MARIANA CAROLINE TOCANTINS ALVIM

ETHANOL STRESS RESPONSES OF *Kluyveromyces marxianus* CCT 7735 REVEALED BY PROTEOMICS AND METABOLOMICS ANALYSES

Thesis presented to the Universidade Federal de Viçosa as part of the requirements of the Agricultural Microbiology's Postgraduate Program, to obtain the title of *Doctor Scientiae.*

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APPROVED: May 30th, 2017.

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BIOGRAFIA

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ABSTRACT

ALVIM, Mariana Caroline Tocantins, D.Sc. Universidade Federal de Viçosa, May, 2017. **Ethanol stress responses of *Kluyveromyces marxianus* CCT 7735 revealed by proteomics and metabolomics analyses**. Advisor: Wendel Batista da Silveira. Co-advisors: Denise Mara Soares Bazzolli and Humberto Josué de Oliveira Ramos.

Non-*Saccharomyces* yeasts, such as *Kluyveromyces marxianus*, have called attention as promising strains for bioethanol production. *K. marxianus* displays desirable physiological characteristics for bioethanol production, such as capacity to assimilate pentoses and disaccharides beyond sucrose – present in the agro-industrial and forest residues – and thermotolerance. However, *K. marxianus*, contrary to *Saccharomyces cerevisiae*, is not tolerant to high ethanol concentrations. Moreover, its physiological responses to ethanol are not well elucidated; therefore, characterizing its physiological responses under ethanol stress is pivotal to apply this knowledge into metabolic engineering approaches to construct strains tolerant to ethanol. Thus, the aim of this work was to determine the ethanol responses of *K. marxianus* CCT 7735 subjected to 1 and 4 h of ethanol stress through both protein and metabolic profiles. It was observed that at 1 h of stress, although glycolytic enzymes and alcohol dehydrogenases were more abundant lactose and ethanol are not consumed by the yeast. On the other hand, at 4 h the abundance of proteins involved in carbon pathways and protein translation was reduced. In addition, there was an increase in the abundance of proteins and metabolites related to the response to oxidative and osmotic stresses, as well as to heat shock proteins. Interestingly, at 1 h of exposure to ethanol, plasma membrane ATPase was highly abundant, likely to counteract the proton motive force dissipation, which is related to the influx of protons due to increased permeability caused by ethanol. However, at 1 and 4 h of stress, respectively, the level of the metabolites trehalose, ergosterol and some amino acids reduced. Therefore, the results obtained in this study provide important information to improve the understanding of the ethanol responses displayed by *K. marxianus*.

RESUMO

ALVIM, Mariana Caroline Tocantins, D.Sc., Universidade Federal de Viçosa, maio de 2017. **Respostas de *Kluyveromyces marxianus* CCT 7735 ao estresse por etanol reveladas pelas análises proteômica e metabolômica**. Orientador: Wendel Batista da Silveira. Coorientadores: Denise Mara Soares Bazzolli e Humberto Josué de Oliveira Ramos.

No contexto de produção de bioetanol, leveduras não-*Saccharomyces*, como *Kluyveromyces marxianus*, vêm recebendo atenção especial. *K. marxianus* apresenta características fisiológicas desejáveis para a produção desse biocombustível, tais como capacidade de assimilar pentoses e dissacarídeos além da sacarose – presentes nos resíduos agroindustriais e florestais – e termotolerância. Entretanto, *K. marxianus* não tolera altas concentrações deste álcool, ao contrário da levedura convencional *Saccharomyces cerevisiae*. Além disso, as respostas fisiológicas dessas leveduras ao etanol não são bem elucidadas; portanto, faz-se necessário caracterizá-las, tendo como perspectiva a aplicação deste conhecimento em estratégias de engenharia metabólica para a obtenção de linhagens tolerantes a este álcool. Assim, este trabalho teve como objetivo determinar, via perfis proteico e metabólico, as respostas apresentadas por *K. marxianus* CCT 7735 ao etanol por 1 e 4 horas. Foi observado em 1 h de estresse que, embora as enzimas da via glicolítica e álcool desidrogenases sejam mais abundantes, lactose e etanol não são consumidos pela levedura. Por outro lado, em 4 h a abundância de proteínas envolvidas nas vias de consumo de carbono e no processo de tradução reduziu. Além disso, houve aumento na abundância de proteínas e metabólitos relacionados com respostas aos estresses oxidativo e osmótico, bem como de proteínas de choque térmico. Curiosamente, em 1 h de exposição ao etanol, a abundância da ATPase de membrana plasmática aumentou, o que possivelmente ocorreu como uma resposta adaptativa a fim de evitar a dissipação da força próton-motiva que está associada ao aumento da permeabilidade causada pelo etanol. No entanto, em 1 e 4 h de estresse, respectivamente, o nível dos metabólitos trealose, ergosterol e de alguns aminoácidos diminuiu. Portanto, os resultados obtidos neste estudo forneceram informações relevantes para auxiliar o entendimento das respostas apresentadas por *K. marxianus*, ao estresse por etanol.

GENERAL INTRODUCTION

With the population and the industrialization increase, the energy demand has raised (IEA, 2016). Besides, climate changes are sorely happening and there is a need of developing technologies of renewable energy. Over the last decades, it has been seen an increase of political incentives, researches and production involving renewable fuels, such as bioethanol (JOHANNSDOTTIRA & MCINERNEYB, 2016; GRFA, 2017). According to feedstock used for bioethanol production, it can be designated as first, second or third generation bioethanol. The first generation bioethanol is obtained from the fermentation of carbohydrates available in several crops carried out commonly by the conventional yeast *Saccharomyces cerevisiae*; however, those crops can be used as human food and animal feed (LIMAYEM et al., 2012; MORALES et al., 2015). To avoid the competition of arable land, fresh water and fertilizer requirement, the second generation bioethanol is produced from agroindustrial residues, such as whey and lignocellulosic biomass (THANGAVELU et al., 2016). However, *S. cerevisiae* neither assimilates nor ferments pentoses – as xylose – and certain disaccharides – as lactose – present, respectively, in hemicellulosic fraction of feedstocks and in whey (LEE et al., 2017).

In this context, non-*Saccharomyces* yeasts, such as *Kluyveromyces marxianus*, have called attention as promising strains for bioethanol production, since they present different features compared to *S. cerevisiae*: it can ferment lactose and it grows at elevated temperatures (SILVEIRA et al., 2005; DINIZ et al., 2012; RADECKA et al., 2015). However, most of the non-*Saccharomyces* yeasts are not tolerant to high ethanol concentrations in contrast to *S. cerevisiae*, which can affect the yield and productivity in biofuel industries (COSTA et al., 2014; CHOUDHARY et al., 2016). Therefore, understanding how those yeasts respond to ethanol exposure is important to develop stress-tolerant strains for bioethanol production. In this sense, the first chapter of this thesis is a review dedicated to describing the main ethanol stress responses of non-*Saccharomyces* yeasts compared to those exhibited by the conventional yeast.

Regarding the yeast *K. marxianus*, the genome sequence of the strain CCT 7735 was announced (SILVEIRA et al., 2014) and its transcriptome under ethanol stress has been recently published (DINIZ et al., 2017). Several responses at mRNA level are different between *K. marxianus* and *S. cerevisiae,* mainly

those related to unsaturated fatty acid, ergosterol and amino acids biosynthesis. Nevertheless, to gain more insights regarding the ethanol responses in *K. marxianus*, it is necessary to explore these responses at other molecular level. In this context, the second chapter of this thesis describes its protein and metabolic profiles under ethanol stress (1 and 4 hours of exposure)[1].

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CHAPTER I

Ethanol Stress Response in Yeasts: a comparison between *Saccharomyces cerevisiae* and non-*Saccharomyces* Yeasts

Introduction

Over the last few decades, the growth in both population and industrialization has increased the global demand for energy, which will reach a net number of 103 million barrels oil per day by 2040 (IEA, 2016). Up to now, most of this demand has been met by fossil fuels; however, these resources are finite. Moreover, studies have pointed out that air pollution caused by the release of CO_2 during consumption of these resources is responsible for eight million deaths worldwide annually, as well as for global warming and climate changes which disrupt the balance in the environment and entire ecosystem (LOUGH, 2015; SEINFELD et al., 2012). Over the last few years, in order to avoid increases in CO_2 emissions (as established by the Kyoto Protocol in 1997) and, concomitantly, to reduce rising pollution levels, research into the production of renewable fuels, such as biofuels, has been undertaken resulting in several advances in this field (SAVALIYA et al., 2015).

Nowadays, the main biofuels available are as follows: liquid fuels (bioethanol, biodiesel and pyrolysis oils), gases (biogas) and solids (pellet fuel) (BIBI et al., 2017). Over the last decades, governmental policies have countenanced the rise of biofuel production in many countries and it is expected that the global production of bioethanol, for example, reaches 1234.5 billion liters by 2024 (OECD/FAO, 2015). Brazil and the USA dominate by a wide margin total worldwide bioethanol production and consumption. According to the Renewable Fuels Association (RFA), the first country had estimated bioethanol production around 7,295 millions of gallons in 2016, while the second one had estimated one about 15,330 millions of gallons in the same year (RFA, 2017). The organization also reported that the European Union, China and Canada produced about 1,377, 845 and 436 millions of gallons of ethanol, respectively, in 2016 (RFA, 2017). Thus, these data highlight the growing worldwide concern for the use of sustainable energy. Nevertheless, biofuel production needs to be improved to substantially reduce dependence on fossil fuels or even to replace them. In 2015, 195 countries approved the global change agreement aimed at reducing

greenhouse gas emissions at the Paris Accord in the Conference of Parties (COP21) (JOHANNSDOTTIRA & MCINERNEYB, 2016). Moreover, recently the G20 leaders were called on by the Global Renewable Fuels Alliance to reinforce the first binding climate agreement at COP21 (GRFA, 2017). Therefore, it is expected that new political incentives will increase the production of biofuel even more.

Bioethanol can be either first, second or third generation, according to the feedstock used in its production. The first generation is obtained commonly by the yeast *Saccharomyces cerevisiae*, owing to its ability to efficiently convert the disaccharide sucrose into ethanol and CO_2 (MORALES et al., 2015). Nevertheless, that carbohydrate (available in several crops) can compete with animal feed and human food due to arable land, fresh water and fertilizer requirements (LIMAYEM et al., 2012). Thus, first generation bioethanol has been strongly criticized. To reduce the adverse impacts, manufacturing second generation bioethanol from non-food agro-industrial biomasses has been well explored (GUO et al., 2015; THANGAVELU et al., 2016). However, *S. cerevisiae* is not a thermotolerant yeast, which impairs its use in the production of bioethanol production through a simultaneous process of saccharification and fermentation (SSF), since this process is conducted at elevated temperatures to improve the cellulose hydrolysis with cellulolytic enzymes (saccharification step) (CHOUDHARY et al., 2016) and it cannot assimilate and ferment pentoses present in this feedstock (LEE et al., 2017).

In this context, the yeast *Kluyveromyces marxianus*, which is phylogenetically related to *Kluyveromyces lactis*, has the potential to play an important role in the production of bioethanol, due to the physiological advantages it offers to second generation ethanol (WÉSOLOWSKI-LOUVEL et al. 1996; FONSECA et al., 2008; LANE et al., 2011). For example, *K. marxianus* is able to assimilate and ferment other disaccharides beyond sucrose, such as lactose (from whey) and cellobiose, and it can assimilate pentoses, such as xylose, the major constituent of hemicellulosic fraction (SILVEIRA et al., 2014). The strain CCT 7735, previously designated as UFV-3, indeed, achieves above 90 % of the theoretical yield during the conversion of lactose from whey to ethanol (DINIZ et al., 2014). Moreover, *K. marxianus* tolerates higher temperatures, which is compatible with second generation ethanol production via SSF process (SOUZA et al., 2012; CHOUDHARY et al., 2016). However, *K. marxianus* CCT

7735 does not resist levels above 6% of ethanol in fermentation, in contrast to *S. cerevisiae*, which tolerates ethanol concentrations above 10% (COSTA et al., 2014).

The genome of *K. marxianus* CCT 7735 was determined and announced (SILVEIRA et al., 2014) and its transcriptome under ethanol stress was recently published (DINIZ et al., 2017). Both proteomic and metabolomic data are shown in the second chapter of this thesis. Nevertheless, to date, little is known about the ethanol response in *K. marxianus* as well as is the case of other non-*Saccharomyces* yeasts. Therefore, we present here the knowledge available from studies carried out on these yeasts and *S. cerevisiae*, the conventional yeast whose ethanol response has been more thoroughly investigated.

The Effect of Ethanol on Plasma Membrane and Response Mechanisms

During ethanol stress, plasma membrane is the cell structure which is most affected (MA et al., 2013): it causes its dehydration, promotes movement of water across the membrane and largely alters the volume and surface-to-volume ratio of yeast cells, causing increases in membrane permeability, proton influx and a reduction of intracellular pH (PIPER, 1995; HENDERSON & BLOCK, 2014). Thus, endocytosis and lipid phase transition occur, making the membrane more unstable (ZHENG et al., 2013). In *S. cerevisiae*, there is a relationship between the fatty acid composition of phospholipid membranes and ethanol stress tolerance: the degree of fatty acid unsaturation increases in the plasma membrane during stress (STANLEY et al., 2010; DOĞAN et al., 2014; ARCHANA et al., 2015; CHEN et al., 2016; KIM et al., 2016), turning, almost exclusively, into mono-unsaturated fatty acids, such as oleic acid and palmitoleic acid (YOU et al., 2003; UEMURA, 2012; ZHENG et al., 2013; HENDERSON & BLOCK, 2014). Indeed, the overexpression of genes involved with the increase in the unsaturated fatty acids index in plasma membrane has been reported (Figure 1) (CHANDLER et al., 2004; MARKS et al., 2008; LI et al., 2010; RAMIREZ-CÓRDOVA et al., 2012; ZHENG et al., 2013; KASAVI et al., 2016).

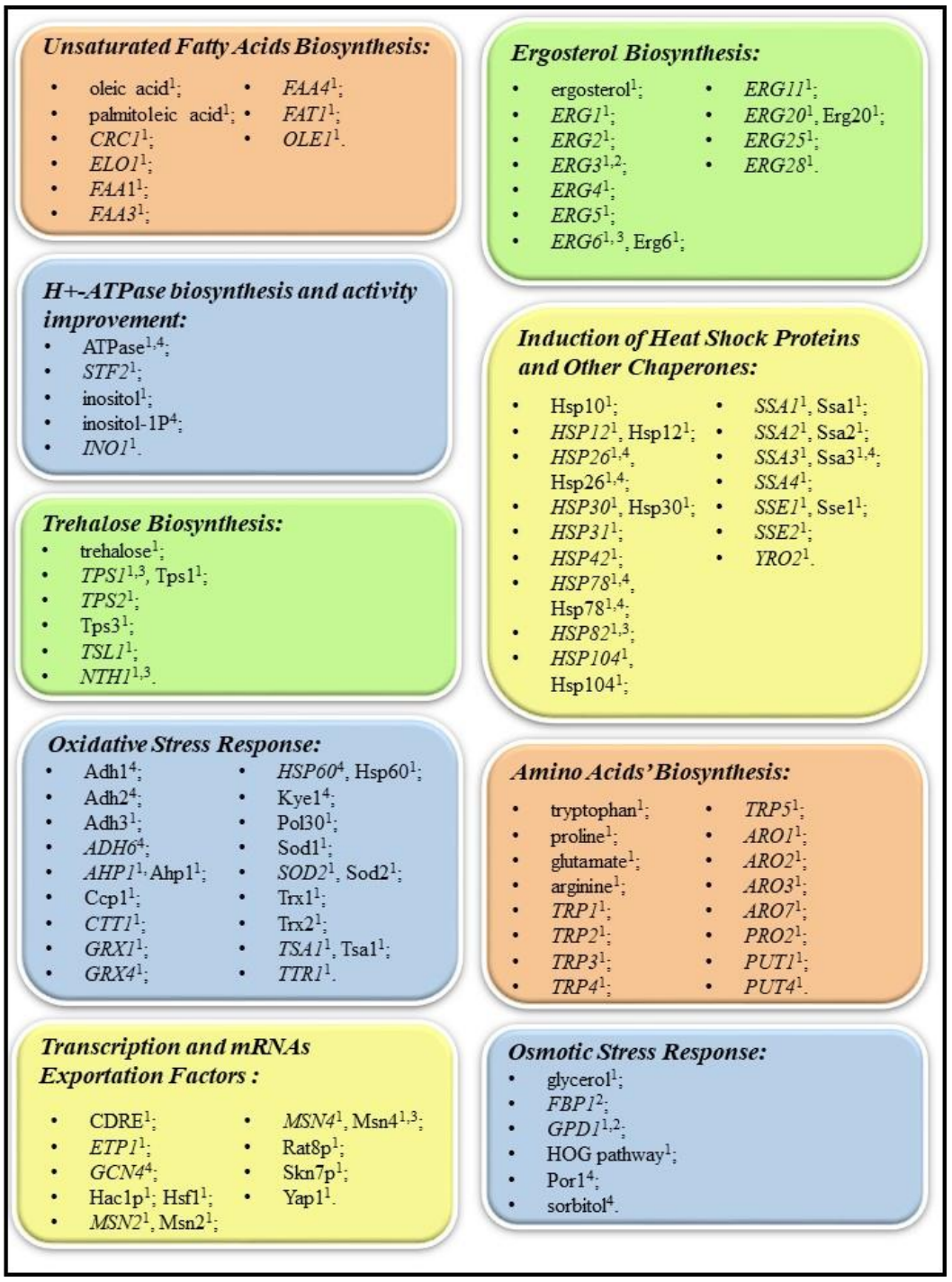

Fig 1. Main genes, proteins, metabolites and pathways induced during the ethanol stress response in yeasts. [1]*Saccharomyces cerevisiae*; [2]*Schizosaccharomyces pombe*; [3]*Brettanomyces bruxellensis*; [4]*Kluyveromyces marxianus*.

