Trends in Biotechnology

CellPress REVIEWS

Review

The Engineering Potential of *Rhodosporidium toruloides* as a Workhorse for Biotechnological Applications

Young-Kyoung Park,[1] Jean-Marc Nicaud,[1] and Rodrigo Ledesma-Amaro[2,*,@]

Moving our society towards a bioeconomy requires efficient and sustainable microbial production of chemicals and fuels. *Rhodotorula* (*Rhodosporidium*) *toruloides* is a yeast that naturally synthesizes substantial amounts of specialty chemicals and has been recently engineered to (i) enhance its natural production of lipids and carotenoids, and (ii) produce novel industrially relevant compounds. The use of *R. toruloides* by companies and research groups has exponentially increased in recent years as a result of recent improvements in genetic engineering techniques and the availability of multiomics information on its genome and metabolism. This review focuses on recent engineering approaches in *R. toruloides* for bioproduction and explores its potential as a biotechnological chassis.

R. toruloides: An Industrial Yeast

Rhodotorula toruloides, previously known as *Rhodosporidium toruloides*, is a red, heterothallic, bipolar yeast that belongs to the Pucciniomycotina (basidiomycetes) [1]. *R. toruloides* is a natural producer of carotenoids (which are responsible for the red color of the cells and provide antioxidant properties), neutral lipids (accumulated under nutrient-limiting conditions as a carbon storage mechanism), and important enzymes. All these compounds are industrially relevant: carotenoids are used as colorants, antioxidants, or vitamin A precursors and are therefore important for the food, feed, and pharma companies; lipids and lipid-derived compounds are good substitutes for petroleum in the production of fuels and chemicals [2]; and enhanced enzymatic activities in this yeast such as L-phenylalanine ammonia-lyase and D-amino acid oxidase are relevant for the pharma and chemical industries.

Therefore, *R. toruloides* has been studied as a potential biotechnological microorganism for many years (since the 1950s) [3]. Physiological studies have also revealed interesting features as an industrial yeast, such as the capacity to grow to high cell density [4] and its ability to utilize a wide range of carbon and nitrogen sources [5], which can be potentially exploited to produce high-value products from low-cost substrates.

Despite the early discovery of the biotechnological potential of this organism, most developments in bioprocesses and applications have been carried out in the past decade, coinciding with the first publication of its genome sequence, followed by the generation of molecular and genetic tools. Such increasing interest by the scientific community may also associated be with the quest to find alternative and environmentally friendly fuel sources.

Trends

Finding the right microorganism is a key issue for a bioprocess. Ideally, it should be a good natural producer, use low-cost substrates, and perform well at large scale. An engineering process is often required to satisfy such requirements.

Lipids and carotenoids are two industrially relevant compounds. Lipids are a good source of fuels and chemicals which could facilitate the replacement of petroleum. Carotenoids are used as ingredients for the food, feed, pharma, and cosmetic industries. *R. toruloides* naturally synthesize high amounts of these compounds, which makes it a good candidate for their production.

R. toruloides has recently emerged as one of the most promising yeasts for bioproduction. In addition to its natural and convenient industrial features, it can now be metabolically engineered to boost production levels and to expand the range of compounds that are produced.

[1]Micalis Institute, Institut National de la Recherche Agronomique (INRA), AgroParisTech, Université Paris–Saclay, 78350 Jouy-en-Josas, France
[2]Department of Bioengineering, Imperial College London, London SW7 2AZ, UK
[@]Twitter: @LedesmaAmaro

[*]Correspondence: r.ledesma-amaro@imperial.ac.uk (R. Ledesma-Amaro).

Today, recent development in efficient genetic and metabolic engineering techniques together with systems biology analysis are boosting the production capacity of *R. toruloides* and are also expanding the industrial products that this yeast is able to synthesize (Figure 1, Key Figure).

This review presents *R. toruloides* as an emerging industrial microorganism. We summarize here the molecular tools, genetic components, and systems analysis, and how they are being used to engineer this yeast to produce an array of different industrially relevant products.

Development of Molecular Tools and Omics Techniques To Engineer *R. toruloides*

In the era of synthetic biology and bioengineering, knowledge of the genome and efficient genetic tractability are essential to improve the natural capacities of an industrial microorganism, which can bring it closer to real-life and economically feasible applications.

In this regard, systems biology including **genomics** (see Glossary) and **metabolomics** have elucidated the genomic organization and metabolic pathways and their regulation in *R. toruloides*, while the development of genetic components (promoters, markers, terminators, etc.) and transformation methods has paved the way towards refined genomic and metabolic engineering approaches. Moreover, the application of fast and reliable screening techniques to *R. toruloides* has facilitated faster selection of beneficial mutants.

Genome-Wide Analysis (Genomics, Transcriptomics, Proteomics, and Metabolomics)

Several complete genome sequences of *R. toruloides* strains have recently been determined [6–9]. These sequences have been used to understand metabolic pathways of interest (Figure 2), genomic responses of this organism to different environmental factors, and the molecular mechanism for lipid accumulation [7]. Metabolomic analysis has been undertaken to understand the metabolism of glycerol and the mechanism for carotenoid production in *R. toruloides* [10]. Another metabolomic study demonstrated catabolite repression of glycerol metabolism by glucose and the importance of oxygen supply for the yield and composition of lipids in this yeast [11]. A **proteomic** study on the lipid production process of *R. toruloides* during different growth phases helped to identify proteins involved in lipid production [12]. In another study, proteome analysis of lipid droplets demonstrated the dynamic nature of 226 proteins, many of which are involved in lipid metabolism, under different conditions [13]. A **multi-omics** study combining genomic, **transcriptomic**, and proteomic analysis identified genes involved in fungi oleaginicity, such as those involved in nitrogen limitation, nitrogenous compound recycling, macromolecule metabolism, and autophagy [7].

Together these researches have expanded our knowledge on *R. toruloides* production pathways and helped to identify target genes for strain engineering.

Molecular and Genetic Tools

Rational genetic engineering is essential to develop *R. toruloides* as a workhorse for various biotechnological applications. The absence of efficient and reliable genetic manipulation tools has been a major obstacle in *R. toruloides*. Since the first report of transformation of *R. toruloides* based on polyethyleneglycol (PEG)-mediated protoplasts was published in 1985 [14], but which was found to be inefficient and unstable, the most commonly used transformation methods are based on ***Agrobacterium tumefaciens*-mediated transformation** (ATMT) [15,16]. A toolset for genetic modification has been developed including constitutive [15,17,18] and inducible promoters [19,20], as well as selectable markers (auxotrophic [21] and antibiotic-based [16]) (Box 1). Unfortunately, homologous recombination, although possible [22], is weak in *R. toruloides*, which reduces the efficiency of targeted integration and gene deletion. To overcome this obstacle, the gene KU70 (which encodes a part of the **KU70/80**

Glossary

***Agrobacterium tumefaciens*mediated transformation (ATMT):** a transformation method using the T-DNA region of a tumor-inducing plasmid for integrating a heterologous DNA fragment into the genome.
CRISPR/Cas9: CRISPR (clustered regularly interspaced short palindromic repeat) and CRISPR associated protein 9 (Cas9) genes are essential in adaptive immunity to bacteria and archaea, enabling the organisms to eliminate invading genetic material. This system has been using as a genome editing and targeting tool in organisms including bacteria, yeast, and mammalian cells.
Electroporation: a transformation method using an electrical field to increase the permeability of the cell membrane.
Genomics: the study of entire genomes (not the study of individual genes and their roles in inheritance). Genomics uses high-throughput DNA sequencing and bioinformatics to assemble and analyze the structure and function of entire genomes.
Golden Gate cloning: DNA assembly techniques where standardized and interchangeable DNA components can be subsequently assembled in a single-step, one-pot reaction by using type II restriction enzymes.
KU70/80 complex: a complex consisting of KU70 and KU80 which is involved in a non-homologous end-joining mechanism that randomly integrates heterologous DNA fragments into the genome.
Lithium acetate/polyethylene glycol (PEG) method: the most commonly used chemical transformation method in yeast that uses a combination of lithium acetate (which makes cells competent by destabilizing the plasma membrane), single-stranded carrier DNA, and PEG.
Metabolomics: a field that quantifies multiple small-molecule types (such as amino acids, fatty acids, carbohydrates, etc.) in cells that are the end-products of cellular processes. Metabolomics is used to study metabolic function and metabolite flux in combination with modeling.
Multi-omics: an integrated approach that combines several types of omics data (such as genomics, proteomics, and

complex, which is required for the non-homologous end-joining system) was deleted in *R. toruloides*, and this improved gene deletion efficiency [23]. More recently, **electroporation** methods have been described [24,25] as well as **lithium acetate/polyethylene glycol** (PEG)-mediated chemical transformation [26,27]. A summary of the different transformation methods is given in Table 1, and genetic elements used as building blocks are summarized in Table 2. In the near future, the development of efficient synthetic biology tools for DNA assembly, such as **Golden Gate cloning**, or genome editing, such as **CRISPR/Cas9**, is expected to boost metabolic engineering approaches in this yeast.

