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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Transcriptome analysis of the thermotolerant yeast *Kluyveromyces marxianus* CCT 7735 under ethanol stress

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Abstract The thermotolerant yeast *Kluyveromyces marxianus* displays a potential to be used for ethanol production from both whey and lignocellulosic biomass at elevated temperatures, which is highly alluring to reduce the cost of the bioprocess. Nevertheless, contrary to *Saccharomyces cerevisiae*, *K. marxianus* cannot tolerate high ethanol concentrations. We report the transcriptional profile alterations in *K. marxianus* under ethanol stress in order to gain insights about mechanisms involved with ethanol response. Time-dependent changes have been characterized under the exposure of 6% ethanol and compared with the unstressed cells prior to the ethanol addition. Our results reveal that the metabolic flow through the central metabolic pathways is impaired under the applied ethanol stress. Consistent with these results, we also observe that genes involved with ribosome biogenesis are downregulated and gene-encoding heat shock proteins are upregulated. Remarkably, the expression of some gene-encoding enzymes related to unsaturated fatty acid and ergosterol biosynthesis decreases upon ethanol exposure, and free fatty acid and ergosterol measurements demonstrate that their content in *K. marxianus* does not change under this stress. These results are in contrast to the increase previously reported with *S. cerevisiae* subjected to ethanol stress and suggest that the restructuration of *K. marxianus* membrane composition differs in the two yeasts which gives important clues to understand the low ethanol tolerance of *K. marxianus* compared to *S. cerevisiae*.

Keywords *Kluyveromyces marxianus* · Transcriptome · Ethanol stress · Membrane

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Introduction

Due to growing concern with both rapid climate change and petroleum consumption, there is a rising demand for biofuels such as bioethanol, which can significantly reduce the consumption of fossil fuels (Goldemberg 2007). Thus, it is crucial to improve the technologies of ethanol production, mainly those that do not compete with food. Among them, bioethanol production from cheap and abundant feedstocks such as whey and lignocellulosic biomass is very attractive. In this sense, the use of yeasts capable of assimilating sugars found in these feedstocks is pivotal (Radecka et al. 2015).

Kluyveromyces marxianus is a hemiascomycetous and homothallic yeast, phylogenetically close to *Kluyveromyces lactis* (Fonseca et al. 2008). Both high metabolic diversity and substantial degree of polymorphism displayed by *K. marxianus* strains are likely related to the different habitats from which they have been isolated, such as plant sources and ecological niches associated with warm-blooded animals, including dairy environments (Silveira et al. 2014). In contrast to *Saccharomyces cerevisiae*, *K. marxianus* is capable of assimilating a variety of sugars, such as lactose, found in whey,

and xylose and arabinose, found in lignocellulosic biomass. For this reason, it has been widely used for the production of biomolecules with economic and biotechnological interest: enzymes such as β-galactosidase, inulinase and pectinase, recombinant proteins, aroma compounds, and ethanol (Bragança et al. 2014; Diniz et al. 2014; Fonseca et al. 2008; Rocha et al. 2010, 2011; Silveira et al. 2005).

Another important feature of *K. marxianus*, also in contrast to *S. cerevisiae*, is thermotolerance, i.e., the capacity of growing at elevated temperatures (>40 °C) (Radecka et al. 2015), which is highly desirable for ethanol production from lignocellulosic biomass through simultaneous saccharification and fermentation process (SSF) (Costa et al. 2014). Moreover, fermentative processes conducted at high temperatures lead to reduction of cooling costs, mainly in tropical countries such as Brazil, as well as minimize the problems associated with contamination. Indeed, several studies have shown the potential of *K. marxianus* for ethanol production from both feedstocks aforementioned.

Over the last decade, our research group has shown the potential of *K. marxianus* CCT 7735, previously designated UFV-3, to produce ethanol from both whey (Diniz et al. 2012; Silveira et al. 2005) and lignocellulosic biomass (Ferreira et al. 2015; Souza et al. 2012). Nevertheless, the main drawback for the use of this strain at industrial level is the low tolerance to high ethanol concentrations, since its growth is strongly inhibited by ethanol concentration higher than 6% (Costa et al. 2014; Silveira et al. 2005).

In *S. cerevisiae*, ethanol affects different cell processes such as fluidity and permeability of membranes, activity of proteins and proton motive force (Voordeckers et al. 2015). In addition, some studies have shown that ethanol tolerance in *S. cerevisiae* is a complex response (Lam et al. 2014; Lahtvee et al. 2016) and the mechanisms involved with the adaptation under ethanol stress are not fully understood (Navarro-Tapia et al. 2016; Voordeckers et al. 2015).

To the best of our knowledge, there are no studies dedicated to elucidate the mechanisms related to ethanol response in *K. marxianus*. Recently, our research group sequenced the genome of *K. marxianus* CCT 7735 (Silveira et al. 2014), opening perspectives of studies regarding the changes in terms of genome-wide gene expression. In order to gain insights with relation to the mechanisms involved with the adaptive responses of this yeast under ethanol stress, we analyzed the alterations of gene expression patterns (transcriptome analysis).

Material and methods

Strain

The yeast strain used in this study, *K. marxianus* CCT 7735, was isolated from a dairy industry of Minas Gerais, Brazil (Silveira et al. 2005), and deposited in the Tropical Culture Collection Tonsello André Foundation, Campinas, São Paulo, Brazil.

RNA isolation

Cells used for RNA isolation were grown at 37 °C with aeration and stirring in the CML (complete minimal) medium. CM [per liter: 6.7 g of yeast nitrogen base without amino acids (Difco Inc., Sparks, MD, USA); 40 mg each of adenine, uracil, and lysine; 10 mg each of arginine, histidine, methionine, threonine, and tryptophan; 60 mg each of phenylalanine and isoleucine; and 50 mg of tyrosine] was prepared and supplemented with 2% lactose. After reaching OD600 nm = 0.8, cells were transferred to 50 mL fresh CML medium and the first aliquot of 10 mL from the culture was collected (0 h) and used as unstressed control. At this moment, ethanol was added to the culture to reach a final concentration of 6% (*v*/*v*). After 1 h and 4 h of stress, aliquots of 10 mL were sampled. Three biological replica were carried out. RNA was extracted from control and stressed samples, treated with DNAse I for 20 min, and purified with Genejet RNA purification kit (Thermo Fisher, Waltham, USA). The RNA concentration was measured with a Qubit-it RNA (Invitrogen, Carlsbad, USA), and the quality was controlled with a 2100 Bioanalyzer according to the user's manual (Agilent Technologies, Santa Clara, USA).