Nevertheless, this effect has not been seen either in *K. marxianus* or in *K. lactis,* a yeast which is closely related phylogenetically. In both species, the degree of fatty acid unsaturation does not increase on exposure to ethanol (HEIPIEPER et al., 2000; DINIZ et al., 2017). Similar results have been observed in other non-*Saccharomyces* yeasts when exposed to ethanol, such as methylotrophic *Hansenula polymorpha* and the wine fermenters *Issatchenkia*

occidentalis and *I. orientalis* (SOOKSAI et al., 2013; ARCHANA et al., 2015). Therefore, this characteristic may be related to the ethanol sensitivity presented by those non-*Saccharomyces* yeasts.

Additionally, another lipid component in yeast plasma membrane – ergosterol – has been demonstrated to play an important role in membrane stabilization by suppressing the phase transition of phospholipid bilayers (DUFOURC, 2008; ABE et al., 2009; VANEGAS et al., 2012). Studies have shown that *S. cerevisiae* cells accumulate this metabolite, induce the expression of genes and proteins related to its biosynthesis under ethanol stress, or the gene deletion results in ethanol sensitivity (Figure 1) (ROSSIGNOL et al., 2009; YOSHIKAWA et al., 2009; PARAPOULI et al., 2010; RAMIREZ-CÓRDOVA et al., 2012; ZHENG et al., 2013; CHEN et al., 2016; NAVARRO-TAPIA et al., 2016). In the wine contaminant yeast *Brettanomyces bruxellensis*, which is ethanol tolerant, the methyltransferase gene involved in ergosterol biosynthesis was overexpressed after long-term ethanol stress (Figure 1) (NARDI et al., 2010). In *Schizosaccharomyces pombe*, ergosterol and oleic acid contents were elevated after the C-5 sterol desaturase gene, from an edible mushroom, had been heterologously expressed in the yeast in order to obtain an ethanol stress tolerant strain (Figure 1) (KAMTHAN et al., 2017). *K. marxianus*, in turn, presented reduction in ergosterol content and the genes related to its biosynthesis were down-regulated under ethanol stress (chapter 2 of this thesis; DINIZ et al., 2017).

Additionally, to counteract increases in proton influx across the membrane, whose permeability is altered, and to avoid cytoplasm acidification, *S. cerevisisae* had the H^+-ATPase activity and its gene expression improved by exposure to ethanol (AGUILERA et al., 2006; FUJITA et al., 2006; YOSHIKAWA et al., 2009; VIANA et al., 2012), since H^+-ATPases can pump protons out of the cell or into the vacuole (Figure 1). This result was also reported when *K. marxianus* was exposed to 3% and 6% of ethanol, respectively (chapter 2 of this thesis; ROSA & SÁ-CORREIA, 1992). Moreover, the molecules (and the genes/proteins responsible for its accumulation) that stimulate the H^+-ATPase activity such as inositol and its precursors are accumulated under ethanol stress by both yeasts (Figure 1) (chapter 2 of this thesis; FURUKAWA et al., 2004; DING et al., 2010; HONG et al., 2010; DERANIEH et al., 2015).

Since ethanol acts detrimentally on the plasma membrane - causing increases in membrane permeabilization and proton influx - cell ion binding is

thought to be facilitated (PETROV et al., 1990). When added to the medium, ions such as magnesium, calcium and potassium provide protection to the *Saccharomyces* species by maintaining the structural integrity of membranes and participating in certain repair mechanisms or cellular functions, such as enzyme activation (NABAIS et al., 1988; DOMBEK et al., 1986; LAM et al., 2014). Despite this, genes, proteins and metabolites related to transporters for those ions did not have their expression significantly changed under this condition in *K. marxianus* (chapter 2 of this thesis; DINIZ et al., 2017).

Heat Shock Protein Induction

It is known that exposure to ethanol and heat shock share significant genetic and functional similarities in yeast cells. In this way, heat shock proteins (HSPs) are frequently overexpressed during the response to ethanol stress in order to repair the damaged proteins and membranes (PIPER, 1995). In *S. cerevisiae*, a range of genes and proteins belonging to HPSs families and other chaperones were reported in this condition (Figure 1) (ALEXANDRE et al., 2001; CHANDLER et al., 2004; ROSSIGNOL et al., 2009; MA & LIU, 2010; ROSSOUW et al., 2010; MORENO-GARCÍA et al., 2014; LAHTVEE et al., 2016; NAVARRO-TAPIA et al., 2016; SANTOS et al., 2017).

In *B. bruxellensis*, under long-terms of fermentation, there was an induction of gene coding for Hsp82 (which is part of the Hsp90 chaperone complex, reacting normally to heat and osmotic shocks), but gene coding for Ssa3 and Ssa4 proteins (Hsp70-homolog proteins involved in the prevention of denatured protein aggregation) were not up-regulated (Figure 1) (NARDI et al., 2010). In *K. marxianus*, it was observed the overexpression of genes or proteins coding for Hsp26, Hsp78 and Ssa3 (which prevent protein aggregation and resolubilizing of insoluble aggregates), demonstrating that this yeast activates a response mechanism when it is stressed with 6% of ethanol (Figure 1) (chapter 2 of this thesis; DINIZ et al., 2017). Curiously, overproduction of Hsp72 in *H. polymorpha,* which also belongs to the Hsp70 protein family, impaired its tolerance to ethanol (TITORENKO et al., 1996).

Trehalose Accumulation

In *S. cerevisiae*, trehalose also protects cell structures against exposure to ethanol. It binds to the polar head groups of both phospholipids as well as

proteins, and prevents their inactivation and denaturation under stressful conditions (MAHMUD et al., 2012). In *S. cerevisiae* exposed to ethanol, the overexpression of genes and proteins involved in the metabolism of trehalose, as well as its synthesis, were reported by several authors (Figure 1) (ALEXANDRE et al., 2001; CHANDLER et al., 2004; KAINO & TAKAGI, 2008; ROSSOUW et al., 2010; ZHENG et al., 2013; CHEN et al., 2016; HASHIM & FUKUSAKI, 2016; KIM et al., 2016; OHTA et al., 2016; SANTOS et al., 2017). In *B. bruxellensis*, there seems to be a role for trehalose under ethanol stress, since the expression of gene encoding neutral trehalase and the synthase subunit of trehalose-6-phosphate synthase was induced (NARDI et al., 2010).

However, under exposure to 6 and 10% ethanol, the intracellular content of trehalose decreased in *K. marxianus* and other non-*Saccharomyces* yeasts: *Clavispora opuntiae*, *Meyerozyma guilliermondii*, *Saccharomyces unisporus*, *S. pombe* and *Torulaspora pretoriensis* (chapter 2 of this thesis; RIBEIRO et al., 1999; GOMES et al., 2002; DINIZ et al., 2017).

Oxidative Stress Response

Another point of interest is that exposure to ethanol leads to oxidative stress in yeast cells by damage the mitochondria and promoting the accumulation of intracellular reactive oxygen species (ROS) (ALLEN et al., 2010; MA et al., 2013). Thus, systems for counteracting the ROS and maintaining the reduced cell environment need to be activated during this condition. They include superoxide dismutase and catalase, and peroxisomes as well as the thioredoxin and glutathione/glutaredoxin systems (MORANO et al., 2012). Indeed, many studies has notably increased expression of genes and proteins that respond to oxidative stress initiated by exposure to ethanol in *S. cerevisiae* (Figure 1) (ALEXANDRE et al., 2001; CHANDLER et al., 2004; ZHOU et al, 2004; PARAPOULI et al., 2010; TEIXEIRA et al., 2011; ZHENG et al., 2013; LAHTVEE et al., 2016; SANTOS et al., 2017). However, metabolites from glutathione metabolism did not accumulate in *K. marxianus* under 6% of ethanol stress (chapter 2 of this thesis).

It should also be noted that further effects from exposure to ethanol seem to be related to oxidative stress response (Figure 1), as follows: the up-expression of gene encoding mitochondrial protein Hsp60 in *S. cerevisiae* and in *K. marxianus* (CABISCOL et al. 2002; LAHTVEE et al., 2016; DINIZ et al., 2017);

the up-regulation of a specific polymerase [proliferating cell nuclear antigen (PCNA)], which functions as a repairing agent of DNA in *S. cerevisiae* (ZHOU et al., 2004); the overexpression of genes/proteins coding for alcohol dehydrogenases in *K. marxianus* and in *S. cerevisiae*, since those enzymes contribute for reducing power generation (chapter 2 of this thesis; ROSSOUW et al., 2010; DINIZ et al., 2017); and finally the enoate reductase 1 overexpression in *K. marxianus*, which is an antioxidant protein induced during the lipid peroxidation (chapter 2 of this thesis).

Osmotic Stress Response

Another effect caused by ethanol exposure is osmotic stress. In response to this stress condition, yeasts synthesize glycerol through high osmolarity glycerol (HOG) pathway (Figure 1), which plays an important role in counteracting the affinity for water of the extracellular osmolite (VRIESEKOOP et al., 2009). In *S. cerevisiae*, the HOG pathway, which is controlled by osmosensors, was induced by ethanol stress and activated the transcription factors Msn2p and Msn4p (MA & LIU, 2010). Additionally, the gene encoding glycerol-3-phosphate dehydrogenase, which acts in glycerol biosynthesis pathway, was up-regulated (ALEXANDRE et al., 2001). Indeed, the metabolomic approach identified an elevated glycerol level in the yeast when it was exposed to ethanol (CHEN et al., 2016). In *S. pombe*, low ethanol concentrations were able to induce the gene expression of glyceraldehyde-3-phosphate dehydrogenase as well as fructose-1,6-bisphosphatase, allowing for growth in glycerol (Matsuzawa et al., 2012).

In *K. marxianus*, in turns, two molecules were related to the osmotic stress response triggered by the stress condition: Por 1, a mitochondrial porine that controls the ion traffic through the organelle, and sorbitol, which seems to act like glycerol (chapter 2 of this thesis).

Biosynthesis of Amino Acids and Translation Process

Certain amino acids, such as tryptophan, proline and, more recently, glutamate and arginine are part of the ethanol stress response, although the reason for this is still unknown (FUJITA et al., 2006; HIRASAWA et al., 2007; CHENG et al., 2016; KIM et al., 2016). Proline appears to protect cells by improving protein and membrane stability and inhibiting protein aggregation during its refolding, as well as lowering the DNA *Tm* and scavenging ROS (TAKAGI,

2008). Arginine seems to protect cells by maintaining the integrity of both the cell wall and the membrane and by controlling the generation of ROS (CHENG et al., 2016). In fact, genes and proteins related to their transport and biosynthesis were overexpressed when *S. cerevisiae* was subjected to ethanol or their deletion resulted in ethanol sensitivity (Figure 1) (HIRASAWA et al., 2007; KAINO & TAKAGI, 2008; DINH et al., 2009; YOSHIKAWA et al., 2009). Furthermore, these amino acids accumulated under this stress condition (KAINO & TAKAGI, 2008; CHEN et al., 2016; CHENG et al., 2016; KIM et al., 2016; OHTA et al., 2016). In *K. marxianus*, it was reported that the up-regulation of gene encoding proteins related to the metabolism of amino acid, specifically branched chain and aromatic amino acids, under ethanol stress, but some amino acid levels decreased, specially the arginine and glutamate ones (chapter 2 of this thesis; DINIZ et al., 2017).

Studies have pointed out that, in turn, the translation process in yeast does not follow an expression standard of ethanol stress over either the short or the long-term. A number of these studies have shown in both *S. cerevisiae* and *K. marxianus* repression of gene and protein expression related to protein synthesis, such as ribosomal proteins and translation factors (chapter 2 of this thesis; CHANDLER et al. 2004; MARKS et al. 2008; DINH et al. 2009; KASAVI et al. 2016; LAHTVEE et al., 2016; DINIZ et al., 2017). On the other hand, other studies have reported the overexpression of these genes and proteins under the same stress condition in *S. cerevisiae* (CHANDLER et al. 2004; ZHOU et al., 2004; YOSHIKAWA et al., 2009; LI et al., 2010; MORENO-GARCÍA et al., 2014). Thus, no trend related to the translation process appears to be associated with ethanol or non-tolerant yeasts, but may just be related to the strain, or time of exposure to ethanol as well as its concentration.

Carbon Flow during Ethanol Stress

Ethanol tolerant yeasts (*S. cerevisiae* strains) display high glycolytic and pentose-phosphate fluxes in order to convert glucose into ethanol, and are active enzymes in the regeneration pathways of NADH/NADPH coenzymes which maintain an adequate redox balance and highly expressed alcohol dehydrogenases, which are necessary to the completion of the alcoholic fermentation process (MA & LIU, 2010; TIAN et al., 2017). On the other hand, in non-ethanol tolerant yeasts, the expression of the most genes/proteins involved in

the central metabolic pathways is highly impaired under ethanol stress and there may be an inhibition of carbon uptake, leading to low growth rates (chapter of this thesis; PIPER, 1995; SILVEIRA et al., 2005; DINIZ et al., 2017). Even so, *K. marxianus* exhibited an up-regulation of the gene that encodes glucose-6-phosphate-1-dehydrogenase and of some glycolytic proteins after 1 h of ethanol exposure (chapter 2 of this thesis; DINIZ et al., 2017). Furthermore, one of its alcohol dehydrogenases was overexpressed, which seems to contribute to a reduction in the production of coenzymes, as aforementioned (chapter 2 of this thesis; DINIZ et al., 2017).

