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Oleaginous yeast for biofuel and oleochemical production

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Current transportation fuels derived from petroleum can also be made from microbial systems. In particular, oleaginous yeast have naturally evolved high flux pathways for fatty acids in the form of neutral lipids, which can be converted into a variety of drop-in fuels. Here, we describe the recent advances in the use of the four most popular oleaginous yeasts for making lipids and other potential fuels – *Yarrowia lipolytica*, *Lipomyces starkeyi*, *Rhodosporidium toruloides*, and *Cutaneotrichosporon oleaginosus*. The paper is divided into three major sections focusing on (1) the important natural complex phenotypes of each yeast; (2) the development of metabolic engineering tools for each yeast; and (3) demonstrations of metabolic engineering in each yeast. At the end of each section, we provide our assessment, of which yeast is most promising in the near and long term for bioenergy production.

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Introduction

Fuels produced from biomass feedstocks have the potential to reduce the net CO_2 generation rate from industrial and transportation combustion processes. Microbial systems are capable of efficiently utilizing biomass feedstocks of varying quality and composition. The most promising microbes for biofuel production are oleaginous yeast, characterized by their significant accumulation of fatty acid in the form of triglycerides, which are useful precursors for conversion to biodiesel, green diesel, and jet fuel [1,2]. The naturally high flux pathways for precursors used in fatty acid biosynthesis can also motivate metabolic engineering of oleaginous yeasts to produce non-native molecules that are potentially useful for biofuels and could have better fuel properties (Figure 1). Other advantages of oleaginous yeast include a broader metabolism of different feedstocks and a wider range of tolerance to operational conditions including pH, inhibitors, and ionic strength. Despite significant efforts to engineer model conventional yeast *Saccharomyces cerevisiae* to produce large quantities of fatty acids that have recently been reported [3], oleaginous yeast continues to outperform engineered non-oleaginous yeast.

This review describes recent advances in the four most promising oleaginous yeasts: *Yarrowia lipolytica*, *Lipomyces starkeyi*, *Rhodosporidium toruloides*, and *Cutaneotrichosporon oleaginosus*. These yeast are compared to one another based on critical factors that influence host selection: (1) the substrates they can natively metabolize, (2) the availability of genetic engineering tools, and (3) the scope of biofuel-relevant products that have been reported. While the nascent nature of these studies precludes any definitive winners, we will provide our thoughts on which host is currently most promising. Of course, the benefits and detractors of any host can and likely will change as more is learned about the native metabolism of these organisms and the availability of genetic engineering tools increases.

