FERNANDO AUGUSTO DA SILVEIRA

ASSESSMENT OF ETHANOL TOLERANCE OF *Kluyveromyces marxianus* CCT 7735 SELECTED BY ADAPTIVE LABORATORY EVOLUTION

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

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APPROVED: August 29th, 2018.

Antônio Galvão do Nascimento

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Wendel Batista da Silveira
(Advisor)

A Deus.

Aos meus pais, Fernando e Shirley.

Aos meus irmãos, Angelo e Magno.

À minha noiva, Elis Marina.

À toda minha família e amigos.

Aos meus mestres.

“Where there is a will, there is a way. If there is a chance in a million that you can do something, anything, to keep what you want from ending, do it. Pry the door open or, if need be, wedge your foot in that door and keep it open”.

Pauline Kael

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BIOGRAPHY

FERNANDO AUGUSTO DA SILVEIRA, filho de Fernando Antonio da Silveira e Shirley Margareth Vieira da Silveira, nasceu no dia 22 de dezembro de 1986, em Ponte Nova, Minas Gerais. Graduou-se em Licenciatura em Ciências Biológicas pela Universidade Federal de Ouro Preto, em janeiro de 2012. Em agosto de 2012, iniciou o curso de mestrado no Programa de Pós-graduação em Microbiologia Agrícola da Universidade Federal de Viçosa, Viçosa, Minas Gerais, submetendo-se à defesa de mestrado no dia 21 de julho de 2014. Em agosto do mesmo ano, iniciou o curso de doutorado neste mesmo Departamento, submetendo-se à defesa de tese no dia 29 de agosto de 2018.

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ABSTRACT

SILVEIRA, Fernando Augusto da, D.Sc., Universidade Federal de Viçosa, August, 2018. **Assessment of ethanol tolerance of *Kluyveromyces marxianus* CCT 7735 selected by adaptive laboratory evolution.** Advisor: Wendel Batista da Silveira. Co-advisors: Hilário Cuquetto Mantovani and Luciano Gomes Fietto.

Whey is a by-product formed during the cheese-making, which presents lactose (4.5-5% w/v), soluble proteins, lipids, mineral salts and other components. It is the most abundant liquid waste generated in the dairy industries, which can lead to the pollution of water bodies due to its high biochemical oxygen demand and chemical oxygen demand. Whey permeate (WP) is a by-product constituted by lactose, produced from whey ultrafiltration. This way, WP can be converted by lactose-assimilating microorganisms, like yeasts, into value-added products. Kluyveromyces marxianus CCT 7735 has been showing a great potential for producing ethanol from lactose. However, its low ethanol tolerance is a drawback to be overcome, i. e., its growth is highly inhibited from 4% of ethanol (v/v). Adaptive laboratory evolution was used to select ethanol-tolerant K. marxianus CCT 7735 strains (ETSs). ETSs were grown under ethanol stress (4%, v/v) for long periods of time, over many generations (310-340 number of generations), in order to select the ethanol-tolerant phenotypes. From the determination of the physiological parameters, ETSs did not present alterations in their fermentative capacity, when compared their parental strains. However, ETS4 strain stood out for displaying a specific growth rate higher than the parental strain under ethanol stress (above 100%) and a specific ethanol production rate superior to all strains evaluated in this work. The metabolomic and fatty acids analysis were carried out with both ETS4 and parental strains to gain insights into the mechanisms related to the acquisition of ethanol tolerance. The accumulation of some amino acids (glutamate, alanine, valine, proline, and leucine) and metabolites of the citric acid cycle (isocitric acid, citric acid, cis-aconitic acid and malic acid) in ETS4 was found to be associated with the acquisition of ethanol tolerance. Furthermore, high fatty acids content and ergosterol in ETS4 compared to the parental strain indicate differences in their plasma membrane composition, which is consistent with metabolite leakage observed in the parental strain. Therefore, the accumulation of amino acids and

citric acid cycle metabolites, as well as the alteration in the fatty acid and ergosterol contents contributed to the acquisition of ethanol tolerance in K. marxianus..

RESUMO

SILVEIRA, Fernando Augusto da, D.Sc., Universidade Federal de Viçosa, agosto de 2018. **Avaliação da tolerância ao etanol de *Kluyveromyces marxianus* CCT 7735 selecionada por evolução adaptativa em laboratório.** Orientador: Wendel Batista da Silveira. Coorientadores: Hilário Cuquetto Mantovani e Luciano Gomes Fietto.

O soro é um subproduto formado durante a fabricação do queijo, constituído por lactose (4,5-5% m/v), proteínas solúveis, lipídios, sais minerais e outros componentes. Ele é o resíduo líquido mais abundante gerado nas indústrias de laticínios, que pode levar à poluição dos corpos de água devido as altas demandas bioquímica e química de oxigênio. O permeado de soro de queijo (PS) é um subproduto constituído por lactose, produzido a partir da ultrafiltração do soro. Portanto, o PS pode ser convertido por microrganismos assimiladores de lactose, como leveduras, em produtos de alto valor agregado. A levedura *Kluyveromyces marxianus* CCT 7735 tem demonstrado um potencial para produzir etanol a partir do açúcar lactose. No entanto, sua baixa tolerância ao etanol é uma desvantagem que precisa ser superada, isto é, seu crescimento é fortemente inibido a partir de 4% de etanol (m/v). A evolução adaptativa em laboratório foi utilizada para selecionar linhagens de *K. marxianus* CCT 7735 tolerantes ao etanol (ETSs). As ETSs foram crescidas sob estresse por etanol (4%, m/v) por longos períodos de tempo, durante muitas gerações (310-340 números de gerações), a fim de selecionar fenótipos tolerantes ao etanol. A partir da determinação dos parâmetros fisiológicos, as ETSs não apresentaram alterações em suas capacidades fermentativas, quando comparadas as linhagens parentais. A linhagem ETS4 se destacou por apresentar uma taxa específica de crescimento maior que a linhagem parental (acima de 100%) sob estresse por etanol e uma taxa específica de produção de etanol mais elevada dentre todas as linhagens avaliadas neste trabalho. As análises dos perfis de ácidos graxos e metabolômica foram realizadas com as linhagens ETS4 e parental para obter informações sobre os mecanismos relacionados à aquisição de tolerância ao etanol. O acúmulo de alguns aminoácidos (glutamato, alanina, valina, prolina e leucina) e metabólitos do ciclo ácido cítrico (ácido isocítrico, ácido cítrico, ácido cis-aconítico e ácido málico) em ETS4 parece estar associado à aquisição de tolerância ao etanol.

Além disso, os altos conteúdos de ácidos graxos e ergosterol na linhagem ETS4 comparado à linhagem parental indicam diferenças entre suas membranas plasmáticas, o que é coerente com o vazamento de metabólitos observado na linhagem parental. Portanto, o acúmulo de aminoácidos e metabólitos do ciclo do ácido cítrico, assim como a alteração nos teores de ácidos graxos e ergosterol contribuíram para a aquisição de tolerância ao etanol em *K. marxianus*.

GENERAL INTRODUCTION

The fast growth of the world's population, expected to reach 9.1 billion in 2040, will increase the energy demand in the next years (IEA, 2017). Nowadays, world primary energy is based on fossil fuel, whose consumption implicates in some issues: (i) oil reserves are non-renewable; (ii) oil prices rise rapidly, and (iii) greenhouse gas emissions (Valdivia et al. 2016; IEA 2017). The use of renewable fuels may help reduce the consumption of fossil fuels. Ethanol, the most used biofuel in the world, is currently produced from sugarcane and corn (Naik et al. 2010). Therefore, its production competes with the production of food. In this sense, the use of alternative feedstocks is pivotal to increase the production of this biofuel in a sustainable way. Whey permeate (WP), a by-product produced from dairy industries, which contains lactose, has been employed on industrial scale to produce ethanol in New Zealand (Anchor Ethanol, Fonterra Cooperative Group), Ireland (Carbery Group) and Germany (Leppersdorf, Theo Müller) (Barile et al. 2009; Guimarães et al. 2010; Prazeres et al. 2012).

Non-*Saccharomyces* yeasts have been used for WP fermentation, since the conventional yeast *Saccharomyces cerevisiae* does not assimilate lactose as the sole carbon source (Yadav et al. 2015; Parashar et al. 2016). Therefore, *Kluyveromyces marxianus* has stood out due to its capacity to convert lactose to ethanol. Nevertheless, *K. marxianus*, in contrast to *S. cerevisiae*, does not tolerate high ethanol concentrations (Silveira et al. 2005). Most of the studies involving ethanol stress in yeasts were performed with *S. cerevisiae*.

Recently, the adaptive laboratory evolution (ALE) methodology has been employed to select microorganisms with phenotypes capable of withstanding to stress conditions (Dragosits and Mattanovich 2013; LaCroix et al. 2017). In this methodology, the cells are grown for long periods of time, under defined environmental conditions (selective pressure), leading to randomized non-directed mutations (Dragosits and Mattanovich 2013; LaCroix et al. 2017).

The main aim of this thesis was the selection of ethanol-tolerant *K. marxianus* strains by using ALE. The thesis was organized in two chapters: the first one is a literature review; while the second one is the manuscript reporting the selection of ethanol-tolerant *K. marxianus* strains by ALE. This manuscript also

reports the physiological characterization of those strains and the metabolic and fatty acid methyl ester profiles of the ethanol-tolerant strain 4, which allowed us to gain insights into the mechanisms involved with the acquisition of ethanol tolerance

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CHAPTER I

Review

Whey permeate

Whey is a by-product formed after coagulation of milk casein during the cheese-making (González-Siso 1996). It represents about 90-95% of the milk volume and retains milk soluble nutrients such as: lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5%), mineral salts (8-10% of dried extract) and other components (lactic and citric acid, vitamins, etc.). Whey is the most abundant liquid waste generated by the dairy industries, estimated at over 10^8 tonnes per year (Koushki et al. 2012). In Brazil, large volumes of whey are produced annually, a total of 2.700 tonnes (Rosana de Oliveira et al. 2017).

Whey is a polluting effluent of water bodies due to its high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which range from 0.6 to 60 g/L and 0.8 to 102 g/L, respectively (Prazeres et al. 2012). Lactose and fat contents are the main responsible for the high COD and BOD. Therefore, from an environmental point of view, it must be treated prior its disposal, which is costly. Normally, its effluent has a low pH (3.0-5.5), although basic pH's have also been found range from 3.3 to 9.0 (Carvalho et al. 2013; Parashar et al. 2016). Over the last years, whey proteins have been commercialized as powdered concentrates and isolates due to their high nutritional value. Their recovery is performed by whey ultrafiltration, generating the whey permeate (WP) as by-product (Barile et al. 2009; Kim et al. 2012). Since the lactose passes through ultrafiltration membranes, whey permeate is constituted by lactose (Koushki et al. 2012; Prazeres et al. 2012). Thus, its disposal also requires treatment. In this context, various studies have used the WP in fermentative processes in order to find promising alternatives for its use at industrial level.

WP can be converted by lactose-assimilating microorganisms, specially yeasts and bacteria, into value-added products. Different microorganisms produce organic compounds and acids by fermentation processes from WP: *Kluyveromyces fragilis* produce glycerol and acetic acid from salted and dried whey (Mostafa 2001); propionic acid was produced by a *Propionibacterium acidipropionici* strain (Yadav et al. 2015); and filamentous fungi strains of *Aspergillus niger* can produce gluconic

and citric acids (Yadav et al. 2015). Production of proteins and microbial biomass, designed as single cell protein, has been performed by *Kefir* microbiota at industrial scale from WP (Paraskevopoulou et al. 2003). Continuous nisin production from WP was performed in bioreactors by using *Lactococcus lactis* subsp. *lactis* ATCC 11454 immobilized into natural fiber surfaces (Liu et al. 2005). From WP, under both submerged and solid state fermentation conditions, mosquitocidal toxin was produced by *Bacillus sphaericus* 2362 and *Bacillus sphaericus* 14N1, presenting high mosquitocidal activity against *Culex pipiens*, a species of blood-feeding mosquito vector of diseases (El-Bendary et al. 2008). Under thermophilic conditions, *Bacillus sp*. strains were able to grow and produce biosurfactant from lactose (Joshi et al. 2008). In the same way, lactose present in WP can be converted into ethanol by yeasts. For example, *Kluyveromyces marxianus* and *Candida pseudotropicalis* (*kefyr*) strains have been used for the industrial production of ethanol from WP (Ghaly and El-Taweel 1995; Silveira et al. 2005; Fonseca et al. 2008; Koushki et al. 2012; Yadav et al. 2015).

Ethanol production from whey permeate (WP)

Over the last years, the demand for the production of biofuels such as ethanol has raised quickly. Currently, most of the ethanol fuel is produced from carbohydrates constituents of sugarcane, cereals and seeds, which is designated as first generation process (Taylor 2008; Naik et al. 2010; Sims et al. 2010). Therefore, this production competes with the food production. An alternative to ensure the production in a sustainable way is the use of wastes generated in agroindustries, such as WP. Importantly, it has been used as feedstock on industrial scale to produce ethanol in New Zealand (Anchor Ethanol, Fonterra Cooperative Group), Ireland (Carbery Group) and Germany (Leppersdorf, Theo Müller) (Barile et al. 2009; Guimarães et al. 2010; Prazeres et al. 2012). The WP fermentation requires the use of lactose-assimilating yeasts, however, *Saccharomyces cerevisiae*, yeast used in the first generation process, does not assimilate lactose (González-Siso 1996; Dragone et al. 2011; Prazeres et al. 2012; Yadav et al. 2015; Borges et al. 2016; Parashar et al. 2016). Otherwise, yeasts isolated from dairy environments, as *K. marxianus* stands out as good ethanol producers from lactose (Barile et al. 2009; Kim et al. 2012).

Our research group has demonstrated the potential of *K. marxianus* UFV-3, also designated as CCT 7735, for producing ethanol from WP. Under hypoxic conditions, *K. marxianus* CCT 7735 produced high ethanol amounts from WP (Silveira et al. 2005). Diniz et. al (2013) determined the factors that affect remarkably the ethanol production by *K. marxianus* CCT 7735 from WP. In addition, Ferreira et al. (2015) demonstrated that *K. marxianus* CCT 7735 is able to produce ethanol from a mixture of the cellulosic residue of sugarcane bagasse and ricotta whey, a bio-product from ricotta-making process.

Characteristics of *Kluyveromyces marxianus*

K. marxianus is a non-conventional yeast phylogenetically related to *S. cerevisiae* and *K. lactis (Coté et al. 2004; Silveira et al. 2005; Fonseca et al. 2008; Das et al. 2016; Parashar et al. 2016)*. *K. marxianus* has the generally recognized as safe (GRAS) status by the American food and drug administration (FDA) and the qualified presumption of safety (QPS) by the European Food Safety Authority (EFSA) (Morrissey et al. 2015; Das et al. 2016). *K. marxianus* has been isolated from both dairy products (fermented milks, cheeses, yoghurt and kefir) and non-dairy environments such as fruit (grapes) and soil (plant material) (Lachance 2007; Lane and Morrissey 2010; Morrissey et al. 2015). Interestingly, *K. marxianus* strains isolated from dairy environments are diploid and triploid, whilst those isolated from non-dairy environments are haploid (Ortiz-Merino et al. 2018). The ploidy status probably reflects differences and variation within *K. marxianus*. It has 4 *LAC12* genes, which encode the lactose transporter, nevertheless, non-dairy strains assimilate lactose, but at a low rate. This can be due to either inactivation or low expression of the *LAC12* genes (Varela et al. 2017). Indeed, the *LAC12* alleles are functional only in dairy strains (Ortiz-Merino et al. 2018).

K. marxianus strains are able to assimilate other sugars including glucose, sucrose, fructose, galactose, raffinose, arabinose, cellobiose and xylose, demonstrating their potential for growing in alternative feedstocks as lignocellulosic biomass, whey permeate and by-products from food industries (Kurtzman and Fell 1998; Fonseca et al. 2008; Fonseca et al. 2013). In addition, they produce enzymes of industrial interest such as β-galactosidase, inulinase, β-glucosidase, β-glucanase, endopolygalacturonases, protein phosphatases, carboxypeptidases and

aminopeptidases (Fonseca et al. 2008; Lane and Morrissey 2010; Lopes et al. 2014; Morrissey et al. 2015).

K. marxianus strains have remarkable differences regarding the regulation of sugar metabolism. Glucose repression, for example, is strain-dependent (Lane et al. 2011). Interestingly, these authors did not observe a correlation between glucose repression and fermentative behavior in some *K. marxianus* strains, which is commonly observed in *S. cerevisiae*.

Moreover, there are differences among *K. marxianus* strains regarding the regulation of cellular energy metabolism. *K. marxianus* CCT 7735 was capable of producing ethanol even under aerobic conditions; however, the ethanol yields were not close to the theoretical yield (Raphael H S Diniz, et. al, 2012; Silveira et al., 2005). Crabtree effect is defined as the use of fermentation in aerobiosis at high sugar concentration (Hagman et al. 2014; Pfeiffer and Morley 2014). In *S. cerevisiae*, a Crabtree-positive yeast, alcoholic fermentation is triggered in sugar concentrations above 9 g/L. On the other hand, Crabtree-negative yeasts display a respiratory metabolism under aerobic conditions (Hagman et al. 2014; Henderson and Block 2014a). It is noteworthy that some *K. marxianus* strains are Crabtree-negative (Fonseca et al. 2013).

Silveira et al. (2005) reported in *K. marxianus* CCT 7735 (UFV-3) that both specific lactose consumption rate and specific ethanol production rate were higher under hypoxic and anoxic conditions. Importantly, Diniz et al. (2012) showed that this strain displays high activities of β-galactosidase, pyruvate decarboxylase and Leloir pathway enzymes, which are related to a higher fermentative flow. Moreover, *K. marxianus* has other two physiological features of particular interest for ethanol production: faster growth than other fastest-growing eukaryotes (Groeneveld et al. 2009) and thermotolerance (Lane et al. 2011).

K. marxianus CCT 7735 and its biotechnological applications

K. marxianus CCT 7735 (UFV-3) strain was isolated from regional Brazilian dairy industry and is able to convert the lactose from whey permeate, an effluent generated in dairy industries, to ethanol (Silveira et al. 2005). Other studies carried out in our research group, at Federal University of Viçosa (UFV), have highlighted

its potential for producing ethanol from whey derivatives (Raphael H S Diniz et al., 2013; Ferreira et al., 2015;).

Importantly, *K. marxianus* CCT 7735 also stood out as host cell for recombinant protein production. It was used for the production of the non-structural protein 1 (NS1) of the dengue virus type 1 (Bragança et al. 2014). Moreover, polysaccharides fractions from its cell wall demonstrated antioxidant, antiproliferative and immunostimulatory properties (Galinari et al. 2018).

This strain is also able to produce enzymes of industrial interest. Lopes et al. (2014), described for example, the production of a β-1,3(4)-glucanase by *K. marxianus* CCT 7735 under optimal conditions of 4% glucose (w/v), pH of 5.5 at 35 ºC. Its enzyme demonstrated activity against cell wall of *S. cerevisiae*. Furthermore, Diniz et al. (2013) reported the production of endopolygalacturonase by *K. marxianus* CCT 7735.

