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Review

Engineering Strategies for Efficient Bioconversion of Glycerol to Value-Added Products by *Yarrowia lipolytica*

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Abstract: *Yarrowia lipolytica* has been a valuable biotechnological workhorse for the production of commercially important biochemicals for over 70 years. The knowledge gained so far on the native biosynthetic pathways, as well as the availability of numerous systems and synthetic biology tools, enabled not only the regulation and the redesign of the existing metabolic pathways, but also the introduction of novel synthetic ones; further consolidating the position of the yeast in industrial biotechnology. However, for the development of competitive and sustainable biotechnological production processes, bioengineering should be reinforced by bioprocess optimization strategies. Although there are many published reviews on the bioconversion of various carbon sources to value-added products by *Yarrowia lipolytica*, fewer works have focused on reviewing up-to-date strain, medium, and process engineering strategies with an aim to emphasize the significance of integrated engineering approaches. The ultimate goal of this work is to summarize the necessary knowledge and inspire novel routes to manipulate at a systems level the yeast biosynthetic machineries by combining strain and bioprocess engineering. Due to the increasing surplus of biodiesel-derived waste glycerol and the favored glycerol-utilization metabolic pathways of *Y. lipolytica* over other carbon sources, the present review focuses on pure and crude glycerol-based biomanufacturing.

Keywords: *Yarrowia lipolytica*; glycerol; biodiesel-derived glycerol; engineering; bio-based products

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1. Introduction

Yarrowia lipolytica is an obligate aerobic heterothallic ascomycetous yeast, originally classified as *Candida lipolytica*, detected in oil-polluted soil, marine and hypersaline environments, and as surface microflora of lipid-rich fermented dairy and meat products [1–4]. In nature is commonly encountered in one of the two haploid mating types (MatA or MatB) that grows as budding cells, pseudohyphae, and true septated hyphae [5], although diploid strains are occasionally isolated [3]. In the lab, morphology alters by manipulating growth conditions, such as carbon source, pH, and medium supplementation [6].

Y. lipolytica, unlike *C. albicans* and *C. tropicalis*, is considered non-pathogenic (classified as GRAS) and most strains do not grow above 32 °C [4]. The most attractive trait of this yeast is its ability to robustly grow on diverse carbon sources (from glucose, fructose, xylose, and glycerol to low-cost hydrophobic substrates) and in a wide range of pH and salinity conditions. Owing to these features, *Y. lipolytica* has been a workhorse for more than 70 years for the biotechnological synthesis of various high value-added bio-products such as lipids, organic acids and sugar alcohols, with eco-friendliness, safety, and high purity [7–15].

Besides its excellent secretory capability, the availability of various genetic tools makes *Y. lipolytica* suitable also for the production of industrially relevant enzymes such as lipases, acid and alkaline proteases, and phosphatases [1,3,8,9,16,17]. *Y. lipolytica* possesses an unusually large genome of 20 Mb compared to other yeasts, which range from 10 to 12 Mb. However, this almost two-fold difference does not correspond to higher gene density, which is significantly lower in *Y. lipolytica* (one gene per 3 kb) than in other yeasts

(e.g., one gene per 2 kb in *Saccharomyces cerevisiae*). As expected from its known secretory functions, the secreted proteins that are involved in the metabolism of lipids and fatty acids are over-represented [2,18]. The average identity level of amino acids between *Y. lipolytica* and other yeasts is approximately 50%, and when proteins are arranged based on common presence or absence, only around 40% are found in other ascomycetes. Compared to *S. cerevisiae*, a number of protein families expand in the *Yarrowia* genome, encoding acyl glycerol lipases, sphingomyelinases-like proteins, α-1,4-glucan glucosidases, alkaline extracellular proteases, choline or allantoate transporters, C-22 sterol desaturase, other cytochrome P450 enzymes or NADPH dehydrogenases [19].

An additional important feature that makes *Y. lipolytica* highly interesting is the remarkable native ability to accumulate large amounts of storage lipids, which on average correspond to 36% *w*/*w* of the biomass [8,9,11,20,21]. With efficient utilization of hydrophobic substrates as carbon source, this lipid accumulation can exceed 50% of the cell dry weight (CDW) [13]. The lipid profile consists of free fatty acids (FFA), 80–90% triacylglycerols (TAG) with different chain lengths (11% C16:0; 6% C16:1; 28% C18:1; 51% C18:2) and a small quantity of steryl esters (SE), located in a specialized cellular compartment known as the lipid body (LB) [15,21,22]. It was reported that when stearin (an industrial product mainly containing palmitic and stearic acid) was exogenously fed, stearic acid (C18:0) could also accumulate intracellularly to significant levels [20]. The lipocentric metabolism of *Yarrowia* has been thoroughly studied, mainly for the production of advanced biofuels and oleochemicals from carbohydrate feedstocks [23,24], but also for the production of lipids with composition similar to various exotic fats (e.g., cocoa butter) [20]. Some crucial metabolic traits that give rise to this oleaginous phenotype include a high acetyl-CoA flux, high tricarboxylic acid (TCA) cycle flux, and absence of undesired fermentative capacity [7,25].

This review presents and discusses advances and developments in the biomanufacturing of commercially important biochemicals using *Yarrowia lipolytica* as a microbial cell factory, highlighting the necessity of integrated strain engineering and bioprocessing strategies not only to enhance target biosynthetic routes but also to identify potential biochemical, genetic, and metabolic factors limiting production. Due to the dynamic growth of the biodiesel industry, which generates an increasing surplus of biodiesel-derived waste glycerol, and combined with the metabolic preference of *Y. lipolytica* for glycerol over other carbon sources, the bioprocesses presented herein valorize either pure or crude glycerol. First, this review provides an overview of biomanufacturing lipids and various bio-products, focusing on how fermentation conditions influence physiological and metabolic phenomena. The focus then is on pathway metabolic engineering strategies, often combined with bioprocess optimization. The aim of this work is to emphasize the huge potential of *Yarrowia lipolytica* as a producer strain of an admirable wide spectrum of biochemicals (lipids, organic acids, polyols, terpenoids, polyphenols, etc.) and also the significance of a productive interplay between stain and bioprocess engineering to achieve the maximization of the biosynthetic metabolic fluxes.

2. Glycerol-Based Biomanufacturing Using *Yarrowia*

In nature, glycerol is a constituent of phospholipids in cell membranes and of TAG found in storage lipids. Many microorganisms can naturally utilize glycerol as the sole carbon and energy source through enzymatic degradation catalyzed by native lipases. In particular, yeasts catabolize glycerol using two different pathways. The first is the phosphorylative glycerol catabolic pathway. In this, glycerol is phosphorylated to glycerol-3-phosphate (G3P) by a glycerol kinase (GK; *GUT1* gene) and then converted to dihydroxyacetone phosphate (DHAP) by a mitochondrial bound G3P dehydrogenase (mGPDH; *GUT2* gene) which acts in conjunction with a freely dispersed cytosolic one (cGPDH; *GPD1*,*2* gene) using $FAD^+/FADH_2$ and $NAD^+/NADH$ as cofactors, respectively. The second pathway includes oxidation of glycerol to dihydroxyacetone (DHA) by an NAD^+-dependent glycerol dehydrogenase (GDH; *GCY1* gene) and, after this, phosphorylation to DHAP

by a dihydroxyacetone kinase (DHAK; *DAK1* gene) [26] (Figure 1). It was reported that overexpression of the *GUT1* gene in *Y. lipolytica* results in rapid glycerol assimilation, which is beneficial for downstream processing [27]. The higher degree of chemical reduction compared to e.g., glucose, sucrose, and starch, makes glycerol a suitable substitute for traditional carbon sources used in various biotechnological processes. Workman et al., quantified the cellular performance during growth on glycerol, glucose or a mixture of both in 1 L batch cultivations. The growth rate was 0.24 h^{-1} and 0.30 h^{-1} when glucose or glycerol was the sole carbon, respectively, with the utilization of the latter causing higher oxygen uptake rates, indicating that the import mainly occurred via phosphorylation into G3P [14].

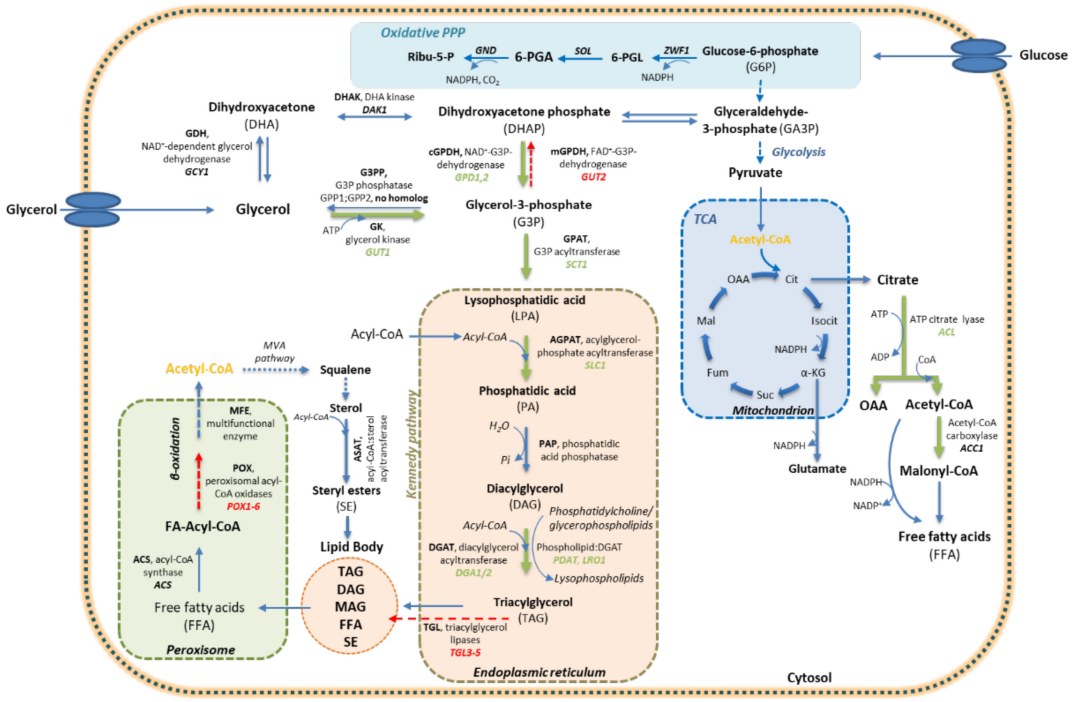

Figure 1. Metabolic pathways and cellular compartments involved in glycerol and glucose metabolism, triacylglycerol (TAG) biosynthesis and degradation. Yeasts catabolize glycerol using two pathways. In the phosphorylative glycerol catabolic pathway, glycerol is phosphorylated to glycerol-3-phosphate (G3P) by a glycerol kinase (GK; *GUT1* gene) and then converted to dihydroxyacetone phosphate (DHAP) by a $FAD^+/FADH_2$-dependent mitochondrial bound G3P dehydrogenase (mGDPH; *GUT2* gene). The second pathway includes oxidation of glycerol to dihydroxyacetone (DHA) by an NAD^+-dependent glycerol dehydrogenase (GDH; *GCY1* gene) and, after this, phosphorylation to DHAP by a dihydroxyacetone kinase (DHAK; *DAK1* gene). The red arrows indicate deleted reactions while the green arrows indicate gene overexpression as discussed in this study. Abbreviations of metabolites are 6-PGL, 6-phosphogluconolactone; 6-PGA, 6-phosphogluconate; Ribu-5-P, ribulose-5-phosphate; Cit, citrate; Isocit, isocitrate; *a*-KG, *a*-ketoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; OAA, oxaloacetate; MAG, monoacylglycerol. Abbreviations of metabolic pathways are TCA, tricarboxylic acid cycle; MVA, mevalonate.

