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Nitrogen as the major factor influencing gene expression in *Yarrowia lipolytica*

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ABSTRACT

Yarrowia lipolytica is an important industrial microorganism used for the production of oleochemicals. The design of effective biotechnological processes with this cell factory requires an in-depth knowledge of its metabolism. Here we present a transcriptomic study of *Y. lipolytica* grown in the presence of glycerol and glucose, and mixture of both at different carbon to nitrogen ratios. It emerged that the transcriptomic landscape of *Y. lipolytica* is more sensitive to the nitrogen availability than to the utilized carbon source, as evidenced by more genes being differentially expressed in lower carbon to nitrogen ratio. Specifically, expression of hexokinase (*HXK1*) is significantly susceptible to changes in nitrogen concentrations. High *HXK1* expression in low nitrogen seems to impact other genes which are implicated in tricarboxylic acid cycle and erythritol biosynthesis. We further show that expression of *HXK1* and two genes belonging to the sugar porter family might be controlled by GATA-like zinc-finger proteins.
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1. Introduction

Microorganisms are under constant influence of ever-changing nutrient concentrations in their environments, hence sensing and adapting to those changes is crucial for survival. As a result of evolution, several mechanisms helping in precise adaptation of microbial physiology to the environment have developed. Via these mechanisms, i.e. signalling pathways, many microorganisms can directly sense changes in the concentrations of various nutrients, such as carbon and nitrogen sources, and adjust their metabolism to the environmental challenges. The cellular metabolism can be adjusted through fine controlled expression of genes involved in utilization of a given nutrient or its derivatives. These events can be observed in complex, often evolutionarily conserved regulatory processes, such as carbon catabolite repression (CCR) and nitrogen catabolite repression (NCR). Most of our current understanding of CCR and NCR comes from observations of investigating these processes in the model yeast *Saccharomyces cerevisiae* as reviewed in [1,2], while only few studies have been devoted to other industrially and environmentally relevant microorganisms such as the oleaginous yeast *Yarrowia lipolytica*.

The strictly aerobic yeast *Y. lipolytica* is a promising cell factory for production of valuable compounds, such as organic acids [3–5], polyhydroxy alcohols [6,7] and aromas [8]. High protein secretion capacity [9] and FDA awarded GRAS status [10] makes the yeast a potential platform for therapeutic protein production [11]. The most prominent characteristic of *Y. lipolytica* is, however, its ability to accumulate large amounts of intracellular lipids that can reach up to 20 % of cell dry weight in wild-type strains [12]. This trait, together with its ability to use industrial wastes as substrates, e.g. crude glycerol [13], as well as a rapidly expanding range of genetic engineering tools [14–16] render *Y. lipolytica* an excellent producer of biofuels and other oleochemicals. To design efficient and cost-effective bioprocesses with this yeast, utilization of inexpensive and sustainable raw materials combined with a deep, system-level understanding of its metabolism are of great importance.

Y. lipolytica is able to use a plethora of cost-attractive carbon sources. Both, hydrophobic and hydrophilic substrates, such as lipids, glycerol or glucose [17,18] are readily utilized by this yeast. Glycerol is a cheap feedstock for bioprocesses due to its significant amounts generated as by-product of soap and biodiesel industries [19,20]. Glucose, on the other hand, is the most abundant monosaccharide found in plant polymers such as starch and cellulose. Unlike the model yeast *S. cerevisiae*, *Y. lipolytica* utilizes glycerol exhibiting much higher growth rates [21]. Moreover, when

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Table 1
Yeast strains used in this study.

Strain	Genotype	Reference
A101	Wild-type	[24]
W29	Wild-type	[10]
PHY94	MatA, *leu2−270, ura3−302*^{b}, *xpr2−322, hxk1, glk1, pTEF-HXK1*	[Hapeta et al., in preparation]

grown in a medium containing glycerol and glucose, glycerol is consumed first or co-consumed with glucose [22] suggesting an as yet unknown regulatory mechanism(s) controlling this behaviour. An attempt to elucidate the background of this phenomenon using whole transcriptome sequencing under carbon limiting conditions has recently been taken [23]. In the current work, we expand on this by considering the interplay between carbon sources and carbon to nitrogen ratios.

2. Materials and methods

2.1. Yeast strains and media

The main strain used throughout this study was the wild type *Y. lipolytica* A101 strain [24]. Strains W29 and PHY94 were used for comparisons in gene expression analysis (Table 1). The strains were routinely maintained in YPD medium consisting of 10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose or glycerol with optional 10 g/L agar (for plates) at 28°C. For long-term storage the strains were kept at −80 °C in 50 % glycerol. Composition of all the minimal media used in this study is listed in Table 2. The YNB (Yeast Nitrogen Base) medium was purchased from Formedium Ltd., Hunstanton, UK. Glycerol and glucose stock solution were of concentration 500 g/L and cold-sterilized through 0.22 μm membranes. A general representation of the performed experiments and strains used is presented in Fig. 1.

2.2. Shake-flask cultures

Cultivations were performed in 250 mL Erlenmeyer flasks with 50 mL YNB medium. Prepared media were inoculated with biomass to an initial OD_{600} of 0.5 and incubated at 28 °C on a rotary shaker with 3 Hz shaking speed. The samples for biomass and substrate utilization analyses were collected at 0, 6, 10 and 24 h of culture. In early exponential growth phase (t = 10 h) 1 mL samples were collected, flash-freezed in liquid nitrogen and kept at −80 °C prior to RNA extraction. Experiments were performed in triplicates.

Table 2
YNB media variants used in this study.

Component	Concentration [g/L]	
	Shake-flasks	
	C/N 20	C/N 40
YNB	1.9	
NH_4Cl	4.47	2.2
Glucose	50	
Phosphate buffer pH 6.8	50 mM	
	Bioreactor batch mode	
	C/N 20	C/N 40
YNB	1.9	
NH_4Cl	1.78	0.89
KH_2PO_4	1	
Glycerol	10.2	
Glucose	10	
	Bioreactor chemostat mode	
	C/N 20	C/N 40
YNB	1.9	
NH_4Cl	4.47	2.2
KH_2PO_4	1	
Glycerol	50	
Glucose	50	
Glycerol + Glucose	25.2 + 24.6	

2.3. Bioreactor cultures

The batch bioreactor cultures were performed in Biostat B Plus fermenters (Sartorius-Stedim, Germany) under following conditions: the working volume of 1500 mL, the temperature of 28 °C, the stirring rate of 10 Hz and the aeration of 0.6 vvm (vessel volume per minute). pH was automatically maintained at pH 6.8 by addition of 40 % (w/v) KOH. Initial OD_{600} was set to 0.5. The experiments were performed in biological duplicates.

2.4. Chemostat cultivations

The chemostat cultivations were carried out in Biostat B Plus fermenters (Sartorius-Stedim, Germany) under following conditions: the working volume of 750 mL, the temperature of 28 °C, the stirring rate of 10 Hz and the aeration of 0.6 vvm (volume of air per volume of liquid per minute). pH was automatically maintained at 6.8 by addition of 40 % (w/v) KOH. The dilution rate was set at 0.05/h. The process was held until the steady state (7–12 media exchanges, depending on applied carbon source), defined as constant biomass and constant remaining substrate concentration in the post-fermentation medium for at least 48 h were achieved. At steady states 10 mL samples were collected, flash-freezed in liquid nitrogen and kept at −80 °C prior to RNA extraction. Each culture variant was carried out in biological duplicate.

