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Glutamate dehydrogenases in the oleaginous yeast *Yarrowia lipolytica*

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Abstract

Glutamate dehydrogenases (GDHs) are fundamental to cellular nitrogen and energy balance. Yet little is known about these enzymes in the oleaginous yeast *Yarrowia lipolytica*. The *YALI0F17820g* and *YALI0E09603g* genes, encoding potential GDH enzymes in this organism, were examined. Heterologous expression in *gdh*-null *Saccharomyces cerevisiae* and examination of *Y. lipolytica* strains carrying gene deletions demonstrate that *YALI0F17820g* (*ylGDH1*) encodes a NADP-dependent GDH whereas *YALI0E09603g* (*ylGDH2*) encodes a NAD-dependent GDH enzyme. The activity encoded by these two genes accounts for all measurable GDH activity in *Y. lipolytica*. Levels of the two enzyme activities are comparable during logarithmic growth on rich medium, but the NADP-ylGDH1p enzyme activity is most highly expressed in stationary and nitrogen starved cells by threefold to 12-fold. Replacement of ammonia with glutamate causes a decrease in NADP-ylGdh1p activity, whereas NAD-ylGdh2p activity is increased. When glutamate is both carbon and nitrogen sources, the activity of NAD-ylGDH2p becomes dominant up to 18-fold compared with that of NADP-ylGDH1p. Gene deletion followed by growth on different carbon and nitrogen sources shows that NADP-ylGdh1p is required for efficient nitrogen assimilation whereas NAD-ylGdh2p plays a role in nitrogen and carbon utilization from glutamate. Overexpression experiments demonstrate that *ylGDH1* and *ylGDH2* are not interchangeable. These studies provide a vital basis for future consideration of how these enzymes function to facilitate energy and nitrogen homeostasis in *Y. lipolytica*.

KEYWORDS

energy metabolism, glutamate dehydrogenase, nitrogen metabolism, oleaginous, *Yarrowia*

1 | INTRODUCTION

The oleaginous yeast *Yarrowia lipolytica* has gained growing interest both as a model organism and as a means for the production of numerous biomolecules (Beopoulos, Chardot, & Nicaud, 2009; Nicaud, 2012). This organism is capable of accumulating significant levels of lipid (>10% dry cell weight) either from hydrophobic precursors such as fatty acids and alkanes (Fickers et al., 2005; Fukuda, 2013) or via de novo biosynthesis from glucose (Beopoulos et al., 2008; Ratledge & Wynn, 2002). Metabolic engineering combined with strategic culture conditions have increased levels of accumulation to nearly 90% dry cell weight (Beopoulos et al., 2009; Blazeck

et al., 2014). Thus, *Y. lipolytica* has been extensively examined as a microbial system for the production of biofuels (Beopoulos, Cescut, et al., 2009; Rakicka, Lazar, Dulermo, Fickers, & Nicaud, 2015) and shows promise as a platform for production of numerous hydrophobic products for nutritional and pharmaceutical uses such as polyunsaturated, hydroxylated, and other specific fatty acids, waxes, carotenoids, and biosurfactants (Ledesma-Amaro & Nicaud, 2016; Mishra et al., 2018; Ratledge & Wynn, 2002; Sabirova et al., 2011; Stamatia et al., 2016). Furthermore, *Y. lipolytica* is also recognized as a potential means for industrial production of citric acid, which the organism secretes into its growth medium (Barth & Gaillardin, 1997; Gonçalves, Colen, & Takahashi, 2014; Hu, Li, Yang, & Chen, 2019; Papanikolaou et al., 2009).

The initial, shared biochemical processes (Figure 1) by which *Y. lipolytica* incorporates carbon from glucose into accumulated lipid or into secreted citric acid are well understood (Barth & Gaillardin, 1997; Papanikolaou & Aggelis, 2011; Papanikolaou et al., 2009). Pyruvate is produced from the glucose via glycolysis, followed by transport into the mitochondrion and conversion to acetyl-CoA. Acetyl-CoA is condensed with oxaloacetate to produce citrate, which may continue in the citric acid cycle (CAC; via reversible isomerization to isocitrate, etc.) or be exported back to the cytoplasm. Induction of both lipid accumulation and citric acid production follows exhaustion of nitrogen in the medium. The present model for the effect is due to a coincident drop in cellular AMP levels. Because AMP is an allosteric activator of isocitrate dehydrogenase, lowered AMP levels result in decreased conversion of isocitrate to α-ketoglutarate. This leads to an elevation in isocitrate and citrate levels within the mitochondrion, and ultimately, citrate is transported into the cytoplasm. The cytoplasmic citrate may be cleaved by the ATP-citrate lyase enzyme, producing acetyl-CoA, which serves as the precursor for de novo fatty acid synthesis and, thereby, intracellular triacylglycerol synthesis. Alternatively, the cytosolic citrate may be secreted into the growth medium.

A large body of investigation has focused upon understanding and engineering of carbon metabolism in *Y. lipolytica* for the purpose of increasing lipid and citrate production, including elucidating the specific pathways, maximizing the availability of reducing equivalents, reducing the amount of lipolysis, and eliminating the diversion of carbon into storage as glycogen (Beopoulos, Cescut, et al., 2009; Bhutada et al., 2017; Coelho, Amaral, & Belo, 2010; Dulermo et al., 2013; Dulermo et al., 2015; Dulermo et al., 2015; Fakas, 2017; Fickers, Marty, & Nicaud, 2011; Fickers, Nicaud, Gaillardin, Destain, & Thonart, 2004; Haddouche et al., 2011; Hardman, McFalls, & Fakas, 2017; Loira, Dulermo, Nicaud, & Sherman, 2012; Papanikolaou et al., 2009; Trebulle, Nicaud, Leplat, & Elati, 2017). Yet, despite the central role of nitrogen starvation in the process, the examination of nitrogen metabolic pathways and their influence on the extent of citrate and/or lipid accumulation has received comparatively less attention. Recent examination of genome-level transcriptional regulation during nitrogen limitation has revealed that lipid accumulation occurs in the relative absence of lipid metabolic regulation at the transcriptional level, whereas genes involved in amino acid biosynthesis are downregulated to increase flux of carbon into lipid (Kerkhoven, Pomraning, Baker, & Nielsen, 2016). Investigation at the level of the genome-wide protein expression and phosphorylation suggests downregulation of β-oxidation processes with a concomitant increase in those yielding acetyl-CoA formation (Pomraning et al., 2016). Additionally, examination of the regulation of lipid accumulation in *Y. lipolytica* overexpressing diacylglycerol acyltransferase (DGA1) suggests a role for 2-isopropylmalate, which is elevated in leucine depletion, in signalling mechanisms for lipid accumulation (Kerkhoven et al., 2017). Finally, a recent study using in silico model design strategies for increased