Natural metabolic flexibility

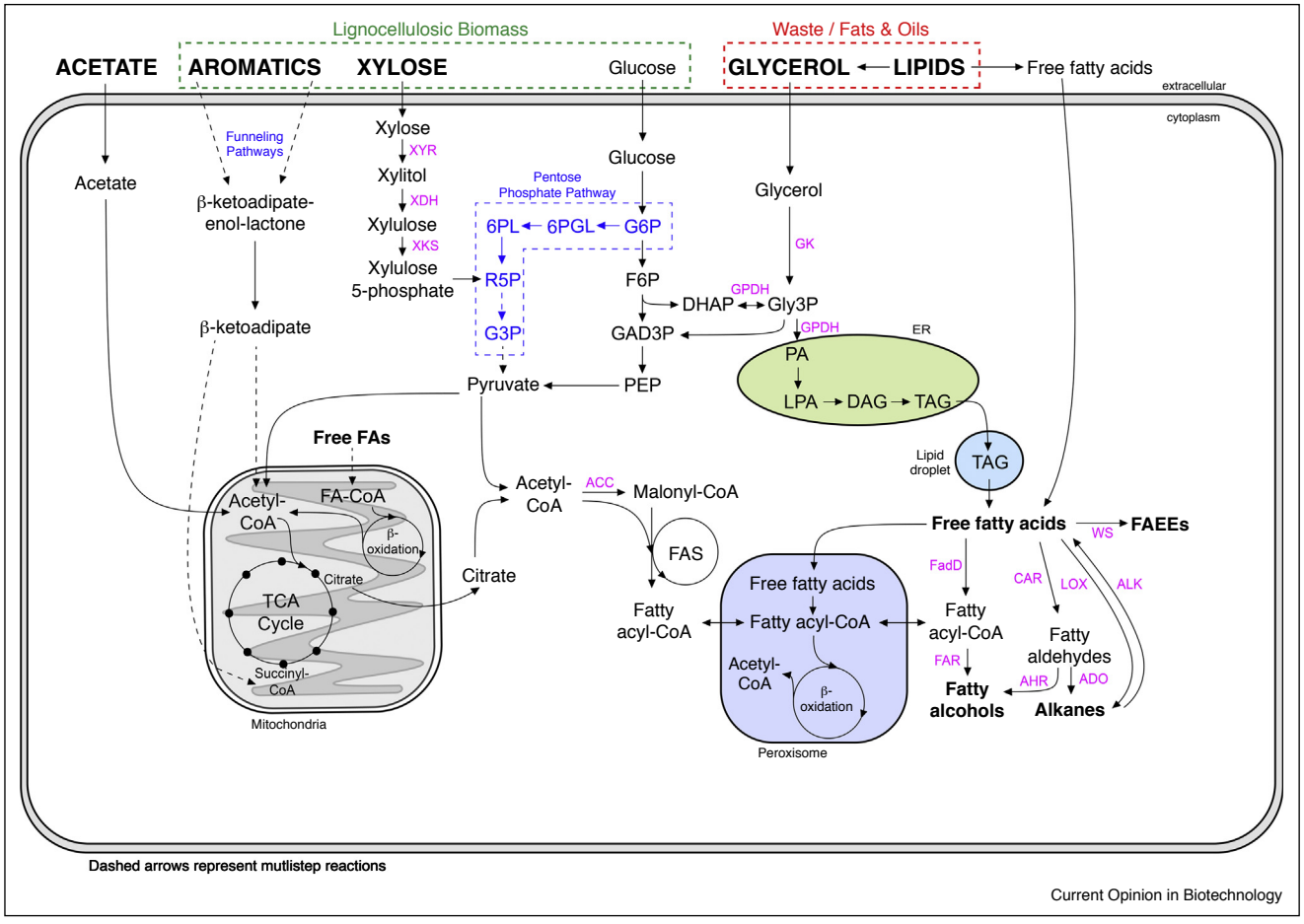
Alternative sugars

The majority of bioprocesses use glucose as a feedstock; however, alternative sugars, such as xylose and arabinose, are economically attractive options over glucose. This encourages interest robust metabolism of a variety of different cellulosic and hemicellulosic hydrolysates. Xylose has received the most attention as it is the major constituent of hemicellulose. The oleaginous yeasts have greater metabolic flexibility than *S. cerevisiae*, with *L. starkeyi* and *R. toruloides* demonstrating more metabolic flexibility than *Y. lipolytica*; however, *C. oleaginosus* clearly has the most diverse sugar metabolism, as is discussed in other review articles [4,5••] (Table 1). In particular, the ability of *C. oleaginosus* to metabolize xylose without catabolite repression and at rates similar to glucose give it an advantage over other oleaginous yeast [5••]. Of the four oleaginous yeast described herein, only *Y. lipolytica* does not readily metabolize xylose as a sole carbon source [6] due it the presence of cryptic xylose metabolism.

Cellulosic and hemicellulosic hydrolysates

Biomass hydrolysates can be an economical way to provide high concentrations of sugars if inhibitory

Figure 1



Pathways for assimilation of various substrates into fatty acid derived biofuels. Glucose, acetate, xylose, glycerol, and aromatics produce acetyl-CoA building blocks for fatty acids. *De novo* fatty acids can be directly shunted from fatty acid biosynthesis or hydrolyzed from triacylglycerol. Introduction of heterologous enzymes can result in fatty alcohols, alkanes, and fatty esters.