Glycerol of abiotic origin is primarily generated from industrial soap and biodiesel manufacturing, where saponification and transesterification of animal and vegetable fats and oils takes place, respectively [26]. Biodiesel-derived glycerol is produced at a ratio of 1:10 crude glycerol to biodiesel, with a varying concentration of pure glycerol from 70 to 98%, and the remaining containing impurities such as fatty acid methyl esters (FAME), fatty acids (FA), methanol, water, soap and ash. Due to the increasingly frequent exploitation of inexpensive substrates for biodiesel production (e.g., waste frying oils), the surplus of glycerol further hampers the biodiesel market [28–30]. Since pure glycerol is laboriously

obtained via an uneconomic purification process, many biodiesel companies are trying to establish new strategies to valorize this valuable feedstock [13,31–33].

2.1. Bioconversion of Glycerol to Lipids

2.1.1. Lipogenesis in Oleaginous Microorganisms

Oleaginous microorganisms utilize both hydrophilic and hydrophobic substrates and accumulate lipids via two different pathways i.e., *de novo* by metabolizing hydrophilic substrates and *ex novo* by fermenting hydrophobic substrates. The *de novo* FA biosynthesis involves the synthesis of FA precursors, such as acetyl-CoA and malonyl-CoA, their elongation, and finally their integration into the Kennedy biosynthetic pathway to form TAG and SE (Figure 1). In yeast, the *de novo* FA synthesis is catalyzed by the cytosolic multidomain FA synthase and the products consist mainly of C16 and C18 FAs that supply the physiological requirements for membrane phospholipid formation [34]. The Kennedy pathway converts diacylglycerols (DAG) into TAG either with phospholipids by the phospholipid:diacylglycerol acyltransferase (PDAT) or with acyl-CoA by diacylglycerol acyltransferase type 1/2 (DGAT1/2), whereas SEs are formed from acyl-CoA by acyl-CoA:sterol acyltransferases (ASAT) [35]. The Kennedy pathway starts with G3P, which is mainly formed by DHAP reduction. G3P is acylated by G3P acyltransferase (GPAT) to lysophosphatidic acid (LPA), after which a second acyl-CoA provides a FA chain to form phosphatidic acid (PA). PA is then dephosphorylated by a phosphatidic acid phosphatase (PAP) to release DAG. Finally, DAG is acylated either by DAG acyltransferase (DGAT1 with acyl-CoA as an acyl donor) or by phospholipid:DAG acyltransferase (LRO1 with glycerophospholipids as an acyl donor) to produce TAG (Figure 1) [36]. On the other hand, the *ex novo* pathway takes up and transports FA, oils, and TAG from the culture medium, and stores them intracellularly either intact or modified. Due to lipotoxicity which can be caused by the accumulation of FFA and sterols, cells have developed mechanisms to store these lipids in their inert forms as TAG and SE, respectively, in the specialized LB [8,37–39].

More than 90% of *Y. lipolytica* lipids accumulate in TAG synthesized via the *de novo* biosynthetic pathway. In oleaginous microorganisms, *de novo* lipogenesis and accumulation is generally induced when an element in the culture medium becomes limiting and the present carbon source is in excess. The most efficient way to control and induce lipid accumulation is that of nitrogen limitation. When *Y. lipolytica* cells sense nitrogen limitation in their environment, the growth rate rapidly decelerates, while the rate of carbon assimilation gradually decreases, redirecting the carbon flux into lipid synthesis and TAG accumulation within LB [40]. Briefly, under nitrogen limitation, a rapid decrease of intracellular adenosine monophosphate (AMP) takes place due to the activity of the AMP deaminase (AMPD), which temporarily provides NH_4^+. Due to the removal of the AMP allosteric activation, the NAD^+ dependent isocitrate dehydrogenase, which converts isocitric acid to *a*-ketoglutarate in the TCA cycle, loses its activity, thereby leading to intra-mitochondrial citric acid accumulation. When the concentration of this intra-mitochondrial citric acid exceeds a critical value, it is secreted into the cytosol. In oleaginous yeasts, cytosolic citric acid is cleaved by ATP-citrate lyase (ACL, encoded by *ACL1* and *ACL2* genes), a key enzyme for FA biosynthesis, into acetyl-CoA and oxaloacetate, with acetyl-CoA being converted by quasi-inverted β-oxidation to FA [41,42]. The FA biosynthesis continues by acetyl-CoA carboxylase (ACC1), which carboxylates acetyl-CoA to malonyl-CoA, and by malic enzyme (MAE) or the pentose phosphate pathway (PPP), both of which generate the required NADPH (Figure 1) [43,44]. Acetyl-CoA and malonyl-CoA are condensed into FA (Claisen condensation), which are then activated to fatty-acyl-CoA, which is responsible for the acylation of the glycerol backbone to produce TAG [45]. *Y. lipolytica*, unlike some other oleaginous microorganisms, does not possess cytosolic MAE [46]; instead, the PPP generates the required cofactor NADPH by an up-regulation of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconolactonase (6PGL) [47].

Apparently, lipid biosynthesis is a complex phenomenon initiated by the activity of three enzymes, i.e., AMPD, ACL, and ACC, which direct carbon flux from the central

carbon metabolism towards the formation of the FA precursors, acetyl-CoA and malonyl-CoA. However, there are many more enzymes involved, which act in many different cellular compartments and undergo multiple levels of transcriptional, post-translational, and feedback inhibition [47]. In addition, lipogenesis requires energy (ATP), reducing equivalents (NADPH), and glycerol head groups. It is reported that acetyl-CoA, ATP, and NADPH are required at an approximate 1:1:2 ratio [48]. Apart from the aforementioned critical biochemical parameters, the biotechnological production of lipids by oleaginous microorganisms has to overcome many challenges. These are:

1. The allosteric inhibition of the lipid biosynthetic pathway by saturated FA [49];
2. The maximum lipid accumulation capacity;
3. The level of dissolved oxygen which has to sustain specific growth rate and cell density at a stable productive state but also prevent the downregulation of lipid biosynthesis [44];
4. The operational factors which impact both lipid content and FA composition, such as type of carbon source, medium composition, pH, temperature, agitation, and aeration [23,50,51].

In general, the ratio of carbon to nitrogen (C/N), pH, and oxygen saturation in the medium appear to be the key factors which influence lipid accumulation [14].

2.1.2. Pure and Crude Glycerol as Carbon Source for Lipid Production by *Yarrowia lipolytica*

The exploitation of the natural ability of oleaginous microorganisms to intracellularly store considerable amounts of lipids is a vigorous biotechnological research field. *Y. lipolytica* is one of the most valuable and frequently employed oleaginous platforms, which was, however, previously used as a protein rather than single cell oil (SCO) producing yeast (Table 1). In addition, the high *ex novo* lipid productivity and accumulation when FA rich substrates were used brings into question its true oleaginous character. Nonetheless, due to the presence of ACL activity, *Y. lipolytica* is considered an oleaginous yeast possessing specific lipid metabolism divergent from other oleaginous yeasts. The fact that it is one of the few yeasts with a sequenced whole genome offers a very convenient platform for developing novel genetic engineering tools and redesigning the lipid biosynthetic pathway [52].

Table 1. Engineering strategies (strain, medium, process) and cultivation systems using pure or crude glycerol as carbon source for the production of lipids by *Y. lipolytica*.

Strain	Carbon Source [1]	Product	Engineering [2]	Strategy	Set-Up [3]	Data from [Ref.]
ACA-DC 50109	CG	41% *w*/*w* lipids	ME	Mixtures of saturated free fatty acids, CG, and glucose	SF	[20]
A101	CG	40% *w*/*w* lipids	SE; PE	Co-overexpression of *GUT1* and *SCT1*; pH 3.0	SF	[36]
A101	CG	12.5% *w*/*w* lipids	SE; PE	Co-overexpression of *GUT1* and *SCT2*; pH 3.0	B	[36]
SM7	CG	35.80% *w*/*w* lipids; lipase activity 38 U/mL	ME	Addition of surfactants and oils in the medium; pH 6.5	SF	[50]
LGAM 5(7)1	CG	43% *w*/*w* lipids	PE	Highly aerated and agitated continuous nitrogen-limited cultures with glycerol concentration in the feed at different dilution rates; pH 6.0	B	[53]

Table 1. *Cont.*

Strain	Carbon Source [1]	Product	Engineering [2]	Strategy	Set-Up [3]	Data from [Ref.]
A101	CG	25% *w*/*w* lipids	ME	Waste products containing various glycerol concentrations of (42–87%); pH 6.0	SF	[54]
SKY7	CG	45.5% *w*/*w* lipids	ME	Glycerol-supplemented media with pH 6.5 and without pH control	B	[55]
SKY7	CG	29.35% *w*/*w* lipids	ME	Sludge media with different sludge solid concentrations; pH 6.8	SF	[56]
DiSVA 347	CG	25.7% *w*/*w* lipids	SS; ME	Glycerol concentration; C/N ratio and time process; pH 6.0	SF	[57]
JMY4086	CG	40% *w*/*w* lipids	SE; PE	Deletion of *POX1–6* and *TGL4* gene, overexpression of *YlDGA2*, *YlGPD1*, *SUC2*, *HXK1*; oxygenation; inoculum densities; pH 3.5	B	[58]
A101	CG	21% *w*/*w* lipids	SE; ME	*DGA1* overexpression; seawater; pH 3.0	B	[59]
NCYC3825	PG	38% *w*/*w* lipids	SE	Genetic construct with 3 heterologous genes (glycerol dehydratase and its reactivator, wide-spectrum alcohol oxidoreductase) under the control of glycerol-induced promoter; pH 4.0	B	[9]
Polh	PG	23% *w*/*w* lipids	SE	Overexpression of ATP:citrate lyase from *Mus musculus* with the multi-copy integration vector pINA1292sp; pH 6.0	SF	[60]
H222	PG	53% *w*/*w* lipids	SE	One-step double gene knock-in and site-specific gene knock-out; *DGA1* and *DGA2* in a *POX*-deleted background; deletion of the *SNF1* lipid regulator; pH $\geq$ 2.5	B	[61]
JMY3580	PG	53.7% *w*/*w* lipids	SE	*DGA2* gene in Q4 strain (Δ*dga1*Δ*dga2*Δ*lro1*Δ*are1*); pH not regulated	SF	[52]
MUCL 28849	PG	38.1% *w*/*w* lipids	ME; PE	Addition of volatile fatty acids; two-stage fed-batch strategy; pH 5.6	B	[62]

[1] CG, crude glycerol; PG, pure glycerol. [2] SE, strain engineering; ME, medium engineering; PE, process engineering; SS, strain screening. [3] SF, Shake flask; B, Bioreactor.

