THÉRCIA ROCHA BALBINO

2- PHENYLETHANOL STRESS RESPONSES IN *Kluyveromyces marxianus* CCT7735

Master's dissertation presented to the Agricultural Microbiology's Graduate Program of the Federal University of Viçosa, as part of the requirements to obtain the title of *Magister Scientiae*.

Advisor: Wendel Batista da Silveira

Co-advisor: Antônio Galvão do Nascimento

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BIOGRAPHY

THÉRCIA ROCHA BALBINO, filha de Soraya das Dores Rocha Balbino e Paulo Roberto Balbino, nasceu no dia 25 de outubro de 1994, em Ponte Nova, Minas Gerais. Graduou-se em Ciências Biológicas pela Universidade Federal de Viçosa, em julho de 2017. Em agosto do mesmo ano, 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 17 de outubro de 2019.

ABSTRACT

BALBINO, Thércia Rocha, M.Sc., Universidade Federal de Viçosa, October, 2019. **2-Phenylethanol stress responses in *Kluyveromyces marxianus* CCT7735.** Advisor: Wendel Batista da Silveira. Co-advisor: Antônio Galvão do Nascimento.

2-phenylethanol (2-PE) is a higher aromatic alcohol that has been used as ingredient in both cosmetic and food industries due to its rose-like aroma and colorless aspect. The 2-PE production by chemical synthesis is undesirable due to the use of harmful precursors and formation of unwanted byproducts. Since the 2-PE extraction from plants and flowers is costly, its production by yeasts is of great interest. Yeasts can synthesize 2-PE from L-phenylalanine (L-Phe) through the Ehrlich pathway or by *de novo* synthesis from sugars through the Shikimate pathway. Recently, *K. marxianus* CCT 7735 was selected as the best producer of 2-PE among yeasts isolated from Brazilian environments. However, *K. marxianus,* like other yeasts, has its growth strongly inhibited by 2-PE concentrations superior to 2.0 g/L. Herein, we evaluated in *K. marxianus* CCT 7735 the damages caused by 2-PE exposure and its adaptative responses. Yeast batch cultures were carried out under nonstress and 2-PE stress conditions. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures and samples were analyzed during 1, 2, 4, 8 and 12 h of cultivation. Under 2-PE stress, the *K. marxianus* growth, glucose uptake, fermentative metabolism, membrane permeability and cell viability were impaired. In addition, both morphology and roughness of *K. marxianus* were altered in response to stress condition. Remarkably, the reactive oxygen species (ROS) increased immediately upon 2-PE exposure. *K. marxianus* CCT 7735 restarted growth after 4 h of stress, suggesting an adaptation to the 2-PE. The adaptive responses included the increase in ergosterol content, changes in membrane fatty acid composition, exopolymer production and elimination of reactive oxygen species. Therefore, our results provided insights to better understand the 2-PE effects on *K. marxianus* and its adaptative responses.

Keywords: Yeast. Aromatic alcohol. Adaptive responses. Morphology. Membrane composition.

RESUMO

BALBINO, Thércia Rocha, M.Sc., Universidade Federal de Viçosa, outubro de 2019. **Respostas de *Kluyveromyces marxianus* CCT7735 ao estresse por 2-feniletanol.** Orientador: Wendel Batista da Silveira. Coorientador: Antônio Galvão do Nascimento.

O 2-feniletanol (2-FE) é um álcool aromático que tem sido usado como ingrediente nas indústrias de cosméticos e alimentícia devido ao seu aroma semelhante a rosa e aspecto incolor. A produção de 2-FE por síntese química é indesejável devido ao uso de precursores tóxicos e formação de subprodutos indesejados. Como a extração de 2-FE a partir de plantas e flores é onerosa, a produção de 2-FE por leveduras é de grande interesse. Leveduras podem sintetizar 2-FE a partir de L-fenilalanina (L-Phe) por meio da via de Ehrlich ou pela síntese *de novo* a partir de açúcares através da via do Shikimato. Recentemente, *K. marxianus* CCT 7735 foi selecionada como a melhor produtora de 2-FE entre leveduras isoladas de ambientes brasileiros. No entanto, *K. marxianus*, assim como outras leveduras, tem o crescimento fortemente inibido por concentrações de 2-FE superiores a 2,0 g/L. Neste trabalho, nós avaliamos em *K. marxianus* CCT 7735 os danos causados pela exposição ao 2-FE e suas respostas adaptativas. Os cultivos das leveduras foram realizados em batelada sob condições de estresse e sem estresse. Sob estresse por 2-FE, o crescimento de *K. marxianus*, consumo de glicose, metabolismo fermentativo, permeabilidade de membrana e viabilidade celular foram prejudicados. A morfologia e a rugosidade de *K. marxianus* também foram alteradas em resposta à condição de estresse. Além disso, a formação de espécies reativas de oxigênio (ERO) aumentou logo após a exposição ao 2-FE. *K. marxianus* CCT 7735 reiniciou o crescimento após 4 h de estresse, sugerindo uma adaptação ao estresse. As respostas adaptativas incluíram o aumento no teor de ergosterol, alterações na composição de ácidos graxos de membrana, produção de exopolímero e eliminação de ERO. Portanto, nossos resultados contribuíram para compreender os efeitos do 2-FE em *K. marxianus* e suas respostas adaptativas.

Palavras-chave: Levedura. Álcool aromático. Respostas adaptativas. Morfologia. Composição de membrana.

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SUMMARY

GENERAL INTRODUCTION

Fragrances and flavor are largely used in beverages, cosmetics and food industries. In 2006, they represented US$ 18 billion per year in the worldwide market (XU; HUA; MA, 2007). 2-Phenylethanol (2-PE) is a higher aromatic alcohol with rose-like aroma, colorless aspect, and has the Generally Recognized as Safe (GRAS) status if produced naturally (SCOGNAMIGLIO *et al.*, 2012). It has been used for producing flavors used as ingredients for some foods, such as cookies and candy, as well as substrate for synthesis of pharmaceutical compounds, such as phenylethyl acetate ester (ETSCHMANN *et al.*, 2002). The 2-PE also contributes to the aroma of wines, beers and soft drinks (CARLQUIST *et al.*, 2015). In addition, it displays antimicrobial activity and therefore potential to be used against phytopathogenic microorganisms (HUA *et al.*, 2014; LESTER, 1965).

The 2-PE can be obtained by extraction from flowers, nevertheless this is an expensive process. Alternatively, the 2-PE can be produced from either chemical synthesis or biosynthesis by microorganisms. The 2-PE obtained from flowers and microbial fermentation is considered natural. In 2006, only 0.5 to 1 ton of 2-PE world market was referent to natural product (ETSCHMANN; SCHRADER, 2006). However, over the last years the consumers have preferred natural 2-PE, because the chemical production involves the use of toxic precursors and generation of unwanted byproducts. The biosynthesis of 2-PE can be performed by yeasts. The yeast *Kluyveromyces marxianus* has stood out as a good 2-PE producer. Its use is also of interest due to the Generally Recognized as Safe (GRAS) status, which is important for industrial applications. Moreover, it is able to assimilate a wide range of sugars (LANE; MORRISSEY, 2010), is thermotolerant, which is a desirable feature in fermentative processes because the cooling costs of bioreactors are reduced. However, the 2-PE hinders the yeast growth, which in turn its production (BOLTEN; WITTMANN, 2008). It has been reported that the stress caused by 2-PE in *S. cerevisiae* leads to the uncoupling between anabolism and catabolism, increase of membrane permeability (SILVER; WENDT, 1967), decrease of the protonmotive force with loss of viability (SEWARD *et al.*, 1996) and overexpression of genes encoding channels for ions, which play important roles in membrane potential balance and up-regulation of mitochondrial proteins genes (JIN *et al.*, 2018).

