivative that *YlURA3*-flanked *AbWS* expression cassette under the control of *TEF1* and *CYC1* terminator.	This work

Table 3. Production of wax esters in engineering *Y. lipolytica* and *E. coli* in this study.

Strain	Culture Scale	WE Production	Glucose	Oleic Acid	Soybean Oil	WCO
Y. lipolytica VSWE1	Shaking Flask	Titer (g/L)	0.12	3.37	2.51	7.58
		Specific Titer (g/g)	0.02	0.19	0.21	0.57
		Productivity (mg/L/hr)	1.00	28.08	20.92	63.17
Strain	**Culture Scale**	**WE Production**	**Glc + Gly**	**Glc + OLA**	**Glc + SO**	**Glc + WCO**
E. coli BL21(DE3) w/ p(FAR-WS)	Shaking Flask	Titer (g/L)	0.10	0.74	0.11	0.20
		Specific Titer (g/g)	0.06	0.38	0.06	0.08
		Productivity (mg/L/hr)	3.33	24.67	3.67	6.67
	1-Liter Fermentation	Titer (g/L)	1.65	3.98	N/A	3.65
		Specific Titer (g/g)	0.08	0.19	N/A	0.19
		Productivity (mg/L/hr)	41.25	99.50	N/A	91.25

Glc: glucose; Gly: glycerol; OLA: oleic acid; SO: soybean oil; WCO: waste cooking oil.

Fig. 1. Construction of WE Biosynthesis pathway in *Y. lipolytica* Po1f Strain. (A) The *ScFAR, MmFAR* or *MhFAR* together with codon-optimized *AbWS* were amplified and cloned into the ARS18-based plasmid. The pTEF1 as strong constitutive promoter was used to control the gene expression. The plasmids were transformed into the Po1f or Po1f-ΔPEX10 (Le3) strain and the Sc-Po1f, Mm-Po1f, Mh-Po1f, Sc-Le3, Mm-Le3 and Mh-Le3 strains were generated. (B) The codon-optimized *HPT* and *URA3* genes were used as selection markers for random integration into the *Y. lipolytica* Po1f strain genome, which were controlled under the *Leu2* minimal promoter and a short synthetic terminator T_{Synth8}. The MhFAR-HPT and WS-URA3 linear cassettes were assembled by overlap extension PCR. Sc: *Simmondsia chinensis; Mm: Mus musculus; Mh: Marinobacter hydrocarbonoclasticus; Ab: Acinetobactor baylyi.*

Fig. 2. Fatty alcohols (A) and wax esters (B) produced by the engineered *Y. lipolytica* Po1f strains in shaking flasks. Co-expression of ScFAR, MmFAR or MhFAR together with AbWS was performed in strain Po1f (Sc-Po1f, Mm-Po1f and Mh-Po1f), Le3 (Sc-Le3, Mm-Le3 and Mh-Le3), SI and DI, respectively. All strains were grown in the nitrogen-limited medium using glucose as main carbon source in flask scale. (C) The SI strain was cultured in the 1-L fed-batch fermentation scale.

Fig. 3. Metabolic Engineering of *Y. lipolytica* for Wax Ester Production. (A) Strategy for Insertion of *MhFAR* and *AbWS* in *Y. lipolytica* by randomly integrated into chromosome. (B) Simplified schematic representation of key fluxes in wax ester synthesis coupled with TAG metabolism in engineered *Y. lipolytica* in the presence of glucose or oils/fats as carbon source. Substrate abbreviations: G6P, Glucose-6-Phosphate; F6P, Fructose-6-phosphate; G3P, Glyceraldehyde-3-phosphate; DHAP, Dihydroxyacetone phosphate; Gly-3-P, Glycerol-3-phosphate; LPA, Lyso-phosphatidic acid; PA, Phosphatidic acid; DAG, Diacylglycerol; TAG, Triacylglycerol; FAR, Fatty acyl-CoA reductase; WS, Wax ester synthase; FFA, Free fatty acids; PPP, Pentose phosphate pathway; FAS, Fatty acid synthesis; TCA cycle, Tricarboxylic acid cycle; ER, Endoplasmic reticulum.

