



**CHALMERS**  
UNIVERSITY OF TECHNOLOGY

## **Yeast based biorefineries for oleochemical production**

Downloaded from: <https://research.chalmers.se>, 2021-03-05 01:36 UTC

Citation for the original published paper (version of record):

Zhang, Y., Nielsen, J., Liu, Z. (2021)

Yeast based biorefineries for oleochemical production

Current Opinion in Biotechnology, 67: 26-34

<http://dx.doi.org/10.1016/j.copbio.2020.11.009>

N.B. When citing this work, cite the original published paper.



ELSEVIER



# Yeast based biorefineries for oleochemical production

Yiming Zhang<sup>1</sup>, Jens Nielsen<sup>1,2,3</sup> and Zihe Liu<sup>1</sup>

Biosynthesis of oleochemicals enables sustainable production of natural and unnatural alternatives from renewable feedstocks. Yeast cell factories have been extensively studied and engineered to produce a variety of oleochemicals, focusing on both central carbon metabolism and lipid metabolism. Here, we review recent progress towards oleochemical synthesis in yeast based biorefineries, as well as utilization of alternative renewable feedstocks, such as xylose and L-arabinose. We also review recent studies of C1 compound utilization or co-utilization and discuss how these studies can lead to third generation yeast based biorefineries for oleochemical production.

## Addresses

<sup>1</sup> Beijing Advanced Innovation Center for Soft Matter Science and Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, 100029, China

<sup>2</sup> Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden

<sup>3</sup> BioInnovation Institute, Ole Maaløes Vej 3, DK2200 Copenhagen N, Denmark

Corresponding authors:

Nielsen, Jens ([nielsenj@chalmers.se](mailto:nielsenj@chalmers.se)), Liu, Zihe ([zihe@mail.buct.edu.cn](mailto:zihe@mail.buct.edu.cn))

Current Opinion in Biotechnology 2021, 67:26–34

This review comes from a themed issue on **Energy biotechnology**

Edited by **Huimin Zhao** and **Yasuo Yoshikuni**

<https://doi.org/10.1016/j.copbio.2020.11.009>

0958-1669/© 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

## Introduction

Fatty acids and their derivatives, also termed oleochemicals, are used as drop-in fuels, lubricants, additives in foods, polymers, cosmetics, and pharmaceuticals, ranging from bulk chemicals to fine chemicals. Traditional production of oleochemicals relies on feedstocks of vegetable oils and animal fats, but microbial synthesis offers sustainable production from a wide range of renewable sources [1]. Besides, it allows for production of non-natural oleochemicals having novel chemical features. Oleochemical synthesis has been well studied and intensively engineered in many microorganisms, including bacterial, yeast and microalgae [2]. This review will only focus on yeast based production of oleochemicals, whereas we refer to other recent reviews on oleochemical production from other microbial cell factories [3,4].

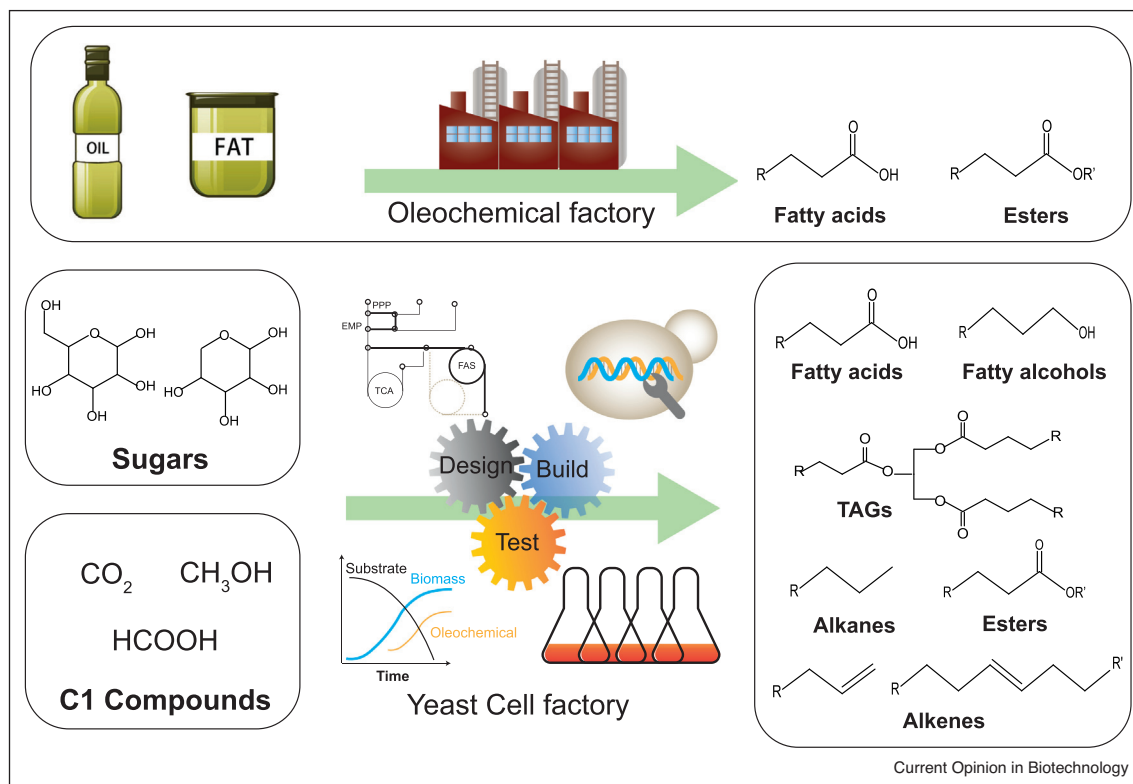
Oleaginous yeast hosts, such as *Yarrowia lipolytica*, *Rhodospiridium toruloides* and *Cryptococcus curvatus*, are often chosen as industrial workhouses for oleochemical production, because of their efficient lipid synthesis and accumulation [5–9]. Non-oleaginous yeasts, such as *Saccharomyces cerevisiae*, is also preferred because of its well-studied cell metabolism and strain engineering tools. So far, a variety of oleochemicals have been synthesized in yeast, including free fatty acids [10], fatty alcohols, triacylglycerols (TAGs) [11], alkanes, alkenes [12], fatty acid methyl-esters (FAMES), fatty acid ethyl-esters (FAEEs) [13], oleoylethanolamide [14] and wax esters [15,16]. Engineering efforts on deregulating lipid metabolism, redirecting carbon flux towards lipid synthesis and rebalancing cytosolic redox factors have been performed to increase the titre, rate and yield of oleochemicals. Meanwhile, discovering enzymes that are capable of synthesizing unnatural or natural oleochemicals with controlled chain length has gained more attention [17,18<sup>\*\*</sup>,19].