To the best of our knowledge, information regarding the 2-PE stress responses in *K. marxianus* are still scarce. As such, the main aim of this work was to evaluate the effects and adaptative responses to the 2-PE stress in *K. marxianus* CCT7735.

This thesis was organized in two chapters: the first one is a literature review that includes the 2-phenylethanol characteristics, global market, 2-PE production, as well as the main characteristics of *K. marxianus.* The second chapter is the manuscript that brings insights about the 2-PE stress responses in *K. marxianus*. The main changes were observed in terms of yeast growth, glucose consumption, metabolism, membrane permeability, cell viability, reactive oxygen species level, morphology, fatty acid methyl ester profile, ergosterol content and exopolymer production. This manuscript allowed us to gain insights about the adaptive responses displayed by *K. marxianus* in response to the 2-PE.

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CHAPTER I

Review

2-Phenylethanol

2-Phenylethanol (2-PE) is a higher aromatic alcohol with rose-like aroma and colorless aspect that has been used as ingredient in both cosmetic and food industries (MARTÍNEZ *et al.*, 2018). If produced naturally, it is considered as Generally Recognized As Safe (GRAS) flavoring agents in food by Flavor and Extract Manufacturers' Association (FEMA), Food and Drug Administration (FDA), Joint Expert Committee on Food Additives (JECFA) and the Council of Europe (COE) (SCOGNAMIGLIO *et al.*, 2012).

Remarkable alkaline stability of 2-PE makes it particularly suitable for soap perfumes. It has also been used for producing flavors used as ingredients for some foods such as cookies and candy. It is also used as substrate for synthesis of pharmaceutical compounds such as phenylethyl acetate ester (ETSCHMANN *et al.*, 2002). In the beverage industry, 2-PE contributes to the aroma of wines, beers and soft drinks (CARLQUIST *et al.*, 2015).

Furthermore, this aromatic alcohol displays antimicrobial effect on phytopathogenic microorganisms, such as *Aspergillus niger, Penicillium notatum*, *Neurospora crassa* and *Aspergillus flavus* (HUA *et al.*, 2014; LESTER, 1965). 2-PE concentrations between 2 and 3 g/L inhibited completely the growth of several species of bacteria such as *Ralstonia solanacearum* (ZHU *et al.*, 2011). It has been demonstrated that the 2-PE inhalation influenced the central nervous system of mice and seems to reduce depression and stress-related diseases, however it may increase anxiety behaviors (UENO *et al.*, 2019).

Global Market

Fragrances and flavor are largely used in beverages, cosmetics and food industries. In 2006, they represented US$ 18 billion per year in the worldwide market (XU; HUA; MA, 2007). In the same year, only 0.5 to 1 ton of 2-PE world market was referent to natural product (ETSCHMANN; SCHRADER, 2006). The global market of 2-PE was estimated at approximately 10,000 tons in 2010 (XU; HUA; MA, 2007). The price of 2-PE chemically synthesized and extracted from flowers is about US$ 5/kg and US$ 1000/kg, respectively (HUA; XU, 2011).

Conde-Báez et al., (2019) showed of an economic point of view that the 2-PE production by *K. marxianus* from whey may be feasible. The maximum 2-PE concentration

obtained was 780 mg/L. Considering the sale of the product as an additive, with the characteristics of fermentation output, the minimum product price was US$207.05 by ton additive. Considering the 2-PE sale as a high purity product, the sales revenue would result in the amount of US$ 1,000,000.

According to a report from QYResearch Group, the prominent companies that participate to the global 2-PE market include BASF SE (Germany), The Dow Chemical (United States), Novorate (China), Tokyo Chemical Industry (Japan), Sigma-Aldrich (United States), Atlantic Richfield Company (United States), Merck Millipore (United States), LyondellBasel (Netherlands).

Extraction and production of 2-PE

2-PE can be obtained by extraction from plants and flowers, chemical synthesis and either biosynthesis or biotransformation by microorganisms (MIERZEJEWSKA *et al.*, 2017). However, most of the flavors are obtained by chemical synthesis and less than 5% is extracted from plants (XU; HUA; MA, 2007).

Chemical synthesis

The chemical synthesis of 2-PE takes place through different routes such as Friedel–Crafts and via the transformation of chlorobenzene (ACHMON; BEN-BARAK ZELAS; FISHMAN, 2014; ETSCHMANN; SELL; SCHRADER, 2004).

Conventionally, 2-PE can be prepared by Grignard synthesis in which chlorobenzene is converted to phenyl magnesium chloride, which in turn reacts with ethylene oxide at 100 °C to generate phenyl ethoxy magnesium chloride. This is decomposed with sulfuric acid to produce 2-PE. Another method for chemical production of 2-PE involves low temperature, Friedel-Crafts alkylation of benzene with ethylene oxide in the presence of anhydrous aluminum chloride. 2-PE is also prepared via reduction of styrene oxide, but are used reducing agents that results in the formation of a mixture of primary and secondary alcohol (RODE *et al.*, 2005).

The chemical synthesis displays important drawbacks such as the use of a corrosive and toxic compound with carcinogenic effect, the formation of unwanted byproducts, which have green-gassy or metallic-chlorine off-odor and the process is often environmentally unfriendly due the use of high temperature, high pressure and strong acid or alkali. Therefore, purification is required to remove them prior to 2-PE commercialization (GAO; DAUGULIS,

2009). However, the price of 2-PE chemically synthetized is lower compared to the 2-PE extraction from flowers.

Extraction of 2-PE from plants and flowers

In nowadays, the European legislation restricts the use of flavors in food, beverages and cosmetics (KIM; LEE; OH, 2014a), thus, the demand for producing natural flavors is rising. According to US and Europe legislation, the "natural" obtainment of 2-PE occurs by its extraction from plants, such as jasmine, hyacinth and mainly rose petals, and production by microorganisms from substrates of natural origin (MIERZEJEWSKA *et al.*, 2017).

2-PE in plants is synthesized from L-phenylalanine (L-Phe) with pyridoxal-5′-phosphate (PLP)-dependent aromatic amino acid decarboxylases (AADC) and phenylacetaldehyde reductases (PAR). AADC transforms L-Phe to phenylacetaldehyde via the Schiff base. Then, phenylacetaldehyde is converted to 2-PE by the action of PAR (HIRATA *et al.*, 2012; ROCCIA *et al.*, 2019).

The extraction of 2-PE from essential oils of flowers and plants results in low concentrations of 2-PE, besides the process is complex and expensive (MARTÍNEZ *et al.*, 2018). Furthermore, flower cultivation is influenced by weather conditions and plant diseases (XU; HUA; MA, 2007). These limitations make difficulty to meet the market demand (MEI; MIN; LÜ, 2009).

2-PE production by microorganisms

The production of 2-PE by microorganisms has advantages such as soft reaction conditions, use of several carbon sources and generation of few byproducts (XU; HUA; MA, 2007). Therefore, there is a rising interest in using microorganisms for producing 2-PE without using toxic compounds. The biosynthesis of 2-PE can be performed by bacteria and fungi. Many yeasts, including *Saccharomyces cerevisiae, Kluyveromyces marxianus, Kluyveromyces lactis, Pichia fermentans, Pichia anomala, Schizosaccharomyces pombe,* and *Hansenula anomala* have the capacity to synthesize 2-PE naturally (ETSCHMANN; SELL; SCHRADER, 2003; FORTI *et al.*, 2015).

