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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.
Yeast based biorefineries for oleochemical production
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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 \textit{Yarrowia lipolytica}, \textit{Rhodosporidium toruloides} and \textit{Cryptococcus curvatus}, are often chosen as industrial workhorses for oleochemical production, because of their efficient lipid synthesis and accumulation [5–9]. Non-oleaginous yeasts, such as \textit{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], oleoylthanolamide [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 [17,18,19].

Yeast based biorefineries can rely on using different renewable feedstocks, for example, glucose from food crops (1st-generation feedstock), xylene and l-arabinose from plant biomass and energy crops (2nd-generation feedstock), and possibly C1 compounds like CO$_2$, methanol 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 xylene, 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]. 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
Oleolchemical production in yeast

Generation of fatty acids from acetyl-CoA

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.

Oleolchemical 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**]. 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**].

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*-dependent glyceraldehyde 3-phosphate dehydrogenase or malic enzyme, or engineering oxidative defense pathways [26**,31**,32].

**Fatty acid synthetase**

Yeast fatty acid synthetases (FASs), Type I FASs, are large proteins with multiple modules of acetyl transferase (AT), malonyl-palmityl 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
oloechemicals are mainly controlled via FAs. Based on biochemical mechanism and structural analysis of FAs complexes, rational protein engineering on FAs from Y. lipolytica, S. cerevisiae, R. toruloides, Mycobacterium vaccae and Aplanocystis tertigulense 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 ± 0.06 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-ACPcs 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 β-oxidation, as shown in Figure 2. Lipid metabolism is tightly regulated in yeast via several regulatory nodes [5,37]. Strategies with blocking β-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 β-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-eicosapentaenoic acid (EPA) [25]. The interconversion between FFAs and different lipids pools offers varied opportunities for synthesis of various oleochemicals.

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

| 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 | Constructing ACL route AnACL1, MmuasACL1, RlME1, MDH3, CTP1† | Enhancing fatty acid synthesis \(\Delta pox1, \Delta faa1, \Delta faa4, \text{FAS}\)† | FB 33.4 g/L, 0.1 g/g | [26**] |
| Glucose | Fatty acids | Yl | Enabling oleochemical synthesis MmarCAR1, BstSp1, EcAHRI, EcFadD† | Optimization of central carbon flux PGII1, IDH2 | SF, 205.4 mg/L, 2.15 g/L | [30] |
| Glucose | Fatty acids | Sc | Increasing fatty acid flux: AnACL1, MmuasACL1, RlME1, MDH3, CTP1†, \(\Delta pox1, \Delta faa5^*\)† | Enabling oleochemical synthesis \(\Delta hfd1, \Delta fpo1, \text{MmarCAR}2^*\)† | SF, 252 mg/L | [19] |
| Glucose | Alkanes/alkenes | Sc | Targeting peroxisomes for oleochemical production Per-McarCAR1, Per-SEAD1, Per-SEFd/FNR† | Enhancing fatty acid flux, ACC1, DGA1†, NADPH supply, CaGAP1, McMCE2† | SF, 3.55 mg/L | [59] |
| Glucose | Alkanes/alkenes | Yl | Targeting ER for oleochemical production ER-AB-FAR1, ER-PMADO† | Enhancing fatty acid flux, ACC1, DGA1†, NADPH supply, CaGAP1, McMCE2† | FB, 98.9 g/L, 0.269 g/g, 1.3 g/L/h | [31**] |
| Glucose | Alkanes/alkenes | Yl | XdhA, Dga1†, NADPH supply, CaGAP1, McMCE2† | Enhancing fatty acid flux, ACC1, DGA1†, \(\Delta pox3^*\)† | B, 1.2 g/L, 0.1 g/g, 0.02 g/L/h | [40] |
| Xylose | Fatty acids | Sc | Enabling oleochemical synthesis TaFAR1† | Enhancing xylose utilization: CaXr1, CxDH1, PpXk1 | FB, 20.1 g/L, 0.08 g/L, 0.19 g/L/h | [44] |
| Xylose | Fatty acids | Yl | Increasing fatty acid flux: \(\Delta pox1-6, \Delta tgl4, \text{GDG}1^*\), DGA2† | Enhancing xylose utilization SxXr1, SxSdh1, YikXk† | FB, 15.0 g/L, 0.08 g/L, 0.19 g/L/h | [43] |
| Xylose | Fatty acids | Yl | Increasing fatty acid flux: \(\Delta pox1-6, \Delta tgl4, \text{GDG}1^*\), DGA2† | Enhancing xylose utilization SxXr1, SxSdh1, YikXk† | FB, 15.0 g/L, 0.08 g/L, 0.19 g/L/h | [43] |
| Agave bagasse | Hydrolysat | Lyp | Increasing fatty acid flux: \(\Delta pox1-6, \Delta tgl4, \text{GDG}1^*\), DGA2† | Enhancing xylose utilization YikXr, YikXh, YikX, AnpPKA, AnACK | FB, 15.6 g/L, 0.34 g/L, 0.19 g/L/h | [45] |

The listed examples report the highest titers for corresponding products within the reviewed publications. Symbols and prefixes: †, overexpression; ††, truncated version; †*, mutated version. General abbreviations: SF, shake-flask; FB, fed-batch bioreactor; B, batch bioreactor.

Species abbreviations: Ab, Acinetobacter baylyi; An, Aspergillus nidulans; Bc, Bacillus subtilis; Ca, Clostridium acetobutylicum; Cs, Candida shehatae; Ot, Candida tropicalis; Ec, E. coli; Mmr, Mycobacterium marium; Mmus, Mus musculus; Mc, Mucor circinelloides; Pm, Prochlorococcus marinus; Pp, Pichia pastoris; Rt, Rhodosporidium toruloides; Sc, Saccharomyces cerevisiae; Se, Synochococcus elongatus; Ss, Schefersomyces stipites; Ta, Tyto alba; Yl, Yarrowia lipolytica. Gene/Enzyme abbreviations: ACC, acetyl-CoA carboxylase; ACP, acetate kinase; ADO, fatty-aldehyde deforming oxygenase; AHR, aldehyde reductase; CAR, carboxylase reductase; DGA, diacylglycerol acyltransferase; FAD, 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, xylanokinase; XPKA, phosphoketolase.

| Reference | | | | | |
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 XDH 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 *Sheffersomyces 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 over-production phenotype via a mating approach, and allowed 1.42 g/L α-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
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, Weinberg 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-arabitol dehydrogenase (LDH), 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**

CO₂ 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, bio-chemical 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 ampliticate 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 CO₂ 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 CO₂ 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 direct 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 requirement 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.

CO₂ 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 CO₂ 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 synthesis glycine from formate and CO₂. 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 CO₂ 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 polysaturated fatty acids [10,69], flavour lactones [16], jojoba-like wax esters [15], and oleylethanolamide [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 CO₂ 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 [NNF19OC1016517], and the Knut and Alice Wallenberg Foundation.
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