Screening Tools (Fast Analysis of Products)

Together with advances in genetic tools for engineering, some fast and reliable screening techniques have been developed to select the best-performing strain and the best culture conditions for each application. Feritas and colleagues [28] applied flow cytometry during *R. toruloides* fermentation to assess yeast carotenoid content and cell viability. More recently, flow cytometry has been used to evaluate lipid and carotenoid production as well as yeast cell size and complexity during culture at different pH [29]. A combination of non-invasive techniques [fluorescent microscopy, flow cytometry, and Fourier transform infrared (FTIR) metabolomics) to better understand the underlying biological processes and how they influence each other.

Proteomics: the large-scale study of proteins, particularly their function and structure. These high-throughput analyses and the quantification of thousands of proteins in cells are used to investigate global proteome interactions and quantify post-translational modifications.

Transcriptomics: the study of the transcriptome, the complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a specific cell using high-throughput methods. Comparison of transcriptomes allows the identification of genes that are differentially expressed in different environments.

Key Figure

Scheme of Engineering Processes with *R. toruloides*

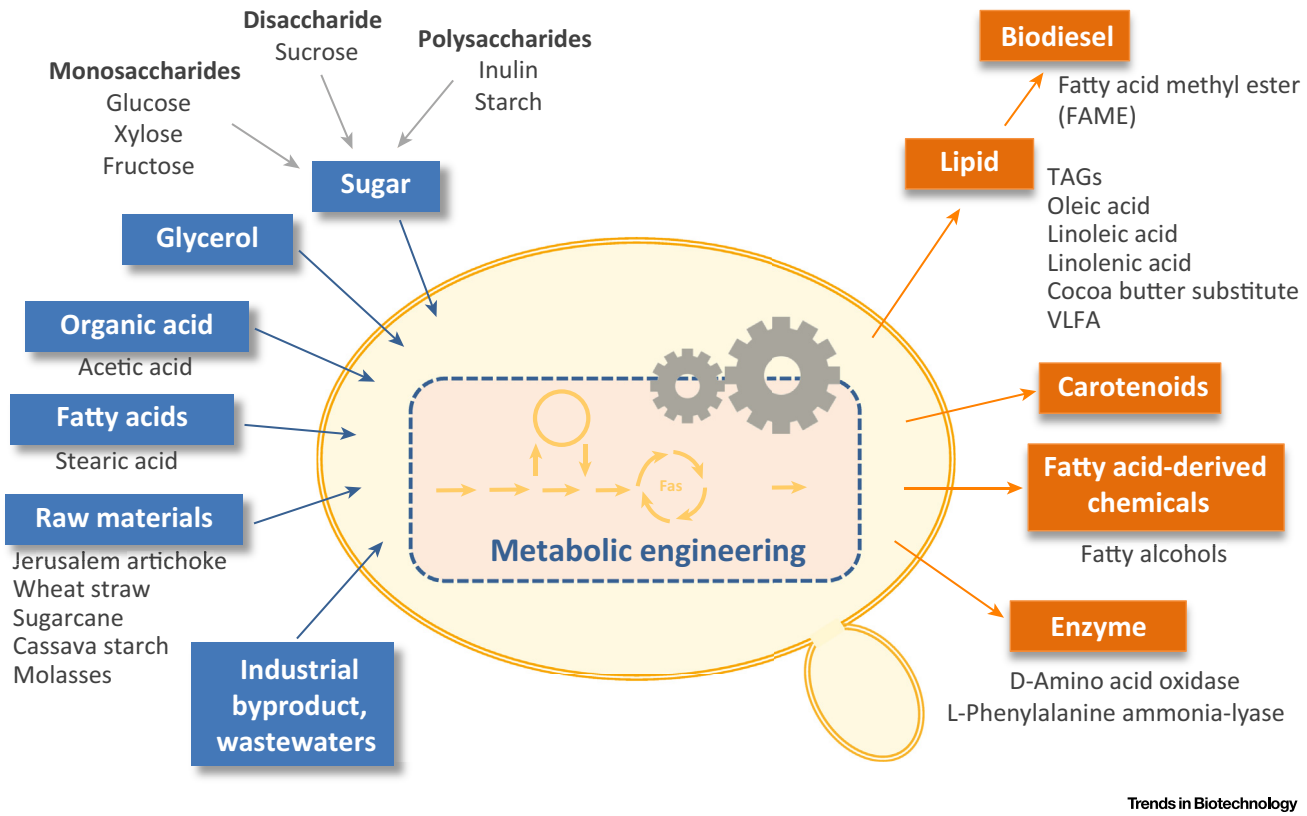


Figure 1. *R. toruloides* can produce industrially relevant compounds (orange boxes) by using different substrates as carbon sources for bioconversion (blue boxes). Some of these compounds have been produced following engineering of *R. toruloides* metabolism. Abbreviations: TAG, triacyl glycerol; VLFA, very long chain fatty acid.