Fig. 4. Feeding Hydrophilic and Hydrophobic Carbon Sources for Wax Ester Production by Engineered *Y. lipolytica* VSWE1 in Shaking Flask. Time course of (A) dry cell weight and (B) wax ester titer from VSWE1 strain after 120hr flask cultivation in the presence of glucose, oleic acid, soybean oil or WCO as carbon source. (C) Lipid accumulation in VSWE1. (D) Wax ester profile of VSWE1 expressing *MhFAR* and *AbWS*. (E) The carbon chain length of wax esters.

Fig. 5. Engineering of *E. coli* BL21(DE3) for Wax Ester Biosynthesis. (A) Construction of expression vector for wax ester production in *E. coli.* Case 1: transformation with pFAR and pWS; Case 2: transformation with pFAR-WS. Case 3: transformation with p(FAR-WS) fusion gene. (B) Wax ester titer and (C) specific titer for 30hr flask cultivation by co-feeding of glucose with glycerol, oleic acid, soybean oil or WCO as carbon source. (D) The composition of wax esters.

Fig. 6. Heterologous Expression of FAR-WS Fused Enzyme in *E. coli* BL21(DE3) for Wax Ester Biosynthesis in Fermentation Scale. Time course of (A) dry cell weight, (B) wax ester titer and (C) specific titer. (D) The composition of intracellular wax esters. Glc: glucose; Gly: glycerol; OLA: oleic acid; WCO: waste cooking oil.

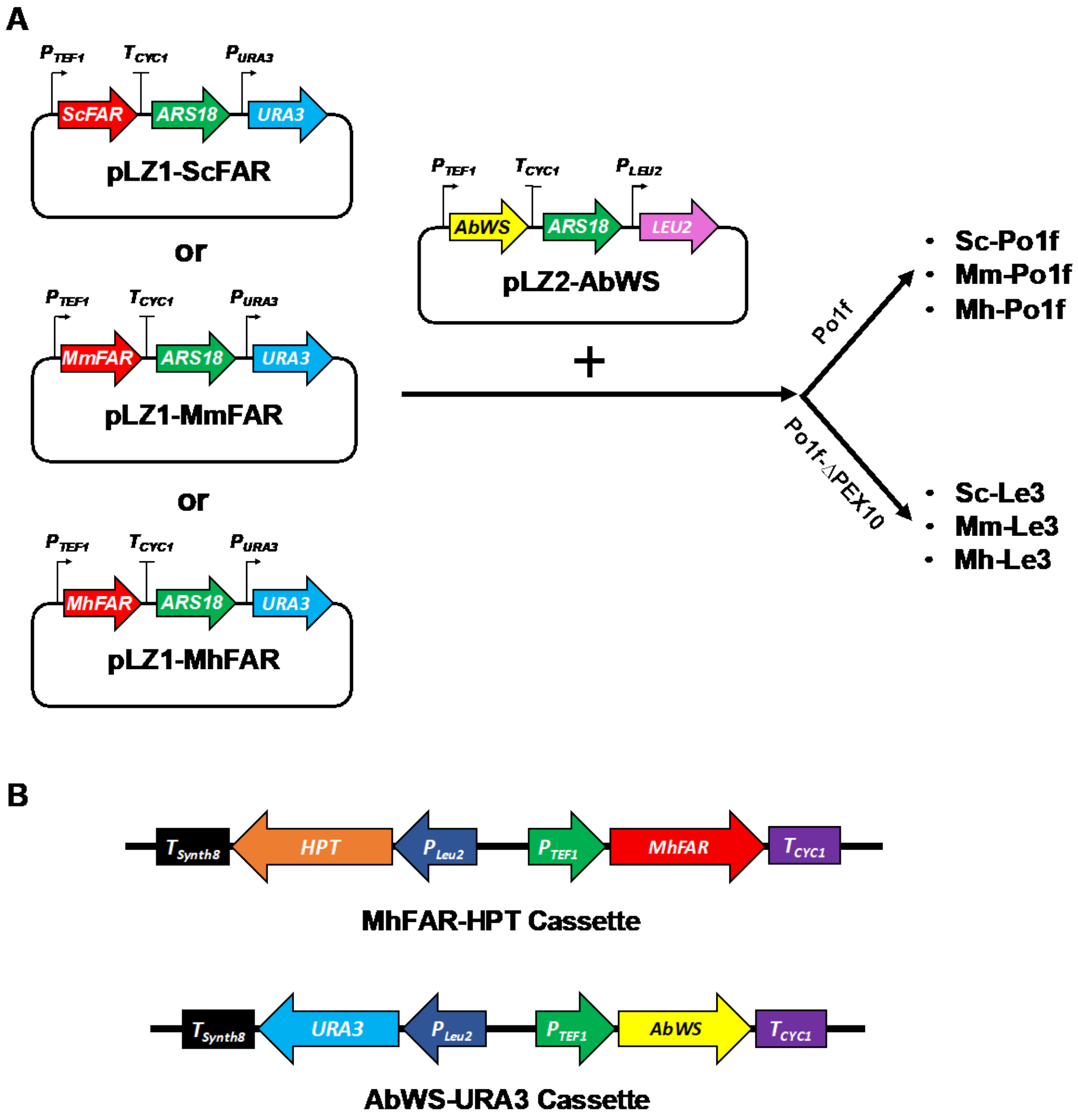
A
P_{TEF1}
T_{CYC1}
P_{URA3}
ScFAR
ARS18
URA3
pLZ1-ScFAR
or
P_{TEF1}
T_{CYC1}
P_{URA3}
MmFAR
ARS18
URA3
pLZ1-MmFAR
or
P_{TEF1}
T_{CYC1}
P_{URA3}
MhFAR
ARS18
URA3
pLZ1-MhFAR
P_{TEF1}
T_{CYC1}
P_{LEU2}
AbWS
ARS18
LEU2
pLZ2-AbWS
+
Po1f
Po1f-ΔPEX10
Sc-Po1f
Mm-Po1f
Mh-Po1f
Sc-Le3
Mm-Le3
Mh-Le3
B
T_{Synth8}
HPT
P_{Leu2}
P_{TEF1}
MhFAR
T_{CYC1}
MhFAR-HPT Cassette
T_{Synth8}
URA3
P_{Leu2}
P_{TEF1}
AbWS
T_{CYC1}
AbWS-URA3 Cassette