byproducts associated with hydrolysis, such as acetic acid, furfural, and 5-hydroxymethylfurural (HMF), can either be avoided or tolerated by microbes. *R. toruloides* and *C. oleaginosus* are capable of metabolizing hydrolysate feedstocks of various biomass sources without need for dilution or detoxification [7], unlike *L. starkeyi* which showed impacted cell growth even with diluted feedstock [8]. When utilizing sorghum stalk and switchgrass hydrolysates, *C. oleaginosus* ATCC 20509 cells metabolized more sugars and accumulated higher lipid and cell titers than *L. starkeyi* ATCC 56304 [9]. The latter strain was also shown to grow on whole bran and corn hydrolysates, though hydrolysis was optimized to prevent inhibitor contamination, rather than sugar release [8]. Curiously, strains of *Y. lipolytica* were able to use xylose in ammonia fiber expansion pretreated cornstover hydrolysates but not as a sole carbon source [10]. Under phosphate-limited conditions in a membrane bioreactor, *C. oleaginosus* DSM 11815 cells accumulated more biomass at the same lipid content and a better lipid productivity while utilizing microalgae hydrolysates as compared to cells grown in the same conditions using a defined medium containing glucose [11]. *R. toruloides* NRRL Y-1091 cells were also able to grow robustly in a two-stage flask culture using enzyme saccharified dilute acid pretreated switchgrass hydrolysate to produce 61% lipids per cell, resulting in 26.2 g/L lipid titer. This strain outcompetes several other strains, including *Y. lipolytica* in terms of biomass, lipid titer, and total substrate utilization, despite the presence of inhibitors [10].

Lignin

Lignin is an underutilized component of lignocellulosic because of the heterogeneity of lignin structure and its depolymerized products. In general, few yeasts are able to grow in the presence of small amounts aromatics due to their toxicity. Even fewer yeast are able to metabolize aromatics. Our recent publication demonstrates

Table 1

Comparison of native metabolic capability and production of lipids for different non-conventional oleaginous yeasts