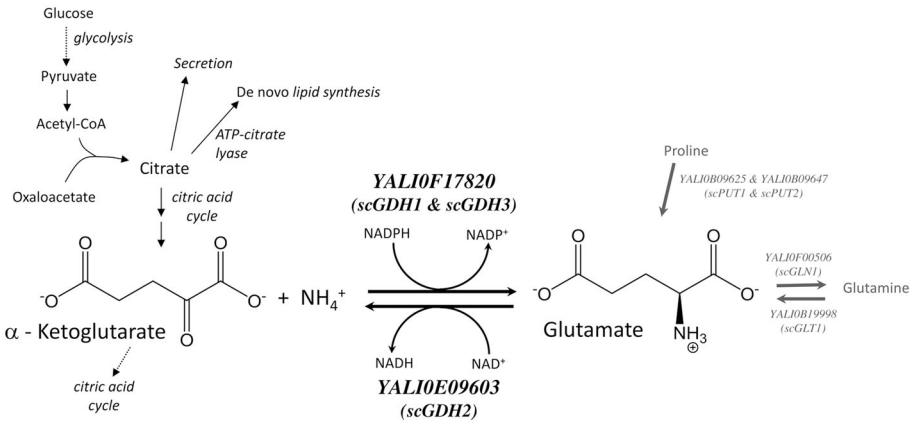

FIGURE 1 Proposed reactions catalysed by *Yarrowia lipolytica* glutamate dehydrogenases (GDHs) in context. Designations of genes identified in *Y. lipolytica* are listed above those of the yeast *Saccharomyces cerevisiae* with which they are homologous. *S. cerevisiae* expresses three GDH genes, two encoding for NADPH-dependent enzymes, which utilize α-ketoglutarate for biosynthesis of glutamate (*scGDH1* and *scGDH3*), and a third encoding a NAD^+-dependent enzyme, which converts glutamate to α-ketoglutarate (*scGDH2*). Potential alternative sources of glutamate formation via glutamine and proline are also included. Figure is adapted from Magasanik and Kaiser (2002), Papanikolaou and Aggelis (2011), and Sieg and Trotter (2014)

production of dicarboxylic acids in *Y. lipolytica* has identified glutamate dehydrogenase (GDH) enzymes as potential engineering targets (Mishra et al., 2018). Such studies implicate a notable role for nitrogen metabolic regulatory mechanisms in the coordinate utilization of carbon and nitrogen in *Y. lipolytica*.

An important link between carbon and nitrogen metabolism is α-ketoglutarate (Figure 1), as it serves both as an intermediate in the CAC and as a point for nitrogen assimilation. A number of studies indicate an important regulatory role for α-ketoglutarate as an indicator and/or signal of carbon and nitrogen status in cells (Araújo, Martins, Fernie, & Tohge, 2014; Chin et al., 2014; Huergo & Dixon, 2015). Nitrogen assimilation via reductive amination of α-ketoglutarate to glutamate is carried out by GDHs, which can also catalyse the reverse, oxidative deamination of glutamate to α-ketoglutarate, which can re-enter the CAC (Figure 1). Because GDH links the CAC and amino acid metabolism, it is recognized as important in the balance of nitrogen and carbon homeostasis in cells (Karaca, Frigerio, & Maechler, 2011; Magasanik, 2003; Plaitakis, Kalef-Ezra, Kotzamani, Zaganas, & Spanaki, 2017). GDHs have been well studied in the yeast *Saccharomyces cerevisiae*, which possesses three GDH-encoding genes (Figure 1). Two NADP-dependent GDHs (NADP-GDHs) are encoded by *scGDH1* and *scGDH3* and catalyse the synthesis of glutamate (Avendano, Deluna, Olivera, Valenzuela, & Gonzalez, 1997; DeLuna, Avendano, Riego, & Gonzalez, 2001; Sieg & Trotter, 2014). The NAD-dependent GDH (NAD-GDH) encoded by *scGDH2* degrades glutamate, producing α-ketoglutarate (Coschigano, Miller, & Magasanik, 1991; Miller & Magasanik, 1990). Carbon and nitrogen sensing at the level of intracellular signalling (Rodkaer & Faergeman, 2014) and the expression of the GDH enzymes (Avendano et al., 2005; Coschigano et al., 1991; Hernandez, Aranda, Lopez, Riego, & Gonzalez, 2011; Magasanik & Kaiser, 2002; Miller & Magasanik, 1990; Miller & Magasanik, 1991; Riego, Avendano, DeLuna, Rodriguez, & Gonzalez, 2002; Zhang et al., 2011) have been extensively studied in *S. cerevisiae*.

The metabolic profile of *S. cerevisiae*, however, differs considerably from that of *Y. lipolytica*. First, *S. cerevisiae* is a facultative anaerobe, whereas *Y. lipolytica* is an obligate aerobe, gaining its energy exclusively via respiration, meaning that the mitochondrion is at the centre of the coordination of energy metabolism in this organism (Dashko, Zhou, Compagno, & Piskur, 2014; Nicaud, 2012). Further, lipid accumulation from citrate via ATP-citrate lyase is absent in *S. cerevisiae* (Boulton & Ratledge, 1981). Although work has been conducted to engineer *S. cerevisiae* for citric acid production (Acourene & Ammouche, 2012), interest has focused more upon the potential of *Y. lipolytica* for this role (Cavallo, Charreau, Cerrutti, & Foresti, 2017; Yalcin, Bozdemir, & Ozbas, 2010). Thus, given the pivotal role of GDH enzymes in carbon and nitrogen metabolism, the present study was focused upon these enzymes in *Y. lipolytica*, with the aim of learning more about their role in nitrogen and energy metabolism. This examination demonstrates that the *YALI0F17820g* and *YALI0E09603g* genes encode NADP- and NAD-GDH enzymes, respectively, that are differentially regulated and serve nonredundant functions in nitrogen and carbon utilization in *Y. lipolytica*.

2 | MATERIALS AND METHODS

2.1 | General growth and culture conditions

The genotypes and sources of *Escherichia coli*, *S. cerevisiae*, and *Y. lipolytica* strains used in this study are listed in Table S1. Propagation of *E. coli* bacteria was carried out on lysogeny broth medium as previously described (Sambrook, Fritsch, & Maniatis, 1989). Rich medium (yeast extract peptone dextrose [YPD]) for yeast contained 1% yeast extract, 2% peptone, and 2% glucose (F. Sherman, 2002). For culturing of plasmid-containing *S. cerevisiae* strains, synthetic complete minus leucine medium (SC-Leu) contained 0.67% yeast nitrogen base without amino acids, 2% glucose, and amino acid supplementation with the exception of leucine (F. Sherman, 2002). *Y. lipolytica* was cultured based upon methods previously described (Barth & Gaillardin, 1996). Minimal yeast nitrogen base medium contained 0.17% yeast nitrogen base without amino acids or ammonium sulfate and 50-mM phosphate buffer (pH 6.8). For most experiments, 1% glucose and 0.5% NH_4Cl were added to give glucose/ammonia medium, which was supplemented with 0.1% leucine and/or uracil as needed. For plates, 1.5–2% agar was added. To test enzyme activity under nitrogen starvation, cells were cultured in medium with 50-mM phosphate buffer (pH 6.8) and 8% glucose (Hardman, Ukey, & Fakas, 2018). To test growth on different carbon and nitrogen sources, 1% sodium glutamate was added to replace NH_4Cl to give glucose/glutamate medium or to replace both NH_4Cl and glucose to give glutamate/glutamate medium. All media reagents were obtained from Sigma Chemical (St. Louis, MO).

