Applied Microbiology and Biotechnology (2018) 102:2509–2523
https://doi.org/10.1007/s00253-018-8813-z

MINI-REVIEW

Critical steps in carbon metabolism affecting lipid accumulation and their regulation in oleaginous microorganisms

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Received: 28 November 2017 / Revised: 23 January 2018 / Accepted: 24 January 2018 / Published online: 8 February 2018

Abstract

Oleaginous microorganisms are able to convert numerous agro-industrial and municipal wastes into storage lipids (single cell oil (SCO)) and are therefore considered as potential biofuel producers. While from an environmental and technological point of view the idea to convert waste materials into fuels is very attractive, the production cost of SCO is not currently competitive to that of conventional oils due to the low productivity of oleaginous microorganisms in combination with the high fermentation cost. Current strategies used to optimize the lipid-accumulating capacity of oleaginous microorganisms include the overexpression of genes encoding for key enzymes implicated in fatty acid and triacylglycerol synthesis, such as ATP-dependent citrate lyase, acetyl-CoA carboxylase, malic enzyme, proteins of the fatty acid synthase complex, glycerol 3-phosphate dehydrogenase and various acyltransferases, and/or the inactivation of genes encoding for enzymes implicated in storage lipid catabolism, such as lipases and acyl-CoA oxidases. Furthermore, blocking, even partially, pathways competitive to lipid biosynthesis (e.g., those involved in the accumulation of storage polysaccharide or organic acid and polyol excretion) can also increase lipid-accumulating ability in oleaginous microorganisms. Methodologies, such as adaptive laboratory evolution, can be included in existing workflows for the generation of strains with improved lipid accumulation capacity. In our opinion, efforts should be focused in the construction of strains with high carbon uptake rates and a reprogrammed coordination of the individual parts of the oleaginous machinery that maximizes carbon flux towards lipogenesis.

Keywords Regulating lipid metabolism · Lipid biosynthesis · Lipid degradation · Competitive pathways · *Yarrowia lipolytica*

Introduction

Oleaginous eukaryotic microorganisms (i.e., fungi, yeasts, and microalgae) and some species of autotrophic and heterotrophic bacteria are on the forefront of the biotechnological research thanks to their ability to accumulate oil (triacylglycerols (TAGs)), so-called single cell oil (SCO), thus the potential to be used as feedstock in the biodiesel manufacture (Li et al. 2008; Meng et al. 2009; Papanikolaou and Aggelis 2011a, b; Röttig et al. 2016). Although the first works on oleaginous microorganisms date back to the 1960s, the largest volume of research has been published in the last 10 years. At this period of time, numerous research projects have been funded in many countries, especially in China, which probably has the world's highest energy demand.

Numerous agro-industrial by-products of low acquisition cost have been considered as substrates for SCO production from heterotrophic oleaginous microorganisms (Papanikolaou and Aggelis 2011b; Qin et al. 2017). For instance, raw glycerol produced during biodiesel production process is a very popular substrate due to its high availability and relational origin, while its conversion into biodiesel may increase productivity and minimize waste production of the biodiesel manufacture (Fig. 1) (Easterling et al. 2009; Ibrahim and Steinbüchel 2009; Papanikolaou and Aggelis 2009; Makri et al. 2010; Chatzifragkou et al. 2011; Nicol et al. 2012; Bommareddy et al. 2015; Moustogianni et al. 2015; Dobrowolski et al. 2016; Gajdoš et al. 2017; de Paula et al. 2017). Despite the low acquisition cost of the raw material, the

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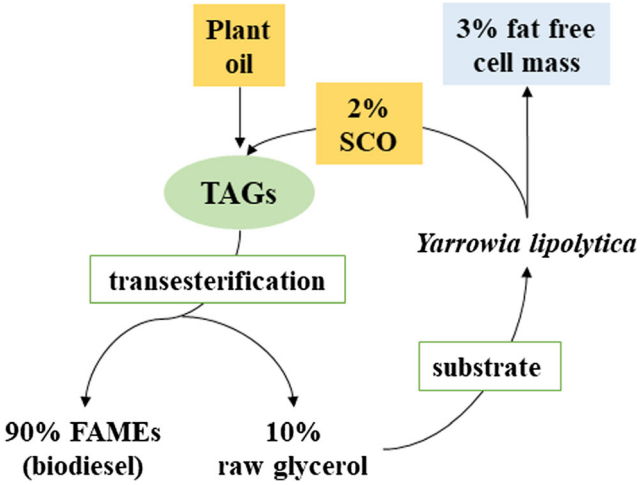

Fig. 1 Conceptual diagram showing the conversion of raw glycerol, produced during biodiesel manufacture, into TAGs that can be further converted into biodiesel

production cost of SCO is very high because of the low productivity of oleaginous microorganisms, and/or the high fermentation cost, principally the cost of fixed facilities such as high-tech bioreactors (Koutinas et al. 2014). Use of oleaginous microalgae can alleviate high fermentation costs, since microalgae can be cultivated in open ponds or natural lakes under non-aseptic conditions using carbon dioxide as substrate (Krienitz and Wirth 2006; Spolaore et al. 2006; Ratledge and Cohen 2008; Priyadarshani and Rath 2012; Bellou et al. 2014a, 2016a). However, in this case, the biomass harvesting cost is very high due to the low cell density required for autotrophic growth that usually does not exceed 400–600 mg/l. High cell densities can be obtained under autotrophic and (mainly) mixotrophic growth conditions in high-tech photo-bioreactors (Ratledge and Cohen 2008; Davis et al. 2011; Sun et al. 2011), but such processes can be cost-limiting. Alternatively, SCO can be effectively produced in a bio-refinery concept, in combination with other biotechnological applications, especially those concerned with the treatment of specific agro-industrial wastes or by-products. These wastes or renewable-type compounds include but are not limited to orange peels (Gema et al. 2002), pear pomace (Fakas et al. 2009), sweet sorghum (Economou et al. 2010; Matsakas et al. 2014), olive mill wastewaters, potentially enriched in low-cost carbon sources (i.e., glucose syrups, glycerol, etc.) (Sarris et al. 2011; Bellou et al. 2014b; Dourou et al. 2016; Arous et al. 2017a; Sarris et al. 2017), second cheese whey (Vamvakaki et al. 2010; Tsolcha et al. 2015), rice hulls (Economou et al. 2011), lignocellulosic sugars and/or hydrolysates (Ruan et al. 2013, 2014; Gardeli et al. 2017), cereals (Čertík et al. 2013), hydrolysates of side streams from wheat milling and confectionery industries (Tsakona et al. 2014, 2016; Arous et al. 2017a).