Transcription and mRNA Exportation Factors

The expression of transcription factors changes in response to ethanol. In *S. cerevisiae*, it has been shown that the up-regulation of several factors, which are involved in the transcription regulation of hundreds of genes, is related to protein folding, detoxification, energy generation, carbohydrate metabolism, and cell membrane organization (Figure 1) (ARAKI et al., 2009; SNOWDON et al., 2009; MA & LIU, 2010; NAVARRO-TAPIA et al., 2016; ZYRINA et al., 2017). The presence of both Msn2p and Msn4p, for example, is necessary to induce genes involved in cellular protection against chronic stresses such as exposure to ethanol (AKHAVAN-AGHDAM et al., 2016). In *B. bruxellensis*, the *MSN4* gene was strongly induced when the yeast was exposed to high ethanol concentrations (Figure 1) (NARDI et al., 2010). In *K. marxianus*, in turn, up-regulation of the gene that encodes the factor GCN4, which activates the expression of amino acid biosynthetic genes (Figure 1) (DINIZ et al., 2017) was observed when it is stressed with 6% of ethanol.

Nevertheless, ethanol damages the integrity of the nuclei membrane, affecting the exporting of mRNA. In *S. cerevisiae*, for example, the poli $(A)^+$ mRNAs were accumulated in nuclei because the factor responsible for their exportation, Rat8p, was accumulated in the nuclei due to a defect in the exporting of Xpo1p/Crm1p exposed to 10% of ethanol (Figure 1) (TAKEMURA et al., 2004; IZAWA et al., 2005). In. *S. pombe*, under the same stress conditions, there was partial inhibition of poli $(A)^+$ mRNA export, which triggered its accumulation in the nuclei (YOSHIDA & TANI, 2005). In *K. marxianus*, no differentiating genic expression was noted that could be attributed to this effect (DINIZ et al., 2017).

Conclusions

The information provided herein regarding the response to ethanol by *K. marxianus* and other non-*Saccharomyces* yeasts is still not enough to reveal all the mechanisms that occur in the cells during this stress condition. Nevertheless, this information points to responses that are different compared to *S. cerevisiae* judging by the low tolerance they exhibit. Exploring non-*Saccharomyces* yeasts in further studies under ethanol stress, including "omics" studies, may provide new insights, and contribute to a more comprehensive understanding of the pathways linked to this stress condition. Besides, considering the fermentative potential of *K. marxianus* CCT 7735 strain, these new insights can be used to construct a stress-tolerant strain for the production of bioethanol.

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CHAPTER II

**Manuscript written according to Molecular Microbiology guidelines.*

Ethanol stress responses of *Kluyveromyces marxianus* (CCT 7735) revealed by proteomics and metabolomics analyses

Keywords: *Kluyveromyces marxianus*, Proteome, Metabolome, Ethanol stress, Membrane permeability, ATPase, Oxidative stress.

SUMMARY

Kluyveromyces marxianus (CCT7735) offers advantages to bioethanol production over *Saccharomyces cerevisie*, including thermotolerance and the ability to convert lactose into ethanol at yields close to the theoretical value. However, contrary to *S. cerevisiae*, it can resist ethanol only to a level of 6% beyond which its use in ethanol production is impaired. Herein we report on the protein and intracellular metabolite profiles of *K. marxianus* at 1 and 4 h under ethanol exposure. We observed that proteins and metabolites from carbon pathways and translation were less abundant, mainly at 4 h of ethanol stress, as evidenced by their low growth rate under this condition. The ergosterol, trehalose and amino acids levels were impaired as well, which contrasts with *S. cerevisiae*. On the other hand, our results show that the abundance of heat shock proteins and proteins and metabolites related to the oxidative and osmotic stresses responses increased. Interestingly, plasma membrane ATPase was highly abundant at 1 h of exposure to ethanol, which likely counteracted the proton influx caused by ethanol. Taken together, the results obtained in this study provide insights into understanding the physiological changes in *K. marxianus* under ethanol stress, indicating possible targets for ethanol tolerant strain construction.

INTRODUCTION

As a result of recent growth in both population and industrialization worldwide, the demand for energy has been continually on the rise (International Energy Agency, 2016). Over recent decades, this demand has been met mainly by fossil fuels; however, their use seems to be associated with climate change. Thus, there is a need for developing renewable energy technology. Indeed, research projects focused on the production of renewable fuels such as biofuels have been attracting increasing interest over the last few years (Savaliya *et al*., 2015), and ethanol is still the most widely produced biofuel in the world. According to the

feedstock used in its production, it can be categorized as either first or second generation. First generation ethanol is commonly produced by the yeast *Saccharomyces cerevisiae* from sugars available in crops which are consumed primarily as either food for human beings or animal feed (Morales *et al*., 2015). Second generation ethanol, in turn, is produced from agroindustrial residues – an attractive and abundant feedstock for bioethanol production (Thangavelu *et al*., 2016). However, *S. cerevisiae* neither assimilates nor ferments pentoses present in this feedstock (Lee *et al*., 2017). Another abundant feedstock that cannot be used for ethanol production by *S. cerevisiae* is whey, the main effluent generated by the dairy industry (Sossna, 2014). This occurs because yeast is not capable of assimilating lactose, the sugar found in whey (Jelen, 2009).

In contrast to *S. cerevisiae*, the yeast *Kluyveromyces marxianus* assimilates lactose and xylose, the main pentose found in hemicellulose, as sole sources of carbon. Thus, it is considered an alternative yeast to *S. cerevisiae* for the production of ethanol from either whey or lignocellulosic biomass (Lane *et al*., 2011; Silveira *et al*., 2014). Furthermore, *K. marxianus* is a thermotolerant yeast, which is a desirable feature, for example, for second generation ethanol production via simultaneous saccharification and fermentation process (SSF) (Costa *et al*., 2014; Silveira *et al*., 2014). Moreover, thermotolerance is also desirable in tropical countries where bioprocessing cooling costs are expensive (Choudhary *et al*., 2016).

Because of these characteristics, our research group has studied the production of ethanol from whey by *K. marxianus* (CCT 7735) over the last few years, previously designated as *K. marxianus* UFV-3 (Silveira *et al*., 2005; Diniz *et al*., 2014; Ferreira *et al*., 2015). This strain displays a capacity for converting lactose into ethanol at yields above 90% of the theoretical value, which appears to be related to the high expression levels of gene encoding enzymes of the Leiloir pathway as well as *LAC4* and *RAG6* that encode β-galactosidase and pyruvate decarboxylase, respectively (Diniz *et al*., 2012; Diniz *et al*., 2014). Nevertheless, *K. marxianus* strains, contrary to *S. cerevisiae*, are not able to grow well in ethanol concentrations of 6% (Silveira et al. 2005; Costa *et al*., 2014). Thus, the main drawback for ethanol production from whey by *K. marxianus* is its low tolerance for ethanol.

The genome of *K. marxianus* (CCT 7735) was announced (Silveira *et al*., 2014) and a transcriptome analysis under ethanol was recently published (Diniz *et*

al., 2017). This study showed that expression of gene encoding proteins related to the glycolytic pathway, translation process and unsaturated fatty acid and ergosterol biosynthesis is highly repressed in this condition, while gene encoding heat shock proteins, alcohol dehydrogenase 6 and proteins relating to the biosynthesis of amino acids are up-regulated. However, some of these responses reveal differences between *K. marxianus* and *S. cerevisiae*, related, in the main, to unsaturated fatty acid, ergosterol and the biosynthesis of amino acids (Alexandre *et al*., 2001; Chandler *et al*., 2004; Fujita *et al*., 2006; Hong *et al*., 2010; Ma & Liu, 2010; Rossouw *et al*., 2010; Stanley *et al*., 2010; Vanegas *et al*., 2012; Doğan *et al*., 2014; Henderson & Block, 2014; Kasavi *et al*., 2016; Kim *et al.,* 2016; Lahtvee *et al*., 2016; Navarro-Tapia *et al*., 2016).

In order to understand the ethanol response in *K. marxianus* (CCT 7735) at the post-transcriptional level, we evaluated its protein and intracellular metabolite profiles at 1 and 4 h of ethanol 6% (v/v) exposure. Thus, the information obtained will be useful to future studies aimed at the construction of an ethanol tolerant *K. marxianus* strain.

RESULTS

Protein profile of K. marxianus *(CCT 7735) under ethanol stress*

In order to investigate the abundance of protein in *K. marxianus* (CCT 7735) under ethanol stress, we analyzed the protein profile of yeast cultures exposed to ethanol. The yeast was cultured in YPL medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) lactose] and on reaching OD_{600} nm = 0.8, *K. marxianus* was stressed with 6% (v/v) ethanol for the following 4 h. Analysis was carried out using three replicates of each culture: at 1 and 4 h of exposure to ethanol, and 0, 1 and 4 h of no exposure (controls). Proteins were extracted, separated by two-dimension electrophoresis (2D-PAGE) and one-dimension electrophoresis (1D-PAGE) and identified by MALDI-TOF/TOF and NanoUHPLC-QTOF mass spectrometers, respectively. Thus, both high and low abundance proteins could be covered.

The results indicate that exposure to ethanol for 1 and 4 h caused significant alterations in the proteomic profile of the yeast. About 2,000 spots were detected in each 2D-PAGE gel stained with Coomassie (Figure S1). Analysis by the Image Master 2D Platinum software program version 7.0 (GE Healthcare, Chicago, IL, United States) revealed 180 intracellular protein spots

changing significantly in all contrasts: 0 h control versus 1 h of ethanol exposure; 1 h control versus 1 h of ethanol exposure; 0 h control versus 4 h of ethanol exposure; and 4 h control versus 4 h of ethanol exposure. A total of 68 spots were successfully identified using MALDI-TOF/TOF mass spectrometer. Among them, 14 and 13 spots were less abundant in cells exposed to ethanol for 1 h and 4 h, respectively, when compared to both controls: to 0 h and 1 h with no exposure to ethanol, and to 0 h and 4 h with no exposure to ethanol. On the other hand, 29 and 12 spots, compared to the same controls, were more abundant in cells exposed to ethanol for 1 h and 4 h, respectively. The protein identifications after 1 h and 4 h were summarized in Figure 1 and Table S1. Additionally, protein isoforms were also identified in 2D-PAGE and Figure 2 shows those with the greatest number of identified spots.

In addition, 227 proteins were identified by the NanoUHPLC-QTOF mass spectrometer: 110 at 1 h of ethanol treatment and 117 at 4 h. However, only 10 and 17 proteins were significantly differently in abundance at 1 h and 4 h, respectively. Among them, eight proteins were less abundant at 1 h and two more abundant at the same point in time. At 4 h, 11 proteins were less abundant and six more abundant. The protein identifications are summarized in Figure 1 and Table S2.

Therefore, taking into account the readings of both mass spectrometers for the analyzed time periods, a total of 53 proteins were identified at 1 h of ethanol exposure and 42 at 4 h. Among them, 27 and 26 were unique proteins at 1 and 4 h, respectively.

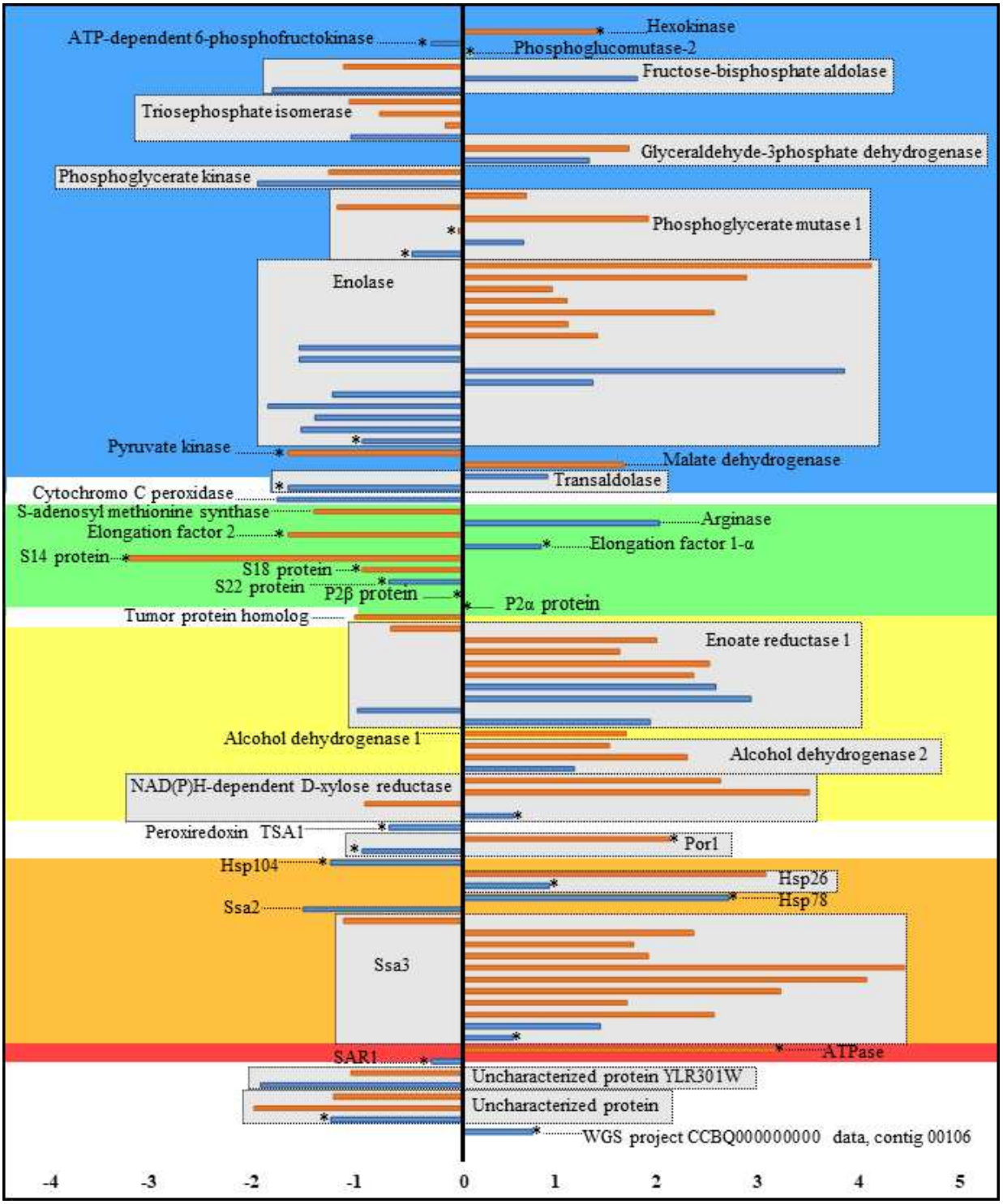

Fig. 1 Proteins more and less abundant in *K. marxianus* (CCT 7735) after 1 h (orange bars) and 4 h (blue bars) of ethanol exposure according to Log_2 Fold Change (X-axis). Isoforms of the same protein were identified in several spots, as seen in gray boxes. Background color indicates the main cellular process related to the proteins identified: blue, carbon metabolism; green, translation process or amino acid metabolism; yellow, oxidation-reduction processes; orange, protein folding; red, ATP biosynthetic process or intracellular protein transport. Asterisks indicate that the protein was identified through NanoUHPLC-QTOF.

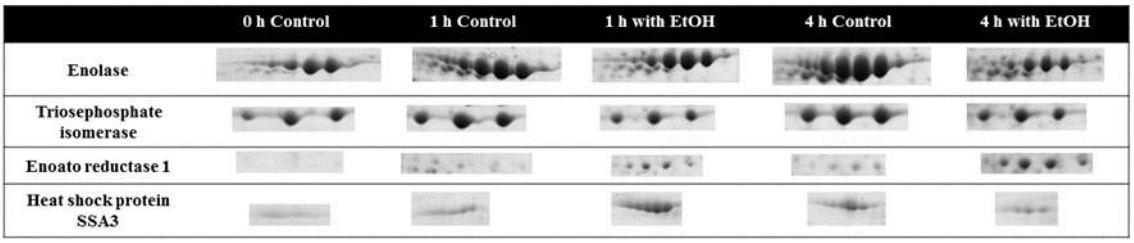

Fig. 2 Main differently abundant proteins that presented isoforms in 2D-PAGE from *K. marxianus* cultures (CCT 7735) exposed to 1 and 4 h of ethanol stress (with EtOH), as well as from control cultures at 0, 1 and 4 h.