RNA sequencing and data processing

The whole transcriptome shotgun sequencing was performed (Sistemas Genómicos, Paterna, Spain) in nine RNA-seq libraries which were sequenced using the SOLiD 5500 XL sequencer (Thermo Fisher Scientific, Waltham, USA). These libraries correspond to the transcriptomes of *K. marxianus* during ethanol exposition (6%) at different time points. Libraries were identified as follows: 0 h (RNA1, RNA4, and RNA7), 1 h (RNA2, RNA5, and RNA8), and finally 4 h (RNA3, RNA6, and RNA9).

The SOLiD XSQ data files were converted to combined CSFASTA + QUAL files using SOLiD System XSQ Tools software (Thermo Fisher Scientific) and were analyzed using CLC Genomics Workbench version 8.5.3 (Qiagen, Aarhus, Denmark). The SOLiD reads were mapped in the genes of *K. marxianus* DMKU3-1042 (GenBank accession AP012213 to AP012221) as a reference genome (alignment parameters length fraction = 0.9; similarity fraction = 0.9; global alignment = yes; color space alignment = yes; color error cost = 3) and the mapping counting data were analyzed using DESeq (Anders and Huber 2010) and other R/Bioconductor packages (Gentleman et al. 2004).

Two analyses were performed. In the first one (RNAseq-analysis_1), all libraries were considered. However, the grouping analysis showed that library RNA3 is different from

the others and it was excluded from the further analysis. The remaining libraries were reanalyzed (RNAseq-analysis_2).

Read counts were used in the differential expression analysis, with the software DESeq (Anders and Huber 2010). Probability values (*p* values) were adjusted for multiple testing using the Benjamini–Hochberg procedure (Benjamini and Hochberg 1995) as implemented in DESeq. All conditions were compared to the reference samples.

Gene set analysis was run using Piano package in R (Väremo et al. 2013) and topGO package in R was used for the enrichment analysis (Alexa and Rahnenführer 2016). GO terms for *K. marxianus* were adapted from Uniprot database (Proteome ID UP000065495). COVAR analysis for the determination of the most significantly changed genes was used as described in Lahtvee et al. (2016).

Fatty acid profile determination and ergosterol quantification

In order to determine the *K. marxianus* fatty acid profile, yeast cells were grown in three batch cultures at 37 °C with aeration and stirring in the YPL (complex) medium [1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, and 2% (*w*/*v*) lactose]. YP was prepared and then supplemented with 2% lactose. After reaching OD600 nm = 0.8, cells were transferred to 50 mL fresh YPL medium and the first aliquot of 10 mL from the culture was collected (0 h) and used as unstressed control. At this moment, ethanol was added to the culture to reach a final concentration of 6% (*v*/*v*). After 1 h and 4 h of stress, aliquots of 10 mL were collected. Samples were harvested at 12,000×*g* at 4 °C for 10 min, and the pellets were lyophilized. The fatty acids in the yeast cells (4–5 mg of dry weight) were saponified, methylated, and extracted following the Sherlock Instant Fame™ User's Guide (Newark, USA). The resulting methyl ester mixtures were separated using an Agilent 7890A gas chromatograph with a flame ionization detector (Agilent Technologies) and identified using the MIDI microbial identification system (Sherlock 6.0 Microbial Identification System, Newark, USA).

To perform the ergosterol measurements, yeast cells were cultured and sampled as mentioned above. Ergosterol extraction was carried out according to Lahtvee et al. (2016) with modifications. Briefly, 1 mL of chloroform/methanol (2:1) solution was added to the lyophilized cells (15–20 mg) and submitted to cellular lysis using TissueLyser II (Qiagen, Hilden, Germany). The extracts were harvested at 12,000×g for 5 min, and the supernatant was removed into new tubes. The first four steps were repeated twice. Two milliliters of 100% (*v*/*v*) chloroform solution was added and homogenized. Two milliliters of 0.73% (*w*/*v*) sodium chloride solution was added, and the solution was harvested at 12,000×*g* for 20 min. Inferior phase was collected and dried in vacuum concentrator (SpeedVac, Thermo Scientific, Vantaa, Finland). Ergosterol standards (0.65 to 3 μg) and the dry extracts from yeast were derivatized with 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide and 50 μL of pyridine. The samples were heated at 70 °C for 30 min.

The GC–TOF/MS analysis was performed on gas chromatograph 7890A (Agilent, Waldbronn, Germany) coupled with spectrometer TruTOF (Leco, St. Joseph, USA) equipped with a 30 m × 0.32 mm × 0.25 μm column (DB-35 MS, Agilent Technologies). Oven temperature program was as follows: initial 200 °C held for 1 min, then ramped at 40 °C/min to 310 °C and held for 5 min. The temperature of the split-less injection was 290 °C and 1 μL of each sample was injected. Acquisition was performed at mass range *m*/*z* 225–450, acquisition rate 20 spectra/s. Spectra were collected at electron ionization (EI) 70 eV. The transfer line temperature and ion source temperature were set at 270 and to 290 °C, respectively. The acquisition of chromatographic data was performed by means of Chroma TOF software (Leco).

Analyses of Student's *t* test were carried out from the results obtained in fatty acid and ergosterol measurements, using with 5% significance level. All analyzes were performed in RStudio© software version 3.3.2 (Boston, MA, USA).

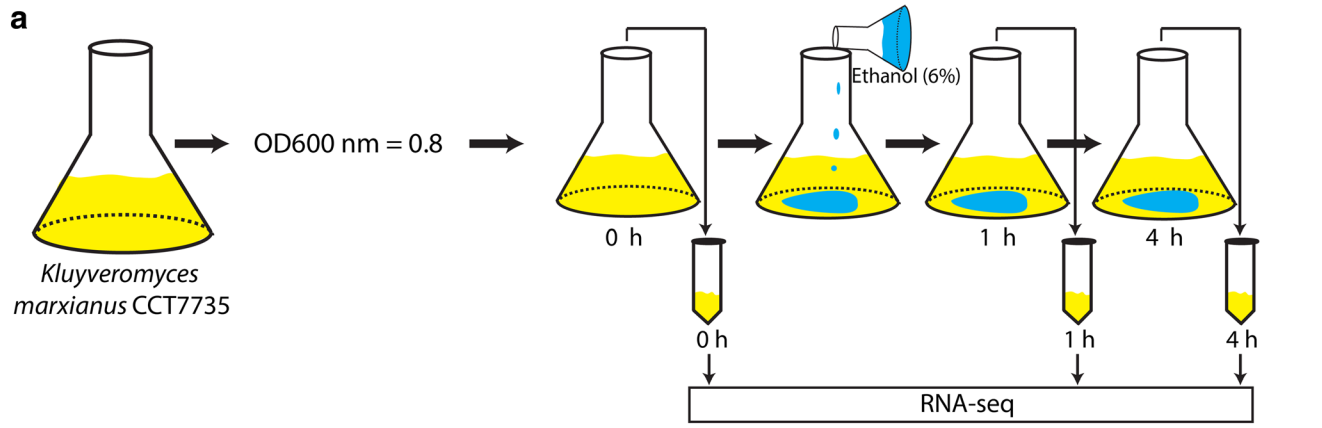
Availability of data and materials

The combined CSFASTA + QUAL files containing the sequenced reads have been deposited in NCBI's Sequence Read Archive (SRA) under accession number SRP101503 and associated to the BioProject accession PRJNA377894 and BioSample accession SAMN02928772.