Research on oleaginous microorganisms is currently focused on the optimization of lipogenic machineries through genetic manipulation of essential enzymes involved in lipid metabolism, as well as of fermentation processes leading to efficient conversion of the various carbon substrates into SCO. Genetic manipulations, involving more than 20 genes, have targeted the upregulation of lipid biosynthetic pathways (i.e., biosynthesis of the building blocks of TAGs and TAG assembly) and the downregulation of enzymes involved in lipid degradation. However, such efforts in SCO production have yet to lead to pilot-scale level projects for production of SCO suitable as biodiesel feedstock. The only large-scale applications existing at the present time are those concerned with the production of SCO-containing polyunsaturated fatty acids (PUFAs) in high concentrations (Ratledge 2013; Bellou et al. 2014a, 2016a). Other uncommon lipid production by oleaginous species may be considered for large-scale applications in the future (Papanikolaou and Aggelis 2010; Fillet et al. 2017; Zhu et al. 2017; Wu et al. 2017).

In the current review article, we describe essential biochemical processes that occur during the life cycle of oleaginous microorganisms with emphasis on recently discovered biochemical properties of oleaginous microorganisms affecting lipogenesis. We then discuss current approaches for the optimization of the lipid-accumulating capacity of oleaginous microorganisms.

Biochemical events during the life cycle of oleaginous microorganisms: fundamentals and recent findings

The life cycle of oleaginous microorganisms growing in high C/N ratio media, where the carbon source is glucose and similarly metabolized substrates, is characterized by three distinct physiological phases, namely the balanced growth phase, the oleaginous phase, and the reserve lipid turnover phase.

During the balanced growth phase, in which all nutrients are found in excess in the growth environment, the oleaginous microorganisms convert the carbon source into cell mass, rich in proteins and polysaccharides, while restricted quantities of lipids, mainly polar lipids such as phospholipids and glycolipids that are essential for the construction of cell membranes, are synthesized (Dourou et al. 2017). Glucose and similar substrates are metabolized via either the Embden-Meyerhof-Parnas (EMP) glycolytic pathway or the pentose phosphate pathway (PPP), which act competitively to each other due to their common substrate (Fig. 2). Both of these pathways, as well as the Krebs cycle, generate biosynthetic precursors and nucleotides involved in the production of energy (i.e., NADH from EMP pathway) or in the production of reducing power (i.e., NADPH from PPP) that are essential for the biosynthesis of various macromolecules. Phosphofructokinase (PFK) is an EMP key enzyme that catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. PFK activity, controlled by the cellular energy

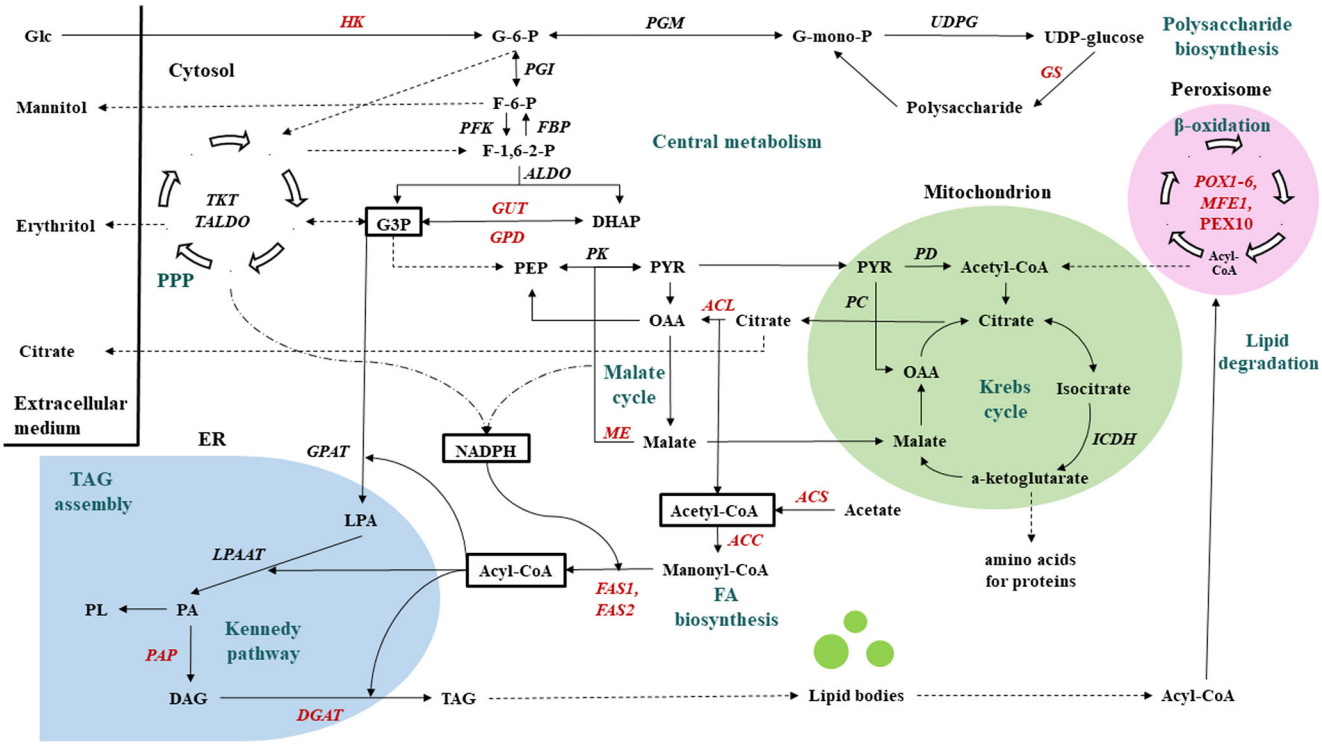

Fig. 2 A simplified overview of central metabolism pathways in oleaginous microorganisms with emphasis to FA and TAG biosynthesis and degradation. Competitive pathways are also depicted. Manipulated genes are shown in red (modified by Dourou et al. 2017). Abbreviations: (i) enzymes: ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; ACS, acetyl-CoA synthetase; ALDO, aldolase; DGAT, diacylglycerol acyltransferase; FAS, fatty acid synthase; FBP, fructose-1,6 biphosphatase; GPAT, glycerol-3-phosphate acyltransferase; GPD and GUT, isoforms of G3P dehydrogenase; GS, glycogen synthase; HK, hexokinase; ICDH, NAD+ dependent isocitrate dehydrogenase; LPAAT, lyso-phosphatidic acid acyltransferase; ME, malic enzyme; MFE, b-oxidation multifunctional enzyme; PAP, phosphatidate phosphatase; PC, pyruvate carboxylase; PD, pyruvate dehydrogenase; PEX, peroxisomal protein; PFK, phosphofructokinase; PGI, glucose-6-phosphate isomerase; PGM, phosphoglucomutase; PK, pyruvate kinase; POX, acyl-CoA oxidases; TALDO, transaldolase; TKT, transketolase; UDPG, UDP-glucose pyrophosphorylase; (ii) intermediate metabolites/substrates: DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; Glc, glucose; LPA, lysophopshatidic acid; OAA, oxaloacetate; PA, phosphatidic acid; PEP, phosphoenolpyruvate; PL, phospholipids; PYR, pyruvate; TAG, triacylglycerols

quotient and various catabolic products, regulates carbon flux towards either the EMP pathway or the PPP.

