Systems-level approaches for understanding and engineering of the oleaginous cell factory *Yarrowia lipolytica*

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October 20, 2020

Abstract

Concerns about climate change and the search for renewable energy sources together with the goal of attaining sustainable product manufacturing have boosted the use of microbial platforms to produce fuels and high-value chemicals. In this regard, *Y. lipolytica* has been known as a promising yeast with potentials in diverse array of biotechnological applications such as being a host for different oleochemicals, organic acid and recombinant protein production. Having a rapidly increasing number of molecular and genetic tools available, *Y. lipolytica* has been well studied amongst oleaginous yeasts and metabolic engineering has been used to explore its potentials. More recently, with the advancement in systems biotechnology and the implementation of mathematical modeling and high throughput omics data-driven approaches, in-depth understanding of cellular mechanisms of cell factories have been made possible resulting in enhanced rational strain design. In case of *Y. lipolytica*, these systems-level studies and the related cutting-edge technologies have recently been initiated which is expected to result in enabling the biotechnology sector to rationally engineer *Y. lipolytica*-based cell factories with favorable production metrics. In this regard, here, we highlight the current status of systems metabolic engineering research and assess the potential of this yeast for future cell factory design development.

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Keywords: lipid accumulation, cell factory, metabolic engineering, systems biology

Introduction

Limited fossil fuel resources as well as the growing environmental concerns associated with their extraction necessitate a substitute for the traditional chemical synthesis of valuable chemicals. As a renewable and sustainable alternative, microbial biotechnology has been proven efficient to produce high value chemicals

(Timmis et al., 2017). Accordingly, an array of microorganisms, from bacteria to yeasts, fungi and microalgae can be utilized as cell factories to produce a wide range of bioproducts. Among these, *Yarrowia lipolytica* is increasingly gaining popularity as a biotechnologically relevant cell factory to the extent that it is already regarded to as the most promising nonconventional yeast in bioproduction industry (Markham & Alper, 2018). Most notably, *Y. lipolytica* is a well-known oleaginous yeast which can be used to produce lipids and fatty acid-derived bio-based compounds (Blazeck et al., 2014).

Generally, the emerging interest in *Y. lipolytica* among both industry and academia is further rooted in other advantages such as having GRAS (generally recognized as safe) status (Groenewald et al., 2014), availability of various tools for genetic manipulation (Madzak, 2018), tolerance to different industrial conditions such as different pH values, higher salt concentration or the presence of organic acids, high capacity to channel nutrients to commodity chemicals via the efficient flux through the TCA cycle (Bilal et al., 2020), and also high potentials in biosynthesis and secretion of proteins (Madzak et al., 2004). Besides being a desirable host for bioproduction, *Y. lipolytica* is also a model organism for research on the biology and physiology of dimorphism, hydrophobic substrate utilization, protein secretion and more importantly, lipid metabolism (Beckerich et al., 1998; Beopoulos, Chardot, et al., 2009; Domínguez et al., 2000; Fickers et al., 2005).

Given the broad use of *Y. lipolytica* as an important microorganism with biological relevance and biotechnological applications, significant efforts have been made to expand the knowledge on this yeast. More recently, with the advancements in omics techniques, a quantitative systems-level insight into cellular organizations has made possible to make valuable contributions to bioprocessing (Campbell et al., 2017). Apparently, being able to analyze the entire genome as well as globally quantifying all cellular components and their interactions, does not only expand our knowledge on the biology of the system under study, but also greatly improves the quality of cell factory design. In general, there are multiple biotechnologically important metabolites produced by *Y. lipolytica* and the application of systems metabolic engineering is in its infancy for this yeast. Therefore, it is beneficial to provide an assessment of the state-of-the-art, to evaluate which approaches have let to promising results and where future research should be directed to. Herein, recent advances in the implementation of systems metabolic engineering in optimizing *Y. lipolytica* as a cell factory is being reviewed.

Y. lipolytica as a cell factory

The industrial application of *Y. lipolytica* has over half a century of history that began with the discovery of its high lipolytic and proteolytic activity. These features enable the yeast to grow on a diverse range of lipid or protein rich materials, where many of them can be considered as cheap substrates. The high capacity of lipase secretion was leveraged on by British Petroleum from 1950s through the 1970s, to pioneer the production of single cell protein (SCP) from*n*- alkanes as substrate (Groenewald et al., 2014). SCP production based on *Y. lipolytica* is still available as animal feed additive but on a smaller scale (Ritala et al., 2017).