Yeasts can synthesize 2-PE from L-phenylalanine (L-Phe) through the Ehrlich pathway (Figure 1). In this pathway, L-Phe is transaminated to phenylpyruvate by transaminase Aro9p and then phenylpyruvate is decarboxylated to phenylacetaldehyde by decarboxylase Aro10p. The genes encoding these enzymes are expressed only when the

nitrogen source is not plentiful. In the last reaction, phenylacetaldehyde is reduced to 2-PE by alcohol and formaldehyde dehydrogenase (JIN *et al.*, 2018).

In *S. cerevisiae*, there are six genes involved in dehydrogenation, five are alcohol dehydrogenases Adh1p, Adh2p, Adh3p, Adh4p and Adh5p, while one is a formaldehyde dehydrogenase (Sfa1p) (JIN *et al.*, 2018).

The enhance of the enzyme activity of aminotransferase or decarboxylase in the Ehrlich pathway has been applied to improve 2-PE accumulation, since the flux from L-Phe to phenylacetaldehyde is the limiting step for high 2-PE biosynthesis (WANG *et al.*, 2019).

Despite the use of L-Phe allows to achieve higher 2-PE concentration, its production from cheap sugars is desirable of an economic point of view. (KIM; LEE; OH, 2014b).

The biosynthesis of 2-PE from sugars occurs by *de novo* synthesis through the Shikimate pathway (Figure 1). Initially, the phosphoenolpyruvate and erythrose-4-phosphate formed in glycolysis and pentose phosphate pathway, respectively, are condensed to chorismate, which in turn follows the Shikimate pathway, resulting in phenylpyruvate synthesis. Phenylpyruvate is converted into 2-PE by Erlich pathway.

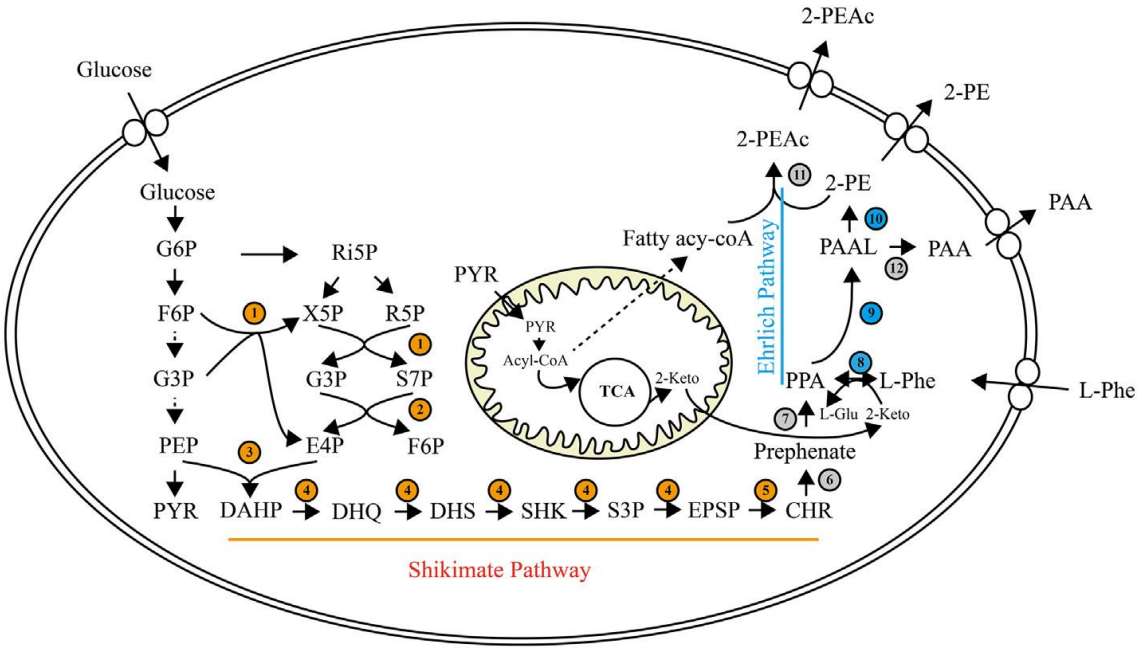

Fig. 1 Metabolic pathway for 2-PE production in yeasts. 2-PE can be derived from L-Phe via the Ehrlich pathway (Blue) or synthesized de novo from glucose via the phenylpyruvate pathway, which is a combination of the Shikimate (Yellow) and Ehrlich pathways. G6P, Glucose 6-p; F6P, Fructose 6-p; G3P, Glyceraldehyde 3-p; PEP, Phosphoenolpyruvate; Ri5P, Ribulose 5-p; X5P, Xyulose 5-p; R5P, Ribose 5- p; S7P, Sedoheptulose 7-p; E4P, Erythrose 4-p; F6P, Fructose 6-p; DAHP, 3-deoxy-D-arabinoheptulosonate 7-p; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; SHK, Shikimate; S3P, Shikimate 3-p; EPSP, 5-enolpyruvyshikimate 3-p; CHR, Chorismate; PYR, Pyruvate; 2-KG, 2-Ketoglutaric acid; L-Glu, LGlutamate; L-Phe, L-phenylalanine; PPA, Phenylpyruvate; PAA, Phenylacetic acid; PAAL, Phenylacetaldehyde; 2-PE, 2-Phenylethanol; 2-PEAc, 2-Phenlethylacetate. 1. Transketolase Tkt1; 2. Transaldolase Tal1; 3. Phospho-2-dehydro-3-deoxyheptonate aldolase Aro3/4; 4, Pentafunctional AROM polypeptide Aro1; 5, chorismate synthase Aro2; 6, Chorismate

mutuase Aro7; 7, Prephenate dehydratase Aro7; 8, Aminotransferase Aro8/9; 9, Decarboxylase Aro10; 10, Dehydrogenases Adh and Saf1; 11, Alcohol acetyltransferase Atf1, 12, Aldehyde dehydrogenase Ald. Source: Wang *et al.* 2019.

Kluyveromyces marxianus

The *K. marxianus* yeast was able to produce 2-PE from alternative substrate, such as whey (CONDE-BÁEZ *et al.*, 2019) and grape must (GARAVAGLIA *et al.*, 2007). The yeast *K. marxianus* has Generally Recognized as Safe (GRAS) status, which is important for industrial applications. Moreover, it is able to assimilate a wide range of sugars, including glucose and xylose, constituents of lignocellulosic biomasses, and lactose, sugar found in whey (DINIZ *et al.*, 2017). Thus, these abundant feedstocks can be used for biotechnological production of 2-PE by *K. marxianus*. Furthermore, *K. marxianus* is a thermotolerant yeast, which is a desirable feature in fermentative processes because the cooling costs of bioreactors are reduced, high acid-tolerance and short doubling time. This yeast produced 780 mg/L of 2-PE from whey with addition of L-Phe (1/g/L) (CONDE-BÁEZ *et al.*, 2019).