Biosynthesis of TAG by *Y. lipolytica* is well studied, but most processes utilize glucose, with a pH around 6.0. Glycerol tolerance for oleaginous microorganisms is strain-dependent, and the concentration of carbon source should be fine-tuned to achieve a high

growth rate and lipid yield [41,50]. For instance, to study the kinetics of lipid production in *Y. lipolytica* strain IMUFRJ 50682, Das et al., tested media with varying amounts of pure glycerol as carbon source and a fixed amount of ammonium sulfate (3 g L^{-1}) as nitrogen source. With an increase of the C/N ratio, lipid accumulation increased to 4 g L^{-1}, followed by substrate inhibition. The best lipid formation rate and titer were obtained with an initial glycerol concentration of 93 g L^{-1}. While lipid production was high, citric acid production in the medium was negligible. The authors showed that minimization of citric acid synthesis via appropriate medium development based on nitrogen starvation and supplementation (thiamine and uracil) could increase lipid accumulation in mere batch cultivation without any genetic modification [42]. Similarly, using *Y. lipolytica* SKY7, Kuttiraja et al., searched for the optimal glycerol concentration (34.4 to 168.2 g L^{-1}) and C/N ratio (25 to 150) to maximize lipid production. An initial glycerol concentration of 112.5 g L^{-1}, C/N molar ratio of 100, and 5% v/v inoculum supplementation were optimal for biomass and lipid production. Based on the above optimal parameters, lipid concentration of 43.8% w/w with a biomass concentration of 14.8 g L^{-1} was achieved. The glycerol uptake also increased with the increase in glycerol concentration. At low C/N ratio, glycerol consumption was found to be high (79.43 g L^{-1} on C/N 25) whereas consumption decreased when the C/N ratio was raised to 150 (40.8 g L^{-1}) [63].

In *Y. lipolytica* fermentations, the use of biodiesel-derived crude glycerol without purification was considered tedious due to the impurities contained therein which could influence biochemical pathways and limit the production [64]. Only a few published works describe its use as a sole carbon source and the initial studied products were citric acid and lipids [53,65]. Instead of pure glycerol, Dobrowolski et al., used samples without any prior purification from five different waste products; each contained various concentrations of crude glycerol (42–87%). The best results for lipid production were obtained using glycerol from fat saponification that reached 1.69 g L^{-1} (25% w/w) with a biomass yield of 0.17 g g^{-1} in flasks. Batch cultivation in a bioreactor resulted in enhanced lipid production of 4.72 g L^{-1} with a biomass yield 0.21 g g^{-1}, showing that crude glycerol from soap production could be efficiently converted to SCO by *Y. lipolytica* without any prior purification [54]. In another study that also used crude glycerol, the lipid production by *Y. lipolytica* SKY7 was investigated with and without pH control [55]. The pH influences the cell membrane and concomitantly the carbon assimilation mechanisms. Lipid and citric acid production improved with pH control (pH 6.5) and biomass concentration between the two tested conditions were similar. In the pH-controlled experiments, the biomass and lipid concentration reached 18 and 7.78 g L^{-1} (45.5% w/w), respectively, with a lipid yield of 0.179 g g^{-1} after 60 h. At this time point, lipid degradation was observed in the pH-controlled reactor, whereas this phenomenon occurred after 84 h with uncontrolled pH. Apart from lipids, citric acid was also produced in both fermentations but at a lower concentration in uncontrolled pH. The authors stated that controlling the pH enhances the lipid production by 15% compared to pH-uncontrolled fermentation and favors citric acid production in late fermentation stage [55].

Interestingly, Ram et al., reported that the inhibition by crude glycerol compared to pure glycerol was negligible when they optimized the concentration of wastewater sludge solids mixed with crude glycerol in fermentation media to produce lipids. Their results indicated that 20 g L^{-1} of sludge solids with 40 g L^{-1} of crude glycerol resulted in the highest lipid content, 29.35% w/w, after 96 h [56]. Similarly, a lipid content of 35% w/w was achieved when crude glycerol was supplemented with olive oil in a study that used various surfactants and oils as inducers to enhance lipid production by *Y. lipolytica* [50]. It seems that the utilization of crude glycerol when mixed with other waste materials overcomes the negative effects exerted by the contained impurities and alleviates the scale-up of these fermentation processes [64]. Mixtures of crude glycerol with co-substrates such as saturated FFA also altered the profile of lipids produced by *Y. lipolytica*. Papanikolaou et al., by using mixtures of crude glycerol, stearin, and glucose, emulated the profile of cocoa fatty acids (more than 60% w/w stearic and palmitic acid). The combination of crude glycerol with

stearin not only resulted in higher lipid synthesis than that of glucose and stearin, but the lipids also contained significant amount of stearic acid (50–70% *w*/*w*) and lower amounts of palmitic, oleic, and linoleic acids (15–20; 7–20; 2–7% *w*/*w*, respectively) [20].

2.2. Bioconversion of Glycerol to Various Bio-Products

Apart from lipids, glycerol is also successfully utilized by *Y. lipolytica* for the synthesis of other bulk and fine chemicals and can potentially be used for their industrial production. Citric acid production is a well-studied case (Table 2). Due to the fact that it represents more than 70% *w*/*w* of all food acidulates and that its demand will increase, new renewable substrates are required for its biotechnological production [66]. It was reported that when crude glycerol was used, both *Y. lipolytica* growth and citric acid production were similar to those achieved on glucose, while glycerol uptake rate was higher. Although nitrogen-limited media were employed, growth was not accompanied by production of lipids but instead by citric acid excretion, and when high initial glycerol concentration was applied, citric acid was produced up to 35 g L^{-1} [53]. In another study, fermentations were performed in flask-batch submerged cultures under conditions favoring production of citric acid and/or lipids, i.e., high initial C/N molar ratio and crude glycerol concentration (30 g L^{-1}). Despite the impurities present in the crude glycerol, significant biomass formation and complete glycerol uptake were observed, while considerable quantities (10–12 g L^{-1}) of citric acid were secreted into the medium. In contrast, small quantities of cellular lipids (5–7% *w*/*w*) were produced [66]. By using cheap crude glycerol, Rzechonek et al., also produced citric and isocitric acid at pH 3 to prevent bacterial contamination that is of great importance for industrial processes. Engineered *Y. lipolytica* strain overexpressing *GUT1* and *GUT2* produced citric/isocitric acid at a final total concentration of 75.9 ± 1.8 g L^{-1} after 7 days of batch production, with 42.5 ± 2.4 g L^{-1} isocitric acid, one of the highest concentrations obtained from a waste substrate [67].

Table 2. Engineering strategies (strain, medium, process) and cultivation systems using pure or crude glycerol as carbon source for the production of citric acid by *Y. lipolytica*.

Strain	Carbon Source [1]	Product	Engineering [2]	Strategy	Set-Up [3]	Data from [Ref.]
LGAM S(7)1	CG	35 g L^{-1} citric acid	ME	High C/N media; pH ≥ 5.0	SF	[65]
A101	CG	75.9 g L^{-1} citric/isocitric acids; 42.5 g L^{-1} isocitric acid	SE; ME	Co-overexpression of *GUT1* and *GUT2*; pH 3.0	B	[67]
ACA-YC 5031	CG	54.0 g L^{-1} citric acid; secondary metabolites	ME	Addition of phenolic compounds; different NaCl concentrations; pH 5.0–6.0	SF	[68]
NG40/UV7	CG	112.0 g L^{-1} citric acid	ME; PE	Addition of inorganic nutrients (nitrogen, phosphorus, or sulfur); pulsed addition of glycerol; pH 5.0	B	[69]
Wratislavia AWG7	CG	157.5 g L^{-1} citric acid	SE; PE	Acetate negative mutants; glycerol concentration; pulsed addition or constant feeding rate; pH 5.5	B	[70]
A-101-1.22	CG	112.0 g L^{-1} citric acid	SE; PE	Acetate negative mutants; batch, repeated batch, cell recycle regimes; pH 5.5	B	[71]

Table 2. *Cont.*

Strain	Carbon Source [1]	Product	Engineering [2]	Strategy	Set-Up [3]	Data from [Ref.]
W29	CG	81.11 g L^{-1} citric acid; 24.90 g L^{-1} malic acid	ME	Optimization of the carbon and nitrogen source; pH 4.0	B	[72]
A-101-B56-5	PG	57.15 g L^{-1} citric acid; invertase	SE; ME	SUC^{+} transformants; sucrose, mixture of glucose and fructose, glucose or glycerol; pH 6.8	B	[73]

[1] CG, crude glycerol; PG, pure glycerol. [2] SE, strain engineering; ME, medium engineering; PE, process engineering; SS, strain screening. [3] SF, Shake flask; B, Bioreactor.

The efficient assimilation of glycerol by *Y. lipolytica* was also demonstrated for the production of erythritol, a sugar alcohol used as a low-calorie sweetener. Tomaszewska et al., used pure and crude glycerol for citric acid and erythritol biosynthesis using wild type and acetate negative mutants in fermentations with varying pH values (3.0–6.5). They observed that lower pH inhibited citric acid and simultaneously enhanced erythritol formation, while, interestingly, the addition of NaCl to the medium as well as the use of crude glycerol (containing salt) also enhanced erythritol production. They stated that the activity of enzymes involved in erythritol biosynthesis (i.e., enzymes of PPP, transketolase and erythrose reductase) was increased by low pH and NaCl presence in the medium [74]. In a recent study, 13 species belonging to the *Yarrowia* clade were isolated from different biotic and abiotic environments (e.g., food, seawater, termite intestine) and tested for erythritol, mannitol and arabitol production using crude glycerol from the biodiesel and soap industries. *Yarrowia divulgata* and *Candida oslonensis* turned out to be particularly efficient polyol producers, producing large amounts from both soap-derived glycerol (59.8–62.7 g L^{-1}) and biodiesel-derived glycerol (76.8–79.5 g L^{-1}). It is equally important that the protein and lipid contents of their biomass (around 30% and 12%, respectively) were also high enough to be considered suitable for animal feed [75]. Medium engineering, examining various parameters affecting erythritol production from glycerol by *Y. lipolytica* Wratislavia K1, was performed by Rywińska et al., Ammonium sulfate, monopotassium phosphate, and sodium chloride were identified as critical medium components that affect the ratio of polyols produced, with optimized concentrations being 2.25, 0.22, and 26.4 g L^{-1}, respectively. The optimum C/N ratio was found to be 81:1. In the optimized medium with 100 g L^{-1} crude glycerol, the strain produced 39.3 g L^{-1} erythritol, which corresponded to 0.40 g g^{-1} yield [76].

For fodder yeast production, Juszczyk et al., similarly compared the biomass production of 21 *Y. lipolytica* strains isolated from different environments grown on pure and crude glycerol. After analysis of technological process parameters and biomass chemical composition, they identified a strain as most suitable for biomass production that gave 11.7 and 12.3 g L^{-1} biomass with 1.30 and 1.37 g L^{-1} h^{-1} productivity, respectively, when pure and crude glycerol (25 g L^{-1}) was used. Surprisingly, higher biomass production was achieved on biodiesel-derived crude glycerol even at low pH (under pH 4.0), thus, additionally preventing bacterial contamination [77].

Biodiesel-derived crude glycerol was tested also for the synthesis of pyruvic acid that is widely used in the production of polymers, pharmaceuticals, and food additives. The selected strain *Y. lipolytica* VKM Y-2378 synthesized pyruvic acid, accompanied by *a*-ketoglutaric acid as by-product that was minimized by medium engineering (i.e., growth limitation by thiamine, ammonium sulfate as nitrogen source, addition of glycerol, 28 °C, pH 4.5, and aeration between 55 and 60% saturation). The selected strain, cultivated under optimal conditions in a bioreactor with crude glycerol, synthesized 41 g L^{-1} pyruvic acid with a yield of 0.82 g g^{-1} [78].