Starting from 2000s and following the development of molecular and genetic tools for engineering the yeast, research studies as well as commercial productions have been oriented towards leveraging the abilities of the yeast to produce more than 100 different recombinant proteins, polyalcohols, aromas, emulsifiers and various organic acids (primarily citric acid) from industrial waste or by-products (Ledesma-Amaro & Nicaud, 2016; Markham & Alper, 2018; Miller & Alper, 2019). In addition, the oleaginous phenotype of *Y. lipolytica* has been of interest as it renders the yeast a promising oleaginous cell factory that can produce oils and fatty acid-derived compounds. Two products, New Harvest EPA oil and Verlasso(r) salmon are commercial examples of this feature and contain omega-3 fatty acids for which there have been many clinical studies showing a wide range of health benefits (Xie et al., 2015). Generally, oils and fatty acid-derived compounds from *Y. lipolytica* can be used as *e.g.* biodiesel as well as ingredients for food and cosmetic industries (Miller & Alper, 2019).

Since the lipid biosynthesis is one of the most important features of*Y. lipolytica* as a cell factory, a brief overview of general lipid metabolism will be given (also depicted in Figure 1) while the details can be found elsewhere (Fakas, 2017; Lazar et al., 2018). Oleaginous strains of *Y. lipolytica* are capable of accumulating

large amounts of lipids in form of a dynamic storage compartment called lipid body (LB) which mainly accommodates neutral lipids such as triacylglycerols (TAGs) (Beopoulos, Chardot, et al., 2009). Metabolism of lipid biosynthesis and accumulation is well studied in *Y. lipolytica* , comprehensive understanding of which is vital for opting any further systems metabolic engineering approach to improve the production. Accumulated lipid can be biosynthesized whether via *de novo* or *ex novo* routs. *de novo* lipid biosynthesis is induced by metabolism imbalance upon an essential nutrient limitation in the growth media. Following by nitrogen starvation and in the excess of carbon source, intracellular adenosine monophosphate (AMP) is used to produce ammonium. Since AMP is an allosteric activator for isocitrate dehydrogenase, its rapid decrease ultimately downregulates the TCA cycle and in turn, citrate accumulates in mitochondria (Lin & McAlister-Henn, 2003). Excess citrate will then be cleaved into acetyl-CoA in cytosol which will be converted to malonyl-CoA used to produce different fatty acids (FAs) by further elongation and desaturation (Yuzbasheva et al., 2019). Finally, TAGs are formed using the activated FAs via the Kennedy pathway in the interface of ER and LBs (Beopoulos, Cescut, et al., 2009). *ex novo* pathway is an alternative for lipid accumulation based on the ability of *Y. lipolytica* to grow on a wide range of hydrophobic carbon sources (such as FAs, oils and TAGs) and with the help of lipases and surfactants (*e.g.* Liposan) which in turn facilitates the surface-mediated transport of the substrate. If the substrate is in excess, they will be stored as LBs comprising TAG and steryl ester (SE). Whether produced *de novo* or *ex novo* , the accumulated lipids can be utilized as energy source upon starvation.

Other than elucidating the metabolic or regulatory pathways of a metabolite from a microbial cell factory, availability of well-established genome modification techniques is an essential factor for optimizing microbial cell factories. In addition to wide availability of basic genetic engineering tools (*i.e.* host strains and markers, vectors, promoters, terminators and replication elements) and conventional genetic modification methods, there is a continuing effort in developing modern gene/genome editing systems for *Y. lipolytica* which can greatly speed up the application of this yeast in systems biotechnology (comprehensively reviewed in (Ganesan et al., 2019)). Briefly, from 2016, several efficient CRISPR–Cas9 systems for single and multigene editing as well repression or activation of genes in *Y. lipolytica* are being developed (Gao et al., 2016; C. Schwartz et al., 2017, 2018; C. M. Schwartz et al., 2016; Z. Yang et al., 2020). More recently, a dual purpose CRISPR-Cpf1 system has been suggested that is capable of simultaneous gene disruption and gene regulation in *Y. lipolytica* (Ramesh et al., 2020). Furthermore, a suite of genetic tools (EasyCloneYALI) has been generated that facilitates transformation protocols by providing a series of pre-designed plasmids and oligos (Holkenbrink et al., 2018). Although there is still significant opportunity to further improve CRISPR-based systems in *Y. lipolytica* in terms of multi-gene editing efficiency or the need for a high-throughput screening technology after genome editing, the existing systems provide a suitable platform to be coupled with the systems level tools to further promote the systems metabolic engineering process.