2.5. RNA extraction

The RNA for transcriptome sequencing was obtained by disrupting the cells using Mikro-Dismembrator U ball mill (Sartorius AG, Göttingen, Germany) and metal beads, followed by extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol. RNA for qRT-PCR were extracted using Total RNA Mini kit (A&A Biotechnology, Gdynia, Poland) according to the provided protocol. In both cases, extracted RNA was checked for quality using Biochrom WPA Biowave DNA spectrophotometer (Biochrom Ltd., Cambridge, UK).

2.6. Quantitative real-time PCR

Extracted RNA samples were treated with DNase (A&A Biotechnology, Gdynia, Poland) and reverse transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). The obtained cDNA samples were then used for qPCR reaction using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and primers listed in Table S1 on the PCRmax Eco 48 thermal cycler (Illumina, San Diego, CA). Expression of genes was standardized to the expression of actin (*YlACT1, YALI0D08272g*) gene.

2.7. RNA sequencing

The RNA samples from chemostat cultivations were sequenced by Novogene (Beijing, China) using HiSeq 4000 next-generation

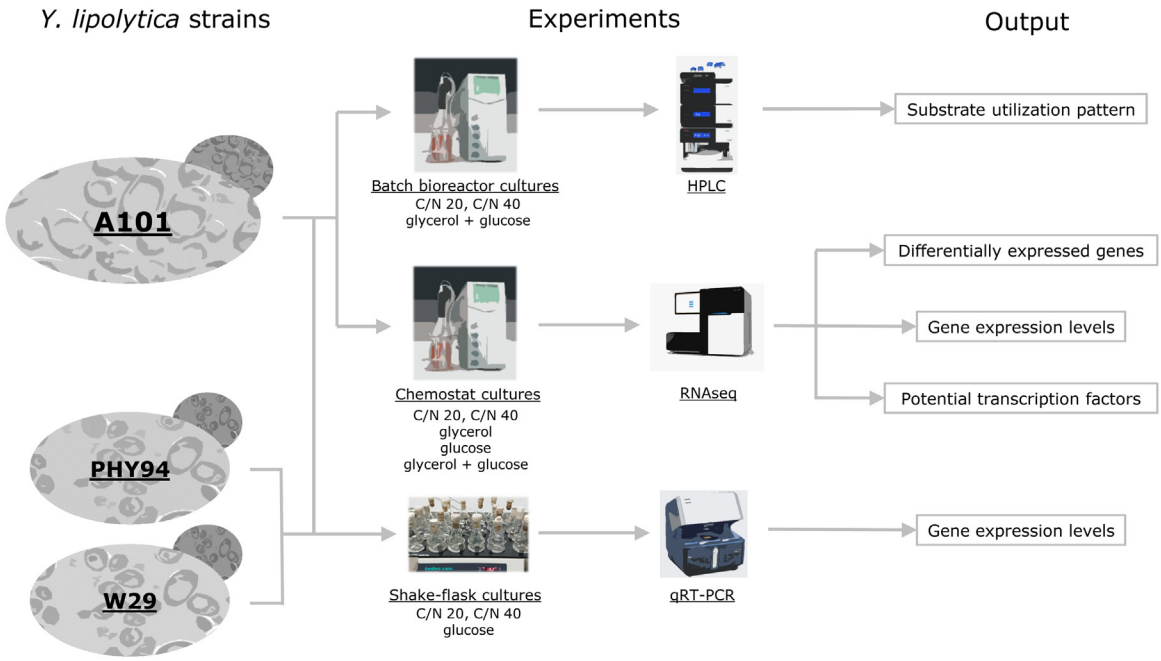

Fig. 1. Experimental design. The *Y. lipolytica* A101 strain was used in all experiments in this study, the PHY94 and W29 strains were used for qRT-PCR. See Materials and methods section for more details.

sequencer (Illumina, San-Diego, CA) in paired end mode. The obtained reads were of length 150 bp.

2.8. Bioinformatics analysis

The RNAseq reads were checked for quality using FastQC 0.11.9 [25] and the 12 bp adapter regions were trimmed by Trimmomatic 0.39 [26]. Subsequently, the reads were mapped to *Y. lipolytica* CLIB122 genome (GenBank assembly identifier: GCA_000002525.1) using STAR Aligner 2.7.2b [27] and quantified with the featureCounts of Subread package 2.0.0 [28]. The count table was used to identify differentially expressed genes with DESeq package [29]. In order to determine potential transcription factors controlling gene expression in *Y. lipolytica*, 1 kb sequences up-stream of chosen genes were retrieved from NCBI database. The YALI0 gene identifiers were used as an input for YEASTRACT + database [30]. The pathways were reconstituted using KEGG pathway database [31].

2.9. Analytical methods

Substrate utilization was determined using Dionex UltiMate 3000 HPLC instrument (Dionex-ThermoFisher, UK) equipped with a Carbohydrate H + column (Thermo Scientific, Waltham, MA) coupled to an UV (λ = 210 nm) and RI (Shodex, Ogimachi, Japan) detectors according to standards. The column was eluted with 25 mmol trifluoroacetic acid at 65 °C and a flow rate of 0.6 mL/min.

The growth was monitored by measuring optical density at 600 nm using SmartSpec Plus spectrophotometer (Biorad, Hercules, CA). Additionally, the dry biomass during bioreactor cultures was determined gravimetrically after cells being filtered through 0.22 μm membranes and dried at 105 °C using laboratory balance with moisture analyzer (RADWAG, Radom, Poland).

2.10. Calculations

The biomass yield was calculated as follows:

$$\frac{Yx}{s} = -\frac{[Xf] - [Xi]}{[Sf] - [Si]}$$

Where:

Yx/s – biomass yield coefficient

[Xi], [Xf] – initial and final biomass concentration, respectively [g/L]

[Si], [Sf] – initial and final biomass concentration, respectively [g/L]

Substrate utilization and specific substrate utilization rates were calculated for linear utilization curves using following equations:

$$Q = \frac{[Sb] - [Sa]}{tb - ta}$$

$$q = \frac{Q}{Xavg}$$

Where:

Q – substrate utilization rate [g/L/h]

q – specific substrate utilization rate [g/g/h]

[Sa], [Sb] – substrate concentration at the beginning and end of linear substrate utilization, respectively [g/L]

ta, tb – time of culture at the beginning and end of linear substrate utilization, respectively [h]

Xavg - average of biomass concentration at ta and tb

3. Results

3.1. Substrate utilization pattern

In oleaginous yeasts such as *Y. lipolytica,* a low availability of nitrogen in the cultivation medium activates lipid accumulation and/or citric acid secretion [32,33], while more nitrogen promotes cells division rapidly generating biomass [34,35]. In this study we aimed to investigate how two readily available substrates, namely glycerol and glucose, are utilized at different carbon to nitrogen ratios (C/N). Therefore, we performed bioreactor batch cultures of a wild-type *Y. lipolytica* A101 strain at two different C/N ratios (20 and 40) in a minimal YNB medium with a mixture of glucose and glycerol. In both experiments, nitrogen availability had no effect on

