



Metabolic switch points enabling targeted lipid accumulation in oleaginous and non-oleaginous yeasts: A comparative review

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ABSTRACT

Oleaginity is a unique property exhibited by only a few organisms to store the excess carbon in the form of lipids under exceptional circumstances. This property is regulated by an intricate interplay of cellular metabolism and external environmental/culture parameters. This review highlights the mechanisms at cellular level that divert the carbon flux toward lipid accumulation in oleaginous yeast/fungi and compares these to their ethanol synthesizing counterparts. Reported information on the influence of culture conditions, media and stress factors on lipogenesis and lipid accumulation have been investigated considering the intricate interplay among various metabolic pathways. The contribution of reductants, enzymatic manoeuvring of carbon flux, roles of carriers/transporters, transcription regulators along with other key cytosolic or mitochondrial enzymes for lipid synthesis and accumulation have been investigated to understand the differential behaviour of oleaginous and ethanologenic yeast. The switch points that differentiate oleaginous and non-oleaginous yeast strains, e.g. Crabtree vs non-Crabtree metabolism, NADPH source, Nitrogen-triggered TOR/SNF1 signalling, ACL-dependent acetyl-CoA supply, have been reviewed. Although the roles of key enzymes such as malate enzyme (ME) and ATP: citrate lyase (ACL) and variations in carbon/nitrogen ratios of the culture medium have been critically reviewed before, the oleaginous behaviour in yeast in comparison with ethanologenic yeast and the mechanistic switch points involved are being reviewed here. The review provides a foundation for innovative research designs for directing the abundant acetyl-CoA flux of oleaginous yeast via intelligent strain/media design, for large-scale industrial applications, by using the information on switch points and metabolic interplay of pathways.

Abbreviations: ACC, acetyl-CoA carboxylase; ACL, ATP:citrate lyase; ACP, acyl carrier protein; ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ALE, adaptive laboratory evolution; AMP, adenosine monophosphate; ATP, adenosine triphosphate; C/N, carbon-to-nitrogen ratio; C/P, carbon-to-phosphorus ratio; C/S, carbon-to-sulfur ratio; CoA, coenzyme A; DO, dissolved oxygen; DNA, deoxyribonucleic acid; EMP, Embden-Meyerhof-Parnas pathway; ER, endoplasmic reticulum; FA, fatty acid; FAD, flavin adenine dinucleotide; FAS, fatty acid synthase; FFA, free fatty acid; G6P, glucose-6-phosphate; GDH, glutamate dehydrogenase; GLN, glutamine synthase; HMF, hydroxymethylfurfural; HMP, hexose monophosphate pathway; ICDH, isocitrate dehydrogenase; INO1, inositol-1-phosphate synthase; ME, malic enzyme; MPC, mitochondrial pyruvate carrier; MPL1, perilipin-like protein; NAD⁺, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NCR, nitrogen catabolite repression; PA, phosphatidic acid; PDC, pyruvate decarboxylase; PFK, phosphofructokinase; Pi, inorganic phosphate; PI, phosphatidylinositol; PL, phospholipid; PPP, pentose phosphate pathway; PUFA, polyunsaturated fatty acid; RNA, ribonucleic acid; SNF1, serine/threonine protein kinase Snf1; TAG, triacylglycerides; TCA, tricarboxylic acid cycle; TOR, Target of Rapamycin; TORC1, Target of Rapamycin Complex 1.

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1. Introduction

Every living organism synthesizes lipids to build and maintain its cellular membranes and other cellular functions. However, some microorganisms accumulate lipids significantly more than 20% of their dry cell mass as triacylglycerides (TAGs). Oil/TAG accumulation is reported mainly in some fungi, yeasts, and algal species, and such organisms are termed 'oleaginous'. A few most explored oleaginous yeasts are *Yarrowia lipolytica*, *Lipomyces starkeyi*, *Rhodotorula* sp., and *Cryptococcus curvatus*/*Aptotrichum curvatum*, reportedly with lipid accumulation as high as up to 40–70% of their dry cell biomass (Nguyen et al., 2025a; Singh et al., 2018). The profile of TAGs accumulated by oleaginous yeasts matches that of plant oils (Alvarez et al., 2019; Lei et al., 2024). On the other hand, some conventional yeasts e.g. *Candida utilis* and *Saccharomyces cerevisiae* under the same culture conditions are unable to accumulate even 5–10% of oil.

In oleaginous yeast, lipid accumulation usually commences upon the exhaustion of a particular nutrient (mainly nitrogen) in the fermentation medium, when cell proliferation stops, leading to assimilation of the surplus carbon (e.g., glucose) as TAGs (Henne and Cohen, 2026; Salvador López et al., 2022). Investigation of key enzymes contributing to fatty acid (FA) synthesis and accumulation showed varying expression levels in different yeast strains. This phenomenon raises several key questions: How can only a few microorganisms accumulate oil? And how some oleaginous species can store more lipid than others? The mystery remains somewhat undeciphered!

In this context, the malate enzyme (ME) and ATP: citrate lyase (ACL) are two essential enzymes studied extensively for regulating lipid accumulation (Ratledge, 2014). ACL activity has been strongly correlated with the ability to accumulate lipid in several fungi, yeast, and other oleaginous microorganisms (Wynn et al., 1999). However, the mere possession of the ACL activity under high carbon over nitrogen (C/N) ratios could not trigger lipid accumulation in ethanologenic food yeast *Saccharomyces* sp. (Kocharin et al., 2013). Interestingly, it is absent in the oleaginous yeast *Y. lipolytica* classified under ascomycetes (Groenewald et al., 2014). Thus, even though ACL and ME were considered a precondition to start lipid accumulation, the presence of only these genes might not be the sole contributing factor. This finding points out toward the lacunae that lie in understanding the important role of other pathways, enzymes, genes, and intermediates in controlling the degree of lipid biosynthesis in different organisms.

This review critically investigates the modulation of lipogenesis in oleaginous yeast that differ from non-oleaginous yeast. The cellular metabolism of various oleaginous yeasts, e.g., *Rhodospiridium*, *Mortierella*, and *Yarrowia* sp., were thoroughly analyzed and compared to non-oleaginous *Saccharomyces*, and *Candida* sp. During alcohol synthesis and lipogenesis, the C flux is controlled and channelized via different routes and is governed by reductants and other key players. Understanding the mechanism of switch points that further impacts the lifestyle of yeast, enables rational strain design for biofuel and oleochemical production. This review uniquely integrates metabolic, redox, and regulatory switch points into a unified comparative framework for understanding and improvising lipogenesis in yeast using genomics, proteomics and metabolomics studies.

2. Metabolic pathways in oleaginous and non-oleaginous yeasts

Oleaginicinity is facilitated by an interplay of metabolic pathways and regulatory mechanisms for availing precursors, enzymes, and energy resources for lipid accumulation (Alvarez et al., 2021). Following carbon uptake, a cascade of reactions leads to lipid production in oleaginous yeast cultivated in various media having different compositions. Among the renewable alternatives, glucose followed by xylose are the simplest and preferred carbon sources for all yeast species. These moieties permeate through the cell membranes and start the first step of respiration in yeasts through Embden-Meyerhof-Parnas (EMP) or glycolysis

pathway (Fakas, 2017). The intermediates and the products further link to several essential pathways e.g. tricarboxylic acid (TCA), fatty acid synthesis pathway, as well as amino acid metabolism, which are associated with TAG biosynthesis depending upon the cellular physiology (Abeln and Chuck, 2021). The hexose monophosphate/pentose-phosphate pathway (HMP/PPP) runs parallel to glycolysis for NADPH and ribose-5-phosphate formation, and the β -oxidation pathway, that results in breakdown of fatty acids into acetyl-CoA units for providing energy and intermediates. This HMP pathway is also linked to TAG biosynthesis and cellular stress responses for extra NADPH requirements. The interaction of these pathways has been integrated diagrammatically in Fig. 1.