Finally, the exploitation of glycerol and its superiority compared to glucose as a carbon source for the production of isoprenoids (e.g., β-carotene and limonene) is highlighted by other studies [79]. For readers interested in this topic, the recent reviews by Ma et al., 2019, Arnesen et al., 2020, and Zhang et al., 2022 are highly recommended [80–82].

3. Pathway Metabolic Engineering Strategies to Improve Glycerol-Based Bioprocesses

The genomic and metabolic traits of *Y. lipolytica* facilitated metabolic engineering efforts and established the yeast as a robust platform for optimizing existing pathways and introducing new ones for cost-effective production of chemicals and fuels. Using metabolically engineered *Y. lipolytica* strains, DuPont (Wilmington, DE, USA) patented and commercialized the production of the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (US2009/0093543A1). A few years later, the engineering strategy was described in detail [83]. Carotenoids are also industrially produced by engineered *Y. lipolytica*. This technology was developed by Microbia (Lexington, MA, USA) and then obtained by DSM (Heerlen, The Netherlands) [84]. The engineering strategies applied to enhance the production of high value-added compounds mainly focus on: (i) increasing the availability of biosynthetic precursors; (ii) increasing the availability of redox and energy equivalents; (iii) shutting down consuming/degradation pathways; and (iv) removing inhibitory intermediates. These strategies are often coupled with optimization of the process conditions to maximize the performance of the yeast and concomitantly also productivity and yield of the bioprocess.

3.1. Lipids

Given that SCOs can serve as functional oils and as feedstock for the biodiesel industry, SCO production by *Y. lipolytica* takes the lion's share of the research conducted. Engineering strategies of *Y. lipolytica* to improve lipid production focus on enhancing the lipogenic pathway flux, shutting down lipolysis and β-oxidation, and increasing NADPH supply [85,86]. However, the identification of gene targets is challenging due to the complexity of lipid synthesis that is subject to multiple layers of regulation. The study of Silverman et al., demonstrated that the overall kinetics of lipid synthesis is susceptible to a plethora of factors that should be properly balanced to fully optimize the SCO biosynthesis. Working with glucose and acetate as a sole carbon source, they examined whether the overexpression of 44 native genes involved in glycerolipid synthesis, FA synthesis, central carbon metabolism, NADPH generation, regulation, and metabolite transport enhances lipid production in *Y. lipolytica*. Their results showed that a diverse subset of genes was effective at individually influencing lipid production, sometimes in a substrate-dependent manner. In the case of glucose, the most productive strain overexpressed the diacylglycerol acyltransferase (DGAT) *DGA2* gene, increasing lipid titer, cellular content, and yield by 236, 165, and 246%, respectively, compared to the control strain. On acetate, the acylglycerol-phosphate acyltransferase (AGPAT) *SLC1* gene was overexpressed, leading to increased lipid titer, cellular content, and yield by 99, 91, and 151%, respectively. Increased lipogenesis was attributed to the overexpression of genes encoding enzymes that directly catalyze lipid synthesis, and genes such as the G3P dehydrogenase (GPDH) *GPD1* that produces glycerol head groups and the 6-phosphogluconolactonase (6PGL) *SOL3* from the oxidative PPP that increases NADPH availability for FA synthesis [47].

3.1.1. TAG Biosynthetic Pathway

Beopoulos et al., redirected carbon flux toward lipid synthesis in *Y. lipolytica* W29 by inactivating the oxidation of G3P into DHAP (*GUT2* deletion), thus increasing G3P availability for lipogenesis via the Kennedy pathway that synthesizes TAG by an initial acylation of the G3P backbone with an acyl-CoA (Figure 1). The additional deletion of *POX1-6* genes (Δ*pox1-6*) which catalyze the limiting step of peroxisomal β-oxidation led to a four-fold increase in lipid content, reaching 42% of CDW. However, the Δ*gut2* strain did not grow on glycerol as the sole carbon source, probably because it is not likely that another enzyme catalyzes DHAP formation from G3P in glycerol medium [87]. To

further investigate the role of the G3P shuttle in lipid accumulation, Dulermo et al., used different mutations in the G3P shuttle pathway (*GPD1* overexpression or *GUT2* deletion) to manipulate the G3P concentration. They showed that in a Po1d genetic background (Leu$^-$ Ura), *GPD1* overexpression, *GUT2* inactivation or both mutations together increased G3P 1.5-, 2.9-, and 5.6-fold, respectively, increasing TAG accumulation. They also showed that each strain with an increased G3P concentration also had a decreased glycerol concentration. Analysis of the genes involved in glycerol metabolism revealed that *Y. lipolytica* does not possess a gene for glycerol-3-phosphatase (*GPP1*, *GPP2*), thus indicating that G3P synthesis competes with glycerol synthesis and that a distinctive glycerol metabolism is committed to G3P and TAG synthesis, possibly contributing to its oleaginous character. Similarly, G3P shuttle dysfunctions coupled to an inactive *β*-oxidation pathway (deletion of *POX1-6* or *MFE1* genes) increased TAG and FFA. Transcriptional analysis of these strains revealed that the high levels of lipids (up to 65–75% of CDW) resulted from overexpression of genes involved in TAG synthesis (*SCT1* encoding G3P acyltransferase, *DGA1* encoding acyl-CoA diacylglycerol acyltransferase) and the repression of genes involved in TAG degradation (*TGL3* and *TGL4*) (Figure 1). Their findings suggest that the availability of G3P and FFA limits TAG synthesis, and that the G3P shuttle and the *β*-oxidation pathway regulate the expression of genes involved in TAG homeostasis. They conclude that the synergistic contribution of acyltransferase gene expression to G3P synthesis is required for high levels of TAGs and lipid accumulation in *Y. lipolytica* [88]. The amplification of FFA production was also achieved by engineering *Y. lipolytica* W29 to uncouple the G3P shuttle and eliminate glycerol metabolism. Under lipogenic conditions with a limiting supply of glycerol, the triple mutant strain Δ*gpd1*Δ*gut2*Δ*pex10* redirected the carbon influx towards the synthesis of unsaturated FA that accumulated in the culture medium up to 2.03 g/L [89]. For additional reading regarding TAG biosynthesis through esterification of FA to glycerol backbone in which two precursors, FA and G3P, are required, the review by Xue et al., is strongly recommended. This review describes the interplay among glycerol metabolism, G3P synthesis and lipid production shedding light on how G3P affects lipid production by modulating glycerol metabolism [90].

In the study conducted by Gajdoš et al., *Y. lipolytica* JMY3580 was constructed by overexpressing the *DGA2* gene in the previously engineered quadruple Δ*dga1*Δ*dga2*Δ*lro1*Δ*are1* strain to increase lipid accumulation. In *Y. lipolytica*, three diacylglycerol acyltransferases encoded by *LRO1*, *DGA1*, and *DGA2* genes are involved in lipid formation (Figure 1). Mutant strains were successively obtained by disrupting one or more genes in the wild-type Po1d strain to construct Δ*lro1*, Δ*dga1*, Δ*are1*, Δ*dga2*, Δ*dga2*Δ*are1*, Δ*dga1*Δ*lro1*Δ*are1*, and the quadruple Δ*dga1*Δ*dga2*Δ*lro1*Δ*are1*. With the single deletions they studied the impact of the loss of a single acyltransferase gene for TAG and/or SE accumulation, whereas with the double and triple deletions, the functions of ARE1 (acyl-CoA:sterol acyltransferase) and DGA2. Finally, the quadruple mutant was employed to study whether other genes possibly contribute to lipid synthesis. The JMY3580 strain, with a reconstructed TAG synthesis pathway, accumulated over 40% of lipids in biomass, while in wild type strain did not exceed 20% on a glycerol-based medium (C/N 90). In fed-batch grown cells, when glycerol was added during cultivation, lipid accumulation above 50% was achieved. Fatty acid composition was also altered when DGA2 was the only diacylglycerol acyl-transferase, with an especially low percentage of linoleic acid present [52].

Engineering the TAG biosynthetic pathway can also be performed to tailor-make the lipid pool, owing to the different FA composition required by each application. Dobrowolski et al., enhanced the lipid synthesis from glycerol (both pure and crude) by overexpressing the *GUT1, SCT1*, and *DGA1* genes (Figure 1). They found that *SCT1* overexpression resulted in a 10-fold increase in linoleic acid (18:2) synthesis and improvement of the polyunsaturated FA pool, while co-expression of *GUT1* and *SCT1* yielded high production of mono- and polyunsaturated FA (MUFA and PUFA) from crude glycerol. Low pH (3.0) was the most suitable for TAG biosynthesis using crude glycerol. When *SCT1* was overexpressed, a high content of PUFA was accumulated, mainly linoleic acid (C18:2), with

more than 20% in the total FA pool in the shake-flask study. Overexpression of the *DGA1* gene redirected the synthesis of FA to stearic acid (C18:0) and resulted in high production both at pH 6.0 and 3.0. Simultaneous overexpression of *GUT1* and *SCT1* yielded a three-fold improvement in C18:2 synthesis from crude glycerol and at low pH [36].

3.1.2. Precursor Metabolites

Apart from G3P and FFA, acetyl-CoA is also a key lipid biosynthetic precursor with which the *de novo* FA synthesis starts [91]. In *Y. lipolytica*, acetyl-CoA can be generated in cytosol, mitochondria and peroxisome [92]. When *Y. lipolytica* senses nutritional limitation (e.g., nitrogen starvation with an excess of glycerol), citric acid accumulates in mitochondria and then is secreted to the cytosol. Cytosolic citric acid is then converted to acetyl-CoA and oxaloacetate by ATP-citrate lyase (ACL) (Figure 1) [91], with the former providing the first two carbons for the *de novo* FA synthesis. The FA chain is then being elongated via the addition of malonyl-CoA (endoplasmic reticulum) or acetyl-CoA units (mitochondria). Malonyl-CoA is generated by acyl-CoA carboxylase (*ACC1*), while the mitochondrial acetyl-CoA is generated by the breakdown of accumulated lipids via the β-oxidation pathway [93]. Though ACL converts citrate to acetyl-CoA for lipid accumulation, *Y. lipolytica* secretes most of the citrate into the culture medium so that the *de novo* lipids synthesis in wild type strains is limited, and most of them accumulate less than 20% *w*/*w* lipids [60].