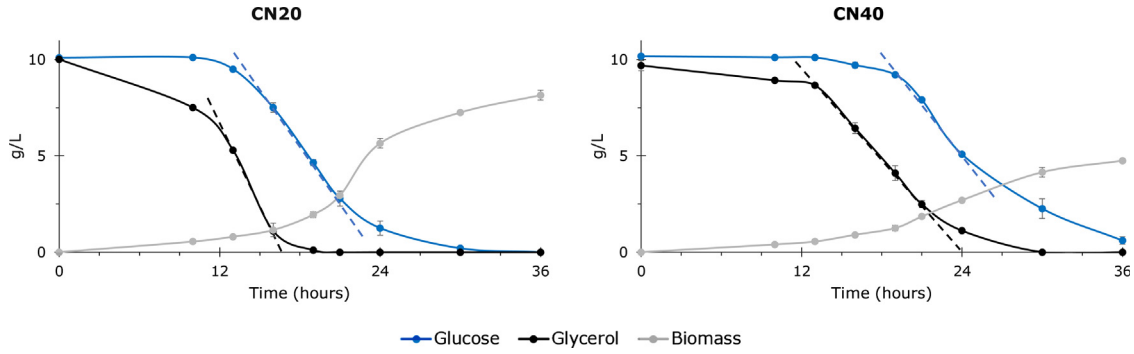

Fig. 2. Growth and substrate utilization by *Y. lipolytica* during bioreactor cultivation in YNB medium supplemented by mixture of glycerol and glucose with C/N 20 and C/N 40 ratio. Dashed lines represent linear utilization of glycerol (in black) and glucose (in blue). Bioreactors were performed in duplicate (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Growth parameters of *Y. lipolytica* growing in YNB medium with a mixture of glycerol and glucose at C/N 20 and C/N 40 ratio.

	$Y_{X/S}$ [g/g]	Q_{gly} [g/L/h]	q_{gly} [g/g/h]	Q_{glc} [g/L/h]	q_{glc} [g/g/h]	q_{gly}/q_{glc}
CN20	0.40	1.39	1.42	0.94	0.46	3.09
CN40	0.25	0.77	0.64	0.83	0.42	1.52

the substrate utilization pattern - glycerol was used prior to glucose (Fig. 2). However, at both C/N ratios, slow glucose utilization could be seen when glycerol was above 5 g/L. Below that concentration glucose consumption accelerated substantially regardless C/N. As expected, more biomass was generated in higher nitrogen concentration (C/N 20) as reflected by the yield coefficient values ($Y_{X/S}$, Table 3). Both glycerol and glucose utilization rates (Q) for linear utilization curves were higher at C/N 20 than at C/N 40 (Fig. 2; Table 3). Additionally, a steep slope of glycerol utilization at C/N 20 was observed and the q_{gly} was *ca*. 2.2-fold higher than at C/N 40.

3.2. Transcriptional changes during growth on glucose, glycerol and a mixture of glucose and glycerol at C/N 20 and C/N 40

To elucidate the effect of C/N ratio on carbon utilization, wild-type *Y. lipolytica* A101 strain was cultivated in bioreactors under chemostat conditions where nitrogen was the growth limiting nutrient at the selected C/N ratios. In all experimental variants the cells were grown at the same dilution rate (specific growth rate) of 0.05/h. This dilution rate was selected as it has previously been shown as optimal for directing carbon flux towards lipid accumulation [36]. Throughout the cultivations, samples were taken for analysis of OD_{600}, substrate and biomass concentrations (Fig. S1). At steady state, defined as a constant biomass and substrate concentration in the culture medium, samples were rapidly taken for RNA extraction and subsequent RNA sequencing and data analysis.

The normalized counts from RNA sequencing were used for quantification of the transcript levels. The purpose of this analysis was to assess the impact of two factors on the transcriptomic landscape of *Y. lipolytica*, where the first factor was the carbon source and the second - nitrogen availability (C/N 20 and C/N 40). Three carbon source conditions were analyzed – glycerol, glucose and an equimolar blend of glucose and glycerol. The glycerol culture was set as a reference condition. The $p \leq 0.05$ and log2 (fold-change) ≥ 1.5 were used as cut-offs. The results showed, that at C/N 20 ratio in YNB glucose medium, 30 genes were up- and 50 were downregulated compared to the glycerol cultured cells (Fig. 3A). At C/N 40 ratio, 21 genes were subjected of up- and 12 of downregulation by glucose. The presence of glycerol in the mixed cultures at C/N 20 caused 35 genes to be upregulated (5 more than in the glucose culture) and 38 to be downregulated (12 less than in glucose culture), while at C/N 40 only 2 genes were differentially expressed, from which both were downregulated. The complete list of differentially expressed genes is available in supplementary materials (File S1).

Next, we were interested to identify those genes that were differentially expressed on both glucose and a mixture of glucose and glycerol. Thus at C/N 20, from 80 and 73 genes, 41 were common and differentially expressed due to the presence of glucose (Fig. 3B). In this group, the *YHT1* (hexose transporter) and *ALK1* (n-alkane inducible cytochrome P450) were found. Similar comparison at C/N 40 revealed no overlapping genes.

Subsequently, the impact of nitrogen availability on gene expression was assessed by comparing glucose and mixed cultures at C/N 20 and 40. The comparison of glucose cultures shown 3 genes to be differentially expressed at both C/N 20 and 40 (Fig. 3C). When mixed cultures were taken for comparison, the 2 downregulated genes at C/N 40 were also present in the group of 73 differentially expressed genes at C/N 20 (Fig. 3C). In both subsets, the *YHT4* gene encoding another hexose transporter was found.

By comparing the downregulated genes at C/N 20 (see Fig. 3A, blue bars), it transpired that 57 genes were negatively affected by glucose, of which 31 genes were downregulated in both mixed and glucose-only cultures (Fig. 4A). Among these 31 consistently downregulated genes (Supplementary File S1) was *ABC3*. On the other hand, there were 8 genes upregulated in both carbon source conditions (Fig. 4B, File S1), among which most notably *YHT1* and *ALK1*. The *YHT4* and *CIT1* (mitochondrial 2-methylcitrate synthase) genes were among those upregulated by glucose alone and the group of 27 genes in which *ALK2* (n-alkane inducible cytochrome P454) and *LIP8* (triacylglycerol lipase precursor) genes were present, were upregulated in the mixed culture.

At C/N 40, only the *YHT4* gene was downregulated in both carbon sources (Fig. 4C), while no genes were upregulated in mixed cultures, leaving a set of 21 genes upregulated in glucose-only cultures, including *ALK2* (Fig. 4D).

3.3. Targeted-transcriptome analysis reveals major impact of nitrogen on gene expression

To gain a more detailed understanding on the transcriptomic behaviors, a targeted expression analysis was carried out, for which groups of genes responsible for relevant biological processes were selected. The expression levels of genes encoding for sugar and glycerol transport, kinases, glycerol metabolism, the tricarboxylic acid cycle and erythritol metabolism are depicted in Fig. 5.