2.1. Regulation of carbon flux: switch points for oleaginous or ethanologenic behaviour

Ethanol or fatty acid synthesis in yeast is regulated by the central carbon flux through the availability of metabolic enzymes and cellular redox equivalents. Upstream of the pyruvate synthesis, a high pyruvate kinase activity diverts the carbon flux through pyruvate, toward fatty acid synthesis in yeast (Evans and Ratledge, 1985) (Fig. 1). Pyruvate being the end-product of glycolysis, is centred at the heart of the metabolic flux to confine the cellular metabolism for lipid synthesis (Evans and Ratledge, 1985). While the activity of pyruvate kinase is regulated through citrate and phosphofructokinase (PFK), the mitochondrial citrate concentration controls the downregulation of the TCA cycle by feedback inhibition through isocitrate dehydrogenase (ICDH) activity. The cytoplasmic efflux of citrate, resulting from this, promotes the acetyl-CoA carboxylase (Acc1) activity for fatty acid biosynthesis as shown in Fig. 2. Acc1 is a rate limiting enzyme that can catalyse the formation of malonyl CoA from acetyl CoA in the cell cytosol. During the alcoholic fermentation phase, the yeast cell shifts the carbon flux toward the Crabtree effect through pyruvate (Osorio-González et al., 2023). At this cross-side of the pathway, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) perform a highly indispensable activity in ethanologenic yeasts. Tiukova et al. (2019) illustrated the importance of carbon sources (e.g., glucose or xylose) used for reducing the PDC expression by two-fold (Tiukova et al., 2019). Subsequently, a decreased ADH expression (Rhto_03062) was observed in the presence of both the carbon sources in *R. toruloides* (Tiukova et al., 2019). Oleaginous yeast diverted the carbon flux toward lipid assimilation, unlike non-oleaginous yeast where PDC and ADH activity were observed. Given a specific pyruvate dehydrogenase complex activity, the citrate synthase and ATP-citrate lyase (ACL) activity are preferably high in oleaginous yeasts (Brandenburg et al., 2021). Table 1 shows the differences between ethanologenic and oleaginous yeast strains based on gene expression studies. The ethanol producing strains could produce increased amounts of fatty acids by deleting suitable genes e.g. pyruvate decarboxylase (*pdh*), whereas the oleaginous strains produced ethanol by expressing certain sets of genes e.g. *pdh* & alcohol dehydrogenase (Zhang et al., 2021). A constant supply of acetyl-CoA is important for continuous fatty acid synthesis in oleaginous fungi through these enzyme complexes. The PDC, acetaldehyde dehydrogenase and acetyl-CoA synthetase pathways are comparatively less energy efficient and hence less favoured in oleaginous yeast metabolism (Zhang et al., 2007). On the other hand, ACL activity is high in some oleaginous yeast species (*L. starkeyi*, *Y. lipolytica*, etc.) for converting mitochondrial citrate to acetyl CoA, required for lipogenesis as shown in Table 1.

The ADH and ALDH activity are not directly involved in the glycolysis or lipogenesis in oleaginous yeasts. However, the catalytic activity of ADH in ethanologenic yeasts facilitates the conversion of aldehyde (or acetaldehyde formed through pyruvate) to alcohol, by oxidizing NADH to NAD⁺ (Fig. 1). In few microbes, the ALDH enzyme reduces the aldehyde to acetate despite generating alcohols (Table 1). Although glycolysis and the TCA cycle feed primary intermediates for generating acetyl CoA (Figs. 1, 2), external stress factors also regulate the carbon

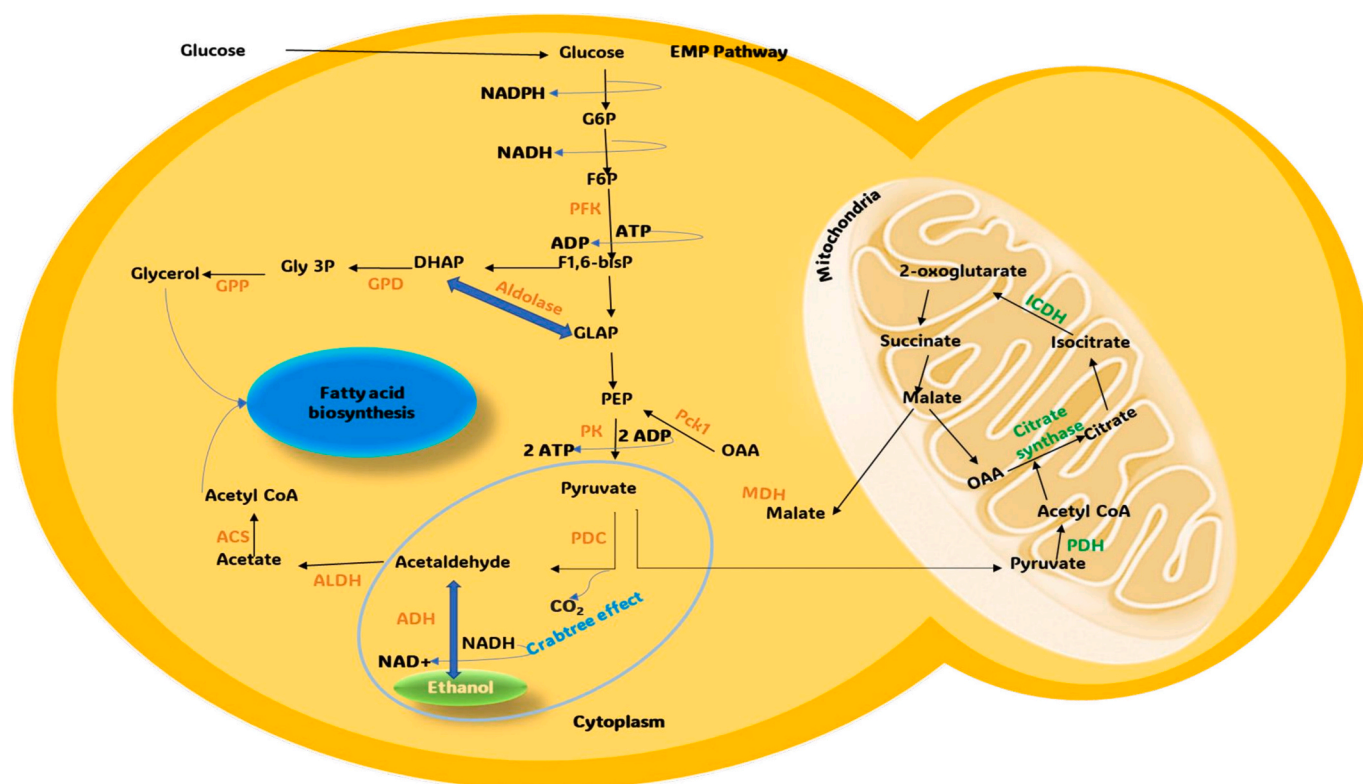


Fig. 1. Role of glycolysis in supplying key intermediates for lipid biosynthesis. It further shows the branching of lipid biosynthesis and ethanol production from pyruvate, positioned at the interface of the mitochondrial matrix, membrane, and cytoplasm. Subcellular localization has been inferred from metabolic pathways described in *S. cerevisiae* and oleaginous yeasts. The Crabtree effect and acetyl-CoA generation from acetate are also depicted, highlighting carbon flux directed toward lipid biosynthesis in oleaginous yeasts and ethanol production in non-oleaginous yeasts.

flux, affecting the fatty acid or alcohol synthesis in yeast. The explicit activity of acetyl-CoA synthase (ACS) reported in non-oleaginous *S. cerevisiae*, uniquely converts this acetate production into cytosolic acetyl-CoA (Fig. 1) (Sato et al., 2021). Such catalytic activity is not common in other yeasts. This could be a plausible role of ALDH and ADH in yeasts as mediators for handling acetate inhibition against lignocellulosic biomass hydrolysate-mediated fermentation (Qi et al., 2017).

Investigations strongly indicated stress-mediated alcohol synthesis in oleaginous yeast *R. toruloides* (Vajzovic et al., 2012). The deletion of ADH and ACS1 and overexpression of critical genes (ScADH1 and ScPDC1) in non-alcohol fermenting yeast *Yarrowia lipolytica*, remained unsuccessful in triggering ethanol synthesis under anaerobic conditions (Gatter et al., 2016). On the other hand, despite a lack of ethanol-producing pathways, low ethanol production levels could be reported under oxygen-limited conditions in *Lipomyces starkeyi* (Pomraning et al., 2016). This suggests that irrespective of cognizance targets of genetic engineering to switch the non-oleaginous metabolism of yeast toward lipogenesis, more detailed metabolomic and proteomic evaluations are required. The advent of metabolic engineering has further helped to understand the role of specific elements that shift the carbon flux in yeasts toward ethanol biosynthesis or oleaginicacy upon targeted gene deletions (Yu et al., 2018a, 2018b; Hu et al., 2019). This can be related to upregulation of specific pathways and enzymes that occurs in all yeasts during lipogenesis. The proteomic and transcriptomic analysis of *Rhodospiridium toruloides* M18 mutant revealed a five-fold expression of genes linked to glycolysis, like phosphoglucomutase (PGM), aldehyde dehydrogenase (ALDH), phosphoenolpyruvate carboxykinase 1 (Pck1) during lipogenesis as a stress tolerance to furfural, HMF, vanillin, etc. present in the sugar stream when lignocellulosics are digested by physico-chemical means (Qi et al., 2017).

