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Research Article

Glucose-mediated regulation of glycerol uptake in *Rhodospiridium toruloides*: Insights through transcriptomic analysis on dual substrate fermentation

Rhodospiridium toruloides is a robust oleaginous yeast that can accumulate lipids to more than 70% of its dry cell mass. Even though it is extensively studied for its fermentation of substrates like glucose and glycerol, limited information is available about its metabolism of mixture of glucose and glycerol. During growth on mixture of glucose and glycerol a typical diauxic growth and higher lipid yields were observed. To understand this phenomenon, RNA-seq analysis was implemented to study the gene expression profiles during growth on mixtures mainly to elucidate regulation of glycerol metabolism. Insights into lipid biosynthesis on mixed substrates are provided at a systems level. Among others, transcriptional profiles showed that glycerol might be produced intracellularly and glycerol kinase (GUT1) and glycerol 3-phosphate dehydrogenase (GUT2) enzymes were not downregulated in the presence of glucose. Transcriptomic analysis also showed that the regulation of glycerol uptake in the presence of glucose at transcriptional level is different from that observed in *Saccharomyces cerevisiae*.

Keywords: Catabolite repression / Glycerol metabolism / Lipid / Mixed substrate / Transcriptome

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1 Introduction

Over the last years more focus has been directed toward converting alternative, cheaper, and waste carbon sources to value-added products in developing a sustainable industry. A considerable interest has been paid to valorize glycerol as it is the main by-product from biodiesel production whereas sugars (especially glucose) are established substrates in industrial bioprocesses [1–3]. Oleaginous microorganisms, especially yeasts have proven to be promising in producing microbial oils from cheap carbon sources like raw glycerol and lignocellulosic biomass hydrolysate [4,5]. *Rhodospiridium toruloides* can accumulate lipids up to 70% of its cell mass under certain conditions. It has been shown that *R. toruloides* utilizes glucose, glycerol, xylose, and arabinose separately with highest preference toward

glucose [6–9]. Simultaneous utilization of substrates by *R. toruloides* has not been reported until we recently reported about its metabolism on mixture of glucose and glycerol [10]. Similar to *Saccharomyces cerevisiae*, *R. toruloides* was shown to exhibit catabolite repression and glycerol was consumed only after when glucose was limiting. In fact, diauxic growth was observed when grown on glucose and glycerol mixtures [10]. This phenomenon hinders *R. toruloides* to be used for industrial processes using complex substrates. Moreover, it was shown recently, that growth on a mixture of both the substrates enhances lipid production by this strain [10]. The regulation behind glycerol metabolism in the presence of glucose in *S. cerevisiae* and other fungal species have been well studied [11–13]. Systems biology based approaches significantly helped to understand and engineer *S. cerevisiae* which is now one of the important microbial hosts implemented for several industrial processes based on alternative carbon sources like xylose and glycerol [14,15].

In this study we aimed at the identification of regulatory patterns of gene expressions during metabolic shift from glucose growth to glycerol growth conditions in *R. toruloides*. RNA-seq

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based transcriptome analysis was performed on samples withdrawn from a culture of *R. toruloides* at four different phases. Insights into the regulation of glycerol metabolism as well as lipid metabolism are provided. This work provides an in-depth profiling of the transcriptome of *R. toruloides* grown on mixtures of glucose and glycerol which could facilitate further optimization of this potential oleaginous yeast.

2 Materials and methods

2.1 Strain and media

The *Rhodospiridium toruloides* DSMZ 4444 was kindly provided by Dr. Seraphim Papanikolaou, Agricultural University of Athens, Greece. The medium for seed cultures and fermentations is similar to that described elsewhere [10]. Briefly, culture medium contained (per liter): KH_2PO_4 7 g; Na_2HPO_4 2.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5 g; CaCl_2 0.15 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.15 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.06 g and $(\text{NH}_4)_2\text{SO}_4$ 0.5 g; yeast extract 0.5 g. For mixed substrate cultivations 1:1 mixture (30 g/L each) of glucose and glycerol were added. All chemicals used were purchased from Carl Roth GmbH, Germany.

2.2 Cultivations

Seed cultivations were performed in shake flasks at 30°C, 180 rpm for 24 h. Controlled batch fermentations were performed in DASGIP fermenters (DASGIP Eppendorf, Jülich, Germany). The 1.5 L capacity fermenter vessels with an initial working volume of 1 L were used. Agitation and temperature were maintained at 400 rpm and 30°C, respectively. pH was maintained at 6.0 by automatic addition of 5M NaOH and 2M HCL. Dissolved oxygen was maintained at 50% air saturation throughout the experiment by adjusting the oxygen concentration in the inlet gas.