2.2 | Expression of *Y. lipolytica* genes in *gdh*-null *S. cerevisiae*

Y. lipolytica genes *YALI0F17820g* (*ylGDH1*) and *YALI0E09603g* (*ylGDH2*), predicted to lack introns and encode potential GDHs (Kersey et al., 2018; D. J. Sherman et al., 2009), were amplified by polymerase chain reaction (PCR; for specific primer sequences, see Table S3) from DNA of *Y. lipolytica* strain INAG34815 (Table S1). The resulting 1.4-kb (*ylGDH1*) and 3.0-kb (*ylGDH2*) PCR fragments were gel purified, blunted using a mixture of polymerase and kinase (Quick Blunting Kit, NE Biolabs, Ipswich, MA), and ligated into the dephosphorylated *Sma1* site of the *S. cerevisiae* pBEVY-L vector (Miller, Martinat, & Hyman, 1998) under the constitutive GPD/ADH1 promoter with leucine selection. The proper insertion of the fragments was confirmed by restriction analysis. Accuracy of the coding sequences was confirmed by sequencing (Iowa Institute of Human Genetics, Iowa City, IA) and comparison with sequences in the *Y. lipolytica* genome database (Kersey et al., 2018; D. J. Sherman et al., 2009). These pBEVY-L-ylGDH1 and pBEVY-L-ylGDH2 plasmids, as well as the empty vector, were then transformed into a *gdh*-null *S. cerevisiae* strain, ASY20A (Table S1), using the Yeastmaker™ yeast transformation system 2 (Clontech-Takara Bio, Kyoto, Japan), and Leu+ transformants were selected. Wild-type BY4742 (Table S1) was also transformed with the empty vector as a control. For

determination of GDH activity (see below), strains were grown to late log phase in SC-Leu medium.

2.3 | Deletion of GDH genes in *Y. lipolytica*

Cassettes for the deletion of *ylGDH1* (YALI0F17820g) and *ylGDH2* (YALI0E09603g) in *Y. lipolytica* were created essentially as described (Fickers, Le Dall, Gaillardin, Thonart, & Nicaud, 2003). Fragments (~1 kb) containing the upstream/promotor (P) region or downstream/terminator (T) regions of the *ylGDH1* and *ylGDH2* genes were generated by PCR using genomic DNA from JMY2341 or JMY2394 strains (Table S1) and the P1/P2 and T1/T2 primer pairs (Table S3). The primers were designed so that an *I-SceI* endonuclease site was introduced to the downstream end of each P fragment and the upstream end of each T fragment. Overlap extension PCR was used to join the P and T fragments into one PT fragment, which was blunted (see above) and cloned into dephosphorylated pRS306 vector (Sikorski & Hieter, 1989) at the *Sma1* site to yield the pRS306-ylGDH1-PT and pRS306-ylGDH2-PT vectors (Table S2). The *LEU2ex* marker was cleaved from the JME2563 vector with *I-SceI* and ligated into the *I-SceI* site of pRS306-ylGDH1-PT to produce the plasmid carrying the *gdh1::LEU2ex* cassette (pRS306-ylGDH1-PLT; Table S2), and the *URA3ex* marker was cleaved from the JME1047 vector with *I-SceI* and ligated into the *I-SceI* site of pRS306-ylGDH2-PT to produce the plasmid carrying the *gdh2::URA3ex* cassette (pRS306-ylGDH2-PUT; Table S2). The *gdh1::LEU2ex* and *gdh2::URA3ex* cassette fragments were amplified from pRS306-ylGDH1-PLT and pRS306-ylGDH2-PUT, respectively, by PCR using the corresponding P1 and T2 primers (Table S3), gel purified, and transformed into JMY2494, a Leu− Ura− *Y. lipolytica* strain capable of homologous recombination but not nonhomologous end-joining DNA repair (Verbeke, Beopoulos, & Nicaud, 2013). Following homologous recombination, a Leu+ prototroph from the *gdh1::LEU2ex* transformation was designated YLT1 (*gdh1Δ* or *gdh1::LEU2ex; Leu+ Ura−*), and a Ura+ prototroph from the *gdh2::URA3ex* transformation was designated YPT7 (*gdh2Δ* or *gdh2::URA3ex; Leu− Ura+*; Table S1). Transforming fragments carrying the *URA3ex* or *LEU2ex* marker into YLT1 and YPT7, respectively, resulted in creation of prototrophic *gdh1Δ* strains YPT20 and YPT37 (*gdh1::LEU2; Ura+ Leu+*) and prototrophic *gdh2Δ* strains YPT25 and YPT35 (*gdh2::URA3ex; Leu+ Ura+*; Table S1). A prototrophic double *gdh1Δ gdh2Δ* mutant was made by transforming the *gdh2::URA3ex* fragment into YLT1 and isolating Ura+ transformants (*gdh1::LEU2 gdh2::URA3ex; Ura+ Leu+*). All deletions were confirmed by PCR analysis (for primer sequences, see Table S3). Strains and genotypes are listed in Table S1.

2.4 | Overexpression of genes in *Y. lipolytica*

Constructs for making *Y. lipolytica* strains overexpressing *ylGDH1* and *ylGDH2* under the control of the constitutive TEF promoter were created in the JMP62 expression vectors (Table S2) JME1047-*URA3ex* or JME2563-*LEU2ex* (Dulermo et al., 2017; Lazar et al., 2013). The *ylGDH1* and *ylGDH2* coding sequences were amplified from the pBEVY-L-ylGDH1 and pBEVY-L-ylGDH2 plasmids with primers that introduced a *BamH1* restriction site to the upstream end and an *AvrII* site to the downstream end of the coding sequence (ylGDH1-Bam-for/ylGDH1-Avr-rev and ylGDH2-Bam-for/ylGDH2-Avr-rev; see Table S3). The BamH1/AvrII-ylGDH1 and BamH1/AvrII-ylGDH2 PCR fragments were inserted into JME1047 and JME2563 cleaved with *BamH1* and *AvrII* to place the genes under control of the TEF promoter, creating JME1047-ylGDH1, JME1047-ylGDH2, JME2563-ylGDH1, and JME2563-ylGDH2 (Table S2). Cassette fragments for expression by genomic integration (ylURA3ex-pTEF-ylGDH1, ylURA3ex-pTEF-ylGDH2, ylLEU2ex-pTEF-ylGDH1, ylLEU2ex-pTEF-ylGDH2) were liberated from the plasmids with *Not1* enzyme. YLT1 (*gdh1::LEU2ex; Leu+ Ura−*) was transformed with ylURA3ex-pTEF-ylGDH1 or ylURA3ex-pTEF-ylGDH2 followed by selection of Ura prototrophs (Leu+ Ura+). YPT7 (*gdh2::URA3ex; Leu− Ura+*) was transformed with ylLEU2ex-pTEF-ylGDH1 or ylLEU2ex-pTEF-ylGDH2 followed by selection of Leu prototrophs (Leu+ Ura+). Resulting prototrophs were then screened for GDH activity (see below) to confirm overexpression, which was defined as possessing GDH activity of twice that found in wild-type cells (data not shown). This yielded *Y. lipolytica gdh1Δ* strains overexpressing either *ylGDH1* (YPT15 and YPT16) or *ylGDH2* (YPT18 and YPT31) and *gdh2Δ* strains overexpressing either *ylGDH*1 (YPT24 and YPT34) or *ylGDH*2 (YPT22 and YPT38). Strains and genotypes are listed in Table S1.