The specific representation of this metabolic shift from respiration to aerobic fermentation can be understood through the crabtree effect in

wild-type baker's yeast. The glycolysis pathway serves as the terminal electron acceptor, instead of oxygen even in oxygen-enriched conditions (De Deken, 1966; Imura et al., 2020). In *Saccharomyces cerevisiae* NAD⁺/NADH ratio is maintained by converting acetaldehyde to ethanol (Fig. 1). A high glucose concentration in the growth medium is managed in an energy-efficient way. The major influence of the crabtree effect is levied upon metabolites linked to NADH synthesis. Furthermore, the higher glucose consumption rates of *S. cerevisiae* compared to its counterpart crab negative *Candida utilis* suggested a better pathway to flux excess glucose for ethanol and glycerol production (Theobald et al., 1997; Van Dijken and Scheffers, 1986). Several engineering approaches have been adapted to redirect carbon flux for lipid production by completely blocking the ethanologenesis, but the trials failed, thus inflicting severe growth defects in *S. cerevisiae* (Van Hoek et al., 1998, 2000). A combined approach employing metabolic engineering with adaptive laboratory evolution (ALE) successfully reprogramming the conventional bacterial pyridine nucleotide transhydrogenase enzyme capable of converting NADH to NADPH was used. The heterologous expression of this enzyme in ethanol fermenting yeast was successful to provide NADPH for fatty acid synthesis with a simultaneous release of extra NADH. This methodology applied in an engineered strain showed re-designed glycolysis and HMP pathways producing 33.4 g/l FFAs (Yu et al., 2018a, 2018b). The significance of redox power was thereby deduced as the second most significant regulatory power for driving the lifestyle of a yeast toward lipid accumulation, after C flux control. In oleaginous yeast the carbon flux is directed toward lipid synthesis where pyruvate kinase and ICDH have regulatory roles, whereas, in ethanologenic yeast the carbon flux is directed toward the crabtree effect via pyruvate and PDC and ADH play key roles. As also indicated in Table 1, oleaginous yeast strains e.g. *R. toruloides* and *L. starkeyi* produced low levels of ethanol under stress conditions, but not *Y. lipolytica*.

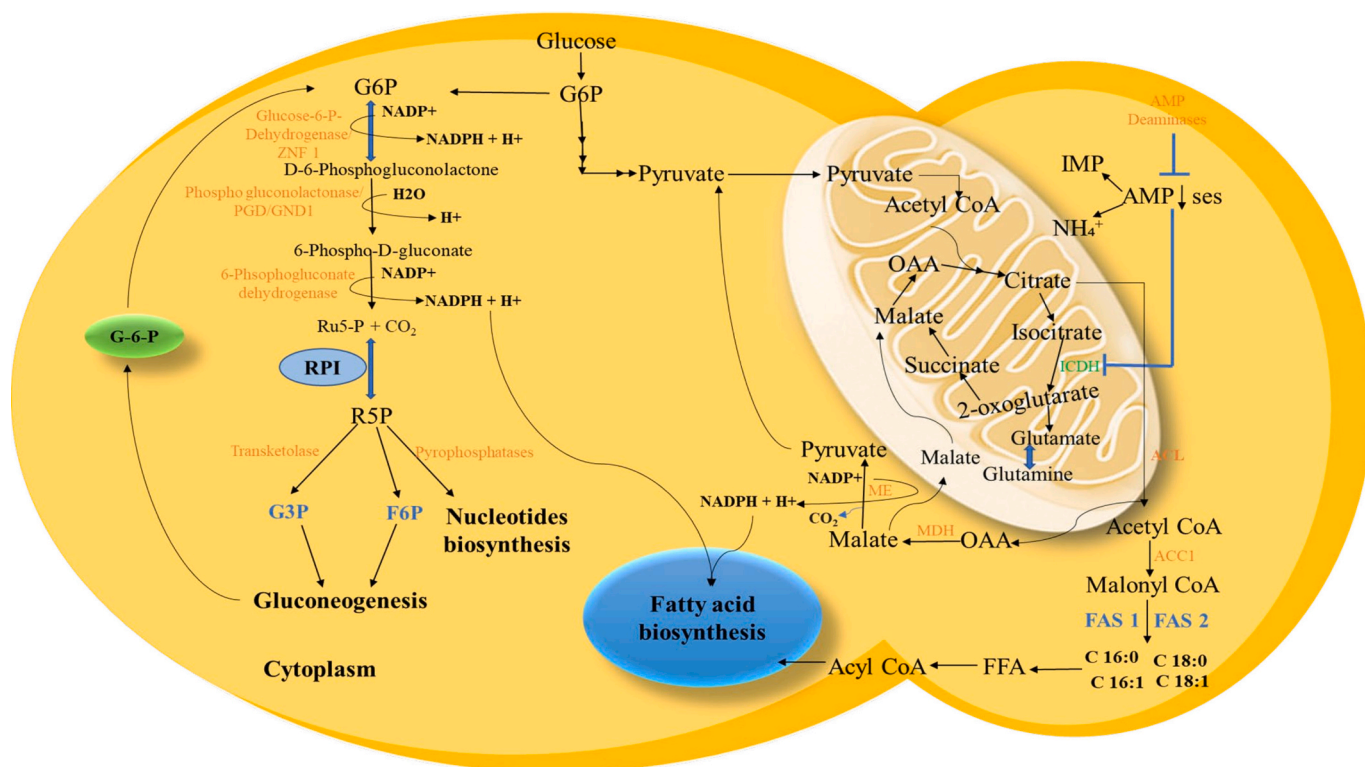


Fig. 2. Hexose monophosphate pathway involved in the generation of reducing equivalents (NADPH) and acetyl-CoA for lipid biosynthesis. It illustrates the regulation of gluconeogenesis and nucleotide synthesis during the phases of lipid accumulation and cellular growth, respectively. It also depicts citrate and malate efflux and influx in relation to malic enzyme activity under nitrogen-limiting conditions. This process is regulated by AMP deaminase activity, which diverts excess carbon toward lipogenesis.

2.2. Role of reductants in lipogenic pathways

Role of reductants like NADH, NADPH, etc. and coordination between specific enzymes e.g. malic enzyme (ME), ATP-citrate lyase (ACL) and fatty acid synthase (FAS) controls the lipogenic pathways in oleaginous and non-oleaginous yeast (detailed in Section 3.1). Lipid synthesis inside the cell remains incomplete without the crucial role played by reductants, supported by an extensive and varied repertoire of ATP, and carbon (Table 1). Other than this, the enzymes isocitrate dehydrogenase (ICDH), ACL, ME, and FAS are bound to the regulation of redox supply in oleaginous yeasts. Not only reductants, but a typical 1:2 acetyl-CoA: NADPH molar ratio is a prerequisite for the TAG synthesis (Ratledge, 2004). Each glycolysis reaction generates two NADH molecules, which is insufficient to provide adequate amounts of NADPH for lipogenesis. Redox engineering of synthetic pathways meant for converting NADH produced during the glycolysis of sugars, to NADPH resulted in an improved lipid yield (Qiao et al., 2017), further indicating that enhanced supply of reductant can potentially elevate lipid synthesis/accumulation. The HMP pathway has been well elucidated for its role in facilitating NADPH supply in the cytoplasm of yeasts e.g., *R. toruloides*, *L. starkeyi*, and *Y. lipolytica* (He et al., 2018; Ratledge, 2014; Wasylenko et al., 2015). The ^{13}C metabolic flux analysis in oleaginous *Y. lipolytica* revealed an accelerated glucose-6-phosphate (G6P) source back to oxidative HMP to meet the NADPH requirement for enhanced lipid synthesis (Fig. 2) (Wasylenko et al., 2015). However, unlike ethanologenic *S. cerevisiae*, a higher degree of lipid accumulation has been reported in wild type *S. cerevisiae* D5A strain due to a supported NADPH supply via glyoxylate cycle, rather than the TCA cycle. The upregulation of HMP and glyoxylate cycle pathway genes is sufficient to induce lipid synthesis by providing reducing equivalents even in the absence of ACL function (He et al., 2018). The generation of reductants due to the activity of certain enzymes and pathways have been explored

and discussed hereafter.

2.2.1. Fatty acid biosynthetic pathway

During de novo fatty acid biosynthesis, the simultaneous lipid backbone is structured by transferring acyl-residues from an acyl-carrier protein (ACP) with the help of an intrinsic acyltransferase to coenzyme A (CoA). The reaction is catalysed by cytosolic fatty acid synthase (FAS 1&2) resulting in long-chain acyl-CoAs. The first and rate-limiting reaction of acetyl-CoA carboxylation is catalysed by acetyl-CoA carboxylase (ACC, encoded as ACC1 and HFA1) for fatty acid synthesis in yeasts (Fig. 2). The ACC1 overexpression has been targeted in many studies by steering acyl-CoA flux toward lipid biosynthesis under nitrogen stress (Davis et al., 2000; Tang et al., 2015; Tehlivets et al., 2007). The conserved ACP domains in red yeast contribute toward improving the polyunsaturated fatty acid fraction in total lipid (Jiang et al., 2008). Similarly, 17% lipid production was obtained in oleaginous *S. cerevisiae* D5A strain due to the overexpression of ACC1 and FAS 1&2 (He et al., 2018).