Generally, *Y. lipolytica* is a biotechnologically important yeast in which the metabolism and the biotechnologically important metabolites production pathways have been studied. Moreover, the genomes of several strains have been sequenced, which together with the increasing omics data production as well as the current availability of plethora of genetic engineering and genome editing techniques (Holkenbrink et al., 2018; Madzak, 2018), has offered perspectives for systems metabolic engineering *Y. lipolytica* as a potential biotechnological workhorse.

Systems metabolic engineering of *Y. lipolytica*

The functional genomics revolution and increasing ease of omics data generation and computational analysis aids in understanding the mechanisms of complex biological systems and the prediction of their dynamic properties via systems biology. This paradigm of studying a cell as a whole is being efficiently implemented to provide a holistic view of the functioning of microbial cell factories with the goal of rational re-designing or creating new cellular functions in these organisms.

Under the moniker of metabolic engineering, modifications of biochemical pathways have been used for decades to achieve desired phenotypes such as overproduction of a metabolite in cell factories, either using random mutagenesis or other conventional molecular genetic engineering tools. However, only after the

development of the methods and tools in the discipline of systems biology has it become practical to approach the metabolic engineering challenges from a systems perspective. The development of an optimal strain via systems metabolic engineering can be performed through two major approaches: omics data-driven (top-down) and biomolecular model-driven (bottom-up). While the bottom-up approach starts with the reconstruction of an accurate mathematical model of a specific biomolecular subsystem (*i.e* metabolic, gene regulatory and signaling networks, or combinations thereof) using existing biological knowledge; the top-down approach begins with extracting information from relevant omics datasets (*e.g.* metabolomics, proteomics, transcriptomics, fluxomics). Figure 2 illustrates these strategies as well as the classic metabolic engineering.

During the past decade, there have been multiple studies on biomolecular model analysis and omics data generation that aimed to improve the understanding and production of many valuable metabolites in *Y. lipolytica* . Genome scale metabolic models (GEMs) are the most commonly studied biomolecular networks, the popularity of which arises not only from their innate nature which directly considers the analysis of the metabolite production phenotype, but also from the availability of powerful constraint-based modeling methods. As GEMs can provide a global view of cellular metabolism, they are influential tools in systems metabolic engineering. Currently, there have been six GEMs published for *Y. lipolytica* by different research groups, where each model includes improvements over previously published models. All six GEMs have been used for evaluating metabolic engineering strategies with a major focus on over-producing lipids, *e.g.* triacylglycerols (TAGs) and to a lesser extent for the increased dicarboxylic acids production (Kavščček et al., 2015; Kerkhoven et al., 2016; Loira et al., 2012; Mishra et al., 2018; Pan & Hua, 2012; Wei et al., 2017).

Since obtaining high-quality omics data has become progressively easier, interest in applying data-driven approaches for acquiring systems-level knowledge on *Y. lipolytica* have increased (Ledesma-Amaro & Nicaud, 2016). While the availability of complete genome sequences reveal its metabolic pathways and functional potential, various transcriptomics and a few proteomics analyses have provided useful information about the dynamics of biological processes, such as the transition from growth phase to the lipid accumulation phase as well as its regulatory mechanisms (Morin et al., 2011). There have also been a few metabolomics and lipidomics analyses in *Y. lipolytica* , which further provide information on the metabolic pathways under study by incorporating the changes in metabolite concentrations (Zhao et al., 2015).

As individual omics analyses (e.g. transcriptomics *or*metabolomics) are not always able to fully elucidate the behaviors of a cellular system, combining different omics data (e.g. transcriptomics*and* metabolomics) may result in a more precise picture of the biological system being studied. Moreover, integration of various types of omics data following a bottom-up approach can boost the predictive capability of the model (Gu et al., 2019). Such combinatory approaches have been applied in a few studies in metabolic engineering of *Y. lipolytica* .

Generally, examples of systems-level understanding as well as metabolic engineering efforts in *Y. lipolytica* powered by systems biology knowledge and tools will be discussed below in relative sections. Furthermore, Table 1 summarizes the systems-level optimizations of lipid-derived as well as non-lipid products in *Y. lipolytica.*

Lipids and fatty acid-derived products

Various studies have been performed to gain a systems-level understanding of lipid metabolism with focus on three main questions: the main source of NADPH that lipid accumulation demands; gene regulation that coincides with lipid accumulation; and transcription factor networks that are underlying the expressional changes during lipid accumulation as will be detailed below. In many cases, such studies have not only expanded our knowledge on various aspects of lipid metabolism, but also yielded proposed targets for genetic engineering for improved lipid production.