Yeast based biorefineries can rely on using different renewable feedstocks, for example, glucose from food crops (1st-generation feedstock), xylose and L-arabinose from plant biomass and energy crops (2nd-generation feedstock), and possibly C1 compounds like CO<sub>2</sub>, methane or methanol (3rd-generation feedstock) [20]. Glucose is the preferred carbon source for yeast, but use of 1st-generation feedstock for oleochemical production threatens food supply. Utilization of xylose, the second most abundant monosaccharide in lignocellulose after glucose, has been studied for years to avoid food competition [21]. Utilization of L-arabinose from 2nd generation feedstock has also been studied for oleochemical production [1,22<sup>\*</sup>]. Recently, utilization of 3rd-generation feedstock has started to be considered even though it is a challenging alternative for microbial synthesis and oleochemical production [23]. This review highlights recent advances in yeast based biorefineries towards production of fatty acids and their derivatives and end with prospects of utilization C1 compounds for microbial synthesis of oleochemicals (Figure 1).

## Engineering on synthesis of oleochemicals

Feedstocks can be assimilated into the central carbon metabolism, including Embden–Meyerhof–Parnas (EMP) pathway, pentose phosphate (PP) pathway and tricarboxylic acid (TCA) cycle, to provide the building block acetyl-CoA and redox factor NADPH for fatty acid synthesis. Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC), which is directly used for fatty acid synthesis and elongation catalysed by fatty acid synthase in a cyclic manner. Engineering for

Figure 1



Sustainable feedstocks are utilized in yeast-based biorefineries for production of various oleochemicals. Yeast cell factories are exploited and optimized for oleochemical production via the Design-Build-Test cycle pipeline.

oleochemical production has been comprehensively reviewed in [5,24,25], and we therefore here focus on recent key points of particular interests for oleaginous and non-oleaginous yeasts, as summarized in Table 1.

### Acetyl-CoA pool and NADPH supply

Generation of cytosolic acetyl-CoA varies between oleaginous and non-oleaginous yeasts. In non-oleaginous yeasts, cytosolic acetyl-CoA is synthesized via the pyruvate dehydrogenase bypass, which is a branch of the fermentative pathway with strong competition with ethanol pathway. In oleaginous yeast, cytosolic acetyl-CoA is shuttled from the mitochondria acetyl-CoA pool via a citrate-malate antiport cycle, and under nitrogen starvation TCA activity decreases and excess citrate is shuttled into the cytosol, which is converted to acetyl-CoA via ATP citrate lyase. Therefore, in oleaginous yeasts under nitrogen starvation high flux through the acetyl-CoA precursor allows for high lipid accumulation and advances oleochemical production, compared with non-oleaginous yeasts. When similar pathways were engineered into *S. cerevisiae*, that is, construction of acetyl-CoA shuttling cycle and deletion of fermentative pathway, cell metabolism was reported to convert from alcoholic fermentation to lipogenesis [26<sup>••</sup>]. Moreover, increasing the acetyl-CoA

pools of oleaginous yeasts further enhanced lipid production under conditions with both high and low C/N ratios, confirming that efficient supply of cytosolic acetyl-CoA is required for high-level lipid synthesis [27–29,30<sup>•</sup>].

Cytosolic NADPH is either predominantly generated from the oxidative PP pathway in most yeasts, or primarily through cytosolic malic enzymes (MEs) in some oleaginous yeasts [25]. Increasing NADPH availability has successfully promoted fatty acid synthesis in both *S. cerevisiae* and *Y. lipolytica* via increasing PP pathway flux, decreasing glycolysis flux, expressing NADPH<sup>+</sup>-dependent glyceraldehyde 3-phosphate dehydrogenase or malic enzyme, or engineering oxidative defense pathways [26<sup>••</sup>,31<sup>••</sup>,32].

### Fatty acid synthetase

Yeast fatty acid synthetases (FASs), Type I FASs, are large proteins with multiple modules of acetyl transferase (AT), malonyl-palmitoyl transferase (MPT), keto synthase (KS), ketoacyl reductase (KS), dehydratase (DH), enoyl reductase (ER), enoyl reductase (ER) and acyl carrier protein (ACP) [33]. In yeasts, FASs catalyze the synthesis of a range of acyl-ACPs (mainly C16 and C18) from acetyl-CoA and malonyl-CoA, and it is suggested that the chain length of

Table 1

| Metabolic engineering strategies for oleochemical production in yeasts |                 |      |   |                                    |           |
|--|-----------------|------|---|------------------------------------|-----------|
| Feedstocks   | Products        | Host | Goals and genetic modifications   | Titer, yield, productivity         | Reference |
| Glucose  | Fatty acids     | Sc   | <b>Constructing ACL route</b> AnACL $\uparrow$ , MmusACL $\uparrow$ , RtME $\uparrow$ , MDH3 $\uparrow$ , CTP1 $\uparrow$<br><b>Enhancing fatty acid synthesis</b> $\Delta$ pox1, $\Delta$ faa1, $\Delta$ faa4, FAS $\uparrow$<br><b>Optimizing central carbon flux</b> PGI1 $\downarrow$ , IDH2 $\downarrow$ | FB 33.4 g/L, 0.1 g/g               | [26**]    |
| Glucose  | Fatty alcohols  | Yl   | <b>Enabling oleochemical synthesis</b> MmarCAR $\uparrow$ , BsSfp $\uparrow$ , EcAHR $\uparrow$ , EcFadD $\downarrow$   | SF, 205.4 mg/L<br>B, 2.15 g/L      | [30*]     |
| Glucose  | Fatty alcohols  | Sc   | <b>Increasing fatty acid flux:</b> AnACL $\uparrow$ , MmusACL $\uparrow$ , RtME $\uparrow$ , MDH3 $\uparrow$ , CTP1 $\uparrow$ , $\Delta$ pox1, FAS $\uparrow$<br><b>Enabling oleochemical synthesis</b> $\Delta$ hfd1, $\Delta$ tpo1, MmarCAR $\uparrow$   | SF, 252 mg/L                       | [19]      |
| Glucose  | Alkanes/alkenes | Sc   | <b>Targeting peroxisomes for oleochemical production</b> Per-MmarCAR $\uparrow$ , Per-SeADO $\uparrow$ , Per-SeFd/FNR $\uparrow$  | SF: 3.55 mg/L                      | [59]      |
| Glucose  | Alkanes/alkenes | Yl   | <b>Targeting ER for oleochemical production</b> ER-AbFAR $\uparrow$ , ER-PmADO $\downarrow$   | SF: 23.3 mg/L                      | [30*]     |
| Glucose  | Lipids          | Yl   | <b>Increasing fatty acid flux</b> , ACC $\uparrow$ , DGA $\uparrow$<br><b>Increasing NADPH supply</b> , CaGapC $\uparrow$ , McMCE2 $\uparrow$   | FB, 98.9 g/L, 0.269 g/g, 1.3 g/L/h | [31**]    |
| Xylose   | Fatty alcohols  | Sc   | <b>Increasing fatty acid flux</b> ACC $\uparrow$ , FAS $\uparrow$ , $\Delta$ trpd3<br><b>Enabling oleochemical synthesis</b> TaFAR $\uparrow$<br><b>Enhancing xylose utilization:</b> CsXR $\uparrow$ , CtXDH $\uparrow$ , PpXK $\uparrow$  | B, 1.2 g/L, 0.1 g/g, 0.02 g/L/h    | [40]      |
| Xylose   | Lipids          | Yl   | <b>Increasing fatty acid flux</b> $\Delta$ pox1-6, $\Delta$ tg14, GDP1 $\uparrow$ , DGA2 $\uparrow$ ,<br><b>Enhancing xylose utilization</b> SsXR $\uparrow$ , SsXDH $\uparrow$ , YlXK $\downarrow$   | FB, 20.1 g/L, 0.08 g/g, 0.19 g/L/h | [44]      |
| Xylose   | Lipids          | Yl   | <b>Enhancing xylose utilization</b> SsXR $\uparrow$ , SsXDH $\uparrow$ , SsXK $\downarrow$<br><b>Starvation-enabled adaptive evolution</b>  | B, 15.0 g/L, 0.08 g/g, 0.19 g/L/h  | [43]      |
| Agave bagasse hydrolysate  | Lipids          | Yl   | <b>Increasing fatty acid flux</b> $\Delta$ pox1-6, $\Delta$ tg14, GDP1 $\uparrow$ , DGA2 $\uparrow$ ,<br><b>Enhancing xylose utilization</b> YlXR, YlXDH, YlXK, AnXPKA, AnACK   | FB, 16.5 g/L, 0.34 g/g, 0.19 g/L/h | [45]      |