As regards the less abundant proteins at 1 h after ethanol exposure, most of them belong to the glycolytic pathway, although the number of less abundant proteins in this cellular process has been lower than the high abundant proteins (Figure 1 and Table S1). Translation was the second most evident process at 1 h, followed by response to oxidation-reduction processes (Figure 1, Tables S1 and S2). Similarly, the most abundant proteins at 1 h are related to oxidation-reduction processes, glycolytic pathway and protein folding (Figure 1, Tables S1 and S2).

Considering the less abundant proteins at 4 h, the glycolytic process was also the more evident one, corresponding to half of the identified proteins, followed by protein folding and translation (Figure 1, Tables S1 and S2). On the other hand, the most abundant proteins at 4 h of stress are related to glycolytic process, oxidation-reduction processes and protein folding (Figure 1, Tables S1 and S2).

Metabolite profile of K. marxianus *(CCT 7735) under ethanol stress*

The metabolite profile of *K. marxianus* (CCT 7735) under ethanol stress was also investigated. The yeast was cultured as previously mentioned, using three replicates of each culture: 1 and 4 h of exposure to ethanol, and 0, 1 and 4 h without exposure to ethanol (controls). Next, intracellular metabolites were identified by GC–TO/MS. Principal components analysis (PCA) was carried out in order to decrease data dimensionality. PCAs explained the alterations in all proposal contrasts: 0 h control versus 1 h of ethanol exposure (Figure 3A); 0 h control versus 4 h of ethanol exposure (Figure 3B); 1 h control versus 1 h of ethanol exposure (Figure 3C); and 4 h control versus 4 h of ethanol exposure (Figure 3D). In all of them, two different groups were clearly formed – one of them from yeast cells exposed to ethanol and another one from cells unstressed with ethanol. Hence, the spatial separation of each group showed the difference

between the samples exposed or not to ethanol, *i.e.*, the ethanol stress changed expressively the metabolic profile of *K. marxianus*.

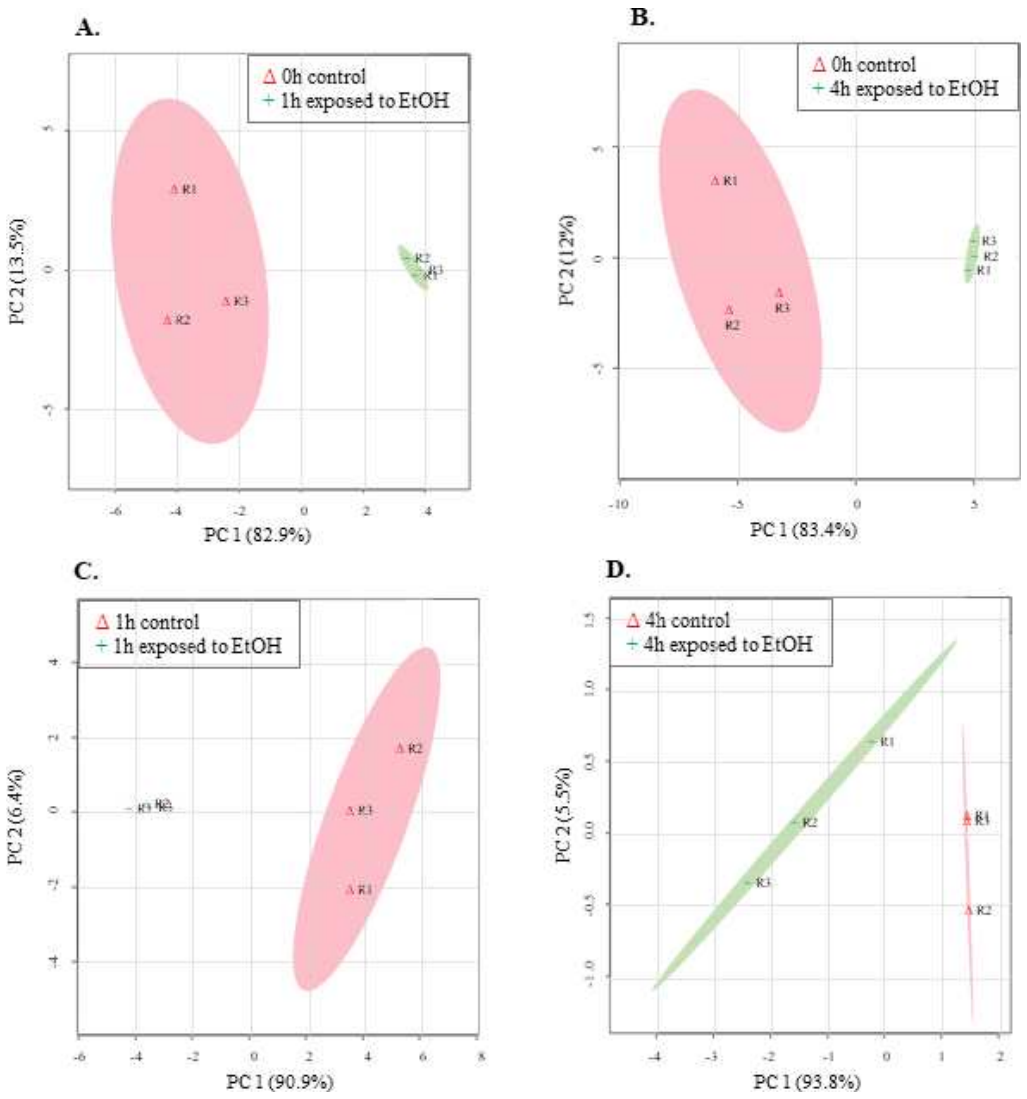

Fig. 3 Principal components analysis (PCA) of the metabolites from *K. marxianus* (CCT 7735) exposed or not to ethanol (EtOH). Three biological replicates (R1, R2 and R3) were used for each treatment.

Metabolomic analysis identified 107 different intracellular metabolites (Table S3) with significant differences (p-value < 0.05) between the aforementioned contrasts. After 1 h of ethanol stress, 61 metabolites had their level reduced and only one had its level elevated as regards the controls from 0 and 1 h without ethanol exposure. At 4 h, the level of 79 metabolites decreased and the level of 4 others increased when compared to the controls from 0 and 4 h without ethanol stress. The pathway of each significant metabolite identified was established by entry with KEGG compound ID for *K. lactis* (the yeast specie closest to *K. marxianus*) at the KEGG database (http://www.kegg.jp/). In Figure 4, the main pathways affected by the ethanol exposure of *K. marxianus* are

highlighted. At 1 h of ethanol stress, the metabolic pathways that were most impaired were the biosynthesis of amino acids (kla01230), carbon metabolism (kla01200) and aminoacyl-tRNA biosynthesis (kla00970) (Figure 4A). Similarly, at 4 h of ethanol stress, the most negatively affected metabolic pathways were the biosynthesis of amino acids (kla01230), pentose phosphate pathway (kla00030) and carbon metabolism (kla01200) (Figure 4B).

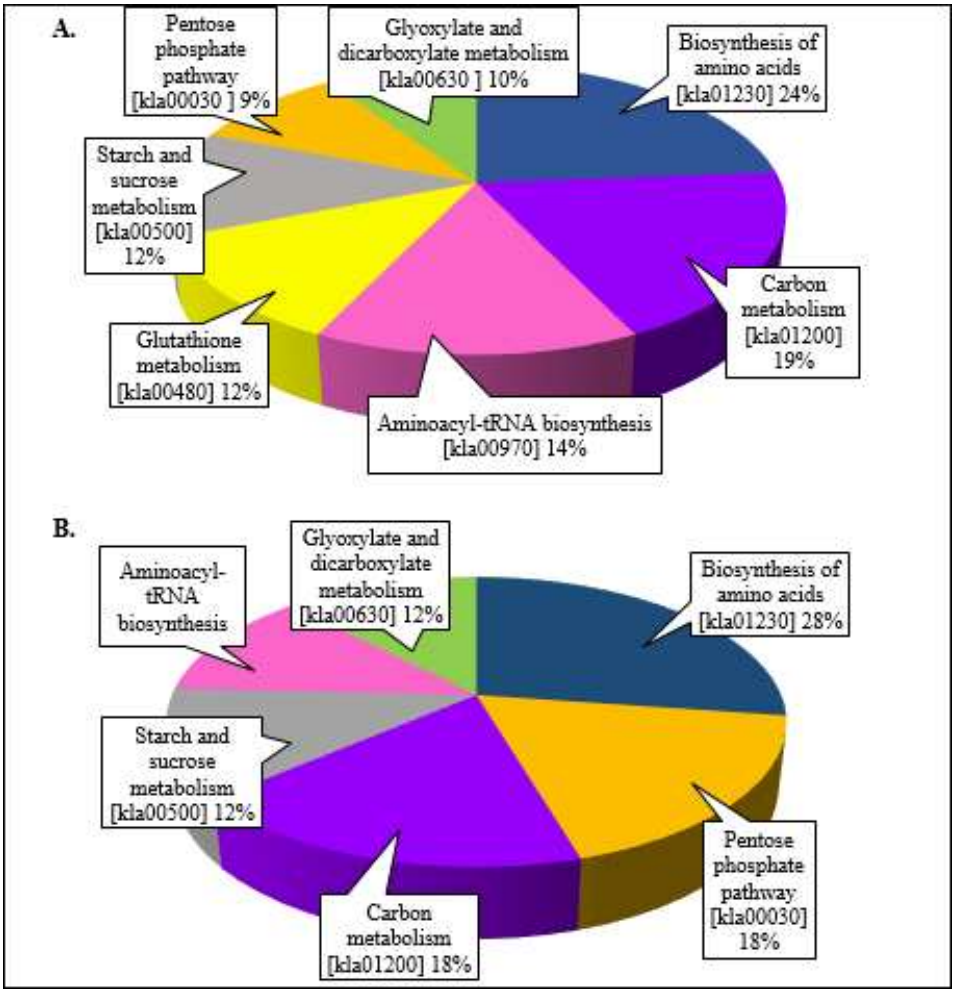

Fig. 4 Main pathways related to the less abundant metabolites in *K. marxianus* (CCT 7735) at **A.** 1 h and **B.** 4 h of ethanol stress obtained from the KEGG database.

Effect of ethanol on carbon metabolism

The differentially abundant proteins at 1 h and 4 h of ethanol stress belong mainly to the glycolytic process (Figure 1, Tables S1 and S2). At 1 h of ethanol stress, most of them had increased abundance, while at 4 h, most of them were less abundant. Enolase and triosephosphate isomerase were highlighted on account of the number of spots identified: 15 and 3, respectively (Figure 2). It should be noted that the presence of several spots for the aforementioned glycolytic enzymes suggests the occurrence of post-translation modifications.

Indeed, these isoforms exhibited modifications in at least one unique peptide (such as oxidation or carbamethylation - data not shown), which caused changes in the gels (Figure 2).

Additionally, the metabolomic data revealed that carbon metabolism and pentose phosphate pathway were impaired under ethanol stress, mainly after 4 h of exposure (Figures 4A and 4B). D-glucose and D-galactose had their abundance reduced at 1 and 4 h of ethanol stress (Table S3), suggesting that lactose uptake was impaired. Indeed, high-performance liquid chromatography (HPLC) analysis indicated that the extracellular content of lactose in the yeast cells exposed to ethanol did not significantly change over time (Figure 5), which is consistent with the low intracellular level of both D-glucose and D-galactose. Furthermore, two alcohol dehydrogenases (Adh1 and Adh2) were more abundant in this period (Table S1 and Figure 1). However, we observed that ethanol was not significantly consumed during the period of stress (Figure 5) and extracellular acetate concentration did not change (data not shown). At 4 h, in turn, most of the glycolytic enzymes were less abundant (Table S1, S2 and Figure 1) and only Adh2p had more abundance (Table S1 and Figure 1).

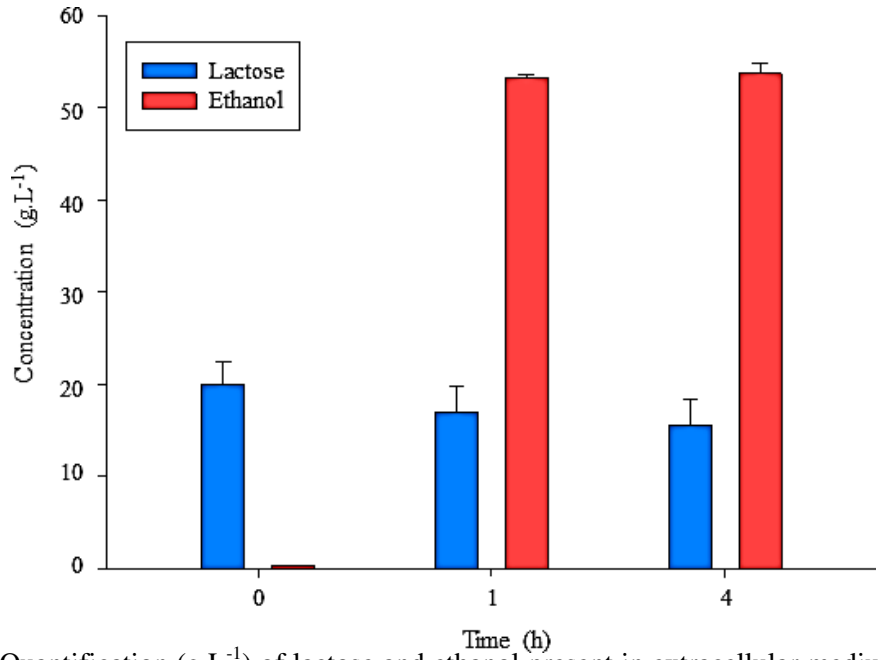

Fig. 5 Quantification ($g.L^{-1}$) of lactose and ethanol present in extracellular medium of the cultures of *K. marxianus* at 0, 1 and 4 hours of ethanol exposure. The measurements were obtained by HPLC analysis. Three biological replicates were used and Student's *t*-test was applied considering a *p*-value < 0.05. The concentration of all the compounds presented no significant differences over time.

It should be noted that the reduction of triosephosphate isomerase abundance could lead to accumulation of dihydroxyacetone phosphate, which could be converted into glycerol 3-phosphate. In turn, glycerol 3-phosphate could be converted into either diacylglycerol 3-phosphate or glycerol. The glycerol concentration did not change over time, indicating that glycerol does not, in fact, accumulate in response to ethanol (data not shown).

Effect of ethanol on translation process

Translation was another process related to less abundant proteins (Figure 1, Tables S1 and S2). Protein reduction abundance was seen in both periods analyzed, although at 1 h it was more prominent (Figure 1 and Table S2). It covered proteins of 40S ribosomal subunit (such as S14, S18 and S22), proteins of 60S ribosomal one (P2-α and P2-β) and elongation factor 2. Additionally, the protein, S-adenosyl methionine synthase - related to amino acid biosynthesis - was less abundant and arginase – involved in arginine catabolic process - was more abundant (Table S1 and Figure 1). As regards metabolomic analysis, the metabolite level from phenylalanine metabolism increased at 4 h of ethanol stress, indicating that the amino acid was hydrolyzed (Figure 4C and Table S3). Moreover, metabolites from the biosynthesis of amino acids, aminoacyl-tRNA biosynthesis and arginine and glutamate levels decreased in both analysis periods (Figures 4A and 4B and Table S3). These results indicate that processes related to translation were impaired during the stress condition imposed.

Effect of ethanol on oxidative and osmotic stress response

Turning to the oxidation-reduction processes, enoato reductase 1 (butanoate + $NAD^+ \leftrightarrow$ 2-butenoate + NADH) is the most representative enzyme, corresponding to nine and six identified spots from 1 and 4 h, respectively (Figures 1 and 2 and Table S1). It is likely that the presence of these spots indicates the occurrence of post-translation modification. Indeed, oxidation or carbamethylation were detected in at least one unique peptide of these isoforms. This protein was able to respond to oxidative stress since in other yeasts it is related to this kind of protection.