Fig. 1

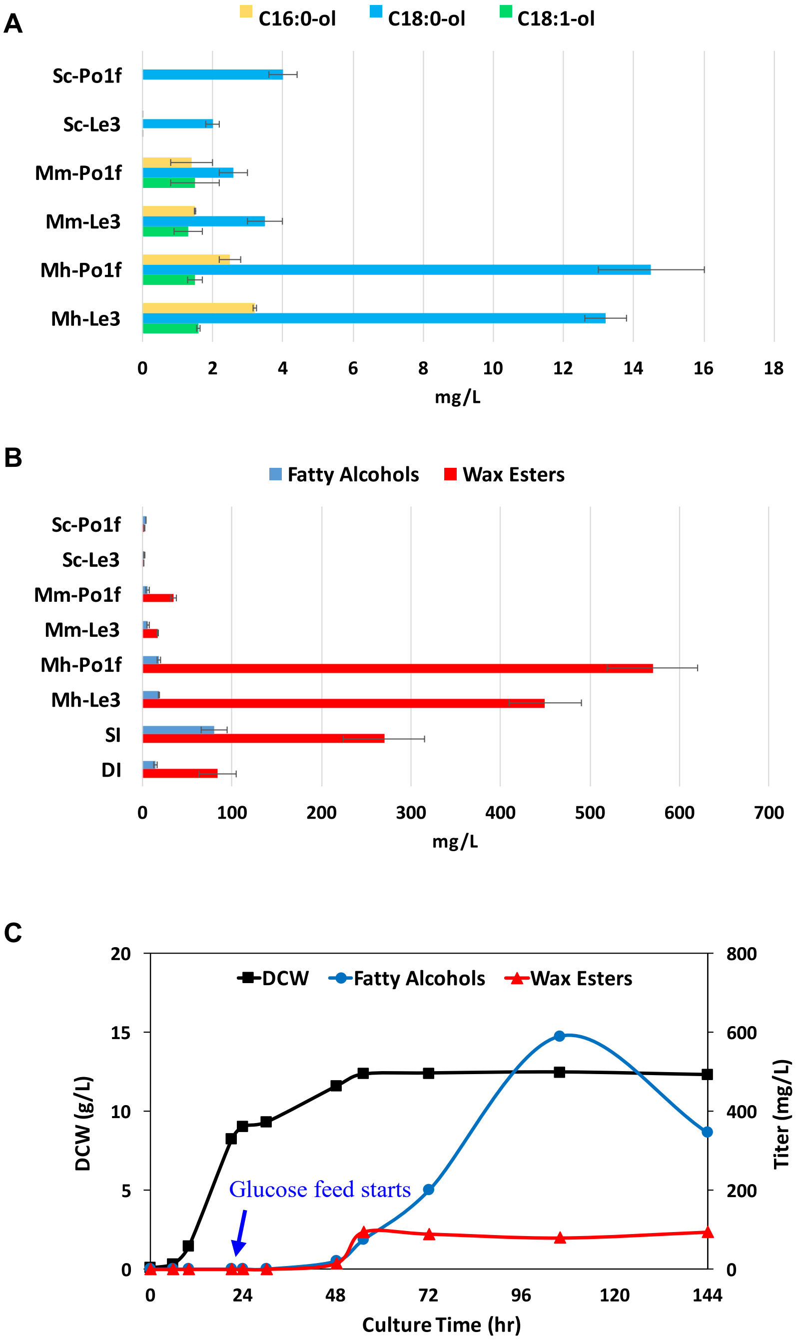
A
C16:0-ol
C18:0-ol
C18:1-ol
Sc-Po1f
Sc-Le3
Mm-Po1f
Mm-Le3
Mh-Po1f
Mh-Le3
0
2
4
6
8
10
12
14
16
18
mg/L
B
Fatty Alcohols
Wax Esters
Sc-Po1f
Sc-Le3
Mm-Po1f
Mm-Le3
Mh-Po1f
Mh-Le3
SI
DI
0
100
200
300
400
500
600
700
mg/L
C
DCW
Fatty Alcohols
Wax Esters
Glucose feed starts
DCW (g/L)
Titer (mg/L)
20
15
10
5
0
800
600
400
200
0
0
24
48
72
96
120
144
Culture Time (hr)