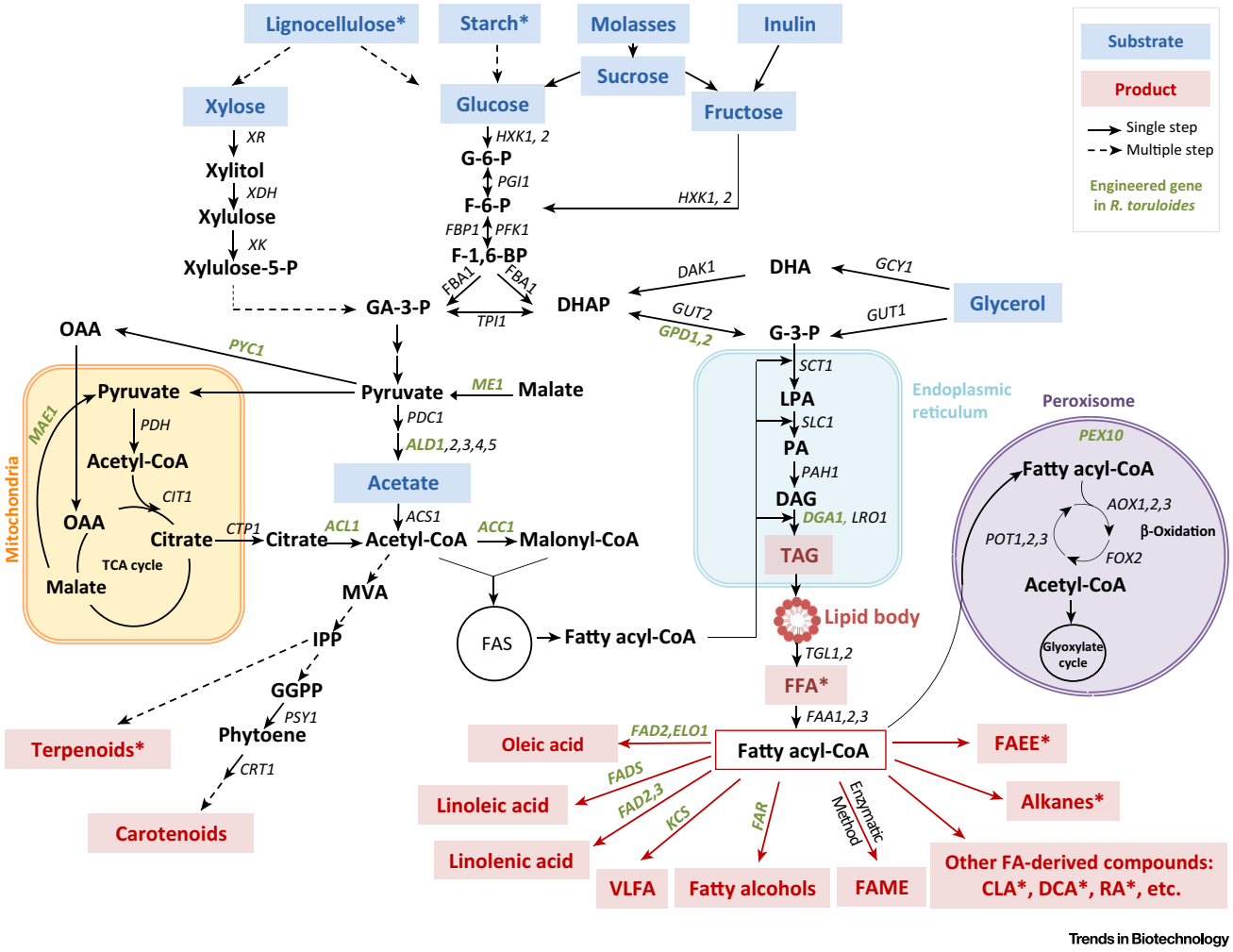


Figure 2. Relevant Metabolic Pathways for Biotechnology in *R. toruloides*. Metabolic pathways are shown, including the metabolites (joined by arrows; a single arrow means one step while a broken arrow means several steps) and the enzymes (named in the middle of the arrow) involved. The commonly used names for metabolites and enzymes are used as abbreviations. Cellular compartments are also indicated: endoplasmic reticulum, mitochondria, lipid body, and peroxisome. Blue boxes indicate carbon sources, while red boxes indicate industrially relevant products. Enzymes named in green correspond to genes that have been already engineered. Asterisks (*) indicate potential substrates/products to target in future engineering approaches. Abbreviations: CLA, conjugated linoleic acid; DCA, dicarboxylic acid; FA, fatty acid; FAME, fatty acid methyl ester; FFA, free fatty acid; FAEE, fatty acid ethyl ester; RA, ricinoleic acid; TAG, triacylglycerol; VLFA, very long chain fatty acid.

microspectroscopy] has been successfully applied to monitor lipid accumulation from crude glycerol over fermentation time [30]. All these techniques monitor lipid accumulation in real time, avoiding time-consuming lipid extraction steps. The application of these screening tools in novel approaches will facilitate the use of high-throughput experiments to select favorable mutations and optimum growth conditions for developing more efficient production processes.

Biotechnological Applications of *R. toruloides* and Their Improvement via Metabolic Engineering

The natural production capacities of *R. toruloides* have previously been enhanced by optimizing fermentation conditions [4,29,31]. More recently, metabolic engineering techniques have boosted the production titers, yields, and productivities of lipids and carotenoids, and have expanded the range of synthesized products, including industrial compounds not naturally produced by this yeast such as some lipid-derived compounds.

Box 1. Genetic Blocks for Synthetic Biology in *R. toruloides*

Synthetic biology is an emerging field of research that seeks to treat biology as an engineering discipline. The development of reliable, efficient, standardized, and robust tools to manipulate biology is one of the major challenges of synthetic biology. For example, the development of fast and combinatorial DNA assembly toolkits such those based on the Golden Gate system allow advanced engineering modifications to be carried out. Although such a toolkit has not yet been developed for *R. toruloides*, traditional genetic engineering techniques have identified a set of genetic components that can be used for engineering this red yeast (see Table 2 in main text).

Recently, a gene cassette carrying the *URA3* auxotrophic marker (isolated from *R. toruloides*) was constructed [20], and several antibiotic resistance markers such as *HYG, NAT,* and *BLE* (which confer resistance to hygromycin, nourseothricin, and bleomycin, respectively) have been developed [15]. Several constitutive promoters have been isolated from *R. toruloides*, including *GPD1*, *FBA1*, *PGK1*, *PGI1*, *TPI1*, *ACC1*, *FAS1*, and *LDP1* [14,16,17]. Inducible promoters such as *DAO1*, *NAR1*, *ICL1*, *CTR3*, and *MET16* are also available [18,19]. The discovery of novel genetic blocks together with the standardization of vectors and plasmids will facilitate the proliferation of rational design strategies to engineer *R. toruloides*.

Neutral Lipids

Microbial lipids have emerged in recent years as alternative sources of fuels and chemicals [32–39]. They present several advantages over plant oils, such as faster growth, lower land use, better adaptability to market needs, and the use of different substrates. *R. toruloides* naturally accumulate high amounts of lipids (up to 65% of dry cell weight, CDW) in the form of triacylglycerols (TAGs) under nitrogen-limited conditions (Box 2 and Table 3); TAGs from microorganisms or plants can be easily used for biodiesel production.

The composition of fatty acids from *R. toruloides* strongly depends on the strain, the carbon/nitrogen ratio in the substrate, the composition of the medium, and the environmental conditions, as recently reviewed by Xu and Liu [5]. In general, the major fatty acids from *R. toruloides* are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2).

Many efforts to improve the lipid production in *R. toruloides* have been made by optimizing the culture media [29,31,40–43], with cultivation modes including fed-batch [30,44] and continuous fermentation [45,46]. Pilot-scale lipid production by *R. toruloides* has also been reported

Table 1. Transformation Methods Developed for *R. toruloides*

Method	Strain		Integration	Expressed gene	Deleted gene	Selective marker	Transformation efficiency	Refs
Protoplast/PEG	*R. toruloides*	MS7013 (*PAL*$^-$)	Random	*RtPAL*		Phenylalanine	1000 transformants/μg DNA	[14]
ATMT	*R. toruloides*	ATCC10657	Random	*RtGFP*		Hygromycin	1000 transformants/plate	[15]
ATMT	*R. toruloides*	ATCC10657	Targeted	*HPT3*	*KU70*	Hygromycin	5.2%	[23]
ATMT	*R. toruloides*	ATCC10657ΔKU70e	Targeted	*HPT3*	*CAR2*	Hygromycin	75.3%	[23]
ATMT	*R. toruloides*	NP11	Random	*HYG*		Hygromycin	1000 transformants/10^5 input cells	[16]
ATMT	*R. toruloides*	NP11	Random	*NAT*		Nourseothricin	140 transformants/10^5 input cells	[16]
ATMT	*R. toruloides*	NP11	Random	*BLE*		Bleomycin	70 transformants/10^5 input cells	[16]
ATMT	*R. toruloides*	NP11	Targeted	*HYG*	*CRT*	Hygromycin	2%	[22]
Electroporation	*R. gracilis*	ATCC26217	Targeted	*ShBLE*	*RgURA3*	Zeocin	40 CFU/μg DNA	[24]
Electroporation	*R. toruloides*	NP11	Random	*BLE*		Bleomycin	1000 CFU/μg DNA	[25]
LiAc/PEG	*R. toruloides*	DMKU3-TK16	Random	*ShBLE*		Zeocin	25 transformants/μg DNA	[26,27]
LiAc/PEG	*R. toruloides*	DMKU3-TK16	Random	*ShBLE*		Zeocin	–	[26,27]
LiAc/PEG	*R. toruloides*	DMKU3-TK16	Targeted	*ShBLE*	*URA3*	Zeocin, 5-FOA	–	[26,27]

Table 2. Genetic Elements Used as Building Blocks for Engineering *R. toruloides*