In the same process, there are the previously mentioned alcohol dehydrogenases Adh1 and Adh2. The former was more abundant at 1 h of ethanol stress, while the level of the latter had increases in both periods (Figure 1 and

Table S1). The reaction catalyzed by these enzymes produces NADH molecules, which can help the cell to eliminate the reactive oxygen species (ROS) formed when exposed to ethanol.

Finally, the NAD(P)H-dependent D-xylose reductase was identified in several spots, some of which presented modifications in at least one unique peptide (such as oxidation or carbamethylation - data not shown). Although this enzyme presented low abundance in a number of spots, most of them had the protein level increased (three spots) at 1 and 4 h of exposure to ethanol (Figure 1 and Table S1).

On the other hand, the abundance of metabolites that participate in glutathione metabolism, which is important in ROS elimination, decreased at 1 h (Figure 4A and Table S3).

Another effect caused by exposure to ethanol is osmotic stress. Here, the mitochondrial outer membrane protein porin 1 - Por1, which can be related to mitochondrial osmotic stress response - was more abundant at 1 h of ethanol stress (Table S2 and Figure 1). Moreover, the sorbitol level was unusually high at 4 h (Table S3). This compound is a compatible solute, resulting from galactose metabolism (Figure 4C) which can assist the yeast cell in its response to osmotic stress.

Effect of ethanol on heat shock protein abundance and trehalose accumulation

With regard to the protein folding process, the heat shock proteins (HSPs), such as Hsp26, Hsp78 and HSP 104 as well as other chaperones Ssa2 and SsaA3 - were differentially abundant. Most of them were more abundant at 1 and 4 h of ethanol stress (Tables S1 and S2). At 2D-PAGE, 12 out of 68 spots were identified as protein involved in protein folding (Figures 1 and 2).

On the other hand, the trehalose level, which is associated with ethanol tolerance, decreased at 1 h of stress (Table S3).

Effect of ethanol on plasma membrane

The plasma membrane ATPase was highly abundant at 1 h of exposure to ethanol (Table S2 and Figure 1), suggesting its pivotal role in counteracting the proton influx caused by the increase in membrane permeability. To verify the membrane permeability alteration caused by ethanol, we evaluated whether propidium iodide staining was capable of binding to DNA using a flow cytometer.

The analysis pointed out that the plasma membrane of *K. marxianus* was damaged at 1 h of ethanol exposure (Figure 6). Therefore, the increase in abundance of plasma membrane ATPase seems to be a way of responding to ethanol stress.

Moreover, the protein Small COPII coat GTPase Sar1 was less abundant at 4 h of ethanol stress (Table S2 and Figure 1). This protein is required in vesicle transport and mediates the exit of newly synthesized plasma membrane ATPase from the endoplasmic reticulum. This could explain the ATPase non-abundance increase at 4 h. Indeed, in this period, plasma membrane permeability is highly restored, since cell viability (Figure 6) and culture OD_{600nm} (data not shown) increase compared to the controls.

Finally, the ergosterol level, a membrane compound associated with ethanol tolerance in *S. cerevisiae*, decreased at 4 h of ethanol stress (Table S3).

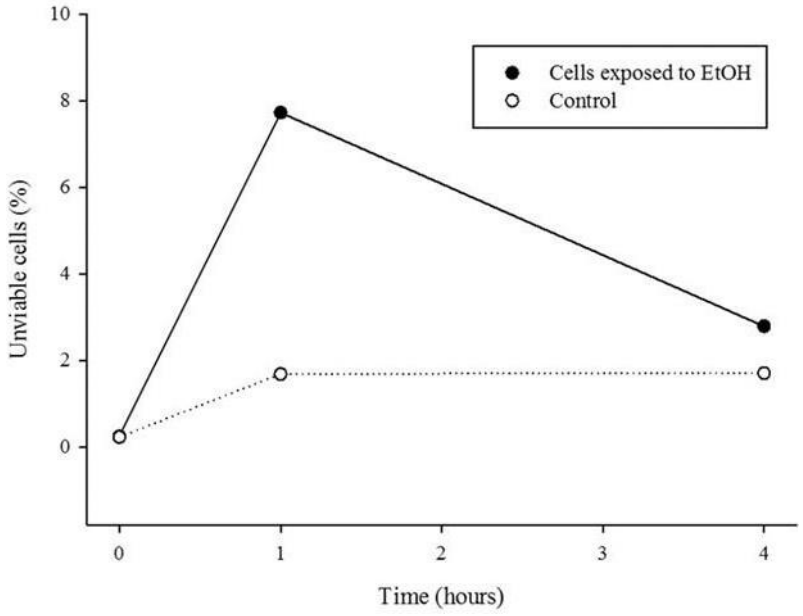

Fig. 6 Percentage of unviable *K. marxianus* cells stained by PI related to membrane permeability caused by ethanol (EtOH) at 0, 1 and 4 hours of ethanol exposure or not, obtained by flow cytometer analysis. Ten thousand cells (10,000 events) were counted for each treatment.

DISCUSSION

K. marxianus showed different responses at 1 and 4 h on exposure to ethanol. It is interesting to note that most of the differentially abundant proteins presented isoforms at 2D-PAGE, mainly glycolytic enzymes and HSPs (Figure 2). These isoforms exhibited modifications in at least one unique peptide (such as oxidation or carbamethylation - data not shown), which caused changes in the gels. Interestingly, *S. cerevisiae* also presented differential abundance of several

isoforms of proteins such as enolase, phosphoglycerate mutase and HSPs in response to long-term fermentations, which lead to ethanol stress (Trabalzini *et al.*, 2003; Rossignol *et al*., 2009). In addition, studies have pointed out that the occurrence of isoforms, as well as their differential abundance, is related to post-translation modifications (Sinclair *et al*., 2006; Perrot *et al*., 2009).

As regards carbon metabolism, at 1 h of ethanol stress most of the glycolytic enzymes were more abundant while at 4 h most of them were less abundant (Figure 1, Tables S1 and S2). On the other hand, the metabolites had decreased levels in both periods of analysis, mainly at 4 h of stress (Figures 4A and 4B and Table S3). In addition, there was no significant lactose nor ethanol consumption over the period of stress (Figure 5). The absence of lactose uptake is likely related to proton motive force disruption and a reduction in the ATP pool, since this sugar is transported into the cell by a proton symport (de Bruijne *et al.*, 1988). The lack of lactose transport may have triggered a "pseudo-starvation" state in the yeast cells, similar to that presented by *S. cerevisiae* when it was exposed to 1 h of ethanol 5% (v/v) (Chandler *et al*., 2004). In this state, despite the carbon availability in the medium, the reduced intracellular levels of D-glucose and D-galactose may have induced the increase of most glycolytic enzymes abundance (Stanley *et al*., 2010). However, the deficiency of cofactors of these enzymes, such as ATP, caused by the proton motive force disruption, may have prevented lactose consumption. Indeed, the level of some metabolites related to glycolytic process decreased in this period (Table S3). At 4 h, the aforementioned enzymes were less abundant, indicating that the processes involving them are not active. Chandler *et al*. (2004) observed similar results after 3 h of ethanol exposure regarding the expression of those enzymes.

In transcriptomic analysis of *K. marxianus* subjected to ethanol stress, most of the genes related to carbon metabolism had their expression repressed in both analyzed periods (Diniz et al., 2017). Interestingly, the expression of glycolytic genes in *S. cerevisiae*, contrary to *K. marxianus*, increases at both the transcriptional and post-transcriptional level under ethanol stress in the majority of studies (Alexandre *et al*., 2001; Dinh *et al*., 2009; Rossignol *et al*., 2009; Parapouli *et al*., 2010; Zheng *et al*., 2013; Chen *et al.*, 2016; Navarro-Tapia *et al*., 2016). These results are consistent with the fact that ethanol tolerant yeasts such as *S. cerevisiae* display accelerated glycolytic and pentose-phosphate pathways, in

order to convert glucose into ethanol and to maintain an adequate redox balance (Hong *et al*., 2010; Ma & Liu, 2010; Zheng *et al*., 2013; Tian *et al*., 2017).

Furthermore, proteomic analysis revealed that the translation process is impaired in *K. marxianus* under ethanol stress, since ribosomal proteins and enzymes from the biosynthesis of amino acids were less abundant at both 1 and 4 h of stress, as well as the arginase being more abundant at 4 h (Figure 1, Tables S1 and S2). Consistent with this, metabolomic data yielded evidence suggesting that the level of metabolites generated from the biosynthesis of amino acids and Aminoacyl-tRNA biosynthesis decreased and that phenylalanine catabolism increased (Figure 4 and Table S3). These results are in agreement with the transcriptomic data, which revealed the down-regulation of genes related to translation, including those that encode to 40S ribosomal protein S18 (Diniz *et al.*, 2017). Thus, the transcriptomic, proteomic and metabolomic data are consistent with the lowest specific growth rate of *K. marxianus* (CCT 7735) under ethanol stress (Silveira *et al*., 2005). Similar results were observed in the ethanol-stressed *S. cerevisiae* (PMY1.1), *i.e.*, down-regulation of the genes related to translation (Chandler *et al*., 2004). It should be pointed out that the period of ethanol exposure was similar to that adopted in our work. Nevertheless, most of the studies point out that genes related to ribosome biosynthesis and translation process are up-regulated in conventional yeast (Dinh *et al*., 2009; Li *et al*., 2010; Kasavi *et al*., 2016).

We still observed, however, in *K. marxianus* that the amino acid level, specifically that of arginine and glutamate, decreased in response to ethanol. In *S. cerevisiae*, it has been reported that a number of amino acids such as proline, tryptophan and, more recently, arginine and glutamate play a protective role against ethanol stress (Fujita *et al*., 2006; Hirasawa *et al*., 2007; Kaino & Takagi, 2008; Dinh *et al.*, 2009; Chen *et al.*, 2016; Cheng *et al.*, 2016; Hashim & Fukusaki, 2016; Kim *et al*., 2016). It is probable that this difference is related to lower ethanol tolerance of *K. marxianus* compared to *S. cerevisiae*. Therefore, the overexpression of gene encoding enzymes involved with both arginine and glutamate biosynthesis may improve ethanol tolerance in *K. marxianus*.

Furthermore, ethanol stress triggers a dramatic increase in ROS in yeast cells, which can promote lipid peroxidation and compromise protein and DNA conformation (Du & Takagi, 2007; Morano *et al*., 2012). Therefore, proteins able to counteract these effects should be activated during this condition. Here, several

ways of maintaining the reduced cell environment and avoiding possible damage were observed.

Firstly, enoate reductase 1 was more abundant in both analysis periods (Table S1, Figures 1 and 2). This protein is known as an old yellow enzyme (OYE) which has flavin-dependent oxidoreductase activity and, because of this, it is an important biocatalyst in the generation of a variety of pharmaceuticals, fragrances, and chemicals (Toogood *et al*., 2010; Raimondi *et al*., 2011). In *K. marxinaus* and *K. lactis*, the OYE protein, encoded by *KYE1* gene, presents an identity ranging from 76% to 99% with the protein Oye2 of *S. cerevisiae* (Raimondi *et al*., 2010). The physiological role of OYEs still remains uncharted, but a number of studies indicate that it may be to act as a potent antioxidant protein in *S. cerevisiae* (Odat *et al*, 2007). Furthermore, Trotter *et al*. (2006) showed that its expression is induced by acrolien exposure, which is a reactive aldehyde formed during lipid peroxidation. Since exposure to ethanol causes lipid peroxidation, it is reasonable to admit that in *K. marxianus* the enoate reductase 1 high abundance is related to the oxidative stress response induced by ethanol. Oye2p high abundance in *S. cerevisiae* was reported in only two proteomic analyses carried out during either the exponential or stationary phases in the wine fermenting process (Rossouw *et al*., 2010; Moreno-García *et al*., 2014).

The induction of enzymes that increases the reducing power in yeast cells is an important response to counteract the ROS generated during ethanol stress (Morano *et al*., 2012). In the present study, Adh 1 was more abundant at 1 h of ethanol stress while Adh2 was abundant in both periods (Table S1 and Figure 1). These enzymes contribute to the generation of NADH when they catalyze the ethanol oxidation of ethanol into acetaldehyde (Wills, 1976). On the other hand, Diniz *et al.* (2017) observed *ADH6* up-regulation only; the other alcohol dehydrogenases were down-regulated. The differences between data can be due to post-translational modifications. In *S. cerevisiae*, the high abundance of alcohol dehydrogenases is frequently reported (Rossouw *et al*., 2010), as evidence of their importance in ethanol tolerant yeasts (Ma & Liu, 2010).

Interestingly we identified that NAD(P)H-dependent D-xylose reductase, which is responsible for the interconversion between xylitol and xylose, was more abundant even in the absence of xylose in the culture medium (Table S1 and Figure 1). Consistent with this result, Schabort *et al.* (2016) observed that *K. marxianus* (UFS-Y2791) presented up-regulation of NAD(P)H-dependent D-

xylose reductase gene even when it was cultured in medium without xylose. Furthermore, a constitutive expression of this enzyme was seen in *K. lactis* at both mRNA and protein levels (Billard *et al*., 1995). Since it is involved with reducing power generation (Wang *et al*., 2007), it probably plays an important role against oxidative stress caused by ethanol. Thus, the result observed in our study indicates that enzyme synthesis induction is not exclusively dependent on the xylose presence in the medium and, since it was more abundant when subjected to ethanol stress, it seems to be important in NAD(P)H generation.

Another consequence of ethanol exposure in *S. cerevisiae* is osmotic stress (Ma & Liu, 2010). Here, a protein that could be related to the osmotic stress response was more abundant at 1 h of ethanol exposure: the mitochondrial outer membrane protein porin 1 - Por1 (Table S2 and Figure 1). This protein is the major porin in yeasts and is involved in the formation of mitochondrial pore. The pore can maintain mitochondrial integrity and assembly during the trafficking of charged molecules across the outer mitochondrial membrane, and counteract osmotic stress (Sánchez *et al*., 2001). Thus, in *K. marxianus* Por1 abundance seems to be important for osmotic stability of yeast mitochondria, which was affected by ethanol. Diniz *et al.* (2017) did not report *POR1* up-regulation, but, in *S. cerevisiae*, Por1 was more abundant and related to ethanol tolerance (Moreno-García *et al*., 2014).

Furthermore, at 4 h of ethanol stress, the sorbitol level increased (Table S3). In the ethanologenic bacterium *Zymomonas mobilis*, this compound was related to ethanol tolerance since it improves bacterium growth, acting as a compatible solute under ethanol stress (Sootsuwan *et al*., 2013; Zhang *et al*., 2015). In *S. cerevisiae*, even though sorbitol has not been reported as an ethanol tolerance mechanism, its elevated level was observed when subjected to this stress condition (Kim *et al.,* 2016). Sorbitol synthesis is involved with galactose metabolism (Figure 4C) and its high content seems to be associated with osmotic stress imposed by ethanol.

It is well known that exposure to ethanol and heat shock share similarities in yeast cells, since hydrophobic interactions within native proteins are disestablished, and their functional conformation is altered leading to protein aggregation. This induces association with heat shock proteins (HSPs) as well as other chaperones, in order to fold and repair the damaged proteins (Piper, 1995). In this study, the elevated abundance of Hsp26, Ssa3 and Hsp78 was highly noted

at 1 and 4 h of ethanol stress (Figure 1 and Tables S1, S2). Hsp26 is a member of the small HSPs family that binds to unfolded proteins and prevents their aggregation in a co-operative manner (Burnie *et al*., 2006). SSa3 is a Hsp70-homolog protein that also prevents the aggregation of denatured proteins by binding to exposed hydrophobic regions (Hasin *et al*., 2014). Hsp78, in turn, is the major mitochondrial chaperone component, conferring tolerance and/or protection on proteins by resolubilizing them from already formed insoluble aggregates and assisting in their degradation by proteases (Leidholda *et al.*, 2006; Lewandowska *et al*., 2009; Voos, 2009). Interestingly, Diniz *et al.* (2017) also observed the up-regulation of *HSP26* and *HSP78* genes. In *S. cerevisiae*, the high abundance of Hsp26, Hsp78, Ssa2 and Ssa3 under ethanol stress has also been reported (Rossignol *et al*., 2009; Rossouw *et al*., 2010; Moreno-García *et al*., 2014; Santos *et al*., 2017). Therefore, the abundance increase of heat shock proteins seems to be a common response to ethanol in yeasts, in both tolerant or non-tolerant species.