2.5 | Determination of GDH activity

GDH activities in lysates were measured in the presence of excess α-ketoglutarate and ammonia by monitoring the oxidation of NADPH or NADH spectrophotometrically as previously described (Doherty, 1970). Lysates were prepared from cultures grown in indicated medium to late log phase (unless otherwise indicated) as described previously (Sieg & Trotter, 2014; Trotter et al., 2005). Aliquots were stored at −80°C and thawed once on the day of the assay. Protein concentrations in the lysates were determined using the BCA (bicinchoninic acid) method (Sigma Chemical, St. Louis, MO). Extinction coefficients were determined by calibration with pure NADPH or NADH.

2.6 | Growth analysis

Strains were pregrown overnight in YPD, standardized at an OD_{600} of 0.1 at time zero, and OD_{600} monitored over time. For microtiter analysis, cells were inoculated into 200 µl of the specified medium in 96-well plates. The plates were placed in a Biotek Synergy MX microtitre plate reader (Biotek Instruments, Colmar, France), where they were agitated constantly at 28°C, and the OD_{600} was measured every 30 min. Growth was analysed by standard methods (Widdel, 2007). Exponential rate for the linear growth interval between 5 and 12 hr was calculated by the equation $r = (\ln OD_2 - \ln OD_1) \div (t_2 - t_1)$, and doubling time (DT) was then calculated by the equation $DT = (\ln 2) \div r$.

3 | RESULTS

3.1 | *YALI0F17820g* and *YALI0E09603g* encode probable GDHs in *Y. lipolytica*

Wild-type strains of *Y. lipolytica* are capable of growth on synthetic medium without amino acid supplementation, as well as with glutamate as sole carbon and nitrogen sources (Barth & Gaillardin, 1996). Thus, we reasoned that the genome of the organism encodes for one or more GDH enzymes. Indeed, examination of the annotated *Y. lipolytica* genome (Kersey et al., 2018; D. J. Sherman et al., 2009) revealed two potential GDH encoding genes based upon homologies to other GDHs. One of these, *YALI0F17820g*, encodes a 458 amino acid protein with sequence highly homologous to the similarly sized NADP-GDH proteins encoded by the *scGDH1* (YOR375C; 454 amino acids) and *scGDH3* genes (YAL062W; 457 amino acids) of *S. cerevisiae* (Engel et al., 2014). Multiple sequence alignment (Goujon et al., 2010; Larkin et al., 2007; Sievers et al., 2011) of the Yali0F17820p predicted amino acid sequence showed an alignment score of 68.94 with that of scGdh1p and an alignment score of 68.49 with scGdh3p. The scGdh1p and scGdh3p amino acid sequences have an alignment score of 87.00 (see Figure S1). Comparison of Yali0F17820p with amino acid sequences in the Protein Data Bank (PDB) structural database (Berman et al., 2000) further revealed an alignment score of 73.5 between Yali0F17820p and the 460 amino acid NADP-GDH protein of the filamentous fungus *Aspergillus niger*, anGDH (Prakash, Punekar, & Bhaumik, 2018). Inspection of the alignment revealed that the crucial residues reported in anGDH to be involved in binding of α-ketoglutarate and the $NADP^+$ cofactor, as well as those directly involved in catalysis, are all conserved in Yali0F17820p (see Figure S3). Thus, bioinformatics provides a compelling argument that Yali0F17820p, which will be referred to henceforth as ylGdh1p, is a NADP-GDH.

The second potential GDH-encoding gene noted in the *Y. lipolytica* database is *YALI0E09603g*, which encodes a protein of 987 amino acids. Alignment (Goujon et al., 2010; Larkin et al., 2007; Sievers et al., 2011) of the predicted Yali0E09603p amino acid sequence with that of the *S. cerevisiae scGDH2* gene (YDL215C; 1092 amino acids), which encodes a NAD-dependent-GDH (Engel et al., 2014), resulted in an alignment score of 42.65 (see Figure S2). Examination for homologues in the PDB structural database revealed that the C-terminal ~500 amino acids of Yali0E09603p has homology to the thermostable NAD-GDH of the hyperthermophilic archaebacterium *Pyrobaculum islandicum*, piGDH, which is a much smaller 421 amino acids (Bhuiya et al., 2005). A closer look at the substrate binding sites reported for the piGDH protein (Bhuiya et al., 2005) indicates that six of 10 residues involved in glutamate binding and five of 12 involved in NAD^+ binding are apparently conserved in Yali0E09603p (see Figure S4), further supporting its possible function as a NAD-GDH. Although parallel sequence analysis for Yali0E09603p, which will be termed ylGdh2p, is less convincing, it provided sufficient impetus for further investigation as a probable NAD-GDH.

3.2 | *Y. lipolytica* expresses one NADPH-GDH and one NADH-GDH

Investigation was then pursued to establish the molecular functions of the putative *Y. lipolytica* GDHs. First, the *ylGDH1* and *ylGDH2* genes were heterologously expressed in *S. cerevisiae*. Fortunately, neither gene possesses an intron (Kersey et al., 2018; D. J. Sherman et al., 2009), so each was easily amplified by PCR and inserted into the *S. cerevisiae* pBEVY-L expression vector under control of the GPD/ADH1 promoter and leucine selection (Miller et al., 1998). The expression constructs were then introduced into a *gdh*-null mutant *S. cerevisiae* that lacks all measurable GDH activity (Sieg & Trotter, 2014). Assay of GDH activity in the presence of the NADPH cofactor (Figure 2a) demonstrated that introduction of the

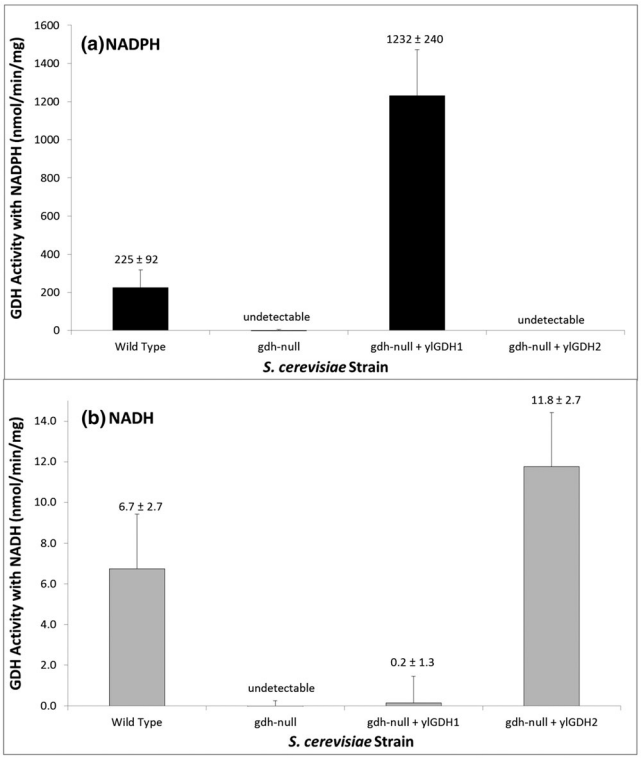

FIGURE 2 Glutamate dehydrogenase (GDH) activity in *gdh*-null *Saccharomyces cerevisiae* mutant cells is restored by introduction of *YALI0F17820g* (*ylGDH1*) or *YALI0E09603g* (*ylGDH2*) from *Yarrowia lipolytica*. The genes were each cloned into a *S. cerevisiae* expression vector (p-BEVY-L) under the control of the constitutive promotor of the *scADH1* gene and leucine selection (Miller et al., 1998). These constructs were then introduced into a *gdh*-null *S. cerevisiae* mutant, which shows no measurable GDH activity in the presence of (a) NADPH or (b) NADH (*gdh*-null; ASY20A) when compared with a parental strain (wild type; BY4742). The plasmid carrying *ylGDH1* (*gdh*-null + ylGDH1) restored GDH activity with (a) NADPH but not (b) NADH, whereas the *ylGDH2* plasmid (*gdh*-null + ylGDH2) did not restore GDH activity with (a) NADPH but did with (b) NADH. Strains were grown to saturation in synthetic complete minus leucine medium, and the data are expressed as nmol NAD(P)H oxidized per minute per milligram protein ± the *SD* from at least three separate experiments