As previously mentioned, *K. marxianus*, contrary to *S. cerevisiae*, is thermotolerant. This feature is relevant for decreasing contamination risks and costs associated to the cooling of industrial bioreactors (Hasunuma & Kondo, 2012). Furthermore, thermotolerance is desirable for the production of ethanol from lignocellulosic biomass via simultaneous saccharification and fermentation (SSF) processes (Costa et al. 2014). *K. marxianus* CCT 7735 was able to ferment a cellulosic hydrolysate from sugarcane bagasse in the SSF processes at 42 °C (de Souza et al. 2012).

In this context, the *K. marxianus* CCT 7735 was also used to produce ethanol from a mixture of glucose plus xylose, sugars found in cellulose and hemicellulose polymers, respectively. Ethanol production was always higher in glucose plus xylose than in glucose as the sole carbon source (Dos Santos et al. 2013). In addition, this strain was capable of producing ethanol from a mixture of sugarcane bagasse hydrolysate and ricotta whey (Ferreira et al. 2015).

Recently, *K. marxianus* CCT 7735 was selected as the best producer of 2-phenylethanol, a higher aromatic alcohol used in the beverages, pharmaceutical and cosmetics industries, among yeasts isolated from Brazilian environments, (Lima et al. 2018).

Ethanol stress in yeasts: targets and responses

Various factors such as temperature, pH, sugar concentration and oxygen level may affect the ethanol production by yeasts (Pronk et al. 1996; Hoek et al. 1998; Soccol et al. 2005). In addition, high concentrations of ethanol achieved from high sugar concentrations, cause damages in yeasts, impairing their fermentative performance. The ethanol stress severely decreases their specific growth rate and cell viability (Piper 1995; Zhao and Bai 2009a; Ma and Liu 2010). The main targets of ethanol in yeasts are cellular membrane, hydrophobic and hydrophilic proteins and endoplasmic reticulum (Stanley et al. 2010). Moreover, high ethanol concentrations also induce osmotic and oxidative stress response in yeasts (Zhao and Bai 2009a; Ma and Liu 2010; Stanley et al. 2010).

S. cerevisiae is more tolerant to the ethanol than other yeasts. Most of the studies involving ethanol stress in yeasts have been performed with this species, although responses to ethanol tolerance were described for other non-*Saccharomyces* yeasts (Stanley et al. 2010; Snoek et al. 2016). The main processes involved in the responses to the ethanol stress are: (1) unsaturated fatty acids biosynthesis; (2) ergosterol biosynthesis; (3) H^+-ATPase biosynthesis and activity; (4) trehalose biosynthesis; (5) amino acids biosynthesis; and (6) induction of heat shocks proteins (Zhao and Bai 2009a; Ma and Liu 2010; Stanley et al. 2010).

Genes that had the expression changed in response to ethanol are described in Table 1. Despite the advances achieved in recent years, the mechanisms involved with ethanol responses are still not fully understood.

Table 1. Cellular functions and number of genes generally involved to ethanol tolerance in yeast cells. Adapted from Snoek et al. (2016) and Stanley et al. (2010).

Cellular function	Number of genes
Biosynthesis	43
Cell cycle	17
Cytoskeleton	18
Mitochondrion	22
Morphogenesis	14
Nucleic acid binding	12
Protease activity	4
Protein transport/Vacuole	45
Signal transduction	4
Transcription	25
Transport	11
Unknown function	41
Total	256

Cellular membrane is the main structure involved in ethanol stress. Ethanol alters its permeability leading to both dissipation of the transmembrane electrochemical potential and acidification of the cytoplasm (Ma & Liu, 2010; Pampulha & Loureiro-Dias, 1989; Zhao & Bai, 2009). Ethanol divides in the lipid-water interface forming hydrogen bonds with the lipid molecules. At low/intermediate ethanol concentrations, ethanol molecules are divided into the lipid bilayers of the membrane, increasing its fluidity due to the increase in the spacing between the lipids heads, at the water-lipid interface. On the other hand, high ethanol concentrations cause an increase of lipid interdigitations in cellular membrane, which in turn reduces its thickness up to 30% (Henderson and Block 2014b). In addition, the interdigitations of membranes lead to dissipation of proton motive force. Indeed, it has been reported that genes involved in the synthesis of mannoprotein biosynthesis, β-glucan synthase and sensor stress-activated kinase (PKC1-MPK1), pathways related in maintenance of cell wall integrity and organization, were up-regulated (Ma and Liu 2010).

Ergosterol ensures the membrane stabilization by suppressing or reducing the amount of lipid interdigitations and the transition phase of phospholipid bilayers (Vanegas et al. 2012). The most common esterified fatty acids of yeasts are palmitic acid ($C_{16:0}$), and stearic acid ($C_{18:0}$) (saturated fatty acids); and palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$) (mono-unsaturated fatty acids) (Henderson & Block,

2014; Lahtvee et al., 2016). Among them, the oleic acid is the most important to counteract the problems caused by ethanol in the cellular membrane. It can mitigate the membrane destabilization by high ethanol concentration, ensuring the hydrophobic match of the integral membrane proteins (You et al. 2003; Ma and Liu 2010; Henderson and Block 2014b). In *S. cerevisiae*, the amount of mono-unsaturated fatty acids was higher in ethanol-tolerant strains, which is related to its capacity to withstand the damages provoked by ethanol. Furthermore, in response to ethanol, the concentration of ergosterol increases in *S. cerevisiae* (Stanley et al. 2010; Vanegas et al. 2012; Caspeta et al. 2014; Caspeta and Nielsen 2015). However, in *K. marxianus* there was no increase of both unsaturated fatty acids and ergosterol under ethanol stress, which seems to be related to its lower tolerance compared to *S. cerevisiae* (Diniz et al. 2017). Likewise *S. cerevisiae*, in *Brettanomyces bruxellensis*, an ethanol-tolerant yeast, genes encoding enzymes involved with ergosterol biosynthesis were overexpressed under ethanol stress (Nardi et al. 2010).

Considering that the permeability of cell membrane increases under ethanol stress, H^+-ATPase activity is enhanced to avoid the intracellular acidification by protons accumulation; it pumps protons to the outside or into organelles, as vacuoles, through the hydrolysis of ATP (Aguilera et al. 2006; Ma and Liu 2010; Vanegas et al. 2012). Therefore, this enzyme has a protective effect maintaining intracellular homeostasis, which is an important response to ethanol. In *S. cerevisiae* the H^+-ATPase activity enhances at high ethanol concentrations, contrary to *Pichia membranifaciens* and *Torulaspora delbruechkii*. However, *P. membranifaciens* and *T. delbruechkii* were isolated from earlier stages of wine fermentation, even so they exhibit sensitivity to ethanol stress (Aguilera et al. 2006; Vanegas et al. 2012). In *K. marxianus*, Alvim et al. (2018) demonstrated that the abundance of H^+-ATPase protein increased after 1 h of ethanol exposure, highlighting its importance in the ethanol stress conditions (submitted manuscript).

The accumulation of some metabolites also plays an important role. Upon ethanol exposure, *S. cerevisiae* accumulates intracellularly trehalose, which avoids oxidation of the fatty membrane acids, protein denaturation and incorrect protein folding (Doğan, Demirci, Aytekin, & Şahin, 2014; Ma & Liu, 2010; Zhao & Bai, 2009). In *B. bruxellensis*, genes encoding enzymes of trehalose biosynthesis were

induced upon ethanol exposure (Nardi et al. 2010). In ethanol-stressed cells of *K. marxianus* after 4 h of ethanol exposure, the trehalose levels were higher compared to non-stressed cells. Thus, the trehalose synthesis seems to be an adaptive response to ethanol (Alvim et al. 2018) (submitted manuscript). However, in *Torulaspora preteorensis* and *Clavispora opuntiae,* the intracellular concentration of trehalose did not increase in response to ethanol (Ribeiro et al. 1999).

Overall, ethanol stress impairs the protein biosynthesis and folding. In response to high ethanol concentrations, heat shock proteins (HSPs) are overexpressed to avoid the protein unfolding and to protect the cell membrane against damages caused by ethanol. In *S. cerevisiae*, the *HSP70*, *HSP26* and *HSP104* genes are induced under ethanol stress (Piper 1995; Ma and Liu 2010; Lahtvee et al. 2016; Navarro-Tapia et al. 2016; Santos et al. 2017). Likewise, gene-encoding HSPs were up-regulated in *K. marxianus* under ethanol exposure (6% v/v) (Diniz et al. 2017). Likewise, Alvim et al. (2018) also demonstrated that Hsp26, Hsp78 and HSP 104 proteins were more abundant during ethanol stress in *K. marxianus* (submitted manuscript). Expression analysis of HSPs genes encoding in *B. bruxellensis* demonstrated that *HSP82* was up-regulated, but *SSA3/4* (*HSP70* family) was not activated in response to ethanol (Nardi et al. 2010).

Beyond trehalose, amino acids accumulations are also involved with ethanol tolerance. Either biosynthesis or uptake of some amino acids have been considered an adaptive response to ethanol (S. Kim et al., 2016; Ma & Liu, 2010; Saharan, Kanwal, & Sharma, 2010). It has been demonstrated that proline accumulation at high ethanol concentration conferred ethanol tolerance (Ma and Liu 2010; Kim et al. 2016). Hirasawa et al. (2007) reported by *S. cerevisiae* that the overexpression of genes involved in tryptophan biosynthesis increased its ethanol stress tolerance. In addition, Kim et al. (2016) showed that high concentration of tyrosine improved the ethanol tolerance. Furthermore, the overexpression of genes encoding enzymes of arginine biosynthesis led to its intracellular accumulation, which in turn contributed for maintaining the integrity of both cell wall and cellular membrane (Cheng, Du, Zhu, Guo, & He, 2016). It has been reported that glutamate accumulation is also related to ethanol stress responses in *S. cerevisiae* (Chen, Zheng, Yi, Wang, & Li, 2016; S. Kim et al., 2016). Likewise, metabolites associated with its metabolism, such as glutamine and citrulline, accumulated in ethanol tolerant-strains upon

ethanol exposure (Zhao and Bai 2009b; Ma and Liu 2010; Doğan et al. 2014), indicating their relation with ethanol tolerance. On the other hand, in *K. marxianus*, genes related to metabolism of aromatic and branched chain amino acids were up-regulated, although the levels of the arginine and glutamate decreased in cells exposed to ethanol after 1 and 4h (Diniz et al. 2017). Importantly, Alvim et al. (2018) (submitted manuscript) observed that both arginine and glutamate concentrations enhanced under ethanol stress at 8 and 12h, suggesting that they are important to counteract the ethanol damages in late periods of exposure. Under ethanol stress, yeast cells can be subject to oxidative stress and cell damage, due to high intracellular levels of reactive oxygen species (ROS). It has been reported that ROS cause cell damage. Indeed, *S. cerevisiae* controls the level of ROS, accumulating molecules as glutathione and activating enzymes such as superoxide dismutase and catalase (Chandler et al. 2004; Ma and Liu 2010; Saharan et al. 2010; Lahtvee et al. 2016; Santos et al. 2017). In *K. marxianus*, the Hsp60 (mitochondrial protein) seems to be related to ethanol exposure (Diniz et al. 2017). It should be pointed out that ethanol stress may causes redox imbalance in microbial cells. Indeed, in ethanol-containing enviroments, *S. cerevisiae* activates genes involved in glycerol biosynthesis, consequently enhancing its intracellular level and overcoming the ethanol-stress condition. Glycerol is synthesized for controlling redox imbalance induced by ethanol production, i. e., glycerol synthesis regenerate the oxidized cofactor NAD^+ (Vriesekoop et al. 2009).

Adaptive laboratory evolution (ALE)

Adaptive Laboratory Evolution (ALE) is a strategy to promote and study evolution of microbial populations. It has been used to select microbial cell factories more tolerant to stress conditions found in fermentative processes, as well as with improved nutrient uptake and productivity of metabolites (Portnoy et al. 2011; Dragosits and Mattanovich 2013; Pennisi 2013; LaCroix et al. 2017). During ALE studies, randomized non-directed mutations accumulate in genomes of microorganisms, leading to the changes desirable for using them in bioprocesses (Barrick et al. 2009; Portnoy et al. 2011; Dragosits and Mattanovich 2013; LaCroix et al. 2017; Tokuyama et al. 2018).

In ALE experiments, cells are grown under defined conditions for a long period of time, that is, over many generations, in order to select the adapted phenotypes and non-targeted genetic modifications (Portnoy et al. 2011; Dragosits and Mattanovich 2013; LaCroix et al. 2017). The selective pressures (environmental conditions of cultivation) imposed to the culture direct the outcome of the evolution process. Several cultivation conditions can be established: alternative carbon sources, nutrients depletion and stress conditions such as temperature, pH, salts and exposure to high concentrations of fermentation products. Indeed, this method has been a valuable tool for improving secondary metabolic pathways; uptake rate of various carbon sources; metabolite production; and increasing in the specific growth rate; and tolerance to various types of stress, commonly caused by industrial processes (Lenski et al. 1991; Lenski and Travisano 1994; Bennett and Hughes 2009; Caspeta et al. 2014; Lang and Desai 2014; Novo et al. 2014; Jeong et al. 2016). Table 2 shows some works that used ALE for improving yeast phenotypes.

ALE has some important advantages: simple demand of culture medium nutrients; ease of laboratory growing; low costs and simple handling. Since *K. marxianus* displays a high growth rate, it can be grown for hundreds of generations within weeks or months (Portnoy et al. 2011; Dragosits and Mattanovich 2013; LaCroix et al. 2017). There are several ALE works carried out employing cultures with yeasts (Table 2).

Table 2. Published works applying the ALE methodology in yeasts. Different environmental conditions (selective pressures) are highlighted according to the research aims (Adapted from Dragosits & Mattanovich, 2013).

Yeast	Environmental condition	Reference
***Saccharomyces cerevisiae* SC288**	glucose limitation	Wenger et al. 2011, *Plos Genet*;
Saccharomyces cerevisiae	xylose fermentation	Shen et al. 2012, *Appl Microbiol Biotechnol*;
***Saccharomyces cerevisiae* Δ*jen*1**	lactate, synthetic medium	de Kok et al. 2012, *FEMS Yeast Res*;
***Saccharomyces cerevisiae* FY2**	lignocellulosic hydrolysate tolerance	Almario et. al 2013, *Biotechnol Bioeng*;
***Saccharomyces cerevisiae* CEN-PK**	several abiotic stresses	Çakar et. al 2005, *FEMS Yeast Res*;
Candida albicans	fluconazole	Selmecki et al. 2009, *J Biotechnol*;
	ethanol tolerance; glucose assimilation	Alper et. al 2006, *Science;*
Saccharomyces cerevisiae	NaCl 0.5 M, medium plus	Dhar et. al 2011, *J Evol Biol*;

BY4741	galactose	
	oxidative stress and salts	Dhar et. al 2013, *Mol Biol Evol*;
***Saccharomyces cerevisiae* W303-1A**	ethanol 6 – 8% (v/v)	Avrahami-Moyal et al. 2012, *FEMS Yeast Res*;
***Saccharomyces cerevisiae* CEN.PK113-7D**	high temperatures (>40 ºC)	Caspeta et al. 2014, *Science*;
***Saccharomyces cerevisiae* RWB218**	acetic acid tolerance (>5 $g.L^{-1}$)	Wright et al., 2011, *FEMS Yeast Res*;
***Saccharomyces cerevisiae* CEN.PK2-1C**	lactic acid production (112 $g.L^{-1}$)	Baek et al. 2015, *Applied Microbiol Biotechnol*.

Decades ago, the identification and characterization of random mutations in genomes of microorganisms selected by ALE was a laborious task. However, the raise of the new sequencing technologies in recent years allowed to access them easily nowadays (LaCroix et al., 2017). Several mutations types have been described: single nucleotide polymorphisms (SNPs) as insertions and deletions in small scale (indels); insertion sequence (IS); and deletions and amplifications of large genomic regions. Among them, SNPs are the most frequent (61%), followed by deletions (29%), insertions (7%) and IS (3%) (Dragosits & Mattanovich, 2013; Wielgoss et al., 2011).

Generally, batch and continuous culture are the two main types of cultivation regimes used in ALE. Several studies have shown that ALE conducted in batch cultures is suitable for improving the desirable phenotypes in microbial cells (Barrick et al. 2009; Dragosits and Mattanovich 2013; LaCroix et al. 2017). In batch culture, cells are propagated in sequential and serial passages in culture flasks. After the cell reaches the exponential growth phase, an aliquot of this culture is transferred to a new flask containing fresh culture medium. At regular intervals (usually on a daily) and generation numbers, cells are propagated until to reach the desired attribute. Batch cultivation has some advantages over continuous culture such as the use of simple and low-cost equipment and use of various growth conditions in parallel. However, there are some disadvantages: environmental conditions alterations (pH, oxygen and nutrient availability) and variations in population density and growth rate (Barrick et al., 2009; Dragosits & Mattanovich, 2013; LaCroix et al., 2017; Portnoy et al., 2011; Scott-Phillips & Kirby, 2010).

Continuous culture (chemostat) is based on controlling the growth rate of a microbial population by shifting the flux at which the fresh medium is added into the

bioreactor. Thus, this cultivation regime has an advantage over batch culture: the growth rate and population density of the microorganisms remain constant. In addition, environmental conditions such as nutrient availability can be controlled during the laboratory evolution (Dragosits & Mattanovich, 2013; Jeong et al., 2016). The main bottlenecks are the operating costs, which exceed the costs of batch cultivation, as well as risks of contamination. In chemostat, a limiting nutrient condition is used to control the growth rate. Contrary to chemostat, the nutrient concentration is not a limiting factor in batch. However, the passages must occur before the cells reach the stationary phase of growth, i. e., predominantly in the exponential phase (Barrick et al., 2009; Dragosits & Mattanovich, 2013; Jeong et al., 2016; LaCroix et al., 2017; Reyes, Gomez, & Kao, 2014).

Microorganisms evolved under limitation of essential nutrients can present some reduction of their fitness in non-limiting conditions, which is known as trade-offs (Bennett & Hughes, 2009; Dragosits & Mattanovich, 2013; Portnoy et al., 2011). The trade-offs are related to the ability of a microorganism to optimize different traits simultaneously, under different selective pressures caused by environmental conditions (King, Ishihama, Kori, & Ferenci, 2004; Portnoy et al., 2011). Similarly, SPANC balance (Self-Preservation and Nutritional Competence) describes the intrinsic ability of cells to keep their permanence in the environment, competing by the available resources. However, microorganisms or populations show trade-offs under important characteristics due to environmental heterogeneity (Elena and Lenski, 2003). *S. cerevisiae* evolved-strain increased the galactose uptake, however trade-offs were observed in glucose-grown cells (Hong et al., 2011). Several genetic mechanisms may produce fitness loss: antagonistic pleiotropy, a particular mutation could be beneficial in a certain condition, but prejudicial in other environments; accumulation of mutations, which a mutation substituted by genetic drift could be neutral as well as deleterious; and independent adaptation, where microorganisms can substitute beneficial mutations for neutral in different environments (Elena & Lenski, 2003; King et al., 2004).

