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A New Biomanufacturing Platform for Bioconversion of Plant Oils into High-Value Products

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Abstract

The United States produces about 20 million tons of plant oils every year, which is about twice as much as the total sugar production. While more and more sugars are used to make fuels, chemicals, and value-added bioproducts via industrial biotechnology, plant oils (mainly vegetable oils) are usually considered as common agriculture commodities for food and feed applications, which generate low or limited economic values. To significantly increase plant oil's economic values and expand its applications, a new biomanufacturing platform emerges that employs a metabolically engineered yeast, *Yarrowia lipolytica*, to directly convert plant oils into high-value products, such as wax esters, long-chain diacids, carotenoids, and omega-3 fatty acids. This paper summarizes the recent progresses that have been made in metabolic engineering and bioprocess engineering of *Y. lipolytic*a and the future directions to achieve efficient bioconversion of plant oils into various high-value products.

Keywords: Bioprocess Engineering; Biomanufacturing; High-Value Products; Metabolic Engineering; Lipids; *Yarrowia lipolytica*

Introduction

Plant oils are important agricultural commodities from palm, soybean, corn, and other oil crops, with an annual production of approximately 20 million tons in the US, which is about twice as much as the total sugar production [1]. While sugars are widely used in biotechnology industry to make fuels, chemicals, and value-added bioproducts [2,3], plant oils are primarily used for food, feed, or nutritional applications with low or limited economic values. To significantly increase economic values of plant (mainly vegetable) oils, a new biomanufacturing platform is emerging recently that uses a metabolically engineered yeast, *Yarrowia lipolytica*, to directly convert lipid substrates into a series of high-value products such as carotenoids, long-chain diacids, wax esters, Polyhydroxyalkanoates (PHAs), and Hydroxylated Fatty Acids (HFAs) [4,5]. In addition, the wild type *Y. lipolytica* is well-known for production of food-grade citric acid [6,7], and the yeast was also successfully engineered to produce omega-3 fatty acids and the project was commercialized in DuPont [8,9]. These products also became part of the high-value product portfolio.

Here we use four representative example products, including wax esters, long-chain diacids, omega-3 fatty acids, and carotenoids, to elucidate recent progresses and future directions in establishing a new biomanufacturing platform that employs metabolically engineered *Y. lipolytica* to make a series of high-value products from plant oils (Figure 1). The following technical fields will be discussed in this paper: (1) understanding oil uptake and fatty acid metabolism in *Y. lipolytica*, (2) cell morphology engineering for hydrophobic substrate utilization, (3) metabolic pathway engineering for each specific product, and (4) fermentation and bioreactor engineering.

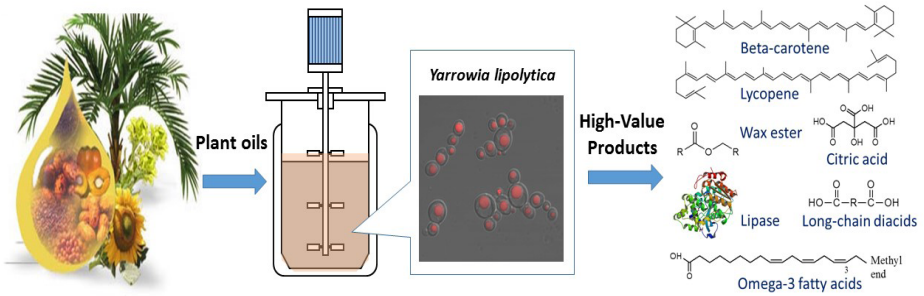

Figure 1: An overview of the biomanufacturing platform that combines intracellular metabolic engineering of *Y. lipolytica* and extracellular fermentation and bioreactor engineering for bioconversion of plant oils into high-value products.

Oil uptake and Fatty Acid Metabolism

Understanding oil uptake and fatty acid metabolism is extremely important before we start to engineer a strain for bioconversion of lipids into high-value products. Some nonconventional yeast, especially *Y. lipolytica*, have a unique advantage of being the host strain for lipid bioconversion since they are originally isolated from the environment where they are able to assimilate fatty acids for cell growth and energy maintenance [5,10,11].