ylGDH1-containing plasmid into the *gdh*-null mutant conferred activity to a level over five times that expressed in wild-type *S. cerevisiae* carrying the empty vector, whereas the activity in the mutant carrying the *ylGDH2*-containing plasmid showed no activity above that found in the mutant carrying the empty vector. Measurement of GDH activity with the NADH cofactor (Figure 2b) revealed a very different pattern. The *gdh*-null mutant with the *ylGDH1*-containing plasmid had no GDH activity, whereas that with the *ylGDH2*-containing plasmid exhibited an activity at about two times that in wild-type *S. cerevisiae*. These data clearly substantiate the predicted molecular functions for the proteins encoded by *ylGDH1* and *ylGDH2* as NADP- and NAD-GDHs, respectively.

Identification of GDH encoding genes in the *Y. lipolytica* genome revealed only two plausible candidates (Kersey et al., 2018; D. J. Sherman et al., 2009). In order to ascertain whether *ylGDH1* and *ylGDH2* are indeed the only GDH enzymes in *Y. lipolytica*, the consequences of gene disruption were examined. NADP- and NAD-GDH activities were measured in wild-type *Y. lipolytica* and in strains carrying a disruption in either only *ylGDH1*, only *ylGDH2*, or in both genes. The *ylGDH1* disruption (*gdh1Δ*) eliminated GDH activity with the NADPH cofactor (Figure 3a), but activity with the NADH cofactor was not different from wild type (Figure 3b). In contrast, the *ylGDH2* disruption (*gdh2Δ*) results in loss of GDH activity with NADH (Figure 3b) while leaving measurable activity with NADPH at about 30% of wild type (Figure 3a). This lower level of NADP-GDH activity in *gdh2Δ* suggests important coordination of these activities. Disruption of *ylGDH1* and *ylGDH2* (*gdh1Δ gdh2Δ*) completely abolishes both NADP- and NAD-GDH activities (Figure 3a,b). Taken together, the bioinformatics (above), heterologous expression experiments (Figure 2), and disruption strain results (Figure 3) provide persuasive evidence that *ylGDH1* encodes a NADP-GDH, *ylGDH2* encodes a NAD-GDH, and these two gene products account for all of the NAD(P)-GDH activity in *Y. lipolytica*.

3.3 | GDH activities in *Y. lipolytica* are differentially expressed

The NADP- and NAD-GDH enzymes are generally considered to have different roles in yeast; NADP-GDH serves in nitrogen assimilation, and NAD-GDH functions in traffic of glutamate carbons into the CAC (Campero-Basaldua et al., 2017; Freese et al., 2011; Magasanik, 2003; Magasanik & Kaiser, 2002). To resolve whether ylGdh1p and ylGdh2p have diverse functions, their activities in wild-type *Y. lipolytica* under different growth conditions were measured. Activity of ylGDH1p in the presence of NADPH and ylGDH2p with NADH in logarithmically growing cells in rich medium was comparable; however, once saturation or stationary phase was reached, ylGdh1p was elevated to an activity of about three times that of ylGdh2p (Figure 4a). In addition, after a 2-day culture under glucose-rich, nitrogen starvation conditions, the ylGdh2p activity was decreased to 25% of the level observed on rich medium, making ylGdh1p activity even more dominant at 12 times that of ylGdh2p (Figure 4a). These data indicate that both ylGdh1p and ylGdh2p are regulated during growth on rich medium; ylGdh1p becomes more active as nutrients decrease, whereas ylGdh2p is unchanged or reduced.

To further study probable distinct functions of ylGdh1p and ylGdh2p, GDH activities were determined after growth to late log phase in synthetic medium containing different carbon and nitrogen sources (Figure 4b). As was observed in glucose-rich, YPD medium

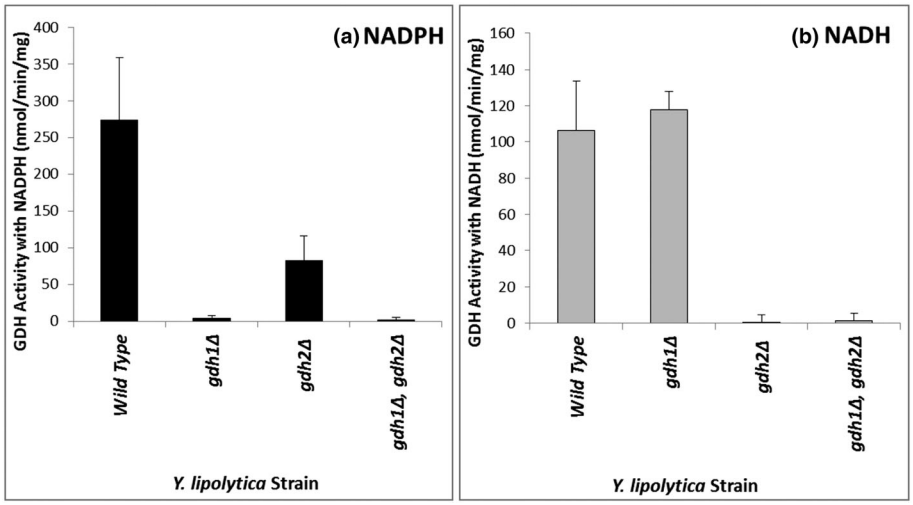

FIGURE 3 Glutamate dehydrogenase (GDH) activities of *Yarrowia lipolytica* with disruptions in the *ylGDH1* (*YALI0F17820g*) and/or *ylGDH2* (*YALI0E09603g*) genes. The *ylGDH1* and *ylGDH2* genes were disrupted by homologous recombination in *Y. lipolytica* carrying a defect in nonhomologous end-joining DNA repair (Verbeke et al., 2013). Disruption of the *ylGDH1* gene (*gdh1Δ*) eliminates the GDH activity in the presence of NADPH (a), but activity in the presence of NADH is unaffected (b). Disruption of *ylGDH2* (*gdh2Δ*) abolishes GDH activity in the presence of NADH (b), whereas significant activity in the presence of NADPH remains (a). Disruption of both (*gdh1Δ*, *gdh2Δ*) leaves cells with no measurable GDH activity. The activities were measured in cells grown to saturation on yeast extract peptone dextrose medium and are expressed as nmol NAD(P)H oxidized per minute per milligram protein ± the *SD* from at least four separate experiments