Results

Overall gene expression in response to ethanol exposure

In order to gain insights regarding the mechanisms involved with the ethanol tolerance in *K. marxianus*, we analyzed changes in its transcriptome in response to a sudden ethanol stress (Fig. 1a). The RNA-seq was applied for triplicate time-course experiments as follows: upon reaching OD600 nm = 0.8, *K. marxianus* was stressed with 6% ethanol during the following 4 h. Samples were harvested for RNA sequencing at three time points: 0 h, no ethanol exposition; and under ethanol stress, after 1 and 4 h. Variations in gene expression were analyzed through three comparisons (Fig. 1b; Supplementary Table S1): the first one between 1 and 0 h, the second one between 4 and 0 h, and the third one between 1 and 4 h. A total of 402 genes were differentially expressed at the first comparison



b

	1 h vs 0 h	4 h vs 0 h	4 h vs 1 h
DE genes (p<0.001)	402	1085	127
Upregulated	145	506	47
Downregulated	257	579	80

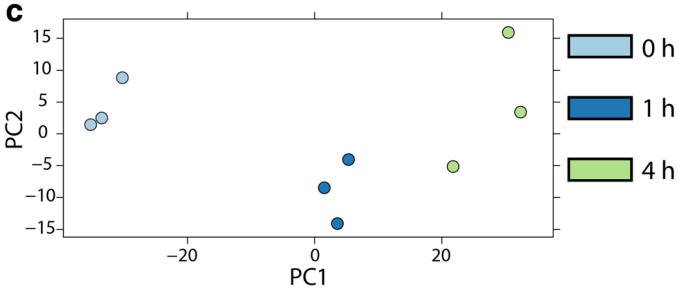


Fig. 1 Overall gene expression in response to ethanol stress. **a** Cultivation of *Kluyveromyces marxianus* CCT 7735 to isolate RNA for transcriptomics analysis. Samples were collected at 0 h (unstressed control), 1 h, and 4 h of ethanol stress. **b** Number of differently expressed genes through the three comparisons: 1 versus 0 h, 4 versus 0 h, and 1 versus 4 h (Benjamini–Hochberg-adjusted p value <0.001). **c** Transcriptome data used for the principal component analysis, showing those three comparisons clustered distinctively; time of sampling is color coded accordingly: 0 h is *light blue*, 1 h is *dark blue*, and 4 h is *green* (color figure online)

(1 versus 0 h), where 145 and 257 genes were up- and downregulated, respectively (Benjamini–Hochberg-adjusted p value <0.001; Fig. 1b). Regarding the second comparison, a higher number of genes (1085) were differentially expressed. Among them, 506 genes were upregulated and 579 genes were downregulated (Fig. 1b). Finally, the third comparison resulted in 127 differentially expressed genes, where 47 and 80 genes were up- and downregulated, respectively (Fig. 1b).

Principal component analyses showed that those three comparisons clustered distinctively, being separated on the first principal component which described 91% of the existing differences in the transcriptional profile (Fig. 1c). It should be noted that the third comparison is more spread out compared to both the first and the second comparison, which is mainly characterized on the second principal component, describing 5% of the total expression changes. These results indicate that the observed differences in terms of gene expression are mainly related to the treatment with ethanol.

Gene set analysis for the determination of significantly DE gene ontology terms

First, we were interested in elucidating the major occurring changes in transcriptional profile in response to ethanol stress in *K. marxianus*, and therefore, gene set analysis (GSA) was applied. GSA determines significantly differentially expressed gene clusters based on provided gene ontology (GO) groups. We used ontology provided by UniProt database and determined the groups which were significantly changing their expression either after 1 or 4 h of applied stress (adj. p value <0.05). Although, there was a larger number of differentially expressed genes at the fourth hour of the experiment compared to the first time point, we were able to detect more significantly differentially expressed GO groups at the first hour. The main changes in response to the ethanol stress occurred among translation processes, glycolytic processes, metal ion (iron) binding, oxidoreductase activity, and fatty acid biosynthetic processes/transmembrane transport (Fig. 2a, b).

The ethanol stress affects the translational machinery

Five out of 14 and 4 out of 10 significantly differentially regulated GO groups (adj. p value <0.05) after 1 and 4 h of stress adaptation, respectively, were related to translation (ribosome, small ribosome subunit, structural constituent of ribosome, translation, large ribosomal subunit). In general, we observe that after 4 h under ethanol stress, the genes that encode 37S rRNA, 40S and 60S ribosomal proteins, and translation initiation factors 3a, 3e (eIF3a,