2.3 Metabolite analysis

Biomass and lipid quantifications were performed as described previously [10]. Quantification of glucose, glycerol, and organic acids was carried out using high-performance liquid chromatography (HPLC; Kontron Instruments, UK) with separation on an Aminex HPX-87H column at 60°C with 0.005 M H_2SO_4 and detection via refractive index or by UV absorption at 210 nm. Ammonia concentration in the supernatant was determined by photometric measurements using a kit from Macherey Nagel, Germany.

2.4 RNA extraction and sequencing

For whole transcriptome sequencing, samples from the mixed substrate (1:1 mixture of glucose and glycerol) cultivations were withdrawn at four specific stages (glucose with nitrogen excess, glucose with nitrogen limitation, diauxic shift and glycerol growth phase). Approximately 3×10^8 cells were collected by

centrifugation (5000 rpm, 2°C for 5 min) and the biomass was immediately resuspended in RNeasy[®] Stabilization Solution (Life technologies, Ambion). The total RNA was extracted using the RiboPure[™] RNA purification kit for yeast (Life technologies, Ambion). The quality and quantity of the RNA was assayed using a BioAnalyzer (Agilent, USA). About 2 ng of total RNA was used for sequencing based on Illumina HiSeq 2000 platform. Library construction was performed using the Illumina TruSeq kit at BGI Tech, Hong Kong.

2.5 RNA-seq data analysis

The generated pair-end reads (FASTQ format) were checked for their quality using FastQC tool. The files were groomed to meet the Sanger-scaled quality values with ASCII offset 33. The groomed paired-end reads were then mapped to the reference genome of *R. toruloides* NP11 [16] using Tophat2 [17]. The gene and transcript expression levels were estimated and normalized based on FPKM (fragments per kilobase of exon per million fragments mapped) which were estimated using Cufflinks [18] with standard parameters and a *p*-value cut-off set to ≤ 0.05 . Gene ontology (GO) analysis was performed by using Blast2GO [19,20]. Graphical representation was performed using cummeRbund Bioconductor package in R studio. All raw sequence reads and processed data are deposited into the NCBI Gene Expression Omnibus database with accession number: GSE74307.

3 Results and discussion

3.1 Transcriptional analysis of *R. toruloides* grown on mixed substrate fermentation

The oleaginous yeast *R. toruloides* was reported to be an excellent lipid producer under nitrogen-limited conditions, as it can accumulate lipid over 70% of its cell dry weight along with a cell density over 100 g/L [21]. Moreover, this strain exhibited a good tolerance to inhibitory compounds found in lignocellulosic biomass hydrolysate [22] or raw glycerol [23], and therefore has been considered as a powerful and sustainable lipid producer [21,24]. Most studies regarding lipid production with *R. toruloides* were based on trials on mono-substrate fermentation [23,25]. Production of lipids with mixed substrates have only been reported using C5 and C6 sugars [6], and a very few reports have dealt with mixture of glucose and glycerol [10]. As shown in Fig. 1, almost 60% lipid content was reached after 168 h cultivation on dual substrate fermentation, suggesting the accumulation of reserve materials other than lipids (e.g. polysaccharide and internal sugars [26]). In fact, the regulation of glycerol utilization is well studied and documented in certain fungal species [13,27]. In all *Ascomycetes*, it has been observed that glycerol utilization is regulated by similar regulatory mechanisms and conserved regulatory binding sites at transcriptional level. In *S. cerevisiae*, for example, glucose is the preferred carbon source and the presence of glucose represses the uptake of glycerol. Catabolite repression is also observed in *R. toruloides* which is a *basidiomycete*, where a typical diauxic growth was observed during mixed substrate fermentations [10] (Fig. 1).

Figure 1. Growth, lipid production, and substrate consumption profiles of *R. toruloides* on a mixture of glucose and glycerol. Values shown are the mean and standard deviation from samples from two biological experiments. Samples withdrawn for RNA-seq analysis is marked with red squares on the biomass curve. Samples withdrawn correspond to four different phases: (i) on glucose + N-excess, (ii) on glucose + N-limited, (iii) diauxic shift, (iv) on glycerol.