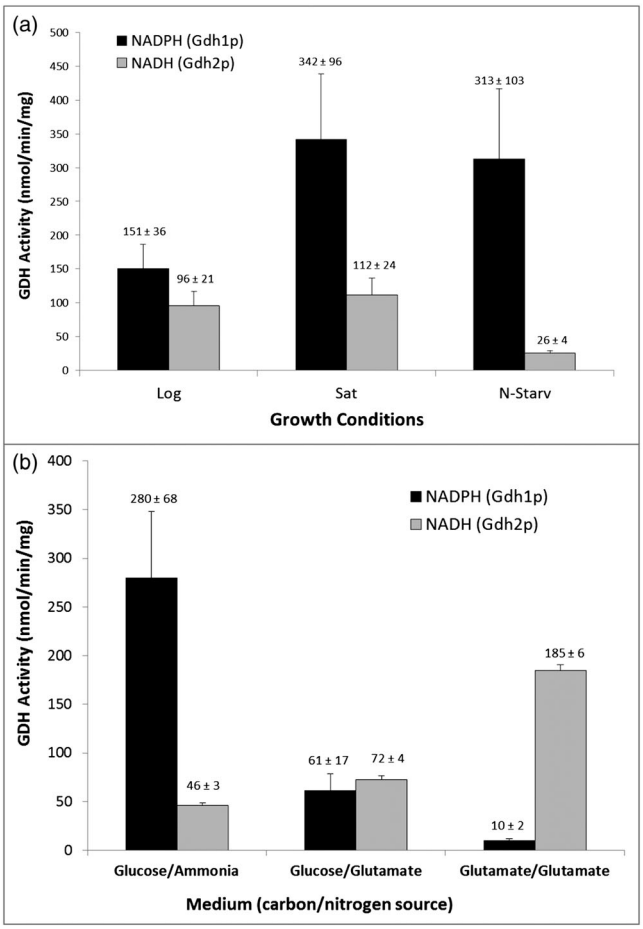

FIGURE 4 *Yarrowia lipolytica* NADP- and NAD-GDH activities are differentially expressed. Glutamate dehydrogenase (GDH) activity was determined with NADPH or NADH as cofactor from cell lysates prepared (a) during logarithmic growth on YPD (Log), after saturation on YPD (Sat), and after 43- to 44-hr culture in nitrogen starvation medium (N-Starv) or (b) during late log phase growth with different sources of carbon and nitrogen. The data are expressed as nmol NAD(P)H oxidized per minute per milligram protein ± the *SD* from at least four separate experiments

(Figure 4a), when cells were grown on synthetic medium with glucose carbon and ammonia nitrogen sources (glucose/ammonia), ylGdh1p activity was highest with sixfold higher activity than ylGdh2p (Figure 4b). Interestingly, when glucose was retained as the carbon source and ammonia nitrogen was replaced with glutamate (glucose/glutamate), ylGdh1p activity was lowered to about 22%, and ylGdh2 was increased to 150% of the activity in ammonia grown cells. Finally, when glutamate was provided as sole carbon and nitrogen sources (glutamate/glutamate), ylGdh1p activity was barely detectable at less than 16% of the activity in glucose/glutamate, whereas ylGdh2p activity was induced a further 250%. These data clearly allow the inference that the ylGdh1p and ylGdh2p enzymes in *Y. lipolytica* have distinct functions. ylGDH1p is apparently the primary GDH, especially when assimilation of ammonia nitrogen is occurring, whereas ylGdh2p seems important to glutamate catabolism.

3.4 | ylGdh1p is important for ammonia nitrogen assimilation, and ylGdh2p is vital for glutamate catabolism

Further elucidation of the cellular roles of ylGdh1p and ylGdh2p was investigated by examining the growth phenotype of prototrophic (Leu+ Ura+) GDH deletion strains on media with different carbon and nitrogen sources (Figure 5). In line with a primary role of ylGdh1p in nitrogen assimilation, strains lacking ylGdh1p (*gdh1Δ*) display impaired growth in comparison with wild type regardless of carbon or nitrogen source. The *gdh1Δ* impairment was most notable when cells were grown on glucose/ammonia medium (Figure 5a); the DT of the *gdh1Δ* strain (9.8 hr) was 2.5-times that of the wild-type strain (3.8 hr). Saturation, as observed by maximum OD_{600}, was reached at a lower density for the *gdh1Δ* strains as compared with wild type (Figure 5a). Similar but less noticeable trends were observed on glucose/glutamate medium (Figure 5b; DT of *gdh1Δ*, 4.8 hr; wild type, 3.7 hr) and glutamate/glutamate medium (Figure 5c; DT of *gdh1Δ*, 5.9 hr; wild type, 4.5 hr). Lower saturation density for the *gdh1Δ* strain as compared with wild type is even discernible when grown on YPD (data not shown).

Loss of ylGdh2p (*gdh2Δ*) altered growth in a very different manner. Growth of wild-type strain and the strains carrying the *gdh2Δ* disruption on different nitrogen sources was indistinguishable, as seen on glucose/ammonia (Figure 5a; DT of *gdh2Δ*, 3.9 hr; wild type, 3.8 hr)

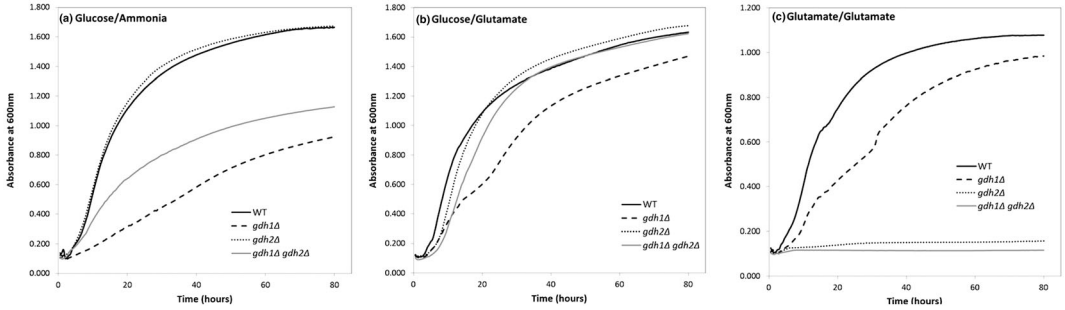

FIGURE 5 Growth of *Yarrowia lipolytica* with *ylGDH1* (*gdh1Δ*) and/or *ylGDH2* (*gdh2Δ*) disruptions in medium containing different carbon and nitrogen sources. Strains were grown in medium containing glucose carbon and ammonia nitrogen (a), glucose carbon and glutamate nitrogen (b), or glutamate as both carbon and nitrogen sources (c). The data are representative of at least three separate growth evaluations and are an average of at least four biologically independent samples

and glucose/glutamate (Figure 5b; DT of *gdh2Δ*, 3.4 hr; wild type, 3.7 hr), signifying that ylGdh2p does not participate in nitrogen assimilation. In contrast, loss of ylGdh2p completely eliminated the ability of cells to use glutamate as carbon source. The DT of *gdh2Δ* cells on glutamate/glutamate medium was 14 times longer than that of wild type (Figure 5c; DT for *gdh2Δ*, 64 hr; wild type, 4.5 hr). These data support a primary role for ylGdh2p in the catabolism of glutamate, likely via its conversion to α-ketoglutarate and routing into the CAC.