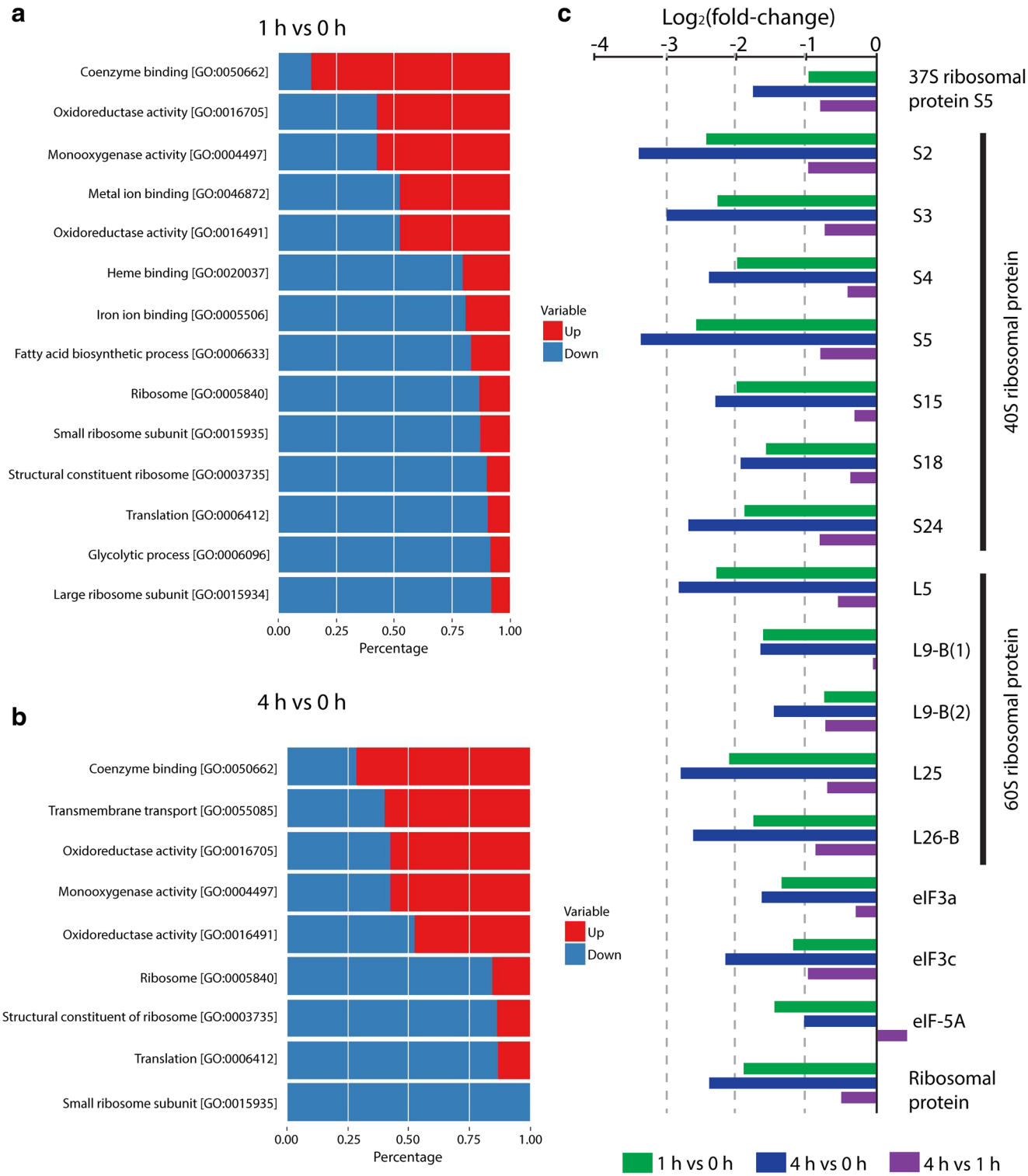


Fig. 2 Gene ontology (GO) groups whose genes were significantly differently expressed in *Kluyveromyces marxianus* in response to **a** 1 h and **b** 4 h of ethanol stress when compared to 0 h (unstressed control), determined by gene set analysis (GSA). **c** The expression of genes [Log_2(FC)] encoding proteins related to translation process in the yeast between the three comparisons: *green bars* represent the comparison between 0 and 1 h; *blue bars* represent the comparison between 0 and 4 h; and *purple bars* represents the comparison between 1 and 4 h (color figure online)

eIF3e), and 5A (eIF5A) were negatively regulated in response to ethanol (Fig. 2c). In addition, genes encoding the L5 and L25 proteins of the 60S ribosomal subunit were highly downregulated.

The ethanol stress decreases the metabolic flow through metabolic central pathways

Gene ontology group glycolytic processes (GO: 0006096) were significantly downregulated as most of the gene-encoding enzymes of the glycolytic pathway were downregulated during the studied time-range after the ethanol exposure (Fig. 2a, b). In accordance to the reduced specific growth rate under ethanol stress condition, the glycolytic flux is strongly reduced, which in turn leads to a decrease in the energy generation. Curiously, we detected the upregulation of the gene that encodes a probable 6-phosphofructo-2-kinase. Additionally, we also observed that some genes that encode enzymes of tricarboxylic acid (TCA) cycle such as aconitate hydratase, succinyl-CoA ligase subunit β, and fumarate reductase had their expression repressed while pyruvate carboxylase was found to be upregulated. In order to illustrate the changes in glycolysis and TCA cycle, we explored at individual gene scale the differential expression in both pathways (Fig. 3).

Besides glycolysis, the Leloir pathway, responsible for lactose/galactose assimilation, was negatively affected (Fig. 4a), since some of the genes that encode enzymes of this pathway such as galactokinase and UDP-galactose-4-epimerase were downregulated in response to ethanol (Fig. 4a; Supplementary Table S2). According with the downregulation of genes aforementioned after ethanol exposure, these results are consistent with the downregulation of genes involved in translational process and also with the low specific growth rate of *K. marxianus* CCT 7735 under ethanol stress (Silveira et al. 2005).

Additionally, we observed a high expression of the gene *ZWF1*, which encodes glucose-6-phosphate-1-dehydrogenase, suggesting that the metabolic flux from glucose-6-phosphate is directed toward pentose phosphate pathway in order to increase NADPH regeneration (Fig. 3). This can be associated to the oxidative stress response, since ethanol exposure also elicits this stress response (Gasch et al. 2000). Interestingly, the *ADH6* gene that encodes a NADP-dependent alcohol dehydrogenase had its expression upregulated, contributing also to the regeneration of NADPH (Fig. 4b). Contrary to the upregulation of *ADH6*, the expression of other genes that encode alcohol dehydrogenase was decreased, highlighting decreased metabolic flow to ethanol (Fig. 4b).

The ethanol stress affects fatty acid biosynthesis

The fatty acid biosynthetic processes gene ontology group (GO: 0006633) was significantly downregulated under the ethanol stress conditions. This GO group consists of 12 genes out of which 8 of them showed significant downregulation at least at one of the studied conditions compared to