In most oleaginous yeasts, ACL and malic enzyme (MAE) provide for FA synthesis, acetyl-CoA and NADPH, respectively. In the study of Dulermo et al., the corresponding genes were inactivated in *Y. lipolytica* W29 strains to analyze the role of these key enzymes in FA synthesis. Inactivation of *ACL1* decreased FA synthesis by 60 to 80%, corroborating its key role in FA synthesis; while, the inactivation of *MAE1* had no effect, excluding MAE as a major key enzyme for FA synthesis [94]. Similar conclusions were reported in the study of Zhang et al., who expressed in *Y. lipolytica* an *ACL* gene from *Mus musculus* (domestic mouse) using the mono-copy pINA1312sp and the multi-copy pINA1292sp integration vectors. The authors chose this heterologous gene due to its low Km value for citrate (about 0.05 mM) after their observation that the affinity of ACL for citrate significantly differs among oleaginous and non-oleaginous microorganisms. In the oleaginous *Lipomyces starkeyi*, which reaches 65% lipid content, the Km value for citrate is 0.07 mM; however, in the non-oleaginous *Aspergillus niger*, a commercial citrate producer, Km value is 2.5 mM. In their experiments with *Y. lipolytica*, a high Km value (3.6 mM) was determined, probably explaining why *Yarrowia* can produce both fatty acid and citrate, and why homologous overexpression of *ACL* did not improve lipid accumulation. In addition, they supported the supposition that an ACL with a low Km value may be effective for cleaving citrate, as it would then provide more acetyl-CoA for FA synthesis rather than the majority of the citrate being secreted into the culture medium. The expression of *ACL* gene from *Mus musculus* enhanced lipid content from 7.3% to 11–23% *w*/*w* of the CDW, while growth was slightly affected. The amount of the secreted citrate decreased by 32%, in a transformant with the highest ACL activity, suggesting that a low Km greatly enhances acetyl-CoA formation for lipid accumulation in *Y. lipolytica* [60].

Even though many engineering efforts have focused on increasing the rate of FA synthesis and the supply of pathway precursors, none of them dealt with one major bottleneck of FA flux, i.e., the allosteric inhibition of FA biosynthetic enzymes by saturated FA. The work by Qiao et al., identified a novel metabolic regulator through the reverse engineering of mammalian fat-storing tissue, i.e., the delta-9 stearoyl-CoA desaturase (SCD). The overexpression of this regulator enriched the intracellular monosaturated FA which in turn promoted both cell growth and FA sequestrations into neutral lipids. Simultaneous overexpression of the lipogenic *SCD*, *ACC1*, and *DGA1* in *Y. lipolytica* yielded an engineered strain with fast cell growth and lipid overproduction, including high carbon to lipid conversion yield (84.7% of theoretical maximal yield), high lipid titers (55 g L^{-1}), enhanced tolerance to glucose and cellulose-derived sugars. Using 1.5 L bioreactor and crude glycerol without prior detoxification step to remove fatty-soap and methanol (150 g L^{-1} crude glycerol),

the engineered strain YL-ad9 strain displayed excellent growth and lipid production, in contrast to the wild type, which barely grows in high glycerol concentrations. After 84 h, glycerol was completely depleted, the lipid concentration reached 35 g L^{-1}, and overall TAG production yield was 0.23 g g^{-1} [49].

3.1.3. Engineering Redox and Energy Equivalents

Engineering redox and energy equivalents is also a strategy to increase lipogenesis in *Y. lipolytica*. NADPH is mainly generated via the PPP [25,40,95]. In addition, the MAE catalyzed decarboxylation of malate to pyruvate is proposed as another important NADPH generator. Zhang et al., however, did not observe any significant improvement in NADPH regeneration in *Y. lipolytica* overexpressing the *MAE* gene, suggesting the presence of alternative routes [46]. Given that NADPH can limit FA synthesis, it was suggested that capturing most of the electrons generated from substrate catabolism could possibly increase the FA conversion yield, and therefore increase substrate-to-product yields [85]. To achieve this goal, Qiao et al., employed the conversion of NADH to NADPH by engineering 13 strains of *Y. lipolytica* with synthetic pathways that convert glycolytic NADH into the lipid biosynthetic precursors NADPH or acetyl-CoA. The three synthetic pathways tested were:

1. The $NADP^+$-dependent GPD (cofactor switch by using $NADP^+$-dependent GPDs i.e., GapC from *Clostridium acetobutylicum* and GPD1 from *Kluyveromyces lactis*);
2. The pyruvate/oxaloacetate/malate which that converts 1 mol NADH to 1 mol NADPH at a cost of 1 mol ATP (by using the $NADP^+$-dependent malic enzyme MCE2 from *Mucor circinelloides*);
3. The non-oxidative glycolytic pathway which yields 3 mol acetyl-CoA from 1 mol glucose, bypassing the Embden-Meyerhof-Parnas (EMP) pathway (by co-expressing phosphoketolase from *Leuconostoc mesenteroides* and phosphate acetyltransferase from *Clostridium kluyveri*).

Their established quantitative model pinpointed the yield of the lipid pathway as a crucial factor that determines the overall process yield. The best-engineered strain achieved a productivity of 1.2 g L^{-1} h^{-1} and a process yield of 0.27 g fatty acid methyl esters/g glucose that is 25% improvement compared to previously engineered strains. Due to the decreased NADH oxidization by aerobic respiration, the oxygen requirements of the highest producer were reduced [85].

The work of Silverman et al., evaluated three potential NADPH sources that could contribute to fatty acid synthesis, i.e., the oxidative PPP, a cytosolic specific $NADP^+$ isocitrate dehydrogenase, and a $NADP^+$ specific glutamate dehydrogenase. As mentioned above, the overexpression of the only native malic enzyme isoform, MAE1, was shown to have no effect on the lipid synthesis in *Y. lipolytica*. It is suggested that in *Y. lipolytica* the MAE1 may be specific mainly for NAD^+ and not $NADP^+$. *SOL3* (6-hosphogluconolactonase), catalyzing the intermediate step of the oxPPP, was the only gene of this group that significantly enhanced lipid synthesis when overexpressed. Specifically, *SOL3* overexpression increased the lipid titer, content, and yield on glucose by 23%, 18%, and 27%, respectively, over the control strain. It should be noted, that in the oxPPP, SOL3 does not generate NADPH, but the reactions before and after generate, catalyzed by *ZWF1* and *GND1,* respectively. When using acetate as carbon source, *SOL3* overexpression significantly increased the lipid content (97% over control), but dramatically reduced growth rate; thus, yielding a lower overall titer. It seems that on acetate, *SOL3* overexpression directs carbon flux into lipids synthesis and not into non-lipid biomass formation. Interestingly, the overexpression of oxPPP NADPH producing genes did not significantly increase lipid production [47].

The work of Park et al., studied how synergistic co-feeding of carbon sources which generate ATP and NADPH can advance bio-product synthesis, overcome undesirable substrate preferences and improve carbon reduction. Their study's concept was that the direct access of substrate mixtures to multiple pathways might optimize the biosynthetic requirements balancing carbon, ATP, and reducing agents. The controlled co-feeding of 'dopant' substrates

was applied to two divergent organisms i.e., *Moorella thermoacetica* and *Y. lipolytica.* More specifically, glucose doping in *M. thermoacetica* triggered CO_2 reduction (2.3 g g_{CDW}^{-1} h^{-1}) into acetate by boosting ATP synthesis via pyruvate kinase, whereas doping *Y. lipolytica* with gluconate accelerated acetate-driven lipogenesis (0.046 g g_{CDW}^{-1} h^{-1}) by obligatory NADPH synthesis via the PPP. With this synergistic co-feeding, the CO_2-derived lipids were produced with 38% energy yield, demonstrating the great potential of CO_2 conversion into valuable bioproducts. *Y. lipolytica* was then cultured on acetate in a fed-batch set-up where limiting quantities of glucose, fructose, glycerol or gluconate were continuously fed as 'dopant' substrates to intensify reductive metabolism. The cells simultaneously consumed acetate and the supplemented carbon, with acetate remaining the primary carbon source. When gluconate was used for doping, the lipogenesis rate was two times faster than that of the acetate-only control. Experiments with ^{13}C gluconate revealed that the obligatory NADPH synthesis via a recursive oPPP was responsible for the observed synergy with acetate [48].

3.2. Polyols

Sugar alcohols or polyols (e.g., mannitol, erythritol, xylitol, sorbitol, maltitol, lactitol and isomalt) are a group of noncyclic hydrogenated carbohydrates finding applications in the food and pharmaceutical industries, and as building blocks for the synthesis of value-added compounds, such as polymers (e.g., polyurethane foam). The wide variety of applications renders them valuable chemicals with constantly increasing demand. Polyols, apart from being a carbon source for many microorganisms, also take part in the regulation of coenzymes and in osmoregulation. Owing to these metabolic functions, many bacteria, fungi and yeasts naturally produce polyols. For readers further interested in the conversion of renewable feedstocks to sugar alcohols by yeasts, the recent review of Erian and Sauer [96] is highly recommended. *Y. lipolytica* has been successfully used for the production of erythritol (1,2,3,4-butanetetrol) (Table 3), i.e., a sugar alcohol with sweetening properties used as a food additive (E968), and various metabolic engineering strategies have been proposed.

Table 3. Engineering strategies (strain, medium, process) and cultivation systems using pure or crude glycerol as carbon source for the production of polyols by *Y. lipolytica*.

Strain	Carbon Source [1]	Product	Engineering [2]	Strategy	Set-Up [3]	Data from [Ref.]
Wratislavia K1	CG	40.7 g L^{-1} erythritol; 65 g L^{-1} citric acid	SE; ME	Acetate negative mutants; pH 3.0 for erythritol, pH 5.5/6.5 for citric acid; NaCl	B	[74]
Y. divulgata	CG	59.8–62.7 g L^{-1} polyols from soap-derived; 76.8–79.5 g L^{-1} from biodiesel-derived glycerol	SS	13 tested species of the *Yarrowia* clade; pH 3.0	B	[75]
Wratislavia K1	CG	100.65 g L^{-1} polyols	SE; PE	Overexpression of heterologous *SUC2* and native *GUT1;* step-wise fed-batch culture with molasses and CG; pH 3.0	B	[97]
Wratislavia K1	CG	110 g L^{-1} citric acid; 81 g L^{-1} erythritol	SE; PE	Acetate negative mutant; fed-batch; pH 5.5	B	[98]

Table 3. *Cont.*

Strain	Carbon Source [1]	Product	Engineering [2]	Strategy	Set-Up [3]	Data from [Ref.]
Wratislavia K1	CG	170 g L^{-1} erythritol	SE; ME	Acetate negative mutant; effect of pH (2.5–6.5); pH 3.0	B	[99]
A UV'1; A-15; Wratislavia K1	CG	80 g L^{-1} erythritol (Wratislavia K1); 27.6 g L^{-1} Mannitol (A UV'1, A-15)	SS; ME	Addition of NaCl; pH 3.0	B	[100]
LFMB 19	CG	7.1 g L^{-1} mannitol	SS	Eukaryotic microbial strains (yeasts and Zygomycetes molds); pH 5.0–6.0	SF	[101]
2,021,417	CG	150 g L^{-1} erythritol	SE	Overexpression of *GUT1*, *GUT2*, and *TKL1;* Deletion of *EYD1*; pH 3.0	B	[102]
Wratislavia K1	PG	132 g L^{-1} erythritol	ME	Concentrations of ammonium sulfate, monopotassium phosphate, and sodium chloride; C/N ratio; pH 3.0	B	[76]
A101	PG	63.9 g L^{-1} citric ; 93 g L^{-1} citric ; 78 g L^{-1} erythritol	SE	Co-overexpression of *GUT1* and *GUT2*; effect of pH (3.0 or 6.0)	B	[27]
MK1	PG	58 g L^{-1} erythritol; 62.5 g L^{-1} erythritol	SE	Overexpression of *TKL1*, *TAL1*, *ZWF1*, and *GND1*; pH 3.0	SF; B	[103]
Po1d	PG	80.6 g L^{-1} erythritol	SE	Overexpression of *GUT1* and *TKL1;* disruption of *EYK1*; pH 3.0	B	[104]
Wratislavia K1	PG	201.2 g L^{-1} erythritol	ME; PE	Addition of vitamins and nitrogen sources; batch and fed-batch; pH 3.0	B	[105]

[1] CG, crude glycerol; PG, pure glycerol. [2] SE, strain engineering; ME, medium engineering; PE, process engineering; SS, strain screening. [3] SF, Shake flask; B, Bioreactor.