The listed examples report the highest titers for corresponding products within the reviewed publications. Symbols and prefixes:  $\uparrow$ , overexpression;  $\downarrow$ , downregulation;  $\uparrow$ , truncated version; \*, mutated version. General abbreviations: SF, shake-flask; FB, fed-batch bioreactor; B, batch bioreactor. Species abbreviations: Ab, *Acinetobacter baylyi*; An, *Aspergillus nidulans*; Bs, *Bacillus subtilis*; Ca, *Clostridium acetobutylicum*; Cs, *Candida shehatae*; Ct, *Candida tropicalis*; Ec, *E. coli*; Mmar, *Mycobacterium marinum*; Mmus, *Mus musculus*; Mc, *Mucor circinelloides*; Pm, *Prochlorococcus marinus*; Pp, *Pichia pastoris*; Rt, *Rhodospiridium toruloides*; Sc, *Saccharomyces cerevisiae*; Se, *Synechococcus elongatus*; Ss, *Scheffersomyces stipites*; Ta, *Tyto alba*; Yl, *Yarrowia lipolytica*. Gene/Enzyme abbreviations: ACC, acetyl-CoA carboxylase; ACK, acetate kinase; ADO, fatty-aldehyde deformylating oxygenase; AHR, aldehyde reductase; CAR, carboxylic acid reductase; DGA, diacylglycerol acyltransferase; FAR, fatty acyl-CoA-ACP or fatty acyl-ACP reductase; FAA, fatty acyl-CoA synthetase; FAS: fatty-acid synthase; Fd/FNR, ferredoxin and ferredoxin/NADP + reductase; GPD, glycerol-3-phosphate dehydrogenase; ME, malic enzyme; POX, fatty-acyl coenzyme A oxidase; Sfp, phosphopantetheinyl transferase; XR, xylose reductase; XDH, NADH-dependent xylitol dehydrogenase; XK, xylulokinase; XPKA, phosphoketolase.

oleochemicals are mainly controlled via FASs. Based on biochemical mechanism and structural analysis of FAS complexes, rational protein engineering on FASs from *Y. lipolytica*, *S. cerevisiae*, *R. toruloides*, *Mycobacterium vaccae* and *Aplanochytrium kerguelense* enabled production of short/medium chain fatty acids (S/MCFAs, C6-C12) in *S. cerevisiae* [18\*\*,34,35], with several mutations introduced into KS, AT and MPT or heterologous thioesterase (TE) domain introduced to FAS. Engineered fungal and bacterial FAS with mutated KS and TE domains were co-expressed in a strain with enhanced MCFA tolerance and optimized carbon flux redirection, resulting in production of  $2.87 \pm 0.06 \text{ g L}^{-1}$  MCFAs in fed-batch cultivations [18\*\*].

Moreover, heterologous FASs from *R. toruloides* and *M. vaccae* have been overexpressed in *S. cerevisiae* to produce very long chain fatty acids and fatty alcohols [36], or enhance fatty acid synthesis [26\*\*].

### Lipid metabolism

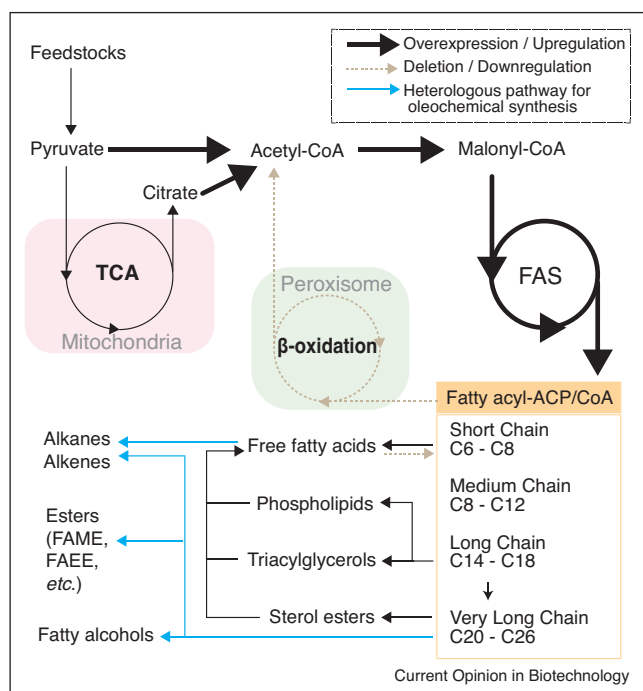
Fatty acyl-ACPs synthesized from FAS are esterified to form three major lipid classes, including triacylglycerols

(TAGs), sterol esters (SEs) and phospholipids (PLs), or released as free fatty acids (FFAs), or degraded to acetyl-CoA via  $\beta$ -oxidation, as shown in Figure 2. Lipid metabolism is tightly regulated in yeast via several regulatory nodes [5,37\*]. Strategies with blocking  $\beta$ -oxidation and down-regulating fatty acid activation and storage lipid formation in *S. cerevisiae* resulted in accumulation of FFAs and PLs [37\*], which ensured high flux into synthesis of downstream oleochemicals, including fatty alcohols, alkanes, alkenes and oleoylethanolamide (OEA) [14,21,26\*\*]. In *Y. lipolytica*, blocking  $\beta$ -oxidation was also performed to enhance lipid synthesis, and in combination with expressing heterologous enzymes for fatty acid synthesis, fatty acyl-ACPs were redirected to synthesis FFAs instead of TAGs, as successfully demonstrated in production of omega-3-icosapentanoic acid (EPA) [25]. The interconversion between FFAs and different lipids pools offers varied opportunities for synthesis of various oleochemicals.