Trehalose is a compatible solute related to the protection of proteins and plasma membrane during stress conditions (Mahmud *et al*., 2012). However, the trehalose level decreased in *K. marxianus* (CCT 7735) at 1 h under ethanol stress (Table S3). Another study also verified the decrease of trehalose in *K. marxianus* with high ethanol concentration (Ribeiro *et al*., 1999). At the transcriptional level, the expression of genes related to trehalose biosynthesis was not altered (Diniz *et al.*, 2017). In contrast, *S. cerevisiae* increased the abundance of enzymes involved in its biosynthesis under ethanol stress (Rossouw *et al*., 2010; Santos *et al*., 2017). Indeed, it accumulates trehalose in response to ethanol (Chen *et al.*, 2016; Hashim & Fukusaki, 2016; Kim *et al*., 2016; Ohta *et al*., 2016). This difference may also be related to lower ethanol tolerance of *K. marxianus* as compared to *S. cerevisiae*, since this yeast does not have enough ATP to synthesize trehalose under this stress condition, since the pathways related to energy generation seem to be inactive. Thus, metabolic engineering strategies might focus on increasing trehalose synthesis in *K. marxianus*, in order to improve its tolerance under ethanol stress.

The yeast plasma membrane has been considered the main target of ethanol. Thus, its integrity has been severely impaired, leading to proton influx and reduction of intracellular pH (Henderson & Block, 2014). In order to counteract this influx, *S. cerevisiae* H^+-ATPase pumps protons out of the cell using the free energy of ATP hydrolysis to generate a pH gradient (Ferreira *et al*.,

2001; Aguilera *et al*., 2006; Sampedro *et al*., 2014). In spite of gene encoding ATPase were not being up-regulated in *K. marxianus* (CCT 7735) (Diniz *et al.*, 2017), we observed in the present study that its abundance at the protein level was elevated at 1 h of ethanol stress (Table S2 and Figure 1). Rosa & Sá-Correia (1992) verified that the ATPase activity in *K. marxianus* increased in response to 3% of ethanol. Thus, the plasma membrane ATPase induction reveals an important response mechanism of *K. marxianus* to ethanol stress. Since the enzyme consumes ATP to pump protons out of the cell, the biomass formation is impaired, which is in agreement with the low specific growth rate observed under ethanol stress (Silveira *et al.*, 2005).

In *S. cerevisiae*, H^+-ATPases and the genes encoding them have been induced under ethanol stress (Alexandre *et al*., 2001; Fujita *et al*., 2006; Dinh *et al*., 2009; Navarro-Tapia *et al*., 2016). Interestingly, molecules that improve their activity, such as inositol, are reported as well (Ding *et al*., 2010; Hong *et al*., 2010; Ohta *et al*., 2016). This molecule, which is present in certain glycerophospholipids, such as phosphatidylinositol, alters the membrane composition, and it was reported that this change, caused by the increase of inositol level, leads to increases in ATPase activity (Furukawa *et al*., 2004).

Here, the inositol-1-phospate level was elevated at 4 h of ethanol stress and had a surprising Log_2 Fold change of 14 (Table S3 and Figure 4C). This metabolite is a constituent of phospholipids and an inositol precursor, which can enhance the plasma membrane ATPase activity, as previously mentioned. However, the protein Small COPII coat GTPase Sar1, which is required in the transport of vesicles and mediates the exit of newly synthesized plasma membrane ATPase from the endoplasmic reticulum (Ferreira *et al*., 2001; Venditti *et al*, 2014), was less abundant at 4 h of exposure to ethanol in *K. marxianus* (Table S2 and Figure 1). Although the differential regulation of this protein was not observed in *K. marxianus* at the transcriptional level (Diniz *et al.*, 2017), its reduced abundance might be associated with the lack of plasma membrane ATPase increased abundance at 4 h. Indeed, the plasma membrane permeability of this yeast was highly restored when compared to the controls (Figure 6). Thus, during this period of ethanol stress, *K. marxianus* appears to control intracellular acidification and to reach pH homeostasis. In *S. cerevisiae*, in turns, Sar1 protein was not reported during ethanol stress, but when inositol was limited in the medium, yeast cells leaked intracellular metabolites such as nucleotides,

phosphate and potassium membrane during this stress condition (Furukawa *et al*., 2004).

The ergosterol decreased in *K. marxianus* at 4 h under ethanol stress. However, Diniz *et al.* (2017) observed that the ergosterol concentration did not alter when exposed to ethanol. We believe that this difference is related to the methodology used, since these authors employed target analysis to measure the ergosterol concentration, which is a more precise method. Nevertheless, it should be pointed out that both results are in contrast to those obtained for *S. cerevisiae*. Studies have shown that *S. cerevisiae* increases the synthesis of ergosterol under ethanol stress in order to stabilize the plasma membrane, the main target of ethanol (Vanegas *et al*., 2012; Chen *et al*., 2016). Based on these results, we hypothesize that the overexpression of gene encoding enzymes of the ergosterol biosynthesis pathway in *K. marxianus* may improve its tolerance to high ethanol concentrations.

Protein and intracellular metabolite profiles of the thermotolerant yeast *Kluyveromyces marxianus* under 1 and 4 h of ethanol stress were evaluated. Summarizing, the abundance of proteins involved mainly in carbon pathways at 4 h and translation process proteins as well as the ergosterol, trehalose and amino acids level were impaired, corroborating the low growth rate of this yeast grown under ethanol stress. On the other hand, *K. marxianus* seems to respond to the stress by increasing the abundance of proteins and metabolites that counteract the oxidative and osmotic stresses as well as heat shock proteins. Interestingly, the plasma membrane ATPase was highly abundant at 1 h in order to replenish the proton gradient. In general, proteomic and metabolomic results present some differences from the transcriptomic ones, which are expected since post-translational modifications can occur. Thus, the results obtained in this study provide complementary insights to understanding metabolic and physiological changes which are found in *K. marxianus* under ethanol stress, suggesting possible targets for the construction of an ethanol tolerance yeast strain.

EXPERIMENTAL PROCEDURES

The experiments were performed at the Laboratory of Microbial Physiology and at the Center for Analysis of Biomolecules, both at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Microorganism's strain and maintenance

The yeast *Kluyveromyces marxianus* (CCT 7735) was used. It had previously been designated as *K. marxianus* (UFV-3), isolated from a dairy plant in Minas Gerais (Silveira *et al*., 2005) and deposited in the Tropical Culture Collection Tonsello André Foundation, Campinas, São Paulo, Brazil. The cells were maintained in YP medium [1% (w/v) yeast extract and 2% (w/v) peptone] and 50% glycerol (v/v) at -80 °C, or in YPD solid medium [2% (w/v) agar and 2% (w/v) glucose] at 4 °C.

Culture conditions and ethanol stress

K. marxianus (CCT 7735) was activated in YPD medium and incubated at 37 ºC, 200 rpm for 16 h, in order to obtain the inoculum. It was harvested at 3,000 x *g* at 4º C for 5 min and the pellet was washed twice with 0.01% (w/v) peptone water. Then, *K. marxianus* was cultured in three batch cultures in YPL [2% (w/v) lactose, pH 5.5], with 0.2 as initial optical density (OD_{600nm}), determined by microplate spectrophotometer (Multiskan GO, Thermo Scientific, Helsinki, Finland) at absorbance at 600 nm. The cultures were incubated at 37 ºC with a stirring rate of 200 rpm so as to reach an absorbance value of 0.8. This step involved a 400 mL aliquot being collected. Next, in order to expose the yeast to ethanol stress, ethanol was added to reach a final concentration of 6% (v/v) and the cultures were incubated. At 1 and 4 h of ethanol exposure, 400 mL aliquots were sampled, frozen with liquid nitrogen and maintained at -80 ºC. In parallel, three control cultures of *K. marxianus* were treated under the aforementioned conditions without the addition of ethanol.

Protein extraction and quantification

The samples were unfrozen and submitted to protein extraction. Two protein extraction methods were performed: the first one in order to obtain intracellular proteins (high abundant proteins) and the second one to extract membrane proteins (low abundant proteins). Intracellular proteins were obtained according to Zhao *et al*. (2014) protocol, with modifications. Approximately 1 g of wet cell was washed with phosphate-buffered saline (PBS buffer - 137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and resuspended in an extraction buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) Triton X-100, 1 mM PMSF (phenylmethylsulfonyl fluoride)]. The suspension was

submitted to cellular lysis using TissueLyser II (Qiagen, Hilden, Germany), followed by sonication. The cell extract was harvested and the supernatant collected.

Membrane proteins, in turn, were obtained in accordance with Cox *et al*. (2008) protocol, with modifications. Approximately 0.5 g of wet cell was washed in a PBS buffer and resuspended in 1 mL of an extraction buffer (1 M sorbitol, 40 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The suspension was incubated at 30 ºC for 10 min. To remove the cell wall, 1 mg of zimolase per gram of cell was added and incubated at 30 ºC for 30 min. Spheroplasts were collected by centrifugation at 500 x g for 10 min. Then they were resuspended in a lysis buffer (200 mM sorbitol, 50 mM HEPES, pH 7.0, 1 mM PMSF) and incubated on ice for 1 h. Spheroplasts were lysed by homogenization using a glass mortar and pestle. They were harvested at 1,000 x g for 5 min, the supernatant was transferred to a new tube and was then harvested at 13,000 x g for 20 min to separate the membrane fraction. This fraction was solubilized in 100 µL of binding buffer [1% (w/v) SDS, 20 mM NaH_2PO_4 pH 7.4, 100 mM NaCl, 10 mM imidazole, 20% (v/v) glycerol] and stirred for 30 min.

In both cases, the protein quantification was performed according to the Bradford method (1976).

Protein separation

Intracellular proteins were separated through two-dimensional electrophoresis (2D-PAGE), while proteins obtained from the second extract were separated by one dimensional electrophoresis (1D-PAGE).

For the separation of proteins in the first dimension of the 2D-PAGE (isoelectric focusing - IEF), intracellular proteins were solubilized in rehydration solution [DeStreak Rehydration Solution and 3% (v/v) immobilized pH gradient (IPG) buffer pH 3 to 10 (both from GE Healthcare, Chicago, IL, United States)]. For the preparative gels, aliquots of 1,000 µg of proteins were loaded into the mixture and centrifuged at 12,000 x g for 1 min at 20 °C. The supernatants were used for rehydration in immobilized pH Gradient (IPG) strips (GE Healthcare, Chicago, IL, United States). The IPG strips of 24 cm, pH 3-10, were rehydrated in 450 µL of the protein solution for 12 h at room temperature in the Immobiline DryStrip Reswelling Trays (GE Healthcare, Chicago, IL, United States). The IEF

were performed using the Ettan IPGphor III System (GE Healthcare, Chicago, IL, United States) at 20 °C. The following running conditions were used in the Ettan IPGphor III System: 1: step and hold at 200 V for 10 h; 2: step at 500 V for 3 h; 3: gradient at 1,000 V for 800 Vh; 4: gradient at 10,000 V for 20,000 Vh; 5: step at 10,000 V for 30,000 Vh; 6: step and hold at 5,000 V for 8 h. After the IEF was completed, the strip gels were stored in equilibration tubes (GE Healthcare, Chicago, IL, United States) at -80 °C to proceed to the second dimension.

Next, focused IPG strips were equilibrated for 30 min in an SDS equilibration buffer solution [6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol (v/v), 2% SDS (v/v), 0.002% bromophenol blue (v/v) and 1% DTT (v/v)], and for an additional 30 min in the same equilibration buffer using 2.5% iodoacetamide instead of DTT. After equilibration, proteins were separated in the second dimension on 10% SDS-PAGE gels at 8° C. Electrophoresis was performed at constant power in two steps. The first step consisted of 10 mA per gel being applied for 45 min, and the second step of 40 mA per gel for 5 h 30 min – 7 h. After the electrophoresis procedure, the gels were immersed in fixation solution with 50% (v/v) methanol and 10% (v/v) acetic acid overnight. Next, the gels were stained following the Colloidal Coomassie Blue G-250 (Merck, Darmstadt, Germany) procedure (GE Healthcare, 2004) for three days for protein detection. The stained gels were transferred to a solution of 10% acetic acid (v/v), 30% ethanol (v/v), and 60% double-distilled water, which was exchanged twice in 1 h until subsequent image analysis.

The separation of proteins by 1D-PAGE was carried out under the same conditions as the aforementioned SDS-PAGE. However, the duration of the gel run was sufficient only to separate the samples for 2 cm in the gels.

Gels analysis

The 2D-PAGE and 1D-PAGE gels were scanned on an Image Scanner using Lab Scan software version 6.0 (GE Healthcare, Chicago, IL, United States) at 300 dots per inch. Spot detection and quantification were carried out by the Image Master 2D Platinum software program version 7.0 (GE Healthcare, Chicago, IL, United States). As for the 2D-PAGE gels, the parameters for spot detection were as follows: smooth factor 2.0, saliency 20.0 and minimal area 51 pixels. The reference gel was used taking into account the greater number of spots and then it was used for matching corresponding protein spots between gels. For

comparative image analysis, the images were grouped, after the intensity of the individual spots, and they were analyzed and compared within and between the image treatment groups of treatments ("0 h control", "1 h control", "1 h with ethanol", "4 h control" and "4 h with ethanol"). The changes in spot patterns revealed by computer-based image analysis were individually investigated and confirmed. Student's *t*-test was used considering a *p*-value < 0.05 and 1.5 of fold change. Thus, the spots related to differently abundant proteins under ethanol stress were selected and subjected to protein identification through a MALDI TOF/TOF mass spectrometer.

Protein digestion

The protein spots selected in 2D-PAGE were cut out from gels using pipet tips of 1,000 µL and extracted from gels by gentle suction. In 1D-PAGE, in turn, 2 cm of samples were completely cut into 10 gel fractions (0.2 cm each one) in order to digest all proteins contained in the samples. In both cases, gel piece proteins were digested according to the Shevchenko *et al*. (2007) protocol: gel pieces were dehydrated twice with 50% acetonitrile (v/v) and 25 mM ammonium bicarbonate, pH 8.0, and dried at room temperature overnight. Then, dehydration was performed twice with the first solution. After that, gel pieces were washed with 100% acetonitrile (v/v) twice and they were dried in vacuum concentrators (SpeedVac, Thermo Scientific, Vantaa, Finland). Thus, gel pieces were reduced with 65 mM dithiothreitol (DTT) at 56 ºC for 30 min. Trypsinization was performed using a modified method based on Shevchenko *et al*. (2007). Gel pieces were alkylated with 200 mM iodoacetamide and 100 mM ammonium bicarbonate pH 8.0 for 30 min at room temperature and then digested with 25 $ng.\mu L^{-1}$ of trypsin solution (Trypsin from porcine Pancreas, proteomics Grade, Sigma-Aldrich, St. Louis, MO, United States). A solution with 10% acetonitrile (v/v) and 40 mM ammonium bicarbonate was added. In-gel tryptic degradation was carried out overnight at 37 °C. Subsequently, the samples were sonicated for 10 min, their supernatants were removed and a solution with 5% formic acid (v/v) and 50% acetonitrile (v/v) was added. After 15 min of reaction at room temperature, the samples were sonicated for 2 min. Finally, the samples were dried in vacuum concentrators and then stored at -20 ºC for subsequent analysis.

Protein identification using MALDI-TOF/TOF

Protein identification was carried out using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/TOF Ultraflex III, Bruker Daltonics, Bremen, Germany). The samples were solubilized in 12 μL of 0.1% TFA (v/v). One μL of each sample was applied to the matrix solution α-cyano-4-hydroxycinnamic acid (CHCA) [at 10 $\mu g.mL^{-1}$ in 50% acetonitrile (v/v) and 0.1% TFA (v/v)] to the MALDI-TOF/TOF steel plate (MTP 384, Bruker Daltonics, Bremen, Germany). The MALDI-TOF spectra was calibrated according to external standards (Peptide Calibration Standard II, Bruker Daltonics, Bremen, Germany).