Strain	Substrate	Sugar/substrate concentration (g/L)	Inhibitor concentration (g/L)	Substrate consumed (g/L)	Lipid titer (g/L)	Lipid accumulation (%)	Reactor type	Ref.
Rhodosporidium toruloides								
DSM-4444	DDAP-EH corn stover hydrolysate	100	n.d.	100	23.3 ± 1.8	60.8 ± 1.1	B	[13]
NRRL Y-1091	Switchgrass hydrolysate	215	5.29[a]	5.75[a]	26.2 ± 1.4	61.5[a]	F	[10]
AS. 2.1389	Acetic acid	20	NA	20	2.1[a]	48.2	F	[57]
CCT 0783	Juice from enzymatically saccharified sweet sorghum	140	NA	140	13.77	33.1	F	[58]
IFO 0880	Ionic liquid pretreated corn stover hydrolysate	73.8	NA	n.d.	n.d.	~27	B	[12••]
Lipomyces starkeyi								
ATCC 56304	Sorghum stalk hydrolysate	50	0	48 ± 0.7	0.16 ± 0.0	44 ± 2.0	F	[9]
ATCC 56304	Switchgrass hydrolysate	50	0	38 ± 0.9	0.17 ± 0.0	39 ± 0.1	F	[9]
CBS 1807	Birch wood hemicellulose hydrolysate	42	0.56	42	8.02	51.3	B	[7]
NBRC10381	Glucose/xylose mixture	100	NA	100	20.3	83.6	F	[59]
NBRC10381	Glucose	50	NA	50	13.6	79.6	F	[59]
NBRC10381	Xylose	50	NA	50	13.9	85.1	F	[59]
AS 2.1560	Crude glycerol supplemented with sodium stearate	70.1[a]	8.4[a]	70.1	12.5	42.9	B	[60]
ATCC 56304	Whole wheat bran hydrolysate	n.d.	0	51.3 ± 1.6	6.37[a]	37.3 ± 1.3	F	[8]
ATCC 56304	Whole corn bran hydrolysate	n.d.	~0.5%	61.7 ± 1.9	7.83[a]	33.3 ± 1.1	F	[8]
ATCC 58680	Para-hydroxybenzoic acid	0.5	NA	n.d.	4.65[a]	51.3 ± 2.51	F	[61]
ATCC 58680	Vanillin	0.5	NA	n.d.	3.49[a]	42.6 ± 1.87	F	[61]
ATCC 58680	Syringaldehyde	1.0	NA	n.d.	2.93[a]	38.6 ± 1.95	F	[61]
ATCC 58680	Furfural	0.4	NA	n.d.	3.40[a]	39.1 ± 1.83	F	[61]
ATCC 58680	HMF	1.0	NA	n.d.	4.08[a]	45.5 ± 1.94	F	[61]
Cutaneotrichosporon oleaginosus								
ATCC 20509	Untreated waste office paper	11.89 ± 0.21	0.29 ± 0.03	n.d.	1.39 ± 0.16	22.0 ± 0.33	F	[62]
ATCC 20509	Treated waste office paper	24.49 ± 0.24	0.14 ± 0.04	n.d.	5.75 ± 0.21	37.8 ± 0.45	F	[62]
ATCC 20509	Crude glycerol + 1.4% (w/v) methanol	62.7	14.0	n.d.	20.78	48.09	B	[14]
ATCC 20509	Sorghum stalk hydrolysate	50	n.d.	45 ± 0.7	13.1 ± 0.7	60 ± 2.5	F	[9]
ATCC 20509	Switchgrass hydrolysate	50	n.d.	46 ± 1.1	12.3 ± 0.2	58 ± 2.6	F	[9]
ATCC 20509	Volatile fatty acids (ratio 15:5:10)	30	NA	27.45	4.93	56.85	F	[63]
DSM 11815	Microalgae hydrolysate	40.6	NA	n.d.	30.6	53	MB	[11]
ATCC 20509	VFAs derived from rice straw hydrolysates	8.12	NA	n.d.	n.d.	n.d.	F	[15]
ATCC 20509	Resorcinol	13	NA	13	1.64 ± 0.2	69.5 ± 4.0	F	[5••]
ATCC 20509	Municipal sludge + crude glycerol + peptone	70	n.d.	n.d.	16.4	40.3	B	[64]
ATCC 20509	DDAP-EH corn stover hydrolysate	100	n.d.	100	21.4 ± 3.6	63.1 ± 3.7	B	[13]
Yarrowia lipolytica								
MTYL037	Acetate	50	n.d.	n.d.	2[a]	25.5[a]	B	[65]
ATCC 8662	Corn oil	20	n.d.	18	5.4	60	B	[66]
A101	Crude glycerol (soap production waste)	150	11.38[a]	150[a]	4.72	20	B	[67]
A101	Fructose	100	n.d.	n.d.	2.19	13	F	[68]

Table 1 (*Continued*)

Strain	Substrate	Sugar/substrate concentration (g/L)	Inhibitor concentration (g/L)	Substrate consumed (g/L)	Lipid titer (g/L)	Lipid accumulation (%)	Reactor type	Ref.
W29	Galactose and glucose (1:1)	20	n.d.	n.d.	1.92	17.2	F	[69]
S0678	Glucose	30	n.d.	n.d.	1.88[a]	13.6	F	[70]
MUCL 28849	Glycerol (40) with acetic, propionic, and butyric acid (0.2)	40.2	n.d.	n.d.	16.5	40.22	B	[71]
ACADC 50109	Industrial waste fat	15	n.d.	n.d.	6.8	54	B	[72]
W29	Oleic acid	20	n.d.	n.d.	2.44[a]	48.9 ± 0.7	F	[73]
MUCL 28849	Pure glycerol	80	n.d.	n.d.	16.11	38.15	B	[71]
Po1g	Sugarcane bagasse hydrolysate	20.02[a]	0.57[a]	19[a]	6.68	58.5	B	[74]

NA = Category not applicable.
n.d. = No data reported.
[a] Denotes values calculated by reported data.