### Oleochemical tolerance and cell fitness

High tolerance towards oleochemicals is required for efficient producing strains. Transporter engineering and

Figure 2



Yeast based biorefineries for oleochemical production utilizing 1st generation feedstock glucose. Carbon flux is redirected to fatty acid synthesis by engineering central carbon metabolism and lipid metabolism with upregulated (as shown in bold arrows) and downregulated (as shown in dashed grey arrows) reactions, and a variety of oleochemicals are synthesized by expression of heterologous pathways (as shown in blue arrows).

adaptive evolution are usually adopted for improving strain tolerance towards toxic biochemicals. For example, expression of a fatty acid transporter FATP1 from human in *S. cerevisiae* mediated fatty acid uptake and facilitated fatty alcohol export, leading to enhanced fatty alcohol production and improved cell growth [38]. Moreover, protein engineering on a membrane transporter Tpo1 involved in tolerance against C10 fatty acid enhanced MCFA production by 1.3 fold, while adaptive evolution enhanced MCFA production by 1.7 fold [18\*\*]. Meanwhile, adaptive evolution was adopted in balancing oleochemical production and cell fitness, which successfully enhanced production of free fatty acids [26\*\*].

### Alternative feedstocks for oleochemical production

Compared with 1st-generation biorefineries, efficient feedstock uptake and utilization is usually a major issue for 2nd-generation biorefineries, in particular as most yeasts do not naturally use pentoses and other components from biomass. There has therefore been much work on engineering yeasts for utilization of pentoses and more recently C1 feedstocks like methanol and carbon dioxide.

### Xylose utilization

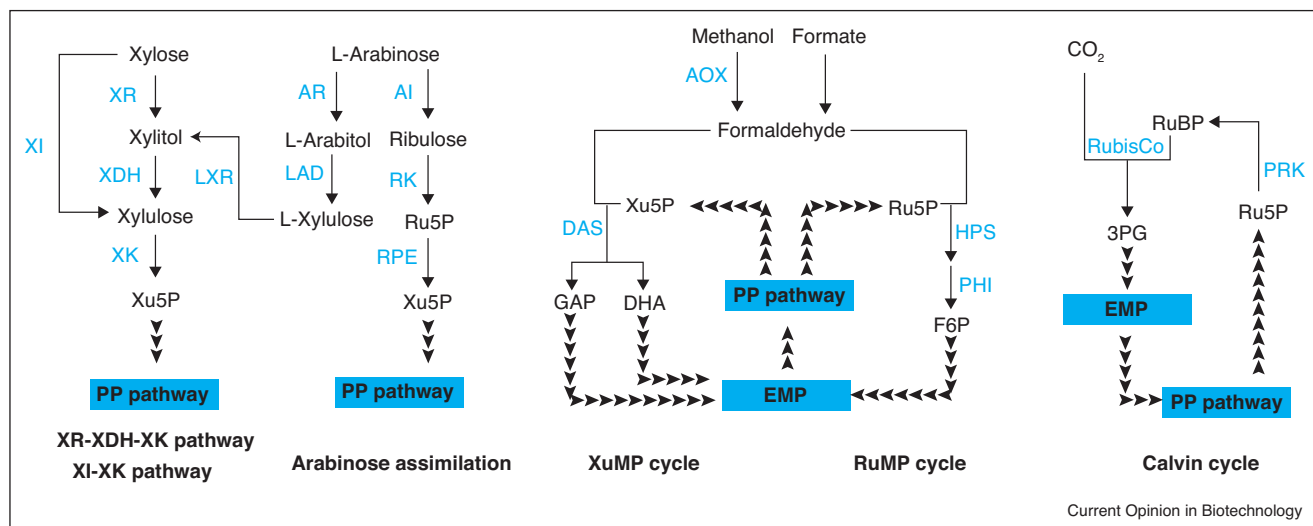
Xylose can be converted to xylulose-5-phosphate (Xu5P) via two different assimilation pathways. One is composed of NAD(P)H-dependent xylose reductase (XR), NADH-dependent xylitol dehydrogenase (XDH) and xylulokinase (XK), and the other is relying on xylose isomerase (XI) and XK. Xu5P is then channelled via the PP pathway or phosphoketolase (PK) pathway into central carbon metabolism for cell growth and biochemical synthesis (Figure 3).

Recently, engineering efforts have been performed in non-xylose metabolizing yeasts to enable oleochemical production [1,39]. For example, in a fatty alcohol producing strain of *S. cerevisiae*, expression of XR from *Candida shehatae*, XDH from *Candida tropicalis* and XK from *Pichia pastoris* resulted in higher yields on xylose compared to glucose during batch and fed-batch cultivations [40].

In *Y. lipolytica*, the dormant pentose metabolism resulted in poor and unstable xylose utilization [41,42]. Expression of XR, XDH and XK from *Scheffersomyces stipitis* together with starvation adaptation enabled efficient xylose utilization and lipid production of 15 g/L [43]. In another study, expression of XR, XDH from *S. stipitis* and native XK in an engineered lipid producing strain resulted in lipid production of 20.1 g/L [44]. By overexpressing heterologous and native xylose utilizing enzymes, *Y. lipolytica* was reported to produce lipids with a high productivity (0.185 g/L/h) and a high yield (0.344 g lipids/g sugars) in xylose-rich agave bagasse hydrolysate [45]. A recent study demonstrated that *Y. lipolytica* could combine the xylose utilization phenotype with the metabolite overproduction phenotype via a mating approach, and allowed 1.42 g/L  $\alpha$ -linolenic acid production on xylose [46].