MS analysis was conducted using the Reflective Positive Peptide method. The following parameters were applied: laser repetition rate at 50 Hz, ion source voltage 1 to 20 kV, ion source voltage 2 to 18.3 kV, ion source lens voltage 6.75 kV and number of shots: 200. Using the FlexAnalysis software program, version 3.3 (Bruker Daltonics, Bremen, Germany), the peptide masses were searched against the NCBI and SwissProt database, version 54.4, with 0.5 error using the Mascot program, version 2.4.0 (http://www.matrixscience.com; Matrix Science, London, United Kingdom), and MASCOT Peptide Mass Fingerprinting database search. The initial search variables for MS analysis allowed for a single trypsin missed cleavage, no restriction on protein mass, carbamidomethyl (C) for fixed modifications, oxidation (M) for variable modifications, peptide mass tolerance of 0.2 and the taxonomic search space was restricted to *K. marxianus*.

Protein identification by MS/MS analyses (MALDI-TOF/TOF), was made, in turn, using the LIFT method and the processing data set was carried out using the FlexAnalysis software program, version 3.3, and the Biotools software program, version 3.2 (Bruker Daltonics, Bremen, Germany). The peptide ions were searched against the NCBI and SwissProt database version 54.4 ($p < 0.05$) using the Mascot program, version 2.4.0 (http://www.matrixscience.com), and MASCOT MS/MS Ion Search database. The search variables for MS/MS analysis allowed for a single trypsin missed cleavage; peptide charge 1+; carbamidomethyl (C) for fixed modifications; oxidation (M) for variable modifications; 0.02 peptide mass tolerance; 0.5 MS/MS tolerance and the taxonomic search space was restricted to *K. marxianus*.

Finally, the results obtained by MASCOT were validated using the Scaffold software program, version 3.6.4 (Proteome Software Inc., Portland, OR,

United Stataes), with a 90% probability for peptide and protein identification, minimum 1 unique peptide and *K. marxianus* (CCT 7735) database.

Protein identification using NanoUHPLC-QTOF

The tryptic peptides from the excisions were solubilized in 70 μL of 0.1% formic acid solution (v/v), and they were stored in tubes. The tubes were agitated for 20 seconds and centrifuged by 6,000 x g for 10 min. After centrifugation, 50 μL of the solution were transferred to a vial. Next, 20 μL were used for analysis through Nano Liquid Chromatography – Mass Spectrometry (nano LC-MS) using the nanoACQUITY UPLC system (Waters, Milford, MA, USA), containing a trap column and a capillary column ProteCol GHQ303 C18 3,0 μm – 300 μm × 150 mm, operating at a flow rate of 4.5 $\mu L.min^{-1}$.

The eluted peptides were injected automatically into the mass spectrometer micrOTOF QII® (Bruker Daltonics, Bremen, Germany), working in online mode, with the aid of a microESI ionization needle. This step consisted of mobile phase solutions being used for the gradient program as follows: (A) water and 0.1% formic acid (v/v) and (B) acetonitrile and 0.1% formic acid (v/v). The following gradient program was used: a linear rising ramp starting at 10% increasing to 50% (B) for 30 min, 50% (B) for 5 min, linear rising ramp starting at 50% increasing to 90% of (B) for 3 min, 90% (B) for 2 min, linear gradient descent starting at 90% decreasing to 10% (B) for 3 min, followed by maintaining steady at 10% (B) for 3 min.

The scanning of ions for MS1 spectra in positive mode was carried out for masses ranging between 300 and 1500 m/z, and between 70 and 2000 m/z for the MS2 spectra. Data were acquired over 46 min in each LC-MS/MS analysis, using the Hystar software program, version 3.2 (Bruker Daltonics, Bremen, Germany), and the spectra were processed through the Data Analysis software program, version 4.0 (Bruker Daltonics, Bremen, Germany), using the default settings for proteomics. The mass spectrometer was operated in self-MSn mode, which collected MS2 spectra for the most intense ions in each whole scan spectrum, excluding ions with simple loads. List peaks were generated in the mascot generic format (mgf) by the Data Analysis software program.

The proteins were identified by the Mascot software program, version 2.4.0 (http://www.matrixscience.com), by the following parameters: cleavage with trypsin, one missed cleavage, fixed modification of carbamidomethylation in

cysteine, variable modification of methionine, 30 ppm MS/MS tolerance and 0.02 peptide mass tolerance. Protein quantification was carried out using the label-free approach by means of the Scaffold software program, version 3.6.4 (Proteome Software Inc., Portland, OR, United Stataes), with a 90% probability of peptide and protein identification, and a minimum of 2 unique peptides and the *K. marxianus* (CCT 7735) database. In order to determine the differential abundance between identified proteins, Student's *t*-test was carried out considering a *p*-value < 0.05 and 1.5 match count.

Metabolite Extraction and Derivatization

To obtain the metabolic profile presented during ethanol stress, the *K. marxianus* yeast (CCT 7735) whether exposed to ethanol or not had its metabolism interrupted after sampling by quenching according to Canelas *et al*. (2008). An aliquot of 1 mL of each sample was added to 1 mL of cold methanol 70% (v/v, -80 ºC). Cells were harvested at 12,000 x g at 4 ºC for 10 min and frozen at -80 ºC.

In order to extract the metabolites, the method described by Villas-Bôas *et al*. (2005) with modifications was used. Contents of 1.5 mL of extraction buffer (water: methanol: chloroform – 1:2.5:1) and 60 µL of the internal standard ribitol (0.2 $mg.mL^{-1}$) were added to the samples. They were homogenized in a vortex and agitated in a ThermoCell Mixing Block MB-101 (Bioer Technology Co., Hangzhou, China) at 950 rpm and 4 ºC for 30 min. After this, the samples were harvested at 6,000 x g and 4 ºC for 10 min, and 1 mL of supernatant was transferred to new tubes. Then, 750 µL of ultrapure water were added and the samples were harvested at 6,000 x g and 4 ºC for 15 min. Subsequently, 50 µL of supernatant were transferred to new tubes and submitted to drying in vacuum concentrators (SpeedVac, Thermo Scientific, Vantaa, Finland) for 30 min.

Derivatization of the metabolic extracts was carried out through a silylation: trimetylsylation reaction described by Lisec *et al.* (2006). The contents of 40 µL of methoxyamine (methoxylamine-hydrochloride 20 $mg.mL^{-1}$) dissolved in pyrimidine were added to the dried samples and homogenized in ThermoCell Mixing Block MB-101 at 950 rpm and 37 ºC for 2 h. Then, 70 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with a standard retention index mixture (FAMES, 20 µL/mL) were added to the samples and agitated in a

ThermoCell Mixing Block MB-101 at 950 rpm and 37 ºC for 30 min. The samples were harvested at 6,000 x g for 10 min.

Metabolite identification

For analysis of the intracellular metabolites, 100 µL of each sample were transferred to a gas chromatography system 7890A (Agilent Technologies, Santa Clara, CA, United States), using a capillary column DB-35MS, 30 m x 0.32 mm x 0.25 µm (Agilent Technologies, Santa Clara, CA, United States), coupled to a mass spectrometer TruTOF HT TOFMS (LECO Instruments, Saint Joseph, MI, United States): GC-TOF/MS. For the chromatographic processing and spectral deconvolution masses, the ChromaTOF software program, version 4.50.8.0 (LECO Instruments, Saint Joseph, MI, United States) was used, and the spectral masses library of compounds derived from trimethylsilyl (TMS), obtained from the Max Planck Institute of Molecular Plant Physiology (Potsdam, Germany) (http://csbdb.mpimp-golm.mpg.de/csbdb). Peak assignment was verified using the TargetSearch software program (Cuadros-Inostroza *et al.*, 2009). The areas from the chromatographic peaks to the fragmented ions were verified, normalized by the area of the peak corresponding to ribitol and corrected by the optic density (OD_{600nm}) of each culture.

The data obtained were exported to the MetaboAnalyst software program, verison 3.0 (http://www.metaboanalyst.ca/faces/home.xhtml), to carry out a principal components analysis (PCA). In the PCA graphic representations, the group of biological samples is based on similarities and differences presented in a metabolic data set, such as the following contrasts: 1 h of ethanol exposure versus 0 h control; 4 h of ethanol exposure versus 0 h control; 1 h of ethanol exposure versus 1 h control; and 4 h of ethanol exposure versus 4 h control. Furthermore, in the MetaboAnalyst software program, Student's *t*-test was applied to metabolites identified in the contrasts above in order to analyze their biological significance, considering a p-value < 0.05. The pathway of each identified significant metabolite was established by entering with KEGG compound ID for *K. lactis* (the yeast specie closest to *K. marxianus*) at the KEGG database (http://www.kegg.jp/).

Lactose and ethanol measurements

Samples collected from *K. marxianus* (CCT 7735) cultured at 0 h without ethanol exposure (control) and at 1 and 4 h under ethanol stress were filtered using a 0.22 μm membrane in order to determine lactose and ethanol concentrations present in the culture medium. This required high-performance liquid chromatography - HPLC (Shimadzu LC 20AT, Shimadzu Corporation, Kyoto, Japan) which was used coupled with a refractive index detector and the ion exclusion column Aminex HPX-87H (300 x 7.8 mm) (BIO-RAD Laboratories Inc, Hercules, CA, United States) at 30 ºC. The analyses were carried out applying the mobile phase with 0.005 M sulfuric acid and a constant flow of 0.7 $mL.min^{-1}$. Sample retention times were compared to the retention time and spectral characteristics of the lactose and ethanol standards. The *t*-test considering a *p*-value < 0.05 was applied to the ethanol and lactose concentrations obtained by the R software program, version 3.3.1.

Membrane permeability analysis

A flow cytometer was used for evaluating cell viability related to membrane permeability caused by ethanol, from cells stained by propidium iodide (PI). PI is known to bind to nucleic acids when the cell membrane has affected permeability (Marza *et al.*, 2002). 10^6 cells from a culture sampled at 0, 1 and 4 h of exposure to 6% ethanol and from control cultures were resuspended in 1 mL of PBS buffer (137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Ten μL of propidium iodide (PI) stock solution (1 $mg.mL^{-1}$) were added to 1 mL cell suspension just prior to the analysis. A BD FACSVerse flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA) was used. For all cells, calibration was carried out by selecting a population of disaggregated cells without budding. Ten thousand cells (10,000 events) were counted for each treatment. Fluorescence was detected using the 568/42 nm filter that excites the sample with a 488 nm laser.

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SUPPLEMENTAR MATERIAL

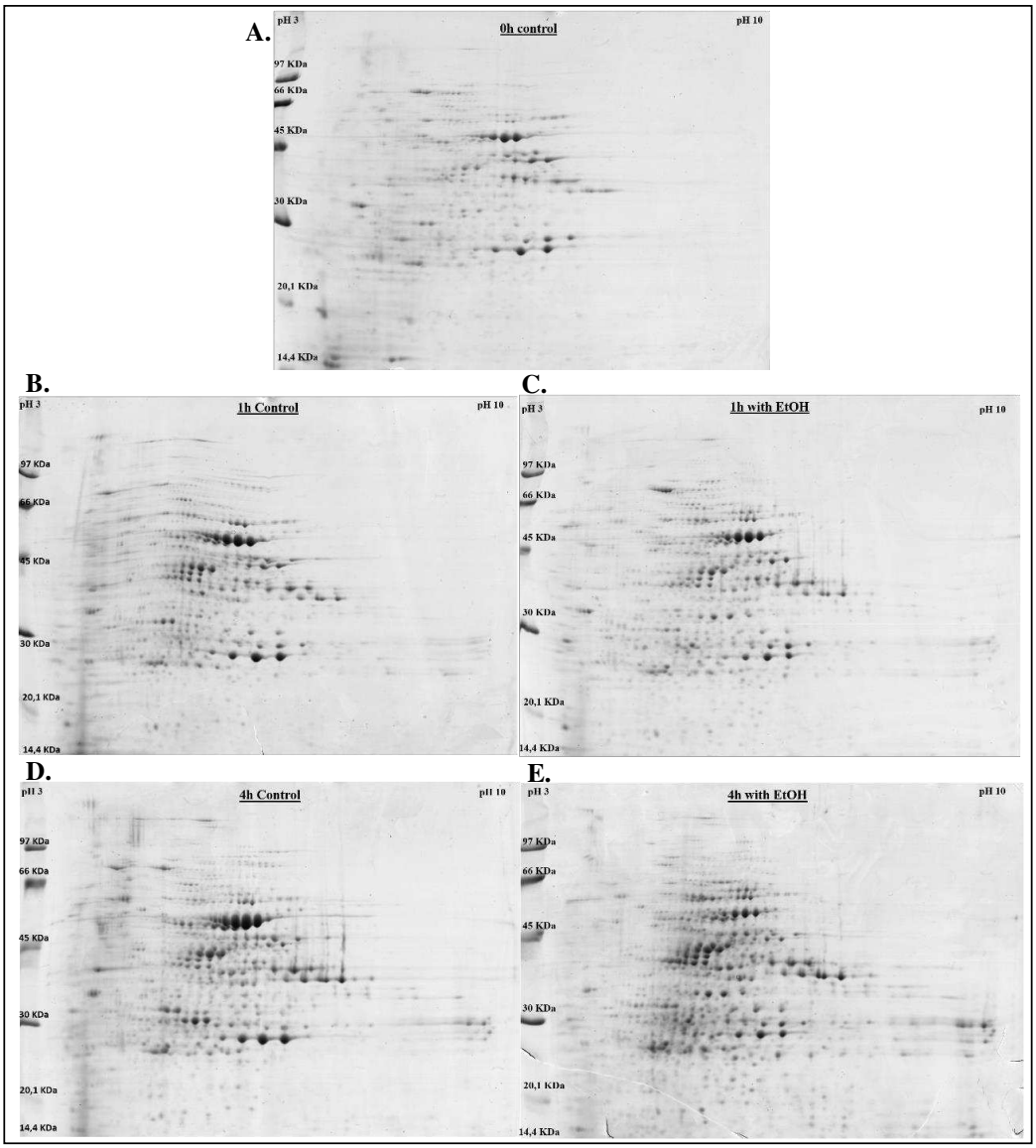

Fig. S1 Intracellular protein expression profile by typical 2D-PAGE images from *K. marxianus* (CCT 7735) at **a.** 0 h control, **b.** 1 h control, **c.** 1 h of ethanol exposure, **d.** 4 h control and **e.** 4 h of ethanol exposure.

Table S1 Differently expressed proteins in ethanol stressed *K. marxianus* (CCT 7735). The proteins were identified by MALDI-TOF/TOF. Red rows represent more abundant proteins and green rows less abundant proteins, according to Log_2 Fold Change.