Lipid biosynthesis has a high NADPH demand that could theoretically be provided through various metabolic pathways. Based on a comparison of flux distribution obtained by ^{13}C metabolic flux analysis in two strains of *Y. lipolytica* , one of which was engineered to produce lipids at roughly twice the yield of the wild strain, Wasylenko *et al.* concluded that the oxidative pentose phosphate pathway (PPP) is the almost exclusive

source of lipogenic NADPH when *Y. lipolytica* is grown on glucose (Wasylenko et al., 2015). However, when cultured on acetate, another flux distribution analysis by Liu *et al.* showed the importance of gluconeogenesis on lipogenic NADPH synthesis (Liu et al., 2016).

To reveal if lipid accumulation is transcriptionally regulated, the first transcriptomics analysis of *Y. lipolytica* during its metabolic shift from growth to lipid accumulation indeed showed significant difference between the expression profile of actively dividing and lipid accumulating cells (Morin et al., 2011). However, the genes directly governing fatty acid synthesis were not transcriptionally regulated, but rather, the authors hypothesized that lipid accumulation is a passive consequence of the rerouting of carbon fluxes. Upon nitrogen deprivation a cascade of transcriptional events is triggered that result in an increased supply of precursor and cofactors necessary for the synthesis of lipids. Subsequent transcriptomics as well as multiomics studies confirmed this hypothesis and elucidated the regulatory role of protein and amino acid metabolism in lipogenesis rather than the transcriptional regulation of lipid metabolism itself. By integrating proteome, phosphoproteome and metabolome data, Pomraning*et al.* suggested posttranslational modification of key lipogenesis enzymes as the governing regulatory mechanism (Pomraning et al., 2016). According to later studies, genes related to amino acid metabolism were appeared to be downregulated upon nitrogen limitation, while alternative nitrogen supply pathways such as protein turnover and autophagy genes were upregulated among which an important factor that affects lipid synthesis was reported to be the metabolism of leucine (Kerkhoven et al., 2016, 2017). In an independent research where links between disruption of the *MHY1* gene, involved in dimorphic transition in *Y. lipoytica* , and lipid accumulation have been studied, transcriptome data from the *MHY1* mutant strain showed decreased amino acid biosynthesis while lipid accumulation increased by 13% based on cell dry weight (G. Wang et al., 2018).

To elucidate if regulatory networks are underlying lipid accumulation, Trébulle *et al* inferred a genome-wide co-regulatory network using CoRegNet on the basis of the gene expression data during lipid accumulation under nitrogen limitation (Trébulle et al., 2017). According to their reconstructed regulatory network, network modules representing different transcriptional programs, from growth to lipid accumulation phases, along with the most influential new transcription factors (TFs) and co-regulators have been identified. Further, the role of the top candidates on lipid accumulation were validated experimentally using a high throughput overexpression system (Leplat et al., 2018). Based on the identified regulators and target genes, their findings again support the hypothesis that lipid accumulation is a consequence of change in carbon fluxes rather than an enhanced lipid metabolism (Trébulle et al., 2017).

Beyond employing systems-level approaches to gain understanding on lipid metabolism, it has been used to find systems-level solutions that favor the oleaginous phenotype in terms of improving its yield, productivity and titer as well as broadening the substrate range. Nonetheless, most studies that have improved lipid production can be classified as classical metabolic engineering, via overexpression of genes that are directly involved in lipid biosynthesis, deletion of those involved in lipid degradation, expressing genes required for the consumption of different carbon sources, balancing cofactors, as well as a combination of these strategies which are comprehensively discussed elsewhere (Abdel-Mawgoud et al., 2018; Das et al., 2020; Lazar et al., 2018). While rationally designed classical metabolic engineering has been able to push lipid accumulation to laudable levels, systems-level approaches would be able to also reach the uncharted territories of the lipogenesis metabolic landscape that would harbor a strain that is robust, versatile and pushes lipid accumulation to its limits. Systems metabolic engineering efforts in *Y. lipolytica* have therefore aimed to approach the theoretical maximal yield via understanding the less intuitive limiting factors and based on conclusions formulated in omics and model-based studies.