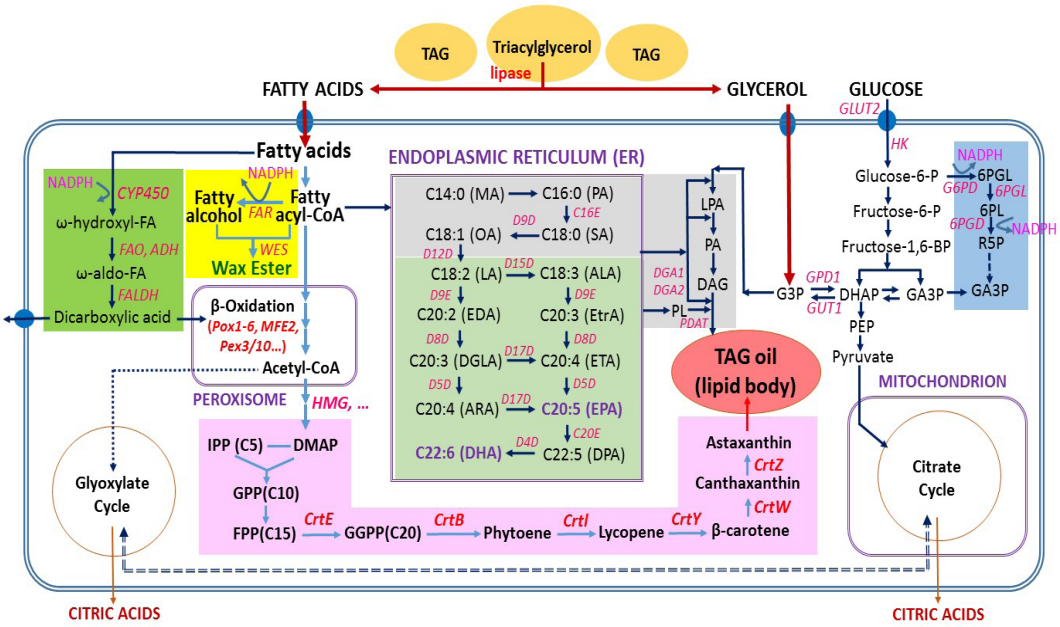

Figure 2: An overview of metabolic engineering strategies in *Yarrowia lipolytica* for production of high-value products from triacylglycerol oil (Filled blue ovals: transport enzymes. Abbreviations: ADH, alcohol dehydrogenase; C16E and C20E, C16 and C20 elongase; CrtB, phytoene synthase; CrtE, GGPP synthase; CrtI, phytoene desaturase; CrtW, β-carotene ketolase; CrtY, lycopene cyclase; CrtZ, β-carotene hydroxylase; CYP450, cytochromes P450 enzyme; D4D, D8D, D9D, D12D, D15D, & D17D, Δ-4, Δ-8, Δ-9, Δ-12, Δ-15, & Δ-17 desaturase, respectively; D9E, Δ-9 elongase; DAG, diacylglycerol; DGA1 & DGA2, DAG acyltransferase; DMAPP, dimethylallyl diphosphate; FALDH, fatty aldehyde dehydrogenase; FAO, fatty alcohol oxidase; FAR, fatty acid reductase; FPP, farnesyl pyrophosphate; G6PD, glucose-6-phosphote dehydrogenase; GA3P, glyceraldehyde3-phosphate; GGPP, geranylgeranyl pyrophosphate; GLUT2, glucose transporter 2; GPP, geranyl pyrophosphate; GPD1, glycerol-3-phosphate dehydrogenase; GUT1, glycerol kinase; HK, hexokinase; HMG, HMG reductase; LPA, lysophosphatidic acid; MFE2, peroxisomal multi-functional enzyme; PA, phosphatidic acid; PDAT, PL and DAG acyltransferase; PEP, phosphoenolpyruvate; Pex3 and Pex10, peroxisome biogenesis gene 3 and 10; 6PGD, 6-Phosphogluconate dehydrogenase; 6PGL enzyme, 6-Phosphogluconolactonase; 6PGL intermediate, 6-phospho-gluconolactone; PL, phospholipid; Pox1~Pox6, acyl-CoA oxidase 1~6; TAG, triacylglycerol; WES, wax ester synthase).

The *Y. lipolytica* cells have hydrophobic cell surfaces and can secret surfactants and emulsifiers that facilitate lipid droplets to attach to cell surfaces and then to be consumed by cells [10,12]. The yeast cells can import the extracellular Free Fatty Acids (FFAs) and then metabolize the substrate inside cells through the β-oxidation process (see also Figure 2). Since plant oils are usually in the form of Triacylglycerol (TAG), the oil uptake process starts at degrading plant oils into FFAs and glycerol, which is catalyzed by lipases released from *Y. lipolytica* cells [10]. The biochemical steps in oil decomposition, fatty acid transport, and intracellular metabolism processes are controlled by the key genes encoding lipase formation, FFA activation, transport, and degradation, and peroxisome function, which have been identified and reviewed earlier [13-16]. Further research and overexpression some of the key genes are expected to promote cell growth and improve oil uptake and bioconversion.