Following the balanced growth phase, de novo lipid accumulation occurs during the oleaginous phase. Carbon excess and depletion of at least one essential nutrient, usually nitrogen (but also sulfate, phosphate, or magnesium), in the growth medium are required to trigger the onset of the oleaginous phase (Papanikolaou and Aggelis 2011a; Kolouchová et al. 2016; Bellou et al. 2016b; Shen et al. 2017). Under nitrogen starvation conditions, cell proliferation is interrupted but oleaginous cells continue to assimilate the carbon source producing storage lipids, mainly TAGs. High uptake rate of the carbon source results in increased C/N ratio in yeast cells, which is a major factor correlated to lipogenesis. Recently, a *Yarrowia lipolytica* strain with enhanced lipid body (LB) formation and content was constructed with deletion of the *MIG1* gene, which encodes for a major repressor of the glucose catabolism (Wang et al. 2013). Likewise, boosting glucose catabolism through glycolysis (e.g., overexpressing the *ylHXK1* gene encoding for hexokinase) also improves lipid yield in the same yeast (Lazar et al. 2014). Moreover, specific peptides, such as tomato peptides, directly affect carbon uptake rate resulting in enhanced lipid accumulation in *Cunninghamella echinulata* (Fakas et al. 2008). Lipogenic ability also depends on the type of the available nitrogen source (e.g., ammonium sulfate, yeast extract, or more complex organic sources). Nitrogen availability depends on the ability of the microorganism to release NH_4^+, which affects the C/N ratio in the cytoplasm (Bellou et al. 2016b).

At the pathway level, nitrogen exhaustion in the culture medium leads to a rapid decrease of intracellular AMP, which is temporarily used as source of NH_4^+. AMP depletion results in inhibition of the mitochondrial NAD^+-dependent isocitrate dehydrogenase (NAD^+-ICDH), enzyme allosterically activated by AMP (Ratledge and Wynn 2002). ICDH inhibition is critical in signaling the onset of lipogenesis (Papanikolaou et al. 2004b; Arous et al. 2016), since the disturbance of the Krebs cycle at this step induces an intra-mitochondrial accumulation of citric acid that is then excreted to the cytoplasm in exchange with malate. In the cytoplasm, citric acid is

cleaved to acetyl-CoA and oxaloacetate by the ATP-dependent citrate lyase (ACL), a key lipogenic enzyme. A similar mechanism is used by the ascomycetous yeast *Debaryomyces etchellsii* for acetyl-CoA synthesis in asci and free ascospores (Arous et al. 2016). Posttranscriptional regulations of the abovementioned key lipogenic enzymes occur in *Y. lipolytica* and probably in other oleaginous microorganisms. Specifically, transcription of the genes *ACL1* and *ACL2* (both encoding for ACL) and ICDH is observed under both oleaginous and non-oleaginous conditions, but lipid accumulation occurs only when low or zero ICDH activity and high ACL activity are detected in the cytoplasm (cell-free extract) (Bellou et al. 2016b).

Below a critical ACL activity, citrate is excreted in the growth environment (as in the case of citric acid producing microorganisms) or, alternatively, accumulated in the cytoplasm inhibiting glycolysis at the level of PFK. In the latter case, phosphorylated sugars (i.e., glucose-6P) are accumulated in the cytoplasm and used as building blocks in polysaccharide biosynthesis (Tchakouteu et al. 2015a, b; Dourou et al. 2017; Gardeli et al. 2017). Recent research has shown that even in oleaginous microorganism polysaccharides are produced inside the cells (Shen et al. 2017), especially during the first growth steps, and are converted under certain conditions into TAGs during the oleaginous phase (Bellou and Aggelis 2012; Tchakouteu et al. 2015a; Dourou et al. 2017; Gardeli et al. 2017). Also, citric acid is commonly excreted in the growth environment of oleaginous microorganisms during lipogenesis (Dourou et al. 2017). These data suggest a deficient coordination between citric acid production and lipid biosynthesis leading to citric acid accumulation in the cytoplasm, which may further induce carbon outflow towards metabolic pathways competitive to lipogenesis. Furthermore, during growth on glucose or similarly catabolized compounds, low molecular weight metabolites, such as acetic acid, mannitol, and erythritol, may be excreted into the growth medium as a microbial response to the nitrogen limitation, instead of storage lipid accumulation that commonly occurs. Excretion of such metabolites has been recorded in *Y. lipolytica* strains and to a lesser extent in *Rhodotorula glutinis* (Papanikolaou et al. 2008, 2009, 2017a, b; Makri et al. 2010; Chatzifragkou et al. 2011; Karamerou et al. 2017).

The acetyl-CoA produced from citric acid is converted into long-chain acyl-CoA by a multienzyme protein, the FA synthase (FAS) with the expense of enormous quantities of reducing power (i.e., NADPH). In bacteria and microalgae, as well as in non-oleaginous eukaryotic microorganisms, such as *Saccharomyces cerevisiae*, acetic acid is the precursor of acetyl-CoA in the cytosol instead of citric acid. In species of *Rhodococcus*, and probably in other oleaginous bacteria, NADPH is provided by the PPP (Spaans et al. 2015). In typical oleaginous eukaryotes, NADPH is provided by the malic enzyme (ME) reaction (i.e., the conversion of malate into pyruvate), which takes place under non-growth conditions (Bellou et al. 2016b). In *Y. lipolytica*, a non-conventional yeast and model oleaginous eukaryotic organism, and other oleaginous species lacking cytoplasmic ME activity, PPP is the main donor of reducing power, instead of the ME reaction (Ratledge 2014; Dulermo et al. 2015; Wasylenko et al. 2015). Even in *Rhodosporidium toruloides*, which possesses cytoplasmic ME activity, PPP contributes by more than 60% to NADPH production (Bommareddy et al. 2015). In *Y. lipolytica*, small quantities of organic nitrogen ensuring growth conditions, thus active PPP, are required for lipid accumulation (Bellou et al. 2016b). This yeast is able to accumulate more than 40% lipid in the dry cell mass, growing in continuous culture (Papanikolaou and Aggelis 2002), while low lipid accumulation occurs in batch culture under non-growth conditions (Makri et al. 2010). In autotrophically growing oleaginous microalgae, although ME activity is detected (Bellou and Aggelis 2012; Bellou et al. 2014a), the main donor of reducing power seems to be the ferredoxin NADP reductase of photosystem I.