Kluyveromyces marxianus CCT 7735 and its biotechnological potential

The *K. marxianus* CCT 7735 strain was selected as the best producer of 2-PE among yeasts isolated from Brazilian environments (DE LIMA *et al.*, 2018). In this work, both 2-PE titer and specific production rate increased under optimized condition, that is, 30 g/L of glucose and 4.0 g/L of L-phe at 30 ºC. Therefore, *K. marxianus* CCT 7735 can be used in further works to scale-up the process of 2-PE production.

This yeast is also able to produce ethanol and grow at higher temperatures (COSTA *et al.*, 2014). *K. marxianus* exhibits high fermentative metabolism in high concentrations of cheese whey permeate and low oxygen levels (SILVEIRA *et al.*, 2005). Under hypoxic condition, this high fermentative metabolism is related to higher expression of genes of the Leloir pathway, *LAC4, LAC12, RAG6*, which encode β-galactosidase, lactose permease and pyruvate decarboxylase, respectively. (DINIZ; SILVEIRA; PASSOS, 2012). The higher expression of the *LAC12* genes was associated with the increase of specific lactose consumption and specific ethanol production rates (PAIVA *et al.*, 2019). Moreover, *K. marxianus* CCT 7735 was able to produce high ethanol concentrations from a mixture of sugarcane bagasse hydrolysate and ricotta whey (FERREIRA *et al.*, 2015), from elephant grass (CAMPOS *et al.*, 2019), and sugar cane bagasse (SOUZA *et al.*, 2012). However, *K. marxianus* CCT7735 had the growth inhibited from 4% (v/v) of ethanol at 42ºC (COSTA *et al.*, 2014).

The 2-PE also inhibits the *K. marxianus* CCT7735 growth, at 2.5 g/L the specific growth rate decreased by 64% (DE LIMA *et al.*, 2018). This is a drawback that needs to be circumvented for its use at the industrial level.

Inhibitory effect of 2-PE

Concentrations of 2-PE between 2.0 and 3.0 g/L inhibit the growth of several species of bacteria and fungi (LESTER, 1965). In *Saccharomyces cerevisiae*, the 2-PE was twenty times more toxic than ethanol in low concentrations of 2-PE, that is, 25 mM, which is equivalent to 1.87 M of ethanol (SEWARD *et al.*, 1996).

The exposure of *Neurospora crassa* to 2-PE caused inhibition of growth and synthesis of ribonucleic, deoxyribonucleic acids and protein. Its stress condition restricted the accumulation of L-leucine, L-tryptophan, or α-aminoisobutyric acid in germinated conidia and partially inhibited the glucose uptake (LESTER, 1965). In *Escherichia coli*, 2-PE also caused the disruption of cell membrane and inhibited DNA synthesis (TREICK; KONETZKA, 1964).

STARK *et al.*, 2003 observed that the 2-PE reduces the respiratory capacity and causes the metabolic uncoupling of catabolism and anabolism in *S. cerevisiae* and can also increase the fluidity of cell membrane, reduce uptake of both glucose and amino acids. Besides, they observed changes in morphology resulting in elongated shape when the yeast was cultivated in addition to L-phenylalanine.

S. cerevisiae exposed to the 2-PE showed an up-regulation of channels for ions which plays important roles in membrane potential balance, and mitochondrial proteins, (JIN *et al.*, 2018). In addition, there is in that yeast a decrease in membrane proton-pumping capacity concomitantly with loss of viability (SEWARD *et al.*, 1996), increase in cell membrane permeability (SILVER; WENDT, 1967). Due to growth inhibition caused by 2-PE, some strategies such as In Situ Product Recovery (ISPR) have been applied to remove the 2-PE from culture medium. Other strategies used are two-phase extraction, In Situ Product Adsorption (ISPA) and solvent immobilization (WANG *et al.*, 2019).

Final remarks

The 2-PE is a product with great importance in various industries such as cosmetic and food industries. The biotechnological production of this aromatic alcohol is promising and therefore should be improved in order to attend the demand of global market. An important drawback associated with the 2-PE production by yeasts is the its inhibitory effect. As such,

the study of responses to the stress caused by 2-PE in yeasts is pivotal to better understand the mechanisms of adaptation apply them in metabolic engineering strategies to improve the yeast robustness.

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CHAPTER II

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

2-phenylethanol stress responses in *Kluyveromyces marxianus* CCT 7735

ABSTRACT

2-phenylethanol (2-PE) is a higher aromatic alcohol with a rose-like aroma. It is used in the cosmetic and food industries as flavoring and displays potential for use as an antifungal. Interest in the biotechnological production of 2-PE from yeast has been growing due to the non-use of toxic compounds and the generation of few by-products. *Kluyveromyces marxianus* CCT 7735 exhibits potential to be used in 2-PE production; however, its growth has been strongly inhibited by this aromatic alcohol. Herein, we evaluated at *K. marxianus* CCT 7735 the effects caused by 2-PE exposure and its adaptive responses. The stress condition was created by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures and samples were analyzed over 1, 2, 4, 8, and 12 h of cultivation. The 2-PE stress impaired yeast growth, glucose uptake, fermentative metabolism, membrane permeability and cell viability. Moreover, this stress condition provoked alterations in both morphology and surface roughness. The reactive oxygen species (ROS) increased immediately on exposure to 2-PE. Changes in membrane fatty-acid composition, ergosterol content, exopolysaccharides production and reduction of the ROS levels appear to be the result of adaptive responses in *K. marxianus*. Our results provided insights into better understanding the effects of 2-PE on *K. marxianus* and its adaptive responses.

Keywords: yeast, aromatic alcohol, adaptive responses, morphology, membrane composition.

Introduction

2-phenylethanol (2-PE) is a higher aromatic alcohol that has been used as an ingredient in both the cosmetic and food industries due to its rose-like aroma and colorless aspect (Martínez et al. 2018). It is also used as a substrate for synthesizing other aromatic compounds such as phenylethyl acetate ester (Etschmann et al. 2002). Furthermore, 2-PE has been used in the biocontrol of pathogenic fungi such as *Aspergillus niger, Penicillium notatum*, *Neurospora crassa* and *Aspergillus flavus* (Lester 1965; Hua et al. 2014).

2-PE is found in the essential oils of flowers; however, its extraction from them is expensive. Moreover, this aromatic compound can be produced by chemical synthesis from benzene, which is a toxic compound with a carcinogenic effect. This synthesis is often environmentally unfriendly due to the use of strong acids or alkalis, high temperature and pressure. Furthermore, unwanted by-products are formed during the process (Gao and Daugulis 2009).

By contrast, the biotechnological production of 2-PE is sourced from compounds which are non-toxic, and is carried out under soft conditions that generates few by-products (Hua and Xu 2011). The biosynthesis of 2-PE can be derived from yeasts such as *Saccharomyces cerevisiae*, *Pichia fermentans* and *Kluyveromyces marxianus* (Huang et al. 2001; Serp et al. 2003; Conde-Báez et al. 2019). Yeasts can synthesize 2-PE from L-Phenylalanine (L-Phe) through the Ehrlich pathway or by *de novo* synthesis from sugars through the Shikimate pathway.

K. marxianus is a yeast that displays important characteristics for industrial applications such as ability to assimilate a wide range of sugars, including glucose and xylose, constituents of lignocellulosic biomasses, and lactose, sugar found in whey (Diniz et al. 2017). *K. marxianus* CCT 7735 was identified as the best producer of 2-PE among the yeasts isolated from Brazilian environments, producing 174 mg/L of 2-PE in medium without L-Phe, using

de novo synthesis and was able to produce 3.44 g/L of 2-PE after 72 h of growth in an optimized condition, which was achieved by the addition of 4 g/L of L-Phe, 30 g/L of glucose and was maintained at 30 ºC (de Lima et al. 2018). However, *K. marxianus*, like other yeasts, has its growth strongly inhibited by 2-PE concentrations in excess of 2.0 g/L (Lester 1965).