The growth phenotype of the double mutant strain further supports different roles for ylGdh1p and ylGdh2p in nitrogen assimilation and glutamate catabolism, respectively. Comparison of the growth of the *gdh1Δ* single mutant with that of the *gdh1Δ gdh2Δ* double mutant on medium with different nitrogen sources reveals that, rather than further impairing growth, the additional loss of ylGdh2p improves growth. The DT of the *gdh1Δ* single mutant on glucose/ammonia was 9.8 hr, whereas that of the *gdh1Δ gdh2Δ* double mutant was 5.7 hr (Figure 5a). And, the DT of the *gdh1Δ* single mutant on glucose/glutamate was 4.8 hr, whereas that of the *gdh1Δ gdh2Δ* double mutant was 3.7 hr (Figure 5b). One interpretation of this result is that by eliminating the channelling of glutamate carbons into energy metabolism, the loss of ylGdh2p activity increases their availability for nitrogen assimilation. In contrast, the double loss of ylGdh1p and ylGdh2p on glutamate/glutamate medium does not improve growth. As discussed above, the *gdh2Δ* disruption nearly abolished growth on this medium. Deletion of *ylGDH1* in this background to make the *gdh1Δ gdh2Δ* double mutant further impairs growth on glutamate/glutamate medium (Figure 5c; DT of *gdh2Δ*, 64 hr; *gdh1Δ gdh2Δ*, 73 hr). Thus, these analyses of growth phenotypes provide clear evidence that ylGdh1p functions in the incorporation of nitrogen and ylGdh2p has a disparate role in the utilization of glutamate carbon.

3.5 | ylGdh1p and ylGdh2 are not interchangeable

Complementation analysis was conducted to further verify the distinct functions of ylGdh1p and ylGdh2p in *Y. lipolytica*. For these experiments, the wild-type *ylGDH1* or *ylGDH2* genes were added back and overexpressed under the regulation of the constitutive TEF promoter by genomic integration in the disruption strains (see Section 2). As observed before, growth of *gdh1Δ* strains on glucose/ammonia medium was significantly impaired as compared with wild type (Figure 5a). As expected, the diminished growth of *gdh1Δ* was completely corrected by *ylGDH1* overexpression (*gdh1Δ + GDH1*); the DTs of wild type and overexpressor were indistinguishable (Figure 6a; DT of wild type, 3.8 hr; *gdh1Δ*, 10.4 hr; *gdh1Δ + GDH1*, 4.0 hr). In contrast, overexpression of *ylGDH2* (*gdh1Δ + GDH2*) was only able to partially complement the *gdh1Δ* growth defect (Figure 6a; DT of wild type, 3.8 hr; *gdh1Δ*, 10.4 hr; *gdh1Δ + GDH2*, 7.9 hr). This limited effect could be due to altering the availability of carbons for other routes of nitrogen assimilation. Next, the effect of overexpression on the glutamate/glutamate growth defect of the *gdh2Δ* disruption strain (Figure 5c) was explored. Overexpression of wild-type *ylGDH2* (*gdh2Δ + GDH2*) was effective at alleviating the *gdh2Δ* growth defect (Figure 6b; DT of wild-type, 4.5 hr; *gdh2Δ*, 33.2 hr; *gdh2Δ + GDH2*, 5.1 hr). On the other hand, *ylGDH1* overexpression (*gdh2Δ + GDH1*) was entirely ineffectual at complementing the *gdh2Δ* growth defect (Figure 6b; DT of wild-type, 4.5 hr; *gdh2Δ*, 33.2 hr; *gdh2Δ + GDH1*, 36.7 hr). These complementation studies allow the unmistakable conclusion that ylGdh1p and ylGdh2p serve unique, non-interchangeable functions in *Y. lipolytica*.

4 | DISCUSSION

GDHs sit at a pivotal spot where energy/carbon and amino acid/nitrogen metabolism converge (Figure 1), meaning these enzymes could have a significant role to play in coordination of these pathways (Karaca et al., 2011; Plaitakis et al., 2017). This study has provided the first in-depth investigation of GDH enzymes in the oleaginous yeast *Y. lipolytica*. The *YALI0F17820g* gene of *Y. lipolytica*, here referred to as *ylGDH1*, was listed in the annotated genome database as a probable NADP-GDH based upon homologies to known enzymes (Kersey et al.,

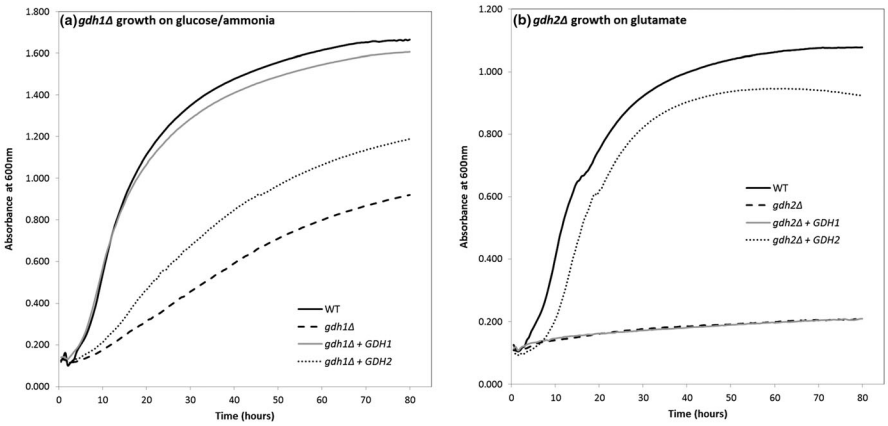

FIGURE 6 *ylGDH1* and *ylGDH2* are not interchangeable. The effect of *GDH1* or *GDH2* overexpression on the (a) glucose/ammonia growth phenotype of the *gdh1Δ* strain (see Figure 5a) and (b) the impaired growth on glutamate of the *gdh2Δ* strain (see Figure 5c) was examined. The data are representative of at least three separate growth evaluations and are an average of at least four biologically independent samples

2018; D. J. Sherman et al., 2009). Indeed, the deduced amino acid sequence of *ylGDH1* possesses significant similarity to the NADP-GDH enzymes of the budding yeast *S. cerevisiae* (Figure S1) and complete conservation of vital active site amino acid residues deduced via structural analysis of the NADP-GDH from *A. niger* (Figure S3). Our experimental results demonstrate that this catalytic activity in *Y. lipolytica* is fulfilled by the product of only one gene, *ylGDH1* (Figure 3), that possesses homology (>68%) to both products of NADP-GDH-encoding genes in *S. cerevisiae*, *scGDH1* and *scGDH3* (Figure S1). The *scGDH1* and *scGDH3* genes of *S. cerevisiae* are two of the many homologous gene pairs that arose as a result of whole-genome duplication thought to have occurred sometime between the genus *Kluyveromyces* and the evolution of *Saccharomyces* (Wolfe & Shields, 1997). Because the *Yarrowia* clade predates *Kluyveromyces* and the whole-genome duplication (Freese et al., 2011; Wolfe & Shields, 1997), it is not surprising to find only one gene encoding a NADP-GDH in this organism. The NADP-GDH enzymes in *S. cerevisiae* carry out reductive amination of α-ketoglutarate to glutamate (Magasanik, 2003). During growth when glucose is plentiful, the enzyme encoded by *scGDH1* is predominant. However, after exhaustion of glucose or in ethanol-grown cells, the enzyme encoded by *scGDH3* is most prevalent (DeLuna et al., 2001). Our results show that in rich (YPD) or synthetic glucose/ammonia media, ylGdh1p activity is high and independent of relative glucose concentration (Figure 4). But, when ammonia nitrogen is replaced with glutamate nitrogen (glucose/glutamate or glutamate/glutamate), ylGdh1p activity is significantly lowered (Figure 4b), suggesting that glutamate is a negative regulator of ylGdh1p. Whether the decreased NADP-GDH activity in glutamate-grown *Y. lipolytica* is a result of gene expression or allosteric regulation remains unanswered. The lower NADP-Gdh1p activity in the *gdh2Δ* deletion strain (Figure 3a) further supports a close sensitivity of this enzyme to glutamate equilibrium in *Y. lipolytica*. Yet, this negative regulatory effect on NADP-GDH in *Y. lipolytica* is in stark contrast to the glutamate-resistant NADP-GDH activities found in *S. cerevisiae*, *Lachancea kluyveri*, and *Kluyveromyces lactis* (Freese et al., 2011; Riego et al., 2002). Despite this regulatory difference, such as *S. cerevisiae* lacking *scGDH1* (Miller, S. M. & Magasanik, 1990; Sieg & Trotter, 2014), the loss of *ylGDH1* causes impaired growth on glucose/ammonia synthetic medium (Figure 5a), supporting a role in nitrogen assimilation.