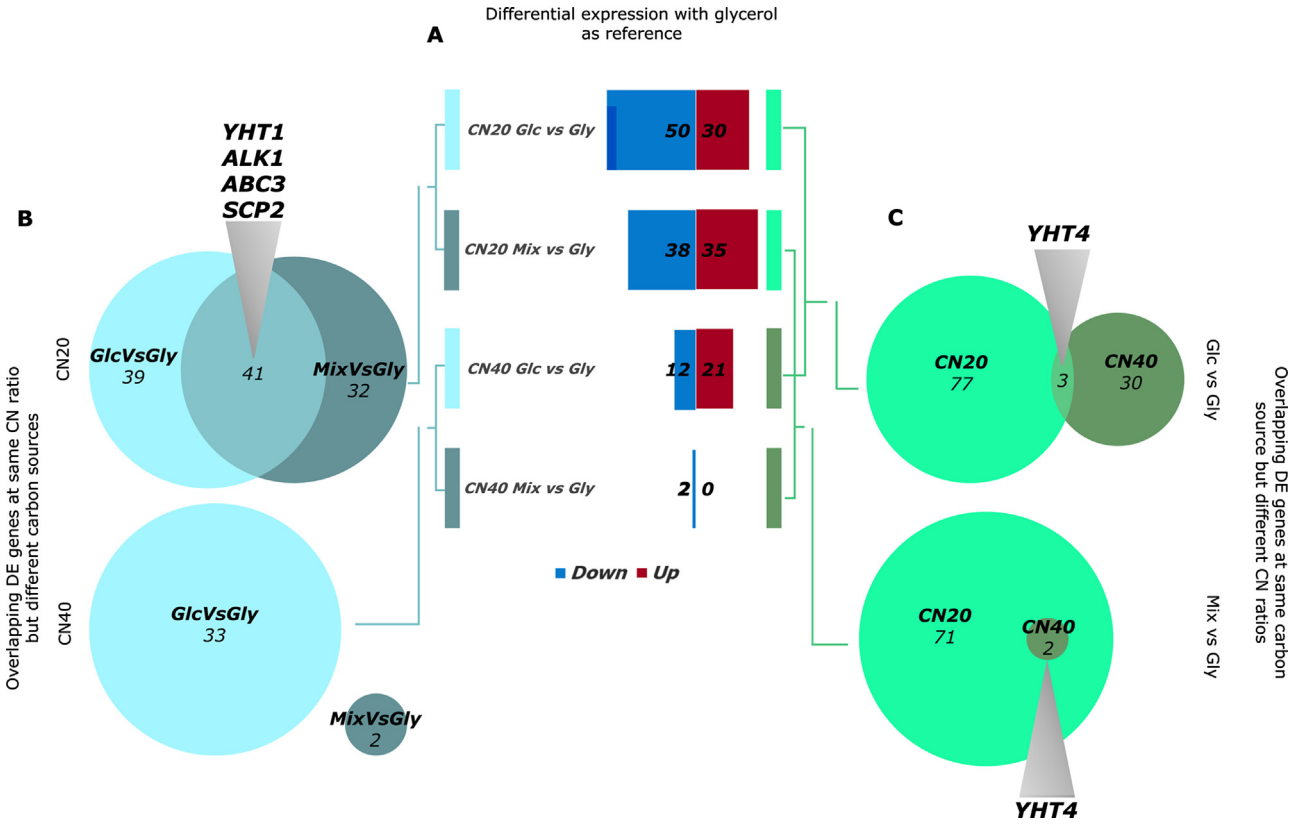

Fig. 3. Up- and downregulated genes, and number of overlapping genes in the experimental conditions. The complete list of differentially expressed genes is available in File S1 (Supplementary Materials).

A first step in the metabolism of glucose and glycerol is their internalization through the plasma membrane. The most highly expressed known hexose transporters in all conditions were *YHT1* and *YHT4*. Five other genes from the sugar porter family, namely *YALI0B21230g*, *YALI0F6776g*, *YALI0F18084g* and *YALI0F23903g* showed similar expression pattern to *YHT1* and *YHT4*, however, the expression levels were much lower than for *YHT1* (Fig. 5A). Regarding glycerol transport, the *Y. lipolytica FPS1* (*YALI0F00462g*) aquaglyceroporin homologue showed almost constant, high expression levels across all conditions but with an increased expression on glycerol at C/N 40.

The consequence of glycerol and glucose uptake into the cell is their phosphorylation by relevant kinases, that allows their incorporation into the central carbon metabolism. The main glycerol phosphorylating protein in *Y. lipolytica* is a *YALI0F00484g* encoding glycerol kinase *Gut1* which expression was higher at C/N 40 and the highest in cells cultivated on glycerol (Fig. 5B). The *GUT1* gene is an element of the glycerol-3-phosphate pathway, which consist of two other genes, *GUT2* and *GPD1*. Both of the later genes showed rather similar expression profiles. It has been proposed that *Y. lipolytica* has a secondary glycerol dissimilation pathway [23,37]. According to the current state of knowledge, this still dubious DHA pathway could be potentially encoded by 10 genes of which two (*DAK2*, *GCY15*) showed higher expression than the others in the present study (Fig. 5B).

In *Y. lipolytica* glucose is phosphorylated by either glucokinase *Glk1* or a hexokinase *Hxk1*. While expression levels of *GLK1* remained relatively constant across all conditions, the *HXK1* gene exhibited drastically increased expression at C/N 40 compared to at C/N 20, appearing as the strongest signal of all analyzed genes in this work (Fig. 5C). Additionally, the *YALI0D16357g* gene encoding phosphofructokinase showed a more moderately increased expression at C/N 40 with the highest expression in the presence of glucose. These changes could support a higher flux in glycolysis when nitrogen is less abundant. We were also interested to investigate the expression levels of *Y. lipolytica SNF1* and *MIG1* homologues, which in *S. cerevisiae* form the core of the SNF1/MIG1 pathway: components of the glucose repression mechanisms. The expression of *SNF1* was not dependent on the carbon source, however slightly higher expression was observed when cells were grown in lower nitrogen concentration (Fig. 5D).

Particularly increased expression was observed for *YALI0C24101g* gene encoding pyruvate carboxylase *PYC1* at C/N 40 (Fig. 5E). Notably, expression of *ACO1*, encoding aconitase, was the highest at C/N 40 with a particularly strong signal on glucose, while *CIT2* (citrate synthase) showed over 3-fold lower expression on glycerol than on glucose at C/N 40. The genes encoding enzymes further in the TCA cycle, such as those responsible for succinate to fumarate conversion (succinate dehydrogenase complex, *SDH*) showed in general low expression compared to the other TCA genes, however, increased expression was observed during cultivation on glucose at C/N 40 (Fig. 5E).

Y. lipolytica is also well known for its overproduction of erythritol, therefore, we checked expression level of genes encoding erythritol biosynthesis pathway (Fig. 5F). Four genes from this pathway revealed significant differences in expression on glucose at C/N 20 and C/N 40. These genes, namely *TAL1*, *GCY15*, *ARA12* and *GRE3* (also annotated to the DHA pathway), encoding transaldolase and three erythrose reductases, respectively, showed lower expression when less nitrogen was available. A similar pattern was observed for genes from two other pathways – *DAK2* associated with glycerol metabolism, as well as *IDH1*, *IDH2* and *ICL1* involved in the tricarboxylic acid cycle (TCA).