Fig. 2

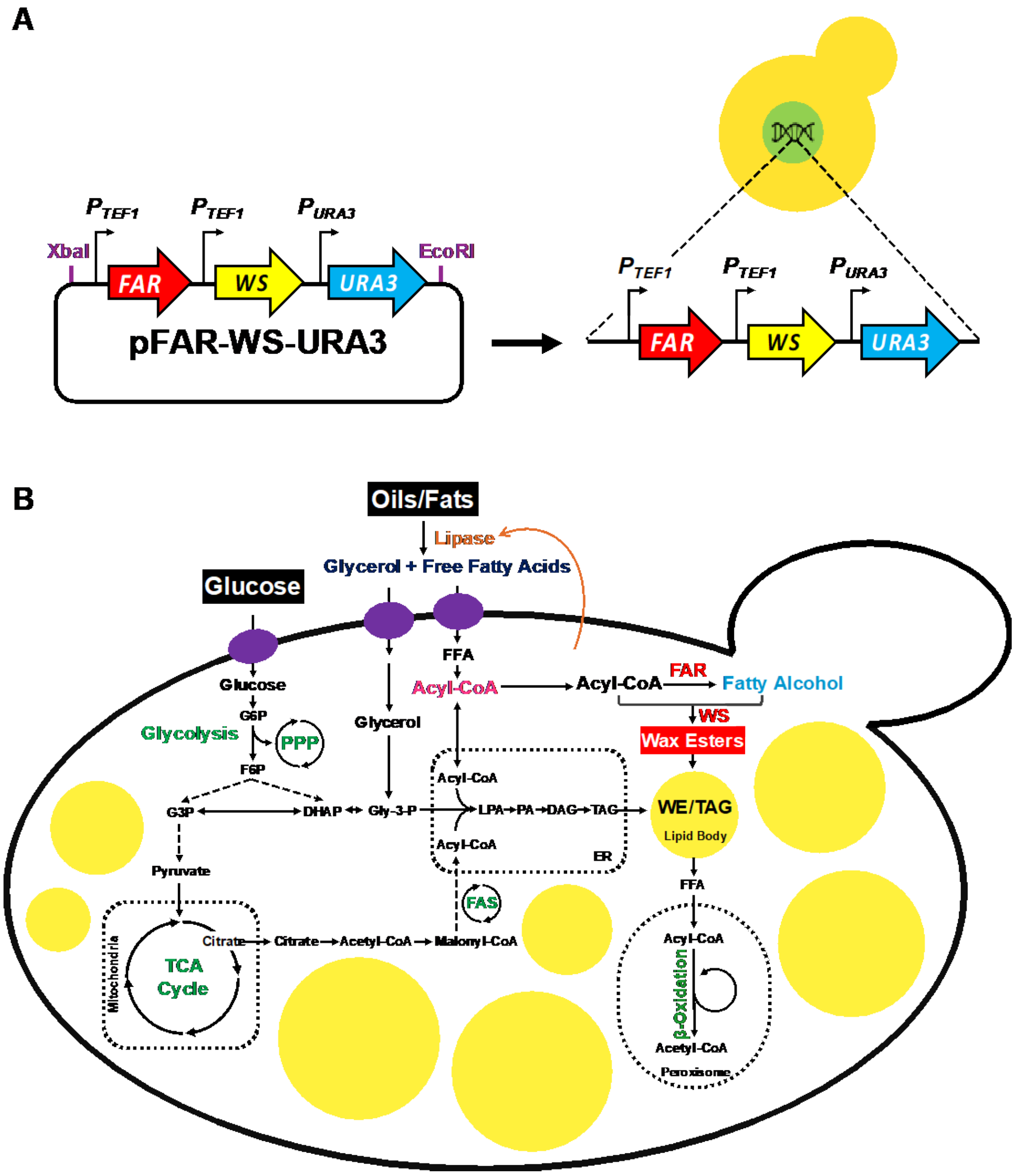
A
P TEF1
P TEF1
P URA3
XbaI
EcoRI
FAR
WS
URA3
pFAR-WS-URA3
P TEF1
P TEF1
P URA3
FAR
WS
URA3
B
Oils/Fats
Lipase
Glycerol + Free Fatty Acids
Glucose
FFA
Glucose
Acyl-CoA
Acyl-CoA
FAR
Fatty Alcohol
WS
Wax Esters
G6P
Glycolysis
PPP
F6P
Glycerol
G3P
DHAP
Gly-3-P
Acyl-CoA
LPA
PA
DAG
TAG
Acyl-CoA
ER
WE/TAG
Lipid Body
FFA
Pyruvate
FAS
Citrate
Citrate
Acetyl-CoA
Malonyl-CoA
TCA Cycle
Mitochondria
Acyl-CoA
β-Oxidation
Acetyl-CoA
Peroxisome