Supporting Information Table 1 shows a comparison between substrate utilization, biomass, and lipid production at the different growth phases. Since, the underlying regulation of glycerol utilization has not yet been studied in *R. toruloides*, samples were collected as marked in Fig. 1, and transcriptional analyses were performed. Four different phases (glucose + N-excess phase, glucose + N-limited phase, diauxic shift phase and glycerol phase) with two biological replicates (total of 8 samples) for each phase were used for analysis. Over 3 million pair-end sequence reads were obtained for each sample. These sequenced reads had > 85% mapping rate to *R. toruloides* NP11 genome. Out of the 7958 genes that were expressed, about 1900 and 800 genes were significantly upregulated and downregulated, respectively when compared between the glucose N-limited phase over the glucose N-excess phase (Supporting Information Figs. 1–3). When glycerol phase was compared with the glucose N-limited phase, about 800 and 500 genes were upregulated and downregulated, respectively, whereas in the diauxic shift phase comparing over glucose-N-limited phase, about 200 and 400 genes were upregulated and downregulated, respectively. Most regulation of genes was observed during the transition from glucose-N-excess phase to glucose-N-limited phase highlighting the major changes in metabolic and biological processes with response to nitrogen starvation. To classify the predicted functions of the transcripts, GO terms were assigned using Blast2GO. The GO terms which were over-represented in each phases are classified into three different categories namely, biological processes, molecular functions and cellular processes are shown in Supporting Information Figs. 4–6. Significantly enriched GO terms in each phase are shown in Supporting Information Table 2. In the following sections, the level of gene expression and their functional classification were compared among the three different phases.

3.1.1 Transcriptional responses to nitrogen starvation (glucose-N-limited versus glucose-N-excess phase)

3.1.1.1 Genes involved in stress response, ammonium uptake, and cell growth. Limitation of ammonium is known to induce lipid accumulation in oleaginous yeast [28–31]. In fact, the transcriptional changes induced by nitrogen limitation in *R. toruloides* and *Y. lipolytica* have already been studied previously on glucose as mono-substrate [16, 32]. Significant changes in gene expression levels associated to GO terms related to biological functions (metabolic processes, cellular localization, transmembrane transport, etc., see Supporting Information Fig. 3) are identified in the nitrogen-limited phase. Identification of highly upregulated genes involved in ammonium transport and metabolism (ammonium transmembrane transporter (RHTO_01680), urea carboxylase (RHTO_01350), glutamate synthase (RHTO_00025, a key enzyme in ammonia assimilation), several transport proteins, amino acid permeases (RHTO_00398, RHTO_01882)), stress response genes (RHTO_01497), or Phytanoyl-CoA dioxygenase family proteins (RHTO_07234; RHTO_07235) were observed. Genes encoding vacuolar proteases, ATPases, autophagy-related genes were also found to be upregulated (Supporting Information Fig. 7). The upregulation of these genes indicates degradation of macromolecules and organelles. Turnover of intracellular components on the onset of nitrogen limitation can be also observed from the elevated mRNA levels of α -mannosidase (AMS1) and other vacuolar lipases. These observations indicate that N-deprivation induces homeostatic responses, including activation of the glutamine synthesis pathway and increase in the cell capacity to utilize trace amounts of nitrogen resources, or redistribution of the intracellular nitrogen such as increase in amino acid metabolism as reported by Yang et al. [33].

Several transcriptional factors and regulatory proteins were also differentially expressed after ammonium limitation. Transcriptional factors related to TFIH subunits were downregulated. TFIH is not only required for transcription but also plays central roles in the fundamental processes of DNA repair and cell cycle control. Several repressors of transcription (TUP1 like) were upregulated during this phase. Stress response proteins like UBI4, CTT1 were significantly upregulated (> 3-fold) during this phase. Furthermore, a decrease in growth rate after nitrogen limitation coincided with the transcriptomic response of *R. toruloides* with significant downregulation of gene expressions related to ribosome biogenesis (RHTO_04839), nucleotide and nucleic acid metabolic processes, genes related to cellular metabolism, protein synthesis, transcriptional, and translational factors.

