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

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*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-attrative 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
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<sub>600</sub> 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-frozen in liquid nitrogen and kept at −80 °C prior to RNA extraction. Experiments were performed in triplicates.

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<sub>600</sub> 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 (vessel volume 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-frozen 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 Micro-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, YALJ0D08272g) gene.

2.7. RNA sequencing

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

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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 - 302b, xpr2-322, hsk1, pTfH-1XIK | [Hapeta et al., in preparation] |

### Table 2

YNB media variants used in this study.

| Component  | Concentration [g/L] | Shake-flasks | C/N 20 | C/N 40 | Bioreactor batch mode | C/N 20 | C/N 40 | Bioreactor chemostat mode | C/N 20 | C/N 40 |
|------------|---------------------|--------------|--------|--------|-----------------------|--------|--------|--------------------------|--------|--------|
| YNB        |                     |              | 1.9    | 2.2    |                       |        |        |                          |        |        |
| NH₄Cl      |                     |              | 4.47   | 2.2    |                       |        |        |                          |        |        |
| Glucose    |                     |              | 50     |        |                       |        |        |                          |        |        |
| Phosphate  |                     |              | 50 mM  |        |                       |        |        |                          |        |        |
| pH 6.8     |                     |              |        |        |                       |        |        |                          |        |        |
| YNB        |                     |              | 1.78   | 0.89   |                       |        |        |                          |        |        |
| NH₄Cl      |                     |              | 1.78   | 0.89   |                       |        |        |                          |        |        |
| KH₂PO₄     |                     |              | 1      |        |                       |        |        |                          |        |        |
| Glycerol   |                     |              | 10     |        |                       |        |        |                          |        |        |
| Glucose    |                     |              | 10     |        |                       |        |        |                          |        |        |
| Glycerol + |                     |              | 25.2 + | 24.6   |                       |        |        |                          |        |        |
| Glucose    |                     |              |        |        |                       |        |        |                          |        |        |

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This content appears to be a research paper discussing the use of yeast strains A101, W29, and PHY94 in various cultivation conditions, including shake-flask and bioreactor cultures. It also covers the methods used for RNA extraction and sequencing, with specific details on the equipment and procedures employed. The tables present the concentrations of various components used in the media and the conditions for RNA extraction and qPCR analysis. The paper highlights the use of Y. lipolytica A101 in initial studies and the expansion of these approaches to understand carbon-nitrogen interactions.
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 v0.11.9 [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, Oginami, 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:

\[
\frac{Y}{S} = \frac{[Y]}{[S]} = \frac{[X_f] - [X_i]}{[S_f] - [S_i]}
\]

Where:

- \(Y/X\) biomass yield coefficient
- \([X_i], [X_f]\) initial and final biomass concentration, respectively [g/L]
- \([S_i], [S_f]\) initial and final substrate concentration, respectively [g/L]

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

\[
Q = \frac{[S_f] - [S_i]}{t_b - t_a}
\]

\[
q = \frac{Q}{X_{avg}}
\]

Where:

- \(Q\) substrate utilization rate [g/L/h]
- \(q\) specific substrate utilization rate [g/g/h]
- \([S_a], [S_b]\) substrate concentration at the beginning and end of linear substrate utilization, respectively [g/L]
- \(t_a, t_b\) time of culture at the beginning and end of linear substrate utilization, respectively [h]
- \(X_{avg}\) average of biomass concentration at \(t_a\) and \(t_b\)

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 cell 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
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_XS, 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 ≤ 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.
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 GDP1. 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 Hxt1. While expression levels of GLK1 remained relatively constant across all conditions, the HXX1 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, encodingaconitate, 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).
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 – YALIO04730g and YALIO001111g (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 YALIO022882p and YALIO017886p. The primary structure of YALIO022882p shows some similarities with a S. cerevisiae CzF3 transcriptional repressor, while the sequence of YALIO017886p is similar to S. cerevisiae Gat1
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 HK1, YAL0C04730g and YAL0D01111g 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 halarchaeon *Halofex 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 reprogramming 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 glycan 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).
Recently, a transcriptomic analysis of two \textit{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 \textit{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 \textit{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 \textit{YHT1} and \textit{YHT4} play a major role in glucose transport, however, their expression was also detected in glycerol cultures, what might indicate that sugar transporters in \textit{Y. lipolytica} are not as strictly controlled as in \textit{S. cerevisiae} [42]. The strongest signal from \textit{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 STLI. We observed a very weak expression of STLI, but the expression levels of gene encoding Fps1 (YIFPS1, 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 YIFPS1 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 YIFPS1 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 YIFPS1 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 (YIGK1) and a hexokinase (YIHkx1), 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 YIHxk1 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 YIHxk1 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 glucose 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 culturing 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 HKX1 was strongly upregulated at C/N 40 we were curious if any of the analyzed genes showed similarity to the HKX1 expression pattern, as often genes with similar expression profiles share common transcription factors. Two genes encoding proteins belonging to the SP family, namely YALI0004730g and YALI0001111g, 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 genes 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 HKX1, YALI004730 g and YALI001111 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 (HKX1) 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 HKX1 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.

Author 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.biortech.2020.e00521.

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