Fig. 3

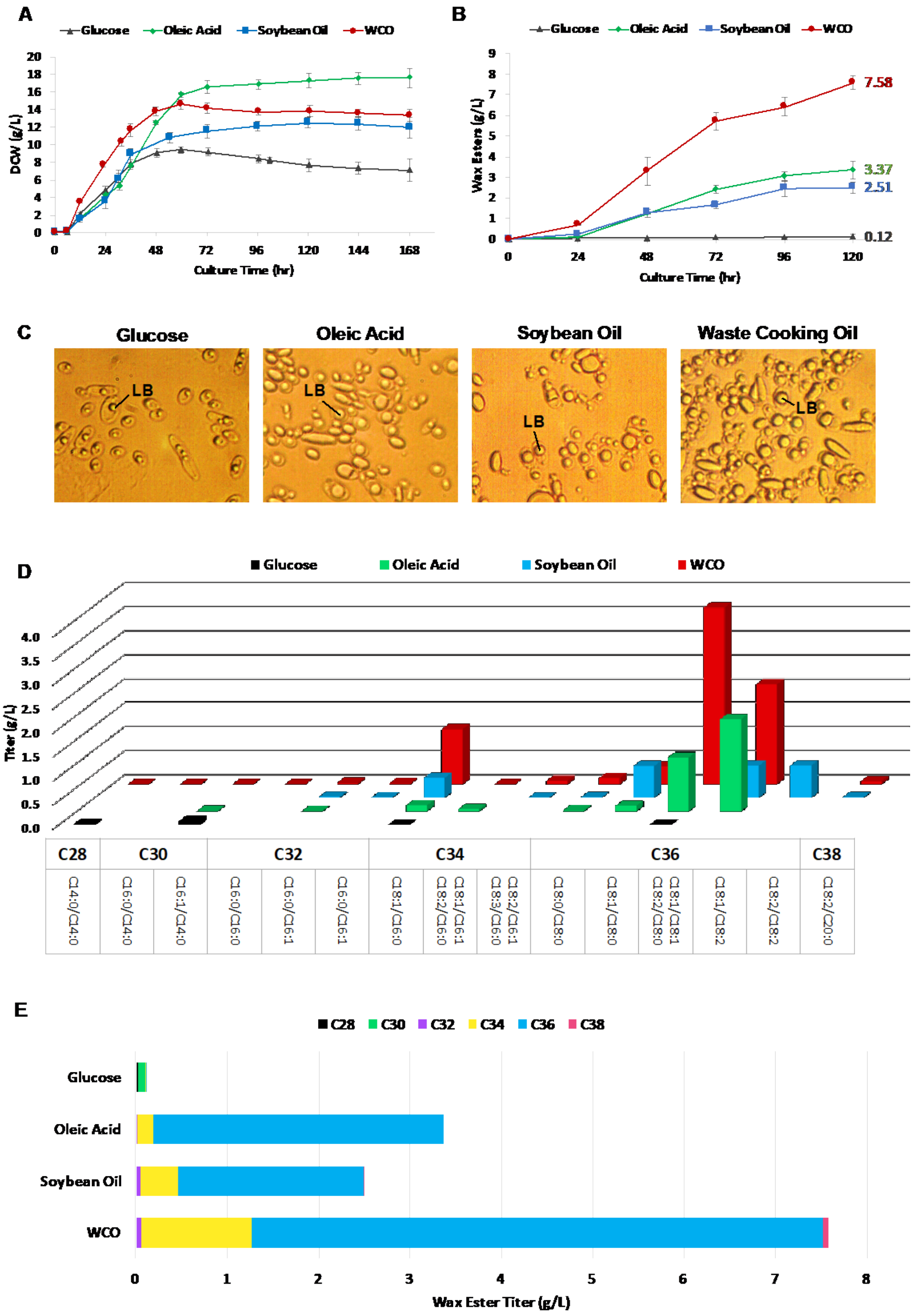A
Glucose
Oleic Acid
Soybean Oil
WCO
DCW (g/L)
0
2
4
6
8
10
12
14
16
18
20
0
24
48
72
96
120
144
168
Culture Time (hr)
B
Glucose
Oleic Acid
Soybean Oil
WCO
Wax Esters (g/L)
0
1
2
3
4
5
6
7
8
9
0
24
48
72
96
120
Culture Time (hr)
7.58
3.37
2.51
0.12
C
Glucose
Oleic Acid
Soybean Oil
Waste Cooking Oil
LB
LB
LB
LB
D
Glucose
Oleic Acid
Soybean Oil
WCO
Titer (g/L)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
C28
C30
C32
C34
C36
C38
C14:0/C14:0
C16:0/C14:0
C16:1/C14:0
C16:0/C16:0
C16:0/C16:1
C16:0/C16:1
C18:1/C16:0
C18:2/C16:0
C18:1/C16:1
C18:3/C16:0
C18:2/C16:1
C18:0/C18:0
C18:1/C18:0
C18:2/C18:0
C18:1/C18:1
C18:1/C18:2
C18:2/C18:2
C18:2/C20:0
E
C28
C30
C32
C34
C36
C38
Glucose
Oleic Acid
Soybean Oil
WCO
0
1
2
3
4
5
6
7
8
Wax Ester Titer (g/L)