Oleaginous yeasts that can naturally grow on xylose appear to be promising candidate hosts for 2nd-generation biorefineries of oleochemical production, including *R. toruloides* [47] and *C. curvatus* [48]. However, limited knowledge of xylose metabolism and engineering tools have restricted their application in oleochemical production. A recent study on comparative analysis of *R. toruloides* revealed that cells grown on xylose achieved almost 50% lower growth rate and sugar consumption rate, lower final biomass yield whereas similar final cellular lipid content [49\*]. Proteome analysis then identified a number of putative sugar transporters for xylose and glucose and suggested that xylose import might be the limiting step during xylose conversion into lipids. NADPH regeneration relied primarily on the PP pathway, and may also involve malic enzyme, alcohol dehydrogenases and aldehyde dehydrogenases. The PK pathway with higher efficiency and carbon conservation, however, seemed to have limited role in xylose conversion into lipids, possibly due to the inefficient xylose

Figure 3



Utilization pathways of pentoses and C1 compounds. Abbreviations used: XR, xylose reductase; XDH, xylitol dehydrogenase; XK, Xylulokinase; XI, xylose isomerase; AR, Arabinose reductase; LAD, L-arabinitol 4-dehydrogenase; LXR, L-xylulose reductase; AI, L-arabinose isomerase; RK, L-ribulokinase; RPE, L-ribulose-5-P-4-epimerase; DAS, dihydroxyacetone synthase; AOX, alcohol oxidase; HPS, 3-hexulose-6-phosphate synthase; PHI, 6-phospho-3-hexuloisomerase; Xu5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate; GAP, glyceraldehyde-3-phosphate; DHA, dihydroxyacetone.

uptake. These findings are valuable for developing lipid production processes on xylose-containing substrates and further optimization of xylose utilization.

Other alternative routes for xylose catabolism, such as the XI pathway, Dahms pathway, Weimberg pathway, and synthetic pathways, may also be useful for future optimization in oleochemical production [21,50]. Besides, engineering strategies to facilitate the conversion of xylose to ethanol in *S. cerevisiae*, like improving xylose uptake, balancing redox factors, as well as evolutionary engineering and transcriptional factor engineering, could also contribute to engineer yeast for oleochemical production from xylose [51,52].

### L-arabinose utilization

L-arabinose is another abundant pentose in lignocellulose feedstock, and it can be assimilated into the central carbon metabolism via two different routes in prokaryotes and eukaryotes (Figure 3). The prokaryotic route involves arabinose isomerase (AI), L-ribulokinase (RK) and L-ribulose-5-P-4-epimerase (RPE), and the eukaryotic involves arabinose reductase (AR), L-arabinitol dehydrogenase (LAD), L-xylulose reductase (LXR), XDH and XK [53].

Expression of heterologous utilization pathways with subsequent evolution engineering in *S. cerevisiae* enabled efficient assimilation of L-arabinose, as well as efficient co-fermentation of xylose and L-arabinose [54]. A recent study found that the underlying mechanism of fast co-

fermenting capacity of L-arabinose and xylose was a high number of copies of the L-arabinose utilization pathways, which will benefit oleochemical production [55]. In *Y. lipolytica*, the dormant L-arabinose assimilation pathway was identified through transcriptomic and metabolic analyses, and it can be activated by overexpression of pentose transporters, XDH and LAD, shedding light on oleochemical production on 2nd generation feedstock [22\*].

### Methanol utilization

CO<sub>2</sub> and methanol are gaining increasing interests as 3rd-generation feedstocks for bioproduction, because of their abundance in nature and cheap price, as well as the urgent need to reduce the threat of the global warming and human reliance on fossil fuels [20,56,57]. However, biochemical production with these C1 compounds is still challenging due to low efficiencies of utilization pathways and high demands of energy and reducing power. Currently, C1 compound utilization pathways are still under evaluation with and without utilization pathways of other feedstocks.

*Pichia pastoris* can grow on methanol as the sole carbon and energy source. During methanol cultivation, peroxisomes amplify and dominate the cell volume of *P. pastoris* [58], which make it a promising methanol utilizing host for oleochemical production, as enhanced alkane titres were achieved when the biosynthetic pathway was targeted to the peroxisomes of *S. cerevisiae* [59]. In a recent study, *P. pastoris* can also be converted into an autotroph strain with CO<sub>2</sub> as the carbon source and methanol as the

energy source, by engineering the methanol assimilation pathway, xylose monophosphate (XuMP) cycle (Figure 3) or called as dihydroxyacetone (DHA) cycle, to a CO<sub>2</sub> fixation pathway [60\*\*]. With the efficient CRISPR-Cas9 mediated genome editing toolkit developed in *Hansenula polymorpha* [61], this thermotolerant methylotrophic yeast could also be a promising chassis for oleochemical production utilizing methanol.

To directly utilize methanol in *S. cerevisiae*, the XuMP cycle from *P. pastoris* was reconstructed in *S. cerevisiae*, and the resulting strain could consume 1.04 g/L methanol, with slow growth and 0.26 g/L pyruvate produced [62]. Meanwhile, expression of the ribulose monophosphate (RuMP) cycle, another methanol assimilation pathway identified in methylotrophic prokaryotes (Figure 3), failed to result in methanol utilization for cell growth in both *S. cerevisiae* and *Y. lipolytica* [62,63]. Clearly, the poor cell growth and slow methanol utilization imposes requirements for more engineering work. This may require improved subcellular expression to reduce the toxicity of the intermediate formaldehyde or establish efficient regeneration of xylose-5-phosphate.

### CO<sub>2</sub> utilization

The enzymes ribulose-1,5-bisphosphate carboxylase (RuBisCO) and phosphoribulokinase (PRK) from the Calvin cycle have been expressed in *S. cerevisiae*, and increased the ethanol yields on both glucose and xylose. Besides, the decreased accumulation of by-products glycerol and xylitol suggested that CO<sub>2</sub> could be used as an external electron acceptor to balance cytosolic redox factors [64,65].

The synthetic reductive glycine pathway has also been demonstrated functional in *S. cerevisiae* by overexpressing endogenous enzymes to synthesize glycine from formate and CO<sub>2</sub>. The pathway with high activity, high affinity and tolerance of formate suggested *S. cerevisiae* might be especially suitable for formate utilization [66]. Recent studies found that CO<sub>2</sub> can be efficiently converted to formate with electrochemical and photochemical methods [67], and these findings may enable yeast based 3rd biorefineries for oleochemical production.

### Conclusion and perspectives

Much progress has been achieved in both oleaginous yeasts and non-oleaginous yeasts utilizing glucose, including production improvements and oleochemical portfolio expansions. However, current titres, rates and yields of most bulk oleochemicals cannot meet the requirements of commercial production. A promising strain for commercial production is the engineered *Y. lipolytica*, which can produce FAMES with a high titre, yield and rate of 98.9 g/L, 1.3 g/L/h and 0.27 g/g glucose, respectively [31\*\*]. With omega-3 production in *Y. lipolytica* commercialized by DuPont [68], biosynthesis of

high-value oleochemicals have attracted more attention, and more enzymes and pathways for novel oleochemicals have been evaluated, including polyunsaturated fatty acids [10,69], flavour lactones [16], jojoba-like wax esters [15], and oleoylethanolamide [14].