A simultaneous supply of both acetyl-CoA and NADPH is necessary for fatty acid synthesis. This is handled by ATP-citrate lyase (ACL) and malic enzyme (ME) in oleaginous yeast (Fig. 2) and their activities differ from organism to organism. In non-oleaginous yeast *S. cerevisiae*, the lack of ACL activity prevents it from using the acetyl-CoA from the TCA cycle and hence further lipid accumulation. In *S. cerevisiae* D5A, unlike other oleaginous yeasts, the downregulation of TCA cycle genes under nitrogen limited conditions could not provide citrate for acetyl CoA in the cytosol (He et al., 2018). A downregulation of carnitine acetyltransferase encoding genes reduces the transport of cytoplasmic acetyl-CoA into mitochondria retaining it in the cytosol for lipid synthesis (He et al., 2018). These outcomes strongly suggest the importance of ACL activity alongwith reductants in the cytoplasm of oleaginous yeast for regulating lipid accumulation.

Table 1

Differential expression of key genes for ethanol and lipid biosynthesis in non-oleaginous and oleaginous yeast strains. The functions of various gene(s), reductants, TOR/SNF1 pathways, crabtree effect varies in ethanologenic and oleaginous species and may vary with different genera of oleaginous yeast/fungi. Here *Saccharomyces cerevisiae* (ethanol producing) has been compared with major oleaginous yeast i.e. *Yarrowia lipolytica* and *Rhodospiridium* sp. and to *M. alpina* (oleaginous fungi).

Pathways/genes	Ethanologenic yeast		Oleaginous yeast	
	<i>Saccharomyces cerevisiae</i>	<i>Yarrowia lipolytica</i>	<i>Rhodotorula/Rhodospiridium</i> sp.	<i>Mortierella alpina</i>
ATP: citrate lyase (Acl)	Absent in some species; overexpression enhances FA synthesis (Tang et al., 2015; Kim et al., 2018)	Overexpression enhanced FA synthesis and gene disruption decreased lipid content (Zhang et al., 2014; Liu et al., 2015)	Overexpression enhanced FA synthesis (Zhu et al., 2012)	Not reported
Acetyl-CoA carboxylase 1 (Acc1)	Overexpression increased FA synthesis (Tehlivets et al., 2007; Runguphan and Keasling, 2014)	Overexpression doubled lipid content	ACC1 overexpression with HMG-CoA reductase repression increased 57% lipid content (Chaturvedi et al., 2021)	Not reported
Glucose-6-phosphate dehydrogenase (ZWF)	overexpression increased isobutanol production (Feng et al., 2017)	ZWF1 and ACBP co-expression improved the lipid content (Yuzbasheva et al., 2017)	upregulation of ZWF1 under nitrogen limiting conditions	Not reported
ME (ME1) Isoforms	Overexpression improved NADPH flux in the cytoplasm (Nouaille et al., 2004) and C4 di-carboxylic acid production (Zelle et al., 2011)	no effect on lipid accumulation (Beopoulos et al., 2011a, 2011b)	over expression improved oil production (Bandhu et al., 2019; Li et al., 2013)	overexpression increased fatty acid content, especially arachidonic acid (Hao et al., 2014)
Crabtree effect TOR/SNF1 (Target of Rapamycin/Sucrose Non-Fermenting) pathway	Positive, Enhances growth rate SNF pathway adapts yeast cells to glucose limitation (Hedbacker, 2008)	Crabtree-negative yeast deletion of SNF genes lead to high lipid accumulation, even in nitrogen available conditions (Seip et al., 2013b)	Crabtree-negative TOR and SNF1 pathways regulate the switch from cellular growth to lipogenesis during stress conditions (Correa-Romero et al., 2023)	Not reported SNF pathway exists for regulating anabolic pathways, e.g. lipogenesis, under scarce glucose condition (Chang et al., 2020)
Nitrogen response mechanism	supplementation of Inorganic nitrogen enhanced the growth kinetics; effect on aromatic profiles was strain-dependent (Ramírez-Aroca et al., 2026).	Higher hexokinase (HXK1) expression was seen in low nitrogen conditions affecting TCA cycle (Hapeta et al., 2020)	Lipidomic studies suggested lipid remodelling during N limitation; Phospholipids converted to storage lipids (Mishra et al., 2024)	KNO ₃ promoted the pentose phosphate pathway, <u>malic enzyme</u> and <u>isocitrate dehydrogenase</u> and enhanced arachidonic acid formation (Yu et al., 2018a, 2018b).
Mitochondrial carriers	mitochondrial inner membrane citrate carrier (Ctp1) exports citrate from mitochondria to cytosol (Castegna et al., 2010)	Transporter (Yhm2) facilitates citrate transportation its deletion reduced lipid accumulation (Yuzbasheva et al., 2019)	Overexpression of mitochondrial pyruvate carrier (Mpc) enhanced fatty acid synthesis (Bricker et al., 2012)	Not reported

The number of ACL genes varies among yeast species. The *R. toruloides* NBRC10032 possess only one gene, oleaginous *Saccharomycotina* sp., two, *T. fermentans*, eight, *A. oryzae*, six and *R. opacus*, seven genes of ACL (Shen et al., 2016). Enhanced lipid accumulation was reported through ACL overexpression in *R. toruloides* (Zhu et al., 2012). The catalytic activity of ACL for obtaining fatty acid is evident in a non-conventional *Starmerella bombicola* yeast, better known for its sphorolipid production. The SbACL1 deletion negatively affected the specific growth rate of *S. bombicola* resulting in extra- and intracellular citrate accumulation. Similarly in *Y. lipolytica*, an enhanced ACL activity resulted in lipid accumulation (Ochoa-Estopier and Guillouet, 2014), while its inactivation alone, decreased the fatty acid synthesis by 60–80% (Dulermo et al., 2015; Liu et al., 2015).

Aligned with the ACL function, the NADP dependent malic enzyme (ME) generates reducing equivalent NADPH through malate carboxylation to form pyruvate for fatty acid synthesis in oleaginous yeast (Fig. 2) (Wynn et al., 1999). The inhibition of ME activity in oleaginous fungal strain *Mucor circinelloides* decreased the cellular fatty acid content from 24% to 2% (Wynn et al., 1999). While alternatively, its heterologous overexpression in the *M. circinelloides* strain R7B, from *Mortierella alpina* resulted in 2.5-fold and *Rhodotorula mucilaginosa* IIP132 resulted in 1.18-fold increase in fatty acid content (Bandhu et al., 2019; Zhang et al., 2007). The pivotal role of ME in enhancing NADPH production during fatty acid biosynthesis was thus established. ME activity, however, was not observed as a common pathway in all oleaginous species for generating NADPH which can contribute toward FA synthesis e.g., *Lipomyces starkeyi* (Takaku et al., 2020). At the same time, the overexpression of ME was not essentially enhancing the lipid accumulation in *Yarrowia* sp. (Beopoulos et al., 2011a, 2011b). The existence of a robust alternative NADPH-generating system other than ME dedicated to lipid synthesis was therefore strongly indicated. In different oleaginous fungi and yeast, a rather integrated NADPH production pathway

exists via malic enzyme or glyoxylate cycle or HMP pathway and connected to amino acid synthesis, especially under nitrogen-deficient conditions. Triacylglycerol production is thereby a concerted mechanism, rather than resulting from a discrete pathway. Several recent studies revealed that leucine auxotrophy caused a 2.5-fold decrease in cellular fatty acid content, while its genic expression restored the content in *M. circinelloides* R7B (Rodríguez-Frómata et al., 2013). Despite concurrent ME overexpression and increased activity, the fatty acid content remained unchanged. Participation of the leucine metabolic pathway for acetyl-CoA generation during fatty acid synthesis in *M. circinelloides* played a crucial role, other than ME activity (Hao et al., 2014; Kohlhaw, 2003; Rodríguez-Frómata et al., 2013). A significant relevance of leucine synthesis pathway and lipogenesis was also observed (Fig. 2).

2.2.2. Amino acid metabolism

There is strong connection between amino acid catabolism and lipid synthesis pathway as recent studies demonstrated that amino acids provide substantial carbon for the TCA cycle, acetyl-CoA and NADPH generation, driving de novo lipogenesis in yeast cells (Cai et al., 2022).

The decreased levels of amino acid metabolism during the stationary phase of the microbial growth diverts carbon flux from amino acid and nucleotide pathways toward lipid biosynthesis by providing carbon skeletons i.e., acetyl-CoA. Especially, during nitrogen stress, several nitrogen molecules are generated through recycling, breakdown and redistribution of non-essential amino acids, proteins stored as essential amino acids and intermediate molecules by the glutamate/glutamine pathway (Fig. 2). An upsurge in glutamate concentration enhances the TCA cycle enzyme activities to supply amino acids or nucleotides as biosynthetic building blocks. The same is not observed in non-oleaginous yeasts (Vorapreeda et al., 2012). This indicates a plausibility of intersection between fatty acid biosynthesis and amino acid

metabolic pathways, where acetyl-CoA provided by amino acid metabolic pathway feeds fatty acid synthesis (Fig. 2).