The only partial effect on glucose/ammonia growth also suggests that, like *S. cerevisiae*, *Y. lipolytica* possesses other pathways for the glutamate synthesis and/or nitrogen assimilation, perhaps via proline or glutamine metabolism (Sieg & Trotter, 2014). The observation that *Y. lipolytica* is capable of growth in medium containing glutamate (Figure 5c), aspartate, or proline as sole carbon and nitrogen sources (Freese et al., 2011) is an indication of alternative modes of nitrogen assimilation in this organism. Indeed, both glutamine synthetase activity and glutamate synthase activity have been observed in *Y. lipolytica* (Il'chenko, Cherniavskaia, Shishkanova, & Finogenova, 2003; Kerkhoven et al., 2016). And, as noted in Figure 1, the *YALI0F00506g* and *YALI0B19998g* genes in the *Y. lipolytica* genome encode proteins with similarity to *S. cerevisiae* glutamine synthetase, encoded by *scGLN1* (YPR035w), and glutamate synthase, encoded by *scGLT1* (YDL171c), respectively (Kersey et al., 2018; D. J. Sherman et al., 2009; Kerkhoven et al., 2016; data not shown). Further, genome analysis indicates that *Y. lipolytica* also possesses proteins similar to the proline oxidase (*PUT1*/YLR142W) and Δ-1-pyrroline-5-carboxlyate dehydrogenase (*PUT2*/YHR037w) of *S. cerevisiae* (Kersey et al., 2018; D. J. Sherman et al., 2009; Engel et al., 2014; data not shown), which facilitate proline utilization (Brandriss & Magasanik, 1979). For a better understanding of nitrogen assimilation in *Y. lipolytica*, these alternative paths to glutamate formation merit future investigation.

In the genome database, the *Y. lipolytica YALI0E09603g* gene, here noted as *ylGDH2*, was predicted to be a NAD-GDH (Kersey et al., 2018; D. J. Sherman et al., 2009). Alignment analysis of deduced amino acid sequences demonstrates a notable homology (>45%) to the scGdh2p of *S. cerevisiae* (Figure S2). However, alignment to the most similar structure available in the PDB database (Berman et al., 2000) showed limited conservation of residues with those in the active site of the NAD-GDH of *P. islandicum* (Figure S4). In *S. cerevisiae*, the NAD-GDH enzyme encoded by the *scGDH2* gene carries out oxidation of glutamate back to α-ketoglutarate and ammonia, allowing the organism to utilize glutamate as sole nitrogen source in glucose/glutamate medium (Miller & Magasanik, 1990). The enzyme activity data (Figures 2 and 3) and growth phenotype of the *Y. lipolytica* deletion mutant (Figure 5) establish an analogous catalytic function for the *ylGDH2* gene product. In *S. cerevisiae*, expression of the *scGDH2* gene is tightly controlled, being highly expressed in fermentation or limiting glucose conditions and in the presence of glutamate and downregulated in ammonia and glutamine medium even with high glucose (Coschigano et al., 1991; Miller & Magasanik, 1991). *Y. lipolytica*'s NAD-ylGdh2p activity is low in ammonia medium and elevated when glutamate replaces ammonia (Figure 4), suggesting similar nitrogen regulation. Nevertheless, NAD-ylGdh2p activity is not elevated upon culture saturation (Figure 4), suggesting different carbon regulation of *ylGDH2*. Because *Y. lipolytica* is an obligate aerobe that gains its energy exclusively via respiration rather than a facultative anaerobe like *S. cerevisiae* (Dashko et al., 2014; Nicaud, 2012), one would anticipate disparate modes of carbon regulation in these organisms. Additionally, *S. cerevisiae* is one of many yeast species unable to utilize glutamate as sole carbon source, presumably because the level of NAD-GDH enzyme activity is insufficient (Freese et al., 2011; Large, 1986). Yet, *Y. lipolytica* strains, even those carrying a *ylGDH1* deletion, grow well with glutamate as sole carbon and nitrogen sources (Figures 5 and 6). Thus, ylGdh2p appears to work in glutamate catabolism not only for nitrogen assimilation but also for channelling of carbon into energy metabolism. Finally, as has also been observed in *S. cerevisiae* (Magasanik, 2003; Miller & Magasanik, 1990), our results indicate that the NADP-ylGdh1p and NAD-Gdh2p enzymes in *Y. lipolytica* are nonredundant and unable to replace one another (Figure 6).

5 | CONCLUSION

These studies aimed at investigating the molecular and cellular functions of the proposed GDH-encoding genes of the nonconventional,

oleaginous yeast *Y. lipolytica*. Our experiments have shown that the gene product of *YALI0F17820g* (*ylGDH1*) is the only expressed NADP-GDH enzyme in this organism, which plays a central role in the incorporation of ammonia nitrogen into glutamate. Also, we established that the *YALI0E09603g* (*ylGDH2*) gene encodes the single NAD-GDH activity that apparently catalyses the reverse reaction *in vivo*, making it important to utilization of nitrogen from glutamate, as well as the routing of glutamate carbons to energy metabolism via the CAC. Our data here show a primary role for ylGdh1p during nitrogen and carbon limitation. Yet, it also suggests that ylGdh2p has a role in liberation of both nitrogen and carbon from cellular glutamate. With the knowledge and the tools we have generated, a number of additional experimental areas can now be investigated, including the mechanisms of *ylGDH1* and *ylGDH2* regulation, the effects of their overexpression, and identification of other pathways for glutamate biosynthesis in *Y. lipolytica*. An area of future interest is what role the balance between ylGdh1p and ylGdh2p function plays in regulation of the CAC in *Y. lipolytica* and how is this altered during nitrogen starvation, which leads to lipid accumulation and citric acid production. This is of particular interest, as GDH is known as an important control point for energy metabolism in higher organisms, where it has been implicated not only in ammonia homeostasis but also in $NAD(P)^+$/NAD(PH) balance and the anaplerotic delivery of α-ketoglutarate to the CAC to allow lipid biosynthesis (Karaca et al., 2011; Owen, Kalhan, & Hanson, 2002; Plaitakis et al., 2017). It stands to reason that GDH enzyme function may also play an important role in regulating the provision of carbon to the CAC in *Y. lipolytica*, thereby playing a hitherto unappreciated regulatory role in the production of lipid and citric acid by this organism.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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