Component		Description	Refs
Selective marker	*URA3*	Uracil auxotrophic marker	[21]
	HYG	Resistance to hygromycin	[16]
	NAT	Resistance to nourseothricin	[16]
	BLE	Resistance to bleomycin	[16]
Constitutive promoter	*GPD1*	Glyceraldehyde-3-phosphate dehydrogenase	[15]
	FBA1	Fructose 1,6-biphosphatealdolase	[17]
	PGK1	Phosphoglycerate kinase	[17]
	PGI1	Glucose 6-phosphate isomerase	[17]
	TPI1	Triose phosphate isomerase	[17]
	LDP1	Perilipin/lipid droplet protein 1	[18]
	ACC1	Acetyl-CoA carboxylase 1	[18]
	FAS1	Fatty acid synthase β subunit	[18]
Inducible promoter	*THI5*	4-Amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase, induced by (–) thiamine, repressed by thiamine	[20]
	THI4	Thiamine thiazole synthase, induced by (–) thiamine, repressed by thiamine	[20]
	CTR31	Copper transporter, induced by (–) copper, repressed by copper	[20]
	DAO1	D-amino acid oxidase, induced by D-alanine (70 mM)	[19]
	NAR1	Nitrate reductase, induced by nitrate, repressed by ammonium	[20]
	ICL1	Isocitrate lyase 1, induced by acetate, repressed by glucose	[20]
	CTR3	High-affinity copper transporter, induced by (–) copper, repressed by copper	[20]
	MET16	3′-Phosphoadenylsulfate reductase, induced by (–) methionine, repressed by methionine	[20]
Terminator	*GPD1*	Glyceraldehyde-3-phosphate dehydrogenase from *R. toruloides*	[26,27]
	NOS	Terminator of *Agrobacterium tumefaciens* nopaline synthase gene	[15]
	HSP	Heat shock protein 18.2 of *Arabidopsis thaliana*	[22]
	35S	Transcriptional terminator of cauliflower mosaic virus gene *35S*	[19]
	SV40	Simian virus 40 Poly(A) sequence	[19]

[47], where 0.44 g/L·h of lipid productivity was obtained using sugarcane juice and urea, which is significantly higher than in microalgae *Chlorella* sp. (0.32 g/L·day) [47].

Metabolic engineering approaches aimed at improving lipid production have lately been carried out in parallel to whole-genome sequencing and the development of genetic tools in *R. toruloides*. Zhang and colleagues [48] increased lipid production a factor of 1.95 (16 g/L of total lipids from glucose in shake flask culture) in *R. toruloides* by overexpressing acetyl-CoA carboxylase (ACC1) and diacylglycerol acyltransferase (DGA1), which catalyze the first and last steps in triacylglycerol biosynthesis. The same research group further engineered the strain by overexpressing stearoyl-CoA desaturase (SCD), and reported an increase in lipid titers by a factor of 1.42 from glucose in fed-batch growth [49].

Specific High-Value Lipids

One of the advantages of microbial lipids is that the producer microbes can be engineered to be enriched in tailor-made, high-value lipids which are normally not produced or are produced in low amounts. This engineering normally consists of introducing or overexpressing enzymes that can modify the naturally produced lipids.

Box 2. Performance of *R. toruloides* Compared with Other Natural Producers of Lipids and Carotenoids

R. toruloides is an organism that possesses several desirable industrial properties, such as the ability to grow on low-cost substrates at high concentrations, and performs well in pilot scale. Moreover, this yeast is a good natural producer of carotenoids and lipids, two industrially relevant compounds. In fact, as summarized in Table 3 (main text), it is one of the best producers ever found for such products. On the one hand, *R. toruloides* is able to produce 65% of the dry cell weight as lipids under nitrogen-limited conditions, which is much more than the most commonly used yeast for this task, *Y. lipolytica*, which accumulates around 30% without additional engineering. However, the development of engineering techniques is more advanced in *Y. lipolytica,* where efficient metabolic engineering approaches have resulted in up to 90% lipid accumulation. On the other hand, *Lipomyces starkeyi* naturally produces similar amounts of lipids as *R. torouloides*, but the manipulation of this yeast is far less developed.

R. toruloides also produces carotenoids at levels similar to those of the top producer organisms, including *Rhodotorula*, *Sporidiobolus*, and *Phaffia* (see Table 4 in main text). Importantly, *R. toruloides* is the most advanced in terms of the genetic engineering tools available, which may allow a further increase in carotenoid production titer, yields, and productivity in the near future.

Oils with high levels oleic acids show excellent thermal and oxidative stability. For this reason they can be used to develop biolubricants, hydraulic fluids, and oils for electrical transformers; these high oleic acid-containing oils have traditionally been produced from plants. Overexpression of elongase (ELO1) and deletion of Δ12 desaturase (FAD2) in *R. toruloides* permitted an increase in oleic acid content (23%) compared to the parent strain [50].

Linoleic acid is an essential fatty acid that humans are unable to synthesize. It has been overproduced in *R. toruloides* by expressing Δ12-fatty acid desaturase (FADS) from *Fusarium verticillioides* and *Mortierella alpina* [51]. The relative linoleic acid content increased fivefold and the final linoleic acid titer reached 1.3 g/L.

α-Linolenic acid (ALA, C18:3) is an ω3 fatty acid that plays important roles in several aspects of human health but is not synthesized by the human body. Shorter-chain ω3 fatty acids such as ALA are precursors for the production of longer-chain ω3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In *R. toruloides*, inactivating aldehyde dehydrogenase (ALD1) led to 1.2-fold higher ALA production, and a further (3.74-fold) increase, with a total ALA content of 49% of total fatty acids, was achieved by introducing Δ12 desaturase (FAD2) from *Mortierella alpine* and ω3 desaturase (FAD3) from *Aleurites fordiiek* [52].

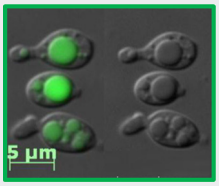
Very long-chain fatty acids (VLCFAs) such as erucic acid (C22:1) and nervonic acid (C24:1) are important renewable feedstocks in plastic, cosmetics, nylon, and lubricant industries. Fillet and colleagues [53] produced VLCFA-rich oils by introducing 3-ketoacyl-CoA synthases (KCS) from different plants into *R. toruloides*. After growing in 7 L bioreactors, the engineered *R. toruloides* strain produced the best titers of VLCFA reported so far (5.8 g/L of erucic acid and 7.9 g/L of nervonic acid, 27% of total fatty acids).

Microbial production of cocoa butter, a main component of chocolate, is recently gaining interest owing to increasing market demand. Six different yeasts including *R. toruloides* were cultivated to study cocoa butter production [54]. *Trishosporon oleaginosus* and *R. toruloides* presented a high proportion of triacylglycerol (TAG) 1,3-dipalmitoyl-2-oleoyl glycerol (POP) (C16:0–C18:1–C16:0) and 1-palmitoyl-3-stearoyl-2-oleoyl-glycerol (POS) (C16:0–C18:1–C18:0), the main lipids in cocoa butter. This work suggests that these two yeasts are promising cocoa butter-like lipid producers.

Lipid-Derived Compounds

Fatty acid-derived products are recently attracting interest because of their numerous commercial applications. Fatty acid methyl esters (FAMEs) can directly be used as biodiesel

Table 3. Comparison of the Lipid Production Capacity of Different Oleaginous Organisms

	Microorganism	Biomass (g/L)	Lipid content (% w/w)	Substrate	Cultivation mode	Refs
 **Figure**. Fluorescence microscopy image of the lipid bodies of *R. toruloides* stained in green with BODIPY.	*Rhodotorula toruloides*	15.8	65.2	Glucose	Batch	[32]
	Yarrowia lipolytica	6.5	30.7	Glycerol	Batch	[33]
	Lipomyces starkeyi	20.5	61.5	Glucose + xylose	Batch	[34]
	Magnusiomyces capitatus	15.4	43.1	Glucose	Batch	[35]
	Candida sp. 107	18.1	37.1	Glucose	Continuous	[36]
	Cutaneotrichosporon curvatus	118.0	25.0	Glycerol	Fed-batch	[37]
	Cutaneotrichosporon curvatus	10.2	33.2	Glucose	Batch	[38]
	Rhodotorula glutinis	22.2	25.0	Glycerol	Batch	[39]

because their physical properties are similar to those of conventional diesel. Crude lipids from *R. toruloides* have been converted into FAMEs by enzymatic transesterification with a biodiesel yield of 67.2% (after 24 h reaction) [42]. A novel and simplified method, base-catalyzed methanolysis, achieved a 97.7% FAME conversion yield from *R. toruloides* lipids [55]. These works demonstrate that *R. toruloides* lipids can be a potential feedstock for biodiesel production.