The fatty acid transport mechanism is still not clear, as pointed out in the previous reviews [10,17]. For cell growth, *Y. lipolytica* cells may have different preferences for fatty acid substrates with various number of double bonds and chain lengths [10,17]. It was found that more double bonds in fatty acids (more unsaturated) gave faster cell growth and substrate utilization [18,19]. For example, Papanikolaou et al. [19] observed that the uptake rates of unsaturated oleic (C18:1) and linoleic acids (C18:2) were significantly higher than those of the saturated palmitic (C16:0) and stearic (C18:0) acids. Since the fatty acid metabolism rate is also dependent on the carbon chain length, this can be regulated by two fatty Acyl-CoA Synthetase (ACS I and II). Typically, ACS I and ACS II convert the long-chain and short-chain fatty acids, respectively, into fatty acyl-CoAs in the cytosol, which are further transported into the peroxisome for β-oxidation [20,21].

After FFAs are transported inside the cells, they are degraded through both β- and ω-oxidation pathways, with β-oxidation being the major pathway for the yeast cells to gain energy for growth on a lipid substrate [4,5,11,21]. The β-oxidation can be considered as a reverse process of fatty acid synthesis. Each β-oxidation cycle releases two carbons from the FA backbone and generates one acetyl-CoA. The β-oxidation is first catalyzed by acyl-CoA oxidases encoded by six POX genes (POX1~6), with each having a different preference for the fatty acid with a specific chain-length. The *Y. lipolytica* cells are not able to grow on lipids/fats if all POX genes are completely knocked out [12,22,23]. After that, β-oxidation are further catalyzed by MFE (multifunctional enzyme), which is encoded by a single MFE gene. The β-oxidation can also be completely stopped by deletion of the single MFE gene [24,25]. The last step of β-oxidation is catalyzed by the thiolase POT1. All the β-oxidation steps take place in the peroxisome, thus mutations in any genes that are responsible for peroxisome structure and function may also completely block the β-oxidation activities. In the previous work, this strategy was applied to improve omega-3 EPA production by deletion of PEX3 or PEX10 in *Y. lipolytica* [8, 26].

The intracellular fatty acids can also be degraded through ω-oxidation pathway in Endoplasmic Reticulum (ER) (see also the green-background pathway in Figure 2) [27]. The ω-oxidation starts with the oxidation of the carbon at ω-position (i.e. the furthest to carboxylic group) of a fatty acid, which is catalyzed by FAH (fatty acid ω-hydroxylase) contained in CYP450 (cytochrome P450). After the fatty acid is converted into ω-hydroxy fatty acid, it is further converted to the fatty aldehyde with the catalysis by ADH (Fatty Alcohol Dehydrogenase). The fatty aldehyde is eventually converted to the Dicarboxylic Acid (DCA) by FALDH (fatty aldehyde dehydrogenase). However, the produced DCAs can be exported out as a product, which was how ω-oxidation pathway was used to make long-chain Dicarboxylic Acids (LCDAs) [28]. Otherwise, DCA is further utilized by cells and converted to acetyl-CoA through β-oxidation process in peroxisome.

Engineering Cell Morphology to Improve Lipid Uptake

Unlike any conventional yeasts, *Y. lipolytica* exhibits dimorphic cell shapes. It has been considered as a suitable model for studying the yeast-to-hypha transition that exhibits various morphological forms [29-30]. The cell morphology of *Y. lipolytica* is subject to change from unicellular yeast form to multicellular filamentous form (pseudohyphae or septate true hyphae) in a stressful environment with nutrient limitation, high osmotic pressure, or other non-preferred growth conditions [29-30]. It is believed that the cell morphology engineering may help to increase the specific surface area of *Y. lipolytica* cells to reach more nutrients or to secret the product in a stressful environment.

Regulation of the yeast-to-hypha transition in *Y. lipolytica* depends on the environmental and physiological conditions and genetic characteristics. Since the *Y. lipolytica* morphology may subject to self-transition as environmental conditions change, the obtained morphology status cannot be steadily maintained. To achieve a stable cell morphology that is more favorable to lipid uptake and bioconversion, gene regulation is suggested through either positive or negative regulators in *Y. lipolytica*. Positive gene regulators, including YlMHY1, YlBEM1 and YlRAS2 genes, induce the mycelial growth, while negative gene regulator such as YlZNC1 and YlTPK1 regulate cellular morphology to yeast-form during the dimorphic transition [31-35].