Accordingly, using a bottom-up approach through the analysis of a manually reconstructed GEM for *Y. lipolytica*(*i* MK735), Kavšček *et al.* suggested a positive effect of oxygen limitation on lipid production in the productive phase giving the hypothesis that reduced aeration rate, which is also favored in industry, might induce lipid accumulation. Moreover, to eliminate citrate excretion, a reduced glycolytic flux was calculated while maintaining lipid synthesis rate. Combination of these findings were used to design a fermentation strategy which resulted in 80% increase in the lipid content of biomass and more than fourfold

yield improvement (Kavšček et al., 2015). In a later model-based approach, various gene knockouts and overexpression targets for improved TAG biosynthesis were predicted by analyzing a comprehensive GEM reconstructed by Wei*et al.* Although not experimentally verified, gene modification strategies mostly being involved in glycolytic and amino-acid metabolic pathways were predicted to yield over 55% increase on TAG production (Wei et al., 2017). In a most recent model-based study, a modified version of *i* MK735 was implemented to analyze flux distributions of *Y. lipolytica* in nitrogen-limited conditions as well as identify metabolic engineering strategies for improving lipid production. Since the cellular objective of growing cells during nitrogen limitation is not obvious, an environmental version of minimization of metabolic adjustment (eMOMA) was used instead of the conventional flux balance analysis (FBA) that requires an assumption of the cellular objective *e.g.* maximized biomass production. This modeling approach predicted multiple novel and non-intuitive targets for metabolic engineering while experimental verification of one of the knockout candidates involved in one-carbon/methionine metabolism (annotated as methylenetetrahydrofolate dehydrogenase) showed 1.45-fold increase in lipid accumulation (M. Kim et al., 2019).

Noteworthy is that the current GEM-based analyses did not include any regulatory information. The integration of further omics data may lead to a more reliable prediction of gene modification targets *in vitro* and decrease the false positive predictions. Moreover, top-down omics-based approaches have not only augmented the biological knowledge of lipid biosynthesis, but also in some cases non-experimentally verified suggestions for production improvement have been concluded from these analyses. Of the non-intuitive examples of these suggestions is the role of cell wall biosynthesis genes in lipid production, shown by Pomraning *et al.* where cell wall thickening was observed upon the exhaustion of extracellular carbon source (Pomraning et al., 2015). Accordingly, a combination of top-down and bottom-up approaches would therefore be recommended.

3.2. Specific oleochemicals

While classic and systems metabolic engineering efforts continue to improve lipid production in *Y. lipolytica* by maximizing flux through TAG biosynthesis, production of specific valuable oleochemicals are being studied in this yeast mostly via synthetic pathway construction.

Many commercially interesting oleochemicals, *e.g.* medium-chain lipids, are less common in natural resources and are therefore ideal candidates for systems and synthetic biology-based production due to their high value (Blazeck et al., 2014). These oleochemicals can be used as nutraceuticals or pharmaceuticals, such as polyunsaturated fatty acids (PUFAs) or fatty alcohols (Ledesma-Amaro & Nicaud, 2016). Substantial progress has been made towards heterologous production of such oleochemicals in *Y. lipolytica* , but only recently have conclusions from systems-level analysis been implemented to improve the production of non-native oleochemicals. Most of these efforts are through using the top-down strategy. For instance, in a study by Dahlin*et al.,* a lipid degradation-deficient strain of *Y. lipolytica* was used as host organism to express copies of codon-optimized fatty acyl–CoA reductase from *Marinobacter algicola.* The final fatty alcohol producer strain was evaluated by transcriptomics, metabolomics and fluxomics analysis. According to the results, unlike *S. cerevisiae* , no elicited stress response was detected in *Y. lipolytica* indicating the non-toxicity of fatty alcohols in this strain. Moreover, potential limiting factors upstream of acetyl–CoA, such as glucose uptake, glucose phosphorylation, conversion to fructose-6-phosphate/fructose-1,6-bisphosphate as well as pyruvate and citrate were indirectly predicted based on metabolomic analysis (Dahlin et al., 2019). Transcriptomics of another fatty alcohol producing *Y. lipolytica* aided to significantly improve the production upon identifying the upregulation of glycolysis genes in response to higher fatty alcohol production. This result was leveraged to optimize the selection of suitable promoters for fatty acyl-CoA reductase expression, to coordinate the fatty alcohol production with glycolysis (Zhang et al., 2019). Similar procedure has been performed to produce pentacyclic triterpenoid oleanolic acid in *Y. lipolytica* . Based on intracellular metabolome data, a direct precursor of the triterpenoid was found to be accumulating. Guided by the analysis of differential transcriptional levels of genes, it transpired that enhancement of electron transfer by fusion expression of P450 enzyme and the last step of the biosynthetic pathway could be used to improve substrate conversion efficiency by 28% (Li et al., 2020).