Fig. 4

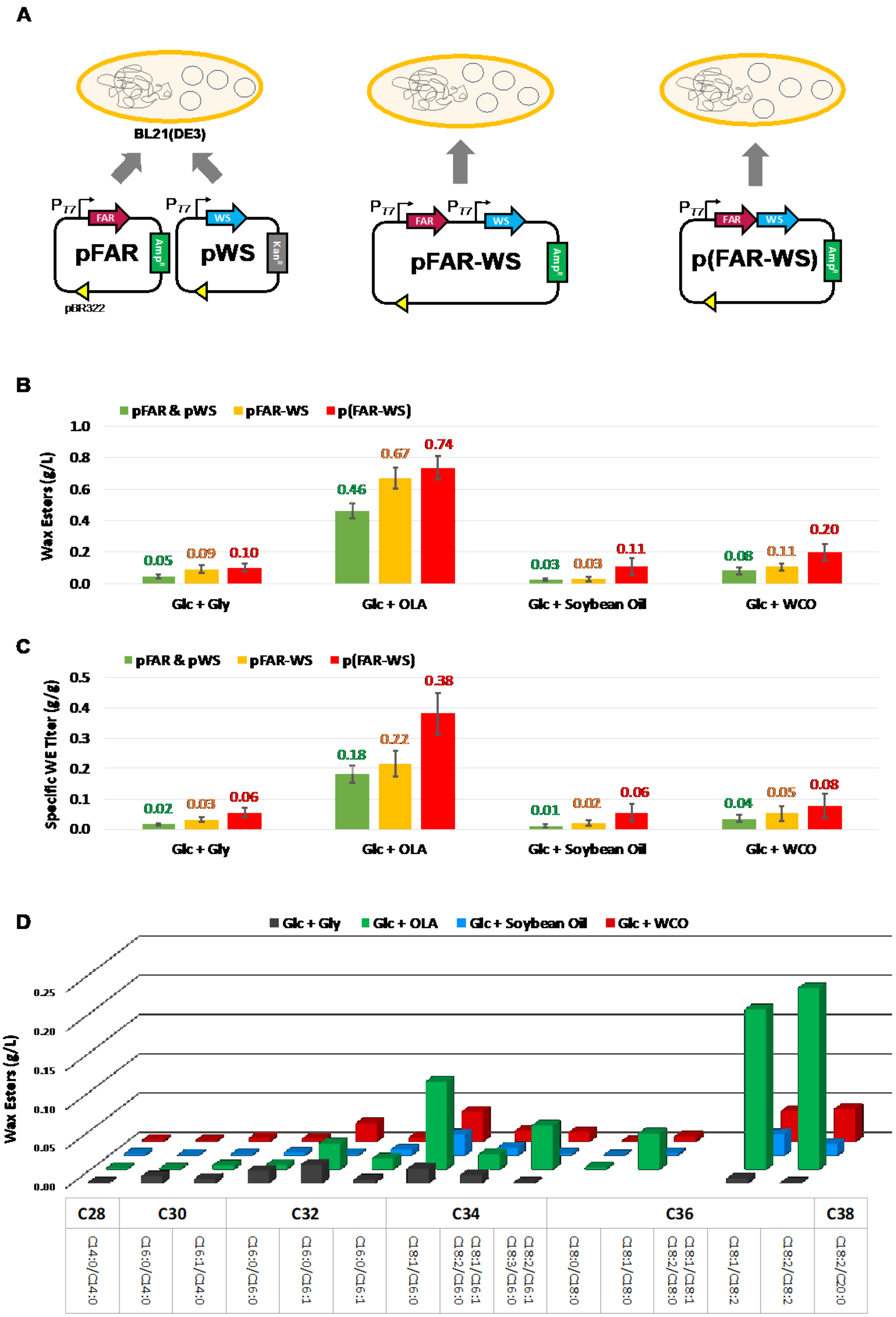
A
BL21(DE3)
P_{T7} FAR
P_{T7} WS
pFAR
Amp^R
pWS
Kan^R
pBR322
P_{T7} FAR P_{T7} WS
pFAR-WS
Amp^R
P_{T7} FAR WS
p(FAR-WS)
Amp^R
B
pFAR & pWS
pFAR-WS
p(FAR-WS)
Wax Esters (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
0.05 0.09 0.10
0.46 0.67 0.74
0.03 0.03 0.11
0.08 0.11 0.20
Glc + Gly
Glc + OLA
Glc + Soybean Oil
Glc + WCO
C
pFAR & pWS
pFAR-WS
p(FAR-WS)
Specific WE Titer (g/g)
0.5
0.4
0.3
0.2
0.1
0.0
0.02 0.03 0.06
0.18 0.22 0.38
0.01 0.02 0.06
0.04 0.05 0.08
Glc + Gly
Glc + OLA
Glc + Soybean Oil
Glc + WCO
D
Glc + Gly
Glc + OLA
Glc + Soybean Oil
Glc + WCO
Wax Esters (g/L)
0.25
0.20
0.15
0.10
0.05
0.00
C28
C30
C32
C34
C36
C38
C14:0/C14:0
C16:0/C14:0
C16:1/C14:0
C16:0/C16:0
C16:0/C16:1
C16:0/C16:1
C18:1/C16:0
C18:2/C16:0 C18:1/C16:1
C18:3/C16:0
C18:2/C16:1
C18:0/C18:0
C18:1/C18:0
C18:2/C18:0 C18:1/C18:1
C18:1/C18:2
C18:2/C18:2
C18:2/C20:0