In addition to the gene regulators from the yeast, the genes controlling the bacteria morphology can also be adopted into the *Y. lipolytica* system. For example, it was found in bacteria that cytoskeletal filament bundles such as MreB control the cell morphology and determine whether the cell takes on a spherical or a rod-like shape [36-38]. The MreB gene can mechanically reinforce

the cell wall and prevent the onset of instability. In addition to MreB, CreS (encoding crescentin) is another gene with similar function, when CreS in *Caulobacter crescentus* was deleted, the cell morphology was changed from its characteristic curved shape to new straight rods [39].

Metabolic Engineering for Bioconversion of Lipids into High-Value Products

The *Y. lipolytica* yeast is able to efficiently degrade oil/lipid substrate into fatty acids, and then convert the fatty acids into many high-value products [4,5]. Here we select wax esters, carotenoids, long-chain diacids, and omega-3 fatty acids as the representative examples, and summarize the metabolic engineering strategies to achieve a production at high titer, rate, and conversion yield (see Figure 2).

Wax Ester

Wax Esters (WEs) are a type of esters that are formed by combining one fatty acid with one fatty alcohol and releasing one water molecule:

$$R\text{-}COOH + R'\text{-}OH \rightarrow R\text{-}COO\text{-}R' + H_2O$$

WEs can be used as high-performance lubricants, cosmetics, candles, printing inks, and coating materials [4,5,40, 41]. The major source of the WEs on the current market is from mineral oil [5]. Some other natural sources for WEs include whale sperm oil and jojoba oil, but they are usually too expensive to be widely used. Therefore, microbial synthesis of WEs becomes more attractive. Previous studies have shown that *Mycobacterium* [42], *Rhodococcus* [43], *Acinetobacter* [44], and *Marinobacter* [45] strains were able to synthesize Bio-WEs by using petroleum hydrocarbons [46] and gluconate [47] as carbon source.

The metabolic pathway for biosynthesis of WE from fatty acids is relatively simple as compared to the pathways for other products (see the yellow-background pathway in Figure 2). The free fatty acids are first converted to fatty acyl-CoA with the catalysis of fatty acyl-CoA synthetase. Then the fatty acyl-CoA is further converted to fatty alcohol by FAR (fatty acyl-CoA reductase). This step requires consumption of NADPH. WE finally synthesized by WES (wax ester synthase) to combine fatty acyl-CoA with fatty alcohol and release the CoA unit [5]. Since *Y. lipolytica* has high efficiency of degrading TAG oil into FFAs, import the FFAs, and partially convert FFAs to fatty alcohol, it is expected that high-yield production of WE can be achieved by overexpression of both FAR and WES and minimizing other byproduct pathway in the yeast [5].

Long-Chain Dicarboxylic Acids

The Long-Chain Dicarboxylic Acids (LCDAs) can be used as monomers to make PA6-16 and PA6-18 nylon polymers, which have special physical properties with advantages over classic nylons, such as more hydrophilic resistance, far less brittle, and higher tolerance of tensile stress [48-50]. Currently, most industrial Dicarboxylic Acids (DCAs), especially for those short-chain DCAs, are produced via chemical routes in high yields by oxidation under high-pressure and high-temperature conditions [51]. An alternative method of producing DCA of C12 chain length (dodecanedioic acid, DDDA) is to use an oxidative cleavage of cyclic oligomers of butadiene [52]. However, for producing long-chain DCAs (LCDAs, i.e. DCAs with C10 or longer carbon chain length), the production costs via chemical processes significantly increase as the carbon chain length of DCA rises due to coproduction of numerous byproducts [53], which also makes the product recovery and purification more difficult [54]. Therefore, production of LCDAs from microbial fermentation processes become more technically achievable and economically attractive [55].

There were some earlier efforts of engineering *S. cerevisiae* [56] and *E. coli* [57] to make DCAs. However, since these strains do not have efficient pathways for fatty acid synthesis, fatty acid transport, and ω-oxidation that are required for DCA synthesis, heterologous expression of key genes for DCA synthesis only led to production of DCAs at very poor titers (< 0.5 g/L) or even non-detectable [56-58]. On the contrary, the oleaginous yeast, including *Candida tropicalis* and *Y. lipolytica*, have the unique advantage of using hydrophobic substrate such as alkanes and fatty acids to make DCAs through ω-oxidation, which is a subsidiary pathway of the β-oxidation process. Currently, the majority of LCDAs are produced in China from alkanes by fermentation with *C. tropicalis* [54, 58-60]. One of the most significant progresses in DCA production by *C. tropicalis* was achieved by a group of scientists in Institute of Microbiology, Chinese Academy of Sciences, where the strains had been successfully mutated by traditional physical and chemical methods to produce more than 130 g/L dodecanedioic acid (DDDA, or C-12 dDCA) from dodecane, which were then commercialized in several companies in China [59]. Picataggio et al. [54] improved the *C. tropicalis* strain by amplifying the ω-oxidation and block the β-oxidation pathway, which led to an increase of DCA production from 95 g/L to 140 g/L in bench scale fermentation.