The long-chain acyl-CoA synthesized in the cytoplasm is transported to the endoplasmic reticulum (ER) and esterified with glycerol-3P (G3P), generating structural (phospholipids, glycolipids) and storage (TAGs) lipids. PUFAs are synthesized by the action of ER-localized desaturases and elongases. Generally, lipids produced by fungi and microalgae are more unsaturated than those of yeasts, containing PUFAs of medicinal interest such as γ-linolenic acid (GLA), dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentanoic acid (EPA). Bacteria usually synthesize specialized lipids, such as poly(3-hydroxybutyrate) and other polyhydroxyalkanoates (PHAs) (Steinbüchel 1991; Steinbüchel and Valentin 1995), while TAGs are not commonly utilized as storage material. Exceptions are members of the actinomycetes group, such as *Mycobacterium*, *Streptomyces*, *Nocardia*, and *Rhodococcus* species and cyanobacteria, such as *Nostoc* species, which are able to accumulate substantial amounts of TAGs (Alvarez et al. 1996; Alvarez et al. 2000; Alvarez and Steinbüchel 2002; Janßen et al. 2013). The mechanism of fatty acid (FA) biosynthesis is strongly conserved between bacteria and eukaryotes, while the archaea synthesize isoprenoid-derived lipids. The FAs synthesized by bacteria are similar to those synthesized by eukaryotes, except that the bacterial FAs are generally shorter and lack polyunsaturation, while the monounsaturated FAs of the C18 group have different double-bond positions. Also, some bacteria are able to synthesize branched chain FAs (Cronan and Thomas 2009).

Besides TAGs, sterol esters (SEs) also participate into LB structures, serving as an intracellular pool of sterols. Sterols are essential components of the membranes of all eukaryotic organisms controlling membrane fluidity and permeability. The biosynthetic precursor of sterols is squalene, which is

converted to lanosterol in fungal cells (Mercer 1984). Subsequently, lanosterol is converted to ergosterol via a sequence of reactions which may differ among eukaryotes (Barrero et al. 2002). Ergosterol is the major sterol in ascomycetes and basidiomycetes, while some green algae also contain large quantities of ergosterol (Mejanelle et al. 2000; Patterson 1969). Zygomycetes produce ergosterol as a major sterol accompanied by dihydro-ergosterol (Ratledge and Wilkinson 1988). Besides ergosterol, the presence of other sterols, such as ergosta-5,7,9(11) 22-tetraen-3β-ol, ergosta-7,22-dien-3β-ol, ergosta-7,22,24(28)-trien-3β-ol, and episterol, was confirmed in *C. echinulata* ATHUM 4411 (Fakas et al. 2006).

After the exhaustion of the extracellular carbon source, or due to low uptake rate, the oleaginous microorganisms utilize their own storage lipids as energy source for maintenance purposes or as intracellular carbon source for the production of new lipid-free material, provided that essential nutrients are available in the growth medium (Aggelis et al. 1995; Alvarez et al. 1996, 2000; Makri et al. 2010; Papanikolaou and Aggelis 2011a; Janßen and Steinbüchel 2014; Dourou et al. 2017). *Y. lipolytica* cultivated on less preferred carbon sources, such as saturated fats, degrades its own storage lipids (Papanikolaou and Aggelis 2003). Cellular lipid degradation in *Y. lipolytica* also occurs when cultivated on glycerol (Makri et al. 2010; Bellou et al. 2016b) and in oleaginous fungi like *Aspergillus niger*, *C. echinulata*, and *Umbelopsis* (*Mortierella*) *isabellina* cultivated on glucose, glycerol, and other sugar-based media (Papanikolaou et al. 2004a; André et al. 2010; Vamvakaki et al. 2010), due to the low uptake rate of the carbon source. However, the addition of carbon source in the growth medium during lipid turnover may suppress, partially at least, lipid degradation (Bellou et al. 2016b). In the case of microalgae, lipid turnover may occur in opaque cultures or under CO_2 starvation conditions (Bellou and Aggelis 2012). TAG lipases and steryl-esters hydrolases are involved in lipid degradation, while the released FAs in their activated form are catabolized via β-oxidation process towards acetyl-CoA, which is further catabolized via glyoxylate shunt. Besides its role in energy production through acyl-CoA catabolism, β-oxidation process may serve as a pathway for FA shortening, thus lipid remodeling. In fact, high activities of acyl-CoA oxidases (ACOXs), peroxisomal enzymes that participate in the oxidation of acyl-CoA to enoyl-CoA, have been detected during lipid accumulation in *Y. lipolytica* and *U. isabellina* (Dourou et al. 2017).

Strengthening lipogenesis in oleaginous microorganisms

The SCO production cost is critically affected by the productivity of oleaginous microorganisms, especially by their ability to accumulate lipids (Davis et al. 2011; Koutinas et al. 2014). The specific growth rate and the total cell mass produced per unit of culture volume also considerably affect lipid production cost, but to a lesser extent than the lipid content. In any case, in high cell density cultures, it is very difficult to retain oleaginous microorganisms in a productive state, since under these conditions dissolved oxygen becomes a limiting factor downregulating lipid biosynthesis (Bellou et al. 2014c). Similarly, in microalgae high cell density cultures, both CO_2 and light are shown to be limiting factors.

Several researchers investigated the possibility of oleaginous microorganisms to secrete their own lipids into the culture medium, overcoming limitations associated with cell mass (Liu et al. 2011; Ledesma-Amaro et al. 2016). However, despite the fact that many yeast strains are able to secrete amphiphilic molecules (e.g., Stodola et al. 1967), very few quantities of glycerides are detected extracellularly. According to our own experience, glycerides are found in the growth environment mostly as a result of cell lysis rather than of active transport from the cytoplasm to the extracellular environment.

Approaches for increasing TAG accumulation in oleaginous microorganisms include the upregulation of key enzymes involved in (a) the biosynthesis of building groups used for TAG assembly (i.e., acyl-CoA and G3P) and (b) TAG assembly.