C. oleaginosus ATCC 20509 cells can tolerate high concentrations of 15 different monoaromatic compounds and accumulate up to 69.5% of dry cell weight as lipids when grown on lignin-derived aromatics in a shake flask [5••]. The metabolic flexibility, combined with this yeast's robust tolerance to many inhibitors, may facilitate use of non-sterile fermentation conditions and impure, undefined depolymerized lignin feedstocks for lower bioproduction costs. *R. toruloides* DSM-4444 and IFO 0880 is another yeast that has been shown to metabolize aromatic monomers found in lignin [12••,13]. A direct comparison of the two strains shows only *R. toruloides* DSM-4444 could metabolize the four chosen model lignin monoaromatics as sole carbon sources [13]. Little is currently known about the genetics of aromatic metabolism in these oleaginous yeasts; however, studies such as these are important to inform lignin depolymerization efforts so that one can tailor the organism to the lignin and depolymerization effort or *vice versa*.

Industrial by-products

Crude glycerol is a byproduct to the biodiesel industry requiring valorization to make biodiesel production more economical. The term crude glycerol refers to glycerol contaminated with methanol, which severely inhibits microbial growth. *C. oleaginosus* ATCC 20509 cells achieved highest biomass, lipid production, and lipid content in non-sterilized crude glycerol fermentation medium diluted to contain 1.4% (w/v) methanol [14]. The same strain grown in volatile fatty acids (VFAs) derived from rice straw hydrolysates showed lipid accumulation of 28% of its biomass. Specific growth rate of these cells was 40% higher than those grown in synthetic blends of VFAs. These cells also produced odd-chain fatty acids, which are difficult to find naturally [15]. The authors never state a lipid titer or lipid content for cells grown on VFAs derived from rice straw hydrolysates; however, their observations about fatty acid profiles agree with another report when different ratios of synthetic mixtures of acetic, propionic, and butyric acid are fed to *C. oleaginosus* ATCC 20509 cells [14]. While most microorganisms find the presence of acetic acid, HMF, and furfural to be lethally toxic, *C. oleaginosus* has been shown to both tolerate and metabolize these inhibitors, as was noted in a previous review [16]. Furfural has been noted as the most toxic across many organisms, but *R. toruloides* and *C. oleaginosus* both grow better on hydrolysates compared to analogous synthetic defined media, likely due to metabolism of inhibitors [12••]. Identifying species capable of tolerating hydrolysate inhibitors is important to facilitate the use of non-detoxified biomass hydrolysates as economical feedstocks.

Summary

Totally according to the metabolic flexibility, *C. oleaginosus* and *R. toruloides* are both attractive options for economically favorable biofuel production. In particular, *C. oleaginosus* has been most studied for its growth and lipid accumulation using biomass and lignin hydrolysates, as well as agro-industrial and municipal waste streams. Given the complex phenotypes needed for metabolism of these substrates, *C. oleaginosus* has great promise for biofuel production.

Genetic tractability

Genetic modification

Integration of heterologous gene expression is typically more labor intensive than using episomal plasmids; however, integration is preferred for stable production strains. In the absence of episomal replicating plasmids, integration is the only way to add new genes to oleaginous yeast, which can be accomplished in either a site specific or random manner.

Targeted integration facilitates rational and precise genome engineering that results in comparable

engineered strains. Of the yeasts reported here, *Y. lipolytica* has the most established set of genetic tools for targeted integration [17–19]. Recent developments include a CRISPR-based markerless integration system that was developed to avoid complications associated with random integration [20]. Another CRISPR gene editing tool developed was excision-assisted gene integration, which exploits the recombination mechanism of homology-mediated end joining (HMEJ) to simultaneously integrate a transgene at a large truncation of several thousand base pairs at once [21•]. The EasyCloneYALI system is a markerless gene integration tool taking advantage of CRISPR–Cas9 and non-replicative donor DNA [22]. *L. starkeyi* has a recently established method for homologous recombination based on a *ku70* knockout that inhibits non-homologous end joining required to facilitate robust homologous recombination [23]. In *R. toruloides*, a *ku70* knockout enabled targeted integration and marker recycling through a flippase-mediated system and a robust, arabinose-inducible cre-lox recombination system [24•,25]. More recently, targeted knockouts were achieved in a *ku70* knockout with nourseothricin selection [26••]. Basic transformation procedures for *C. oleaginosus* were only recently reported [27••,28], and thus targeted integration has not yet been reported. Both transformation methods using Agrobacterium-mediated transformation (AMT) or electroporation result in random genomic integrations. Efficiencies were not reported for either; however, protoplasting was not done either study, which could dramatically increase transformation efficiencies and facilitate high throughput screening. Combined with a *ku70* knockout, targeted integration and knockout should be possible.