Fatty alcohols are another group of lipid-derived compounds that have a wide range of applications, including as lubricants, surfactants, solvents, emulsifiers, plasticizers, emollients, thickeners, and fuels. Fatty alcohols can be produced in microorganisms by expressing a fatty acyl-CoA reductase. A fatty acyl-CoA reductase gene (*Maqu 2220*) from *Marinobacter aquaeolei* VT8 has recently been overexpressed in *R. toruloides* to produce over 8 g/L of C16–C18 fatty alcohols [56]. This is the highest microbial titer reported so far (0.1–0.5 g/L in *S. cerevisiae*, 1.7 g/L in *E. coli*) and it suggests that *R. toruloides* is a good platform for the industrial production of such compounds.

Carotenoids

R. toruloides (often referred as 'pink yeast') and other basidiomycetous yeasts [3,57,58] are well-known sources of carotenoids, where up to 90% of the carotenoids produced are torularhodin, torulene, γ-carotene, and β-carotene [3,10] (Box 2), and *Rhodotorula* spp. are among the best producers of carotenoids (Table 4). Carotenoids are commercially used as colorants for food, feed, and cosmetic products [59], and, in addition, β-carotene has pro-vitamin A, antioxidant, anticancer, and immune modulatory activities [60].

Table 4. Comparison of the Carotenoid Production Capacity of Different Organisms

	Microorganism	Substrate	Total carotenoids, mg/g dry cell mass	Total carotenoids, mg/L	Refs
 **Figure**. Precipitated cells of *R. toruloides* where the orange-pink color associated with carotenoids can be clearly seen.	*Rhodotorula glutinis*	Glucose	0.11	0.63	[3]
	Rhodotorula graminis	Glucose	0.16	1.47	[3]
	Rhodotorula toruloides	Glucose (batch)	0.12	1.31	[3]
	Rhodotorula toruloides	Glucose (fed- batch)	0.28	27.5	[61]
	Rhodotorula paludigenum	Glycerol	0.40	2.99	[57]
	Sporobolomyces roseus	Glucose	0.08	0.63	[3]
	Sporidiobolus salmonicolor	Glucose	0.35	0.61	[3]
	Phaffia rhodozyma	Glycerol	0.21	1.48	[58]

Many studies have tried to improve carotenoid production in *R. toruloides* by optimizing the culture conditions. The effect of culture medium pH on carotenoid and lipid production by *R. toruloides* has been studied using flow cytometry. The optimal pH for biomass and lipid production was determined to be 4, whereas the optimal pH for carotenoids was 5 [29]. Therefore, Dias and colleagues [61] reported a dual-stage pH-controlled fed-batch cultivation strategy for increased carotenoid production (0.29 g/L·h). Another work analyzed the importance of culture medium components in lipid/carotene production by response surface methodology (RSM) and observed a 10% (w/v) increase in carotenoid production under optimized osmotic stress [62].

Fewer approaches have attempted physicochemical or insertional mutagenesis to improve carotenoid production. Physicochemical mutagenesis, such as the atmospheric and room temperature plasma (ARTP) and nitrosoguanidine (NTG) methods, has been successfully applied to select a mutant strain based on the colony color, one example of which produced 0.75 mg carotenoids/g CDW in nitrogen-limited conditions [63]. In 2017, Lin and colleagues [64] altered the production profile of carotenoids by creating an insertional mutant library with ATMT. The three mutants selected by color change showed a 2.4-fold increase in γ-carotene, a ninefold increase in torulene, and a 1.7-fold increase in β-carotene.

Rational metabolic engineering has been used to harvest and separate carotenoids, normally accumulated inside the cells, from cultures of *R. toruloides*. In this regard, Lee and colleagues [65] expressed the pleiotropic drug-resistance membrane transporter PDR10 from *Saccharomyces cerevisiae*, which permitted the export of carotenoids to the culture medium and concurrent separation from intracellular lipids. The application of novel synthetic biology tools to modify the carotenoid pathway of *R. toruloides* has enormous potential to increase carotenoid production or to tune carotenoid profile in the near future.

L-Phenylalanine Ammonia-Lyase (PAL)

PAL (E.C.4.3.1.24) catalyzes the conversion of L-phenylalanine to ammonia and *trans*-cinnamic acid. PAL has various industrial and potential medical applications, such as treating phenylketonuria (PKU), a metabolic disorder that results from impaired activity of hepatic phenylalanine hydroxylase [66]. Furthermore, the reverse reaction has been exploited commercially in the production of L-phenylalanine, which is important as an essential amino acid for human nutrition and because of its wide use in the pharmaceutical and food industries [67].

PAL is present in many higher plants, some filamentous fungi, and yeasts [67], but *R. toruloides* has been a major commercial source of this enzyme because of its high PAL activity. Since the gene coding for PAL from *R. toruloides* was cloned and sequenced [68], several strategies have been attempted for efficient and stable production of recombinant PAL (these studies have been reviewed by Cui and colleagues [67]). Recently, a high-resolution crystal structure of PAL was determined [69], allowing structure-based molecular engineering approaches to enhance its therapeutic capacities [70,71]. However, despite the promising characteristics of PAL from *R. toruloides*, it showed low performance in repeat-dose studies even after exhaustive mutagenesis to reduce immunogenicity or to increase stability [72]. Therefore, issues regarding immunogenicity, enzyme production, and mode of delivery remain a challenge for its industrial and medical application.

D-Amino Acid Oxidase (DAAO)

DAAO (E.C.1.4.3.3) is a flavo-enzyme using FAD as the prosthetic group. It catalyzes the oxidative deamination of D-amino acids to the corresponding 2-oxo acids and ammonia, with the simultaneous reduction of molecular oxygen to hydrogen peroxide. DAAO has a remarkable importance in biotechnology because of its use for the deamination of cephalosporin C to

7-aminocephalosporanic acid, a key intermediate for the production of semi-synthetic cephalosporin antibiotics [73]. DAAO has also been used to produce R-keto acids for treating chronic uremia [74] and to develop biosensors for detecting D-amino acids in biological samples [75].

DAAOs from *R. toruloides* (RtDAO) and *Trigonopsis variabilis* are considered to be the most favorable because of their high turnover rates (K_{cat} = 44200 minutes^{-1} for the *R. toruloides* enzyme) and the adequacy of yeasts for biotechnological applications [73]. However, heterologous expression of DAAO from *R. toruloides* in *E. coli* produced inclusion bodies and reduced host cell growth [76]. Alternative strategies based on whole-cell immobilization of *R. toruloides* produced low conversion yields and high amounts of side products [73]. Recently, structure-based protein engineering has allowed the redesign of DAAO specificity, cofactor binding, and stability for DAAO-related applications such as biosensors, biocatalysts, and novel areas of medical, environmental, and agricultural research [77].

Exploring Low-Cost Substrate Utilization

The cost of the media components is a crucial issue in any biotechnological process. The price of the carbon and nitrogen sources can account up to 70–85% of the total cost of the bioprocess [78,79]. Therefore, by using cheap carbon sources, processes that are in principle unfeasible because of the low price of the final product (such as in the case of fuels and bulk chemicals) could become economically viable.

Interestingly, *R. toruloides* can naturally grow on a wide range of carbon sources and it presents good tolerance to inhibitory compounds found in unrefined substrates.

In relation to the carbon sources, most studies aiming at increasing the production of lipids, lipid-derived compounds, or carotenoids have used glucose as the carbon source. However, some articles have explored alternative and cheaper carbon sources such as glycerol, hydrolysates, and waste products. Several studies investigated the feasibility of using crude glycerol (a byproduct of biodiesel preparation) as a carbon source [30,80,81]. Gao and colleagues [81] reported higher lipid content and production from crude glycerol than from pure glycerol, without a negative effect of the impurities that are present in crude glycerol. Other low-cost substrates that have been investigated to produce lipids in *R. toruloides* include, for example, levoglucosan [82], sugarcane juice [47], sugarcane bagasse hydrolysate [83], Brazilian molasses [84], sweet sorghum juice [85], wheat straw hydrolysate [86], cassava starch hydrolysate [87], and Jerusalem artichoke juice [88]. These substrates were recently reviewed by Xu and colleagues [5].