Biosynthesis of building groups used for TAG assembly

Acetyl-CoA is the biosynthetic precursor of acyl-CoA. In eukaryotic oleaginous microorganisms, acetyl-CoA is produced from citrate through the ACL reaction carried out in the cytoplasm (see above). Although it has been proposed that this reaction is not the limiting step in lipogenesis, overexpression of the *ACL* genes increases lipid accumulation in *A. oryzae* (Tamano et al. 2013) and in *Y. lipolytica* (Blazeck et al. 2014; Zhang et al. 2014).

The first committing step in the acyl-CoA synthesis is the conversion of acetyl-CoA into malonyl-CoA, a reaction catalyzed by the acetyl-CoA carboxylase (ACC) and with central role in carbon metabolism. It takes place in the cytosol of heterotrophic or both in the cytosol and plastid of autotrophic microorganisms (Bellou et al. 2014a). Overexpression of *ACC1* in oleaginous yeasts, such as *Y. lipolytica* and *R. toruloides*, significantly increased lipid content (Tai and Stephanopoulos 2013; Zhang et al. 2016).

S. cerevisiae, although a non-oleaginous yeast, is frequently used for studying lipid metabolism (Fakas 2017). This yeast possesses both cytosolic and mitochondrial ACC, encoded by the *ACC1* and *HFA1* genes, respectively. Manipulating ACC at the posttranslational level resulted in increased activity and thereby improved flux through malonyl-CoA-dependent metabolic pathways for the production of chemicals including FA ethyl esters (FAEEs) (Shi et al. 2014). Combined overexpression of *ACC1* from *Lipomyces starkeyi* and *GPD1*, encoding

for glycerol 3-phosphate dehydrogenase (GPDH) (see below), resulted in 63% increase in lipid content in *S. cerevisiae* (Wang et al. 2016). Recently, Besada-Lombana et al. (2017) engineered a constitutively active ACC in *S. cerevisiae* by replacing the serine residue 1157 with alanine, so as to prevent deactivation by phosphorylation. Expression of $ACC1^{S1157A}$ resulted in an increase in total FA production, especially in oleic acid. However, increasing *ACC1* expression has not always had the desirable outcome in FA synthesis, as is the case in *A. oryzae* (Tamano et al. 2013) and in *Cyclotella cryptica* and *Navicula saprophila* (Dunahay et al. 1996; Mühlroth et al. 2013).

Following malonyl-CoA production, FAS catalyzes the elongation of the carbon chain consuming huge quantities of NADPH (i.e., 2 NADPH molecules per C2-unit elongation). NADPH is provided by the ME located in the cytosol or via the PPP. Wynn et al. (1999) suggested that the ME activity plays a crucial role in determining the extent of lipid accumulation in filamentous fungi. Often, overexpression of the ME is a target of genetic engineering for enhanced lipid accumulation. Indeed, overexpression of this gene in *R. glutinis* (Li et al. 2013) and in heterotrophically growing *Phaeodactylum tricornutum* (Xue et al. 2015) significantly improved lipid accumulation. However, overexpression of the *malA* gene (encoding for isoforms III/IV of ME), although it resulted in higher transcript levels and ME activity, was inconsequential for growth or lipid content in *Mucor circinelloides* (Zhang et al. 2007; Rodríguez-Frómeta et al. 2013), which probably means that either the ME reaction is not the rate-limiting step or that NADPH is supplied by another reaction. In *Y. lipolytica*, overexpression of ME did not significantly affect lipid content (Beopoulos et al. 2009; Beopoulos et al. 2011; Zhang et al. 2013). *Y. lipolytica*, similarly to *L. starkeyi*, is suggested to only have mitochondrial ME activity that does not participate in the process of lipid accumulation (Tang et al. 2010; Ratledge 2014). In *Y. lipolytica*, the PPP is the NADPH source (Ratledge 2014; Yang et al. 2014; Dulermo et al. 2015; Wasylenko et al. 2015). In the fungal pathogen *Ustilago maydis*, the activity of ME was lower than that of the cytosolic $NADP^+$-ICDH, the glucose-6-phosphate dehydrogenase (G6PD) and the 6-phosphogluconate dehydrogenase (6PGD), indicating that the ME reaction is not the main source for NADPH (Aguilar et al. 2017). Recently, Safdar et al. (2017) reported that in *Crypthecodinium cohnii*, a heterotrophic dinofagellate, the G6PD contributes more to NADPH production than the ME. In other cases, both PPP and ME reaction play essential roles in metabolism, as independent knockouts of 6PGD and ME in *Synechocystis* sp. resulted in mutants that could not grow under dark heterotrophic conditions (Wan et al. 2017). Therefore, we can conclude that, despite an initial assessment about the unique role of ME reaction in lipogenesis, PPP seems to be an important source of reducing power for many oleaginous microorganisms.

In yeasts and fungi, FAs are biosynthesized by a type I FAS, which is a large multifunctional protein that contains all of the required catalytic sites within domains of two polypeptides. In the algal chloroplast, as well as in bacteria, a type II FAS is found, which is a group of independently acting enzymes that catalytically elongate a growing FA by two carbon units in an iterative pathway (Cronan and Thomas 2009; Blatti et al. 2013; Bellou et al. 2016a). Upregulation of the acyl-carrier protein (ACP) or the 3-ketoacyl- ACP synthase (both essential proteins of FAS) together with overexpression of the fatty acyl-ACP thioesterase (which catalyzes the hydrolysis of acyl-ACP complex), in *Haematococcus pluvialis* improved FA synthesis (Lei et al. 2012). Moreover, overexpression of *FAS1* and *FAS2* (encoding for FAS) along with *ACC1*, in *S. cerevisiae*, resulted in a significant improvement of lipid production over the wild-type (Runguphan and Keasling 2014).

The synthesis of PUFAs requires the presence of specific elongases and desaturases, which act primarily on phospholipid-bound FAs (see below). The implication of these enzymes in the biosynthesis of PUFAs has been extensively reviewed in heterotrophic microorganisms and in microalgae (Bellou et al. 2014a, b, c, 2016a). Microsomal membrane-bound ME is also implicated in PUFA biosynthesis through NADPH production, which is required for FA desaturation (Kendrick and Ratledge 1992). For instance, in *M. circinelloides*, increased ME activity led to an increase in both lipid content and GLA biosynthesis (Zhang et al. 2007). Palmitic and stearic acids are successively desaturated and elongated into the ER in yeasts and fungi or both in the ER and plastids in algae. In the last decade, genetic engineering has resulted in the construction of improved yeast strains (mainly *Y. lipolytica*, but also *S. cerevisiae*) and microalgae. Such research focuses on the construction of strains able to express various desaturases (e.g., D5-, D6-, D8-desaturate), elongases and acyl-transferases, and to synthesize TAGs rich in PUFAs. In particular, production of DGLA, eicosatetraenoic acid, and EPA was achieved in *S. cerevisiae* (Li et al. 2011; Tavares et al. 2011), while GLA, EPA, and DHA have been successfully produced after overexpression of related genes from *M. alpina* and *Thraustochytrium aureum* in *Y. lipolytica* (Chuang et al. 2010; Damude et al. 2014; Xie et al. 2015). Improvement of PUFA content in microalgae after genetic engineering has also been reported (Hamilton et al. 2014; Peng et al. 2014).