It has been reported in *S. cerevisiae* that the stress caused by 2-PE leads to an uncoupling of anabolism from catabolism (Stark et al. 2003), an increase in membrane permeability, a decrease in the protonmotive force with loss of viability (Seward et al. 1996), overexpression of genes encoding channels for ions, which play important roles in membrane potential balance, and up-regulation of mitochondrial protein genes (Jin et al. 2018).

To the best of our knowledge, damage caused by 2-PE in *K. marxianus* and its adaptive responses is yet still to be addressed. As such, our work aimed to evaluate 2-PE stress responses in *K. marxianus* CCT 7735. Under 2-PE stress, this yeast underwent an immediate impact followed by an adaptive response.

Our results helped to better understand the 2-PE effects on *K. marxianus* and its adaptive responses.

Experimental procedures

Yeast strain and culture media

The yeast strain, *K. marxianus* CCT 7735, was used in all experiments. It belongs to the culture collection of the Microbial Physiology of the Department of Microbiology at the Federal University of Viçosa (UFV), and was isolated from a dairy plant in Minas Gerais-Brazil (Silveira *et al.* 2005) then deposited in the Tropical Culture Collection André Tonsello Foundation, Campinas, São Paulo, Brazil. These yeast cell strains were maintained in YP medium (g/L): peptone 20, yeast extract 10, with the addition of glycerol 4% (w/v) at -80 ºC.

Yeast cells were cultured on a Yeast Nitrogen Base (YNB) medium (Sigma Chemical Co., MO, USA) without amino acids but with 6.7 g/L of ammonium sulfate with either 20 or 5 g/L of glucose. To obtain a pre-inoculum, *K. marxianus* CCT 7735 was grown overnight at 30 °C and 200 rpm (New Brunswick, Edison NJ, USA). The YNB-PE medium containing 5 g/L of glucose with 2-PE concentrations ranging from 1.5 to 3.5 g/L was used to evaluate the inhibitory effect of 2-PE on cell growth. As control, the yeast was cultured on YNB medium without 2-PE.

Inhibitory effect of 2-PE on cell growth

The inhibitory effect was expressed as the ratio of the specific growth rate in YNB with 2-PE ($\mu_{Experiment}$) over the specific growth rate in the media without 2-PE ($\mu_{Control}$). The 2-PE concentration that inhibited the yeast growth, and allowed for achieving biomass content to carry out the analyses was selected for further cultivations.

Batch cultures

First, batch cultures were carried out to determine the yeast growth phases and the times to carry out sampling. The cultivations performed in 1.2 L bioreactors (New Brunswick BioFlo/CelliGen 115) containing 0.8 L of YNB medium with 20 g/L of glucose. *K. marxianus* CCT 7735 was inoculated in the bioreactor to obtain the optical density at 600 nm (OD_{600}) of approximately 0.2. Yeast cultures were grown at 30 °C, air flow was set to be 1.5 vessel volume per minute (vvm), dissolved oxygen (DO) was maintained at 30% and pH was 5.5. Agitation was controlled by adjusting the percentage of dissolved oxygen present in the culture medium. Yeast cultures were evaluated under control conditions, both without 2-PE (non-stress), and with 2-PE addition (stress condition). The samples were initially harvested from the exponential growth phase, t=0,1, 2, 4, 8 and 12 hours, in triplicates (n = 3), of both growth conditions.

Determination of specific growth rate and dry weight

Cell growth was monitored by measuring the OD_{600}, using a UV-visible spectrophotometer (BECKMAN DU series 600). Biomass content and specific growth rate (μ) were determined as previously established by da Silveira et al. (2018).

Membrane permeability analysis

A BD flow cytometer (Becton Dickinson, Mississauga, ON, CA-FACSVerse™) was used for evaluating membrane permeability and cell viability as a protocol established by Alvim et al. (2019) with some alterations. with a number of alterations. Cells from batch cultures were sampled between 0 and 12 h under stress and non-stress conditions and stained with propidium iodide (PI).

Reactive oxygen species analysis

The level of ROS in non-stressed and stressed cells was measured using the oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR, USA). 10^6 cells withdrawn from batch cultures were resuspended in 1 mL of YNB medium and 10 μM DCFDA were added. The cells were incubated at 30 °C for 30 min and at 200 rpm in the dark. Next, the samples harvested were centrifuged at 28,000 *g* for 10 min, and stained cells were resuspended in YNB/PE medium and incubated for 0–12h at 30 °C at 200 rpm. The fluorescence of the oxidized DCFDA probe was measured using a BD flow cytometer (Becton Dickinson, Mississauga, ON, CA-FACSVerse™). A minimum of 10,000 cellular events was collected at each point (0, 1, 2, 4, 8 and 12 h). Fluorescence was detected using the 530 nm filter that excites the sample with a 488 nm laser.

Morphological analysis

The effect of 2-PE on yeasts cell morphology was evaluated using an Atomic Force Microscopy (AFM). The cells harvested from batch cultures were centrifuged at 28,000 *g* for 10 min and resuspended in ultrapure water till reaching approximately OD_{600} of 10. Cells were scattered in glass sheets of 1 cm^2. After drying, the sheets were analyzed using the atomic force microscope (NT-MDT, Ntegra Prima, Russia) so as to obtain topography measurements of the cells by intermittent contact to minimize the deformation risk and allow for higher lateral resolution. The measurements were taken on a scale of 5 to 20 μM.

Negative staining

The exopolymer presence was analyzed by placing a drop of negative stain (India Ink, Acrilex, Brazil) on the glass microscope slides. Afterwards, 20 μL of cells harvested from batch cultures were smeared in the dye 1:1 (v/v) in water. The slides were air dried for 7 min and the smear saturated with 0.01% methylene blue (w/v) for 1 min. The pictures were taken in different fields with a high-resolution microscope (Olympus BX51, Olympus Corporation of the Americas, USA). The percentage of exopolymer area was measured using the freely available image processing program ImageJ (https://imagej.nih.gov/ij/).

Fluorescent-lectin binding assays

10 μL of samples harvested from batch cultures at 0h and 12h under non-stress and 2-PE stress conditions were smeared on glass slides and allowed to dehydrate. The cells were fixed for fifteen minutes at room temperature in 4% paraformaldehyde (v/v). The slides were washed three times in sodium phosphate buffer (PBS) 0.1 M at pH 7.2 and stained with solution containing PI and fluorescein-isothiocyanate (FITC) conjugated lectin from *Triticum vulgaris* (Sigma Aldrich Co, USA) at final concentration of 20 and 50 μg/ml, respectively. After forty-five minutes incubation at room temperature, the slides were washed 3 times in

PBS with a final wash in deionized water and were observed at M5000 EVOS fluorescence microscope (Thermo Fisher Scientific, China) equipped with 60X objective lens. The fluorescence of PI was observed with 535 nm excitation and 617 nm emission filters. FITC-lectin were observed with 492 nm excitation and 520 nm emission filters and the light intensity was fixed to 0.116. Images were analyzed using freely available image processing program ImageJ (https://imagej.nih.gov/ij/).