Genome-scale engineering

While targeted integration is preferred for rational engineering, random integration is still a powerful tool for genome-scale engineering. Two different transposon-based random mutation/integration methods have been developed for *Y. lipolytica* to facilitate better strain engineering [29,30]. Recently, a genome-wide CRISPR–Cas9 knockout library was developed and used for both functional genomics and strain engineering [31•]. An AMT based genome-wide insertional screen in *R. toruloides* IFO 0880 [26••] and ATCC 10657 [32] facilitated identification of 150 genes important for lipid biosynthesis and curation of the genome annotation. Such information is required for rational genome engineering to create robust strains for biofuel production. Genome-scale libraries have not been reported for *L. starkeyi* or *C. oleaginosus*.

Promoters

Constitutive promoters allow for continuous heterologous gene expression and have been developed in all four of the yeasts discussed in this review. A number of native and engineered promoters have been published for *Y. lipolytica*, as is discussed in several reviews [33,34]. Constitutive promoters were identified and characterized for *R. toruloides* using AMT and hygromycin selection [35]. Only three strong constitutive promoters were characterized for *C. oleaginosus* [28] and only two such promoters were identified for *L. starkeyi* [36]; however, the availability of transcriptomic data should make further identification of promoters straightforward.

Inducible promoters allow the control of heterologous gene expression. In additional to several well characterized native inducible promoters (*POX2* and *LIP2* are fatty acid inducible; *YAT1* is induced by nitrogen limitation; *ALK1* is alkane inducible), a strong and fatty acid inducible hybrid promoter was developed using repeats of upstream sequences from the *POX2* gene [37•]. A similar approach led to the development of an erythrulose and erythritol-inducible promoter [38]. A set of three strong promoters for *R. toruloides* inducible by nitrogen starvation and cell cycle were characterized [39]. Four minimal inducible promoters were identified for *R. toruloides* CBS 14 and characterized using AMT and an eGFP screen. Each promoter is inducible by a different substrate at different rates and to different maxima. *NAR1* is only 200 bp, shows the highest levels of induced expression, and is tightly repressed by glucose and induced by nitrate. *ICL1* also showed high levels of expression when induced with acetate but was not completely repressed in the presence of glucose. The *CTR3* promoter is strongly repressed by copper and has a medium level of induction. *MET16* has a low level of expression and dynamic range; however, it is very tightly repressed by the presence of methionine [40•]. The promoter for the *LAD* gene was used to control a Cre-Lox recombination system in derivatives of *R. toruloides* CECT 13085 [24•]. No inducible promoters have been identified for *L. starkeyi* and *C. oleaginosus*; however, available transcriptomic data will facilitate promoter identification in various conditions.

Genome-scale metabolic models

By combining multi-omic data with genome-scale models, a high-level, holistic approach to engineering *Y. lipolytica* is possible [41]. Similarly, a genome-scale model of *R. toruloides* has been reported [42,43]. No genome-scale model currently exists for *C. oleaginosus* or *L. starkeyi*. With the exception of *Y. lipolytica*, the relative lack of genetic engineering tools for these systems has limited the use of these genome scale models for strain engineering.

Summary

With regard to the availability of genetic engineering tools, it is clear that *Y. lipolytica* is the most advanced. *R. toruloides* has some genetic engineering capabilities, while *L. starkeyi* and *C. oleaginous* are currently underdeveloped. It is our opinion that genetic engineering tools

are becoming straightforward to develop and that they are less important than the complex phenotypes of these yeast in host selection.