Glycerol-3-phosphate (G3P) is the second component of TAG molecules and therefore has been hypothesized that its availability affects TAG biosynthesis. Dulermo and Nicaud (2011) showed that overexpression of *GPD1* (encoding for a G3P dehydrogenase that is involved in G3P synthesis from DHAP) in *Y. lipolytica* and/or inactivation of *GUT2* (encoding for a different G3P dehydrogenase, which converts G3P to DHAP) result in increased G3P concentration, leading to high

TAG accumulation. In the model microalga *Chlamydomonas reinhardtii*, among the five GPD-encoding genes, *GPD2* and *GPD3* were shown to be induced by nutrient starvation and/or salt stress. Surprisingly, overexpression of *GPD2* had no significant impact on growth, while *GPD3* overexpression resulted in growth inhibition and changes in lipid composition. This suggests the existence of a downstream regulation on glycerolipid metabolism pathway, and thus, engineering of lipid metabolism via GPD modification may also affect these additional downstream effectors (Driver et al. 2017).

TAG assembly

Acyl-CoA is elongated up to 16 or 18 carbon atoms and then moved to the ER and esterified in the glycerol backbone via the Kennedy pathway, in which various acyltransferases are involved. In the first step of TAG assembly, acylation of G3P by the G-3-P acyltranferase (GAT) to yield 1-acyl-G-3-P (lysophospatidic acid (LPA)) is conducted. The LPA is then acylated by lysophosphatidic acid acyltransferase (also named 1-acyl-G-3-P acyltransferase-AGAT) to yield phosphatidic acid (PA), the key intermediate of all glycerophospholipids and TAGs. PA is subsequently dephosphorylated by the PA phosphatase (PAP) to release diacylglycerol (DAG) (Carman and Han 2009). PA dephosphorylation has been considered as the committing step in TAG biosynthesis (Pascual and Carman 2013; Park et al. 2015; Fakas 2017; Hardman et al. 2017).

In the final step of the Kennedy pathway, the DAG is acylated either by diacylglycerol acyltransferase (DGAT) or phospholipid diacylglycerol acyltransferase to produce TAGs. Overexpression of both *DGA1* and *FAA3* (encoding for an acyl-CoA synthetase) in *S. cerevisiae* restores and/or increases lipid biosynthesis (Kamisaka et al. 2013; Greer et al. 2015). Furthermore, overexpression of *DGA1* and *DGA2* in *Y. lipolytica* significantly increases lipid yield and productivity (Tai and Stephanopoulos 2013; Blazeck et al. 2014; Gajdoš et al. 2015; Friedlander et al. 2016), while this upregulation is also beneficial for lipid accumulation in *R. toruloides* (Zhang et al. 2016). The combination of three genetic modifications in *Y. lipolytica*, i.e., overexpression of *DGA1* from *R. toruloides* and of *DGA2* from *Claviceps purpurea* along with deletion of the *TGL3* lipase regulator, significantly increases lipid content (Friedlander et al. 2016). In addition, double overexpression of *ylDGA2* (encoding for DGAT) and *ylGPD1* (encoding for glycerol-phosphate dehydrogenase) genes in a peroxidase and lipase deficient strain of *Y. lipolytica* resulted in increased TAG biosynthesis (Sagnak et al. 2018). Concerning the photosynthetic microorganisms, overexpression of a DGA in the diatom *Phaeodactylum tricornutum* increased neutral lipid content by 35%, as well as PUFA content (Niu et al. 2013), but did not affect either TAGs biosynthesis or lipid profile in *C. reinhardtii* (La Russa et al. 2012).

In *S. cerevisiae*, the genes *ARE1* and *ARE2*, encoding for acyl-CoA:cholesterol acyltransferase-related enzymes, are implicated in SEs biosynthesis (Yang et al. 1996; Yu et al. 1996). Disruption of *ARE1* had nearly no effect on the biosynthesis of SE, whereas deletion of *ARE2* reduced the SE level to approximately 25% of wild type (Yang et al. 1996). Deletion of both genes resulted in the total lack of SE, demonstrating that Are1p and Are2p are the only sterolesterifying enzymes in yeast. Jensen-Pergakes et al. (2001) reported that the role of Are2p is to esterify ergosterol, while the role of Are1p is to esterify sterol intermediates.

After their synthesis in the ER, TAGs and SEs are moved to the cytoplasm forming, in association with phospholipids and proteins, LBs. The size and morphology, as well as the number of LBs per cell, vary considerably among genera and even among closely related species (Arous et al. 2017b). In *R. toruloides*, the LB proteome consists of 226 proteins, many of which are involved in lipid metabolism and LB formation and progress. *R. toruloides LDP1* overexpression in *S. cerevisiae*, encoding for a major LB structural protein, facilitates giant LB formation, suggesting that this protein plays an important role in the regulation of LB dynamics (Zhu et al. 2015). In *Y. lipolytica*, overexpression of specific DGATs affects, besides lipid accumulation, LB formation. Specifically, overexpression of *YlDGA2* (located in a structure strongly resembling the ER) induces the formation of large LBs, while smaller but more numerous LBs are produced when *YlDGA1* (located in LBs) is overexpressed (Gajdoš et al. 2016). Simultaneous deletion of *DGA1*, *LRO1*, *ARE1*, and *ARE2* genes in *S. cerevisiae* completely abolishes LB formation, while typical LB proteins were restricted to the ER in the mutant strain (Sorger et al. 2004). In the oleaginous bacterium, *Rhodococcus jostii* LBs bind to genomic DNA through MLDS (a major LB protein in bacteria), resulting in increased survival rate of the cells under nutritional and genotoxic stress. That indicates that bacterial LBs participate in genome regulation and facilitate bacterial survival under adverse conditions (Zhang et al. 2017).