In order to repurpose the lipophilic properties in *Y. lipolytica*for beta-carotene biosynthesis, Wang *et al* .

designed a strategy of global transcription machinery engineering (gTME) to explore the changes of gene expression levels that benefits carotene production (M. Wang et al., 2018). This strategy had been introduced for other complex cellular genotypes in *S. cerevisiae* (El-Rotail et al., 2017) and*Zymomonas mobilis* (Tan et al., 2016). Accordingly, a library of randomly mutated versions of the key transcription factor Yl-Spt15 were expressed in a strain containing a heterologous beta-carotene biosynthetic pathway. Strains with mutated Yl-Spt15 expression displaying either enhanced or decreased production were analyzed by transcriptomics, and the most dominant differentially expressed genes were experimentally tested and those involved in RNA polymerase, fatty acid metabolism, and specially ketone body metabolism were shown to be beneficial for beta-Carotene production improvement. Although the reported beta-carotene yields were far lower compared to other published yields, their exploited gene targets and pathways can further guide the rational design of *Y. lipolytica* for tuning lipophilic production in combination with improved fermentation design.

3.3. Non-lipid products

Although a major focus in *Y. lipolytica* research is on lipogenesis, this yeast is able to produce other native and non-native industrially important chemicals such as organic acids (OAs) and polyols. The most industrially important non-lipid chemicals are OAs which are direct or indirect intermediates of the TCA cycle. Due to its oleaginous phenotype, *Y. lipolytica* accommodates a highly active TCA cycle. However, since biosynthesis of OAs is highly linked to cellular energy metabolism, reaching efficient production metrics will require more complex engineering approaches. This renders systems metabolic engineering profitable tools to optimize OA production. Nonetheless, also for this group of products has classical metabolic engineering through rational genetic modifications and optimization of fermentation condition shown to enhance the productivity (reviewed in (Finogenova et al., 2005)). Meanwhile, efforts to elucidate the underlying systems-level production mechanisms are crucial to optimize cell metabolism favorable for OA production. In terms of citric acid (CA) production, Sabra *et al.* have focused on transcriptome and fluxome data in a citrate producing strain of *Y.lipolytica* . While controlling the oxygen supply on mixed substrates proved instrumental to improve the citrate production titer more than 3 fold, multi-omics data analysis have provided pentose phosphate pathway and glyoxylate cycle genes as targets for metabolic engineering of CA (Sabra et al., 2017). In another study, after reporting *Y. lipolytica* as the best CA producer among more than 40 yeast strains, Kamzolova *et al* ., studied the underlying overproduction mechanism using growth limitation assessment and enzyme activity measurements which helped in fermentation design for enhanced production (85 g/L) (Kamzolova & Morgunov, 2017). A more recent screening also showed that the production of CA strongly strain dependent, therefore, screening for overproducers and studying the underlying molecular mechanisms is of great importance (Carsanba et al., 2019). Noteworthy is that due to the relations between lipid and CA metabolism, knowledge on the regulation surrounding lipid accumulation as well as nitrogen limitation can have considerable relevance for CA production studies.

The production of succinic acid (SA) in *Y. lipolytica* has also undergone metabolic engineering efforts (Abdel-Mawgoud et al., 2018). Unlike SA producing bacteria, which are among the most successful producers to date, *Y. lipolytica* can tolerate low pH and thrives under acidic conditions. This simplifies downstream processes as the bacterial fermentation broth is rather kept near neutral pH and requires an additional acidification step for SA purification which causes byproduct formation (Ahn et al., 2016). Although high titers have been achieved (160 g/L) in an engineered strain lacking active succinate dehydrogenase (SDH) to prevent oxidation of succinic acid to fumaric acid, low yield and productivity hinders the implementation of *Y. lipolytica* for industrial production. SDH deficient mutants of *Y. lipolytica* are growth defective in cultures using glucose source (Yuzbashev et al., 2010). To circumvent this problem Yuzbashev *et al.* have isolated an improved SA producer from combined induced mutagenesis and metabolic evolution, which could more efficiently utilize glucose (Yuzbashev et al., 2016). ^{13}C flux analysis on SDH deficient mutants was performed to investigate the contribution of various metabolic pathways in SA formation (Yuzbashev et al., 2016), however, to fully reveal the molecular mechanism by which adaptive evolutionary approaches have resulted in a beneficial phenotype would require genome sequence analysis of the evolved and unevolved strains. A further problem hindering improvement of SA production is the accumulation of the by-product acetate (Cui et al., 2017). By metabolic pathway analysis, Cui *et al.* have reported CoA-transferase activity