Efficient xylose utilization would be a step further for commercial production of oleochemicals. An engineered *Y. lipolytica* has demonstrated its potential capacity for using xylose-rich agave bagasse hydrolysates as feedstock resulting in a high lipid yield of 0.344 g/g sugars, titre of 16.5 g/L and rate of 1.85 g/L/h [45]. Non-conventional yeasts, like *R. toruloides* and *C. curvatus*, could also be promising chassis for 2nd-generation oleochemical production. For example, *C. curvatus* could accumulate lipids up to 69.5% of dry cell weight when growing on aromatic substrates, representing one of the promising yeast cell factories for oleochemical production from depolymerized lignin [70]. Although limited genetic engineering tools restrict their usages, multi-omics analysis uncovers their metabolic capabilities of lipid synthesis and possible regulation mechanisms on different feedstocks, which can guide future strain performance on oleochemical production [71,72].

So far it has been demonstrated that *S. cerevisiae* can expand the range of oleochemicals it can produce, and it therefore represents a ready-to-use chassis for development of novel oleochemical synthesis. *Y. lipolytica* also seems to be an attractive chassis for both 1st generation and 2nd generation refineries for oleochemical production due to its higher flux from cytosolic acetyl-CoA towards fatty acid synthesis. The capacity may be further enhanced by introducing the Calvin cycle to utilize CO<sub>2</sub> as an electron acceptor, as previously used to enhance ethanol production and yield with high carbon-conversion and energy-conversion in *S. cerevisiae* [64,65]. Moreover, the PP pathway is highly involved with both the utilization pathways of xylose and C1 compounds, and the previous study found that overexpression of non-oxidative PP enzymes to ensure sufficient pool of ribulose-5-phosphate was required for implementation of the Calvin cycle [65]. Therefore, engineering of the PP pathway to balance the carbon and energy flux is desired for oleochemical production on 2nd-generation and 3rd generation feedstocks.

### Conflict of interest statement

Nothing declared.

### Acknowledgements

This work was supported by National Natural Science Foundation of China [grant no. 21908004, 2019; 21808008, 2018]; the Fundamental Research Funds for the Central Universities [grant no. buctrc201801, 2018], the Novo Nordisk Foundation [NNF10CC1016517], and the Knut and Alice Wallenberg Foundation.

## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Yaguchi A, Spagnuolo M, Blenner M: **Engineering yeast for utilization of alternative feedstocks.** *Curr Opin Biotechnol* 2018, **53**:122-129.
  2. Yan Q, Pflieger BF: **Revisiting metabolic engineering strategies for microbial synthesis of oleochemicals.** *Metab Eng* 2020, **58**:35-46.
  3. Alishah Aratboni H, Rafiei N, Garcia-Granados R, Alemzadeh A, Morones-Ramirez JR: **Biomass and lipid induction strategies in microalgae for biofuel production and other applications.** *Microb Cell Fact* 2019, **18**:178.
  4. Sun T, Li S, Song X, Diao J, Chen L, Zhang W: **Toolboxes for cyanobacteria: recent advances and future direction.** *Biotechnol Adv* 2018, **36**:1293-1307.
  5. Lazar Z, Liu N, Stephanopoulos G: **Holistic approaches in lipid production by *Yarrowia lipolytica*.** *Trends Biotechnol* 2018, **36**:1157-1170.
  6. Kamineni A, Shaw J: **Engineering triacylglycerol production from sugars in oleaginous yeasts.** *Curr Opin Biotechnol* 2020, **62**:239-247.
  7. Spagnuolo M, Yaguchi A, Blenner M: **Oleaginous yeast for biofuel and oleochemical production.** *Curr Opin Biotechnol* 2019, **57**:73-81.
  8. Sreeharsha RV, Mohan SV: **Obscure yet promising oleaginous yeasts for fuel and chemical production.** *Trends Biotechnol* 2020, **38**:873-887.
  9. Park YK, Nicaud JM, Ledesma-Amaro R: **The engineering potential of *Rhodospiridium toruloides* as a workhorse for biotechnological applications.** *Trends Biotechnol* 2018, **36**:304-317.
  10. Gemperlein K, Dietrich D, Kohlstedt M, Zipf G, Bernauer HS, Wittmann C, Wenzel SC, Muller R: **Polyunsaturated fatty acid production by *Yarrowia lipolytica* employing designed myxobacterial PUFA synthases.** *Nat Commun* 2019, **10**:4055.
  11. Bergenholm D, Gossing M, Wei Y, Siewers V, Nielsen J: **Modulation of saturation and chain length of fatty acids in *Saccharomyces cerevisiae* for production of cocoa butter-like lipids.** *Biotechnol Bioeng* 2018, **115**:932-942.
  12. Zhou YJ, Hu Y, Zhu Z, Siewers V, Nielsen J: **Engineering 1-alkene biosynthesis and secretion by dynamic regulation in yeast.** *ACS Synth Biol* 2018, **7**:584-590.
  13. Zhang Y, Nielsen J, Liu Z: **Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived hydrocarbons.** *Biotechnol Bioeng* 2018, **115**:2139-2147.