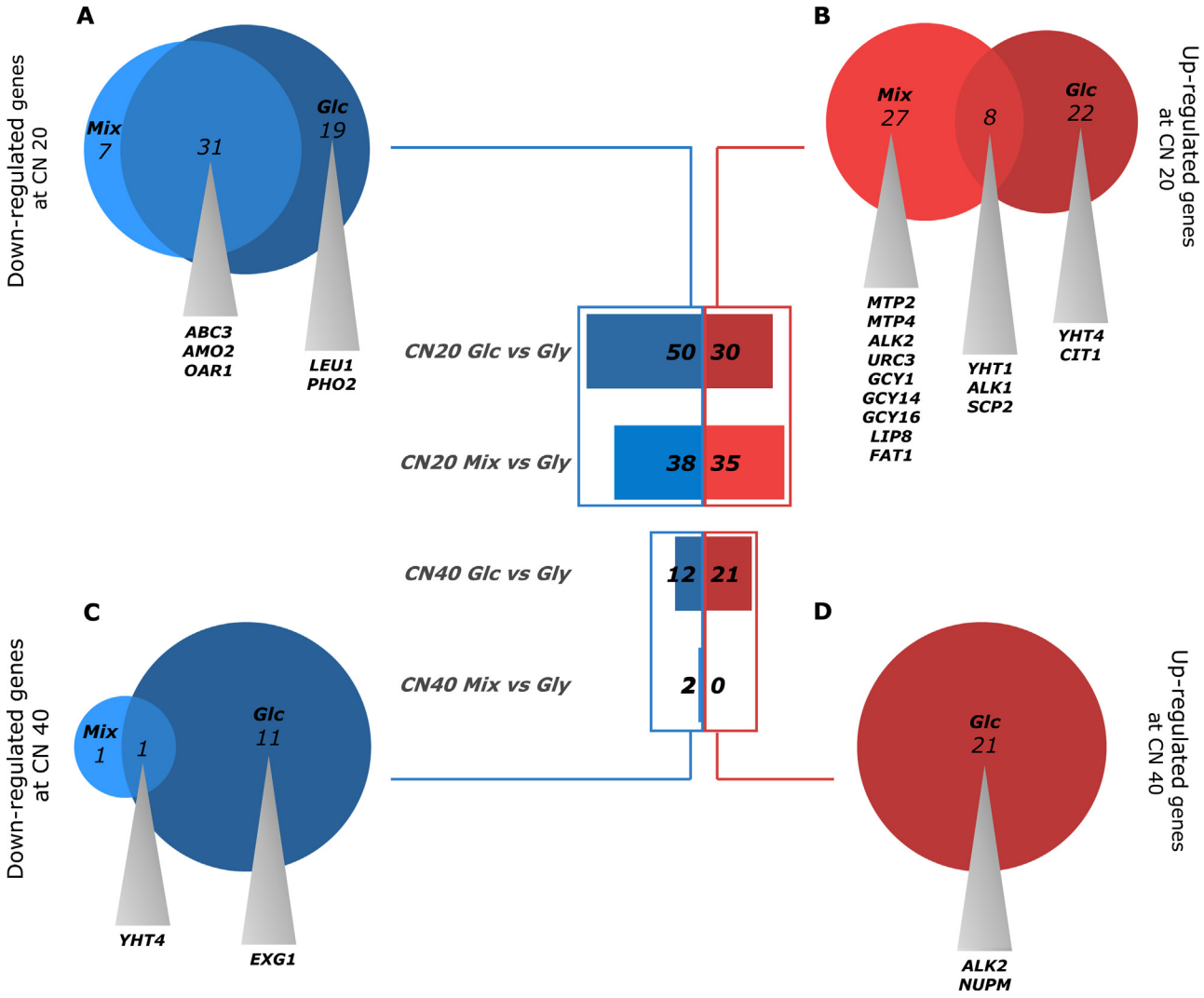

Fig. 4. Up- and downregulated genes at C/N 20 and C/N 40, and number of overlapping genes in experimental conditions. See text and File S1 (Supplemetary Materials) for more details.

3.4. Influence of hexokinase on gene expression levels

The drastic differences in *HXK1* expression could have regulatory effect on other genes, we therefore searched for genes with a correlating expression profiles. In other words, we reasoned that strong *HXK1* expression might influence expression of several genes by an unknown mechanism. The targeted transcriptome analysis described above revealed a set of 8 genes with high expression at C/N 20 and low at C/N 40 (Fig. 6A). To test our hypothesis that hexokinase plays such role we designed an experiment with batch cultures of (i) *Y. lipolytica* A101, (ii) a wild-type W29 as control strain; and (iii) a *HXK1* overexpressing PHY94 strain [Hapeta et al., in preparation]. All cultures were performed in flasks with glucose media at either C/N 20 or C/N 40. The data for W29 are included because the PHY94 strain is a W29 derivative. Samples for analysis were taken from early exponential growth phase (t = 10 h) and subjected to RNA extraction, followed by qRT-PCR analysis. We confirmed the expression level of *HXK1* obtained from RNASeq analysis (Fig. 6B) as well as the levels of *DAK2, GCY15, GRE3, TAL1, ARA12, IDH1, IDH2* and *ICL1* (Fig. 6C). The relative expressions of these genes in A101 strain were similar to those obtained from RNAseq, while the pattern was also observed for the W29 strain. From the obtained data transpires that overexpression of *HXK1* correlates with an increase of *GCY15*, *GRE3*, *IDH1* and *IDH2* expression at C/N 40. In contrary, comparing the PHY94 (overexpressing *HXK1*) to the wild-type strains, the levels of *DAK2*, *ARA12* and *IDH2* seems to be decreased in C/N20. Interestingly, *TAL1* expression was lower at C/N 20 and higher at C/N 40 when *HXK1* was overexpressed, while the *ICL1* remained almost unchanged. The results show a correlation of *HXK1* expression with the transcript levels of the analyzed genes, suggesting a role of hexokinase in the regulation of these genes.

3.5. Expression of hexokinase and two sugar porters might be controlled by zinc finger proteins in Y.lipolytica

Genes with similar expression patterns potentially often share common transcription factors binding to the promoter regions, thereby controlling their expressions. The targeted transcriptome analysis allowed identification of three genes with similar expression profiles, namely *HXK1* and two genes from the sugar porter family – *YALI0C04730g* and *YALI0D01111g* (Fig. 7). Analyzing a 1000 bp sequences upstream to the start codons of these genes as an input for YEASTRACT + database [30] two potential transcription factors have been identified, namely *YALI0C22682p* and *YALI0F17886p*. The primary structure of *YALI0C22682p* shows some similarities with a *S. cerevisiae* Gzf3 transcriptional repressor, while the sequence of *YALI0F17886p* is similar to *S. cerevisiae* Gat1