in mitochondria as the major source of acetic acid. Deletion of the responsible gene, as well as further overexpression of the key enzymes of the oxidative TCA resulted in an improved SA titer and no acetic acid accumulation in defined media (Cui et al., 2017). There have been successful systems metabolic engineering approaches for SA production in other microorganisms, such as GEM-based SA production improvement on *S. cerevisiae* (Agren et al., 2013) as well as other omics-based studies (Otero et al., 2013). Similar approaches could be applied to *Y. lipolytica* to yield industrially feasible SA production levels in this yeast.

α-ketoglutarate (α-KG) is another OA for which *Y. lipolytica*seems a promising production host. The main strategy being used to improve its production is still based on fermentation design as well as a few genetic engineering in the direct biosynthetic enzymes (Guo et al., 2016). Therefore, while systems-level insight is still desired to investigate key metabolic processes influenced and regulated by the known factors that positively affect α-KG production, there are a few studies based on omics data. In a study to improve α-KG production, Guo et *al.* , have assessed the roles of specific transporters (Guo et al., 2015). To this end, they have identified six endogenous putative transporter genes using a comparative genomics approach and by expressing them in an α-KG-producing wild-type strain, the production of this OA has improved while reduced pyruvic acid (a byproduct) concentration was observed. Their study showed whole genome analysis for transporters can be helpful in improving the accumulation of specific organic acids. Another comparative genomics method with the aim of investigating the regulatory mechanisms controlling α-KG production, showed the positive role of genes associated with the regulation of mitochondrial biogenesis and energy metabolism in α-KG production, whereas they have concluded genes related to transformation between keto acids and amino acids as reducers of the accumulation of α-KG (Zeng et al., 2016). As another strategy to accumulate α-KG, is to take advantage of its thiamine-auxotrophic trait. *Y. lipolytica* is unable to biosynthesize thiamine which is the co-factor for α-ketoglutarate dehydrogenase which converts α-KG to succinyl-CoA. Therefore, a culture medium lacking this vitamin will likely favor the accumulation of α-KG when carbon source is in excess. However, enhanced accumulation of α-KG by disruption of genes directly affecting TCA cycle will undoubtedly cause cellular growth and survival defects circumventing which urges deeper molecular knowledge. In this regard, similar omics data-based findings of the study by Walker *et al.* on the effects of thiamine deficiency on cellular metabolism of thiamine auxotroph *Y. lipolytica* which was discussed in lipid production improvement strategies, can be helpful (Walker et al., 2020).

Beyond native OAs, *Y. lipolytica* has been used to produce the non-native itaconic acid (IA). IA is naturally produced by several*Aspergillus* species and is a replacement for petroleum derived products (Bonnarme et al., 1995; Robert & Friebel, 2016). Due to shortcomings with the native producer in terms of poor growth and shear stress sensitivity, its production is studied in alternative hosts such as *Y. lipolytica* . IA is synthesized by cis-aconitic acid decarboxylase (CAD) (Kanamasa et al., 2008) and IA production in*Y. lipolytica* has been established by heterologous expression of CAD (Blazeck et al., 2015) resulting in IA yields of 0.06 g/(g glucose) at natural low pH. Although these studies can serve as a promising stepping-stone toward enhancing IA production in *Y. lipolytica* , to establish this yeast as an industrially competitive IA producer, further efforts are required to improve production metrics. Even while bottom-up systems-level approaches have been used for IA production in other microorganisms (Harder et al., 2016), there is still no report on systems metabolic engineering in *Y. lipolytica* .