Fatty acid methyl ester (FAME's) analysis

Three samples were collected from batch cultures under 2-PE stress and non-stress conditions. They were harvested at 12,000 *g* at 4 °C for 10 min and then the pellets were lyophilized. The fatty acids in the yeast cells (4–5 mg of dry weight) were saponified, methylated, and extracted following the Sherlock Instant Fame™ User's Guide (Newark, USA). The resulting methyl ester mixtures were separated using an Agilent 7890A gas chromatograph with a flame ionization detector (Agilent Technologies, USA) and identified using the MIDI microbial identification system (Sherlock 6.0 Microbial Identification System, Newark, USA).

Ergosterol extraction and determination

Ergosterol was extracted according to Lahtvee et al. (2016), with modifications. Briefly, 0.5 mL of methanol/chloroform (2:1, v/v) solution and two steel beads were added to the lyophilized cells (15–20 mg) and submitted to cellular lysis using TissueLyser II (Qiagen, Hilden, Germany). The extracts were harvested at 4,000 *g* for 10 min, and the supernatants transferred to new tubes. The first four steps were repeated three times. Two milliliters of 100% chloroform solution (v/v) were added and homogenized. Afterwards, 2 mL of 1% sodium chloride solution (w/v) were added, and the solution was harvested at 12,000 *g* for 20 min. Ten microlites of inferior phase were collected and injected into a RP-C18 high-

performance liquid chromatography (HPLC) column. The ergosterol was detected by a UV–visible detector at 282 nm, at 24 ºC, using a 98% methanol (v/v) solution as mobile phase and flow rate of 1.0 mL/min with isocratic elution. External standards varying between 0.01 and 1.0 mg/mL of ergosterol resuspended in a methanol/chloroform (2:1, v/v) solution were used as a calibration curve to quantify its content.

Determination of metabolite concentrations

For glucose, ethanol and 2-PE analysis, supernatant samples obtained from batch cultures were filtered using a 0.22 μM membrane prior to injection into the HPLC (LC-20AT, Shimadzu, Japan).

To measure the 2-PE concentration, 5 μL of samples were injected and passed through a Kinetex RP-C18 column (250×4.6 mm, 5 μm, Phenomenex, California, USA) and detected by a UV–visible detector at 258 nm. Sterile water and methanol (35:65, v/v) were used as a mobile phase, with a flow rate of 0.5 mL/min with isocratic elution.

Glucose and ethanol concentrations were analyzed by injecting 10 μL of samples into an Aminex HPX-87H ion exchange column (300 × 7.8 mm, 9 μm, Bio-Rad, Munich, Germany) coupled to a refraction index RID-20A detector (Shimadzu, Japan), with 5 mM H_2SO_4 as a mobile phase, and a flow rate of 0.6 mL/min, at 60 °C.

Determination of fermentation parameter

Biomass yield ($Y_{X/S}$, g/g) was determined by angular coefficient from a linear regression of the plot dry cell weight (DCW) *versus* glucose consumption (g/L).

Statistical analysis

Statistical analyses were carried out using the R 3.4.2 software program (www.r-project.org). All experiments were conducted in triplicate. The significance level used in the statistical tests was $p = 0.05$.

Results

2-PE concentration effect on *K. marxianus* CCT 7735 growth

K. marxianus CCT 7735 growth was evaluated in a range of 1.5 to 3.5 g/L 2-PE concentration (Fig. 1). Concentrations of 1.5, 2.0, 2.5, 3.0 and 3.5 g/L of 2-PE reduced the specific growth rate by 20.06, 35.15, 64.52, 80.57 and 87.13%, respectively. These results indicate that the 2-PE concentrations of 3.0 and 3.5 g/L imposed a strong stressful condition on *K. marxianus*. Based on this, we selected the 2-PE concentration of 3.0 g/L to identify the 2-PE effects on *K. marxianus* fermentative parameters and its adaptive responses, because despite its strong inhibitory effect, the biomass was enough to carry out the analyses.

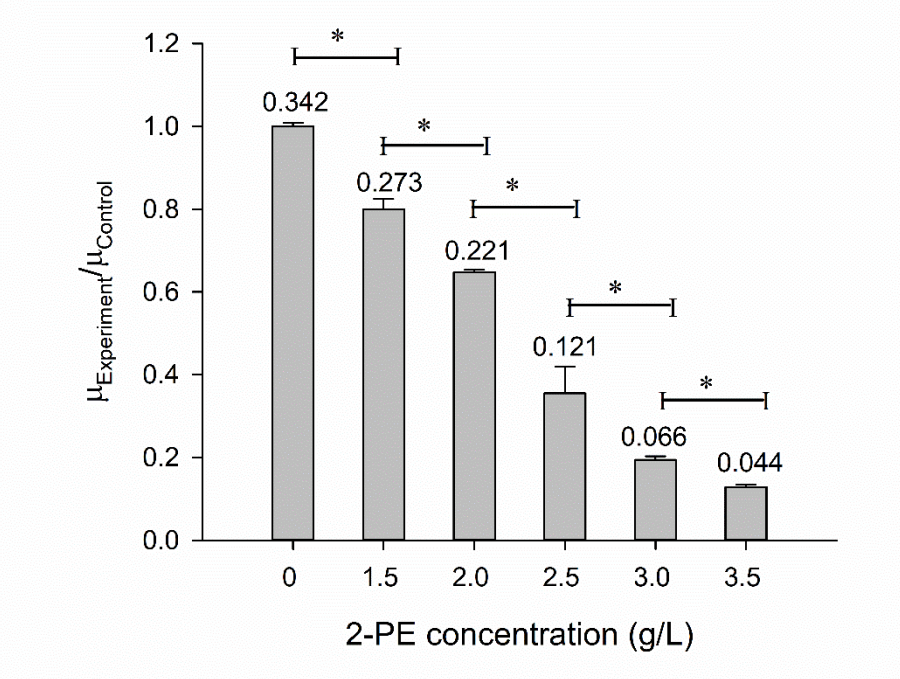

Fig. 1 Inhibition effect of 2-PE on *K. marxianus* CCT 7735 growth. $\mu_{Experiment}$ and $\mu_{Control}$ represent the specific growth rates of the yeast grown in the presence and absence of 2-PE, respectively. The values above the bars indicate the $\mu_{Experiment}$. (*) Means significant difference between $\mu_{Experiment}/\mu_{Control}$ compared to the previous concentration according to Student's t-test ($p = 0.05$)

Effect of 2-PE on glucose consumption, cell yield and fermentative metabolism

During the first two hours of growth, glucose was not consumed by either non-stressed and stressed cells (Fig. 2). Otherwise, there was glucose consumption between 2 and 4 h under non-stressed condition, while the cells subjected to stress condition did not consume this

carbon source. The stressed cells consumed glucose after 4 h of cultivation. However, the glucose consumption rate was higher in non-stressed cells. The glucose was practically fully consumed after 12 h of growth by non-stressed cells, the period in which the stressed cells consumed 68.4% of this sugar (Online Resource 1), providing evidence of 2-PE exposure impairing the glucose consumption.

After 2 h, the non-stressed cells grew better than the stressed cells until the end of cultivation (Fig. 2). The stressed cells grew better after 8 h, indicating an adaptation stage to the stress condition in the earlier hours (Fig. 2b). As expected, the 2-PE stress also resulted in lower biomass yield ($Y_{X/S}$) compared to the non-stressed condition (Online Resource 1).