3.1.1.2 Genes involved in TAG and precursors production.

Genes involved in lipid production were shown to be significantly regulated after nitrogen limitation. Upregulated genes were the ATP citrate lyase (ACL1, 1.3-fold relative to glucose-N-excess phase, (Fig. 2)) acetyl-CoA carboxylase (ACC), and fatty acid synthases (FAS subunits a/b). ACL1 is considered the main supplier of cytosolic Acetyl-CoA for fatty acid synthesis [34]. ACC catalyzes the initial and rate limiting steps of fatty acid synthesis [35]. ACL requires coenzyme A and ATP to form acetyl-CoA in the cytosol. During nitrogen

Figure 2. Mean of expression levels of genes involved in fatty acid synthesis pathway (log2 FPKM values) and standard deviation from two biological samples. Legend: black = glucose-N-excess phase; red = glucose-N-limited phase; blue = diauxic shift; dark cyan = glycerol phase.

limitation, adenylate kinases (ADK1, 2), NADH dehydrogenases, ATP synthase beta chain, and other ATPases displayed differential expression. About twofold upregulation of dephospho-CoA kinase (DPCK, RHTO_04302) which is the final enzyme in the CoA biosynthesis pathway was observed suggesting an increased CoA biosynthesis which can directly or indirectly help for lipid accumulation.

Since the production of one mol TAG (C16:0) requires 48 moles of NADPH, this precursor is one of the limiting factors for fatty acid synthesis [36]. It was assumed that reducing equivalents for biosynthetic purposes could be mainly generated by the pentose phosphate pathway enzymes (glucose 6-P dehydrogenase-ZWF1, 6-phosphogluconate dehydrogenase, 6-PGD), isocitrate dehydrogenase (IDP1), and the cytosolic malic enzyme (ME1) [37]. Interestingly, expression levels of ZWF1 (0.65-fold upregulated) and IDP1 (0.70-fold upregulated) did not change much whereas the expression levels of mitochondrial malic enzyme (MAE1) were significantly upregulated during glucose-N-limited phase (Fig. 3). Moreover, the anaplerotic pyruvate carboxylase gene (PYC) and phosphoenolpyruvate carboxykinase (PCK1) were found to be upregulated significantly after nitrogen limitation activating the futile cycle to regenerate excess ATP during slow growth phase. Malate dehydrogenase (MDH2) in the cytosol was also upregulated significantly during this phase converting malate to oxaloacetate. Interestingly, the cytosolic malic enzyme (ME1) and the mitochondrial malate dehydrogenase (MDH1) were downregulated after nitrogen limitation. The physiological role of malic enzyme is still uncertain; however, from the transcriptional data presented, the malate produced in the cytosol during the glucose-N-limited phase is converted to oxaloacetate which is converted back to PEP by PPC. High expression levels of ALD (aldehyde dehydrogenase) and ACS (acetyl-CoA synthetase) coding genes during this phase indicate that *R. toruloides* also uses an alternative route to produce acetyl-CoA in the cytosol. This could also be beneficial for lipid biosynthesis as ALD1/6 produces NADPH. Mitochondrial malic enzyme (MAE1) is also found to be highly upregulated during this phase but it is not beneficial

for lipid production as NADPH inside the mitochondria cannot be transported into the cytosol.

As expected, the 2-methylisocitrate lyase (ICL2) was also found to be highly upregulated (Fig. 3) suggesting that citrate is converted to succinate and pyruvate in an attempt to fuel the TCA cycle [16]. Aconitase (ACO1) was also significantly upregulated in this phase. In *Yarrowia lipolytica*, the most extensively studied oleaginous yeast, where citrate is produced extracellularly, downregulation of ICL2 was observed after nitrogen limitation. This in fact explains one major difference between *R. toruloides* and *Y. lipolytica* [32]. Moreover, *Y. lipolytica* does not exhibit catabolite repression and can simultaneously assimilate glucose and glycerol, with higher assimilation rate for glycerol than glucose [38].

3.1.1.3 Glycerol metabolism and transport genes in the presence of glucose. Glycerol transport is the first barrier for glycerol utilization in a microbial cell. In the presence of glucose, and irrespective of the medium nitrogen content, glycerol:H⁺ symporter (GUP1) (RHTO_03825) showed similar expressions, whereas expression of STL1 (MFS-sugar transporter/glycerol proton symporter) showed upregulation after nitrogen limitation (Fig. 4A). Moreover, the channel protein glycerol efflux/influx facilitator (FPS1) (RHTO_06134) showed no expression during growth on glucose irrespective of nitrogen limitation and is highly regulated after glucose limitation, suggesting its importance for glycerol uptake in *R. toruloides*. Interestingly, glycerol kinase (GUT1) and mitochondrial FAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GUT2) which are significantly downregulated in *S. cerevisiae* in the presence of glucose [27] were significantly upregulated in this phase (1.4-fold).