  14. Liu Y, Liu Q, Krivoruchko A, Khoomrung S, Nielsen J: **Engineering yeast phospholipid metabolism for de novo oleoylethanolamide production.** *Nat Chem Biol* 2020, **16**:197-205.
  15. Wenning L, Ejsing CS, David F, Sprenger RR, Nielsen J, Siewers V: **Increasing jojoba-like wax ester production in *Saccharomyces cerevisiae* by enhancing very long-chain, monounsaturated fatty acid synthesis.** *Microb Cell Fact* 2019, **18**:49.
  16. Marella ER, Dahlin J, Dam MI, Ter Horst J, Christensen HB, Sudarsan S, Wang G, Holkenbrink C, Borodina I: **A single-host fermentation process for the production of flavor lactones from non-hydroxylated fatty acids.** *Metab Eng* 2020, **61**:427-436.
  17. Liu K, Li S: **Biosynthesis of fatty acid-derived hydrocarbons: perspectives on enzymology and enzyme engineering.** *Curr Opin Biotechnol* 2020, **62**:7-14.
  18. Zhu Z, Hu Y, Teixeira PG, Pereira R, Chen Y, Siewers V, Nielsen J: **Multidimensional engineering of *Saccharomyces cerevisiae* for efficient synthesis of medium-chain fatty acids.** *Nat Catal* 2020, **3**:64-74.
- With rational engineering of FAS and directed evolution against product toxicity, production of MCFAs was improved to 2.87 g/L, and this represents the highest titer reported in eukaryotic microbes.
19. Hu Y, Zhu Z, Gradischnig D, Winkler M, Nielsen J, Siewers V: **Engineering carboxylic acid reductase for selective synthesis of medium-chain fatty alcohols in yeast.** *Proc Natl Acad Sci U S A* 2020, **117**:22974-22983.
  20. Liu Z, Wang K, Chen Y, Tan T, Nielsen J: **Third-generation biorefineries as the means to produce fuels and chemicals from CO<sub>2</sub>.** *Nat Catal* 2020, **3**:274-288.
  21. Zhao Z, Xian M, Liu M, Zhao G: **Biochemical routes for uptake and conversion of xylose by microorganisms.** *Biotechnol Biofuels* 2020, **13**:21.
  22. Ryu S, Trinh CT: **Understanding functional roles of native pentose-specific transporters for activating dormant pentose metabolism in *Yarrowia lipolytica*.** *Appl Environ Microbiol* 2018, **84**.
- The authors revealed a fundamental understanding of the dormant pentose metabolism in *Yarrowia lipolytica*, which guides future metabolic engineering for oleochemical production on 2nd generation feedstocks.
23. Cotton CAR, Claassens NJ, Benito-Vaquerizo S, Bar-Even A: **Renewable methanol and formate as microbial feedstocks.** *Curr Opin Biotechnol* 2020, **62**:168-180.
  24. Hu Y, Zhu Z, Nielsen J, Siewers V: **Engineering *Saccharomyces cerevisiae* cells for production of fatty acid-derived biofuels and chemicals.** *Open Biol* 2019, **9**:190049.
  25. Abdel-Mawgoud AM, Markham KA, Palmer CM, Liu N, Stephanopoulos G, Alper HS: **Metabolic engineering in the host *Yarrowia lipolytica*.** *Metab Eng* 2018, **50**:192-208.
  26. Yu T, Zhou YJ, Huang M, Liu Q, Pereira R, David F, Nielsen J: **Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis.** *Cell* 2018, **174**:1549-1558 e14.
- The authors first demonstrated *Saccharomyces cerevisiae* engineering from alcoholic fermentation to lipogenesis, and reported high-level production of free fatty acids of 33.4 g/L.
27. Donzella S, Cucchetti D, Capusoni C, Rizzi A, Galafassi S, Chiara G, Compagno C: **Engineering cytoplasmic acetyl-CoA synthesis decouples lipid production from nitrogen starvation in the oleaginous yeast *Rhodospiridium azoricum*.** *Microb Cell Fact* 2019, **18**:199.
  28. Yang X, Sun W, Shen H, Zhang S, Jiao X, Zhao ZK: **Expression of phosphotransacetylase in *Rhodospiridium toruloides* leading to improved cell growth and lipid production.** *RSC Adv* 2018, **8**:24673-24678.
  29. Marella ER, Holkenbrink C, Siewers V, Borodina I: **Engineering microbial fatty acid metabolism for biofuels and biochemicals.** *Curr Opin Biotechnol* 2018, **50**:39-46.
  30. Xu P, Qiao KJ, Ahn WS, Stephanopoulos G: **Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals.** *Proc Natl Acad Sci U S A* 2016, **113**:10848-10853.
- Yarrowia lipolytica* was engineered for production of lipids, FAEs, alkanes, fatty alcohols, and fatty acids, which demonstrated its great potentials in oleochemical production.
31. Qiao K, Wasylenko TM, Zhou K, Xu P, Stephanopoulos G: **Lipid production in *Yarrowia lipolytica* is maximized by engineering cytosolic redox metabolism.** *Nat Biotechnol* 2017, **35**:173-177.
- The paper achieved a lipid titer of 98.9 g/L and a nearly theoretical yield through optimization of cytosolic acetyl-CoA supply and NADPH supply.
32. Xu P, Qiao K, Stephanopoulos G: **Engineering oxidative stress defense pathways to build a robust lipid production platform in *Yarrowia lipolytica*.** *Biotechnol Bioeng* 2017, **114**:1521-1530.
  33. Singh K, Graf B, Linden A, Sautner V, Urlaub H, Tittmann K, Stark H, Chari A: **Discovery of a regulatory subunit of the yeast fatty acid synthase.** *Cell* 2020, **180**:1130-1143 e20.