The induction of autophagy under limited nitrogen conditions is another important phenomenon observed in *R. toruloides*. The free fatty acids liberated from cellular residues potentially contribute to alternative acetyl-CoA production for the activation of the mitochondrial β -oxidation (MBO) pathway (Section 4.1). The nitrogen stress could be relieved by free amino acids. Therefore, autophagy also plays a role in making more room for lipid-droplet formation by clearing organelles or residues of cellular metabolism (Rodríguez-Navarro and Cuervo, 2010).

2.2.3. Hexose monophosphate pathway (HMP)

HMP Pathway is a parallel pathway to glycolysis that mainly supplies NADPH, which play a crucial role during lipid synthesis in the intracellular compartment of oleaginous yeast. The role of HMP has been studied exclusively as a major supplier of NADPH for lipid biosynthesis in yeasts (Ruchala and Sibirny, 2021). The pathway runs parallel to glycolysis unidirectionally to generate ribulose-5-phosphate (Ru5P) along with NADPH (Fig. 2). Depending upon the cellular energy requirement, the ribose-5-phosphate (R5P) moiety gets converted into different glycolytic intermediate i.e., glyceraldehyde-3-phosphate (G-3-P), fructose 6-phosphate (F-6-P) or nucleotides precursors via a non-oxidative process. During nitrogen limited and carbon rich conditions the NADPH demand for fatty acid biosynthesis increases. Thus, R-5-P conversion to G-3-P and F-6-P increases through a specific transketolase enzymatic activity that gets converted to G-6-P through gluconeogenesis and feed HMP for even more NADPH production. On the other hand, during a nitrogen-rich growth condition, the R-5-P conversion takes place to produce nucleotide precursors e.g. DNA/RNA via pyrophosphate activity (Fig. 2).

This showed that the nitrogen abundance in the fermentation medium could stimulate glucose metabolism and divert the carbon flux to the glycolytic pathway (Evans and Ratledge, 1985), and the limitation connected the glycolytic cycle to HMP through gluconeogenesis by generating G-3-P and F-6-P metabolites in oleaginous yeast for NADPH production (Akram et al., 2019). In non-lipogenic *S. cerevisiae*, HMP was found to play only a minor role, while glucose catabolism was of greater significance (Bertels et al., 2021). The role of HMP for NADPH production suggest only 2–30% share for glucose dissimilation in non-oleaginous yeasts e.g., *S. cerevisiae* (Suomalainen, 1971). It goes as high as up to 30–50% and 60–80% for *C. utilis* and *Rhodotorula* sp. respectively (Bruinenberg et al., 1983). The role of HMP is important in maintaining a redox balance in *S. cerevisiae* and in this aspect oleaginous yeast differ from others. It has been observed that after ZWF1 (glucose-6-phosphate dehydrogenase) and GND1(6-phosphogluconate dehydrogenase) gene deletion, during a counter cellular glutathione concentration response is demonstrated, to manage NADPH production (Table 1), indicating that the physiological state of the cell is also a determinant of switch between these pathways (HMP shunt pathway to gluconeogenesis and the glycolytic pathway) and is influenced by carbon flux. Yet again not only redox potential of the biochemical pathways, but the lipid accumulation is also governed by process parameters optimized during the cultivation of yeasts. The activation and suppression of specific pathways is indirectly regulated by the process parameters during lipid accumulation.

3. Enhanced oleagenicity: influence of bioprocess parameters

The optimum amounts of carbon, nutrients (mainly nitrogen) and oxygen in a fermentation medium contributes equally for improving oleagenicity. The availability of these factors mobilises crucial precursors and metabolites to stop stalling of certain metabolic pathways and help in shifting the carbon flux and balance the redox potentials. Some key process parameters that influence the generation of lipid or ethanol biosynthesis in yeast have been discussed hereafter.

3.1. Nitrogen limitation

Nitrogen limitation in oleaginous microorganisms starts a cascade of reactions as follows:

- An increased AMP deaminase activity decreases the AMP content in the cytosol and mitochondrion.
- The decrease in AMP content inside the mitochondria impedes AMP-dependent ICDH activity resulting in a lack of isocitrate metabolism and consequent accumulation.
- Aconitase equilibrates it with citric acid, which also accumulates thereof.
- Citrate efflux to the cytosol through the mitochondrial membrane in exchange for malate influx takes place.
- In the cytoplasm, ACL converts this citrate and CoA, utilizing ATP to generate oxaloacetate, ADP + Pi and acetyl-CoA, which enters the fatty acid biosynthesis.
- Oxaloacetate is again converted to malate via malate dehydrogenase activity to counter ion exchange for citrate efflux from mitochondria (Fig. 2).

The transcriptional profiles of oleaginous *S. cerevisiae* D5A reveals nitrogen deprivation as a trigger to upregulate the genes of nitrogen metabolism, fatty acid biosynthesis, amino-acid metabolism, glycolysis, TCA cycle, and glyoxylate cycle as compared to non-oleaginous strain. Other than enhanced neutral lipid storage, phospholipid storage also increases to maintain cellular integrity. An increased phospholipid conversion is one of the mechanisms to accumulate increased lipid content in yeast (Czabany et al., 2008). Substantially low differences of phospholipid transcript levels between oleaginous and non-oleaginous yeasts indicate this may not be the strong probability for higher lipid accumulation in different strain backgrounds. Other than the availability of an optimum nitrogen source, a specific C/N ratio is also an integral aspect of enhanced lipid accumulation in oleaginous yeasts. The expression of nitrogen assimilating genes (GLN1 and GLN2) is up-regulated during N-limiting conditions in *Y. lipolytica*. Metabolic and lipidomic profiling of carbon flux at genome scale, under high C/N ratio in *Y. lipolytica* revealed that lipid accumulation is associated with transcriptional regulation of amino acid biosynthesis and not lipid metabolism. *Rhodospiridium kratochvilovae* HIMPA1 was cultivated in glucose synthetic medium with different nitrogen (1 g/l and 0.1 g/l) and phosphorus (0.05 g/l and 0.1 g/l) limited regimes. Among various N and P-limited regimes, the highest lipid content ($60.34 \pm 0.69\%$) was obtained with simultaneous N and P limitation (0.1 g/l N and 0.05 g/l P) (Patel et al., 2017).

These results are similar to a notable ethanol production observed in non-oleaginous *S. cerevisiae* under nitrogen limitation. As a stress response mechanism, the upregulation of nitrogen catabolite repression, and ammonia transporter orthologue (MEP2) genes have been observed in *S. cerevisiae*. Similar results were observed in chemostat cultures of oleaginous *R. toruloides*, along with upregulation of genes linked to amino-acid permeases, central nitrogen metabolism machinery i.e., glutamine synthase (GLN), glutamate dehydrogenase (GDH), urea and urate degradation cell (Zhu et al., 2012). They directly enhanced a direct acetyl CoA supply in the cytoplasm through an upregulated gene expression. Thus, the metabolic contribution of amino acids, e.g., leucine, phenylalanine, and tyrosine to generate more NADPH and acetyl-CoA for lipid metabolism could be strongly suggested (Wang et al., 2013).

3.2. Sulphate and phosphate limitation

The multiomics analyses of the oleaginous yeast *R. toruloides* (Wang et al., 2018), shed light on a less studied phosphate limited lipid accumulation. An inorganic phosphate (Pi) limitation facilitated up-regulation of triacylglycerol biosynthesis by plausible

dephosphorylation of AMP that acts as an allosteric activator of the ICDH enzyme (Fig. 2). Another study supported an improvement in biomass yield, up to 70% in yeast *Yarrowia lipolytica* MUCL30108, upon adding potassium phosphate to the medium, yet the total lipid content remained unchanged (Hoarau et al., 2020). However, a high C/P ratio with a high C/N ratio in both oleaginous (*Y. lipolytica*, *R. glutinis*, *T. cutaneum*, *Candida* sp.) and non-oleaginous yeasts (*K. polysporus*, *T. delbrueckii*, *S. cerevisiae*) prompted a high biomass yield and transformed fatty acid composition (Kolouchová et al., 2016). A higher polyunsaturated lipid accumulation was also reported in *T. cutaneum*. Unlike *Y. lipolytica* MUCL30108, a combined effect of phosphorous and nitrogen changed the fatty acid composition of yeast lipid. More specifically a C/N ratio, 30 and C/P ratio, 1043 promoted higher biomass content while a decreased polyunsaturated FAs content (~ 80%) in oleaginous yeast compared to non-oleaginous strains (Kolouchová et al., 2016). Reports on the effect of sulphate limitation on the growth of oleaginous microbes are limited, and this phenomenon needs to be explored further. Only a few findings suggest sulphate limitation has been effective for promoting lipid accumulation up to 58.3% with a very high initial C/S molar ratio of 46,750 in *R. toruloides* Y4 (Wu et al., 2011). These unexplored avenues can support major improvement in lipid accumulation at process optimization levels.