Despite the significant progresses achieved in the *C. tropicalis* strains, the concern of the pathogen feature of the strain (Biosafety Level 2, BSL-2) and the strains' preference of using the fossil oil-derived (non-renewable) alkane substrate made scientists start to switch more attention to *Y. lipolytica*, which is a BSL-1 strain and can efficiently use the renewable vegetable oil substrate [60-62]. In *Y. lipolytica*, FFA is converted into DCA through ω-oxidation process (see the green-background pathway in Figure 2). To achieve an efficient synthesis of LCDA in *Y. lipolytica* from TAG oil, the first thing is to amplify the ω-oxidation pathway [54,61]. The ω-oxidation process starts by oxidization of FFA to the

corresponding ω-hydroxy FA, which is catalyzed by a cytochrome P450 monooxygenase (CYP or ALK) and an NADPH-dependent cytochrome P450 reductase (NCP) [60]. Then the ω-hydroxy FA is further oxidized to fatty-aldehyde with the catalysis by fatty-alcohol dehydrogenases (ADH, mainly in *Candida viswanathii*) or fatty acid oxidases (FAO, mainly in *Y. lipolytica*) [27, 60]. DCA is finally synthesized in the last step by converting the aldehyde group to the carboxyl group, which is catalyzed by a Fatty-Aldehyde Dehydrogenase (FALDH) [60].

To further improve DCA production, any byproduct pathways should be minimized or inactivated, this includes (1) blocking the β-oxidation pathway by deletion of pox1~6 [12,22,23], MFE1 [24,25], or pex3/10 [8,26] genes; and (2) minimizing the TAG oil formation from FFA by deleting DGA1/2 [61]. Since the β-oxidation is blocked for DCA production, other hydrophilic carbon source such as glucose or glycerol needs to be supplied to support cell growth, provide energy maintenance, and generate co-factor NADPH during the fermentation (Figure 2). Though using hydrophilic substrate alone such as glucose can support both cell growth and DCA production, only poor DCA production was achieved due to the overall low efficiency of converting glucose to FFA and FFA to DCA [62]. Therefore, co-feeding glucose (or other hydrophilic substrate) and TAG oil (or other types of lipid substrate) and optimization of the feeding strategy in fermentation should be further explored in future for high-yield and low-cost production of LCDA from oil/lipid substrate that contains long-chain fatty acid unit.

Omega-3 Fatty Acids

It is believed that omega-3 fatty acids (mainly refer to the C20:5, EPA and the C22:6, DHA) have significant health benefits in improving human's heart health, immune function, mental health, and infant cognitive development [8]. The current major source of omega-3 fatty acids is from fish oil, which is limited by its sustainability due to the concerns from overfishing and unpredictable contamination in ocean [8,11]. Microalgae was also used in Martek (now part of DSM) to make omega-3 DHA from glucose at large scale [8,63]. In recent studies from JILES (Japan EPA Lipid Intervention) [64] and AMR01 [65], EPA seemed to have more significant benefits in preventing from major coronary events and reducing triglyceride levels in adult patients with hypertriglyceridemia. Therefore, DuPont has developed a sustainable strategy of producing omega-3 EPA by using metabolically engineered *Y. lipolytica* [8,9].