Erythritol synthesis from glycerol is not redox-balanced due to the net amount of the required oxidized cofactors. However, it is more advantageous than using glucose since erythritol synthesis from glucose consumes redox cofactors that must be replenished by glucose oxidation. When glycerol is used, DHAP is converted by a triosephosphate isomerase (TIM) into glyceraldehyde-3-phosphate (GA3P), which enters into the PPP, where a transketolase (TK) converts it into erythrose-4-phosphate (E-4-P). E-4-P is then dephosphorylated by an erythrose-4-P phosphatase (E4PP) and reduced to erythritol by an erythrose reductase (ER) with NAD(P)H oxidation (Figure 2) [104]. Specific ER(s) catalyze the last step of erythritol synthesis. In the work of Janek et al., a native ER gene (YALI0F18590g) from erythritol-producing yeast *Y. lipolytica* MK1 was identified, overexpressed and characterized. Gene overexpression increased erythritol titer up to 44.44 g L^{-1} (20% over the control), yield on glycerol to 0.44 g g^{-1}, and productivity to 0.77 g L^{-1} h^{-1}. However, low erythritol production was still determined after gene deletion, indicating that other ER(s) might exist in the genome

of *Y. lipolytica*. In addition, the authors pointed out the need to elevate NAD(P)H pool via engineering (overexpression of PPP genes), as ER requires NAD(P)H to reduce erythrose into erythritol [106].

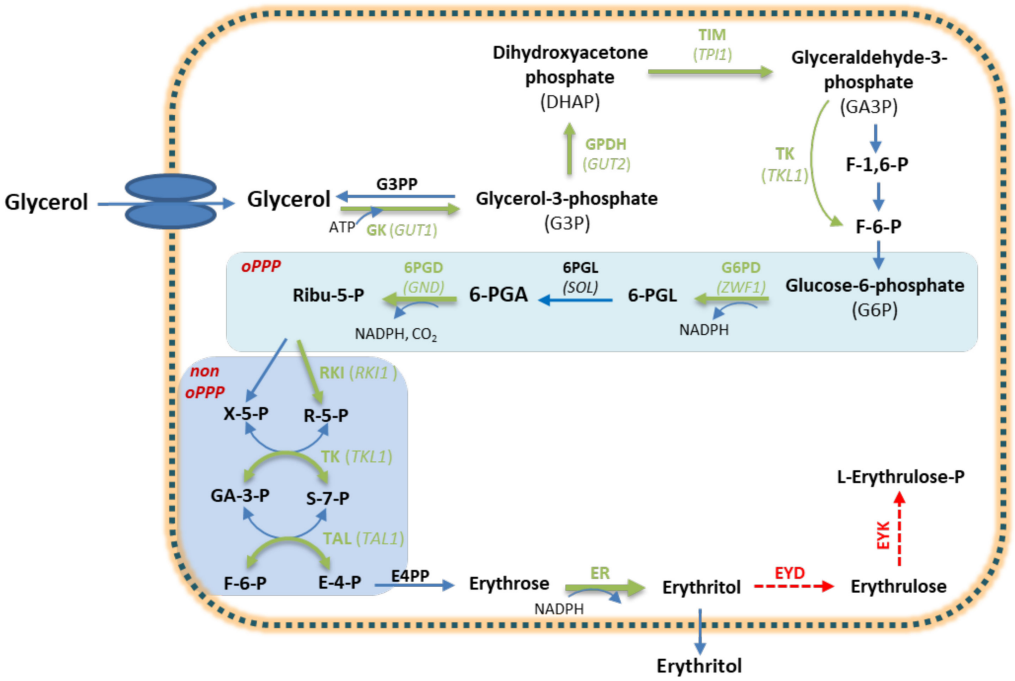

Figure 2. Biosynthetic pathway of erythritol in *Y. lipolytica* using glycerol. The enzymes encoded by corresponding genes are listed as follows: G3PP, G3P phosphatase; GK, glycerol kinase (*GUT1*); GPDH, G3P-dehydrogenase (*GUT2*); TIM, triose-phosphate isomerase (TPI1); TK, transketolase (*TKL1*); G6PD, glucose 6-phosphate dehydrogenase (*ZWF1*); 6PGL, 6-phosphogluconolactonase (*SOL*); 6PGD, 6-phosphogluconate dehydrogenase (*GND1*); RKI, ribose 5-phosphate isomerase (*RKI1*); TAL, transaldolase (*TAL1*); E4PP, erythrose 4-phosphate phosphatase; ER, erythrose reductase; EYD, erythritol dehydrogenase; EYK, erythrulose kinase. Abbreviations of metabolites are: F-1,6-P, fructose-1,6-bisphosphate; F-6-P, fructose-6-phosphate; 6-PGL, 6-phosphogluconolactone; 6-PGA, 6-phosphogluconate; Ribu-5-P, ribulose-5-phosphate; X-5-P, xylulose-5-phosphate, R-5-P, ribose-5-phosphate; GA-3-P, Glyceraldehyde 3-phosphate S-7-P, sedoheptulose-7-phosphate; E-4-P, erythrose-4-phosphate. The red arrows indicate deleted reactions while the green arrows gene overexpression as discussed in this study.

Using low-cost feedstocks such as raw beet molasses and crude glycerol, Rakicka et al., engineered *Y. lipolytica* and evaluated erythritol production. They overexpressed the *S. cerevisiae* SUC2 gene and the native *GUT1* gene in the Wratislavia K1 strain to allow for (i) sucrose assimilation by the mutant strain (wild type strains lack invertase activity being unable to hydrolyze sucrose), and (ii) efficient glycerol utilization due to the *GUT1* overexpression. By applying a step-wise fed-batch culture with molasses (60 g L^{-1}) added at the beginning of the process for yeast growth, and crude glycerol (150 g L^{-1}) added after 24 h of culture for polyol production, they reached a maximum polyol production of 101 ± 4 g L^{-1}, with productivity 1.1 ± 0.9 g L^{-1} h and yield 0.67 ± 0.2 g g^{-1} [97]. Similarly, Mirończuk et al., enhanced glycerol assimilation by co-overexpressing *GUT1* and *GUT2*, resulting in rapid erythritol and citric acid synthesis. The engineered strains were able to utilize 150 g L^{-1} glycerol within 44–48 h in a 5 L bioreactor. Erythritol productivity for *GUT1* overexpression and co-expression of *GUT1* and *GUT2* was 24 and 35%, respectively, over the control strain. Gene co-expression enhanced erythritol productivity from glycerol up to 1.08 g L^{-1} h^{-1} and also the production of citric acid from 4.4 to 64 g L^{-1} (0.69 g L^{-1} h^{-1}) [27]. Using a mutant that overexpressed *GUT1* and *TKL1*, and in which *EYK1* encoding erythrulose kinase (erythritol catabolism) was disrupted, Carly et al., increased erythritol productivity 75% compared to the wild type. Furthermore,

the culturing time needed to achieve maximum concentration reduced by 40%. Due to *EYK1* deletion, the strain was unable to consume the synthesized erythritol, further increasing the process efficiency [104]. On the other hand, Zhang et al., proposed a multiple gene integration strategy to efficiently improve the production of erythritol in *Y. lipolytica* from glycerol. For that, seven genes which are involved in the erythritol synthesis pathway were individually evaluated, with the *TKL1* and *TAL1* genes playing important roles in enhancing erythritol production. The co-expression of four genes (*GUT1*, *TPI1*, *TKL1*, *TAL1*), together with the disruption of the *EYD1* gene (encoding erythritol dehydrogenase), resulted in approximately 40 g L^{-1} erythritol from glycerol. Further enhanced erythritol synthesis was achieved by overexpressing the *RKI1* gene (encoding ribose 5-phosphate isomerase) and the *AMPD* gene (encoding AMP deaminase), suggesting for the first time that these two genes also determine erythritol production in *Y. lipolytica* [107].

Osmophilic microorganisms in response to osmotic stress also produce erythritol [108]. Osmotic stress combined with heterologous expression of sugar alcohol phosphatase (*PYP*, YNL010W) from *S. cerevisiae* also increased sugar alcohol production in *Y. lipolytica*. It was found that osmotic stress increased the expression of genes involved in glycerol catabolism and the PPP, while intracellular metabolites such as amino acids, sugar alcohols, and polyamines produced at higher levels. Overexpression of the heterologous *PYP* gene increased both erythritol production and glycerol consumption, suggesting that this new enzyme creates an orthogonal route for increasing production. The expression of genes encoding the native glycerol kinase and transketolase further increased erythritol production. This strain was able to produce 27.5 $\pm$ 0.7 g L^{-1} and 58.8 $\pm$ 1.68 g L^{-1} erythritol from glycerol during batch and fed-batch growth in shake-flask experiments, respectively. Additionally, the glycerol utilization was increased by 2.5-fold. The engineered strain was also able to efficiently produce erythritol from crude glycerol [109]. Using proteomics to study erythritol production from glycerol under hyperosmotic pressure revealed that enzymes related to osmotic stress response (such as pyridoxine-4-dehydrogenase and the AKRs family) and polyols biosynthesis (such as transketolase and triosephosphate isomerase) had significantly increased expression levels [108].

3.3. Terpenoids

Y. lipolytica possesses a native cytosolic mevalonate pathway (MVA) which supplies the C5 isoprenoid precursors for terpenoid biosynthesis, i.e., isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The synthesis of farnesyl diphosphate (FPP), the C15 direct precursor of sesquiterpenes, follows; thus, only one heterologous gene is required for sesquiterpene biosynthesis. Apart from this, and due to its oleaginous character, high culture density, and large intracellular acetyl-CoA pool, *Y. lipolytica* is a promising sesquiterpene chassis producer. Farnesene is a sesquiterpene typically used as fragrance, flavor and vitamin E/K1 synthetic precursor. Farnesene biosynthesis has been widely studied in *E. coli* and *S. cerevisiae*. Using engineered *S. cerevisiae*, Amyris produced 130 g L^{-1} β-farnesene in industrial bioreactors [110]. Meanwhile, in *E. coli*, Zhu et al., reported *a*-farnesene production up to 1.1 g L^{-1} in fed-batch fermentation, which is the highest production in *E. coli* cells to date [111].