3.3. Oxygen limitation

The rate of oxygen supply is one of the key process parameters that affects the respiration rate and as well as fermentation in yeast (Dasgupta et al., 2017). The levels of oxygen availability after the depletion of nitrogen, plays a critical role in recycling and re-allocation of preformed nitrogen intermediates for increasing biomass growth and lipid accumulation. The metabolomic analysis of *M. alpina* under nitrogen limiting conditions highlighted the importance of optimum oxygen supply for lipid generation. Higher levels of oxygen supply accelerated lipid metabolism with negligible lipid accumulation. A lower supply could be insufficient in activating nitrogen catabolic reactions (Lu et al., 2021). It does not merely affect the lipid accumulation but also determines the fatty acid composition. This effect is unfolded through desaturases and elongases activities, which are regulated by oxygen supply. A medium level oxygen supply under nitrogen limited conditions can result in long chain-PUFA production (Valdebenito et al., 2023). The comparative studies for ethanol production in *S. cerevisiae* under oxygen limiting and non-limiting conditions reported a 23% increase in biomass and ethanol production up to 147 g/l as compared to microaerobic conditions (Alfenore et al., 2004). In oleaginous *Lipomyces starkeyi*, lipid accumulation decreased from 60% to 50% with increasing the agitation rates to 300 rpm (Calvey et al., 2016). This supports the efficiency of a two stage-dissolved oxygen (DO) controlled studies employing *R. glutinis*, which employs lowering the DO in the second phase to improve lipid accumulation (Yen and Zhang, 2011). While in a separate scale-up study using *Y. lipolytica*, DO as a sole controlling parameter yielded a high lipid production of $44.8 \pm 1.2\%$ (w/w) at 30% oxygen saturation (Magdouli et al., 2017). This can be concluded that limiting oxygen levels causes a decrease in oxidative phosphorylation of glucose, leading to a decrease in ATP formation but excessive NADH generation via glycolysis. The imbalance is resolved via alternative reduction reactions that re-oxidize NADH back to NAD⁺, resulting in reduced alcohol accumulation. Parallely, the oxygen limitation also regulates the growth rates regulating the ATP requirement for replication and reduced protein synthesis. Thus, under nitrogen limiting conditions (specifically during the stationary phase) the plausible diversion of excessive carbon into lipid synthesis in oleaginous yeasts is governed by oxygen levels as well. An understanding of a typical mechanism remains unresolved, warranting further research.

3.4. Temperature stress

Growth rate, lipid productivity, and fatty acid composition were all impacted by temperature and glucose levels, making oleaginous yeasts a viable option for producing single-cell oil (Amaretti et al., 2010). It has been reported that a robust oleaginous yeast *Rhodotorula glutinis* ZHK exhibits temperature-dependent metabolic control; at low temperatures (16 °C), exopolysaccharide production is enhanced, whereas at high temperatures (32 °C), carotenoids (torulene, torularhodin) and lipid accumulation are increased (Zhao and Li, 2022). These changes are mainly due to temperature-mediated regulation of the TCA cycle and acetyl-CoA availability, as suggested by multi-omics analyses employed in this study such advanced techniques provide information for improving fermentation and suggest the areas for applying metabolic engineering techniques (Zhao and Li, 2022).

In contrast to this, under carbon surplus and nitrogen limitation, a psychrophilic oleaginous yeast *Rhodotorula glacialis* DBVPG 4785 could grow robustly and accumulate large amounts of lipids (up to 68% of cell dry weight) at temperatures ranging from -3 to 20 °C (Amaretti et al., 2010) Therefore, the temperature stress is species/strain dependent.

3.5. Multi stress

Significant changes in cell wall composition, such as enhanced glucanans, chitin, and stress-associated glycoproteins, were observed in an evolved multi-stress tolerant oleaginous yeast strain of *Rhodotorula toruloides*. These changes resulted in higher rigidity and stress resistance (Antunes et al., 2024). The findings provided direction for creating yeast strains that are industrially resilient and it emphasized on cell wall remodelling as a crucial component of robustness (Antunes et al., 2024).

In a multi stress study, an oleaginous yeast strain *Rhodotorula toruloides* obtained from adaptive laboratory evolution (ALE) showed increased resistance to many inhibitors found in lignocellulosic hydrolysates, which was reinforced by significant genomic and transcriptome remodelling (Antunes et al., 2024). Broad multi-stress resistance is supported by important alterations in cell surface biogenesis and stress-response pathways, underscoring adaptive evolution as a successful tactics for creating reliable industrial yeast cell factories (Antunes et al., 2024).

4. Cellular regulation of lipogenesis and lipid storage

The oleagenicity of yeast is not limited to end-product i.e., TAG or phospholipid synthesis. A crucial cellular strategy is essentially followed to guard this lipid accumulation from lipolysis or lipotoxicity. After synthesis, the neutral lipids are stored in the phospholipid monolayer enclosed, protein-coated specialised organelles termed as lipid droplets or bodies (Fig. 3). The protein present in lipid bodies belong to a diverse family of proteins known as perilipin protein that governs the lipolysis (Bickel et al., 2009). The presence of a perilipin-like protein MPL1 in fungi protected the stored neutral lipids from degradation (Wang and St. Leger, 2007). The MPL1-like perilipins have also been reported in non-conventional oleaginous yeast *Y. lipolytica*. In basidiomycetes yeast *R. toruloides* a new class of perilipin proteins was investigated, which is composed of the amino-terminal perilipin and carboxy-terminal apolipoprotein domains. The volume of each lipid droplet and amount of lipid stored in, are important during the lipid accumulation phase. Transcriptome analysis studies in oleaginous yeasts revealed that an increased lipid content may also be ascribed through a reduced TAG degradation and phospholipid turnover (continuous synthesis and degradation process) under nitrogen deprivation (He et al., 2018; Pomraning et al., 2016). The critical comparisons that make a difference in lipid accumulation must be identified through analysis of upregulation or downregulation patterns of key modulators.

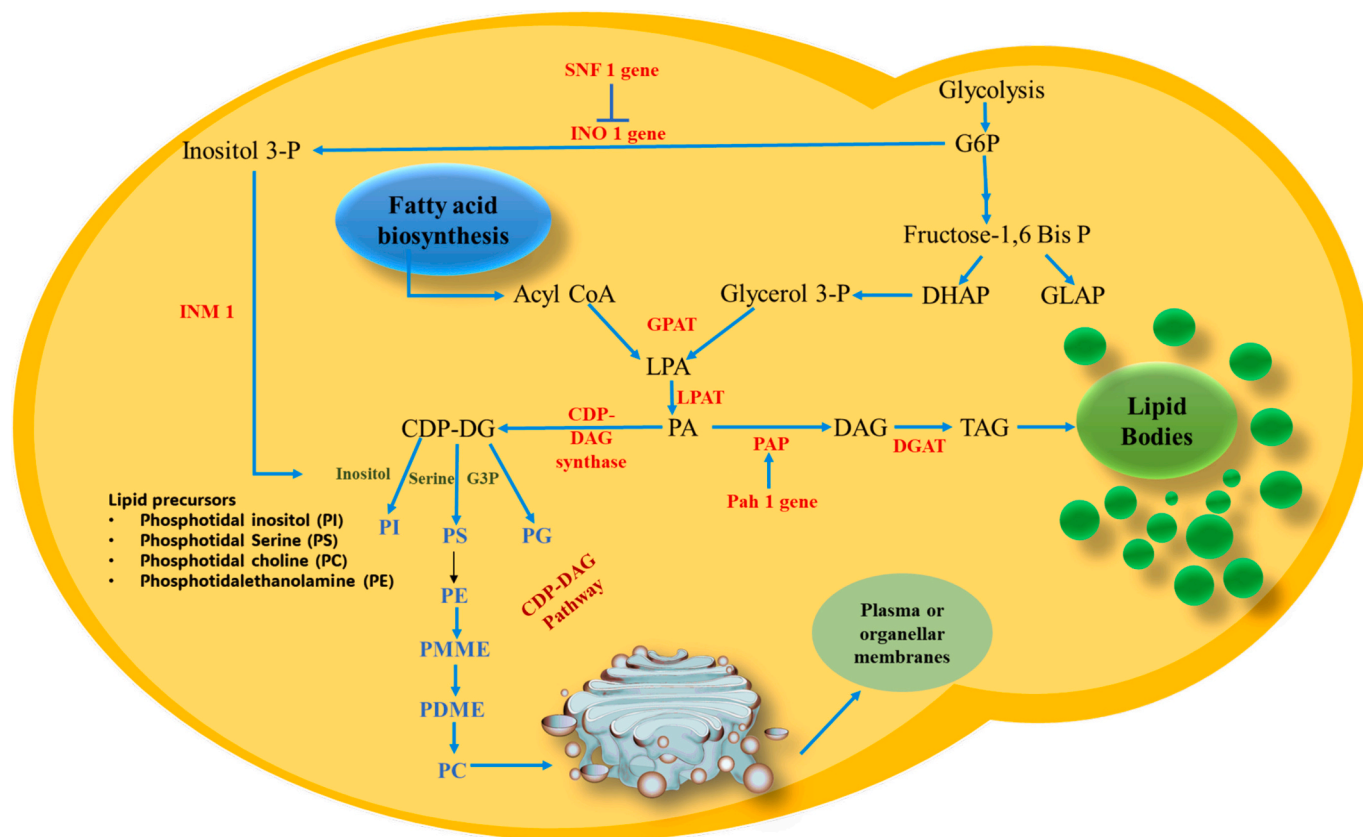


Fig. 3. Regulation of phosphatidic acid in phospholipid and neutral lipid synthesis within the endoplasmic reticulum lumen under both nutrient-limiting and nutrient-rich conditions. The figure further illustrates the transcriptional regulation of inositol linked to glycolysis operating in the cytosol. Membrane lipid transport through the Golgi apparatus and neutral lipid transport through lipid bodies are also depicted via the CDP-diacylglycerol pathway and the Kennedy pathway, respectively.