In spite of ALE can cause loss of fitness due to different environmental conditions, the fitness may be improved due to the selection pressure. Regarding the microorganisms adaptation towards environmental stresses, cross-protection is an effect from selective pressure. It could lead to the increase in microorganisms

tolerance or resistance to stresses, when they are subjected to a second stress condition (Caspeta & Nielsen, 2015; Dragosits & Mattanovich, 2013; Lahtvee et al., 2016). Some studies have shown that cobalt-resistant yeast strains have been resistant to other metal ions and to oxidative and heat stress due to cross-protection (Mitchell et al., 2009). The trade-off and cross-protection are inherent to the process of ALE. Thus, the culture regime (or condition) imposed to the microorganisms, as well as the conditions of selective pressure, define the degree and extent of these phenomena.

Additionally, ALE can be combined with other metabolic engineering strategies such as target genetic changes, gene deletions, sequence insertions into genome regions, induction or repression of promoter regions, and alteration of the gene regulation expression to reach the improved fitness.

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CHAPTER II

**Manuscript written according to Applied Microbiology and Biotechnology guidelines.*

Assessment of ethanol tolerance of *Kluyveromyces marxianus* CCT 7735 selected by adaptive laboratory evolution

Abstract

Kluyveromyces marxianus CCT 7735 shows great potential for producing ethanol from lactose. However, its low ethanol tolerance is a drawback to overcome for its application at an industrial level. Adaptive laboratory evolution was used to select ethanol-tolerant *K. marxianus* CCT 7735 strains (ETSs). The ETS4 strain stood out which displayed a specific growth rate higher than the parental strain under ethanol stress (above 100%) and a specific ethanol production rate superior to all strains evaluated in the present study. Metabolomic and fatty acids analysis were carried out with both ETS4 and parental strains to gain insight into the mechanisms related to acquisition of ethanol tolerance. The accumulation of some amino acids (glutamate, alanine, valine, proline, and leucine) and metabolites of the Citric Acid cycle (isocitric acid, citric acid, cis-aconitic acid and malic acid) in ETS4 was found to be associated with acquisition of ethanol tolerance. Furthermore, high fatty acids content and ergosterol in ETS4 compared to the parental strain indicate differences in their plasma membrane composition, which is consistent with metabolite leakage observed in the parental strain. Therefore, the accumulation of amino acids and TCA metabolites, as well as the alteration in the fatty acid and ergosterol contents contributed to the acquisition of ethanol tolerance in *K. marxianus*.

Keywords: biofuel, whey, chemostat, ethanol stress, metabololomic analysis, cellular membrane

Introduction

Energy crisis and environmental issues have raised the demand for alternative renewable energies such as ethanol, the most commercialized biofuel in the world (Mussatto et al., 2010; Taylor, 2008; Valdivia, Galan, Laffarga, & Ramos,

2016). Currently, ethanol is commonly produced form sugarcane and corn, thus creating a competition between production of biofuels and food. In this context, ethanol production from other feedstocks such as whey and its derivatives have been considered an alternative to increase its production while reducing the impact on the food industry (González-Siso 1996; Prazeres et al. 2012; Parashar et al. 2016). However, *Saccharomyces cerevisiae*, a yeast widely used for ethanol production, is not able to assimilate lactose, the main sugar found in whey (Koushki et al. 2012). On the other hand, *Kluyveromyces marxianus* strains isolated from dairy environments can convert lactose to ethanol. *K. marxianus* CCT 7735, previously designated as UFV-3, has stood out as a good ethanol producer from whey permeate (Silveira et al. 2005; Diniz 2013) and a mixture of sugarcane bagasse hydrolysate and ricotta whey (Ferreira et al. 2015). Nevertheless, in contrast to *S. cerevisiae*, *K. marxianus* is less tolerant to the ethanol. *K. marxianus* CCT 7735 growth is severely impaired in ethanol concentrations above 4% (v/v) (Silveira et al. 2005; Costa et al. 2014). This is the main drawback for the use of this yeast at an industrial level.

In *S. cerevisiae*, the cellular membranes are the main targets of ethanol toxicity. Ethanol alters cell membrane structures by affecting the lipid-water interface, increasing lipid interdigitations and reducing membrane thickness (Ma and Liu 2010; Henderson and Block 2014b; Dong et al. 2015). It should be pointed out that the endoplasmic reticulum and protein structure/function are also targets of ethanol in long-term stress (Ma and Liu 2010; Stanley et al. 2010). Most studies in literature that assess cellular responses to ethanol in yeasts have focused on *S. cerevisiae* (Ma and Liu 2010; Stanley et al. 2010; Snoek et al. 2016). Nevertheless, the mechanisms involved with these responses are not fully understood.

Changes in ergosterol content, the major sterol in yeast membranes, as well as in fatty acids content and composition are important to counteract the damages provoked by ethanol in *S. cerevisiae* (Stanley et al. 2010; Vanegas et al. 2012; Caspeta and Nielsen 2015). In addition, the cell wall integrity and organization of integrated membrane proteins are affected by ethanol stress (Ma and Liu 2010).

In terms of metabolic responses, the overflow of certain metabolites has been related with responses to ethanol in *S. cerevisiae* (Granucci et al. 2015; Reaves et al. 2015). The accumulation of metabolites as proline, leucine, alanine,

glutamate and the tricarboxylic acid (TCA) cycle-related metabolites confers cellular protection against ethanol stress (Zhao and Bai 2009a; Ma and Liu 2010; Stanley et al. 2010; Ohta et al. 2015; Kim et al. 2016).

Recently, Diniz et al. (2017) analysed the *K. marxianus* CCT 7735 transcriptome under ethanol stress (6% v/v). The results revealed that the expressions of genes encoding enzymes of central metabolic pathways were reduced. In addition, they also observed that both ergosterol and unsaturated fatty acids contents did not change upon ethanol exposure in *K. marxianus* CCT 7735. Since in *S. cerevisiae*, the ergosterol and unsaturated fatty acids levels increase in response to ethanol, the authors claimed that these differences might be related to the low ethanol tolerance of *K. marxianus*.

Over the last years, adaptive laboratory evolution (ALE) has been widely used to obtain yeast strains more tolerant to stress conditions found in fermentative processes (Barrick et al. 2009; Dragosits and Mattanovich 2013; Caspeta et al. 2014; LaCroix et al. 2017). In the present work we selected ethanol-tolerant strains of *K. marxianus* by ALE under ethanol stress. The metabolic and fatty acids profiles of unstressed and ethanol-stressed cells provided insights into the mechanisms involved in the acquisition of ethanol tolerance in *K. marxianus*.

Experimental procedures

Yeast strain, maintenance and culture media

Kluyveromyces marxianus CCT 7735 used in this work, previously designated as UFV-3, belongs to the culture collection of Microbial Physiology of the Department of Microbiology at the Federal University of Viçosa (UFV). It is stored in the Tropical Cultures Collection André Tonsello Foundation, São Paulo, Brazil. The yeast was maintained frozen at -80 ºC in YP medium [yeast extract (10 g/L) and peptone (20 g/L)] containing glycerol 50% (v/v) or in YPL agar medium [yeast extract (10 g/L), peptone (20 g/L), lactose (20 g/L) and agar (15 g/L)] at 4 ºC.

For the adaptive laboratory evolution (ALE) experiments, *K. marxianus* CCT 7735 strains were cultivated in a synthetic defined medium (SD), SD agar and SDE. SD consisted of g/L: Yeast Nitrogen Base – YNB – without amino acids (Sigma Chemical Co., MO, USA) 6.7, and lactose 20. To prepare SD agar medium, agar

(15 g/L) was added to the SD medium. The SDE medium was prepared by adding ethanol (4% v/v) to the SD medium.

For chemostat cultivation, we used the defined medium described by Verduyn et al. (1992) containing 20 g/L of lactose (CBS) and CBSE, in which ethanol (4% v/v) was added to the CBS medium.

Adaptive laboratory evolution (ALE): yeast strains, growth media and cultivation conditions

For the ALE experiments, *K. marxianus* CCT 7735 was cultivated in SD agar medium and incubated at 37 ºC for 48 hours. After incubation, four colonies of *K. marxianus* CCT 7735 were randomly selected and grown for one day in 125 mL Erlenmeyer flasks containing 25 mL of SD medium at 37 ºC with a stirring rate of 200 revolutions per minute (rpm) in an orbital incubator. Posteriorly, the cells were harvested at 3,000 *g* at 4 ºC for 5 min, diluted into fresh medium to give an optical density at 600nm (OD_{600}) of about 0.2. The four selected colonies were cultivated in 125 mL Erlenmeyer flasks containing 25 mL of SD plus 4% (v/v) ethanol (SDE), at 37 ºC and 200 rpm up to reach 4-7 generations. One aliquot of the culture was diluted into a fresh SDE medium and transferred to a new Erlenmeyer flask containing SDE at OD_{600} of 0.2 and grown under the conditions aforementioned. Serial passages were performed across 300 generations, a period in which there was a significant increase (above 50%) in the specific growth rate. Subsequently, the ethanol tolerant strains obtained by ALE were stored in YP culture medium with 50% glycerol (v/v) at -80 ºC.

Determination of specific growth rate and dry weight

The cell growth was monitored by measurement of OD_{600}, using a UV-visible spectrophotometer (BECKMAN DU series 600). Biomass was determined by establishing a calibration curve of the dry cell weight (DCW) versus optical density. The yeast was transferred to 10 mL of YNB incubated at 37 ºC at 200 rpm for 18 h and centrifuged at 9,000 rpm at 4 ºC for 10 min. The resulting pellet was resuspended in 6 mL of distilled water. Four 1 mL aliquots of the resuspended yeast were harvested to determine the dry cell weight by drying at 105 ºC over 24 h. In parallel, 1 mL aliquots of the cell suspension were diluted ($1 \times 10^{-2,}$ 2×10^{-2}, 3×10^{-2},

4 x 10^{-2}, 5 x 10^{-2} and 6 x 10^{-2}) and their optical density at OD_{600} were measured. A calibration curve was obtained from linear regression between absorbance (OD_{600}) and DCW (mg/mL). The specific growth rate (μ) was determined by linear regression between values of optical density (600 nm) and time (h) using the R software 3.2.5 (www.r-project.org).

Physiological characterization

The physiological characterization of ethanol-tolerant *K. marxianus* CCT 7735 and parental strains was carried out by determination of both kinetic and fermentative parameters. The pre-inoculums were obtained in 125 mL Erlenmeyer flasks containing 25 mL of SD medium at 37 ºC for 20 h. After incubation, these cultures were centrifuged at 4 ºC and 16,000 *g* for 5 min. The cell mass was washed twice with peptone water (0.1% w/v), diluted to OD_{600} of 0.2. The washed pre-inoculum was inoculated into 50 mL of SD medium in 250 mL Erlenmeyer flasks and incubated at 37 ºC, 200 rpm for 24 h. The cell growth was monitored by measurement of OD_{600} using a UV-visible spectrophotometer (BECKMAN DU series 600). Aliquots of 2 mL were collected every 1 h for the first 12 h. Furthermore, the samples were centrifuged at 4 ºC, 16,000 *g* for 5 min, and then the supernatants were filtered (pore size 0.22 μm). The samples were stored at -20 ºC for both lactose consumption and metabolite production analysis.

Analysis of lactose and primary metabolites

The lactose consumption, ethanol and glycerol production of *K. marxianus* CCT 7735 tolerant and parental strains were determined by high performance liquid chromatography (HPLC) - chromatograph Shimadzu TA-20 (Kyoto, Japan) coupled to refractive index detector and column ion exchange Rezex ROA-Organic acid H^+ 8% (300 x 7.8 mm, Phenomenex, California, USA), temperature of 45 ºC, using 0.005 M sulfuric acid as mobile phase, flow rate of 0.6 mL/min and injection volume of 10 μL. Metabolite quantification was obtained by calibration curves using external standards.

Determination of fermentation parameters

The ethanol yield ($Y_{P/S\ ethanol}$, g/g) was determined by angular coefficient from a linear regression of the plot ethanol concentration (g/L) *versus* lactose consumption (g/L). Cell biomass yield ($Y_{X/S,}$ g/g) was determined by angular coefficient from a linear regression of the plot DCW *versus* lactose consumption (g/L). Specific ethanol production rate (q_p, g/g/h) and specific lactose consumption rate (q_s, g/g/h) were determined by multiplying the respective yields by the specific growth rate (per h). Linear regressions were adjusted at the exponential growth phase. Ethanol volumetric productivity ($Q_{p\ ethanol}$, g/L/h) was calculated by maximum ethanol production divided by time.

Chemostat cultivation

Both ethanol-tolerant and parental strains were cultivated in chemostat in order to identify the metabolic changes in response to ethanol. Exponentially growing cells in CBS medium were inoculated with an initial OD_{600} of 0.2 into 2 L of CBS medium in a 5 L Labfors 5 bioreactor system from INFORS HT (Bottmingen, Switzerland). Air flow was set to 1 vessel volume per minute (vvm) and the dissolved oxygen (DO) levels were kept >15% throughout the experiments. The both temperature and pH were kept constant at 37 ºC and 5.5 by adding either NaOH (10% w/v) or H_2SO_4 (10% v/v), consequently. The cultivation started as a batch culture with a stirring rate of 200 rpm. When the lactose concentration reached 5 g/L, the chemostat mode was started. The chemostat was operated at a dilution rate of 0.05 (per h); the feed medium was the same described for the batch phase, except for lactose concentration, 5 g/L instead 20 g/L. Sampling for intra and extracellular metabolites as well as fatty acids methyl esters (FAMEs) analysis were performed in steady-state through at least two residence times (two culture volumes of fresh medium). After sampling, an ethanol pulse in steady-state was carried out to reach the final ethanol concentration of 4% (v/v). Afterwards, sampling was performed over 25 min every 5 min. This period corresponds to the transition phase. Posteriorly, the feed medium was changed from CBS to CBSE. Upon two residence times under ethanol stress, samples were withdrawn in steady-state for intra (*n* = 5) and extracellular (*n* = 3) metabolites as well as FAMEs analysis (*n* = 3).

Sampling and extraction procedures for intracellular and extracellular metabolite analysis

The procedure adopted in this work was based on the protocol described by Smart et al. (2010). For intracellular analysis, five samples of 30 mL culture were harvested from a chemostat in steady-state, and quickly transferred to centrifuge tubes with cold glycerol-saline solution (3:2) in an ethanol bath at −23 ºC for quenching (Villas-Bôas and Bruheim 2007). Next, they were vigorously mixed and centrifuged at 36,000 *g* for 20 min at −20 ºC using a refrigerated centrifuge. The resulting cell pellets were resuspended in cold glycerol-saline washing solution (1:1) followed by centrifugation as described above. The samples were kept at temperatures below −15 ºC. Afterwards, intracellular metabolites were extracted from the cell pellets by adding cold methanol–water solution (1:1) at −30 ºC and the internal standard (2, 3, 3, 3-d_4-alanine), followed by three freeze–thaw cycles. The extraction steps were carried out at −20 ºC or below. Samples containing intracellular metabolites were freeze-dried and concentrated using a VirTis freeze-dryer from SP Scientific (Newtown Square, PA, USA). The freeze-dried samples were stored at −80 ºC for posterior analysis.

For extracellular analysis, three samples of 10 mL were withdrawn from the chemostat in steady-state and immediately filtered using a membrane filter (0.2 mm pore size). Subsequently, the filtrates were divided into 3 aliquots (2 mL) and 20 μL of 2, 3, 3, 3-d_4-alanine (internal standard) was added to each. Next, the samples were freeze-dried on a VirTis freeze-dryer (SP Scientific, Newtown Square, PA, USA) and stored at −80 ºC.

Derivatization and GC-MS analysis

The metabolites were derivatized as described by Smart et al. (2010), with some modifications. The freeze-dried intracellular and extracellular samples of both ethanol-tolerant and parental strains were resuspended in 400 μL of NaOH (1 M) and transferred to silanized reaction tubes. Subsequently, 334 μL and 68 μL of methanol and pyridine, respectively, were added to the samples. Methylchloroformate (MCF) was used as the derivatizon agent. The derivatized samples were analyzed by using a GC-MS using a GC7890 gas chromatograph

coupled to a MSD5975 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Metabolite identification and data analysis

The deconvolution of GC-MS chromatograms and metabolite identification were carried out by AMDIS software using an in-house MCF mass spectral library. The GC peak values of selected reference ions of identified metabolites were used to determine the relative abundance by ChemStation (Agilent Technologies, Santa Clara, CA, USA). For intracellular metabolites analysis, these values were normalized initially by dividing the metabolite intensities by the abundance of the internal standard (2, 3, 3, 3-d_4-alanine); additionally, the values were divided by the biomass values measured for each sample. For extracellular metabolites, the metabolite intensities detected in the non-inoculated medium samples were first subtracted from respective intensities detected in the extracellular sample. Thereafter, they were normalized by the internal standard and biomass at each sample. The entire data-mining, normalization and analysis were automated in R software as described by Aggio, et al. (2011) and Smart et al. (2010). The relative abundance of each identified metabolite was tested using Student's t-test to determine whether there was statistical significance between unstressed and ethanol-stressed cells.