R. toruloides has been shown to be able to use cheaper nitrogen sources that are alternatives to yeast extract or peptone. Kiran and coworkers [89] tested rapeseed meal (a byproduct of biodiesel production) in combination with crude glycerol as a carbon source to produce lipids in *R. toruloides*. This low-cost medium increased lipid yield by 58% (0.19 g lipid/g glycerol) and lipid production by 21% (19.7 g/L) compared to a medium with yeast extract. In addition, sunflower meal (a byproduct of biodiesel production [90]) and bioethanol wastewater [91] were also suitable for microbial oil production using *R. toruloides*.

An interesting substrate for biochemical production is wastewater such as municipal or distillery waste. These waste effluents contain high levels of organic matter that *R. toruloides* can use. Such processes would at the same time recover wastes and convert them into high-value products. *R. toruloides* is able to grow and produce lipid in real mixed wastewater after optimizing the culture conditions [92]. More recently, a coculture of *R. toruloides* and *Chlorella pyrenoidosa* showed an increase in lipid yield and content as well as efficient removal of soluble chemical oxygen demand, nitrogen, and phosphorus in a mix of wastewaters [93]. To further

reduce the cost of the process, recent work has explored the possibility of using part of the end-culture as a seed culture for the following process without adding extra nutrients. Interestingly, this strategy maintained the same lipid production performance but with ~30% reduction in the materials cost for medium preparation [94]. These studies demonstrate the potential use of *R. toruloides* for wastewater valorization and lipid production in a cost-efficient manner.

Concluding Remarks and Future Trends

Research interest in *R. toruloides* has clearly increased in recent years. As described above, the combination of academic and industrial interest in potential biotechnological applications of this yeast, and the development of tools for engineering it, are generating a vast expansion of the number of laboratories worldwide using *R. toruloides*. This expansion is expected to lead to rapid development both of new applications and more efficient genetic tools (see Outstanding Questions).

Regarding the tools, improvement in transformation efficiency and especially in targeted insertions/gene deletions is a major need in the field. Synthetic biology tools, for example DNA assembly techniques such as the creation of a Golden Gate toolkit with a range of promoters, markers, terminators, insertion sites, and the development of CRISPR/Cas9 systems for genome editing and controlling gene expression, are also expected to be developed soon for this yeast. Such techniques would facilitate the application of automated high-throughput platforms such as bio-foundries in the strain engineering process. The development of new screening techniques and fermentation optimization will also increase production yield, titers, and productivity.

Regarding the applications of *R. toruloides*, it seems clear that further improvements will take place in the production of carotenoids and lipids, especially through metabolic engineering, which has not yet been fully exploited in the pathways leading to these products. As has recently occurred with the production of novel compounds not naturally produced by *R. toruloides*, such as FAMEs or fatty alcohols, new products will be explored in the near future. In particular, those derived from the two pathways with high metabolic flux in this yeast (fatty acid and carotenoid synthesis pathways), such as other fatty acid-derived compounds {e.g., alkanes [95], conjugated linoleic acids (CLA), ricinoleic acids (RAs), γ-decalactone, dicarboxylic acids (DCAs), hexanal, and so on [96]}, other carotenoids (e.g., lycopene, astaxanthin, canthaxanthin [97]), or terpenes (e.g., β-farnesene [98], squalene [99]). Producing these high-value products can easily make the *R. toruloides* bioprocesses more economically feasible.

Another way to reduce the total cost of the process is by engineering the substrate utilization set of *R. toruloides*, as has been already done in other oleaginous yeasts such as *Y. lipolytica* [100]. For example, *R. toruloides* can produce lipids from starch or lignocellulosic hydrolysates, but is unable to directly use the raw substrates as a consolidated bioprocess. This type of strategy will not only allow the growth of the red yeast on cheaper carbon sources where it is naturally unable to grow but also it will allow better use of the resources present in complex media such as biomass hydrolysates (e.g., by co-consumption of sugars or by using all the carbon sources in the broth).

We conclude that *R. toruloides* is an industrial yeast with great potential in the production of commodities and fuels, which, with the help of synthetic biology and metabolic engineering, can help to pave the way towards a global bioeconomy.

Outstanding Questions

Can new efficient and robust tools to rationally engineer *R. toruloides* be developed? Specifically, the development of an efficient transformation method and synthetic biology techniques (such as CRISPR/Cas9, Golden Gate assembly, characterization of new components, etc.) are expected to boost both the use and knowledge of this organism.

Would it be useful to develop more genome-wide techniques? Would a genome-scale metabolic model help to identify novel targets for engineering *R. toruloides*? An available, curated and annotated genome database would help researchers worldwide to work with this yeast.

The genes involved in the pathways leading to the production of lipids and carotenoids have been recently described, and the tools to overexpress and delete genes have been developed. Would it be possible to further increase production titers by performing metabolic engineering on these pathways?

Is it possible to create high yields of new products that are not naturally produced in *R. toruloides* (e.g., alkanes, terpenes, dicarboxylic acids, etc.) by expressing heterologous enzymes?

Can *R. toruloides* be engineered to use low-cost substrates that it naturally is unable to consume, or be improved in its natural substrate assimilation capacities? This could reduce the cost of the processes, which can make a difference for an economically viable industrial setup.

Acknowledgments

R.L.A. received financial support from an Imperial College Research Fellowship.