34. Zhu Z, Zhou YJ, Krivoruchko A, Grninger M, Zhao ZK, Nielsen J: **Expanding the product portfolio of fungal type I fatty acid synthases**. *Nat Chem Biol* 2017, **13**:360-362.
35. Gajewski J, Pavlovic R, Fischer M, Boles E, Grninger M: **Engineering fungal de novo fatty acid synthesis for short chain fatty acid production**. *Nat Commun* 2017, **8**:14650.
36. Yu T, Zhou YJ, Wenning L, Liu Q, Krivoruchko A, Siewers V, Nielsen J, David F: **Metabolic engineering of *Saccharomyces cerevisiae* for production of very long chain fatty acid-derived chemicals**. *Nat Commun* 2017, **8**:15587.
37. Ferreira R, Teixeira PG, Siewers V, Nielsen J: **Redirection of lipid flux toward phospholipids in yeast increases fatty acid turnover and secretion**. *Proc Natl Acad Sci U S A* 2018, **115**:1262-1267
- The authors simplified lipid metabolism of *Saccharomyces cerevisiae* with reduced feedback regulation and redirected carbon flux to free fatty acid and phospholipids, providing insight into yeast based biorefinery for oleochemical production.
38. Hu Y, Zhu Z, Nielsen J, Siewers V: **Heterologous transporter expression for improved fatty alcohol secretion in yeast**. *Metab Eng* 2018, **45**:51-58.
39. Kwak S, Jo JH, Yun EJ, Jin Y-S, Seo J-H: **Production of biofuels and chemicals from xylose using native and engineered yeast strains**. *Biotechnol Adv* 2019, **37**:271-283.
40. Guo W, Sheng J, Zhao H, Feng X: **Metabolic engineering of *Saccharomyces cerevisiae* to produce 1-hexadecanol from xylose**. *Microb Cell Fact* 2016, **15**:24.
41. Ryu S, Hipp J, Trinh CT: **Activating and elucidating metabolism of complex sugars in *Yarrowia lipolytica***. *Appl Environ Microbiol* 2016, **82**:1334-1345.
42. Rodriguez GM, Hussain MS, Gambill L, Gao D, Yaguchi A, Blenner M: **Engineering xylose utilization in *Yarrowia lipolytica* by understanding its cryptic xylose pathway**. *Biotechnol Biofuels* 2016, **9**:149.
43. Li H, Alper HS: **Enabling xylose utilization in *Yarrowia lipolytica* for lipid production**. *Biotechnol J* 2016, **11**:1230-1240.
44. Ledesma-Amaro R, Lazar Z, Rakicka M, Guo Z, Fouchard F, Coq AC, Nicaud JM: **Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose**. *Metab Eng* 2016, **38**:115-124.
45. Niehus X, Crutz-Le Coq AM, Sandoval G, Nicaud JM, Ledesma-Amaro R: **Engineering *Yarrowia lipolytica* to enhance lipid production from lignocellulosic materials**. *Biotechnol Biofuels* 2018, **11**:11.
46. Li H, Alper HS: **Producing biochemicals in *Yarrowia lipolytica* from xylose through a strain mating approach**. *Biotechnol J* 2020, **15** 1900304.
47. Lopes HJS, Bonturi N, Kerkhoven EJ, Miranda EA, Lahtvee PJ: **C/N ratio and carbon source-dependent lipid production profiling in *Rhodotorula toruloides***. *Appl Microbiol Biotechnol* 2020, **104**:2639-2649.
48. Koivuranta K, Castillo S, Jouhten P, Ruohonen L, Penttila M, Wiebe MG: **Enhanced triacylglycerol production with genetically modified *Trichosporon oleaginosus***. *Front Microbiol* 2018, **9**:1337.
49. Tiukova IA, Brandenburg J, Blomqvist J, Sampels S, Mikkelsen N, Skaugen M, Arntzen MO, Nielsen J, Sandgren M, Kerkhoven EJ: **Proteome analysis of xylose metabolism in *Rhodotorula toruloides* during lipid production**. *Biotechnol Biofuels* 2019, **12**:137
- The authors performed comparative analysis of *Rhodotorula toruloides* on glucose and xylose, and the proteomic data are valuable for future development of oleochemical production on xylose.
50. Li X, Chen Y, Nielsen J: **Harnessing xylose pathways for biofuels production**. *Curr Opin Biotechnol* 2019, **57**:56-65.
51. Lee M, Rozeboom HJ, Keuning E, de Waal P, Janssen DB: **Structure-based directed evolution improves *S. cerevisiae* growth on xylose by influencing in vivo enzyme performance**. *Biotechnol Biofuels* 2020, **13**:5.
52. de Paula RG, Antonieto ACC, Ribeiro LFC, Srivastava N, O'Donovan A, Mishra PK, Gupta VK, Silva RN: **Engineered microbial host selection for value-added bioproducts from lignocellulose**. *Biotechnol Adv* 2019, **37** 107347.
53. Lane S, Dong J, Jin YS: **Value-added biotransformation of cellulose sugars by engineered *Saccharomyces cerevisiae***. *Bioresour Technol* 2018, **260**:380-394.
54. Gao M, Ploessl D, Shao Z: **Enhancing the co-utilization of biomass-derived mixed sugars by yeasts**. *Front Microbiol* 2018, **9**:3264.
55. Wang X, Yang J, Yang S, Jiang Y: **Unraveling the genetic basis of fast l-arabinose consumption on top of recombinant xylose-fermenting *Saccharomyces cerevisiae***. *Biotechnol Bioeng* 2019, **116**:283-293.
56. Zhang W, Song M, Yang Q, Dai Z, Zhang S, Xin F, Dong W, Ma J, Jiang M: **Current advance in bioconversion of methanol to chemicals**. *Biotechnol Biofuels* 2018, **11**:260.
57. Cotton CA, Edlich-Muth C, Bar-Even A: **Reinforcing carbon fixation: CO<sub>2</sub> reduction replacing and supporting carboxylation**. *Curr Opin Biotechnol* 2018, **49**:49-56.
58. Pena DA, Gasser B, Zanghellini J, Steiger MG, Mattanovich D: **Metabolic engineering of *Pichia pastoris***. *Metab Eng* 2018, **50**:2-15.
59. Zhou YJ, Buijs NA, Zhu Z, Gomez DO, Boonsombuti A, Siewers V, Nielsen J: **Harnessing yeast peroxisomes for biosynthesis of fatty-acid-derived biofuels and chemicals with relieved side-pathway competition**. *J Am Chem Soc* 2016, **138**:15368-15377.
60. Gassler T, Sauer M, Gasser B, Egermeier M, Troyer C, ●● Causon T, Hann S, Mattanovich D, Steiger MG: **The industrial yeast *Pichia pastoris* is converted from a heterotroph into an autotroph capable of growth on CO<sub>2</sub>**. *Nat Biotechnol* 2020, **38**:210-216
- For the first time *Pichia pastoris* was engineered to grow on CO<sub>2</sub> as the sole carbon source with methanol as the energy source, by replacing methanol assimilation pathway with the Calvin-Benson-Bassham cycle.
61. Wang L et al.: **Efficient CRISPR-Cas9 mediated multiplex genome editing in yeasts**. *Biotechnol Biofuels* 2018, **11**:277.
62. Dai Z, Gu H, Zhang S, Xin F, Zhang W, Dong W, Ma J, Jia H, Jiang M: **Metabolic construction strategies for direct methanol utilization in *Saccharomyces cerevisiae***. *Bioresour Technol* 2017, **245**:1407-1412.
63. Vartiainen E, Blomberg P, Ilmen M, Andberg M, Toivari M, Penttila M: **Evaluation of synthetic formaldehyde and methanol assimilation pathways in *Yarrowia lipolytica***. *Fungal Biol Biotechnol* 2019, **6**:27.
64. Xia PF, Zhang GC, Walker B, Seo SO, Kwak S, Liu JJ, Kim H, Ort DR, Wang SG, Jin YS: **Recycling carbon dioxide during xylose fermentation by engineered *Saccharomyces cerevisiae***. *ACS Synth Biol* 2017, **6**:276-283.
65. Papapetridis I, Goudriaan M, Vazquez Vitali M, de Keijzer NA, van den Broek M, van Maris AJA, Pronk JT: **Optimizing anaerobic growth rate and fermentation kinetics in *Saccharomyces cerevisiae* strains expressing Calvin-cycle enzymes for improved ethanol yield**. *Biotechnol Biofuels* 2018, **11**:17.
66. Gonzalez de la Cruz J, Machens F, Messerschmidt K, Bar-Even A: **Core catalysis of the reductive glycine pathway demonstrated in yeast**. *ACS Synth Biol* 2019, **8**:911-917.
67. Claassens NJ, Cotton CAR, Kopljar D, Bar-Even A: **Making quantitative sense of electromicrobial production**. *Nat Catal* 2019, **2**:437-447.
68. Xie D, Miller E, Sharpe P, Jackson E, Zhu Q: **Omega-3 production by fermentation of *Yarrowia lipolytica*: from fed-batch to continuous**. *Biotechnol Bioeng* 2017, **114**:798-812.
69. Ji X-J, Ledesma-Amaro R: **Microbial lipid biotechnology to produce polyunsaturated fatty acids**. *Trends Biotechnol* 2020, **38**:832-834.

70. Yaguchi A, Robinson A, Mihealsick E, Blenner M: **Metabolism of aromatics by *Trichosporon oleaginosus* while remaining oleaginous.** *Microb Cell Fact* 2017, **16**:206.
71. Kim M, Park BG, Kim EJ, Kim J, Kim BG: **In silico identification of metabolic engineering strategies for improved lipid production in *Yarrowia lipolytica* by genome-scale metabolic modeling.** *Biotechnol Biofuels* 2019, **12**:187.
72. Dahlin J *et al.*: **Multi-omics analysis of fatty alcohol production in engineered yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica*.** *Front Genet* 2019, **10**:747.