4.1. Carriers, precursors, and transcription regulators

Citrate, pyruvate, and malate are significant intermediates linked to TCA cycle that serve as substrates in different set of reactions to commonly assist fatty acid biosynthesis pathways. Few specific importers, and exporters linked to these metabolites present in the boundaries of cell organelles play crucial role to determine the oleaginous property of yeast. The mitochondrial pyruvate carriers and their isomers MPC1, MPC2, and MPC3 were found in the inner mitochondrial membrane of yeasts. The cytoplasmic pyruvate uptake facilitated by overexpression of these importers could lead to 18% increment in fatty acid synthesis (Bricker et al., 2012). However, there is limited information on discrete roles played by these carriers in oleaginous species. Yet the possession of phosphate, pyruvate, and di- and tri-carboxylic anions by mitochondrial carrier system confirms a faster transport across the membrane comparatively (Palmer and Hall, 1972). Furthermore, the extramitochondrial concentration of L-malate is regulated in direct proportion to citrate efflux and L-malate uptake (Fig. 2). In *Candida curvata*, the presence of a saturated translocator was observed to be regulating this transport under citrate saturated intramitochondrial conditions. The malate specific mitochondrial tricarboxylate carriers have long been investigated in 10 different yeast strains (Evans et al., 1983). The L-malate and citrate efflux rate was 2.5-times higher in oleaginous yeasts than the non-oleaginous counterparts. The intramitochondrial citrate concentration was also observed to be 3–4 times higher in oleaginous yeast.

Another category of mitochondrial citrate carriers, Ctp1 and Yhm2, have also been identified and characterized both in *S. cerevisiae* and oleaginous yeast *Y. lipolytica* (Castegna et al., 2010; Kadooka et al., 2019). The lipid concentration decreased upon Yhm2 deletion in

Y. lipolytica (Yuzbasheva et al., 2019). While, in a separate study, the overexpression of citrate carrier genes enhanced lipid biosynthesis in oleaginous fungus *Mucor circinelloides*. Furthermore, the deletion of putative tricarboxylate mitochondrial carriers YICTP1 and YIYHM2 inhibited the growth of *Y. lipolytica* mutants in citrate minimal medium (Yuzbasheva et al., 2019). Growth was restored by glucose supplementation, however, the mutant remained incapable of citric and isocitric acid production resulting in a reduced total neutral lipid production (Yang et al., 2019). Later a heterologous overexpression of YHM2 gene from *Aspergillus niger* to *Y. lipolytica* increased the citric acid production up to 45% (Kirimura et al., 2019; Yuzbasheva et al., 2019). These findings strongly suggest the vital role played by mitochondrial citrate carriers in lipid metabolism by offering the initial substrate of lipid synthesis mechanism. Thus, insights into the metabolic pathways and unexplored gene functions of mitochondrial citrate carriers suggested operational routes for improving citric acid or lipid biosynthesis in oleaginous yeast strains.

In case of the yeasts, as well as other eukaryotes, phosphatidic acid (PA) is one of the key precursors for the synthesis of intermediate molecule phosphatidylinositol (PI). PI serves a precursor for synthesis of several molecules in the downstream pathway for lipid-mediated signalling e.g., inositol polyphosphates, phosphoinositides, and sphingolipids containing inositol etc. The stress conditions such as inositol deprivation regulates the expression of INO1 gene that codes for inositol 3-phosphate synthase (*Ino1p*), and further catalyses inositol synthesis (Henry et al., 2012). Besides inositol and phospholipid biosynthesis pathway, the stress response proteins particularly involved in unfolded protein response gets upregulated during inositol limitation. As revealed through a genome-wide microarray data of wild type cells, the inositol starvation and secretory stress together divert lipid metabolism from

cellular membrane phospholipid synthesis (or cell surface expansion) to TAG biosynthesis synergistically (Gaspar et al., 2008). During nitrogen starvation several precursors, carriers and transcriptional regulators are affected that in turn upregulate lipid biosynthesis.

The nitrogen stress induced for lipid accumulation changes the course of enzymatic actions in oleaginous yeasts as discussed in Section 3.1 and influences the transcriptional levels. Nitrogen starvation inhibits the TORC1 complex (Target of Rapamycin Complex 1) and facilitates the nuclear import and expression of GATA transcription factors (Gln3, Gat1), which were otherwise present in the cytoplasm in nitrogen excess conditions. Thus, nitrogen starvation regulates nitrogen catabolite repression (NCR) related genes through a controlled translocation of GATA transcription factors in *S. cerevisiae* (Beck and Hall, 1999). Eleven putative GATA-type transcription factors were reported in *R. toruloides* for a plausible transcriptional regulation of nitrogen metabolism (Zhu et al., 2012), suggesting similarity in oleaginous and non-oleaginous yeast strains. The transcriptome profiling of oleaginous yeast *Y. lipolytica* shows an upregulation of only FAS1/2, and PYC1 genes while the expression of ACL1, ACC1, ME1, IDH1 and IDH2, remained the same under nitrogen starvation (Morin et al., 2011). An assisted comparative proteomic analysis later suggested a post-transcriptional regulation of these genes. Therefore, lipogenesis in oleaginous yeast could be associated with upregulated glycolysis, increased packaging of lipid droplets, followed by an impaired TCA cycle. Transcription factors and regulators control and regulate FA synthesis and therefore are considered as a significant target for engineering metabolic pathways. The genes encoding transcriptional regulators of the lipid metabolic pathway, e.g., SNF1 (serine/threonine protein kinase), MGA2 (transcriptional activator and functional analogue of mammalian SREBP-1) or, MIG1 (a zinc finger transcriptional repressor) in *Y. lipolytica*, were deleted or mutated to make those non-functional. Such strategies were found to facilitate lipid biosynthesis indicating their role in regulation of synthesis pathway (Liu et al., 2015; Seip et al., 2013a; Wang et al., 2013).

4.2. Prevention of TAG metabolism

Unlike *S. cerevisiae*, mitochondrial β -oxidation (MBO), ACL, and carotenoid biosynthetic pathways function in *R. toruloides*. In comparison to the peroxisomal β -oxidation, the energy recovery is more prominent in the MBO pathway. Most of this recovery is in the form of ATP, using the flavin adenine dinucleotide (FAD) cofactor through the degradation of fatty acids, which couples with the respiratory chain. Moreover, MBO-associated enzymes contribute to acetyl-CoA production by metabolizing branched-chain amino acids. Thus, MBO might provide alternative acetyl-CoA and energy sources for fatty acid synthesis by degrading amino acids and membrane lipids.