References

1. Coelho, M.A. *et al.* (2008) Identification of mating type genes in the bipolar basidiomycetous yeast *Rhodosporidium toruloides*: first insight into the MAT locus structure of the Sporidiobolales. *Eukaryot. Cell* 7, 1053–1061
2. Passoth, V. *et al.* (2017) Lipids of yeasts and filamentous fungi and their importance for biotechnology. In *Biotechnology of Yeasts and Filamentous Fungi* (Sibirny, A., ed.), pp. 149–204, Springer
3. Buzzini, P. *et al.* (2007) Carotenoid profiles of yeasts belonging to the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces*, and *Sporidiobolus*. *Can. J. Microbiol.* 53, 1024–1031
4. Li, Y. *et al.* (2007) High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme Microb. Technol.* 41, 312–317
5. Xu, J. and Liu, D. (2017) Exploitation of genus *Rhodosporidium* for microbial lipid production. *World J. Microbiol. Biotechnol.* 33, 54
6. Kumar, S. *et al.* (2012) Genome sequence of the oleaginous red yeast *Rhodosporidium toruloides* MTCC 457. *Eukaryot. Cell* 11, 1083–1084
7. Zhu, Z. *et al.* (2012) A multi-omic map of the lipid-producing yeast *Rhodosporidium toruloides*. *Nat. Commun.* 3, 1112
8. Morin, N. *et al.* (2014) Draft genome sequence of *Rhodosporidium toruloides* CECT1137, an oleaginous yeast of biotechnological interest. *Genome Announc.* 2, e00641-14
9. Hu, J. and Ji, L. (2016) Draft genome sequences of *Rhodosporidium toruloides* strains ATCC 10788 and ATCC 10657 with compatible mating types. *Genome Announc.* 4, e00098-16
10. Lee, J.J. *et al.* (2014) Metabolomic profiling of *Rhodosporidium toruloides* grown on glycerol for carotenoid production during different growth phases. *J. Agric. Food Chem.* 62, 10203–10209
11. Bommareddy, R.R. *et al.* (2015) Metabolic network analysis and experimental study of lipid production in *Rhodosporidium toruloides* grown on single and mixed substrates. *Microb. Cell Fact.* 14, 36
12. Liu, H. *et al.* (2009) Comparative proteomic analysis of *Rhodosporidium toruloides* during lipid accumulation. *Yeast* 26, 553–566
13. Zhu, Z. *et al.* (2015) Dynamics of the lipid droplet proteome of the oleaginous yeast *Rhodosporidium toruloides*. *Eukaryot. Cell* 14, 252–264
14. Tully, M. and Gilbert, H.J. (1985) Transformation of *Rhodosporidium toruloides*. *Gene* 36, 235–240
15. Liu, Y. *et al.* (2013) Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RtGPD1 and development of genetic transformation method by dominant selection in oleaginous yeast *Rhodosporidium toruloides*. *Appl. Microbiol. Biotechnol.* 97, 719–729
16. Lin, X. *et al.* (2014) Functional integration of multiple genes into the genome of the oleaginous yeast *Rhodosporidium toruloides*. *FEMS Yeast Res.* 14, 547–555
17. Wang, Y. *et al.* (2016) Cloning and evaluation of different constitutive promoters in the oleaginous yeast *Rhodosporidium toruloides*. *Yeast* 33, 99–106
18. Liu, Y. *et al.* (2016) Developing a set of strong intronic promoters for robust metabolic engineering in oleaginous *Rhodotorula* (*Rhodosporidium*) yeast species. *Microb. Cell Fact.* 15, 200
19. Liu, Y. *et al.* (2015) Engineering an efficient and tight D-amino acid-inducible gene expression system in *Rhodosporidium*/*Rhodotorula* species. *Microb. Cell Fact.* 14, 170
20. Johns, A.M. *et al.* (2016) Four inducible promoters for controlled gene expression in the oleaginous yeast *Rhodotorula toruloides*. *Front Microbiol.* 7, 1666
21. Yang, F. *et al.* (2008) Identification of the orotidine-5′-monophosphate decarboxylase gene of the oleaginous yeast *Rhodosporidium toruloides*. *Yeast* 25, 623–630
22. Sun, W. *et al.* (2017) Homologous gene targeting of a carotenoids biosynthetic gene in *Rhodosporidium toruloides* by *Agrobacterium*-mediated transformation. *Biotechnol. Lett.* 39, 1001–1007
23. Koh, C.M. *et al.* (2014) Molecular characterization of KU70 and KU80 homologues and exploitation of a KU70-deficient mutant for improving gene deletion frequency in *Rhodosporidium toruloides*. *BMC Microbiol.* 14, 50
24. Takahashi, S. *et al.* (2014) Genetic transformation of the yeast *Rhodotorula* gracilis ATCC 26217 by electroporation. *Appl. Biochem. Microbiol.* 50, 624–628
25. Liu, H. *et al.* (2017) Fast and efficient genetic transformation of oleaginous yeast *Rhodosporidium toruloides* by using electroporation. *FEMS Yeast Res.* 17, fox017
26. Tsai, Y.Y. *et al.* (2017) Development of a sufficient and effective procedure for transformation of an oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16. *Curr. Genet.* 63, 359–371
27. Tsai, Y.Y. *et al.* (2017) Erratum to: Development of a sufficient and effective procedure for transformation of an oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16. *Curr Genet.* 63, 373
28. Freitas, C. *et al.* (2014) New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodosporidium toruloides* NCYC 921 batch growth. *Process. Biochem.* 49, 554–562
29. Dias, C. *et al.* (2016) Effect of medium pH on *Rhodosporidium toruloides* NCYC 921 carotenoid and lipid production evaluated by flow cytometry. *Appl. Biochem. Biotechnol.* 179, 776–787
30. Signori, L. *et al.* (2016) Assessing an effective feeding strategy to optimize crude glycerol utilization as sustainable carbon source for lipid accumulation in oleaginous yeasts. *Microb. Cell Fact.* 15, 75
31. Wu, S. *et al.* (2011) Microbial lipid production by *Rhodosporidium toruloides* under sulfate-limited conditions. *Bioresour. Technol.* 102, 1803–1807
32. Hu, C. *et al.* (2009) Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. *Bioresour. Technol.* 100, 4843–4847
33. André, A. *et al.* (2009) Biotechnological conversions of bio-diesel-derived crude glycerol by *Yarrowia lipolytica* strains. *Eng. Life Sci.* 9, 468–478
34. Zhao, X. *et al.* (2008) Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. *Eur. J. Lipid Sci. Technol.* 110, 405–412
35. Wu, H. *et al.* (2011) Production of microbial oil with high oleic acid content by *Trichosporon capitatum*. *Appl. Energy* 88, 138–142
36. Gill, C.O. *et al.* (1977) Lipid accumulation in an oleaginous yeast (*Candida* 107) growing on glucose in single-stage continuous culture. *Appl. Environ. Microbiol.* 33, 231–239
37. Meesters, P.A.E.P. *et al.* (1996) High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Appl. Microbiol. Biotechnol.* 45, 575–579
38. Evans, C.T. and Ratledge, C. (1983) A comparison of the oleaginous yeast, *Candida curvata*, grown on different carbon sources in continuous and batch culture. *Lipids* 18, 623–629
39. Easterling, E.R. *et al.* (2009) The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*. *Bioresour. Technol.* 100, 356–361
40. Saran, S. *et al.* (2017) Process optimization for cultivation and oil accumulation in an oleaginous yeast *Rhodosporidium toruloides* A29. *Fuel* 188, 324–331
41. Xu, J. *et al.* (2016) Renewable microbial lipid production from oleaginous yeast: some surfactants greatly improved lipid production of *Rhodosporidium toruloides*. *World J. Microbiol. Biotechnol.* 32, 107
42. Zhao, X. *et al.* (2012) Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *Rhodosporidium toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid. *Bioprocess Biosyst. Eng.* 35, 993–1004
43. Wu, S. *et al.* (2010) Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. *Bioresour. Technol.* 101, 6124–6129