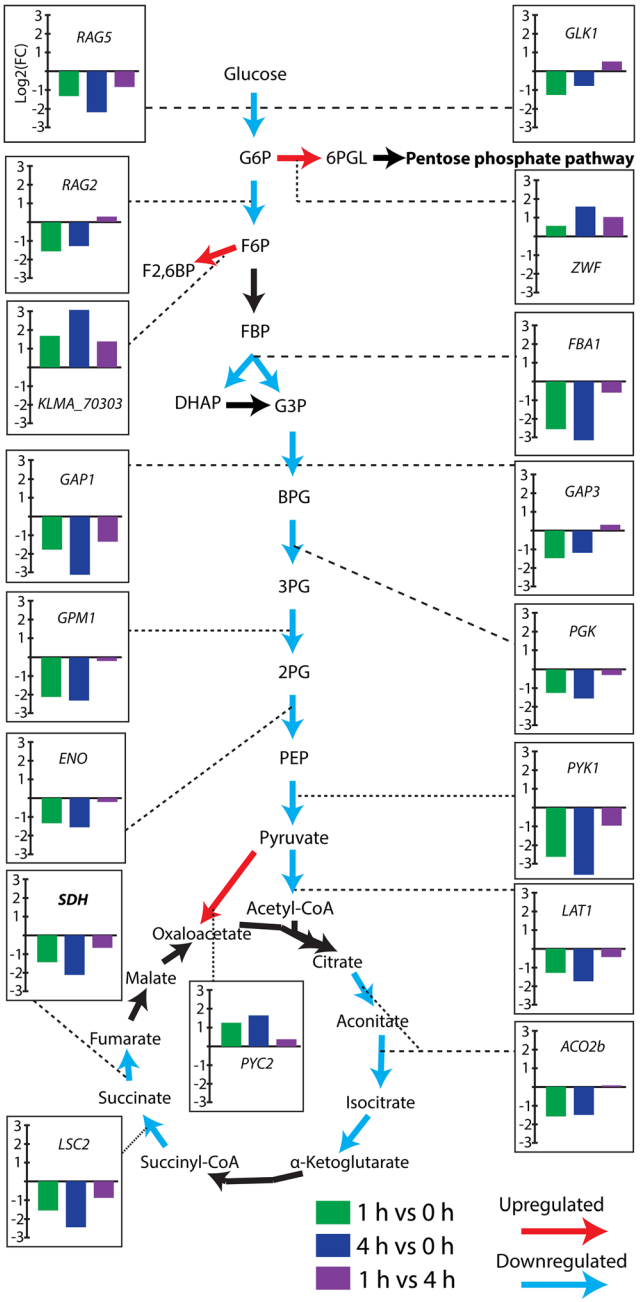


Fig. 3 Changes in the expression of gene-encoding enzymes of the metabolic central pathways. *Arrows* represent enzymes: *blue arrows* indicate enzymes whose gene expression was downregulated; *red arrows* indicate enzymes whose gene expression was upregulated. The expression of the genes [Log_2(FC)] encoding each enzyme is showed in the graphics close to them: *green columns* represent the comparison between 0 and 1 h; *blue columns* represent the comparison between 0 and 4 h; and *purple columns* represent the comparison between 1 and 4 h. *RAG5*, hexokinase; *GLK1*, hexokinase; *RAG2*, glucose-6-phosphate isomerase; *ZWF*, glucose-6-phosphate 1-dehydrogenase; *KLMA_70303*, probable 6-phosphofructo-2-kinase; *FBA1*, fructose-bisphosphate aldolase; *GAP3*, glyceraldehyde-3-phosphate dehydrogenase; *GAP1*, glyceraldehyde-3-phosphate dehydrogenase; *PGK*, phosphoglycerate kinase; *GPM1*, phosphoglycerate mutase; *ENO*, enolase; *PYK1*, pyruvate kinase; *LAT1*, acetyltransferase component of pyruvate dehydrogenase complex; *PYC2*, pyruvate carboxylase; *ACO2b*, aconitate hydratase; *LSC2*, succinyl-CoA ligase subunit β