The β -oxidation cycle in peroxisomes of *S. cerevisiae* could be disrupted by deleting the key *pox1* gene. This strategy, however, did not work to progress the production of fatty alcohols or fatty acid ethyl esters (Runguphan and Keasling, 2014). A combined effect of three strategies i.e., (1) disrupting the acyl-CoA transport to the β -oxidation pathway, (2) avoiding TAG biosynthesis, and (3) nitrogen limiting culture conditions, pushed the carbon flux toward fatty acyl-CoA production. A similar strategy was followed for deleting the genes of peroxisomal β -oxidation to improve the TAG accumulation in *Y. lipolytica*. The first step of metabolism is catalysed by six acyl-CoA oxidases (Aox1–Aox6), encoded by *pox1–6* genes (Imatoukene et al., 2020). The absence of all six *pox* genes rendered a strain that was unable to degrade FAs (Dulermo and Nicaud, 2011). Similarly, the deletion of triacylglycerol lipases involved in TAG remobilization such as TGL3 and TGL4, resulted in improved oleaginicinity in yeast (Dulermo and Nicaud, 2011). Engineered *Y. lipolytica* strain designed by blocking native lipid consumption routes and by enhancing precursor supply, achieved overproduction of lipid (Ghogare et al., 2020; Nguyen et al., 2025b). However, proteomic analysis in *R. toruloides* suggested a more

distinctive peroxisomal β -oxidation in yeast cultivated on xylose as carbon source instead of glucose, upon nitrogen starvation. The levels of enzymes (Rhto_03776, Rhto_03890, Rhto_07118, Rhto_05407, Rhto_06581, Rhto_00300) responsible for peroxisomal oxidation was 8- to 16-fold higher in cells grown in xylose than those grown in glucose, during the exponential growth phase. The levels of some enzymes decreased for peroxisomal oxidation in xylose-grown cells, due to decreased Rhto_04298 and Rhto_00300 activity. Other enzymes e.g., Rhto_05520 and Rhto_02517 showed elevated production when cultivated in glucose to match the trend during lipid production phase. Similar patterns were observed during mitochondrial β -oxidation as well, where expression of Rhto_06738, Rhto_04971, Rhto_00397, Rhto_01625 enzymes were 8–128 times upregulated in cells cultivated in xylose. However, upregulation of Rhto_04971, Rhto_00397, Rhto_05797 in glucose-grown cells, was only 2.3–8 times during lipid accumulation phase. Upregulation in genes for β -oxidation and FA biosynthesis in xylose-grown cells resulted in lower lipid production as compared to glucose-grown cells. Lower biomass yield resulted due to use of xylose as a substrate for growth rather than glucose, again explaining lower levels of lipid production (Tiukova et al., 2019).

In *R. toruloides*, the number of enzymes produced by a cell during TAG biosynthesis did not change significantly, except glycerol-3-phosphate dehydrogenase (Rhto_02273), when either hexose or xylose was used as the carbon source. The enzyme involved in PA biosynthesis, termed as 1-acyl-sn-glycerol-3-phosphate acyltransferase (Rhto_06718), was found to be upregulated during growth in glucose medium. The TAG lipase (Rhto_00361) levels increased during cultivation in xylose, but it was susceptible to fatty acid degradation. However, it is speculated that increasing perilipins might counteract lipase levels to protect lipid droplets from hydrolysis (Tiukova et al., 2019). In *R. toruloides*, the inhibition of TOR signalling or nutrient signalling gave rise to a cascade of processes leading to autophagy, which was proven by the increase in the number and levels of autophagy-related genes when nitrogen-limited conditions prevailed. This resulted in the release of free amino acids and fatty acids due to degradation of proteins and membrane lipid, respectively (Rodriguez-Navarro and Cuervo, 2010) (Fig. 3).

4.3. Cytosolic lipid and membrane phospholipid synthesis

Saccharomyces cerevisiae is a robust model system to infer the molecular biology and biochemistry of cellular lipids, that exist as (1) free fatty acids, (2) membrane lipids, or (3) storage lipids. The PL composition varies as per organellar membrane formation requirement as well as carbon source used for yeast fermentation (Klug and Daum, 2014; Koch et al., 2014; Simons and Sampaio, 2011; Tuller et al., 1999). The PL synthesis takes place from phosphatidic acid (PA), and PA, in turn, is generated from lyso-PA (LPA) that originates from glycerol-3-phosphate (G-3-P), the reaction being catalysed by G-3-P-acyltransferases (GPAT) (Fig. 3). PA may also contribute to PL biosynthesis due to the action of ER-localized cytidine diphosphate diacylglycerol (CDP-DG) synthase to convert it to (CDP-DG), where, CDP-DG acts as the precursor molecule after combining with serine, inositol or G-3-P for the formation of all major phospholipids. Inositol, that is derived from glucose-6-phosphate may be converted to inositol, by inositol-3-phosphate synthase (*Ino1p*) or inositol 3-phosphate phosphatase (*Inm1p*) or directly transported from the environment using inositol permeases (Klug and Daum, 2014). In oleaginous yeast, the repressors of inositol-1-phosphate synthase (*ino1*) control phospholipid synthesis during transcription by binding to cis-regulatory element UAS_{INO} in the promoter region of the *ino1*, during the synthesis of inositol phosphates and inositol-containing phospholipids (Feng et al., 2015) (Fig. 3). The expression of the *ino1* is regulated by a dozen of transcription factors by a family of INO1 regulators to control the expression of this gene (Feng et al., 2015). To understand their significance in gene expression leading to the formation of PLs, the transcription factors that served as negative regulators of the *ino1* e.g., RPD3, OPI1, PAH1, UME6, ITC1, SIN3, MOT1 and ISW2, were knocked

out. Therefore, those could not suppress the expression of INO1 in phospholipid production. The knockout strains Δ MOT1, Δ PAH1, Δ OPI1, and Δ RPD3 showed enhanced hexadecanol production, showing that INO1 was important for PL biosynthesis (Feng et al., 2015).

The type of carbon also affected the formation of lipid bodies or membrane fatty acids. During fatty acid biosynthesis, enzymes involved in phospholipid biosynthesis, e.g., phosphatidate cytidyltransferase (Rhto_01718) and phosphatidyl-*N*-methylethanolamine *N*-methyltransferase (Rhto_03783), were upregulated, when *R. toruloides* was grown on xylose medium (Tiukova et al., 2019). The cells grown in glucose, reported higher levels of phosphatidylethanolamine (formed by Rhto_03399, phosphatidylserine decarboxylase), that is a major molecule of cellular membranes. Also, it was observed that the proteins involved in β -oxidation had increased levels in xylose-fed cells, probably due to a higher ATP demand. Deletion of the β -oxidation pathway would thus be expected to enhance lipid formation and growth in xylose fed cells as compared to glucose in *R. toruloides*. The type of fatty acid accumulated (saturated/unsaturated) was also found to vary among yeast strains. In general, the organisms used for single cell oil production reportedly generate very high contents of few desired fatty acids. For example, *Cryptocodinium cohnii*, used in the Martek process, produced a TGA content rich in DHA (docosahexaenoic acid) (40%–50%) as the sole PUFA of the total fatty acids. The monounsaturated fatty acid found was mainly oleic acid (C_{18:1}) but in a relatively low abundance. The single cell oil generated from oleaginous microbes have this unique characteristic of enrichment of specified categories of fatty acids, which is not observed in plants or animals derived oil. Thus, the enriched fatty acid fractions that can only be met by microbial oils, can be looked for dietary requirement as well. Apart from lipids for use as biofuel feedstock (Ahuja et al., 2023; Paul, 2024), other industrially important products that primarily depend on an efficient supply of acetyl-CoA, e.g. poly-3-hydroxybutyrate (PHB) (Li et al., 2017), terpenoids (Vickers et al., 2017), β -farnesene (Shi et al., 2021), polyketides triacetic acid lactone (Liu et al., 2019) and DHA (Xue et al., 2013) may be produced in large scale using oleaginous yeasts, considering them as robust cell factories (Gu et al., 2023; Shi and Zhao, 2017).

5. Conclusion and future perspectives

Yeast and fungi occur ubiquitously in various niches because they utilize a wide range of substrates for their survival. Their lifestyle choices—especially for lipid or ethanol production—depend on carbon flux regulation, redox balance, nutrient availability, metabolic pathways, redox balance, presence of carriers, precursors, regulators, etc. Oleaginous yeasts are characterized by (i) cytosolic acetyl-CoA generation via ACL, (ii) strong NADPH supply via PPP/glyoxylate pathways, and (iii) nitrogen-triggered TOR regulation, whereas non-oleaginous yeasts rely on fermentative NADH reoxidation and lack efficient lipid-directed carbon flux. Stress factors such as inhibitors, (furfural, HMF, vanillin) activate the expression of particular enzymes (PGM, ALDH, Pck1) during lipogenesis in oleaginous yeast and HMP plus glyoxylate cycle pathway provides reductants for lipogenesis. NADPH production is important for lipogenesis, but resources for supply of NADPH (Table 1) vary among strains. In ethanologenic yeast, overexpression of ACC1, FAS 1&2 overexpression could switch them to oleaginous lifestyle.

The interplay of biochemical pathways also regulates the properties of lipid formed and its localization within the cell, either as cell membrane phospholipid or as oil globule surrounded by a perilipin. Information on metabolic switches may assist the designing of synthetic pathways for high-value end products and pave the path for genetic engineering to suppress competing pathways and redirect carbon flow toward lipid biosynthesis.

Looking ahead, the integration of omics data, systems biology, and machine learning promises a new biotechnological solution such as Data-Driven Synthetic Microbes (DDSM) optimized for sustainable lipid production. AI-assisted metabolic modelling, predictive flux analysis,

and adaptive laboratory evolution driven by computational learning can accelerate strain optimizations for robust yeast cell factories. Future circular bioeconomy challenges will require continued convergence of metabolic engineering and artificial intelligence.

CRedit authorship contribution statement

Debarati Paul: Software, Methodology, Conceptualization. **Neha Bansal:** Writing – original draft, Data curation. **Alok Patel:** Writing – review & editing. **Debansh Ghosh:** Visualization, Supervision, Investigation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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