44. Fei, Q. *et al.* (2016) Enhanced lipid production by *Rhodosporidium toruloides* using different fed-batch feeding strategies with lignocellulosic hydrolysate as the sole carbon source. *Biotechnol. Biofuels* 9, 130
45. Shen, H. *et al.* (2012) Effects of dilution rate and carbon-to-nitrogen ratio on lipid accumulation by *Rhodosporidium toruloides* under chemostat conditions. *Sheng Wu Gong Cheng Xue Bao* 28, 56–64 (in Chinese)
46. Shen, H. *et al.* (2013) Kinetics of continuous cultivation of the oleaginous yeast *Rhodosporidium toruloides*. *J. Biotechnol.* 168, 85–89
47. Soccol, C.R. *et al.* (2016) Pilot scale biodiesel production from microbial oil of *Rhodosporidium toruloides* DEBB 5533 using sugarcane juice: performance in diesel engine and preliminary economic study. *Bioresour. Technol.* 223, 259–268
48. Zhang, S. *et al.* (2016) Engineering *Rhodosporidium toruloides* for increased lipid production. *Biotechnol. Bioeng.* 113, 1056–1066
49. Zhang, S. *et al.* (2016) Metabolic engineering of the oleaginous yeast *Rhodosporidium toruloides* IFO0880 for lipid overproduction during high-density fermentation. *Appl. Microbiol. Biotechnol.* 100, 9393–9405
50. Fillet, S.C. *et al*. Neol Biosolutions. Production of microbial oils with an elevated oleic acid content, WO 2016185073
51. Wang, Y. *et al.* (2016) Overexpression of Δ12-fatty acid desaturase in the oleaginous yeast *Rhodosporidium toruloides* for production of linoleic acid-rich lipids. *Appl. Biochem. Biotechnol.* 180, 1497–1507
52. Liu, Y. *et al*. Temasek Life Sciences Laboratory. Methods for efficient production of polyunsaturated fatty acids (PUFA) in *Rhodosporidium* and *Rhodotorula* species, WO 2016039685 A1
53. Fillet, S. *et al.* (2017) Engineering *Rhodosporidium toruloides* for the production of very long-chain monounsaturated fatty acid-rich oils. *Appl. Microbiol. Biotechnol.* Published online August 15, 2017. http://dx.doi.org/10.1007/s00253-017-8461-8
54. Wei, Y. *et al.* (2017) Cocoa butter-like lipid production ability of non-oleaginous and oleaginous yeasts under nitrogen-limited culture conditions. *Appl. Microbiol. Biotechnol.* 101, 3577–3585
55. Thliveros, P. *et al.* (2014) Microbial biodiesel production by direct methanolysis of oleaginous biomass. *Bioresour. Technol.* 157, 181–187
56. Fillet, S. *et al.* (2015) Fatty alcohols production by oleaginous yeast. *J. Ind. Microbiol. Biotechnol.* 42, 1463–1472
57. Yimyoo, T. *et al.* (2011) Carotenoid production by *Rhodosporidium paludigenum* DMKU3-LPK4 using glycerol as the carbon source. *Kasetsart J.* 45, 90–100
58. Yamane, Y. *et al.* (1997) Influence of oxygen and glucose on primary metabolism and astaxanthin production by *Phaffia rhodozyma* in batch and fed-batch cultures: kinetic and stoichiometric analysis. *Appl. Environ. Microbiol.* 63, 4471–4478
59. Malisorn, C. and Suntornsuk, W. (2008) Optimization of β-carotene production by *Rhodotorula glutinis* DM28 in fermented radish brine. *Bioresour. Technol.* 99, 2281–2287
60. Granado, F. *et al.* (2001) A fast, reliable and low-cost saponification protocol for analysis of carotenoids in vegetables. *J. Food Compos. Anal.* 14, 479–489
61. Dias, C. *et al.* (2015) New dual-stage pH control fed-batch cultivation strategy for the improvement of lipids and carotenoids production by the red yeast *Rhodosporidium toruloides* NCYC 921. *Bioresour. Technol.* 189, 309–318
62. Singh, G. *et al.* (2016) Concomitant production of lipids and carotenoids in *Rhodosporidium toruloides* under osmotic stress using response surface methodology. *Front. Microbiol.* 7, 1686
63. Zhang, C. *et al.* (2016) Combined mutagenesis of *Rhodosporidium toruloides* for improved production of carotenoids and lipids. *Biotechnol. Lett.* 38, 1733–1738
64. Lin, X. *et al.* (2017) Characterization the carotenoid productions and profiles of three *Rhodosporidium toruloides* mutants from *Agrobacterium tumefaciens*-mediated transformation. *Yeast* 34, 335–342
65. Lee, J.J. *et al.* (2016) Engineering *Rhodosporidium toruloides* with a membrane transporter facilitates production and separation of carotenoids and lipids in a bi-phasic culture. *Appl. Microbiol. Biotechnol.* 100, 869–877
66. Hoskins, J. *et al.* (1980) Enzymatic control of phenylalanine intake in phenylketonuria. *Lancet* 315, 392–394
67. Cui, J.D. *et al.* (2014) Biotechnological production and applications of microbial phenylalanine ammonia lyase: a recent review. *Crit. Rev. Biotechnol.* 34, 258–268
68. Gilbert, H.J. *et al.* (1985) Molecular cloning of the phenylalanine ammonia lyase gene from *Rhodosporidium toruloides* in *Escherichia coli* K-12. *J. Bacteriol.* 161, 314–320
69. Calabrese, J.C. *et al.* (2004) Crystal structure of phenylalanine ammonia lyase: multiple helix dipoles implicated in catalysis. *Biochemistry* 43, 11403–11416
70. Wang, L. *et al.* (2005) Structure-based chemical modification strategy for enzyme replacement treatment of phenylketonuria. *Mol. Genet. Metab.* 86, 134–140
71. Gámez, A. *et al.* (2005) Development of pegylated forms of recombinant *Rhodosporidium toruloides* phenylalanine ammonia-lyase for the treatment of classical phenylketonuria. *Mol. Ther.* 11, 986–989
72. Bell, S.M. *et al.* (2017) Formulation and PEGylation optimization of the therapeutic PEGylated phenylalanine ammonia lyase for the treatment of phenylketonuria. *PLoS One* 12, e0173269
73. Pilone, M.S. and Pollegioni, L. (2002) D-amino acid oxidase as an industrial biocatalyst. *Biocatal. Biotransform.* 20, 145–159
74. Fernández-Lafuente, R. *et al.* (1998) The coimmobilization of D-amino acid oxidase and catalase enables the quantitative transformation of D-amino acids (D-phenylalanine) into α-keto acids (phenylpyruvic acid). *Enzyme Microb. Technol.* 23, 28–33
75. Sacchi, S. *et al.* (1998) Determination of D-amino acids using a D-amino acid oxidase biosensor with spectrophotometric and potentiometric detection. *Biotechnol. Tech.* 12, 149–153
76. Zheng, J. *et al.* (2017) Efficient production of D-amino acid oxidase in *Escherichia coli* by a trade-off between its expression and biomass using N-terminal modification. *Bioresour. Technol.* 243, 716–723
77. Pollegioni, L. and Molla, G. (2011) New biotech applications from evolved D-amino acid oxidases. *Trends Biotechnol.* 29, 276–283
78. Meng, X. *et al.* (2009) Biodiesel production from oleaginous microorganisms. *Renew. Energ.* 34, 1–5
79. Bautista, L.F. *et al.* (2012) *Biodiesel from Microbial Oil,* pp. 179–203, Woodhead Publishing
80. Tchakouteu, S.S. *et al.* (2015) Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. *J. Appl. Microbiol.* 118, 911–927
81. Gao, Z. *et al.* (2016) Effect of crude glycerol impurities on lipid preparation by *Rhodosporidium toruloides* yeast 32489. *Bioresour. Technol.* 218, 373–379
82. Lian, J. *et al.* (2013) Fermentation of levoglucosan with oleaginous yeasts for lipid production. *Bioresour. Technol.* 133, 183–189
83. Kitahara, Y. *et al.* (2014) Isolation of oleaginous yeast (*Rhodosporidium toruloides*) mutants tolerant of sugarcane bagasse hydrolysate. *Biosci. Biotechnol. Biochem.* 78, 336–342
84. Vieira, J.P. *et al.* (2014) Microbial lipid production: screening with yeasts grown on Brazilian molasses. *Biotechnol. Lett.* 36, 2433–2442
85. Matsakas, L. *et al.* (2015) High concentrations of dried sorghum stalks as a biomass feedstock for single cell oil production by *Rhodosporidium toruloides*. *Biotechnol. Biofuels* 8, 6
86. Yu, X. *et al.* (2011) Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. *Bioresour. Technol.* 102, 6134–6140
87. Wang, Q. *et al.* (2012) Lipid production from hydrolysate of cassava starch by *Rhodosporidium toruloides* 21167 for biodiesel making. *Renew. Energ.* 46, 164–168

88. Zhao, X. *et al.* (2010) Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. *J. Ind. Microbiol. Biotechnol.* 37, 581–585
89. Uçkun Kiran, E. *et al.* (2013) Microbial oil produced from biodiesel by-products could enhance overall production. *Bioresour. Technol.* 129, 650–654
90. Leiva-Candia, D.E. *et al.* (2015) Biorefining of by-product streams from sunflower-based biodiesel production plants for integrated synthesis of microbial oil and value-added co-products. *Bioresour. Technol.* 190, 57–65
91. Zhou, W. *et al.* (2013) Lipid production by *Rhodosporidium toruloides* Y2 in bioethanol wastewater and evaluation of biomass energetic yield. *Bioresour. Technol.* 127, 435–440
92. Ling, J. *et al.* (2013) Enhancement of lipid productivity of *Rhodosporidium toruloides* in distillery wastewater by increasing cell density. *Bioresour. Technol.* 146, 301–309
93. Ling, J. *et al.* (2014) Lipid production by a mixed culture of oleaginous yeast and microalga from distillery and domestic mixed wastewater. *Bioresour. Technol.* 173, 132–139
94. Ling, J. *et al.* (2016) Cost reduction for the lipid production from distillery and domestic mixed wastewater by *Rhodosporidium toruloides* via the reutilization of spent seed culture medium. *Energy* 13, 135–141
95. Schirmer, A. *et al.* (2010) Microbial niosynthesis of alkanes. *Science* 329, 559
96. Ledesma-Amaro, R. and Nicaud, J.-M. (2016) *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. *Prog. Lipid Res.* 61, 40–50
97. Mata-Gómez, L.C. *et al.* (2014) Biotechnological production of carotenoids by yeasts: an overview. *Microb. Cell Fact.* 13, 12
98. Meadows, A.L. *et al.* (2016) Rewriting yeast central carbon metabolism for industrial isoprenoid production. *Nature* 537, 694–697
99. Pan, J.-J. *et al.* (2015) Biosynthesis of squalene from farnesyl diphosphate in bacteria: three steps catalyzed by three enzymes. *ACS Cent. Sci.* 1, 77–82
100. Ledesma-Amaro, R. and Nicaud, J.-M. (2016) Metabolic engineering for expanding the substrate range of *Yarrowia lipolytica*. *Trends Biotechnol.* 34, 798–809