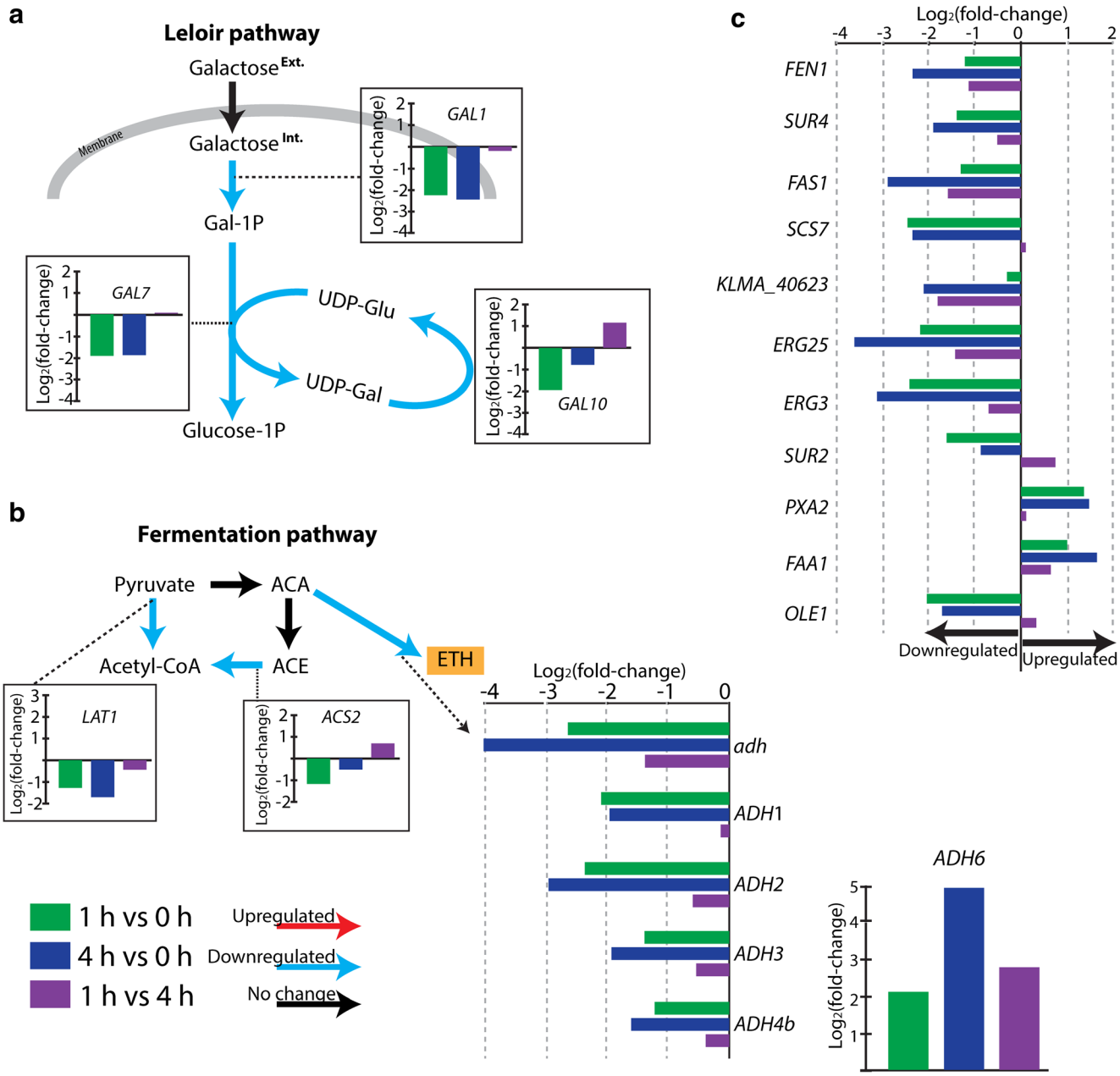


Fig. 4 Changes in the expression of gene-encoding enzymes of **a** the Leloir pathway, **b** the fermentative pathway, and **c** the elongation of fatty acids or ergosterol biosynthesis. *Arrows* represent enzymes: *blue arrows* indicate enzymes whose gene expression was downregulated; *red arrows* indicate enzymes whose gene expression was upregulated; and *black arrows* indicate enzymes whose gene expression did not change. The expression of the genes [Log_2(FC)] encoding each enzyme is showed in the graphics close to them: *green columns/bars* represent the comparison between 0 and 1 h; *blue columns/bars* represent the comparison between 0 and 4 h; and *purple columns/bars* represents the comparison between 1 and 4 h. *GAL1*, galactokinase; *GAL7*, galactose-1-phosphate uridylyltransferase; *GAL10*, bifunctional protein GAL10; *LAT1*, acetyltransferase component of pyruvate dehydrogenase complex; *ACS2*, acetyl-CoA synthetase; *adh*, alcohol dehydrogenase; *ADH1*, alcohol dehydrogenase 1; *ADH2*, alcohol dehydrogenase 2; *ADH3*, alcohol dehydrogenase 3; *ADH4b*, alcohol dehydrogenase 4; *ADH6*, NADP-dependent alcohol dehydrogenase 6; *FEN1*, elongation of fatty acids protein; *SUR4*, elongation of fatty acids protein; *FAS1*, fatty acid synthase subunit β; *SCS7*, ceramide very long chain fatty acid hydroxylase; KLMA_40623, δ (12) fatty acid desaturase; *ERG25*, C-4 methylsterol oxidase; *ERG3*, C-5 sterol desaturase; *SUR2*, sphingolipid C4-hydroxylase SUR2; *PXA2*, peroxisomal long-chain fatty acid import protein 1; *FAA1*, long-chain-fatty-acid—CoA ligase 1; *OLE1*, acyl-CoA desaturase (color figure online)

the reference one. Majority of these genes were related to elongation of fatty acids or ergosterol biosynthesis (Fig. 4c). Other genes related to ergosterol or fatty acid biosynthesis, but not included to the GO group fatty acid biosynthetic processes (e.g., KLMA_20527, ergosterol biosynthetic protein 28; KLMA_10244, acyl-CoA desaturase; KLMA_20392, δ (12) fatty acid desaturase), were also downregulated.

Time-dependent impact of ethanol stress and the most significantly DE genes

For better understanding the time-dependent behavior of the transcriptional data and relate it to the observed differentially expressed GO terms, we clustered the data into five distinctive groups (Supplementary Fig. S1; Supplementary Table S3). Clusters were mainly determined by the level of differential expression and no major groups of genes were determined with a recovery profile during the studied 4 h. We ran functional group enrichment analysis for each cluster and did not find any significant enrichment among the clusters showing upregulation with time. Downregulated genes were divided between three clusters mainly based on the level of differential expression from the reference sample. Enrichments were found among purine nucleotide biosynthesis pathway for the cluster that showed the strongest average downregulation at the fourth hour time point. Two clusters which showed the lowest average expression levels at the first hour time point were significantly enriched in the genes being related to ribosomes and translation (Supplementary Table S1; Supplementary Table S3).

Next, we were interested in the most significantly differentially expressed individual genes under the environmental conditions studied. By combining information from the intensity and fold changes of the individual genes, we determined the most significant DE genes using COVAR analysis. Again, among the top 100, the most significantly downregulated genes were mainly encoding proteins involved in translation and ribosomes. There were no significant enrichments detected among the top 100 upregulated genes; however, five out of the top 10 genes encoded heat shock proteins and a number of genes were related to amino acid metabolism, specifically branched chain and aromatic amino acids; the gene for the transcriptional activator GCN4, which activates the expression of amino acid biosynthetic genes was also included. Gene-encoding heat shock proteins (HSP), such as HSP60, HSP78, and HSP26, were upregulated (Supplementary Table S2), suggesting that ethanol induces the expression of proteins that act in preventing protein aggregation and mediating protein refolding.

K. marxianus transcription profile in response to ethanol compared with *S. cerevisiae*

To elucidate the differences in ethanol response between the well-studied microorganisms *S. cerevisiae* and *K. marxianus*, we reanalyzed the data provided in Li et al. (2010), where stress tolerance of haploid and diploid *S. cerevisiae* was studied. We used GSA to determine the functional groups most affected under the applied ethanol stress conditions between 1 and 0 h (Supplementary Table S4) and between 4 and 0 h (Supplementary Table S5). Very similar to *K. marxianus*, translation was the major group of transcripts downregulated under ethanol stress. Additionally, methyltransferase activity, messenger RNA (mRNA) binding, and nucleotidyltransferase activity were downregulated. Among the upregulated GO functions, we detected lyase activity, cellular amino acid metabolic processes, and oxidoreductase activity. In addition to similar changes among GO groups translation and oxidoreductase activity, studies describing ethanol stress of *S. cerevisiae* have observed the upregulation of the heat shock proteins, proline transporters, and ergosterol biosynthesis enzymes, which together result in the improvement of the stability of proteins and membranes (Li et al. 2010; Navarro-Tapia et al. 2016; Stanley et al. 2010). In contrast to *S. cerevisiae*, *K. marxianus* showed significant downregulation among ergosterol and fatty acid biosynthesis genes. This being one of the major differences between transcriptional regulation in the two organisms in response to ethanol. For the latter reason we decided to detect possible differences in fatty acid and ergosterol composition of *K. marxianus* treated and untreated cells.

Ethanol effect on fatty acid profile and ergosterol content in *K. marxianus*

In *K. marxianus*, we observed that the gene-encoding enzymes related to fatty acid elongation and δ (12) fatty acid desaturase were downregulated (Fig. 4c; Supplementary Table S1), suggesting that the unsaturated fatty acid degree decreases during this condition. To test this experimentally, fatty acid profile was determined in *K. marxianus* cells grown under the same conditions as those used for the transcriptome analyses. Compared with the reference, the content of the most abundant unsaturated fatty acids did not change at 1 and 4 h of stress, although the content of the palmitic acid (16:0) decreased (Fig. 5). This result suggests that the yeast *K. marxianus* CCT 7735 does not alter its unsaturated fatty acid degree under ethanol stress within the first 4 h after ethanol exposure.

Regarding another membrane component—ergosterol—we observed that the gene-encoding enzymes related to its biosynthesis, such as the lanosterol 14-α-demethylase, C-4 methylsteroloxidase, and sterol C-5 desaturase, were downregulated indicating that its synthesis could be decreased in this condition (Fig. 4c). In order to evaluate this result, ergosterol measurements were carried out and we observed that ergosterol level did not change over time (Fig. 6). Taken together, both results suggest that despite of the differences in gene expressions, ethanol exposure does not change fatty acid and ergosterol content in *K. marxianus* during the tested period, indicating a compensation at the post-transcriptional level.