Interestingly, most transcriptional changes, especially stress responses were significantly upregulated after nitrogen exhaustion and the expression of certain regulatory proteins or transcriptional factors did not change significantly after the nitrogen exhaustion. This phenomenon is different to what was observed for *S. cerevisiae* and other fungi [11–13] where no expressions of GUT1, GUT2, and STL1 were observed during the glucose

Figure 3. Transcript levels of genes involved in central carbon metabolism compared between the four phases. Expression levels based on log₂ FPKM values, and the values shown the mean and standard deviation from two biological repeats. Legend: black = glucose-N-excess phase; red = glucose-N-limited phase; blue = diauxic shift; dark cyan = glycerol phase.

growth phase and a significant upregulation of these enzyme expression levels were reported after glucose exhaustion with correlation toward expressions of certain positive regulators like Adr1p and Snf1p. Relatively good mRNA levels of the glycerol utilization and synthesis enzymes were observed in the glucose-N-excess phase (Fig. 4A) which indicates that glycerol utilization/regulation in *R. toruloides* is not similar to that as observed in *S. cerevisiae* and it is regulated through an unknown mechanism or not regulated at the transcriptional level.

3.1.1.4 Glycerol production by *R. toruloides* grown on glucose. NAD-dependent glycerol-3-phosphate dehydrogenase (GPD1) which is an important enzyme in glycerol and lipid synthesis was upregulated (1.1-fold) in this phase, whereas glycerol-3-phosphate phosphatase (GPP1/GPP2) was not identified in the genome of *R. toruloides* so far. However, the 2-deoxyglucose-6-phosphatase (DOG1) (RHTO_04434) which was downregulated shares about 36% similarity in protein sequence with GPP1 of *S. cerevisiae*. Increased expression of GPD1 confirms

that glycerol-3-phosphate being mainly used for lipid synthesis during this phase. Upregulation of glycerol dehydrogenase (GCY1) (0.5-fold) and glycerone kinase (DAK1) (3.1-fold) were also observed during this phase (Fig. 4A) giving a hint that *R. toruloides* might be synthesizing glycerol intracellularly as a response to stress induced by nitrogen limitation. Triose phosphate isomerase (TPI) activity was also downregulated (1.0-fold) during this phase as a response to stress. Indeed, with glucose as the sole carbon source, *R. toruloides* was shown to produce glycerol after nitrogen limitation (Fig. 4B). It should be stressed here, that glycerol production was never reported to occur in oleaginous yeast. Nevertheless, to confirm that glycerol was not consumed but produced during growth on mixture, an experiment with labeled glycerol (U-¹³C₃) and unlabeled glucose was performed. Protein bound amino acid labeling patterns were analyzed and no higher masses (M+3) were observed in the labeling patterns, confirming that glycerol was not consumed in the presence of glucose (data not shown). Interestingly, several osmotic response proteins were also upregulated after nitrogen

depletion. A positive regulator of FPS, pH domain containing protein (RGC2) which binds to FPS1 during nonhyperosmotic conditions [39] and holds the channel for glycerol efflux is highly upregulated during this transition phase which correlates with inactive Hog1 (Table 1). It is known however, that some yeast strains produce glycerol [40, 41] or polyols [42] in response to

high osmotic pressure or after exposure to physiological conditions relevant to stress. All these observations suggest that nitrogen limitation induces several global responses that evolve more dynamically during this transition in *R. toruloides*. It can be concluded that the regulation of glycerol metabolism in the presence of glucose in *R. toruloides* is different compared to

Figure 4. (A) Mean of expression levels of genes involved in glycerol metabolism and biosynthesis ($\log_2$ FPKM values) and standard deviation from two biological samples. Legend: black = glucose-N-excess phase; red = glucose-N-limited phase; blue = diauxic shift; dark cyan = glycerol phase. (B) Kinetic profile of *R. toruloides* grown on glucose as the sole carbon source.