Direct synthesis of biodiesel by bacteria

The biodiesel production process includes the conversion of FAs esterified with glycerol (forming glycerides, mostly TAGs), into FA methyl (or ethyl) esters, and is an energy-dependent process. Kalscheuer et al. (2006) proposed the direct synthesis of biodiesel (FA ethyl esters) by *Escherichia coli*, avoiding TAG production followed by transesterification with methanol or ethanol. This was achieved in recombinant *E. coli* by co-expression of the ethanol production genes from the ethanol-producing fermentative bacterium *Zymomonas mobilis* and the *atfA* gene (encoding wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase) from a strain of *Acinetobacter baylyi*. Similar approaches have been used

by Nawabi et al. (2011) and Sherkhanov et al. (2016) for FA methyl ester production in *E. coli*. Although the microbial production of FA methyl-/ethyl-esters can lead to a suitable technology, it seems that the current yields are not high enough to permit the transfer of this technology to pilot scale.

Repression of storage lipid turnover

Repression of storage lipid catabolism is a strategy to increase lipid content in oleaginous microorganisms and may be approached through inactivation of genes encoding for enzymes involved in FA release from lipid structures (i.e., TAGs and SEs) and/or for enzymes involved in β-oxidation pathway. Deletion of the *ylTGL4* gene, encoding for a lipase attached to the LBs, resulted in an increase in lipid accumulation (Sagnak et al. 2018). In *Y. lipolytica*, inactivation of *POX1-6* genes, encoding for six different acyl-CoA oxidases, which participate in the first reaction of β-oxidation pathway (Mlíčková et al. 2004; Beopoulos et al. 2008; Dulermo and Nicaud 2011), and/or of *MFE1* gene, encoding for an enzyme catalyzing the second step of the β-oxidation pathway (Gajdoš et al. 2015), significantly increased lipid content. Deletion of *ylSNF1*, encoding for SNF1 protein kinase (Seip et al. 2013), also enhances lipid content. Likewise, effective repression of FA oxidation may be achieved through the disturbance of peroxisome biogenesis via *PEX10* deletion (Xue et al. 2013).

In a *Pseudomonas putida* strain that synthesized medium-chain-length PHAs, interruption of the *phaZ* gene encoding for a PHA depolymerase resulted in significantly enhanced biopolymer titer, while high production of citrate was also observed (Poblete-Castro et al. 2014).

Blocking competitive to lipid biosynthesis pathways

An alternative approach towards increasing SCO production involves the manipulation of pathways that energetically compete or directly counteract SCO production. The accumulation of macromolecules rich in energy, such as polysaccharides or PHAs, and/or the secretion of low molecular weight metabolites (e.g., citric acid, polyols) occur in the expense of energy that could be channeled towards SCO production. The biosynthesis of these compounds is obviously competitive to the SCO production, and thus, the partial blocking of these pathways could increase lipid accumulating ability in oleaginous microorganisms.

Lipid vs polysaccharide/PHA accumulation

Although organisms, both unicellular and multicellular ones, are specialized in accumulating specific forms of chemical energy (i.e., TAGs or polysaccharides or PHAs), it has been shown that they do not store all their energy in a single macromolecule. Several oleaginous and non-oleaginous microorganisms are able to accumulate, in addition to lipids, other energy-containing compounds. In particular, several oleaginous yeasts (e.g., *L. starkeyi*, *Cryprococcus curvatus*, *R. torulaides*, and *Y. lipolytica*) (Tchakouteu et al. 2015a, b; Dourou et al. 2017) synthesize, during the first growth phase (i.e., before nitrogen depletion in the growth medium), significant quantities of polysaccharides. Similarly, the oleaginous fungus *U. isabellina* (Dourou et al. 2017; Gardeli et al. 2017) and the microalgae *Chlorella* sp. and *Nannochloropsis salina* (Bellou and Aggelis 2012), synthesize polysaccharides in parallel with lipid accumulation. On the other hand, higher non-oleaginous fungi, such as *Flammulina velutipes*, *Pleurotus pulmonarius*, *Morchella esculenta*, and *Volvariella volvacea* are able to synthesize storage lipids in parallel with polysaccharide synthesis (Diamantopoulou et al. 2012, 2014, 2016). Oleaginous bacteria, such as *R. opacus* and *R. ruber*, synthesize PHAs in parallel with TAGs (Alvarez et al. 1996, 2000).

Studies on carbon metabolism in *Y. lipolytica* and *U. isabellina* revealed that during the balanced and the early oleaginous phases, polysaccharide accumulation is triggered as a result of insufficient enzymatic activity of PFK to drive hexoses towards pyruvate synthesis (Dourou et al. 2017). A clear interconversion of polysaccharides into lipids was observed in both *U. isabellina* (Dourou et al. 2017) and in *Chlorella* sp. (Bellou and Aggelis 2012) during the lipid accumulation phase, while polysaccharide degradation and reconstruction was also observed, as suggested by the high enzymatic activities of phosphoglucomutase (PGM) and fructose-1,6-biphosphatase (FBP). On the contrary, there is no evidence of such inter-conversion between lipids and polysaccharides during the lipid accumulation phase in *Y. lipolytica*. In this yeast, due to non-negligible activities of transaldolase (TALDO), PGM, and FBP, a polysaccharide reconstruction through the PPP and gluconeogenesis may occur. During lipid turnover, in both *Y. lipolytica* and *U. isabellina* high activities of UDPG, PGM, TALDO, and FBP, enzymes involved in polysaccharide biosynthesis, degradation, and reconstruction are detected (Dourou et al. 2017). In the non-oleaginous fungi *P. pulmonarius*, *Agrocybe aegerita*, *Ganoderma applanatum*, and *V. volvacea*, a clear conversion of lipids to polysaccharides during the late growth steps was observed (Diamantopoulou et al. 2012, 2014, 2016).

Blocking enzymes involved in polysaccharide biosynthesis may favor lipid accumulation. Recently, Bhutada et al. (2017) reported that the deletion of *ylGSY1* encoding for glycogen synthase in *Y. lipolytica*, increased biosynthesis of storage lipids. Strikingly, in photosynthetic microorganisms, blocking starch synthesis may be a more effective strategy for improving TAG production than the direct manipulation of the lipid synthesis pathway. The inactivation of ADP-glucose pyrophosphorylase in *C. starchless* led to an increase in TAG content (Li et al. 2010). In addition, lipid yield was

considerably increased by blocking starch synthesis, in *C. pyrenoidosa* and *C. reinhardtii* (Ramazanov and Ramazanov 2006; Work et al. 2010), indicating that shifting carbon flux from starch to lipid synthesis is feasible. Accordingly, we can hypothesize that blocking PHA biosynthesis in bacteria can favor TAG accumulation.