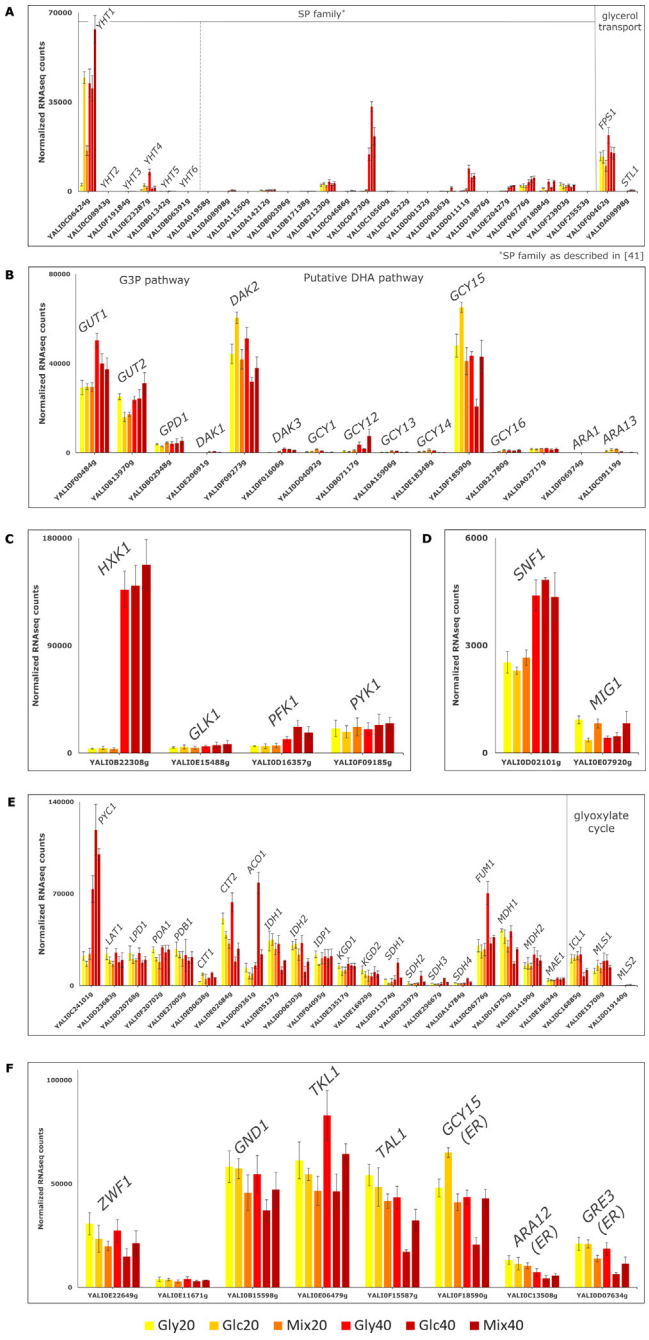

Fig. 5. Targeted transcriptome analysis. A – transporter genes; B – glycerol metabolism genes; C- kinases; D – *SNF1* and *MIG1;* E – tricarboxylic acid cycle genes; F – erythritol biosynthesis genes.

transcription factor for nitrogen regulation. In fact both Gzf3 and Gat1 are GATA zinc finger proteins acting as transcription factors for the nitrogen catabolite repression genes. These two transcription factors in *S. cerevisiae* are the elements of the GATA gene regulatory network and recognize the same core motif in the promoter regions of their gene targets. All three promoter sequences analyzed here contain such motifs (Fig. S2), what indicates that expression of *HXK1, YALI0C04730 g* and *YALI0D01111g* genes could be controlled by Gzf3 and/or Gat1 as indicated by YEASTRACT + database search.

4. Discussion

The present study focused on elucidating the molecular background of glucose and glycerol utilization patterns in the important industrial yeast *Y. lipolytica* and the impact of carbon to nitrogen ratio on its gene expression. One of the unique properties of this yeast is the preferential use of glycerol over glucose [22], a phenomenon described only for one other organism, the haloarchaeon *Haloferax volcanii* [38]. As in this latter microorganism a glycerol catabolite repression has been described, we hypothesized that a similar mechanism exists in *Y. lipolytica* as it has been reported that glycerol represses n-alkane utilization [39]. While some wild-type *Y. lipolytica* strains, show a co-utilization of glycerol and glucose, including the W29 and H222 strains and the A101 strain presented here, other wild-type strains use glucose only when glycerol is completely depleted [23]. In our study a sequential utilization of these two carbon sources was observed, however, glucose uptake began when glycerol concentration dropped below 5 g/L. This suggests a mechanism which senses glycerol (or carbon source in general) in the environment and allows for an efficient cell reprograming for the metabolism of other, less preferable carbon sources such as glucose. The analyzed C/N ratios did not influence the substrate utilization pattern but varying uptake rates of both substrates at tested C/N ratios were observed. Moreover, at C/N 20 a sequential utilization of substrates occurred while at C/N 40 the substrates were co-utilized. This is in concert with other studies at different culture modes, in which glycerol was also used prior to glucose [22,23]. It seems, that nitrogen availability does not influence the unique pattern of substrate utilization in *Y. lipolytica*, however, there are some variations in a mode of its utilization (co-utilization or sequential). We hypothesize, that this phenomenon might be connected to the fact, that in higher nitrogen concentrations (C/N 20) the cellular machinery of *Y. lipolytica* is set for biomass production, while when nitrogen becomes scarce (C/N 40) the cells begin to store available carbon in storage molecules such as triacylglycerols (TAG) and glycogen. This could also explain slightly higher specific glucose utilization rate at C/N 40, because expression levels of genes involved in TAG and glycogen biosynthesis are higher at C/N 40 than at C/N 20 (Fig. S3). Interestingly, this expression pattern was also observed for genes responsible for lipid mobilization and degradation (Fig. S3). Additionally, it has been previously reported that deletion of the glycogen synthase, a key enzyme in glycogen biosynthesis pathway, increases TAG formation in *Y. lipolytica* [40].

The RNAseq analysis allowed for quantification and identification of differentially expressed genes during growth on glycerol, glucose and a mixture of both substrates at two C/N ratios. The number of differentially expressed genes at C/N 20 was higher than at C/N 40, indicating that nitrogen availability has a major impact on gene expression in the chemostat cultures, regardless carbon source. However, a certain glycerol regulatory effect was observed, as its presence in the medium with glucose caused reduction in the number of downregulated genes at both C/N ratios (Fig. 3). In fact, the *ALK1* gene implicated in n-alkane utilization was identified as differentially expressed on all carbon sources at C/N 20. Moreover, this gene was upregulated/derepressed by glucose and at the same time downregulated by glycerol (Fig. 4), what confirms previous findings [39]. In almost all tested conditions, the hexose transporters *YHT1* or *YHT4* were differentially expressed what suggests that glucose transport is particularly susceptible to changes in carbon source quality and nitrogen availability. Further, the *YHT4* was upregulated on glucose at C/N 20 as could be expected by examining the function of its product – a high affinity glucose transport [41]. It is puzzling however, that the same gene was downregulated on glucose at C/N 40 (Fig. 4).

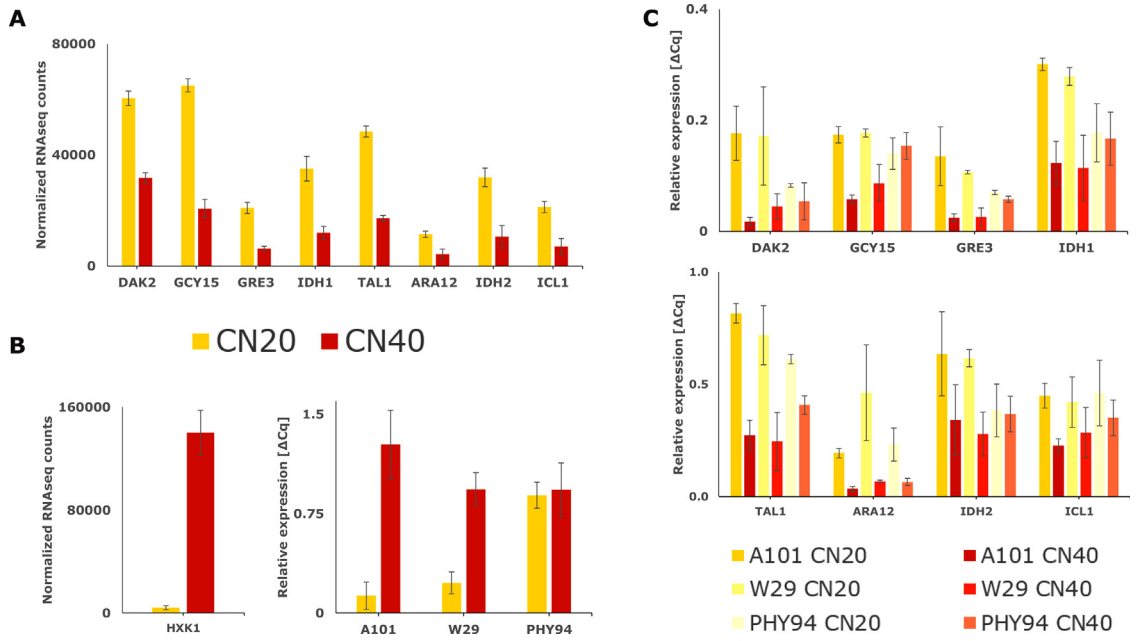

Fig. 6. A. Expression levels in chemostat conditions in A101 strain; B. Expression of hexokinase under chemostat conditions and during shake-flask cultures in A101, W29 and PHY94; C. Expression levels during shake-flask cultivations. For clarity only transcript levels during glucose cultures are shown.