Fatty acids methyl esters analysis

For FAMEs analysis, three samples were withdrawn from the chemostat in steady-state and centrifuged at 2,400 *g* at 4 ºC for 10 min. The supernatant was discarded and 10 mL of cold bidistilled water (4 ºC) was added to cell pellets and stored at -80 ºC. Afterwards, the samples were freeze-dried on a VirTis freeze-dryer (SP Scientific, Newtown Square, PA, USA). Subsequently, the direct-transesterification method was performed. The freeze-dried samples were weighed into glass tubes, 1 and 5 mg of parental and ethanol-tolerant strains, respectively. Next, 2 mL of internal standard solution dissolved in methanol-toluene solution (4:1; v/v) was added. The internal standard concentration (tridecanoic acid - $C_{13:0}$) dissolved in 2 mL of methanol-toluene 4:1 (v/v) ranged from 50 µg to 300 µg. Afterwards, 200 µL of acetyl chloride was added slowly, over a period of 1 min. The

glass tubes containing magnetic stirring bars were closed tightly with the Teflon-lined and heating/stirring into dry block at 100 ºC for 1 h. Posteriorly, these tubes were cooled in cold water at and leakage was checked by slowly adding 5 mL of 6% K_2CO_3 per sample, which was used to stop the reaction and neutralize the mixture. Finally, the samples were vortexed and centrifuged at 22 ºC and 450 *g* for 5 min. The toluene in the upper phase was collected and injected into GC-MS using a GC7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a MSD5975 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Ergosterol analysis

Ergosterol of both ethanol-tolerant and parental strains was extracted according to Lahtvee et al. (2016) with some modifications. Three samples were harvested from chemostat in steady-state and stored at -80 ºC for 24 h. The frozen samples were freeze-dried on a LIOTOP L101 freeze-dryer (Liobras, São Carlos, SP, Brazil). Freeze-dried cells were weighed (40-50 mg) and added to 0.5 mL of methanol/chloroform (2:1) solution and two steel beads. Next, they were subjected to cellular lysis using TissueLyser II (Qiagen, Hilden, Germany). The extracts were centrifuged at 12,000 *g* for 10 min and the supernatants were transferred to new tubes. The first four steps were repeated three times. Briefly, 2 mL of 100% (v/v) chloroform solution were added and homogenized. Subsequently, 2 mL of 1% (w/v) sodium chloride solution were added and centrifuged at 12,000 *g* for 20 min. The inferior phase was collected and transferred to new tubes for drying in the Concentrator plus/Vacufuge® plus (Eppendorf AG, Hamburg, Germany). Dried extracts were resuspended in a methanol-chloroform (2:1) solution. The ergosterol content determination was performed by high performance liquid chromatography (HPLC) - chromatograph Shimadzu TA-20 (Kyoto, Japan) coupled to SPD-10A UV–visible detector at 282 nm and column Kinetex RP-C18 column (250 x 4.6 mm, 5 µm, Phenomenex, California, USA), at 30 ºC, using 98% (v/v) methanol solution as the mobile phase, flow rate of 1 mL/min and an injection volume of 10 µL. Ergosterol was quantified by a calibration curve obtained from external standards (0.01 to 1.0 mg/mL). The data was normalized with biomass.

Pathways analysis

We used MetaboAnalyst (www.metaboanalyst.ca), a web-based data analysis platform, for pathway analysis to predict and compare the activity of different pathways in ethanol-tolerant and parental strains, under chemostat growth conditions tested. It uses the KEEG metabolic pathway database (www.genome.jp/kegg/pathway.html) to identify significantly perturbed metabolic pathways from changes in the metabolite profiles (Xia et al. 2009; Xia et al. 2012; Xia et al. 2015). This tool uses metabolomics data, as well as lists of genes or KEGG orthologs to support integrative analysis, using the relative abundances of intra- and extracellular metabolite profiles to predict which pathways may be active in the yeast. Graphical representations of these results were performed by software R (www.r-project.org).

Results

Adaptive laboratory evolution (ALE) under ethanol stress

Taking into account that the *K. marxianus* CCT 7735 growth is impaired at ethanol concentration of 4% (v/v) (Silveira et al. 2005; Costa et al. 2014), this concentration was used for ALE experiments. We used four different populations from individual clones of *K. marxianus* CCT 7735 wild-type in order to select strains more tolerant to ethanol stress by adaptive laboratory evolution (ALE). Those yeast populations were propagated in sequential passages under ethanol stress. The ALE was stopped when the ethanol-tolerant *K. marxianus* (ETS) strains 1, 2, 3 and 4 had an increase superior to 50% in the specific growth rate compared to the wild-type (parental) strains 1, 2 3 and 4 (Figure 1; Table S1), which corresponded to 340, 310, 328 and 335 number of generations, respectively. The ETS1 and ETS4 strains stood out since their specific growth rates increased above 100%, that is, 127.8% and 101.2%, respectively. The specific growth rates of the ETS2 and ETS3 strains were also superior to their parental strains, 60.33% and 88.5%, respectively, however these increases were lower than those observed for the ETS1 and ETS4 strains. Therefore, the ALE was a suitable strategy to select ethanol-tolerant *K. marxianus* strains. Beyond mutations that lead to the acquisition of the desirable phenotype in microbial cells evolved by ALE, it has been reported that other one

cause trade-off phenotypes (Dragosits and Mattanovich 2013; Caspeta and Nielsen 2015). To address this issue, we evaluated whether the ETS strains had an alteration in their fermentative capacity by determining the physiological parameters of both ETS and parental strains.

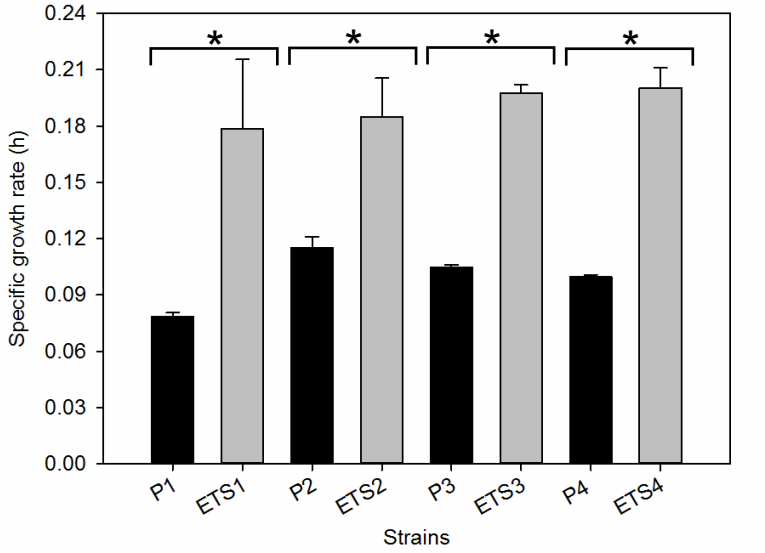
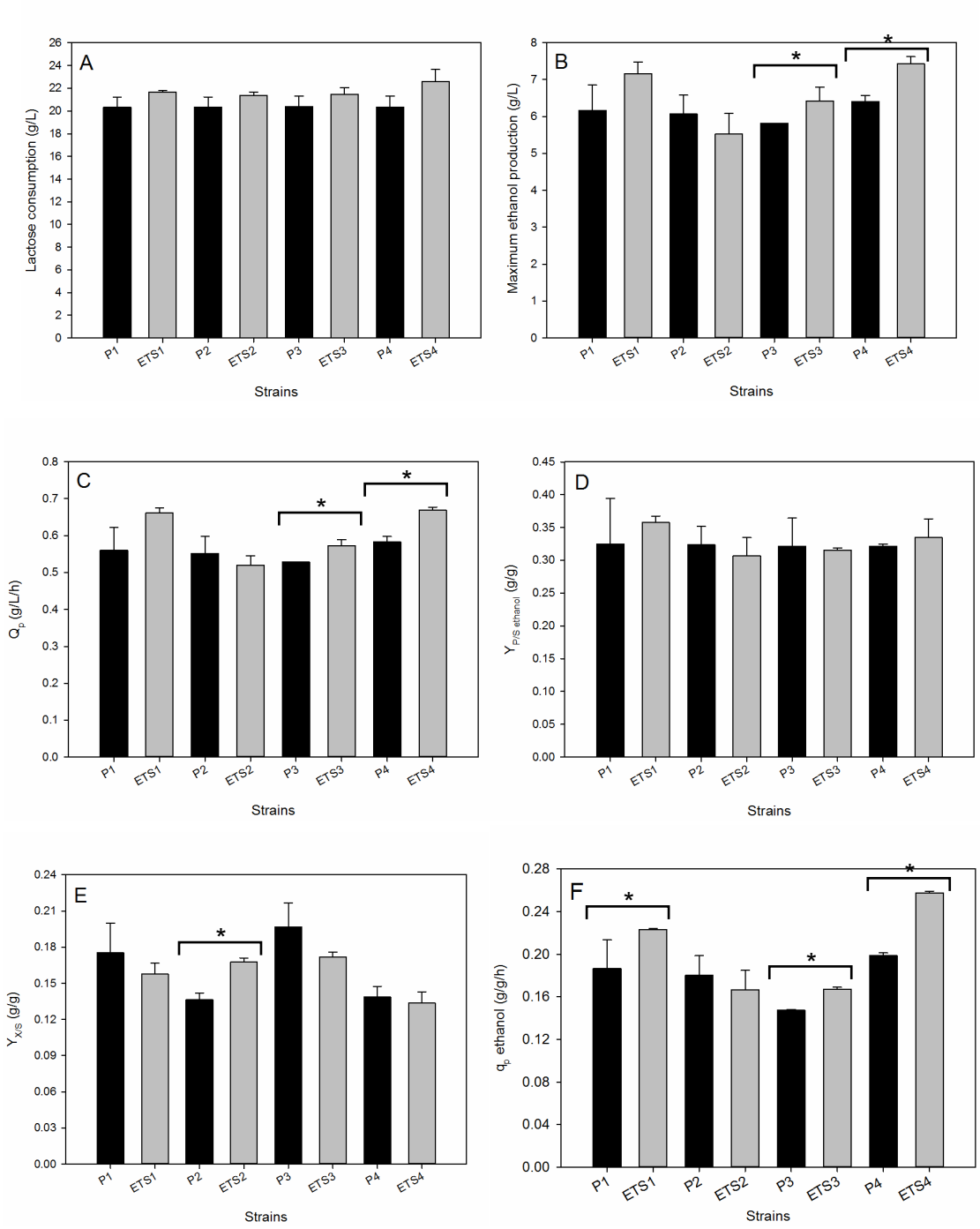


Figure 1. Adaptive laboratory evolution of *K. marxianus* CCT 7735 under ethanol stress (4% v/v). The specific growth rates of the ethanol-tolerant strains (ETS1, ETS2, ETS3 and ETS4) under ethanol stress were higher than of the parental strains (P1, P2, P3 e P4). (*) Means significant difference between treatments according to Student's t-test (p = 0.05).

In general, we observed that the physiological parameters of ETS and parental strains were similar (Figure 2A to 2G; Table S1). However, the ETS1 and ETS4 strains displayed the highest maximum ethanol production and volumetric ethanol productivities (Figures 2B and 2C). In addition, we verified that the specific ethanol production rate presented by ETS4 strain was higher than those obtained by other ETS and parental strains (Figure 2F). The biomass yield of the ETS4 strain was lower than those presented by other ETS strains, which is consistent with the fact that it presented the highest specific ethanol production rate and ethanol titer. Overall, the ETS strains did not present a trade-off for their fermentative capacity, which is pivotal for further application in the ethanol production. Nevertheless, it is important to point out that the ETS4 strain stood out for displaying both a specific growth rate higher than the parental strain in culture medium containing ethanol (above 100%) and the highest specific ethanol production rate compared the other ETS strains. Therefore, we chose the ETS4 strain for further metabolic

characterization in order to gain insights about the mechanisms involved with the acquisition of ethanol tolerance in *K. marxianus*.



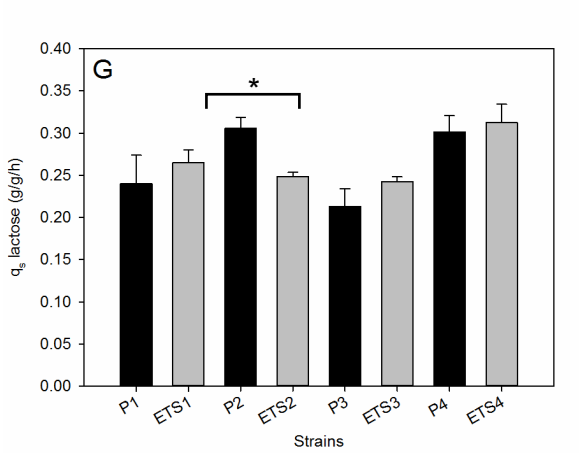


Figure 2. Physiological parameters of ethanol-tolerant *K. marxianus* (ETS) strains (ETS1, ETS2, ETS3 and ETS4) and parental strains (P1, P2, P3 and P4) selected from ALE: (A) lactose consumption (g/L); (B) maximum ethanol production (g/L); (C) ethanol volumetric productivity (Qp; g/L/h); (D) ethanol yield per lactose ($Y_{P/S\ ethanol}$; g/g); (E) biomass yield per lactose ($Y_{X/S}$; g/g); (F) specific ethanol production rate (q_p, g/g/h); and (G) specific lactose consumption rate (q_s, g/g/h). (*) Means significant difference between strains according to Student's t-test (p = 0.05).

Metabolomic analysis

The metabolic profile of both ETS4 and P4 strains was analyzed in chemostat cultures conducted at a fixed dilution rate (0.05 per h) under both ethanol and non-ethanol stress conditions (Figure 3). Under non-ethanol stress condition, ETS4 and P4 strains were grown on a lactose-limited chemostat (5 g/L). Intra- (n = 5) and extracellular (n = 3) metabolomic samples were harvested from steady-state. Subsequently, those strains were pulsed with ethanol 4% (v/v) and then intra- and extracellular metabolomics samples (n = 5) were taken over the transition phase. To establish the ethanol stress, the strains aforementioned were grown on a lactose-limited chemostat with addition of ethanol 4% (v/v). After reaching the steady state, intra (n = 5) and extracellular (n = 3) metabolomic samples were withdrawn.

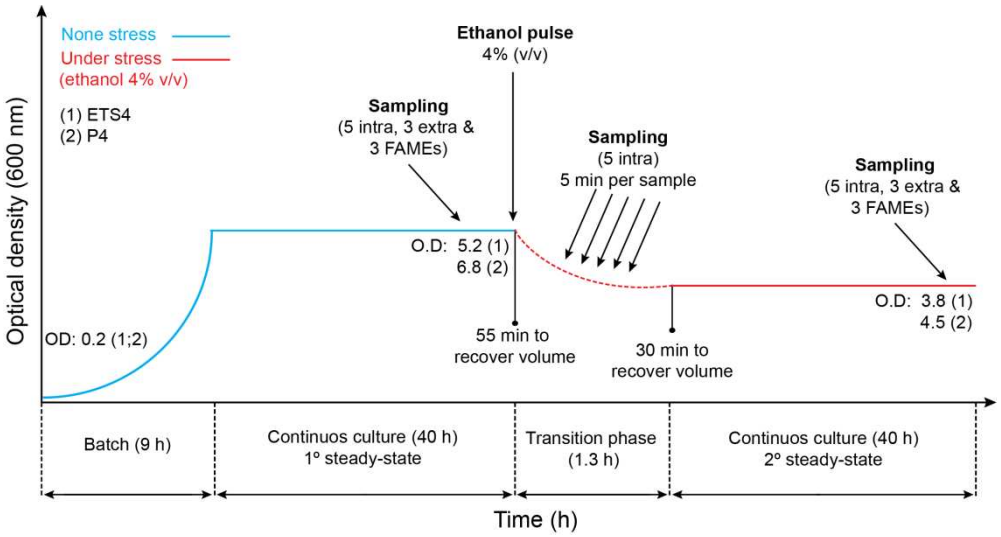


Figure 3. Experiment overview. ETS4 and P4 strains were cultivated in bioreactors on lactose limited chemostat culture starting as 20 g/L of lactose (batch phase), at 37 ºC, 200 rpm, pH 5.5 and 1 vvm of air. The bioreactor was switched to chemostat once lactose reached approximately 5 g/L. The chemostat was carried out with 5 g/L of lactose and 0.05 per h of dilution rate. Ethanol 4% was added to the bioreactor resulting in a transition phase. Upon this phase, the chemostat was carried out with 5 g/L of lactose and ethanol 4% (v/v). Samples for metabolomic (intra and extracellular metabolites) and FAMEs profiles were harvested at the steady-states and transition phase.

We identified 151 intracellular metabolites from chemostat cultures of ETS4 and P4 strains under both ethanol and non-ethanol stress, however 55 metabolites were unidentified (Table S1). For the exometabolome, 141 metabolites and 47 metabolites were identified and unidentified, respectively, under the conditions aforementioned (Table S2). Most of the metabolites identified intra- was detected extracellularly; therefore, we relate them with metabolic responses to the ethanol stress.

The intra- and extracellular profiles of significantly different metabolites from ETS4 and P4 strains cultivated non- and under ethanol stress were statistically compared by principal component analysis (PCA). This analysis reduces the dimensionality of multivariate data allowing visualized them graphically with minimal loss of information (Xia et al. 2012). The four groups formed on both intra and extracellular metabolites PCA plots (Figure 4A and 4B) are composed for two strains under ethanol stress (P4 stress and ETS4 stress) and the same strains under non-ethanol stress (P4 and ETS4). All groups were separated by principal components 1 (PC_1) and 2 (PC_2), which accounted a total variance of 61.83% and 13.80% for intracellular metabolites, 91.30% and 3.03% for extracellular metabolites, respectively. Notably, we observed that the P4 stress strain was in a distinct cluster for both intra- and extracellular metabolites (Figure 4) and that the

ETS4 stress strain was close to ETS4/P4 cluster (Figure 4A). Indeed, we observed an ellipses overlap on ETS4 and ETS4 stress clusters. For PCA analysis, loading scores mean how much each metabolite contributed to the generated clustering, therefore PC_1 contains the higher total variance value, indicating that the components significantly influenced the separation of the strains. β-alanine, cystathionine, threonine, proline and alanine (intracellular metabolites); and malonic acid, leucine, 2-hydroxybutiric acid, lactic acid and nicotinic acid (extracellular metabolites) were the most important metabolites which contributed positively to PC_1. We observed higher levels of those metabolites in P4 and ETS4 and ETS4 stress, suggesting that their decrease in the parental strain subjected to the ethanol stress seems to be related to its lower ethanol tolerance compared to the ETS4.

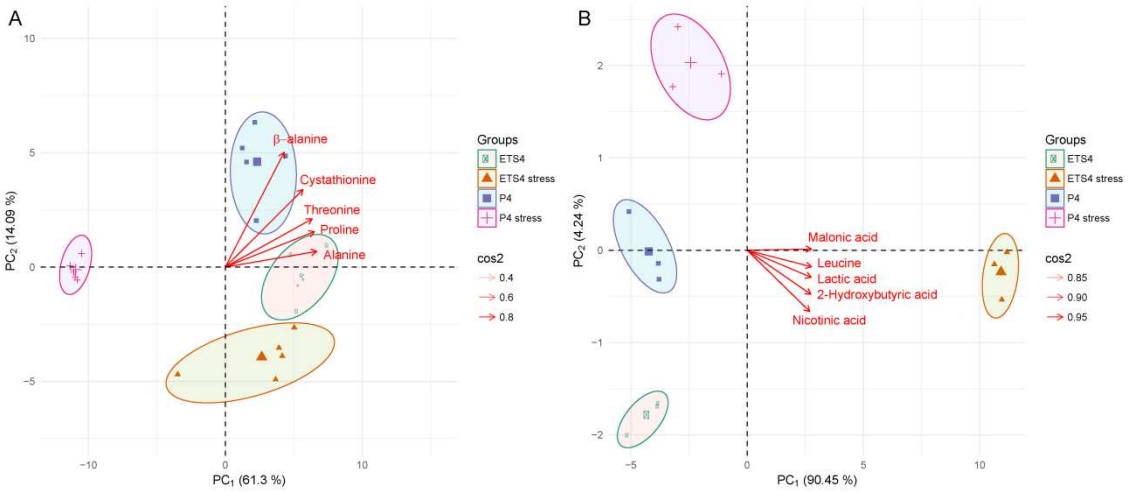


Figure 4. PCA plots of the intra- (A) and extracellular (B) metabolites data for both ETS4 and P4 strains. Experiments were carried out under ethanol (4%; v/v) and non-ethanol stress. Ellipses demonstrate the groups of strains formed. PCA describes the alterations in the formed groups from intra and extracellular metabolites levels statistically different (p = 0.05). Metabolites were identified from in house mass spectral library. The Five metabolites of each condition that most contributed for clustering (autovectors) are highlighted on PCA plot. P4: parental strain under non-ethanol stress; P4 stress: parental strain under ethanol stress; ETS4: ethanol-tolerant strain under non-ethanol stress; and ETS4 stress: ethanol-tolerant strain under ethanol stress.