CA and/or polyol excretion during lipid accumulation

Y. lipolytica strains are capable of secreting low molecular weight secondary metabolites, such as organic acids (mostly citric acid) and polyols (i.e., mannitol, erythritol), under specific growth conditions. Citric acid is an intermediate of lipid biosynthesis and a compound of biotechnological interest (Papanikolaou et al. 2008; André et al. 2009; Gonçalves et al. 2014; Morgunov and Kamzolova 2015; Rakicka et al. 2016). Excess citric acid that cannot be assimilated by the lipogenic machinery is excreted in the growth environment under certain circumstances, probably due to a deficient coordination between citric acid production and its channeling in lipid biosynthesis (see above). Despite the fact that both *ACL1* and *ACL2* are constitutively expressed (Bellou et al. 2016b), and ACL activity is high, in both *Y. lipolytica* and *U. isabellina* (Bellou et al. 2016b; Dourou et al. 2017), citric acid is constantly excreted in the growth environment, indicating that additional to ACL bottlenecks may exist in the lipid anabolic pathway. Thus, it is not surprising that the overexpression of genes involved in TAG synthesis (e.g., *ylDGA2* and *ylGPD1*) in *Y. lipolytica* decreases citric acid production (Sagnak et al. 2018). Kavscek et al. (2015) reconstructed the metabolic network of *Y. lipolytica* using a genome-scale model of this yeast as a scaffold. They successfully designed a fed-batch strategy to avoid citrate excretion during the lipid production phase in *Y. lipolytica* and succeeded to increase the lipid content up to 80% in cell mass, while lipid yield was improved more than fourfold compared to standard conditions.

Sugar alcohols, such as xylitol, mannitol, sorbitol, and erythritol, are considered as osmoprotective agents for plants, fungi, yeasts, and bacteria. Osmotolerant strains of *Y. lipolytica* and other yeasts have been reported to produce some of these molecules in high concentrations, depending on the growth phase and the type of the carbon source in the growth medium (Tomaszewska et al. 2012; Tomaszewska-Hetman and Rywińska 2016; Dourou et al. 2016; Park et al. 2016; Meng et al. 2017; Papanikolaou et al. 2017b; Rakicka et al. 2017). Dulermo et al. (2015) observed a negative correlation between FA and mannitol synthesis in *Y. lipolytica*, indicating that the related biochemical pathways are competitive. Indeed, after inactivation of the *ylSDR* gene, encoding for a mannitol NADPH-dependent dehydrogenase converting fructose to mannitol, the FA content increased by 60% during cultivation on fructose.

Conclusions and future perspectives for improving SCO production

The potential of SCO to be utilized as biodiesel feedstock depends on the reduction of the production cost of SCO, by reducing the fermentation and downstream processing cost, and/or by increasing the relevant microorganism productivity. The construction of oil-overproducing microbial strains is currently approached through the overexpression of genes involved in biosynthesis of building groups used for TAG assembly, mostly acyl-CoA, such as ACC, ACS, ACL, ME, FAS, and in TAG biosynthesis from FAs and G3P, including DGAT, and/or the deletion/inactivation of the genes involved in lipid degradation, such as lipases and acyl-CoA oxidases.

Approaches for strain improvement could include adaptive laboratory evolution (ALE) of wild or engineered strains towards their capacity to rapidly take up the carbon source, presenting a high growth rate during the balanced growth phase and a high lipid accumulation rate during nitrogen starvation, and/or towards optimization of citric acid utilization by the lipogenic machinery. The genetic changes that occur during adaptation are a reflection of the condition under which adaptation occurs (Wenger et al. 2011; Gerstein et al. 2012; Kvitek and Sherlock 2013; Venkataram et al. 2016; Deatherage et al. 2017). Often, adaptation under a certain condition can result in decreased fitness in another environment (Wenger et al. 2011; Deatherage et al. 2017). Considering that and by being aware of the metabolic processes available in our system, we can engineer strains in such a way so as to shift metabolism towards desired pathways. Careful design of the adapting condition(s) can lead to the evolution of desired phenotypes in *Yarrowia*, and also reveal unanticipated targets for further engineering and optimization. Nevertheless, these are extremely complex phenotypes and the search of growth conditions that provide the appropriate selective pressures for such traits to be maintained and/or refined could be an endeavor on its own. In such cases, employing more than one alternating environment as adapting conditions could be a promising strategy (Sandberg et al. 2017).

Adaptive evolution in model systems has revealed a wealth of information on evolutionary dynamics under various conditions and on the respective molecular targets of adaptation. For example, we know that adaptation rates diminish over the course of evolution, mostly due to diminishing fitness advantage that adaptive mutations have as they accumulate (Kryazhimskiy et al. 2014; Wünsche et al. 2017). Consequently, maladaptive strains are expected to have high adaptation rates. Indeed, strong selective pressures can initially result to suboptimal "fixes," such as chromosomal rearrangements that are subsequently optimized (Yona et al. 2012), or fixation of mutator phenotypes that are reverted once fitness is restored (Lynch et al. 2016). The process of

introduction of "novel" sequences in engineered microbes results in reduced fitness compared to their non-engineered ancestors (Chou et al. 2011; Kacar et al. 2017). All these suggest that engineered genotypes, more often than not have many ways to increase organismal fitness; thus, engineered strains can be genetically unstable, accumulate compensatory mutations, and their cultures can be vulnerable to contaminations by fitter strains. However, ALE approaches under appropriate conditions can optimize stability and performance of such strains, without loss of the desirable trait(s). Currently, several studies have employed ALEs for the generation of strains with biotechnological interest. Such efforts have mostly focused on strain optimization, frequently after engineering, for the production of biofuels (Almario et al. 2013; Dragosits and Mattanovich 2013; Wallace-Salinas and Gorwa-Grauslund 2013; Jin et al. 2016; Gong et al. 2017; Horinouchi et al. 2017).

An interesting possibility for strain construction and/or optimization via ALE could arise from adaptation under conditions that promote spatially structured populations. Spatial structure can alleviate clonal interference, thus allowing for exploration of remote genotypes that may be of great interest, as opposed to well-mixed unstructured populations (Nahum et al. 2015; Van Cleve and Weissman 2015). Finally, genetic screening of randomly mutagenized cells could also lead to the generation of interesting strains; however, in that case, the discovery of the causal locus can be challenging.

Funding information We acknowledge support of this work by the project "INVALOR: Research Infrastructure for Waste Valorization and Sustainable Management" (MIS 5002495) which is implemented under the Action "Reinforcement of the Research and Innovation Infrastructure," funded by the Operational Programme "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014–2020) and co-financed by Greece and the European Union (European Regional Development Fund).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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