Table 1. Fold change ($\log_2 > 0.5$) of selected transcription factors and stress response factors in diauxic and glycerol phases relative to glucose-N-limited phase and in glucose-N-limited phase relative to glucose-N-excess phase

Transcript (gene id)	Glucose-N-limited phase	Diauxic shift phase	Glycerol phase	Description
Adr1p (RHTO_03280)	NS	NS	NS	Transcription activator of certain genes after glucose exhaustion in yeast
Cat8p (RHTO_00451)	0.61	1.03	0.64	Putative zinc finger transcription factor (binds to CSRE of gluconeogenic genes)
Tbp1p (RHTO_08020)	NS	NS	−0.50	TATA-box binding protein
Sip4p (RHTO_04578)	1.70	NS	NS	C6 transcription factor
Mig1p (RHTO_07440)	NS	NS	NS	Glucose-mediated repressor
Msn2/4p (RHTO_00480)	1.90	−1.70	−1.70	General stress response
Sko1p (RHTO_07952)	1.60	1.10	1.40	Osmotic and oxidative stress response (bZIP transcription factor)
Snf1 (RHTO_04494)	1.00	0.64	NS	Carbon catabolite de-repressing protein
Hog1 (RHTO_03321)	NS	NS	NS	MAP kinase (osmotic stress response)
Ssk1 (RHTO_07372)	0.52	−0.85	NS	Osmolarity response regulator
Pbs2 (RHTO_02351)	NS	−0.59	−0.61	MAP kinase kinase of HOG1 pathway (osmotic response)
CTT1 (RHTO_01370)	2.30	−1.82	NS	Catalase T (stress response)
RIM15 (RHTO_04687)	0.67	1.30	0.90	Serine/threonine protein kinase (response to nutrient depletion)
GSY1 (RHTO_04065)	0.60	2.50	0.60	Glycogen synthase (stress response)
GAC1 (RHTO_02726)	1.50	1.10	0.60	Glc7p, activator of heat shock proteins
OPI1 (RHTO_04818)	1.11	0.62	0.61	Clock controlled transcriptional regulator (over producer of inositol)
Sho1p (RHTO_00337)	1.45	NS	NS	Osmosensor
RGC2 (RHTO_06926)	2.91	0.87	NS	pH domain containing protein
UB14 (RHTO_01909)	3.17	−1.22	NS	Ubiquitin (stress response)
Hap4p (RHTO_03567)	NS	NS	0.88	Transcriptional activator (Respiratory chain, TCA/glyoxylate and mt ribosome-proteins)
Ino2p/Ino4p (RHTO_03930)	NS	0.61	NS	Transcriptional activator (binds to ICREs)

Values are $\log_2$ (expression ratio).

NS = 'not significant'.

S. cerevisiae at the transcriptional level and more detailed insights into post transcriptional or translational details can resolve the regulation of glycerol utilization in the presence of glucose in *R. toruloides*.

3.1.2 Diauxic shift phase versus glucose-N-limited phase

During the diauxic shift phase, significant upregulation of respiratory mechanism related GO terms, fatty acid metabolism, stress, and electron transport related terms were observed (Supporting Information Table 2). Significantly downregulated are related to protein synthesis, electron transport terms, which is correlated to attenuated growth rate after glucose exhaustion. In general, the readjustment of the metabolism from glycolytic to gluconeogenic operation was recorded (Fig. 3). In the central metabolism, a significant upregulation of aldehyde dehydrogenases (ALD1, 3), acetyl-CoA synthase (ACS1), fructose 1,6-bisphosphatase (FBP1), TCA cycle enzymes (CIT1 and IDP1), glyoxylate cycle enzymes (ICL), and storage compound producing enzyme (glycogen synthase (GSY2)) were observed (Fig. 3). Similar results were also observed with previous studies on *S. cerevisiae* [43]. In general, most of the stress response proteins that were upregulated during the diauxic shift phase (after glucose exhaustion) in *S. cerevisiae*, were upregulated during the nitrogen-limited phase in *R. toruloides* (Table 1) indicating its complexity and uniqueness of transcriptional regula-