Fig. 5

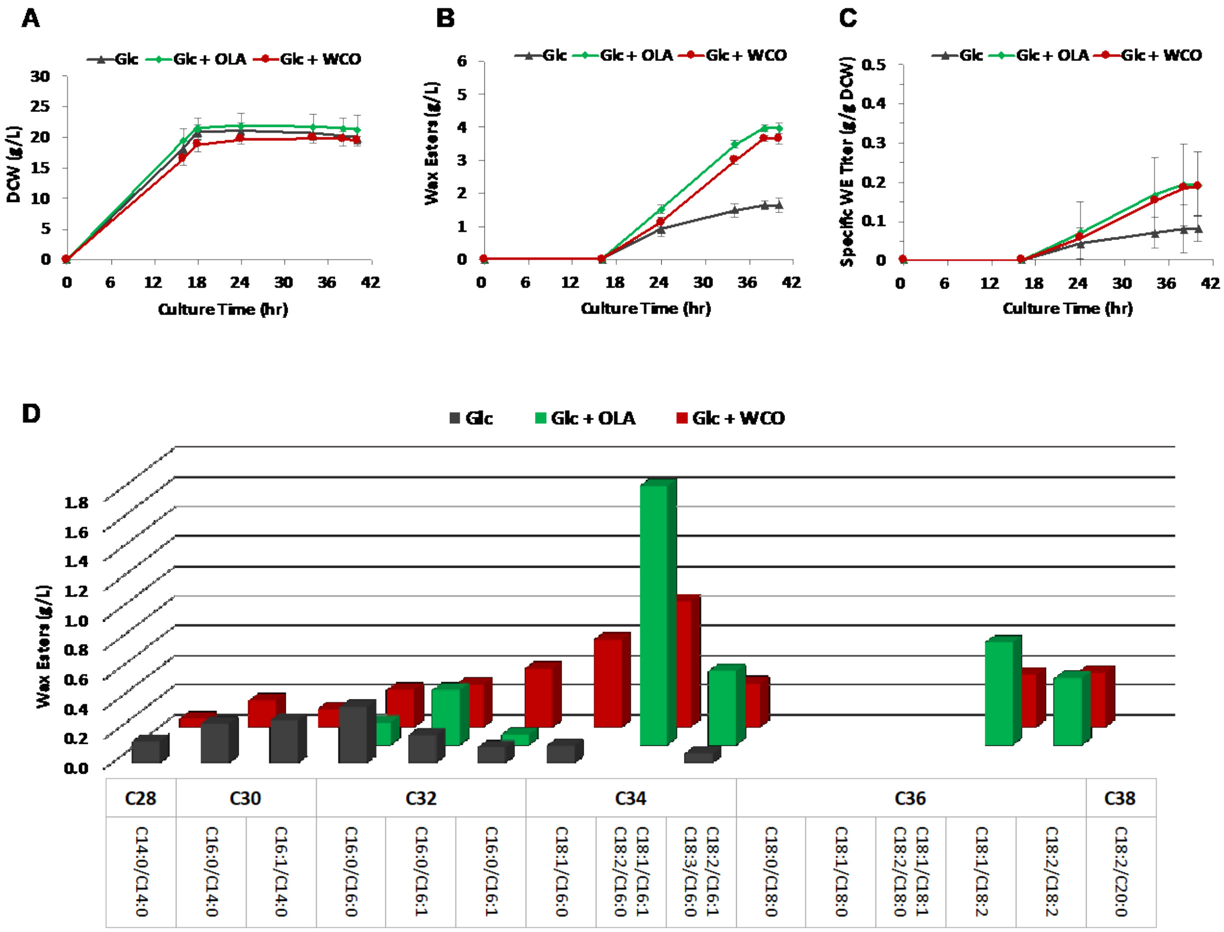
A
Glc
Glc + OLA
Glc + WCO
DCW (g/L)
30
25
20
15
10
5
0
0
6
12
18
24
30
36
42
Culture Time (hr)
B
Wax Esters (g/L)
6
5
4
3
2
1
0
Culture Time (hr)
C
Specific WE Titer (g/g DCW)
0.5
0.4
0.3
0.2
0.1
0
Culture Time (hr)
D
Glc
Glc + OLA
Glc + WCO
Wax Esters (g/L)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C28
C30
C32
C34
C36
C38
C14:0/C14:0
C16:0/C14:0
C16:1/C14:0
C16:0/C16:0
C16:0/C16:1
C16:0/C16:1
C18:1/C16:0
C18:2/C16:0 C18:1/C16:1
C18:3/C16:0 C18:2/C16:1
C18:0/C18:0
C18:1/C18:0
C18:2/C18:0 C18:1/C18:1
C18:1/C18:2
C18:2/C18:2
C18:2/C20:0

Fig. 6