Both omega-3 EPA and DHA can be synthesized in *Y. lipolytica* from C16~C18 fatty acid intermediates through either a Δ6-desaturase pathway (found in algae, mosses, fungi and others) or a Δ9-elongase and Δ8-desaturase pathway (found in euglenoids) [8,66,67]. Though glucose was still the primary carbon source in almost all previous researches for omega-3 biosynthesis, plant oils (or mainly vegetable oils) can potentially replace glucose for the same purpose. The Δ9-elongase and Δ8-desaturase pathway to make EPA and DHA is shown in Figure 2 with light green background. In a summary, this pathway requires overexpression of the following genes to convert C16~C18 fatty acids (the major components in plant oils) to omega-3 EPA (C20:5) [8,26]: (1) a Δ9-elongase to convert linoleic acid (LA, C18:2) to eicosadienoic acid (EDA, C20:2 n-6) and ALA (C18:3) to eicosatrienoic acid (ETrA: C20:3 n-3); (2) a Δ15-desaturase to convert LA (C18:2) to ALA (C18:3); (3) a Δ8-desaturase to convert EDA (C20:2) to DGLA (C20:3) and ETrA (C20:3) to ETA (C20:4); (4) a Δ5-desaturase to convert DGLA (C20:3) to ARA (C20:4) and ETA (C20:4) to EPA (C20:5); (5) a Δ17-desaturase to convert ARA (20:4) to EPA (C20:5) and DGLA (C20:3) to ETA (C20:4); and (6) a C20 elongase and a Δ4-desaturase to convert EPA (C20:5) to DHA (C22:6) (if DHA is desired). The Δ6-desaturase pathway can also be used to make EPA and DHA (not shown in Figure 2), but the difference between Δ6 and Δ9 pathways comes from the first two steps [8]. The first step in Δ6 pathway is converting LA (18:2) to GLA (18:3 n-6) and/or ALA (18:3) to STA (18:4 n-3) [8]. Then the second step is to convert GLA (C18:3 n-6) to DGLA (20:3 n-6) and/or STA (18:4 n-3) to ETA (20:4 n-3) [8]. All these elongation and desaturation steps take place in Endoplasmic Reticulum (ER). Also, all substrates for desaturases and elongases are believed to be catalyzed in the form of phospholipid and acyl-CoAs, respectively [8]. To further improve the omega-3 yield, the metabolic pathways for most byproducts should be blocked. The most important thing for high-yield omega-3 production is that the peroxisome biogenesis should be disrupted by knock-out of PEX gene [26]. In addition, fermentation process development also plays critical roles in further improving the titer, rate, and yield of omega-3 production [9].

Carotenoids

Carotenoids are a type of antioxidants that have shown critical benefits in human and animal health [68]. The most common carotenoids include lycopene, β-carotene, canthaxanthin, and astaxanthin [68]. It was estimated that the market value will reach $1.4 billion in 2018 [69, 70]. Currently, carotenoids are mainly produced either by extraction from natural organisms or by chemical synthesis [26]. Biosynthesis of carotenoids have been recently investigated by metabolic engineering in *E. coli*, *S. cerevisiae*, and *Y. lipolytica* to meet the increased need [68-71].

Carotenoids are bio-synthesized via the mevalonate pathway (see the pink-background pathway in Figure 2). During the biosynthesis process, acetyl-CoA is first converted to two C5 precursors, Isopentenyl Diphosphate (IPP) and Dimethylallyl Diphosphate (DMAPP), which is catalyzed by HMG reductase via multiple steps. Both IPP and DMAPP are then converted into Geranyl Pyrophosphate (GPP, C10), Farnesyl Pyrophosphate

(FPP, C15), and Geranylgeranyl Pyrophosphate (GGPP, C20) via a series steps. These C10~C20 intermediates are finally converted to various carotenoids (C40), which include lycopene, β-carotene, canthaxanthin, and astaxanthin [68-71].

Biosynthesis of carotenoids in *Y. lipolytica* received the interests not only from academia [71,74], but also from major biotechnology companies such as DuPont [72] and Microbia (now part of DSM) [73]. In DuPont's research, the carotenoid synthesis genes CrtE, CrtB, CrtI and CrtY were codon-optimized and then overexpressed in an omega-3 producing strain, which led to a production of 8.9 mg/g total carotenoids in biomass, with 64% being β-carotene [72]. This research also achieved a coproduction of carotenoids and omega-3 EPA in the yeast. Further engineering the *Y. lipolytica* led to significant production of canthaxanthin and astaxanthin [72]. In Microbia's research, *Y. lipolytica* was also engineered to produce β-carotene [73]. In addition, the β-carotene produced by *Y. lipolytica* demonstrated the same quality as compared to other commercial products on the market [73]. Since carotenoids are hydrophobic and soluble in lipids, it was found that increasing lipid content in yeast significantly improved carotenoid production, which could be achieved by block the β-oxidation [74]. For example, lycopene production increased to 16 mg/g biomass by mutation of pox1-pox6 and gut2 in β-oxidation and overexpression of codon-optimized CrtE, CrtB, CrtI, and HMG [74]. Most recently, Gao et al. demonstrated that the engineered *Y. lipolytica* produced up to 4 g/L β-carotene (nearly 50 mg/g in biomass) after further optimization of gene copy number, promoter strength of target genes, and the fermentation conditions [71].

Fermentation and Bioreactor Engineering

After a metabolically strain is constructed, more bioprocess engineering work is needed to achieve a production with high titer, rate, and yield. Typical bioprocess development includes strain screening, fermentation medium and process optimization, and bioreactor engineering [8,75].