tion induced by nitrogen limitation. With respect to the glycerol uptake system, the glucose repressor protein Mig1p in combination with proteins like Cat8p and Sip4p that were differentially expressed in *S. cerevisiae* remain unchanged in *R. toruloides* [27]. In fact, GUT1, GUT2 and several other genes are repressed through the negative repressor Mig1p in presence of glucose in *S. cerevisiae* [43, 44]. However, in *R. toruloides*, the expression of Mig1p and other repressors like Cat8p did not change significantly between the different phases. Additionally, mRNA levels of Adr1p which positively regulates the activities of certain genes (GUT1, ADH2, PUT4, CTA1, etc.) in *S. cerevisiae* and *Aspergillus* [13] species were also found to be unchanged in *R. toruloides* (Table 1). Positive regulator Ino2p/Ino4p which regulates phospholipid biosynthesis and GUT2 and the negative regulator OPI1 of phospholipid biosynthesis were also identified in the genome of *R. toruloides* but the expressions levels of these regulators did not change significantly during the diauxic shift (Table 1). Multiple sequence alignment of upstream sequences (1000 bp) of GUT1 from *R. toruloides* and *S. cerevisiae* did show the consensus binding sites of Mig1p, Adr1p (Supporting Information Fig. 8) but no UAS_{Ino}/OPI1 binding sites were identified. Expressions levels of GUT1, GUT2, and GUP1/Stl1 are significantly induced during this phase but the expression levels of their positive or negative regulators (Mig1p, RGC2, Adr1p) did not change significantly during the diauxic shift compared to the glucose phase. Obviously this indicates that the regulation of the diauxic growth

in *R. toruloides* is through an unknown mechanism that has to be studied in detail.

3.1.3 Glycerol phase versus glucose-N-limited phase

During continued growth on glycerol, significantly enriched GO terms including protein synthesis, rRNA assembly, energy generation processes were observed (Supporting Information Table 2). Most significantly, upregulated terms are related to mitochondrial activity and energy generations whereas significantly downregulated are the genes related to precursor metabolites generation. Interestingly, glycerol metabolism did not show any significant changes compared to the glucose-N-limited phase. mRNA levels of GUT1, GUT2, GPD1, 2 and GCY2 remained unchanged whereas upregulation of FPS1 and STL1 was observed (Fig. 4A). Interestingly, as shown in Fig. 4A, about three-fold downregulation of DAK1 was observed in this phase. GPD1 expression level was slightly downregulated compared to the glucose-N-limited phase. In general, upon glucose limitation, the onset of the glyoxylate cycle operation triggered by an increased expression of the enzymes that catalyze malate synthase and cytosolic citrate synthase reactions were observed (Fig. 3) which was also noticed during diauxic shift in *S. cerevisiae* [45]. Moreover, the upregulation of ACS1 and other cytosolic acetyl-CoA producing enzymes were upregulated in diauxic shift phase in a process to fuel the TCA cycle by providing acetyl-CoA. Significant induction in dicarboxylic transporters which transport succinate, AKG, malate into mitochondrion was induced during this phase which correlates with induced TCA cycle. Furthermore, during growth on glycerol, significant upregulation of phospholipid biosynthesis pathway genes (Supporting Information Fig. 9) was also observed. In *S. cerevisiae*, phospholipid biosynthesis is found to be regulated by the UAS_{ino} mediated regulation [46]. As discussed above the regulators mediated by UAS_{ino} did not show any significant changes at the mRNA level during this glycerol phase. It could be that as the phospholipid formation is also based on the substrate used, more flux entering to the phosphatidic acid acts as a regulating point for initiating phospholipid biosynthesis.

4 Concluding remarks

Rhodospiridium toruloides showed completely different regulation of glycerol metabolism when compared to *S. cerevisiae* in presence of glucose. Glycerol kinase (GUT1) and mitochondrial FAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GUT2) were significantly downregulated in *S. cerevisiae* in the presence of glucose, whereas they were significantly upregulated in presence of glucose after ammonium limitation in *R. toruloides*. Of interest is the conjecture that *R. toruloides* uses glycerol as backup in response to stress by accumulating it intracellularly. In the view of lipid production, certain complex cascade of transcriptional responses could lead to redirection of acetyl-CoA and NADPH for their synthesis which is unique between oleaginous yeasts and detailed study at molecular level is required to understand the underlying regulation which will facilitate efficient engineering of these strains.

Practical application

Rhodospiridium toruloides can accumulate lipids up to 70% of its cell mass under certain conditions. Previous studies showed that lipid yields of this yeast grown on glucose are lower compared to growth on glycerol. Biomass production was significantly more on glucose compared to glycerol. On mixture of glucose and glycerol, *R. toruloides* accumulated lipid with a comparable yield to that observed on glycerol alone and with a higher biomass concentration. However, a typical diauxic growth was observed. In an attempt to understand the underlying regulation of glycerol metabolism in the presence of glucose, transcriptomic analysis was performed on *R. toruloides* grown on glucose and glycerol mixtures which could open doors toward optimization of lipid production.

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