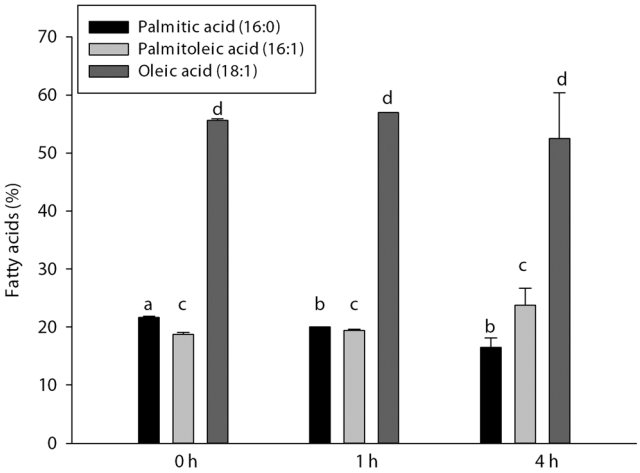


Fig. 5 Ergosterol content per cell mass of *Kluyveromyces marxianus* at 0 h (unstressed control), 1 h, and 4 h of ethanol stress. According to the Student's *t* test (with 5% significance level), *bars followed by the same letter* do not present statistical difference

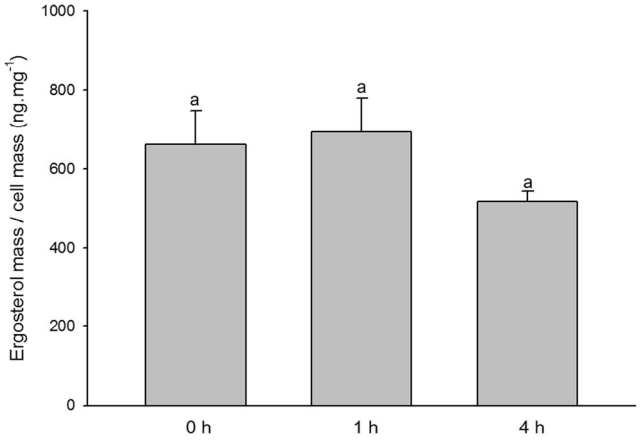


Fig. 6 Fatty acid profile of *Kluyveromyces marxianus* at 0 h (unstressed control), 1 h, and 4 h of ethanol stress. Three of the most common fatty acids in this yeast are presented: palmitic acid (16:0)—*black*; palmitoleic acid (16:1)—*light gray*; and oleic acid (18:1)—*dark gray*. According to the Student's *t* test (with 5% significance level), *bars followed by the same letter* do not present statistical difference

Discussion

Ethanol stress dramatically impairs the growth of *K. marxianus* CCT 7735 (Costa et al. 2014; Silveira et al. 2005). Therefore, if the changes in gene expression are not compensated by post-transcriptional mechanisms, we expected that protein synthesis could be negatively affected under the ethanol stress. Indeed, gene-encoding ribosomal proteins and translation initiation factors were among the top 100 downregulated genes. For example, genes associated with 40S ribosomal proteins—which participate actively in the recognition of mRNAs, allowing the active conformation of initiation complexes—were strongly downregulated. In addition, genes encoding the L5 and L25 proteins of the 60S ribosomal large subunit were strongly downregulated (Fig. 2c). Moreover, it is important to point out that eIF3, whose expression was downregulated, is the initiation factor with the largest influence on translation rate, meaning that its repression is very important to redirect the energy fluxes to other metabolic requirements under ethanol stress. *S. cerevisiae* strains also respond to ethanol stress with downregulation of genes related to protein synthesis, i.e., ribosomal proteins and translation factors (Dinh et al. 2009; Kasavi et al. 2016). It is worth mentioning that most of the *K. marxianus* genes aforementioned also were downregulated in *S. cerevisiae* when it was subjected to similar ethanol stress periods (Chandler et al. 2004). Therefore, the downregulation of genes related to protein synthesis can be associated to significant changes in specific growth rate, which are an unavoidable general consequence of a stress response; however, it does not describe stress-specific salvage mechanisms. Besides, the *K. marxianus* gene *GCN4* was upregulated. This gene encodes a transcriptional activator of amino acid biosynthetic genes, which responds to amino acid starvation. This fact, in turn, is consistent to the events related to the ethanol response in *S. cerevisiae*, since the membrane permeability alteration affects the amino acid input (Piper 1995).

In addition, we observed that the ethanol stress led to downregulation of gene-encoding enzymes of the central metabolic pathways such as glycolytic pathway, Leloir pathway, and TCA cycle (Figs. 3 and 4a), which is consistent with lower energetic requirements associated to the inhibition of *K. marxianus* growth caused by ethanol (Silveira et al. 2005). It has already been reported in *S. cerevisiae* that the increase of membrane permeability caused by ethanol leads to unfavorable effects, such as inhibition of sugar, ammonium and amino acid uptake, leakage of amino acids, potassium, and nucleotides as well as the accentuated proton influx, causing the intracellular acidification (Piper 1995). Taken together, those effects can result in glycolysis inhibition and, consequently, reduction of energy generation. Indeed, we observed that the ethanol stress impaired the lactose consumption in *K. marxianus* CCT 7735 (data not shown), which is coherent with the expected reduction of flux through the central metabolic pathways as long as post-transcriptional mechanisms do not compensate the changes in gene expression. However, it should be noted that the gene encoding for a 6-phosphofructo-2-kinase was upregulated upon ethanol exposure in *K. marxianus*. Interestingly, it was also induced in *S. cerevisiae* after ethanol shock (Alexandre et al. 2001). Indeed, the gene encoding the 6-phosphofructo-2-kinase enzyme in *S. cerevisiae* was identified as one of the genes that conferred resistance to inhibitory ethanol concentrations (Teixeira et al. 2009). This enzyme catalyzes the synthesis of fructose-2,6-biphosphate, which is a positive allosteric effector of the phosphofructokinase (glycolytic enzyme) and a negative allosteric effector of the fructose-1,6-bisphosphatase (gluconeogenic enzyme). It has been pointed out that in *S. cerevisiae*, fructose 2,6-bisphosphate regulates

both phosphofructokinase and fructose 1,6-bisphosphatase in order to avoid futile cycles (Hofmann et al. 1985). Thus, yeast cells do not spend energy in those processes being able to use the ATP for the maintenance of cellular homeostasis during the ethanol stress. Therefore, we interpret that this saving mechanism is conserved in both yeasts.