As regards the fermentative metabolism, maximum ethanol concentration was achieved by non-stressed cells at 8 h of growth (Fig. 2a). By the end of this period, the ethanol had been consumed. Ethanol production was severely decreased by 2-PE exposure. Indeed, maximum ethanol concentration decreased by 25.9% (Online Resource 1). Importantly, the 2-PE concentration did not alter during cultivation under stress, indicating that it was neither produced nor consumed (Fig. 2b).

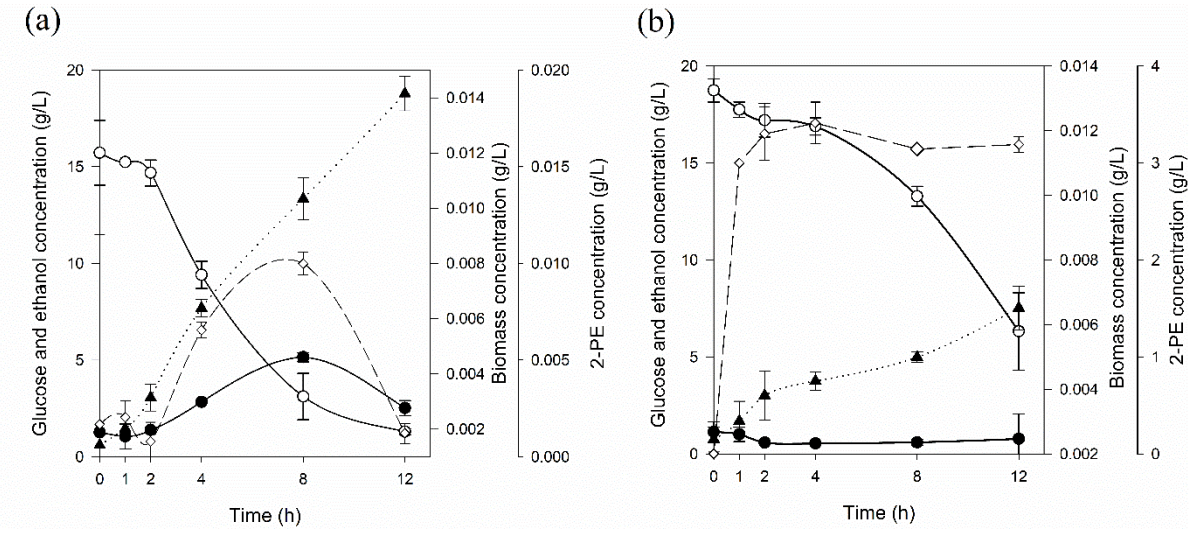

Fig. 2 Effect of 2-PE on glucose consumption, biomass, ethanol and 2-PE production of *K. marxianus* CCT 7735. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures. (a) glucose consumption and production of both ethanol and 2-PE under non-stressed condition. (b) glucose consumption and production of both ethanol and 2-PE under stress condition. (—○—) glucose, (…▲…) biomass, (—●—) ethanol and (---◊---) 2-PE

Effect of 2-PE on membrane permeability and cell viability

Since the inhibitory effect of 2-PE on *K. marxianus* growth was observed, we evaluated its impact on membrane permeability and cell viability. Together with cell viability analysis, the embedding detection of PI, which binds to nucleic acids, allowed us to visualize viable cells which presented increased membrane permeability. This impact was most evident after 4h of 2-PE exposure, and was represented by the largest distribution of cells in the transition range between living and dead cells (Online Resource 2). The 2-PE exposure did not influence cell viability during the first 2 h of stress (Table 1; Online Resource 2). The percentage of cell death after 8 h of stress was two-fold higher than after 4 h of stress. The reduction in cell viability continued to occur up to 12 h under the stress condition. However, this was lower than in earlier periods. At 12 h of stress, we observed 13.89% cell death, whereas, after the same period, cell death was only 2.6% under the non-stressed condition (Table 1).

With regard to cell size, analyzed through forward scatter-Area (FSC-A), the stressed cells recorded an increase over time compared to the non-stressed cells (Online Resource 3). In addition, we evaluated the variations in intracellular complexity through side scatter-Area (SSC-A). Notably, the 2-PE exposure resulted in higher intracellular complexity over 12 h of growth (Table 2).

Table 1 Variations in cell death percentage, cell size (FSC-A) and intracellular complexity (SSC-A) of *K. marxianus* CCT 7735 under non-stress and 2-PE stress conditions. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures. The dead cells were detected through propidium iodide, a probe that entered into cells when the membrane integrity was affected

	Time (h)	% dead cells	FSC-A Mean	SSC-A Mean
Non-stressed cells	0	1.675 ± 0.60	107.671 ± 0.27	56.978 ± 0.43
	1	2.605 ± 0.37	101.276 ± 0.52	51.674 ± 0.31
	2	2.20 ± 0.37	94.931 ± 0.10	49.823 ± 0.39
	4	2.125 ± 0.25	90.266 ± 89.855	52.916 ± 0.63
	8	2.565 ± 0.12	114.354 ± 0.53	52.487 ± 0.72
	12	2.655 ± 0.18	120.139 ± 0.76	58.736 ± 0.43
2-PE stressed cells	0	1.755 ± 0.49	108.115 ± 0.88	53.164 ± 0.58
	1	2.350 ± 0.64	123.854 ± 0.81	61.055 ± 0.85
	2	2.435 ± 0.77	141.411 ± 0.45	65.021 ± 0.44
	4	4.195 ± 0.77	175.206 ± 0.75	79.159 ± 0.67
	8	8.805 ± 0.37	208.618 ± 0.45	104.798 ± 0.73
	12	13.890 ± 0.74	205.130 ± 0.82	110.225. ± 0.63

FSC-A: forward scatter-area; SSC-A, side scatter-area

Effect of 2-PE on reactive oxygen species level

Taking into account that ethanol stress leads to the increase of ROS in yeasts (Jing et al. 2018; Sugiyama et al. 2019), we were interested in evaluating whether the 2-PE also induces the same effect. Importantly, the maximum ROS level was achieved immediately upon 2-PE exposure (time 0 h) (Fig. 3; Online Resource 4). It should be noted that their level reduced during cultivation, and was very similar in both stressed and non-stressed cells after 8 h.

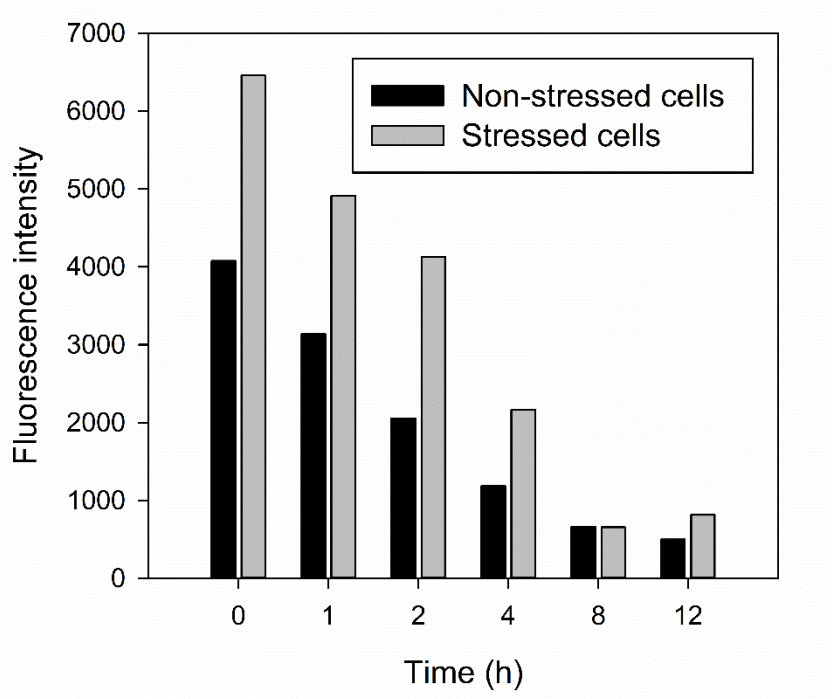

Fig. 3 ROS production of *K. marxianus* CCT 7735 under non-stressed and 2-PE stress conditions. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures. The graph is a representative result of 10,000 events and three experiments