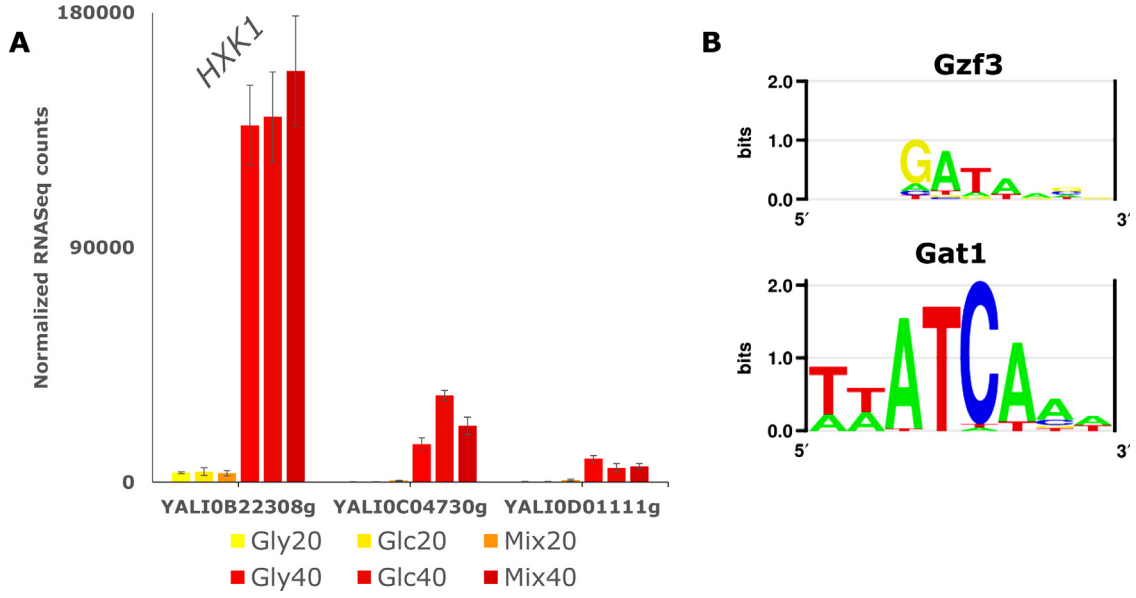

Fig. 7. A. Expression levels of *HXK1* and two sugar porter family genes in presence of various carbon sources and C/N ratios; B. Consensus motifs recognized by Gzf3 and Gat1 transcription factors.

Recently, a transcriptomic analysis of two *Y. lipolytica* strains growing in glucose, glycerol and a mixture of both substrates in carbon limiting conditions has been revealed [23]. In their experimental setup, the W29 strain showed 94 differentially expressed genes when cultures on glycerol and glucose were compared, while in the IBT strain 61 genes were identified as differentially expressed. The same comparison in our study showed 80 and 33 genes at C/N 20 and C/N 40, respectively. This indicates that the transcriptomic landscape of *Y. lipolytica* is strain-dependent and varies widely in different cultivation conditions. Our results show that less changes occur when less nitrogen is available and that its availability has higher impact on gene expression than different carbon sources and further advanced our understanding on the transcriptional changes in various cultivation conditions.

The sugar uptake is not completely resolved in *Y. lipolytica*, as proven by the number of publications on that subject. The most extensive study yet resulted in the identification of 24 proteins belonging to a sugar porter (SP) superfamily, from which 6 (Yht1-Yht6) were identified as hexose transporters. Among these, Yht1 and Yht4 are the most important for glucose uptake [41]. The expression levels presented here confirm that *YHT1* and *YHT4* play a major role in glucose transport, however, their expression was also detected in glycerol cultures, what might indicate that sugar transporters in *Y. lipolytica* are not as strictly controlled as in *S. cerevisiae* [42]. The strongest signal from *YHT1* at C/N 20 was

detected when *Y. lipolytica* was grown in the presence of glucose. A different situation occurred at C/N 40, where the highest expression level was achieved in a mixed cultivation. This contrasts with the previous study, in which *YHT1* was not much affected by the carbon source, however, the cultivations were carried out in carbon-limiting conditions [23]. On the other hand, the upregulation of *YHT4* on glycerol at C/N 40 was observed, a result similar to the IBT strain [23]. Notably, expression of 10 other genes encoding proteins belonging to SP family was detected (Fig. 5A). In this group, two, namely *YALI0C04730g* and *YALI0D01111g* showed an interesting expression pattern – they are expressed almost exclusively at low nitrogen concentration (C/N 40). The *YALI0C04730g* seems to be also upregulated/derepressed by glucose. During identification and characterization of hexose transporters in *Y. lipolytica* there were some evidences of this gene to be fructose transporter, however, the obtained results were inconclusive [41 and Lazar personal communication]. The results presented here show that regulation of substrate uptake depends not only on the presence of carbon source but also on the nitrogen availability and that additional sugar transporters might be functional in *Y. lipolytica* at higher C/N ratio.

In *Y. lipolytica* the glycerol transport into the cell likely occurs via aquaglyceroporin which is an ortholog of *S. cerevisiae* Fps1 or a glycerol/H^+-symporter encoded by a gene orthologous to *STL1*. We observed a very weak expression of *STL1,* but the expression levels of gene encoding Fps1 (Yl*FPS1*, *YALI0F00462g*) was the highest on glycerol (at C/N 40) confirming importance of this transporter for glycerol uptake. This result correlates with the recent study [23], where expression of *YlFPS1* was strongly induced by glycerol but also present, when the yeast grew on glucose and a blend of glucose and glycerol. Until now, no in-depth study on the *YlFps1* has been conducted, but evidence for its glycerol transporting activity was assessed in an experiment to improve glycerol consumption in *S. cerevisiae* [43]. Altogether, the expression data suggests that *YlFps1* could be a major glycerol facilitator in *Y. lipolytica*.