Using the engineered *Y. lipolytica* strain P7, Yang et al., produced approximately 55 mg L^{-1} *a*-farnesene in shake flasks and 260 mg L^{-1} in a bioreactor. Different overexpression vectors harboring combinations of truncated HMG-CoA reductase (*tHMG1*), IPP isomerase (*IDI*), FPP synthase (*ERG20*) and codon-optimized α-farnesene synthase (*OptFS*) genes were constructed and integrated into the genome of *Y. lipolytica* Po1h (Figure 3). The engineered strain produced 57.08 mg L^{-1} α-farnesene, which is a 20.8-fold increase over the initial production of 2.75 mg L^{-1} in YPD medium in shake flasks. Bioreactor scale-up in PM medium led to *a*-farnesene concentration of 260 mg L^{-1} with *a*-farnesene to biomass ratio of 34 mg g^{-1}, which was a 94.5-fold increase over the initial production. This was the first report on *a*-farnesene synthesis in *Y. lipolytica*, albeit using glucose [112]. In their later work, a series of metabolic engineering strategies were employed to facilitate *a*-farnesene accumulation including opti-

mization of the MVA pathway, engineering intracellular acetyl-CoA availability and promoter replacement, leading to engineered *Y. lipolytica* strains producing up to 90 mg L^{-1} *a*-farnesene in shake flask culture. The best metabolic engineered strain was used in shake flasks to determine the optimal carbon source, with glycerol also included (mannitol, glycerol, citric acid, maltose, sucrose, fructose, glucose). The results showed that *a*-farnesene production from glycerol was higher than that from any other carbon source reaching up to 90 mg L^{-1}. Therefore, glycerol was used for feeding after 48 h batch fermentation with glucose in 5 L bioreactors. *A*-farnesene production was significantly enhanced, reaching 2.57 g L^{-1} final concentration and 34 mg g^{-1} yield on biomass, more than 60% higher than those obtained when glucose was used for feeding [79]. It should be noted that the highest terpenoid titer reported in *Y. lipolytica* is 25.55 g L^{-1} after 288 h of fed-batch fermentation with glucose as initial and feeding carbon source. The engineering strategy applied in this case was based on the design of a highly efficient non homologous end-joining (NHEJ)-mediated genome integration, and the enhancement of the intracellular mevalonate synthesis [113].

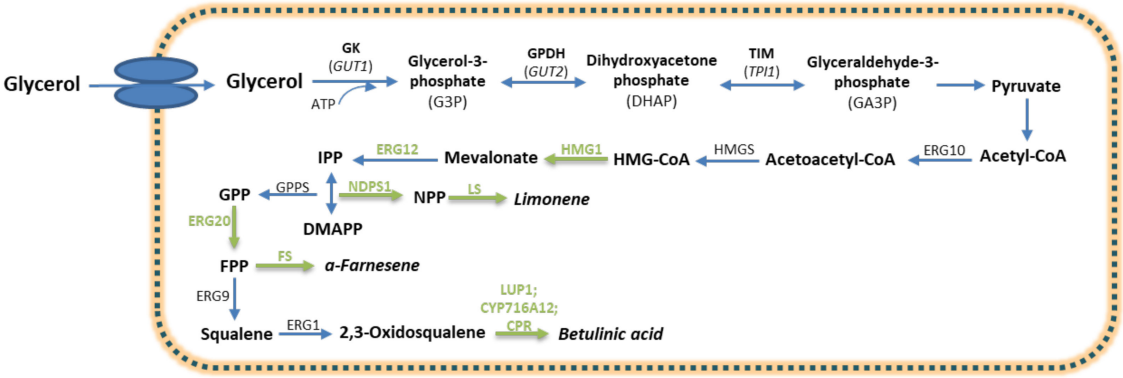

Figure 3. Terpenoids biosynthesis in *Y. lipolytica*. Enzyme abbreviations are: ERG10, acetyl-CoA C-acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase; HMG1, HMG-CoA reductase; ERG12, mevalonate kinase; GPPS, geranyl diphosphate synthase; NDPS1, neryl diphosphate synthase 1; LS, limonene synthase; ERG20, farnesyl diphosphate synthase; FS, farnesene synthase; ERG9, squalene synthase; ERG1, squalene monooxygenase; LUP1, lupeol synthase; CYP716A12, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase. Abbreviations of metabolites are: HMG-CoA, 3-Hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl diphosphate; NPP, neryl diphosphate; FPP, farnesyl diphosphate. Green arrows indicate gene overexpression as discussed in this study.

Y. lipolytica has also been used for limonene production, a valuable monocyclic monoterpene. Limonene biosynthesis has been widely studied in *E. coli* [114,115] and *S. cerevisiae* [116,117]. *Y. lipolytica* possesses a native MVA pathway; however, it cannot produce limonene due to the lack of limonene synthase (LS). Cao et al., constructed a limonene biosynthesis pathway in *Y. lipolytica* and then further engineered this strain to achieve efficient production. Specifically, two genes encoding neryl diphosphate synthase 1 (NDPS1) (*Solanum lycopersicum*) and LS (*Agastache rugosa*) were codon-optimized and heterologously expressed in *Y. lipolytica*. To enhance limonene production, genes of the MVA pathway were overexpressed, either in different copies of the same gene or in combination, with the overexpression of *ERG12* (mevalonate kinase) and *HMG1* (3-hydroxy-3-methylglutaryl-coenzyme A reductase 1) improving limonene production (Figure 3). Finally, with an optimized pyruvic acid and dodecane concentration in their flask culture, a maximum limonene titer and content of 23.56 mg L^{-1} and 1.36 mg g^{-1} CDW were achieved in the final engineered strain Po1f-LN-051, showing an approximately 226-fold increase compared with the initial yield of 0.006 mg g^{-1} CDW [118]. Using the engineered strain Po1f-LN-051, the introduction of an additional *LS* gene copy, further increased limonene production. This engineered strain was used to further optimize production. Various carbon sources

were tested, with glycerol leading to the highest limonene production and citrate selected as an auxiliary carbon source. In fed-batch fermentation with an optimized medium, the engineered strain produced 165.3 mg L^{-1} limonene, which corresponds to the highest yield to date for the production of limonene in *Y. lipolytica* [119].

Glycerol was also used to produce betulinic acid by *Y. lipolytica*, a pentacyclic lupane-type triterpenoid and a potential antiviral and antitumor drug. In this case, the heterologous biosynthesis of betulinic acid was achieved by co-expressing lupeol synthase *opAtLUP1*, NADPH-cytochrome P450 monooxygenase *opCYP716A12*, and NADPH-cytochrome P450 reductase *opAtCPR1* (Figure 3). The engineered *Y. lipolytica* produced 0.32 mg L^{-1} betulinic acid. P450 enzyme fusion and overexpression of key genes from the upstream MVA pathway further increased production to 9.41 mg L^{-1}. Using glycerol further enhanced the production to 16.98 mg L^{-1}, 1.8-fold higher than that obtained with glucose. 26.53 mg L^{-1} betulinic acid was obtained with 40 g L^{-1} glycerol as the sole carbon source, which was comparable to the titer obtained from *S. cerevisiae*, with 50 g L^{-1} glucose in shake flask cultivation. The authors stated that glycerol improved betulinic acid titer by increasing the expression of key genes in the MVA pathway and by increasing the supply of acetyl-CoA [120].

3.4. TCA Cycle Intermediates

The TCA cycle is the principal pathway of the central carbon metabolism that enables aerobic organisms to oxidize organic compounds and provide precursors for biomass formation, energy, and redox equivalents to the cell. Apart from diverting the TCA carbon flux towards the target biosynthetic route and exploiting the produced redox equivalents and metabolic precursors through pathway engineering for strain improvement [121], TCA intermediates *per se*—such as succinic acid (SA) and *a*-ketoglutaric acid (*a*-KG)—are of high biotechnological interest (Table 4).

SA is formed from *a*-KG through *a*-KG dehydrogenase and from isocitric acid through isocitrate lyase (glyoxylate cycle), whereas, succinate dehydrogenase complex (SDH) oxidizes SA to fumaric acid reducing ubiquinone to ubiquinol. *Y. lipolytica* strains with reduced or lost SDH activity, by impairing the *SDH1*/*SDH2* gene or exchanging the native *SDH2* promoter with a weak promoter, accumulated about 4–5 g L^{-1} SA using glycerol as substrate [122,123]. Gao et al., deleted the *Ylsdh5* gene, encoding a subunit of SDH (SDH5) in *Y. lipolytica* Po1f, and evaluated the Δ*Ylsdh5* strain for SA production using crude glycerol as the sole carbon source with respect to its tolerance and productivity. The strain exhibited excellent tolerance to environmental stress using 200 g L^{-1} crude glycerol and produced 43 g L^{-1} SA. Using fed-batch fermentation, highest fermentative SA titer was achieved (160.2 g L^{-1}) with a SA productivity of 0.40 g L^{-1} h^{-1}, spotlighting *Y. lipolytica* as a potent industrial SA producer [124]. Using the Δ*Ylsdh5* mutant strain, Li et al., developed a bioprocess for efficient SA production by *Y. lipolytica*, using an in situ fibrous bed bioreactor (isFBB). They evaluated different agricultural residues such as wheat straw, corn stalk, and sugarcane bagasse to improve the performance of isFBB, with sugarcane bagasse being the best immobilization material. Under the optimal conditions of 20 g sugarcane bagasse, 120 g L^{-1} crude glycerol as carbon source and 4 L min^{-1} of aeration rate, the SA concentration was 53.6 g L^{-1} with an average productivity of 1.45 g L^{-1} h^{-1} and a SA yield of 0.45 g g^{-1}. SA titer increased up to 209.7 g L^{-1} by feeding crude glycerol, which was the highest value ever reported [125]. Although the strain achieved high SA titer at neutral pH from glycerol, it produced acetate, impeding cell growth and SA production yield. For that, the pH had to be adjusted during the fermentation to ensure high succinate titer. At low pH, SA titer was relatively low (5.2 g L^{-1}). Cui et al., further improved the strain phenotype (production and cell growth) by deleting the *Ylach* gene responsible for acetic acid overflow (acetyl-CoA hydrolase) and overexpressing the genes which improve SA formation via reductive carboxylation and oxidative TCA pathway. They identified that the CoA-transfer from acetyl-CoA to SA in mitochondria leads to acetic acid overflow, rather than the pyruvate decarboxylation reaction in SDH negative *Y. lipolytica*. When the

Ylach gene was knocked out, acetic acid formation was eliminated and both SA production and cell growth improved. Furthermore, they studied how the overexpression of genes encoding key enzymes of oxidative TCA, reductive carboxylation, and glyoxylate bypass affects the SA yield and by-products formation. They observed that the overexpression of the phosphoenolpyruvate carboxykinase (*ScPCK*) gene from *S. cerevisiae* and the endogenous succinyl-CoA synthase beta subunit (*YlSCS2*) gene yielded the best performing strain, with a SA titer improved by 4.3-fold. In fed-batch fermentation, this strain produced 110.7 g L^{-1} with a yield of 0.53 g g^{-1} glycerol without pH control. This is the highest SA titer achieved at low pH by yeast reported to date, using defined media [126]. Recently, Billerach et al., characterized the *Y. lipolytica* strains Δ*sdh5* (PGC01003) and Δ*sdh5*Δ*ach1* (PGC202, additional deletion of acetyl-CoA hydrolase 1 gene to avoid acetate synthesis as a co-product) and compared them to the wild type strain W29 in defined medium. By performing fed-batch cultivation with glycerol and under nitrogen deficiency conditions, they investigated how carbon flux can be rerouted toward SA synthesis. The Δ*sdh5* produced 19 g L^{-1} SA with an overall yield of 0.23 g g^{-1} and an overall productivity of 0.23 g L^{-1} h^{-1}, while the Δ*sdh5*Δ*ach1* produced 33 g L^{-1} with an overall yield of 0.12 g g^{-1} and a productivity of 0.57 g L^{-1} h^{-1} [127].

Table 4. Engineering strategies (strain, medium, process) and cultivation systems using pure glycerol as carbon source for the production of organic acids by *Y. lipolytica*.