Besides OAs, *Y. lipolytica* is a promising producer of sugar polyols, and there is a currently strong interest in bio-based production of such value-added functional sugars for use in food and pharmaceuticals industry as sweetener (*e.g* . mannitol and erythritol). Recent progress in the use of *Y. lipolytica* to synthesize these functional sugars, especially the genetic engineering approaches, are comprehensively reviewed elsewhere (Bilal et al., 2020). The importance of systems-level studies in this field has been demonstrated in a study by Yang *et al.* where erythritol production could be enhanced in an osmophilic strain of *Y. lipolytica* by cultivation in an osmotic pressure control fed-batch strategy (L. B. Yang et al., 2014). Proteomics of erythritol production from glycerol in response to osmotic pressure revealed that highly ranked differentially-expressed proteins such as enzymes related to osmotic stress response, the aldo-keto reductases and catalase T had drastically increased expression levels under hyperosmotic pressure (L. B. Yang et al., 2015). This information could be leveraged on for future reverse engineering of *Y. lipolytica* strains for polyols biosynthesis.

Conclusions and perspectives

For decades, *Y. lipolytica* has been known as a promising biotechnological chassis not only for its high oleaginous flux, but also for the production of a variety of native and non-native chemicals. However, despite advances in genome editing tools which have facilitated classical metabolic engineering of *Y. lipolytica,* production metrics for most products still needs improvement. In this regard, systems biology allows a more in-depth understanding of the intricate regulation and metabolism which can further help in identifying non-intuitive strain design targets with the aim of pushing yields to the theoretical limits. This can in turn expand the industrial potential of this yeast. Although several *Y. lipolytica* strain engineering studies have begun to involve omics and model-based approaches, there is significant potential in further use of the systems-level approach. Accordingly, system-level approaches have been used for studying lipid-derived products with some promising results, while non-lipid products have benefited very little from these approaches. Moreover, both bottom-up and top-down approaches are used, although not to the same extend in all the products of *Y. lipolytica* . On the other hand, as the classical metabolic engineering results generally shows, there is not always a linear correlation between the overexpression of a gene and its *in vivo* production improvement which is believed to be caused by various layers of cellular control. To this end, combination of both the top-down and bottom-up approaches seems promising. Particularly useful would be metabolic flux analysis (MFA) which can be performed to measure accurate flux distributions as model simulations are not always able to give predictions with enough certainty. Another obstacle hindering systems-level ME is that many (about 40% in case of *Y. lipolytica* W29) *Y. lipolytica* genes are annotated as hypothetical proteins, which can limit the interpretation of data-driven information as well as predictive efficacy of genome-based models. This need can at least partially be addressed using already available computational approaches such as machine learning that is not only able to accurately predict translation start sites but also functional annotations (G. B. Kim et al., 2020). Artificial neural networks, clustering and other machine-learning algorithms have also shown promising results in biosynthetic pathway prediction for a target chemical (Segler et al., 2018) (useful in non-native chemical productions), prediction of a probability that an enzyme catalyzes a reaction (Mellor et al., 2016) (useful in GEM reconstruction), and different gene expression control parameter prediction such as promoter strength (Jervis et al., 2019) (useful in metabolic engineering). Finally, *Y. lipolytica* is regarded as a promising host for the production of various metabolites, therefore, it is expected that attempts to elucidate systems-level behaviors for one product will also positively impact on this yeast as a future host for other products.

Acknowledgments

We acknowledge funding from the Novo Nordisk Foundation (grant no. NNF10CC1016517). Authors declare no conflict of interest.

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Figure captions

Figure 1. Overview of Lipid metabolism in *Y. lipolytica* .

Solid arrows: chemical conversions and transport reactions, dashed arrows: multiple chemical conversion steps, dotted lines and arrows: representing N-limitation consequences. Abbreviations: AMP Adenosine monophosphate; Cit Citrate; DAG Diacylglycerol; DHAP Dihydroxyacetone phosphate; F6P Fructose 6-phosphate; FA Fatty acid; FBP Fructose 1,6-bisphosphate; Fum Fumarate; G3P Glycerol 3-phosphate; G6P Glucose 6-phosphate; GA3P Glyceraldehyde 3-phosphate; Icit Isocitrate; IMP Inosine monophosphate LPA Lysophosphatidic acid; Mal Malate; Mal-CoA Malonyl coenzyme A; NH4 Ammonium; OAA Oxaloacetate; PA Phosphatidicacid; Pyr Pyruvate; Suc Succinate; TAG Triacyglycerol.

Figure 2. Schematic procedure of classic *vs.* systems metabolic engineering and the major identified engineering targets for lipogenesis in *Y. lipolytica* by each strategy.

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