Upon the uptake, glucose and glycerol are subsequently phosphorylated by kinases, what allows for their incorporation into the central carbon metabolism. Glycerol in *Y. lipolytica* is assimilated via the glycerol-3-phosphate (G3P) pathway, of which *YlGut1* is a glycerol kinase [37,43]. The expression of *GUT1* was higher in presence of glycerol at C/N 40 but remained constant at C/N 20 regardless of the carbon source used and its expression was the highest compared to the other components of the G3P pathway, namely *GUT2* and *GPD1*. This observation correlates with previous reports [23,44]. Alongside the G3P pathway, a presence of a putative catabolic DHA pathway for glycerol uptake in *Y. lipolytica* is still under consideration. This two-step pathway consists of an unidentified glycerol dehydrogenase and a dihydroxyacetone kinase [37,43]. Expression of genes putatively related to this pathway was detected, where the *DAK2*, a dihydroxyacetone kinase ortholog, expression was the strongest. This contrasts with another transcriptomic study conducted in carbon-limiting conditions [23], where two DAK genes showed similar expression, while one was significantly lower expressed compared to the others.

Glucose in *Y. lipolytica* is phosphorylated by a glucokinase (YlGlk1) and a hexokinase (YlHxk1), which phosphorylates also fructose [45,46]. The *GLK1* gene, encoding glucokinase, was expressed in a constant manner across all tested conditions, while surprisingly, *HXK1* gene encoding hexokinase, showed very strong expression at C/N 40 (Fig. 5C). This could explain a higher specific glucose utilization rate (Table 3), suggesting higher flux of carbon through glycolysis. That could also be seen at the expression levels of *PFK1* at C/N 40. However, such drastic differences in *HXK1* expression and similar transcript levels in various carbon source conditions suggest that hexokinase in *Y. lipolytica* may contribute to other cellular processes. Alongside its catalytic functions, it is well known that in *S. cerevisiae* hexokinase 2 (Hxk2) plays a pivotal role in the carbon catabolite repression phenomenon [47], being a component of a Snf1/Mig1 pathway [48]. The unusual behaviour of YlHxk1 may stem from its peculiar structure, as 40 amino acid loop of unidentified function is present in its sequence [46]. This could indicate an as yet to be elucidated regulatory mechanism in which the *YlHxk1* protein is an important component.

As *Y. lipolytica* is well suited for overproduction of Krebs cycle intermediates such as citric acid [4] we were interested to see differences in expression of genes encoding this pathway. As it turned out some of the genes have been upregulated at C/N 40, with the *PYC1* being the most significant example, alongside *CIT2*, *ACO1* and *FUM1* (Fig. 5E). It is generally known, that when *Y. lipolytica* is cultivated at low nitrogen concentration a higher flux through TCA occurs leading to overproduction of some of TCA intermediates such as citric acid [3,4] what could explain these results. The expression levels of *CIT2* and *FUM1* were the highest in presence of glycerol regardless of C/N, confirming that glycerol is a good substrate to use for overproduction of various TCA acids. Moreover, we observed that during cultivation on glucose *ACO1* was more strongly expressed than *CIT2* while the opposite situation took place on glycerol (Fig. 5E). From these findings we hypothesize that when using glucose as sole carbon source *Y. lipolytica* is rather set for growth, while glycerol utilization causes overproduction of citric acid. This could also explain high citric to isocitric ratios (high purity of citric acid) in the A101 strain and its derivatives [6,49,50]. A relatively lower flux through the pentose phosphate pathway upon growth on glycerol in contrast to glucose could also contribute to the overproduction of citric acid, as it is feasible that insufficient NADPH precludes the formation of storage lipids.

Apart from citric acid, *Y. lipolytica* has been developed as a promising host for erythritol biosynthesis, especially from glycerol [6,51], therefore, we were interested to check the expression levels of the erythritol biosynthesis pathway encoding genes. The genes involved in erythritol biosynthesis were generally similarly expressed, however, four genes, namely *TAL1*,*GCY15, ARA12* and *GRE3* exhibited interesting expression patterns at C/N 40. *TAL1* (*YALI0F15587g*) encodes a transaldolase while *GCY15* (*YALI0F18590g*), *ARA12* (*YALI0C13508g*) and *GRE3* (*YALI0D07634g*) encode erythrose reductases [52,53]. Their transcript levels were almost the same when cultured on glycerol and a blend of glycerol and glucose but approximately two-fold lower when cultured on glucose alone, proving that glycerol is a well suited carbon source for production of erythritol. Additionally, the expression data for erythritol biosynthesis pathway suggests that production of this polyol in *Y. lipolytica* might be improved by cultivation in higher nitrogen concentrations, however, this does not correlate with current methods in which high C/N ratios (typically C/N 70) are used [51,54,55].

The same expression pattern as for *TAL1, GCY15, ARA1* and, *GRE3* was observed for other genes not directly related to erythritol biosynthesis, namely *DAK2*,*IDH1*, *IDH2* and *ICL1*. Additionally, on glucose at C/N 20 their expression was noticeably higher than at C/N 40. We were interested to elucidate this pattern with very high expression of hexokinase at C/N 40, so we used a hexokinase overexpressing strain to check for transcript levels of the above genes. Indeed, a clear impact of more highly expressed hexokinase could be observed for *GCY15*, *IDH1* and *IDH2*, as their expression levels at C/N 40 matched that of a wild-type at C/N 20. There is an evidence of *HXK1* involvement in glucose catabolite repression of the *LIP2* gene encoding extracellular lipase in *Y. lipolytica* [56]. Taken together, it is feasible that *HXK1* is an element of a gene regulatory network, or acts in concert with other parts in a transcription controlling machinery, in which expression of genes

depends on the hexokinase abundance. However, this hypothesis should be tested in more detail.

As *HXK1* was strongly upregulated at C/N 40 we were curious if any of the analyzed genes showed similarity to the *HXK1* expression pattern, as often genes with similar expression profiles share common transcription factors. Two genes encoding proteins belonging to the SP family, namely *YALI0C04730 g* and *YALI0D01111g*, were chosen due to strong expression at C/N 40 (Fig. 7). The analysis of promoter sequences revealed that motifs recognized by Gat1 and Gzf3 zinc-finger protein homologues are present. Both of the proteins are GATA-type transcription factors in *S. cerevisiae*, and while Gat1 acts as an activator for nitrogen utilization genes in poor nitrogen conditions [57], Gzf3 act as a repressor competing for binding sites with Gat1 [58,59]. This is puzzling since *HXK1, YALI0C04730 g* and *YALI0D01111 g* are not directly implicated in nitrogen metabolism but in the metabolism of carbon sources. However, with the data presented here one could assume, that these genes are upregulated by Gat1-like transcription factor in poor nitrogen conditions (e.g. C/N 40) and downregulated by Gzf3, when more nitrogen is available (e.g. C/N 20). It will be of great importance for the development of *Y. lipolytica* as even more robust cell factory to analyze these interactions closer.

5. Conclusions

The present study focused on elucidating the molecular background of the unique glucose and glycerol utilization pattern in the yeast *Y. lipolytica* and an influence of the interplay between carbon sources and carbon to nitrogen ratios on the global gene expression using RNAseq based transcriptomics. The results show that glycerol is used prior to glucose regardless nitrogen availability and that C/N ratio is a stronger factor influencing gene expression than carbon source. The major finding of this work is that hexokinase (*HXK1*) expression is highly dependent on nitrogen concentration as at high C/N its expression levels were drastically elevated compared to low C/N ratio. Furthermore, we show that *HXK1* expression in *Y. lipolytica* could be controlled via GATA zinc-finger transcription factors and that hexokinase itself could influence expression of certain genes through an as yet to be discovered mechanism.

Auhtor statement

PH designed the experiment, performed the experiments, wrote the manuscript, EJK designed the experiments, supervised transcriptome analysis, revised the manuscript, ZL designed the experiments, revised the manuscript.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00521.

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