Strain	Carbon Source [1]	Product	Engineering [2]	Strategy	Set-Up [3]	Data from [Ref.]
VKM Y-2378	CG	41 g L^{-1} pyruvate; *a*-ketoglutarate	SE; ME; PE	33 taxonomically different yeast strains; thiamine and biotin concentration; nitrogen source; temperature, aeration, and glycerol concentration; pH 4.5	B	[78]
Po1f	CG	160 g L^{-1} succinic acid	SE; PE	Inactivation of *SDH5*; effect of pH, oxygen supply; pH 6.0	B	[124]
W29	CG	81.11 g L^{-1} citric acid; 24.90 g L^{-1} malic acid	ME	Optimization of the carbon and nitrogen source; pH 4.0	B	[72]
Po1f	PG	110.7 g L^{-1} succinic acid	SE; PE	Deletion of *Ylach;* overexpression of heterologous *ScPCK* from *S. cerevisiae* and native *YlSCS2;* fed-batch fermentation; pH not regulated	B	[126]
Po1f	PG	45.5 g L^{-1} succinic acid	SE; ME	Ts-mutants with blocked SDH activity; medium buffering; $CaCO_3$; pH < 3.5	SF	[123]
WSH-Z06	PG	66.2 g L^{-1} *a*-ketoglutaric acid	ME; PE	Two-stage pH control strategy; constant feeding; pH 3.0	B	[128]
374/4	PG	8.2 g L^{-1} pyruvic acid	SS; ME	Thiamine-auxotrophic strain; thiamine concentration; periodical addition of glycerol; pH 4.5	B	[129]

[1] CG, crude glycerol; PG, pure glycerol. [2] SE, strain engineering; ME, medium engineering; PE, process engineering; SS, strain screening. [3] SF, Shake flask; B, Bioreactor.

Glycerol was also exploited for *a*-KG production, a valuable industrial compound with various medical, pharmaceutical, food and feed, and chemical synthesis applications. The biotechnological synthesis of *a*-KG is particularly attractive not only due to its value (15–20 $/kg in comparison to citric acid 0.6 $/kg), but also due to tedious production and downstream processes [130]. A recent metabolic engineering strategy to enhance *a*-KG production involved the overexpression of genes encoding glycerol kinase (*GUT1*) to increase glycerol phosphorylation, methylcitrate synthase (*CIT1*) to improve oil utilization, and a previously uncharacterized mitochondrial organic acid transporter (*YALI0E34672g*) to study its impact on organic acids secretion. Using a mixed glycerol-oil medium (60 g glycerol/40 g rapeseed oil, C/N/P 87:5:1), the strain overexpressing all three genes produced 53.1 g L^{-1} of *a*-KG with productivity of 0.35 g L^{-1} h^{-1} and yield of 0.53 g g^{-1}, demonstrating the potential of *Y. lipolytica* as an *a*-KG producer [130].

3.5. Polyphenols

Glycerol-based production of polyphenols such as resveratrol, a plant secondary metabolite with multiple health-beneficial properties, has also been studied using *Y. lipolytica*. However, the microbial production process requires laborious engineering to reach commercially viable levels. He et al., constructed reservatol producing *Y. lipolytica* strains by overexpressing bacterial and plant-derived resveratrol biosynthesis genes. Resveratrol is produced either via tyrosine or phenylalanine; therefore, they tested both pathways. They reported that using glucose media, the phenylalanine pathway resulted in slightly higher production, although after supplementation with amino acids, the best producing strain was the one bearing two copies of the tyrosine pathway, reaching 0.085 g L^{-1}. Using glycerol instead of glucose, the highest titer (0.104 g L^{-1}) was achieved with the engineered strain expressing both biosynthetic pathways. The strain with the best performance was then evaluated in a bioreactor where a production of 0.43 g L^{-1} was reached, suggesting that *Y. lipolytica* is a promising host for resveratrol production from glycerol [131]. In another study, the potential of *Y. lipolytica* to produce not only resveratrol but also other shikimate pathway-derived metabolites (*p*-coumaric acid, *cis*, *cis*-muconic acid, and salicylic acid) was explored. In this work, medium optimization was done using mineral medium, YNB and YP with either glucose (20/80 g L^{-1}) or glycerol (20/80 g L^{-1}) as carbon source. For all types of media and concentrations, when 80 g L^{-1} of carbon source were added, only ca. 15–25 g L^{-1} were consumed after 96 h cultivation, and glucose performed better than glycerol. Interestingly, when comparing media containing the same carbon source, cultivation in a mineral medium yielded 33–181% higher titers than in the complex YNB medium. In a small-scale cultivation, the *Y. lipolytica* strain expressing a heterologous pathway produced 52.1 mg L^{-1} resveratrol, whereas the titer increased to 409 mg L^{-1} when the strain was further engineered with feedback-insensitive alleles of key genes in the shikimate pathway and with five additional copies of the heterologous biosynthetic genes. In controlled fed-batch bioreactor, the strain produced 12.4 g L^{-1} resveratrol, the highest reported titer to date for *de novo* resveratrol production, showing that *Y. lipolytica* is an attractive host organism for the production of shikimate pathway-derived metabolites [132].

4. Conclusions

This review summarizes and discusses advances and developments in the biotechnological production of commercially important biochemicals using the yeast *Yarrowia lipolytica*. Towards the goal of building and ensuring a sustainable society, the bioconversion of waste materials into value-added compounds should be in the spotlight, and to that end, the biodiesel-derived waste glycerol is the focal point of the present review. By presenting significant accomplishments that integrate strain, medium, and process engineering, this review articulates the necessity to design production processes in a cross-disciplinary manner in order to achieve previously unattainable targets.

Strain engineering strategies implemented in *Yarrowia* mainly aimed at: (i) enhancing the biosynthetic pathway of interest (overexpression of *GUT1*, *GPD1,2*, *SCT1*, *SLC1*,

DGA1,2, PDAT, *LRO1*, etc.); (ii) increasing the availability of biosynthetic precursors (G3P, FFA, acetyl-CoA, mal-onyl-CoA, 6-PGL, Ribu-5-P, R-5-P, S-7-P, E-4-P, mevalonate, NPP, FPP, etc.); (iii) increasing the availability of redox and energy equivalents (manipulation of PPP, cofactor switch, overexpression of heterologous enzymes, co-feeding of carbon sources); (iv) introducing novel pathways (overexpression of heterologous enzymes e.g., *LS*, *FS*); and (v) shutting down consuming/degradation pathways (β-oxidation, deletion of *GUT2*, *TGL*, *EYD*, *EYK*, etc.). The performance of the producer strain was often further enhanced by medium and process optimization strategies. An optimized cultivation medium will guarantee fast microbial growth, increased production metrics (titer, rate, yield; TRY), and decreased accumulation of unwanted by-products to facilitate downstream processing. Just as importantly, it should be cheap to decrease the production costs and easily transferable to an industrial environment for a commercialized production process. Often, medium engineering can alter or tailor-make the profile of the synthesized bio-products (e.g., biomass-lipids-citric acid ratio, profile of lipids or polyols) by rearranging the involved carbon fluxes, balancing the redox supply or by supplying the required precursors. The herein presented medium engineering strategies involved: (i) fine-tuning of medium composition (C/N ratio, concentration of nutrients); (ii) supplementation with critical nutrients or elements (e.g., fatty acids, amino acids, minerals, vitamins); (iii) selection of the most favorable carbon source (glycerol in the vast majority of cases) or combination of different ones (co-feeding or "doping"); (iv) selection of the nitrogen source (inorganic or organic); and (v) type of medium buffering. The optimization of the bioprocess will then secure an ideal environment for the strain to acquire and utilize all the necessary nutrients in order to support cell growth and proliferation, and as well, the biosynthetic pathways leading to the target compounds. Process engineering strategies involved the optimization of critical process parameters (inoculum density, pH regulation or no regulation, oxygen supply, agitation) and the operational feeding mode (batch, fed-batch, continuous or hybrid process) in order for a high-yielding production process to be established.

The presented examples clearly demonstrated not only the huge potential of glycerol as a favorable sustainable feedstock for *Yarrowia lipolytica* bioprocesses and the outstanding biosynthetic routes leading to a wide spectrum of valuable compounds, but also how a bioprocess design based on integrated strategies can maximize the performance of a producer strain.

5. Future Perspectives

The continuous progress of industrial biotechnology has consolidated microbial processes that valorize renewable feedstocks in the spotlight of a bio-based economy. However, most microorganisms employed in biotechnological applications are evolved by nature to secure their growth and not to synthesize the envisaged industrial relevant compounds. The desired substitution of chemical processes by bio-based ones is still not comprehensively realized, as very few microbial processes are scaled-up for industrial-scale production. In many cases, considerable time and effort should be still devoted to the identification and elimination of bottlenecks in biosynthetic pathways that negatively affect production efficiency and to the fine-tuning of the signaling, metabolic, and regulatory networks to maximize productions metrics (TRY) without losing the robustness and stability of the strain. It seems that the rationalization of successful industrial strain development remains a not-straightforward task, which imperative calls for a system-wide approach that will pursue the system in its full complexity.

Systems metabolic engineering which merges tools and strategies from systems biology, synthetic biology, and adaptive laboratory evolution can facilitate the tailor-making of microbial cell factories and the modulation of their biosynthetic machineries; therefore, transforming them into high-performance production organisms [133]. High-throughput computational and experimental techniques which simplified the generation of omics or multi-omics datasets, advanced software for pathway prediction and design, and genome-scale metabolic modeling, are just a few systems biology technologies that enable a greater

understanding of the underlying complex metabolic functions, identification of engineering targets, and discovery of previously unknown biosynthetic pathways. Nonetheless, in order for systems biology to deliver the best outcomes, in silico studies should be validated by further in vitro and in vivo experimental works, since these latter better reflect the real microbial phenotype under operational conditions. In addition, high-quality genome-scale metabolic models which incorporate supplementary biochemical information (e.g., thermodynamics, enzyme-substrate interactions, post-translational modification, and structure of protein complexes), allowing the analysis of the dynamic interplays of gene-protein-flux, are still in demand [134].

Groundbreaking genome engineering technologies that have revolutionized synthetic biology, such as CRISPR, and tools to control and tune the expression level of promoters or the copy number of plasmids can also be exploited to reroute metabolic fluxes and optimize target biosynthetic pathways. Even if a genetic engineering strategy is carefully designed and executed, the final phenotypic outcome may not be the envisaged one due to the complexity of the microbial metabolism. ^{13}C-Metabolic flux analysis (^{13}C-MFA), a method which utilizes ^{13}C-labeled substrates to determine the labeling incorporation in the downstream metabolites and quantify the intracellular carbon flux distribution in central metabolism, can be employed to greatly assist and rationalize metabolic engineering [135].

It is obvious that we have purposeful and powerful weapons in our arsenal that constantly improve and not only demystify biological processes but also debottleneck pathway limitations and design novel high-yielding biosynthetic routes in a rational manner. Despite the astounding progress which has allowed for the systematic engineering of strains, in most cases the strain performance will still need further improvement to meet the demands of an industrial commercial bioprocess that, at the end of the day, must be competitive, robust, and sustainable. To ensure this, the process design, optimization and scale-up should be an intensive interdisciplinary task that employs concurrent upstream and midstream process development practices. Clearly, a bioprocess design and optimization which relies on the integration of strain and process engineering, focusing on not only the host cellular metabolism but also taking into account the overall bioprocess, will continue to provide attractive and feasible solutions for sustainable products and processes in industrial biotechnology.

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