Engineered products

Substantial work has recently been completed to enhance the lipid production, metabolism, and genome annotation of *R. toruloides*. Several studies have modified endogenous gene expression to enhance lipid biosynthesis [24•,39,44,45]. Overexpression of *ACC1* under the *GAPDH* promoter and *DGA1* under the *ACL* promoter resulted in high lipid production of 61.1% and 43.4%, respectively, on a dry cell weight basis from 70 g/L glucose and 70 g/L xylose. This is a marked improvement over the wild type strain transformed with an empty vector, which produced 31.3% lipids on a dry cell weight basis [45]. Additional overexpression of malic enzyme (*ME*), pyruvate carboxylase (*PYC1*), glycerol-3-P dehydrogenase (*GPD*), and stearoyl-CoA desaturase (*SCD*), and knockout of *PEX10* to impair β-oxidation resulted in 75.6% lipid accumulation [45]. Overexpression of *DGAT1* and *SCD1* genes under control of the native xylose reductase (*XYL1*) promoter coupled to adaptive evolution facilitated better tolerance to growth inhibitors and improved xylose consumption [24•]. Fed-batch cultivation of this engineered strain in a 7 L bioreactor resulted in the highest lipid production in *R. toruloides* with non-detoxified wheat straw hydrolysate to date [24•]. An engineered *R. toruloides* strain was able to accumulate up to 261 mg/L of bisabolene, a terpene precursor to the biofuel bisabolane, using a novel biocompatible ionic liquid-treated corn stover hydrolysate on the bench scale. The same strain accumulated 680 mg/L bisabolene in a high-gravity fed-batch bioreactor using an alkaline pretreated corn stover hydrolysate [12••].

UV-mutagenesis coupled to screening with ethanol, hydrogen peroxide or cerulenin resulted in a strain of *R. toruloides* with improved lipid production titer (1.25 g/L over 0.94 g/L) and rate (51.2 mg/L/h over 9.8 mg/L/h) compared to the original strain. Transcriptional analysis showed overexpression of isocitrate dehydrogenase and malate dehydrogenase, which may enhance lipid production by generating NADPH, a key reducing agent [46].

Only two instances of metabolic engineering have been reported for *C. oleaginosus* due to its lack of genetic engineering tools. In the first, AMT was utilized to produce omega-3 fatty acids and conjugated linolenic acid. While the products are outside the scope of this review, it is a good demonstration of genetic manipulation for non-native fatty acid production and can be applied for production of relevant biofuels. In a recent publication, a set of three exogenous genes were introduced to the yeast genome using electroporation. A metabolic model based on *Y. lipolytica* predicted that the pyruvate dehydrogenase (PDH) bypass was incomplete. Combinatorial genomic integration of acetaldehyde dehydrogenase, pyruvate decarboxylase, and acetyl-CoA synthetase completed the PDH bypass and ultimately resulted in a yield of 0.27 g TAG/g xylose, nearing theoretical maximum yield [28]. This suggests alternate, successful methods to improve lipid production in this yeast.

Y. lipolytica is the most well-studied oleaginous yeast. This non-conventional, model oleaginous yeast is a prolific producer of lipids and has been engineered to funnel that flux into a number of lipid-derived compounds, such as fatty alcohols [47••], alkanes [47••,48], and ketones [49]. Lipids can be produced by *Y. lipolytica* at industrially useful rates, in some cases exceeding 1.2 g/L/hour [50•]. Lipids can be readily converted into methyl or ethyl fatty acid esters through chemical transesterification. Resultant biodiesel mixtures have already been shown to function well in existing diesel engines [51]. Alternative chemical processing of lipids from biomass results in renewable or green diesel, which is no different than petroleum diesel. The critical point is that the leap between lipids and directly usable fuel products is larger than desired, often requiring many engineering steps. This suggests that one possible approach to biofuel production may be a combination of biological and traditional chemical processes, such as bioproduction of lipids and traditional transesterification or cracking.