Several studies have pointed out that production of reactive oxygen species (ROS) increases dramatically under ethanol stress in *S. cerevisiae*, resulting in the oxidative stress (Du and Takagi 2007). ROS, if not counteracted, are injurious to biomolecules such as DNA and proteins and they can lead to lipid peroxidation in yeast cells. Therefore, yeasts present adaptive responses, which help to maintain a reduced-state environment, defending cells against the oxidative conditions caused by ethanol exposure (Morano et al. 2012). In this study, we observed that the alteration of the expression of some genes might be related to the oxidative stress response if post-transcriptional mechanisms do not offset it. Two possible ways to respond to this stress condition, both related to NADPH regeneration, were observed. The first one consists in the upregulation of the gene *ZWF1*, which encodes glucose-6-phosphate-1-dehydrogenase, an enzyme responsible for catalyzing the oxidation of glucose-6-phosphate in the first reaction of the pentose phosphate pathway, where NADPH is regenerated. The high expression of pentose phosphate pathway genes is apparently reminiscent of its natural environment where *Kluyveromyces* genus can be isolated, that is, environments with plant-derived substrates (Fonseca et al. 2008). Curiously, most of the glucose consumed by *K. lactis* is metabolized in the pentose phosphate pathway (González-Siso et al. 1996; Rodicio and Heinisch 2013); in fact, some *K. marxianus* strains present a high flux metabolic through the pentose phosphate pathway similar to *K. lactis* (Bellaver et al. 2004). The second way is the *ADH6* upregulation, since this gene encodes the NADP-dependent alcohol dehydrogenase that also promotes NADPH regeneration. The NADPH produced can be used as reducing agent to reduce oxidized glutathione and thioredoxin, which are required to counteract oxidative stress (González-Siso et al. 2009; Navarro-Tapia et al. 2016). In addition, the upregulation of the *HSP60* gene might also be involved in the oxidative stress response. Indeed, in *S. cerevisiae*, the HSP60 protein, which is a mitochondrial protein, confers protection against oxidative stress (Cabiscol et al. 2002).

Other *K. marxianus* responses to ethanol stress are related to expression of gene-encoding heat shock proteins. Since it is not counterbalanced by post-transcriptional mechanisms, upregulation of gene-encoding HSP26 and HSP78 proteins highlights their role as a salvage mechanism of yeasts under this stress condition. Ethanol destabilizes hydrophobic interactions within native proteins, exposing their hydrophobic regions. This damage, in turn, induces the association to heat shock proteins and, consequently, the activation of HSP genes (Piper 1995). Thus, the chaperone role of heat shock proteins ensures the refolding of inactive and denatured proteins in order to keep the functional conformation of them. Similarly, HSP26 and HSP78 genes were also overexpressed in *S. cerevisiae* under ethanol stress (Alexandre et al. 2001; Chandler et al. 2004; Stanley et al. 2010). It is important to point out that the HSP78 is a mitochondrial protein, indicating that the functions of this organelle are pivotal after ethanol exposure. Indeed, when the ethanol stress was studied under a constant specific growth rate in *S. cerevisiae*, main changes were determined in mitochondria and transmembrane transport (Lahtvee et al. 2016). These changes are usually masked when studied in batch conditions due to the rapid decrease in growth soon after the stress is applied. Moreover, it has been shown that in *S. cerevisiae*, the mitochondrial genome stability is important for ethanol tolerance (Chandler et al. 2004).

Remarkably, cellular membranes are the most affected cell structures by ethanol, because it mainly causes the increase of their permeability—increasing the passive proton influx and dissipating the electrochemical potential gradient maintained across them—as well as the membrane dehydration and rehydration (Piper 1995; Voordeckers et al. 2015). Thus, endocytosis and lipid phase transition can occur, making the membranes more unstable (Zheng et al. 2013). In order to maintain the membrane stability, *S. cerevisiae* increases the degree of unsaturated fatty acids of plasma membrane under ethanol stress (Doğan et al. 2014; Stanley et al. 2010), becoming almost exclusively mono-unsaturated fatty acids, such as oleic acid (Δ9Z-C18:1) and palmitoleic acid (Δ9Z-C16:1) (Lahtvee et al. 2016; Uemura 2012; Zheng et al. 2013). However, the transcriptome data suggest that, in *K. marxianus*, the unsaturated fatty acid biosynthesis may be impaired, since some genes related to their synthesis were downregulated (Fig. 4c; Supplementary Table S1). In order to confirm that, we evaluated the fatty acid profile and found that unsaturated fatty acid degree does not increase in *K. marxianus* (Fig. 5). This represents a significant difference between the two yeasts that could explain the lower adaptation to ethanol stress of *K. marxianus*. It is noteworthy that in *K. lactis*, a yeast phylogenetically close to *K. marxianus* and that also presents low ethanol tolerance, the degree of fatty acid unsaturation is reduced in its presence (Heipieper et al. 2000).

Besides, regarding the changes in the membrane composition of *K. marxianus*, we verified that some gene-encoding enzymes of the ergosterol biosynthesis pathway were downregulated under ethanol stress (Fig. 4c). Moreover, we observed that ergosterol content did not change upon ethanol exposure (Fig. 6). Also, this result is in contrast to the observed in *S. cerevisiae*, i.e., increase of the ergosterol content upon ethanol exposure. Indeed, in *S. cerevisiae*, the genes *ERG1* (squalene epoxidase), *ERG3* (C-5 sterol desaturase), *ERG4* (C-24 (28) sterol reductase), *ERG5* (C-22 sterol

desaturase), *ERG6* (δ (24)-sterol C-methyltransferase), *ERG8* (phosphomevalonate kinase), *ERG11* (lanosterol 14-α-demethylase), *ERG20* (farnesyl pyrophosphate synthetase), and *ERG25* (C-4 methyl sterol oxidase) were overexpressed under ethanol stress (Navarro-Tapia et al. 2016; Zheng et al. 2013). Therefore, the difference between *K. marxianus* and *S. cerevisiae* in terms of ethanol tolerance seems also to be related to the lower ergosterol content displayed by *K. marxianus* in response to the stress. Indeed, the higher ergosterol content in *S. cerevisiae* under ethanol stress appears to suppress the transition phase of phospholipid bilayers and maintaining the membrane thickness (Vanegas et al. 2012). Taking into account the results aforementioned, we hypothesized that absence of alteration in the membrane composition of *K. marxianus* subjected to ethanol stress is associated with its lower ethanol tolerance compared to *S. cerevisiae*.

It is noteworthy that the decrease of the expression of gene-encoding enzymes of both unsaturated fatty acid and ergosterol biosynthesis pathway did not lead to reduction of the content these metabolites, suggesting that a compensatory and transcriptionally independent regulation also works in *K. marxianus*, but it never reaches upregulation as observed in *S. cerevisiae*.

Thus, the results obtained in this study provide insights about the mechanisms of *K. marxianus* that are involved with ethanol response, enabling future metabolic engineering approaches to improve its ethanol tolerance.

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Authors' contributions RHSD, MLM, and NMV executed the bench procedures. PJL, PMPV, and JCV performed the bioinformatics procedures. JCV, PJL, and WBDS analyzed the data. MIGS and MEC supervised the work at the Spanish laboratory and contributed to data interpretation. JCV, MCTA, PJL, and WBDS wrote the manuscript. WBDS designed and supervised the overall of research project. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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