Ethanol Exposure Time	Spot No.	Protein Name	Accession No. [a]	Score [b]	Sequence Coverage (%) [c]	Student's *t*-test [d]	Log_2 (Fold Change)
1 h	12	Glyceraldehyde-3-phosphate dehydrogenase	W0TG93	122	8	0.0275	1.64
	28	Heat shock protein SSA3	W0THY0	246	6	0.0071	-1.19
	31	Heat shock protein SSA3	W0THY0	198	6	0.0057	1.83

38	Triosephosphate isomerase	W0TDE9	136	10	0.0019	-1.13
39	Triosephosphate isomerase	W0TDE9	144	10	0.0049	-0.83
41	Triosephosphate isomerase	W0TDE9	133	10	0.0029	-1.76
53	Phosphoglycerate mutase 1	W0T6W1	419	19	0.0439	0.62
55	Phosphoglycerate mutase 1	W0T6W1	114	7	0.0029	-1.25
64	Heat shock protein 26	W0T939	83	6	0.0012	3
87	Uncharacterized protein YLR301W	W0T702	138	12	0.0448	-1.11
96	Enoate reductase 1	W0T3V3	170	8	0.0484	-0.72
103	Enoate reductase 1	W0T3V3	276	9	0.0037	1.91
104	Enoate reductase 1	W0T3V3	312	16	0.007	1.55
120	Alcohol dehydrogenase 2	W0TDP8	181	11	0.001	1.45
135	SEC14 cytosolic factor	W0TBV4	143	13	0.05	-1.29
139	Uncharacterized protein	W0T9E1	146	13	0.0473	-2.08
149	NAD(P)H-dependent D-xylose reductase	W0T4K1	251	20	0.0005	2.55
151	NAD(P)H-dependent D-xylose reductase	W0T4K1	270	20	0.0001	3.43
182	Fructose-bisphosphate aldolase	W0T9W3	163	8	0.0028	-1.19
193	Malate dehydrogenase	W0TEG9	159	7	0.0022	1.58
194	Enolase	W0T7K9	318	17	0.0015	4.04
205	Alcohol dehydrogenase 1	W0TBC6	198	16	0.0208	1.61
213	Phosphoglycerate kinase	W0T3H5	385	15	0.0008	-1.33
227	Alcohol dehydrogenase 2	W0TDP8	107	5	0.0293	2.22
248	Heat shock protein SSA3	W0THY0	232	4	0.0154	2.28
251	Heat shock protein SSA3	W0THY0	148	4	0.013	1.68
253	S-adenosyl methionine synthase	W0T4F1	143	4	0.0039	-1.48
259	Enolase	W0T7K9	297	12	0.0012	2.8
263	Enolase	W0T7K9	388	16	0.0009	0.88
265	Enolase	W0T7K9	432	15	0.0022	1.02
337	Heat shock protein SSA3	W0THY0	653	16	0.0005	4.37
338	Heat shock protein SSA3	W0THY0	170	7	0.0017	4.00
397	Enolase	W0T7K9	213	6	0.0004	2.48
417	Heat shock protein SSA3	W0THY0	797	18	0.0082	3.14
451	Heat shock protein SSA3	W0THY0	198	7	0.0022	1.62

	464	Enoate reductase 1	W0T3V3	131	6	0.0015	2.44
	488	Heat shock protein SSA3	W0THY0	83	4	0.0006	2.48
	505	Enoate reductase 1	W0T3V3	103	5	0.0066	2.28
	513	NAD(P)H-dependent D-xylose reductase	W0T4K1	251	11	0.0324	-0.98
	557	Enolase	W0T7K9	196	8	0.0278	1.03
	574	Enolase	W0T7K9	270	11	0.0001	1.33
	592	Phosphoglycerate mutase 1	W0T6W1	210	17	0.0059	1.83
	648	Translationally-controlled tumor protein homolog	W0TG01	187	15	0.0408	-1.08
4 h	16	Heat shock protein SSA3	W0THY0	108	4	0.0383	1.35
	33	Heat shock protein SSA2	W0T5M8	123	5	0.0439	-1.59
	39	Triosephosphate isomerase	W0TDE9	144	10	0.0104	-1.11
	53	Phosphoglycerate mutase 1	W0T6W1	419	19	0.0312	0.59
	73	Enolase	W0T7K9	219	7	0.0092	-1.63
	74	Enolase	W0T7K9	273	12	0.0173	-1.63
	86	Uncharacterized protein YLR301W	W0T702	135	14	0.0389	-2.01
	92	Cytochromo C peroxidase	W0TFP1	183	7	0.0029	-1.85
	103	Enoate reductase 1	W0T3V3	276	9	0.0005	2.5
	104	Enoate reductase 1	W0T3V3	312	16	0.0479	2.85
	120	Alcohol dehydrogenase 2	W0TDP8	181	11	0.0028	1.1
	128	Glyceraldehyde-3-phosphate dehydrogenase	W0T9E0	172	8	0.0007	1.24
	182	Fructose-bisphosphate aldolase	W0T9W3	163	8	0.0303	1.72
	183	Fructose-bisphosphate aldolase	W0T9W3	167	8	0.0035	-1.89
	192	Transaldolase	W0TCV6	90	7	0.0221	0.83
	194	Enolase	W0T7K9	318	17	0.0007	3.78
	215	Phosphoglycerate kinase	W0T3H5	469	21	0.0003	-2.04
	222	Enoate reductase 1	W0T3V3	234	8	0.0356	-1.05
	226	Enoate reductase 1	W0T3V3	248	12	0.0035	1.85
	250	Enolase	W0T7K9	473	15	0.0132	1.28
	254	Enolase	W0T7K9	288	12	0.0277	-1.3
	263	Enolase	W0T7K9	388	16	0.0192	-1.94
	266	Enolase	W0T7K9	123	8	0.0154	-1.47
	272	Enolase	W0T7K9	2176	8	0.0072	-1.61
	565	Arginase	W0TCC3	88	7	0.0459	1.94

a. Uniprot accession number.
b. MASCOT score (; Matrix Science, London, United Kingdom).
c. Percentage of amino acid sequence coverage of matched peptides from the validated proteins by the software Scaffold, version 3.6.4 (Proteome Software Inc., Portland, OR, United States).

[d] Student's *t*-test calculated between spot intensity in three different gels of each contrast by Image Master 2D Platinum software version 7.0 (GE Healthcare, Chicago, IL, United States), considering p-value < 0.05 and 1.5 of fold change.

Table S2 Differently expressed proteins in ethanol stressed *K. marxianus* (CCT 7735). The proteins were identified by NanoUHPLC-QTOF. Red rows represent proteins more abundant proteins and green rows less abundant proteins, according to Log_2 Fold Change.

Ethanol Exposure Time	Protein Name	Accession No. [a]	Sequence Coverage (%) [b]	Student's *t*-test [c]	Log_2 (Fold Change) [d]
1 h	40S ribosomal protein S18	W0TCR0	29	0.0019	-1.00
	60S acidic ribosomal protein P2-B	W0T7L8	34	0.0023	-
	60S acidic ribosomal protein P2-α	W0T703	21	0.0049	-
	Plasma membrane ATPase	W0T7K4	8	0.0055	3.10
	40S ribosomal protein S14	P27069	26	0.0069	-3.32
	Mitochondrial outer membrane protein porin 1	W0TJY9	24	0.0110	2.04
	Elongation factor 2	W0T6K4	8	0.0210	-1.74
	Pyruvate kinase	W0TDT2	27	0.0370	-1.74
	Phosphoglycerate mutase 1	W0T6W1	40	0.0380	-0.51
	Hexokinase	W0TF55	19	0.0400	-4.06
4 h	Uncharacterized protein	W0T4X3	12	0.0001	-1.32
	Heat shock protein 26	W0T939	11	0.0009	0.85
	Heat shock protein 78	W0TCP1	4	0.0017	2.63
	40S ribosomal protein S22	W0TDN9	30	0.0076	-0.74
	Heat shock protein 104	W0TAQ8	5	0.0100	-1.32
	Mitochondrial outer membrane protein porin 1	W0TJY9	16	0.0110	-1.00
	Enolase	W0T7K9	38	0.0170	-1.00
	Phosphoglycerate mutase 1	W0T6W1	27	0.0190	-0.51
	Peroxiredoxin TSA1	W0TC44	13	0.0230	-0.74
	NAD(P)H-dependent D-xylose reductase	W0T4K1	9	0.0260	0.49
	Small COPII coat GTPase SAR1	W0TAY6	13	0.0280	-0.32
	WGS project CCBQ000000000 data, contig 00106	A0A0A8L5D7	20	0.0350	0.68
	Transaldolase	W0TCV6	12	0.0380	-1.74
	ATP-dependent 6-phosphofructokinase	W0TDM8	4	0.0430	-0.32
	Elongation factor 1-α	W0TGV8	17	0.0450	0.77
	Heat shock protein SSA3	W0THY0	13	0.0490	0.49
	Phosphoglucomutase-2	W0TBD3	7	0.0490	-

[a] Uniprot accession number.

[b] Percentage of amino acid sequence coverage of matched peptides from the validated proteins by the software Scaffold, version 3.6.4 (Proteome Software Inc., Portland, OR, United States).

[c] Student's *t*-test calculated by the software Scaffold, version 3.6.4., considering p-value < 0.05.

[d] The lack of number in the column Log_2 Fold Change means that the protein was not detected in the sample treated with ethanol.

Table S3 Metabolites of *K. marxianus* (CCT 7735) whose abundance was altered at 1 h and 4 h after ethanol exposure when compared to the controls. The metabolites were identified by GC-TOF/MS. Red rows represent metabolites with elevated level and green rows metabolites with reduced level, according to Log_2 Fold Change.

Class	Metabolite	KEGG ID[a]	Log_2(Fold Change) (1 h)[b]	Log_2(Fold Change) (4 h)[b]
Amines	D-Glucosamine	C00329	-1.38	-1.82
	Diethanolamine	C06772	-	-1.41
	L-Citrulline	C00327	-1.46	-
	3-Methoxytyramine	C05587	-1.05	-2.17
	N-Acetyl-D-glucosamine	C00140	-1.15	-1.56
	N-Acetyl-D-mannosamine	C00645	-1.95	-
	N-Acetylgalactosamine	C01074	-1.54	-1.87
	N-Acetylmuramate	C02713	-	-1.39
	N-Methyltryptamine	C06213	-1.28	-
	Spermidine	C00315	-2.58	-
	Tyramine	C00483	-	-2.32
Amino acids	β-Alanine	C00099	-1.11	-
	β-Alanyl-L-lysine	C05341	-1.64	-
	Glutamate	C00025	-1.43	-
	Glutamine	C00303	-2.73	-1.56
	Isoleucine	C16434	-1.18	-
	L-Arginine	C00062	-1.81	-2.77
	L-Asparagine	C00152	-	-1.17
	L-Cysteine	C00097	-1.38	-
	L-Glutamine	C00064	-	-2.47
	L-Histidine	C00135	-2.24	-
	L-Homoserine	C00263	-	-1.40
	L-Lysine	C00047	-1.39	-2.05
	L-Methionine	C00073	-1.29	-
	L-Selenomethionine	C05335	-	-2.75
	N-Formylmethionine	C03145	-	-1.43
	O-Acetyl-L-serine	C00979	-1.89	-3.40
	Ornithine	C01602	-1.66	-2.41
	5-Oxoproline	C01879	-1.45	-2.25
Lipids	5-Aminopentanoate	C00431	-1.34	-2.65
	Cembrene	C11893	-	-1.65
	Cholesterol	C00187	-6.70	-
	Epigallocatechol	C12136	-	-1.81
	Ergosterol	C01694	-	-2.00
	Estradiol-17α	C02537	-3.77	-
	16-α-hydroxyestrone	C05300	-	-1.58
	Lithocholic acid	C03990	-	-2.51
	Menadione	C05377	-3.50	-3.53
	5-β-Pregnane-3,20-dione	C05479	-1.44	-
	Sphinganine	C00836	-3.61	-3.69
	Stigmasterol	C05442	-2.27	-8.58

	δ-Tocopherol	C14151	3.56	-
Nucleosides	Cytidine	C00475	-	-2.41
	Deoxyguanosine	C00330	-	-1.10
Organic acids	Abscisate	C06082	-1.34	-2.15
	Acetoacetate	C00164	-	-2.96
	4-Aminobutanoate	C00334	-1.11	-1.61
	Aminomalonate	C00872	-	-1.89
	4-Chlorophenylacetate	C03077	-1.01	-1.68
	4-Coumarate	C00811	-2.10	-2.34
	Dehydroascorbic acid	C05422	-2.51	-
	3,4-Dihydroxymandelate	C05580	-	-1.22
	D-Galactarate	C00879	-	-1.42
	Glyceraldehyde	C02154	-2.05	-1.25
	D-Glycerate	C00258	-1.28	-2.12
	D-Gluconate	C00257	-1.60	-2.27
	L-Gulono-1,4-lactone	C01040	-	-1.15
	2-Hydroxyphenylacetate	C05852	-	7.07
	Indolepyruvate	C00331	-	-1.15
	α-Isopropylmalate	C02504	-	-1.26
	Itaconate	C00490	-	-1.29
	Malate	C00711	-2.44	-3.85
	Maleamate	C01596	-1.60	-2.45
	Mesaconate	C01732	-	-1.16
	Oxaloacetate	C00036	-1.22	-
	3-Oxalomalate	C01990	-	-1.05
	2-Phosphoglycolate	C00988	-2.50	-
	4-Pyridoxate	C00847	-2.34	-1.40
	D-Ribonate	C01685	-1.52	-1.98
	Sebacic acid	C08277	-2.02	-2.95
	Sinapate	C00482	-2.67	-3.27
	Trans-Cinnamate	C00423	-1.08	14.39
	Trans-2-Hydroxycinnamate	C01772	-	-1.31
Phosphates	D-Fructose 1-phosphate	C01094	-2.67	-3.20
	D-Glucose 6-phosphate	C00092	-1.22	-2.06
	D-Ribose 5-phosphate	C00117	-1.89	-1.67
	D-Ribulose 5-phosphate	C00199	-	-1.70
	Glyceraldehyde 3-phosphate	C00661	-	-1.34
	Inositol 1-phosphate	C01177	-	14.62
	Phosphoric acid	C00009	-1.04	-
Sugars and sugar alcohols	D-Arabinose	C00216	-2.81	-10.10
	D-Erythrose	C01796	-	-1.30
	D-Fructose	C00095	-	-2.49
	D-Galactose	C00124	-1.34	-2.61
	D-Glucose	C00031	-1.88	-4.04
	D-Psicose	C06468	-1.73	-3.15

	Deoxyribose	C01801	-	-1.36
	Erythritol	C00503	-	-1.51
	Gentiobiose	C08240	-	-1.90
	Isomaltose	C00252	-2.80	-5.65
	Lactulose	C07064	-	-2.17
	Maltose	C00208	-1.00	-
	Palatinose	C01742	-2.61	-
	Raffinose	C00492	-2.44	-
	Sorbitol	C00794	-	8.72
	Trehalose	C01083	-1.31	-
	Xylose	C01394	-	-3.81
Vitamins	Phylloquinone	C02059	-	-1.17
	Pyridoxamine	C00534	-	-2.74
	Pyridoxine	C00314	-1.18	-1.62
Miscellaneous	Ajmaline	C06542	-	-1.81
	Calystegin B2	C10851	-	-2.17
	Cystathionine	C00542	-1.76	-2.24
	Flavanone	C00766	-	-2.55
	Hexachlorobenzene	C11042	-	-1.69
	3-Indoleacetonitrile	C02938	-	-1.16
	Nivalenol	C06080	-1.46	-

a. KEGG compound ID.

b. The lack of number in the column Log_2 Fold Change means that the metabolite was not detected in that time of ethanol stress.

GENERAL CONCLUSIONS

The present thesis aimed, firstly, to outline the main yeast responses to ethanol stress through a mini review. Posteriorly, the experimental responses of *Kluyveromyces marxianus* CCT 7735 at 1 and 4 h of ethanol exposure were presented.

The review presented in the first chapter highlights the available information about the ethanol response in *K. marxianus* and other non-*Saccharomyces* yeasts, which are still scarce. Therefore, it is important to explore those yeasts in further studies under ethanol stress, in order to understand better the pathways involved with this stress condition.

The second chapter pointed out that in *K. marxianus*, in agreement to its low growth rate, the abundance of proteins involved in carbon pathways and translation process were impaired mainly at 4 h of ethanol stress. The level of ergosterol and trehalose decreased as well. Besides, *K. marxianus* seems to respond to the oxidative and osmotic stresses (both caused by ethanol stress) and to increase the heat shock proteins abundance. Moreover, the plasma membrane ATPase was highly abundant at 1 h, in order to replenish the proton gradient.

Taken together, the results obtained here provide complementary insights to understand the physiological changes that occur in *K. marxianus* under ethanol stress. Those results provide insights to construct a stress-tolerant strain for bioethanol production.