We performed a hierarchical cluster analysis (HCA) to analyse the different groups of intra and extracellular metabolites (Figures 5 and 6). Regardless of the strain and stress condition, the intracellular metabolites identified were similar; however their relative abundance was group-dependent. Most of the intracellular amino acids and derivatives, amina/amida compounds and organic acids had their relative abundance strongly reduced in the P4 strain under ethanol stress (Figure 5; Figure S1). Otherwise, the extracellular abundance of all metabolites remarkably increased upon ethanol exposure (Figure 6; Figure S2), suggesting that the ethanol

induced a leakage of metabolites in the parental strain for altering the permeability of its membrane. Interestingly, the relative abundance of the most of intracellular amino acids and derivative in ETS4 stress also reduced, however in a lesser degree than in P4 stress (Figure 5A; Figure S3). In addition, most of amina/amida compounds and organic acids had their relative abundance enhanced under ethanol stress (Figure 5B; Figure S3). Taken together, these results indicate that the membrane permeability of the ethanol-tolerant strain was less affected compared to parental strain. Therefore, the higher level of some intracellular metabolites in the ETS4 stress is probably related to its improved tolerance to ethanol.

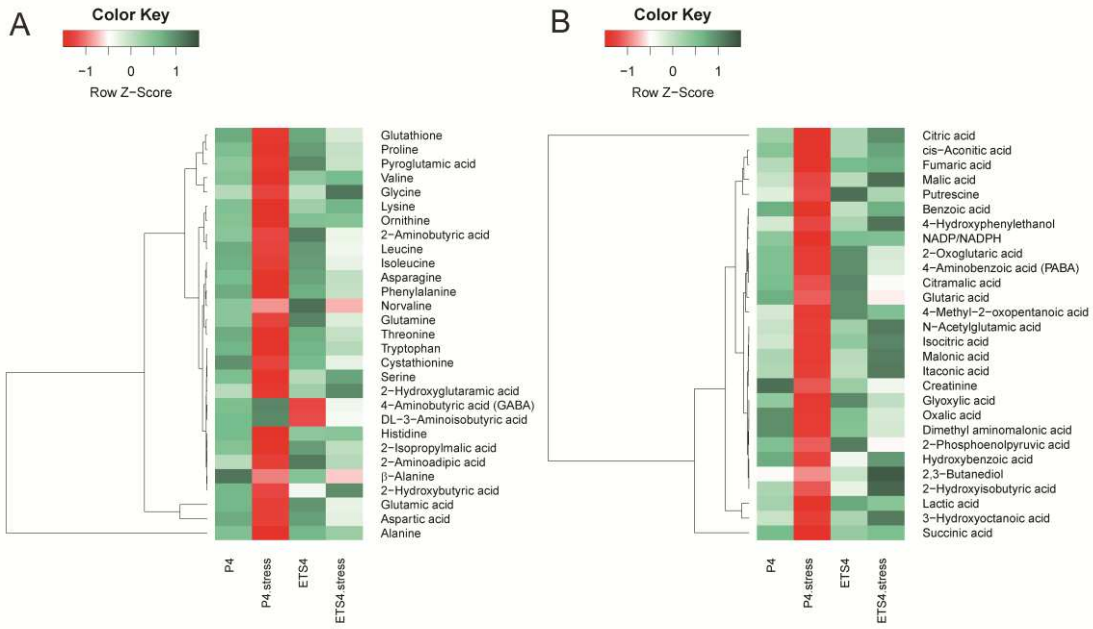


Figure 5. Hierarchical cluster analysis of intracellular metabolites groups: (A) amino acids and derivatives; and (B) amina/amida and organic acids. P4: parental strain under non-ethanol stress; P4 stress: parental strain under ethanol stress; ETS4: ethanol-tolerant strain under non-ethanol stress; and ETS4 stress: ethanol-tolerant strain under ethanol stress. A dendrogram was added to the left side reordering the metabolites according to the set of abundance values under ethanol and non-ethanol stress. The highest (green color) and lowest (red color) abundance values are shown on Row-Z score.

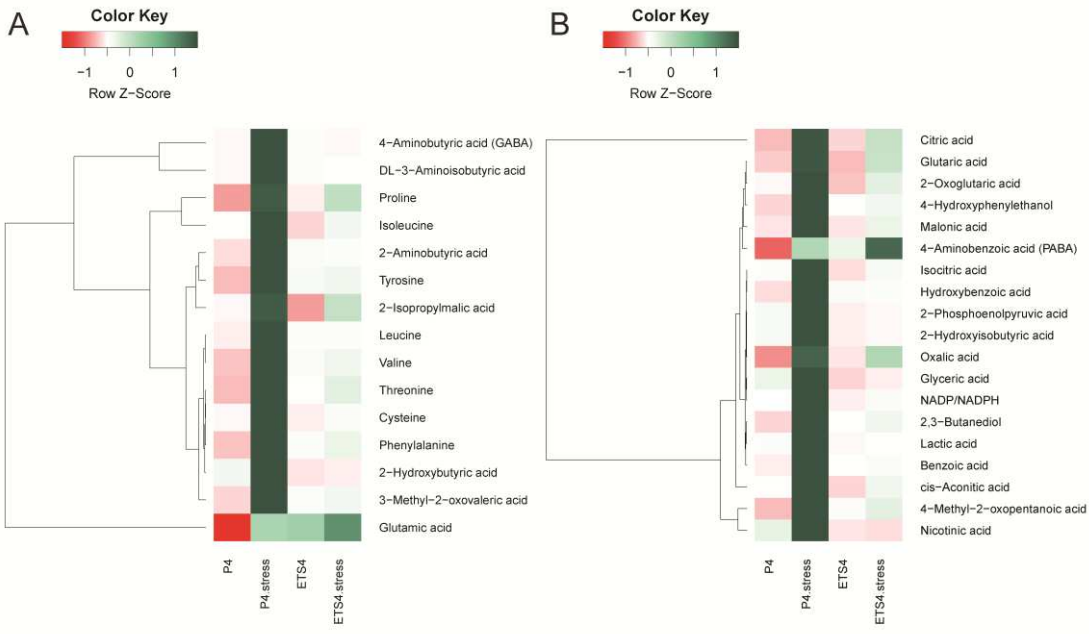


Figure 6. Hierarchical cluster analysis of extracellular metabolites groups: (A) amino acids and derivatives; and (B) amina/amida and organic acids. P4: parental strain under non-ethanol stress; P4 stress: parental strain under ethanol stress; ETS4: ethanol-tolerant strain under non-ethanol stress; and ETS4 stress: ethanol-tolerant strain under ethanol stress. A dendrogram was added to the left side reordering the metabolites according to the set of abundance values under ethanol and non-ethanol stress. The highest (green color) and lowest (red color) abundance values are shown on Row-Z score.

Importantly, some amino acids and derivatives as glutamic acid, alanine, valine; organic acids involved in The Citric Acid cycle (TCA) as isocitric acid, citric acid, cis-aconitic acid, malic acid and other one as malonic acid, benzoic acid and 4-hydroxyphenylethanol had their levels increased intra- and extracellularly. Therefore, those metabolites seem to have been secreted due to the metabolic overflow, indicating that the metabolic fluxes of the pathways involved with their synthesis enhanced (Figures 5 and 6; Figures S3 and S4). We also detected an increase of the levels of some amino acids and derivatives as alanine, alanine, 4-aminobutyric acid (GABA) and DL-3-aminoisobutyric acid both intra- and extracellularly in P4 stress (Figures 5 and 6; Figures S1 and S2), suggesting a metabolic overflow.

FAMEs and ergosterol analysis

We performed the fatty acid methyl esters (FAMEs) profiles from plasma membrane of P4 and ETS4 strains (non- and ethanol stress) by transesterification method and GC-MS analysis in order to evaluate its composition and abundance in response to ethanol.

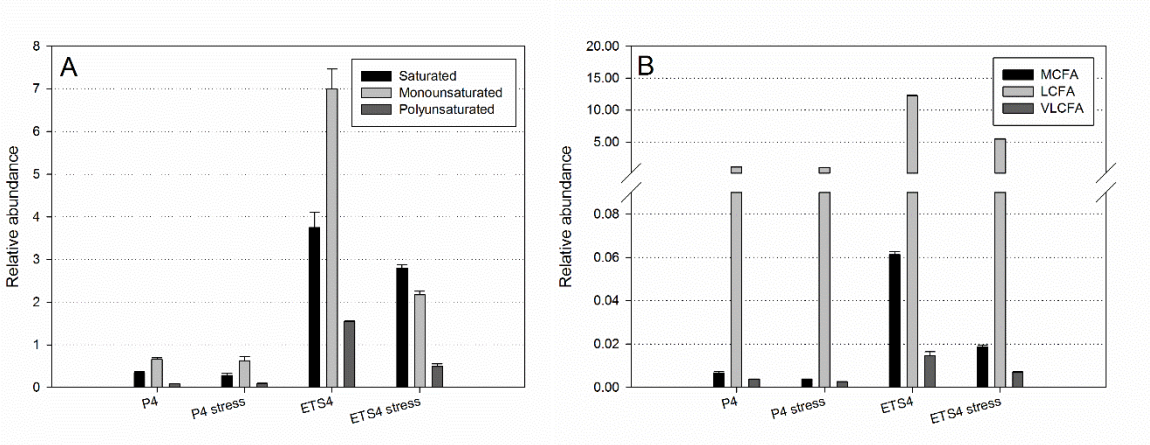


Figure 7. Fatty acids methyl esters profiles of P4 and ETS4 strains under non- and ethanol stress. (A) total of FAMEs (saturated, monounsaturated and polyunsaturated); and (B) length of FAMEs: medium-chain fatty acids (MCFA) ranging from 6 to 12 carbons; long-chain fatty acids (LCFA) ranging from 13 to 21 carbons and very long chain fatty acids (VLCFA) >21 carbons. Relative abundance values from each sample were divided by value of biomass detected in P4 and ETS4 samples (specific relative abundance by biomass).

The membrane fatty acids abundance in the ETS4 strain is remarkably higher than in P4 strain. In P4, the monounsaturated fatty acids were more abundant under both non- and ethanol stress (Figure 7A and 7B). Their abundance were also higher in ETS4 strain, however when it was exposed to the ethanol, the unsaturated fatty acids level decreased. In addition, we detected that the long-chain FAMEs were more abundant in the two strains regardless of the stress condition (Figure 7B). These results are consistent with the fact that most common fatty acids from yeast phospholipids bilayers are long-chain such as palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$) and oleic acid ($C_{18:1}$). In addition, we compared the relative abundance of those fatty acids (Figure 8). According to results shown in Figure 7, their abundance were highest in ETS4 strain, however it decreased once that strains were subjected to ethanol stress. In general, palmitoleic acid and oleic acid (monounsaturated fatty acids) content were higher than palmitic acid and stearic acid (saturated fatty acids). Importantly, there were no statistically significant differences in palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$) and oleic acid levels between P4 and P4 stress. On the other hand, the decrease in palmitic acid, palmitoleic and oleic acid contents in ETS4 stress were statistically different.

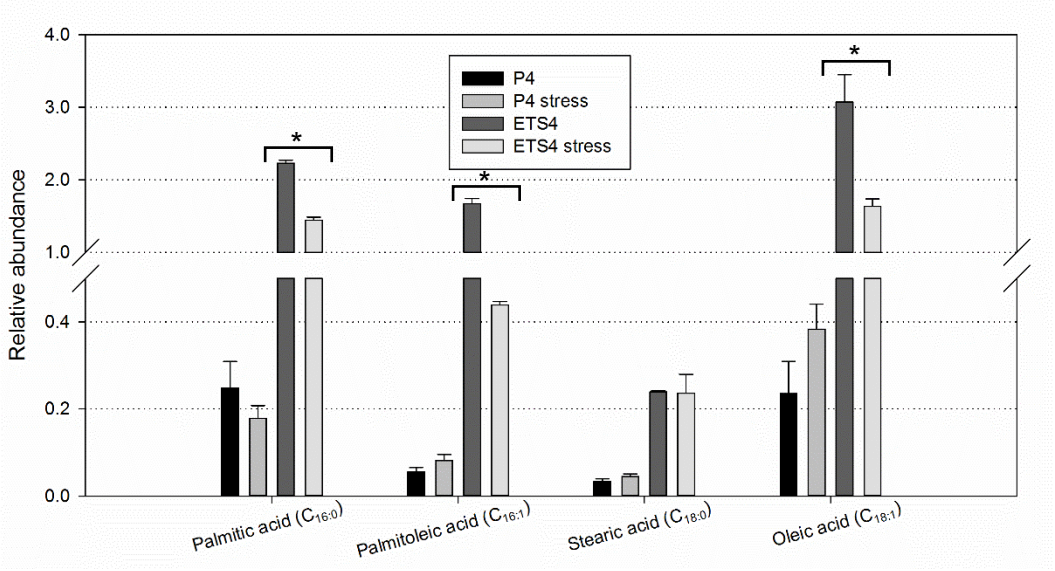


Figure 8. Relative abundances of fatty acids methyl esters of P4 and ETS4 strain under non- and ethanol stress: palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$) profiles. Relative abundance values from each sample were divided by value of biomass detected in P4 and ETS4 samples (specific relative abundance by biomass). (*) Means significant difference between fatty acids according to Student's t-test (p = 0.05).

Ergosterol plays a crucial role in response to ethanol stress in *S. cerevisiae* (Stanley et al. 2010; Vanegas et al. 2012). We evaluated its concentration in both P4 and ETS4 strains under non- and ethanol conditions (Figure 9). Ergosterol was more abundant in ETS4 than in P4 under both conditions. It should be noted that its concentration decreased in both P4 and ETS4 strains under ethanol stress.

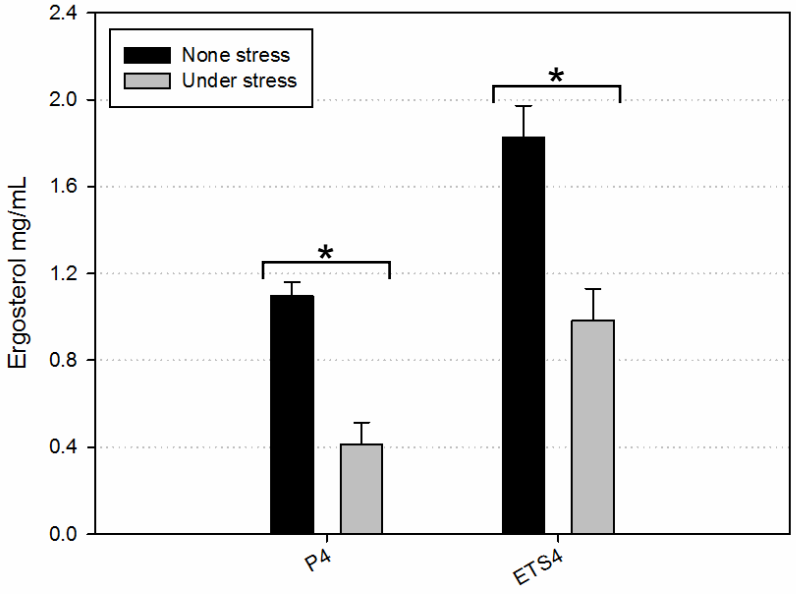


Figure 9. Ergosterol concentration of ETS4 and P4 strains under non- and ethanol stress. (*) Means significant difference between fatty acids according to Student's t-test (p = 0.05).

Metabolic pathways analysis

We analysed the relative abundance of intra- and extracellular metabolite profiles under ethanol and non-ethanol stress to generate a comparative metabolic pathways analysis (Figure 10; Figures S4 and S5). We used the MetaboAnalyst tool (www.metaboanalyst.ca), a web-based site for high-throughput metabolomic data processing and analysis. It utilizes the models from KEGG online database to analyse the changes in intra- and extracellular metabolites.

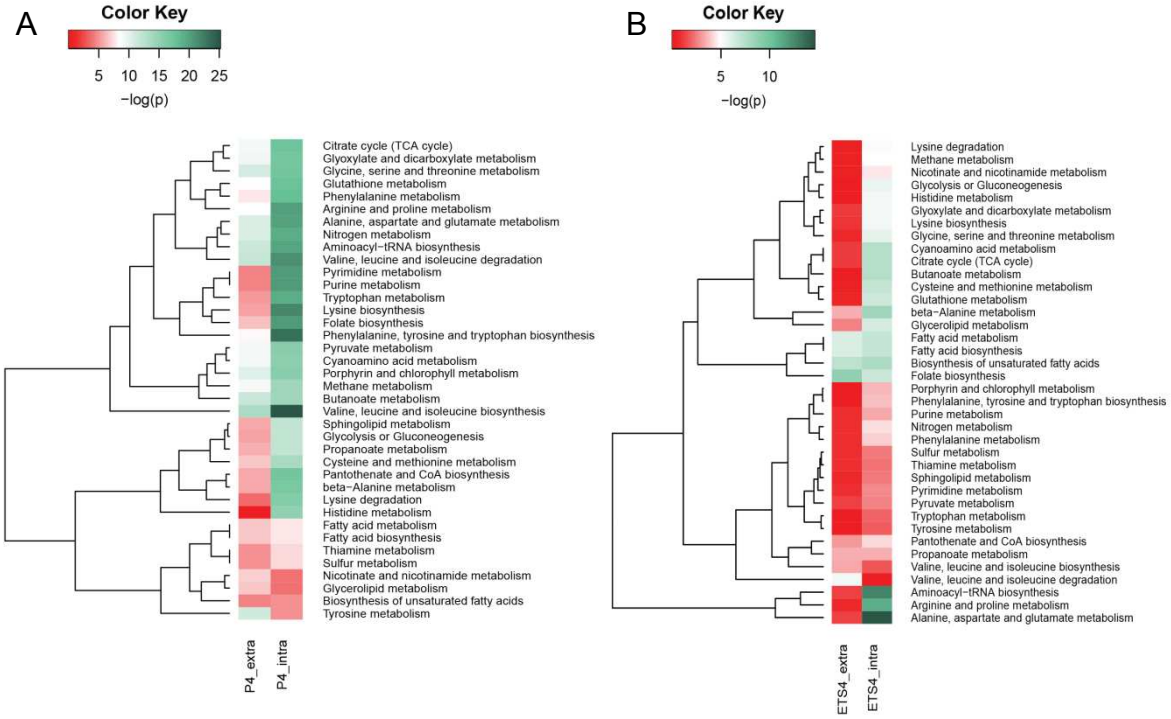


Figure 10. Metabolic pathways activities from intra- and extracellular metabolite profiling data of the P4 (A) and ETS4 (B) strains. The activities of metabolic pathways were compared to ethanol and non-ethanol stress for both strains. Deeper red colors (low values) indicate pathways that were less affected in response to ethanol, while deeper green colors (high values) are metabolic pathways more affected. Only metabolic pathways that were statistically significant change from metabolite profiling are shown (p = 0.05).