In general, new strains are tested in shake flasks and bench scale fermentors. However, shake flasks support only low levels of cell growth (< 10 g DCW/L) and lack precise controls of pH values and Dissolved Oxygen (DO) levels. Bench scale fermentation experiments are time consuming and labor intensive [8,75,76]. Therefore, micro- or mini-bioreactors with independent and precise pH and DO controls become extremely important for high-throughput strain screening [76]. In addition, for each pre-selected strain, these micro- and mini-bioreactor experiments can be used for further optimization of medium and process parameters (T, pH, DO) [76]. For example, in DuPont's omega-3 research project, application of an advanced Micro-24 Bioreactor system significantly improved the efficiency of strain screening [8,75]. Moreover, the performance of a selected strain in Micro-fermentor usually gave the same or very similar conversion yield, specific omega-3 titers, and productivities as compared to those achieved in bench-, pilot-, and commercial-scale fermentors [8,75].

After a strain is constructed and pre-selected, the fermentation medium and process conditions should be optimized under bench-scale fermentation conditions [8,75]. Though minimal medium is preferred for initial medium optimization studies, rich medium with commercially available yeast extract or corn steep liquor should be investigated so that the developed fermentation process can be transferred to large-scale fermentors [8]. The temperature and pH values for *Y. lipolytica* fermentation are typically 30°C and 5.5~7.0, respectively [9]. In addition, *Y. lipolytica* requires oxygen to grow and make products [9]. A typical dissolved oxygen level for *Y. lipolytica* fermentation is within 20~30% [9,77]. This also suggests the cell density in a fermentation process should be carefully designed based on fermentor's capacity for Oxygen Transfer Rate (OTR) so that the entire process does not have serious oxygen limitations.

Most *Y. lipolytica* fermentation processes are operated in fed-batch mode to achieve high product titers [8,11,76]. Also, most large-scale fermentation facilities are built based on the design of batch or fed-batch processes. However, batch and fed-batch were developed from about 70 years ago and cannot meet the current need for high productivities and low capital cost [11,76]. Therefore, there have been many studies on continuous fermentation technologies in the past decades [77-81]. Unfortunately, while these continuous fermentation processes significantly improved productivities, they also led to losses in product conversion yield and/or titers. This is because most continuous fermentation processes are single-stage, which requires one fermentor to provide optimal conditions for both cell growth and product formation. However, product formation for most of *Y. lipolytica* fermentation is non- or partially growth associated, which suggests a single-stage continuous process is difficult to achieve efficient cell growth and product formation at the same time. Recently, a two-stage continuous fermentation process was developed, which consisted of a smaller fermentor (Stage 1) for continuous cell growth and a larger fermentor (Stage 2) for continuous omega-3 production [76]. Since cell growth and product formation were independently controlled under their own optimal conditions in separate stages, the two-stage continuous process successfully doubled the omega-3 EPA productivity, improved EPA concentration by 40%, and maintained the conversion yield very similar [76].

Another important issue has to be addressed for the fermentation processes with oil/lipid substrate is the mixing efficiency in a bioreactor since lipid/oil is insoluble in aqueous medium. *Y. lipolytica* can produce some surfactants and help emulsify oil/lipid in aqueous phase, but the overall mixing efficiency still largely depends on impeller design, power input of agitation, and other bioreactor configurations [82-84]. Oil/lipid substrate has to be well dispersed into small droplets in the

bioreactor vessel so that they become more reachable to cells. An important parameter for oil mixing in bioreactor is oil droplet size distribution in bioreactors, which has been previously investigated by experiments and summarized into empirical equations [85-87]. To help further analyze the mixing efficiency under various bioreactor designs and operating conditions, Computational Fluid Dynamics (CFD) now become a powerful tool to achieve this goal [88-93]. Since the fermentation with oil/lipid substrate is a multiphase (oil, aqueous, gas) system and the bioreactions further increase the complexity, traditional CFD methods and tools become very difficult and inefficient to simulate the oil/lipid fermentation in the bioreactor. To reduce the complexity caused by the bioreactions, a Tanks-in-Series Model (TSM) was previously developed and successfully used in modeling and scale-up of bioreactors with bioreactions in gas-liquid system [88,89]. In TSM models, the effective bioreactor domain is divided into a limited number of interconnected volumes, with each has homogeneous flow properties (turbulence, concentration, and etc.) [88,89]. The TSM models can be extended to the stirred tank bioreactor to simulate the oil/lipid fermentation system. However, the TSM models often fail to accurately predict the mixing behavior since these macroscopic numbers are not able to represent all local flow complexity in a bioreactor. A second approach is to rely upon the combination of CFD and Compartment Model (CM) [90]. The compartment models are also called multi-zone, multi-block, or network-of-zone models [91-93]. For CFD-CM, it consists of solving the turbulent liquid flow without bioreactions by CFD and then, of developing a compartment model based on the CFD results. The fluxes between compartments are computed from CFD velocity fields [90]. Therefore, a CFD-CM is expected in future to accurately simulate, analyze, and design the oil mixing, uptake, and bioconversion in a bioreactor.