Alkanes represent the most direct petroleum replacements as the major constituents of gasoline, diesel, and jet fuel are saturated alkanes. *Y. lipolytica* has been used to produce a number of different alkane species by heterologously expressing different enzymes that can exploit the abundant fatty acid (FA) species present in the yeast. In one case, expression of a lipoxygenase from the soybean plant was used to cleave naturally occurring linoleic acid into pentane and 13-oxo-*cis*-9,*trans*-11-tridecadienoic acid [48]. While this was not an efficient use of carbon, it did represent a proof of concept that *Y. lipolytica* will tolerate the shunting of its native FA species toward heterologous enzymes. A different approach incorporated both a fatty-acyl-CoA reductase and an aldehyde deformylating oxygenase in a two-step reaction to produce a distribution of alkanes from *Y. lipolytica*'s native pool of fatty-acyl-CoAs [47••]. When employing this strategy, the alkane profile produced closely mirrored the chain-length distribution of fatty acids within the yeast. Depending on application, this may be seen as an advantage or disadvantage. Alternatively, choosing enzymes with preferred chain-length specificities can alter the final product profile, as has been done in the production of fatty alcohols [52].

Fatty alcohols, longer chain alkyl alcohols of greater than approximately six carbons, represent another potential

biofuel source due to their compatibility with existing infrastructure and high energy density [53]. Fatty alcohols have been synthesized in *Y. lipolytica* by taking advantage of existing fatty-acyl-CoA pools. When expressed in *Y. lipolytica*, fatty acyl-CoA reductases (FAR) from species such as *Marinobacter aquaeolei* (MaFAR), *Tyto alba* (TaFAR), and *Mus musculus* (MmFAR), are capable of reducing acyl-CoAs to aldehydes and then to fatty alcohols [47••,54]. A single cytosolic fatty acyl-CoA synthetase targeted to the cytosol enhanced the available acyl-CoA pool, resulting in over 200 mg/L total fatty alcohol from a single copy of MaFAR [47••]. Approximately 690 mg/L of hexadecanol was achieved through integration of five copies of TaFAR1 in a Δ*DGA*Δ*FAO1* background strain [54]. Even with such an improvement, the production rate still falls short of that which is necessary for industrial-scale production and replacement of petroleum fuels [55]. By contrast, higher titers and yields was achieved with less engineering in *L. starkeyi* by expressing MmFAR1. Batch cultivation in a bioreactor with glucose resulted in 1.7 g/L fatty alcohols, a 28 mg/g yield. A majority of the fatty alcohol was 1-hexadecanol and 95% secretion. This strain was able to metabolize glycerol and xylose for fatty alcohol production, though this was not tested in the bioreactor [56].

Summary

While *Y. lipolytica* is clearly a well-established organism, the other yeasts discussed in this review naturally produce higher lipid titers and metabolize recalcitrant, heterogeneous, non-conventional feedstocks. Their limited genetic tractability inhibits their broad use in industry; however, their potential to out-produce the industrial standard, *Y. lipolytica*, outweighs the work required to develop novel organisms. While heterologous pathway engineering is challenging, it is becoming more routine. As genetic engineering tool development continues, less tractable organisms will become viable hosts for bioenergy and other products. Prime evidence of this is shown for *R. toruloides* and *L. starkeyi*, where significant advantages are realized compared to *Y. lipolytica*.

Conclusion

It is easy to let the progress made in model systems influence our judgments of future promise for biofuels production. Significant progress has been made using *Y. lipolytica* due to the availability of genetic engineering tools. While *Y. lipolytica* is a better host for fatty acid-based biofuels than *S. cerevisiae*, we propose other oleaginous yeast may be better suited for biofuels production from various low-value feedstocks. Indeed, the other oleaginous yeast described in this paper have a wider appetite for feedstocks and tolerance to inhibitors. The few examples of engineered products show the great promise for non-model oleaginous hosts. Continued development of genetic engineering tools for these systems are likely to pay dividends and lead to more efficient biofuel processes.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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