In P4 stress strain, 12 and 30 out of 38 metabolic pathways that were statistically different from intra- and extracellular metabolites, respectively. In particular, pathways associated with the fatty acids metabolism and biosynthesis, biosynthesis of unsaturated fatty Acids, glycerolipid metabolism and some amino acids and derivatives metabolism were less affected in both intra- and extracellular metabolite profiling (Figure 10A). In the ETS4 stress strain, 19 and 4 out of 38 metabolic pathways were statistically different from intra- and extracellular metabolite profiling, respectively (Figure 10B). Contrary to P4 strain, fatty acids

metabolism and biosynthesis and biosynthesis of unsaturated fatty acids were more affected both intra- and extracellular metabolite profiling, as well as glycerolipid metabolism in intracellular metabolites (Figure 10B). Additionally, ETS4 pathways from intracellular metabolites as Citrate cycle (TCA cycle), glutathione and glutamate metabolism, proline, arginine, lysine and butanoate metabolism were more affected under ethanol stress. According to the FAMEs, PCA an HCA results, those metabolic pathways seem to be involved with the acquisition of ethanol tolerance in *K. marxianus*.

Discussion

K. marxianus CCT 7735 (UFV-3) is capable of converting efficiently lactose from whey permeate to ethanol (Silveira et al. 2005; Diniz et al. 2013; Ferreira et al. 2015). Nevertheless, its low ethanol tolerance is the main drawback for its application at industrial level. In this work, ALE was suitable to select *K. marxianus* CCT 7735 ethanol-tolerant (ETS) strains, highlighting its efficiency in improving the robustness of yeasts. Indeed, it has been considered a valuable tool in metabolic engineering (Dragosits and Mattanovich 2013; Caspeta and Nielsen 2015; LaCroix et al. 2017). The four ETS strains did not show fermentative trade-offs, which is pivotal for industrial applications, however, we selected the ETS4 as stated in results. Its metabolic and FAMEs profiles were characterized, in comparison with the parental strain

Notably, the metabolic profiles were significantly different between the ETS4 and P4 strains. In contrast to P4 strain, we observed higher levels of the metabolites alanine, cystathionine, threonine, proline, leucine and malonic acid in the clustering of the ETS4 and ETS4 stress. Likely these metabolites are associated with the acquisition of ethanol tolerance. Consistent with this, the metabolic pathways of arginine, proline and alanine were activated in the ETS4 strain under ethanol stress (Figure 10B). It is noteworthy that in *S. cerevisiae*, which is more tolerant to the ethanol than *K. marxianus*, the concentration of amino acids such as proline, histidine, leucine, alanine, tyrosine and phenylalanine increases in response to ethanol (Zhao and Bai 2009a; Ma and Liu 2010; Stanley et al. 2010).

Overflow of certain metabolites occurs due to their overproduction inside the cells. Under stress conditions, the overproduction of metabolites can be associated

with an adaptive response. Contrary to P4 strain, the ETS4 strain under ethanol stress displayed intra- and extracellularly higher levels of the following metabolites: isocitric acid, citric acid, cis-aconitic acid, malic acid (TCA cycle intermediates), glutamic acid, alanine and valine (amino acids and derivatives) (Figures 6 and 7), indicating that there was a metabolic overflow. In agreement with these results, TCA cycle was up-activated only in the ETS4 strain (Figure 10B).

Therefore, the accumulation of those metabolites seems to have contributed for the acquisition of ethanol tolerance by the ETS4 strain. Consistent with this, previous studies have related the intracellular accumulation of metabolites of TCA cycle with ethanol tolerance in *S. cerevisiae* (Ohta et al. 2015). Stanley et al. (2010) claimed that the up-activation of TCA cycle in *S. cerevisiae* provides energy to cope with the stress condition.

Importantly, the ETS4 strain accumulated glutamate after ethanol exposure (metabolic overflow), suggesting that it is involved with the acquisition of ethanol tolerance. Consistent with this, we observed that the glutamate metabolism was up-activated. In *S. cerevisiae*, a protective role against damages caused by ethanol stress has been attributed to glutamate accumulation (Chen et al., 2016; E. Ohta, Y. Nakayama, Y. Mukai, T. Bamba, 2015; Kim et al., 2016).

In P4 strain under ethanol stress, the decrease of the most intracellular metabolites was followed by the increase in the extracellular medium, indicating that there was a remarkably metabolite leakage. In agreement with these results, a high efflux of intracellular compounds occurred with *K. marxianus* IGC 2671 exposed to high ethanol concentration (6% v/v). Indeed, the yeast cellular membrane is the main target of ethanol toxicity. Its integrity is lost leading to metabolite leakage (Dong et al., 2015; Henderson & Block, 2014; Salgueiro et al., 1988). Importantly, we did not observe this effect in ETS4 strain, indicating that the ethanol effect on its membrane is less pronounced compared to the membrane of the parental strain (Figures 5 and 6).

With regard to FAMEs analysis, we focused on following features: (1) bond number in the fatty acid chain (saturated, monounsaturated or polyunsaturated); (2) carbon atoms in the fatty acids unbranched chain (medium, long or very long chain fatty acids); and (3) (Figures 7 and 8). Notably, the ETS4 strain had a higher relative abundance of membrane fatty acids than the P4 strain (Figures 7 and 8). In

addition, we did not observe, in contrast to P4, metabolite leakage in ETS4. Taken together, these results indicate that the cellular membrane of the ETS4 strain is less susceptible to the damages provoked by ethanol. The long-chain fatty acids were more elevated in both strains (Figure 7B). In P4 strain (wild-type *K. marxianus* CCT 7735) the ethanol stress did not change the content of both palmitoleic and oleic acids. Likewise, Diniz et al. (2017) also observed that the level of unsaturated fatty acids in batch culture of *K. marxianus* CCT 7735 was not altered upon ethanol exposure. Remarkably, the relative abundances of both palmitoleic acid and oleic acids were higher in ETS4 than in P4. In *S. cerevisiae* ethanol-stressed cells, the high levels of palmitoleic acid and oleic acid has been considered an important adaptive response to avoid the formation of interdigitations, as well as keeping the optimal thickness of yeast membranes (Henderson and Block 2014b; Dong et al. 2015; Kim et al. 2016; Lahtvee et al. 2016). By comparing the metabolic pathways that were more affected by ethanol stress, we showed that fatty acids metabolism and biosynthesis of unsaturated fatty acids in the ETS4 strain are activated under both non- and ethanol stress (Figure 10; Figure S5). This suggests that in the ethanol-tolerant strain, the metabolism of mono-unsaturated fatty acids is up-activated to maintain the plasma membrane integrity, minimizing the damages caused by ethanol.

In *S. cerevisiae*, the ergosterol concentration increases under ethanol stress, which is considered an important adaptive response to ethanol, because it reduces both lipid interdigitations and transition phase of phospholipid bilayers, ensuring the membrane stabilization (Prakash et al. 2011; Vanegas et al. 2012; Caspeta et al. 2014; Caspeta and Nielsen 2015). Contrary to *S. cerevisiae*, its concentration was reduced in both ethanol-tolerant and parental strains. Our results also are in contrast to those obtained by Diniz et al. (2017). They showed that the ergosterol content did not change in batch cultures of *K. marxianus* CCT 7735 exposed to ethanol (6% v/v) after 1 and 4 h. It is noteworthy that we cultivated both ethanol-tolerant and parental strains in chemostat, besides the ethanol concentration used in the feed medium was 4% (v/v) instead 6 % (v/v). Therefore, we believe that the differences of growth conditions should be considered to compare the results aforementioned. Nevertheless, the concentration of ergosterol in the ethanol-tolerant *K. marxianus* strain was remarkable higher than in parental strains, which is

also associated with acquisition of ethanol tolerance, counteracting the damage proved by ethanol.

In conclusion, the ethanol-tolerant *K. marxianus* CCT 7735 strain accumulates amino acids and metabolites of TCA cycle in response to ethanol. In addition, the high content of fatty acids and ergosterol in this strain contributes to maintain the structural integrity of its cellular membrane, reducing metabolite leakage.

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Supplementary material

Table S1. Physiological parameters of ethanol-tolerant *K. marxianus* strains (ETS1, ETS2, ETS3 and ETS4) and parental strains (P1, P2, P3 and P4) obtained from ALE under ethanol stress: specific growth rate under ethanol stress ($\mu_{ethanol}$; h), lactose consumption (g/L), maximum ethanol production (g/L), ethanol production (g/L), ethanol volumetric productivity (Qp; g/L/h), ethanol yield per lactose ($Y_{P/S\ ethanol}$; g/g), biomass yield per lactose ($Y_{X/S}$; g/g), specific ethanol production rate (q_p, g/g/h) and specific lactose consumption rate (q_s, g/g/h).

Strain	$\mu_{ethanol}$ (h)	Lactose consumption (g/L)	Ethanol production (g/L)	Q_p (g/L/h)	$Y_{P/S\ ethanol}$ (g/g)	$Y_{X/S}$ (g/g)	$q_{p\ etanol}$ (g/g/h)	$q_{s\ lactose}$ (g/g/h)
P1	0.08 ± 0.02	20.33 ± 0.89	6.16 ± 0.69	0.56 ± 0.06	0.32 ± 0.07	0.18 ± 0.02	0.19 ± 0.02	0.24 ± 0.03
P2	0.12 ± 0.03	20.34 ± 0.90	6.07 ± 0.51	0.55 ± 0.05	0.33 ± 0.02	0.14 ± 0.01	0.18 ± 0.01	0.31 ± 0.01
P3	0.10 ± 0.01	20.36 ± 0.94	5.81 ± 0.03	0.53 ± 0.03	0.32 ± 0.04	0.20 ± 0.01	0.15 ± 0.01	0.21 ± 0.02
P4	0.09 ± 0.01	20.34 ± 0.97	6.41 ± 0.16	0.58 ± 0.01	0.32 ± 0.03	0.14 ± 0.01	0.19 ± 0.02	0.30 ± 0.02
ETS1	0.18 ± 0.03	21.68 ± 0.14	7.16 ± 0.32	0.66 ± 0.01	0.36 ± 0.01	0.16 ± 0.02	0.22 ± 0.01	0.27 ± 0.02
ETS2	0.18 ± 0.02	21.38 ± 0.27	5.53 ± 0.55	0.52 ± 0.02	0.31 ± 0.03	0.17 ± 0.01	0.17 ± 0.02	0.25 ± 0.01
ETS3	0.19 ± 0.01	21.48 ± 0.56	6.43 ± 0.37	0.57 ± 0.01	0.32 ± 0.03	0.17 ± 0.01	0.17 ± 0.01	0.24 ± 0.03
ETS4	0.20 ± 0.01	22.60 ± 1.06	7.42 ± 0.20	0.67 ± 0.01	0.34 ± 0.01	0.13 ± 0.02	0.26 ± 0.01	0.31 ± 0.02

Table S2. A total of 151 intracellular metabolites identified from chemostat ETS4 and P4 strains cultivation on presence and absence of ethanol using in house mass spectral library.

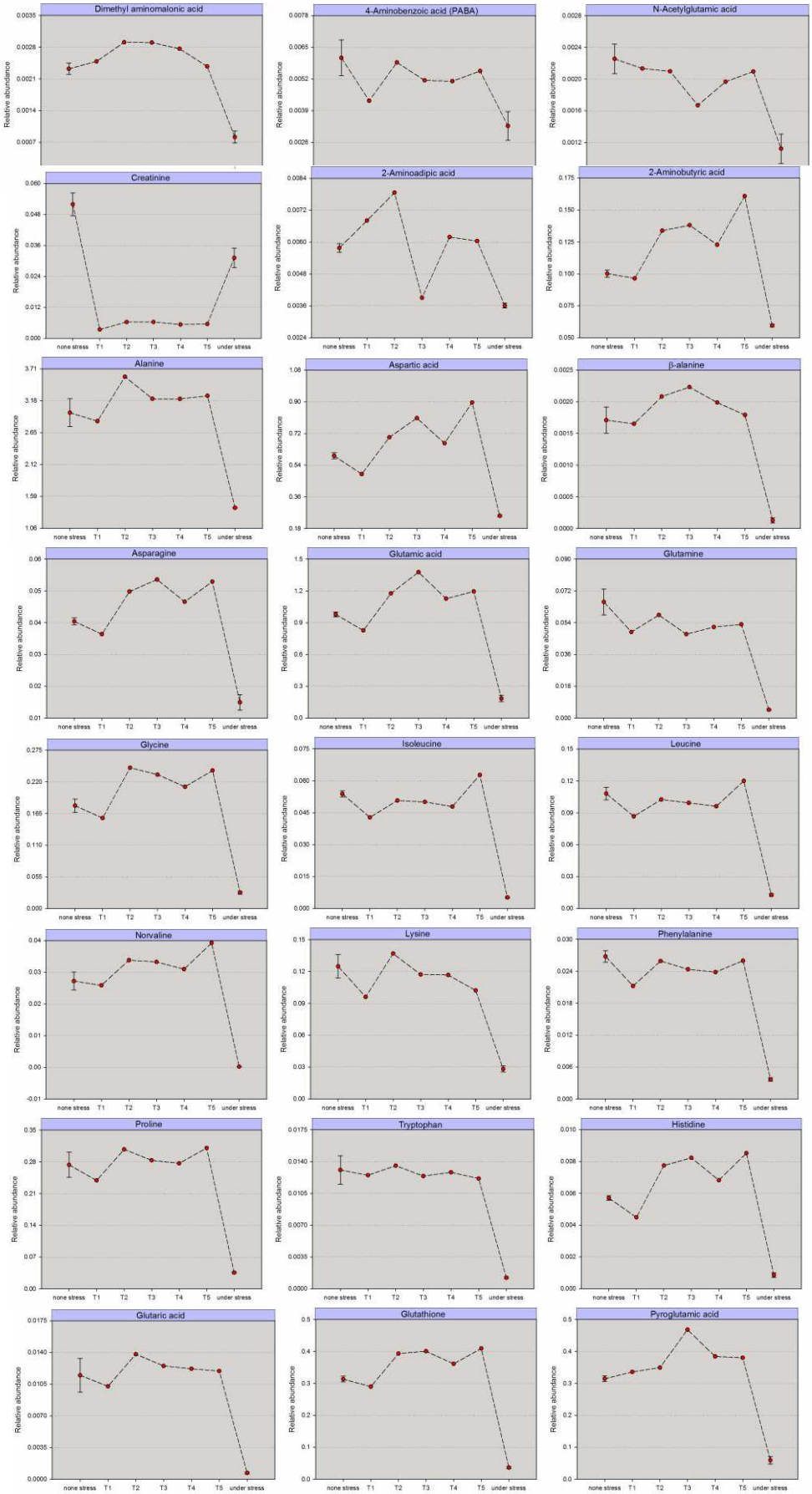
Free fatty acids (22)	
10,12-octadecadienoic acid (C18:2n-10,12c)	Margaric acid (C17:0)
10-Pentadecenoic acid (C15:1n-5c)	Myristic acid (C14:0)
11,14,17-Eicosatrienoic acid (C20:3n-3,6,9c)	Myristoleic acid (C14:1n-5c)
11,14-Eicosadienoic (C20:2n-6,9c)	Octanoic acid (C8:0)
9-Heptadecenoic acid (C17:1n-8t)	Oleic acid (C18:1n-9c)
Arachidic acid (C20:0)	Palmitic acid (C16:0)
Caprinoic acid	Palmitoleic acid (C16:1n-7c)
Conjugated linoleic acid (C18:2n-9,11c)	Pentadecanoic acid (C15:0)
Decanoic acid (C10:0)	Stearic acid (C18:0)
Hexanoic acid (C6:0)	Tridecanoic acid (C13:0)
Linoleic acid (C18:2n-6,9c)	Undecanoic acid (C11:0)
Amino acids and derivatives (31)	
2-Aminoadipic acid	Isoleucine
2-Aminobutyric acid	Leucine
2-Hydroxybutyric acid	Lysine
2-Hydroxyisobutyric acid	Norvaline
2-Isopropylmalic acid	Ornithine
3-Methyl-2-oxovaleric acid	Phenylalanine
4-Aminobutyric acid (GABA)	Proline
Alanine	Pyroglutamic acid
Asparagine	Serine

Aspartic acid	Threonine
β-Alanine	Tryptophan
Cysteine	Tyrosine
Glutamic acid	Valine
Glutamine	2-Hydroxyglutaramic acid
Glycine	Cystathionine
Histidine	
Aminas/Amidas (10)	
4-Aminobenzoic acid (PABA)	Dimethyl aminomalonic acid
NADP/NADPH	Creatinine
Nicotinamide	DL-3-Aminoisobutyric acid
Nicotinic acid	Glutathione
Putrescine	N-Acetylglutamic acid
Organic compounds (25)	
2,3-Butanediol	Malonic acid
2-Phosphoenolpyruvic acid	Oxalic acid
3-Hydroxyoctanoic acid	Succinic acid
4-Hydroxyphenylethanol	2-Oxoglutaric acid
4-Methyl-2-oxopentanoic acid	Azelaic acid
Benzoic acid	BHT (Antioxidant)
Citramalic acid	cis-Aconitic acid
Citric acid	Glutaric acid
Fumaric acid	Glyoxylic acid
Glyceric acid	Hydroxybenzoic acid
Itaconic acid	Isocitric acid
Lactic acid	Salicylic acid
Malic acid	
Others (8)	
2,4-Di-tert-butylphenol	Glycerol
Carbamic acid	Pentadecane
Dodecane	Tridecane
Ethylenediamine tetraacetic acid (EDTA)	Dibutyl Phthalate (DPB)
Unknown (55)	

Table S3. A total of 141 intracellular metabolites identified from chemostat ETS4 and P4 strains cultivation on presence and absence of ethanol using in house mass spectral library.