Here we show some preliminary results of using CFD to address the oil mixing in bioreactors. We have used the CFD software, Ansys Workbench 17.0 – Fluent, to simulate oil droplet distribution in aqueous medium in a 2-L bioreactor. The results (see Figure 3) show that most larger oil droplets exist in the upper region of the reactor vessel. Increasing the agitation speed provides higher power input, breaks up the larger oil droplets into smaller ones, and disperses them into more bottom regions, thus significantly improves the overall (macro- and micro-) oil substrate mixing, which will then lead to faster cell growth and/or higher production formation. The simulation results were consistent with our previous observations in oil fermentation experiments that higher agitation speed significantly improved the citric acid production from the wild-type *Y. lipolytica*. It is expected that the bioreactor CFD will play an extremely important role in establishing the biomanufacturing platform for high-value products from plant oils.

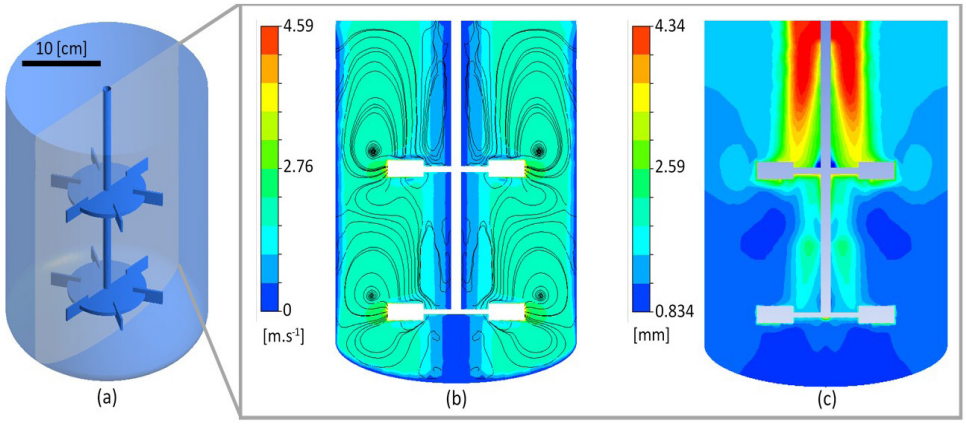

Figure 3: CFD simulation of a 2-L bioreactor with oil droplet distribution in aqueous medium during an oil/lipid fermentation process. (a) the bioreactor geometry for CFD; (b) velocity field and streamlines; (c) the oil droplet size distribution at the vetical plane.

Conclusions

1. Plant oil (mainly vegetable oil) is a non-polluting renewable resource abundant in the US, but its value is limited by its applications in food and feed industry. However, recent progresses in metabolic engineering and bioprocess engineering make it very promising to establish a new biomanufacturing platform for bioconversion of plant oils into high-value products.
2. Among the microbial community, the nonconventional yeast Y. lipolytica, with high capability of oil/lipid uptake and metabolism, stands out as a very promising host strain that can be used to establish the biomanufacturing platform. The technologies required for

efficient bioconversion of oil/lipid into high-value product include (1) the cellular morphology engineering to facilitate oil uptake and product secretion (for extracellular products); (2) metabolic engineering for fatty acid transport and intracellular metabolism; (3) metabolic pathway engineering to maximize a specific product; and (4) fermentation and bioreactor engineering to maximize the tier, rate, and yield of a product.

3. Four representative high-value products derived from fatty acids are discussed, which include wax esters, long-chain dicarboxylic acids, omega-3 fatty acids, and carotenoids. The metabolic engineering strategies for each specific product are reviewed in this paper. Plant oils seemed to have unique advantages over sugars to produce these high-value products with higher titer, rate, and/or yield.

4. Fermentation and bioreactor engineering is extremely important for bioconversion of oil/lipids in bioreactors due to the insolubility of the substrate in aqueous medium. In addition to the regular fermentation optimization experiments, CFD simulation shows very promising features in helping analyze, design, and improve the oil/mixing and bioconversions under each specific bioreactor conditions.

5. Further research of this new biomanufacturing platform will re-shape plant oils from low-value, common agriculture commodities to products with extremely high economic values and critical benefits in improving human health, life, and environment.

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