Free fatty acids (22)	
10,12-octadecadienoic acid (C18:2n-10,12c)	Margaric acid (C17:0)
10-Pentadecenoic acid (C15:1n-5c)	Myristic acid (C14:0)
11,14,17-Eicosatrienoic acid (C20:3n-3,6,9c)	Myristoleic acid (C14:1n-5c)
11,14-Eicosadienoic (C20:2n-6,9c)	Octanoic acid (C8:0)
9-Heptadecenoic acid (C17:1n-8t)	Oleic acid (C18:1n-9c)
Arachidic acid (C20:0)	Palmitic acid (C16:0)
Caprinoic acid	Palmitoleic acid (C16:1n-7c)
Conjugated linoleic acid (C18:2n-9,11c)	Pentadecanoic acid (C15:0)

Decanoic acid (C10:0)	Stearic acid (C18:0)
Hexanoic acid (C6:0)	Tridecanoic acid (C13:0)
Linoleic acid (C18:2n-6,9c)	Undecanoic acid (C11:0)
Amino acids and derivatives (31)	
2-Aminoadipic acid	Isoleucine
2-Aminobutyric acid	Leucine
2-Hydroxybutyric acid	Lysine
2-Hydroxyisobutyric acid	Norvaline
2-Isopropylmalic acid	Ornithine
3-Methyl-2-oxovaleric acid	Phenylalanine
4-Aminobutyric acid (GABA)	Proline
Alanine	Pyroglutamic acid
Asparagine	Serine
Aspartic acid	Threonine
β-Alanine	Tryptophan
Cysteine	Tyrosine
Glutamic acid	Valine
Glutamine	2-Hydroxyglutaramic acid
Glycine	Cystathionine
Histidine	
Aminas/Amidas (8)	
4-Aminobenzoic acid (PABA)	Putrescine
NADP/NADPH	DL-3-Aminoisobutyric acid
Nicotinamide	Glutathione
Nicotinic acid	N-Acetylglutamic acid
Organic compounds (25)	
2,3-Butanediol	Malonic acid
2-Phosphoenolpyruvic acid	Oxalic acid
3-Hydroxyoctanoic acid	Succinic acid
4-Hydroxyphenylethanol	2-Oxoglutaric acid
4-Methyl-2-oxopentanoic acid	Azelaic acid
Benzoic acid	BHT (Antioxidant)
Citramalic acid	cis-Aconitic acid
Citric acid	Glutaric acid
Fumaric acid	Glyoxylic acid
Glyceric acid	Hydroxybenzoic acid
Itaconic acid	Isocitric acid
Lactic acid	Salicylic acid
Malic acid	
Others (8)	
2,4-Di-tert-butylphenol	Glycerol
Carbamic acid	Pentadecane
Dodecane	Tridecane
Ethylenediamine tetraacetic acid (EDTA)	Dibutyl Phthalate (DPB)
Unknown (47)	


Dimethyl aminomalonic acid
4-Aminobenzoic acid (PABA)
N-Acetylglutamic acid
Creatinine
2-Aminoadipic acid
2-Aminobutyric acid
Alanine
Aspartic acid
β-alanine
Asparagine
Glutamic acid
Glutamine
Glycine
Isoleucine
Leucine
Norvaline
Lysine
Phenylalanine
Proline
Tryptophan
Histidine
Glutaric acid
Glutathione
Pyroglutamic acid
Relative abundance
none stress T1 T2 T3 T4 T5 under stress

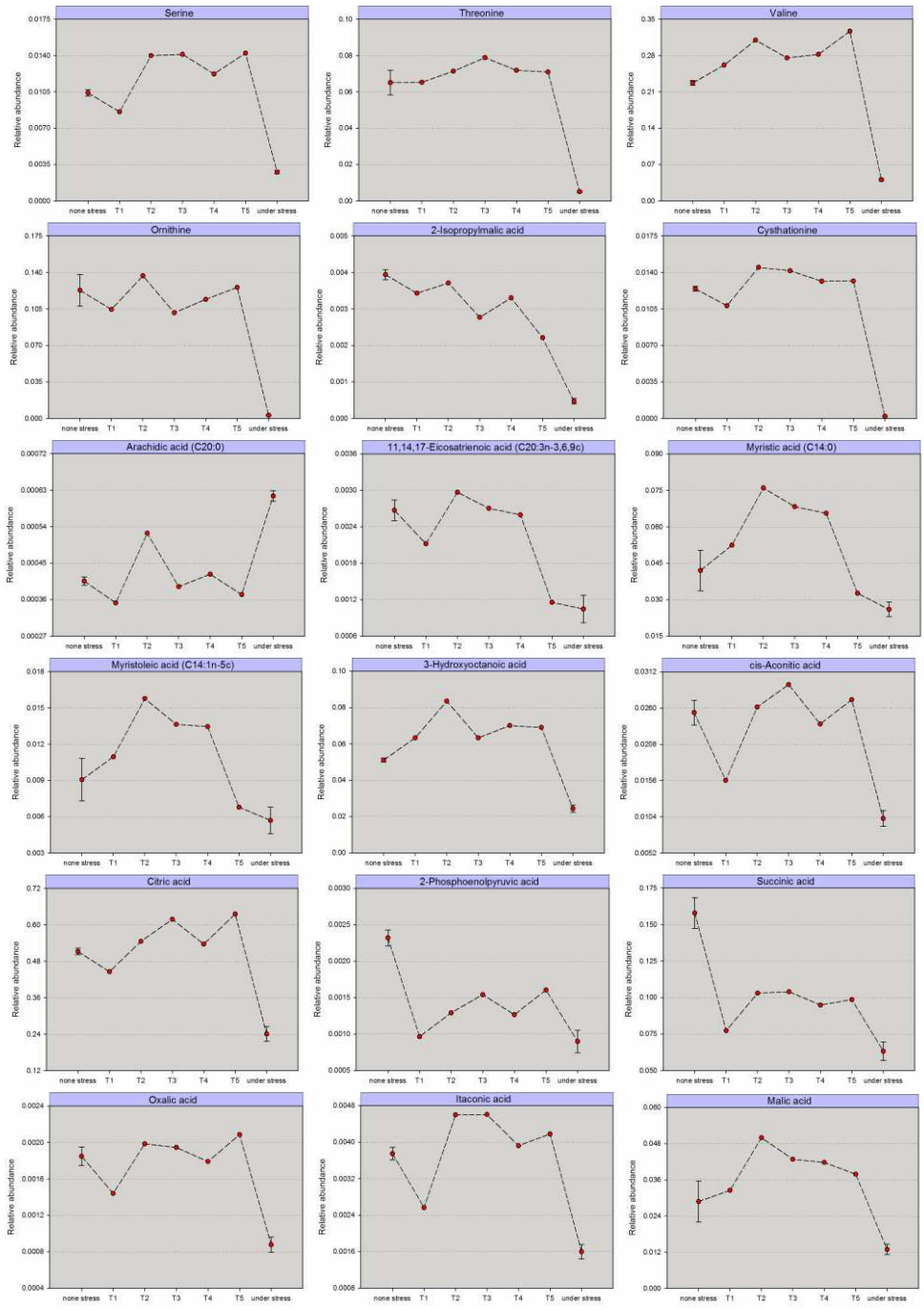
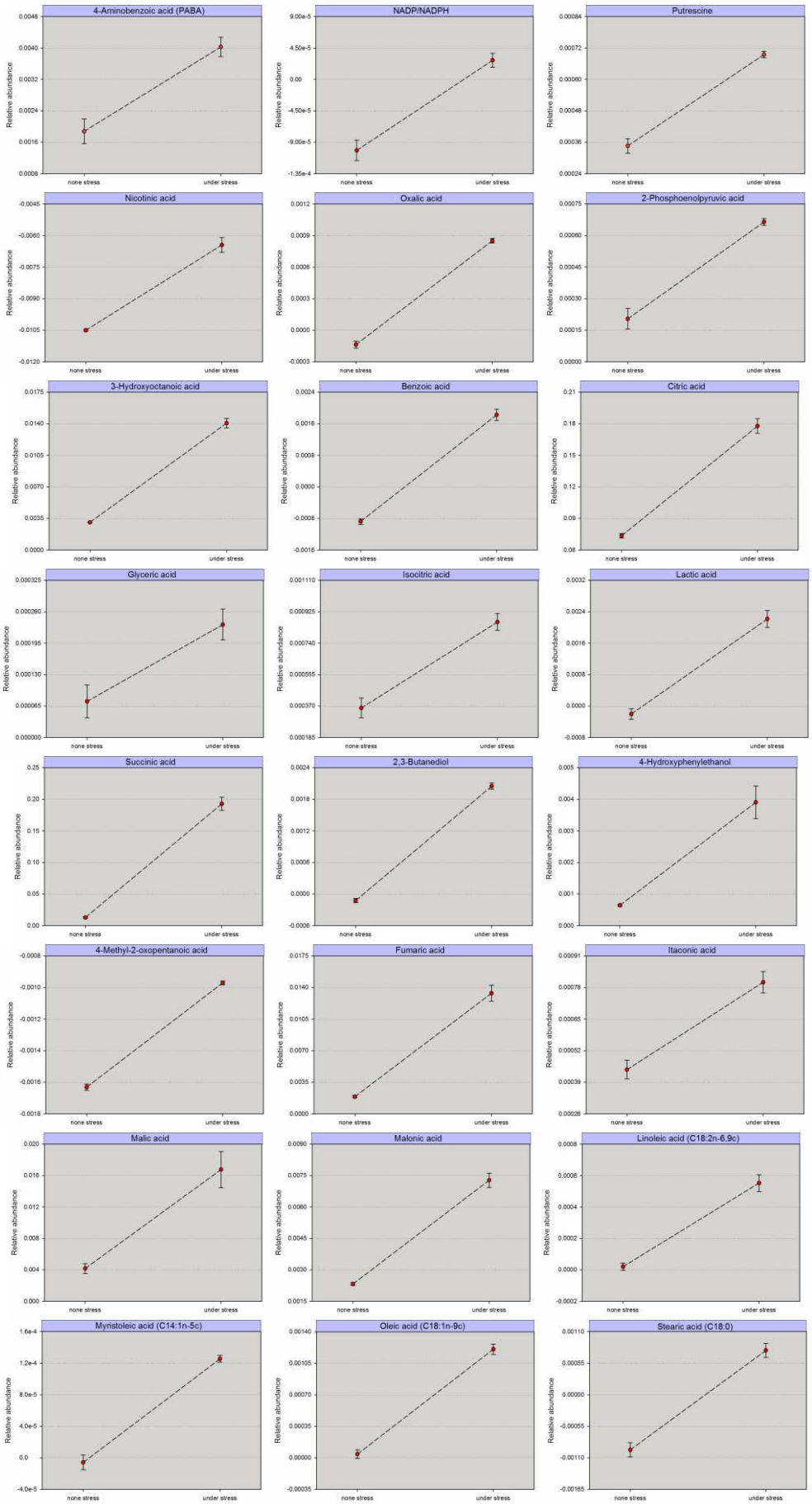
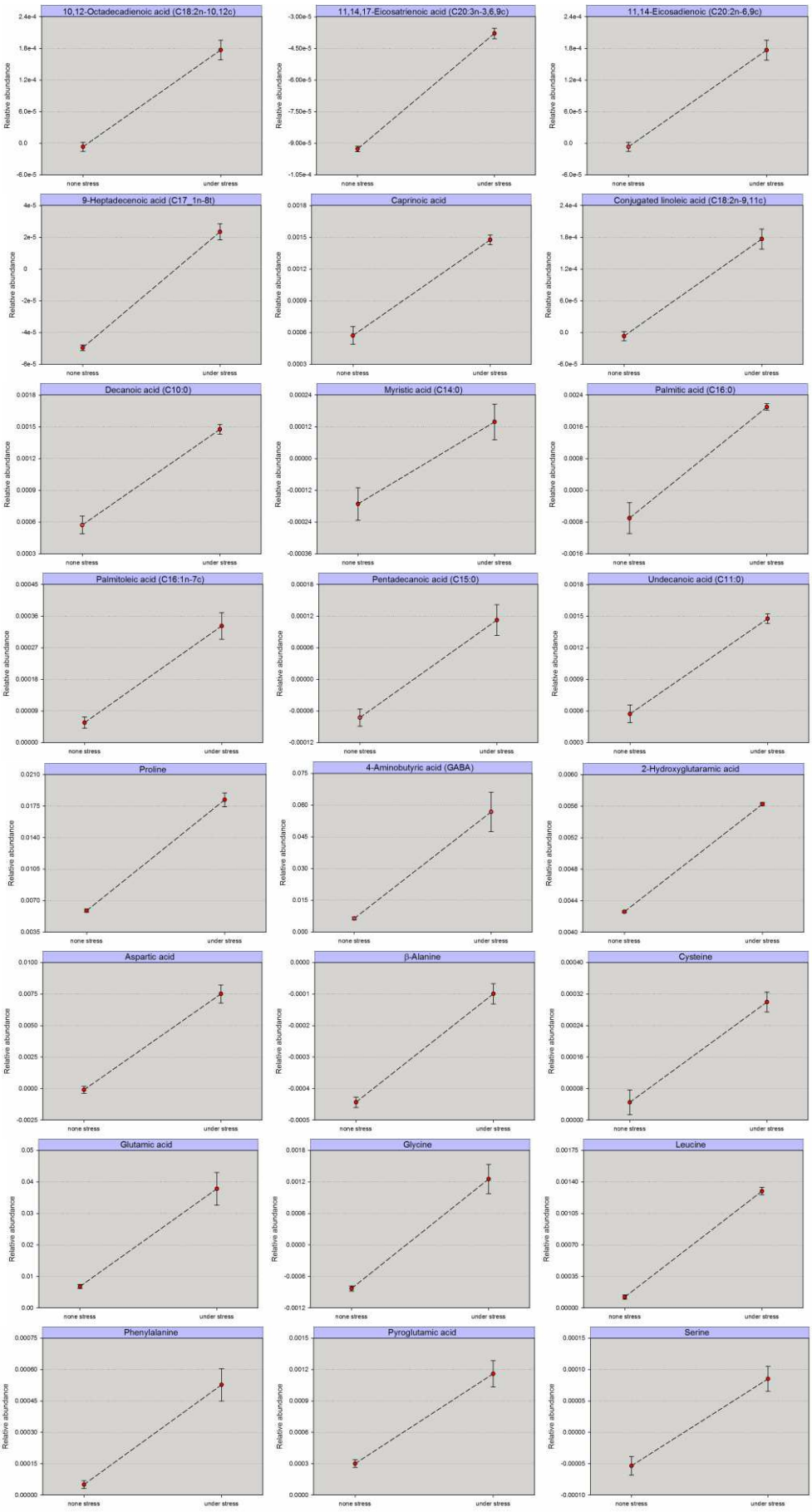


Figure S1. Intracellular metabolites levels changed significantly (p = 0.05) identified from in house mass spectral library in response to ethanol stress, in the parental (P4) cultivation. Metabolite levels shown on Y axes refer to relative abundance obtained from their GC-MS base peaks after normalization by internal standard (2, 3, 3, 3, d_4-alanine) and biomass concentration. None stress: non-ethanol stress; T1 to T5: transition phase after the ethanol pulse; under stress: ethanol stress.


4-Aminobenzoic acid (PABA)
NADP/NADPH
Putrescine
Nicotinic acid
Oxalic acid
2-Phosphoenolpyruvic acid
3-Hydroxyoctanoic acid
Benzoic acid
Citric acid
Glyceric acid
Isocitric acid
Lactic acid
Succinic acid
2,3-Butanediol
4-Hydroxyphenylethanol
4-Methyl-2-oxopentanoic acid
Fumaric acid
Itaconic acid
Malic acid
Malonic acid
Linoleic acid (C18:2n-6,9c)
Myristoleic acid (C14:1n-5c)
Oleic acid (C18:1n-9c)
Stearic acid (C18:0)
Relative abundance
none stress
under stress


10,12-Octadecadienoic acid (C18:2n-10,12c)
11,14,17-Eicosatrienoic acid (C20:3n-3,6,9c)
11,14-Eicosadienoic (C20:2n-6,9c)
9-Heptadecenoic acid (C17_1n-8t)
Caprinoic acid
Conjugated linoleic acid (C18:2n-9,11c)
Decanoic acid (C10:0)
Myristic acid (C14:0)
Palmitic acid (C16:0)
Palmitoleic acid (C16:1n-7c)
Pentadecanoic acid (C15:0)
Undecanoic acid (C11:0)
Proline
4-Aminobutyric acid (GABA)
2-Hydroxyglutaramic acid
Aspartic acid
β-Alanine
Cysteine
Glutamic acid
Glycine
Leucine
Phenylalanine
Pyroglutamic acid
Serine
Relative abundance
none stress
under stress

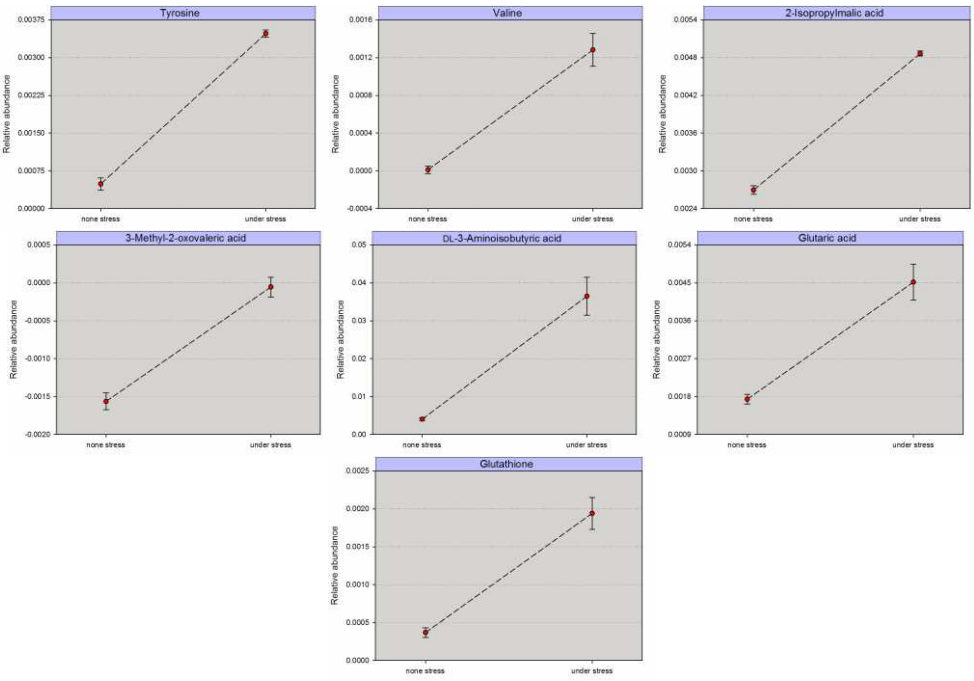
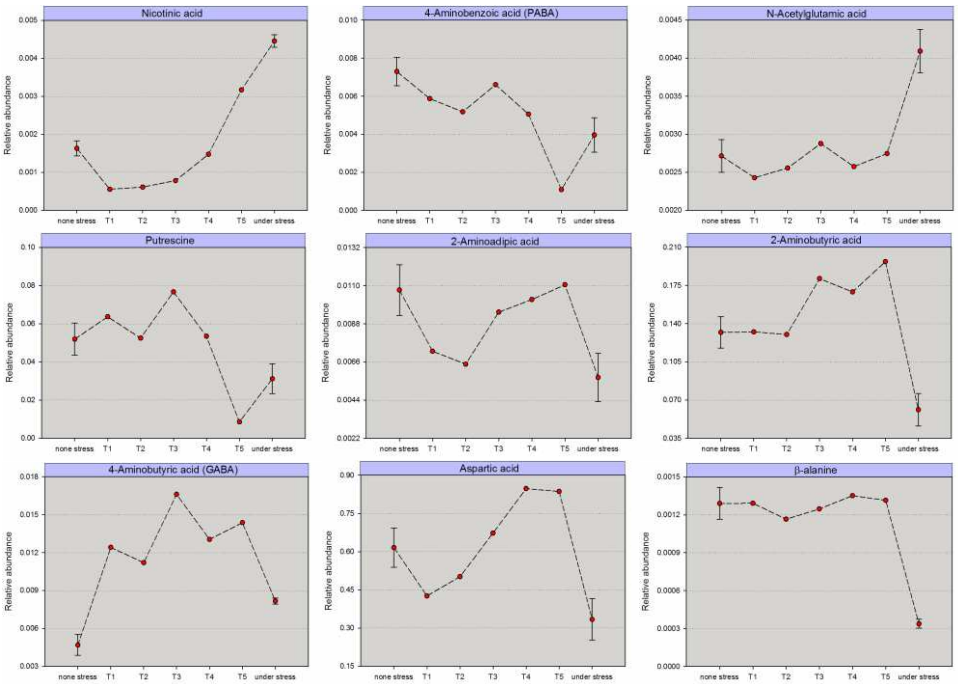


Figure S2. Extracellular metabolites levels changed significantly (p = 0.05) identified from in house mass spectral library in response to ethanol stress, in the parental (P4) cultivation. Metabolite levels shown on Y axes refer to relative abundance obtained from their GC-MS base peaks after normalization by internal standard (2, 3, 3, 3, d_4-alanine) and biomass concentration. None stress: non-ethanol stress; under stress: ethanol stress. Negative values of relative abundance mean consumed extracellular metabolites.



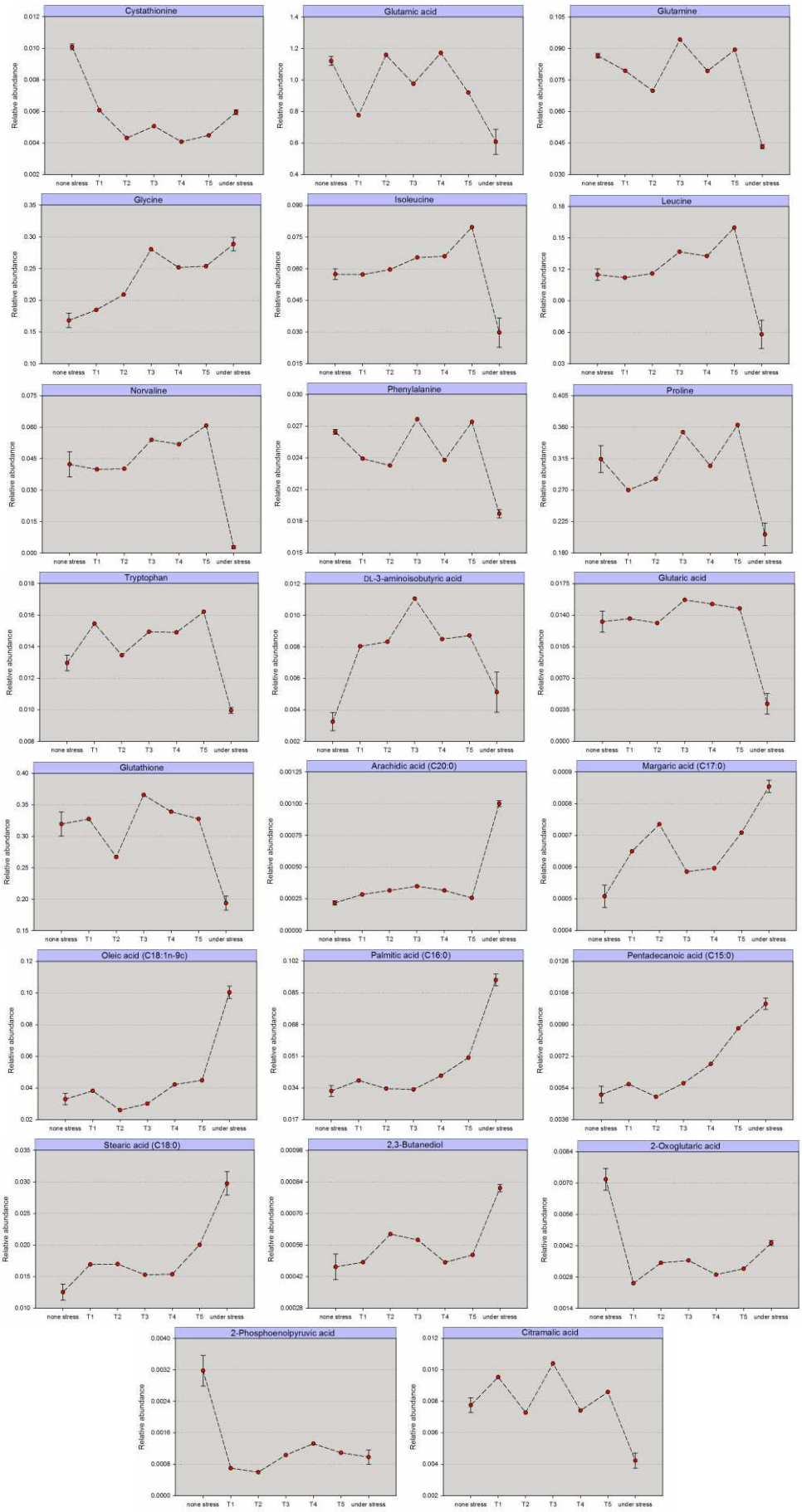

Cystathionine
Glutamic acid
Glutamine
Glycine
Isoleucine
Leucine
Norvaline
Phenylalanine
Proline
Tryptophan
DL-3-aminoisobutyric acid
Glutaric acid
Glutathione
Arachidic acid (C20:0)
Margaric acid (C17:0)
Oleic acid (C18:1n-9c)
Palmitic acid (C16:0)
Pentadecanoic acid (C15:0)
Stearic acid (C18:0)
2,3-Butanediol
2-Oxoglutaric acid
2-Phosphoenolpyruvic acid
Citramalic acid
Relative abundance
none stress T1 T2 T3 T4 T5 under stress

Figure S3. Intracellular metabolites levels changed significantly (p = 0.05) identified from in house mass spectral library in response to ethanol stress, in the ETS4 cultivation. Metabolite levels shown on Y axes refer to relative abundance obtained from their GC-MS base peaks after normalization by internal standard (2, 3, 3, 3, d_4-alanine) and biomass concentration. None stress: non-ethanol stress; T1 to T5: transition phase after the ethanol pulse; under stress: ethanol stress.

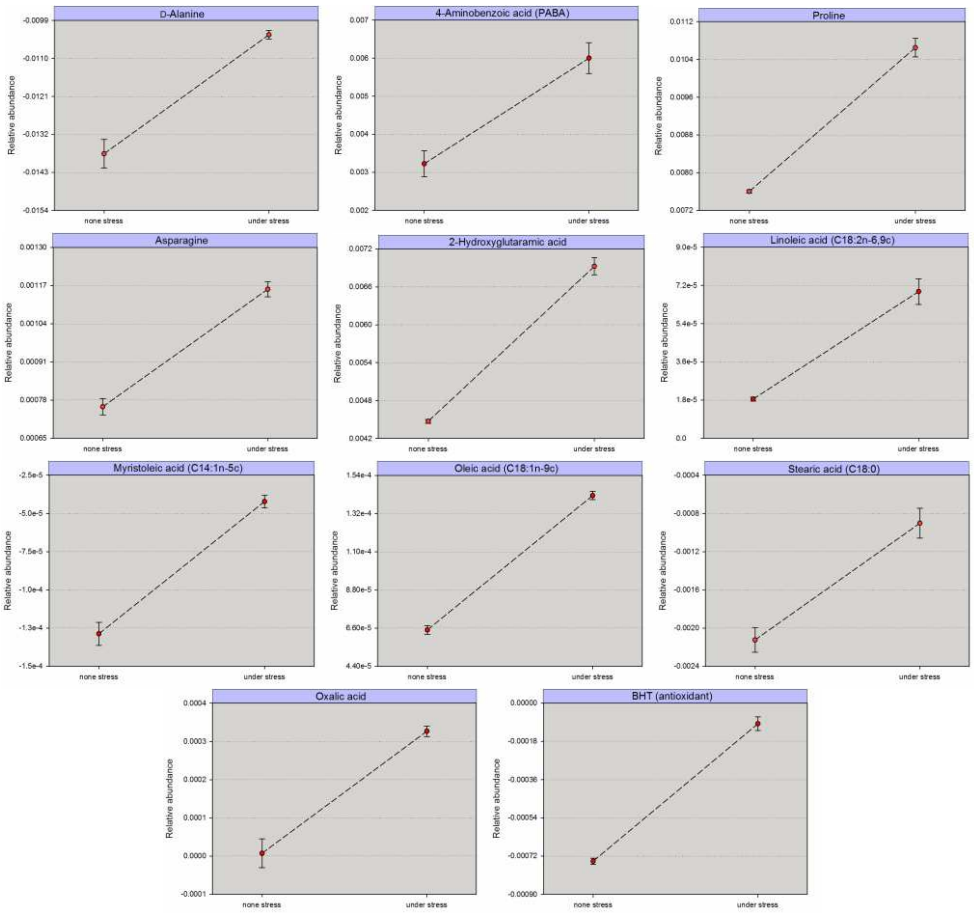


Figure S4. Extracellular metabolites levels changed significantly (p = 0.05) identified from in house mass spectral library in response to ethanol stress, in the ETS4 cultivation. Metabolite levels shown on Y axes refer to relative abundance obtained from their GC-MS base peaks after normalization by internal standard (2, 3, 3, 3, d_4-alanine) and biomass concentration. None stress: non-ethanol stress; under stress: ethanol stress. Negative values of relative abundance mean extracellular consumed metabolites.

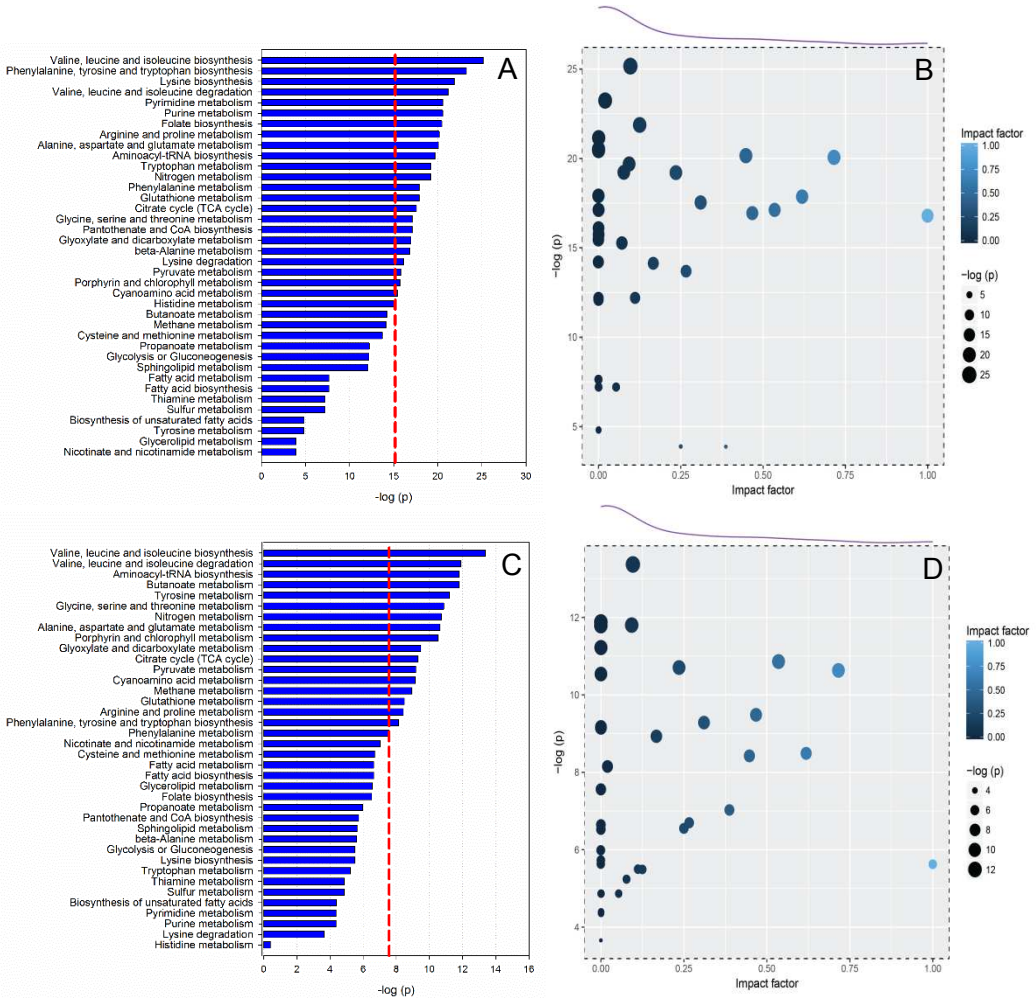


Figure S5. Metabolic pathways activities changed based on intra- and extracellular metabolite profiles of P4 strain under and non-ethanol stress obtained from chemostat culture. (A, C) Barplots show the most changed metabolic pathways (above dashed red line) comparing P4 strains under ethanol and non-ethanol stress, from alterations in relative abundance of intra- and extracellular metabolite profiles, respectively; (B, D) all of metabolites in each metabolic pathway. It Scatterplots contains all the matched pathways arranged by "-log (p)" (p-values) versus "Impact factor" (pathway impact values) of intra and extracellular metabolites, respectively. The node color is based on its p-value and the node radius is determined based on their pathway impact values.

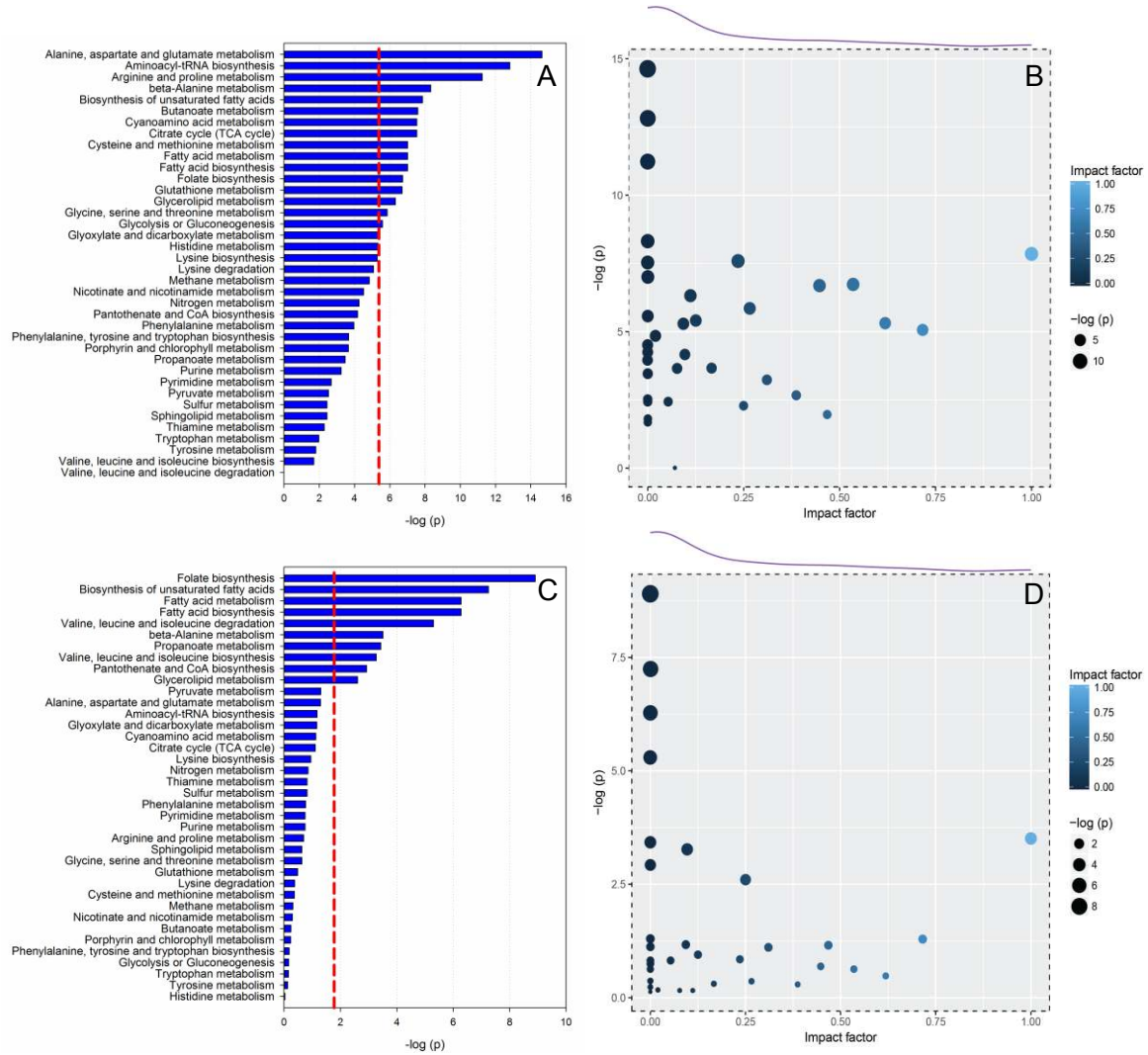


Figure S6. Metabolic pathways activities changed based on intra- and extracellular metabolite profiles of ETS4 strain under and non-ethanol stress obtained from chemostat culture. (A, C) Barplots show the most changed metabolic pathways (above dashed red line) comparing P4 strains under ethanol and non-ethanol stress, from alterations in relative abundance of intra- and extracellular metabolite profiles, respectively; (B, D) all of metabolites in each metabolic pathway. It Scatterplots contains all the matched pathways arranged by "-log (p)" (p-values) versus "Impact factor" (pathway impact values) of intra and extracellular metabolites, respectively. The node color is based on its p-value and the node radius is determined based on their pathway impact values.

GENERAL CONCLUSION

Ethanol-tolerant *K. marxianus* CCT 7735 strains (ETSs) were selected by using adaptive laboratory evolution (ALE). The ETS4 strain displayed a specific growth rate higher than the parental strain under ethanol stress (above 100%) and the highest specific ethanol production rate compared to other ETSs. In response to ethanol stress, ETS4 strain showed some remarkable differences compared to parental strain (non-tolerant): accumulation of some metabolites (amino acids and metabolites of TCA cycle) and high contents of fatty acids and ergosterol. The differences observed between their plasma membranes may explain the metabolite leakage in the parental strain, indicating that the ETS4 strain is less susceptible to the ethanol effects on membrane.

Taken together, these results provided insights into the mechanisms involved with the acquisition of ethanol tolerance in *K. marxianus*.