Effect of 2-PE on cell morphology and exopolymer formation

We analyzed the effect of 2-PE on cell morphology by using AFM. Notably, 2-PE exposure resulted in increase in root mean square roughness (RMS) during the first 2 h of cultivation (Table 2). In contrast, the RMS of non-stressed cells decreased over the same period. Importantly, we were unable to analyze roughness of cells exposed to 2-PE stress at 4, 8 and 12 h due to the presence of a compound on the cell surface, which makes it brighter and more homogeneous (Fig. 4). The presence of this compound in non-stressed cells was less pronounced, which allowed us to evaluate the roughness. Under the non-stressed condition, the RMS continued decreasing up to 4 h, but began increasing at 12 h (Table 2). We confirmed through negative staining that the compound observed on cell surface was an exopolymer (colorless area around the cells). This compound was more pronounced in stressed cells than around non-stressed cells from 4 h of cultivation (Fig. 5; Online Resource 5).

Furthermore, the stressed cells showed a bigger length and smaller width compared to the non-stressed cells at 12 h (Table 2). We did not observe any differences on both length

and width under the non-stressed condition. In addition, the maximal height was similar under both conditions (Table 2). Taken together, these results show that the roughness, length, width, and exopolymer production were altered on exposure to 2-PE.

Table 2 Comparison between cell roughness of *K. marxianus* CCT 7735 under non-stress and 2-PE stress conditions. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

	Time (h)	RMS (nm)	Length mean (nm)	Width mean (nm)	Max (nm)
Non-stressed cells	0	404.12	3,337.01 ± 0.52	1,856.67 ± 0.26	1,417.81
	2	309.66	3,582.33 ± 0.65	2,026.50 ± 0.49	1,638.52
	4	278.91	3,818.17 ± 0.60	2,320.68 ± 0.17	1,678.84
	12	356.20	3,647.67 ± 0.27	2,641.67 ± 0.32	1,723.68
2-PE stressed cells	0	385.498	3,994.01 ± 0.70	2,088.00 ± 0.25	1,953.00
	2	426.12	3,949.50 ± 0.58	2,096.51 ± 0.36	1,705.51
	4	*	4,895.03 ± 0.57	2,080.00 ± 0.15	1,319.43
	12	*	4,590.00 ± 0.32	1,586.67 ± 0.32	1,656.16

RMS: root mean square roughness; Max: maximum height

(*) RMS not detected due to the presence of a compound on cell surface

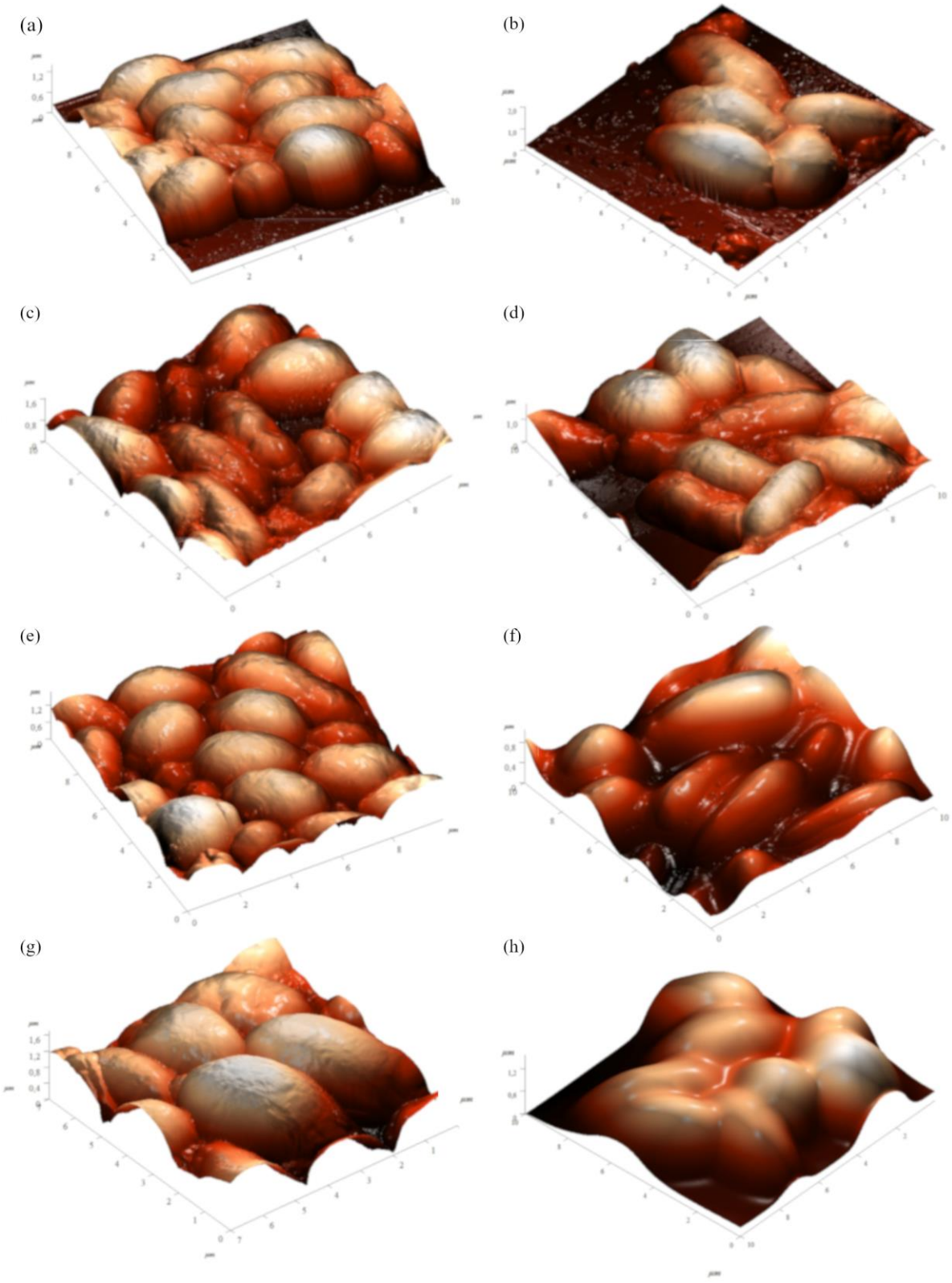

Fig. 4 Cell morphology of *K. marxianus* CCT 7735 under non-stress (left column) and 2-PE stress (right column) conditions, by AFM. The three-dimensional images correspond to yeast cells collected at 0 (a, b), 2 (c, d), 4 (e, f) and 12 h (g, h). The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

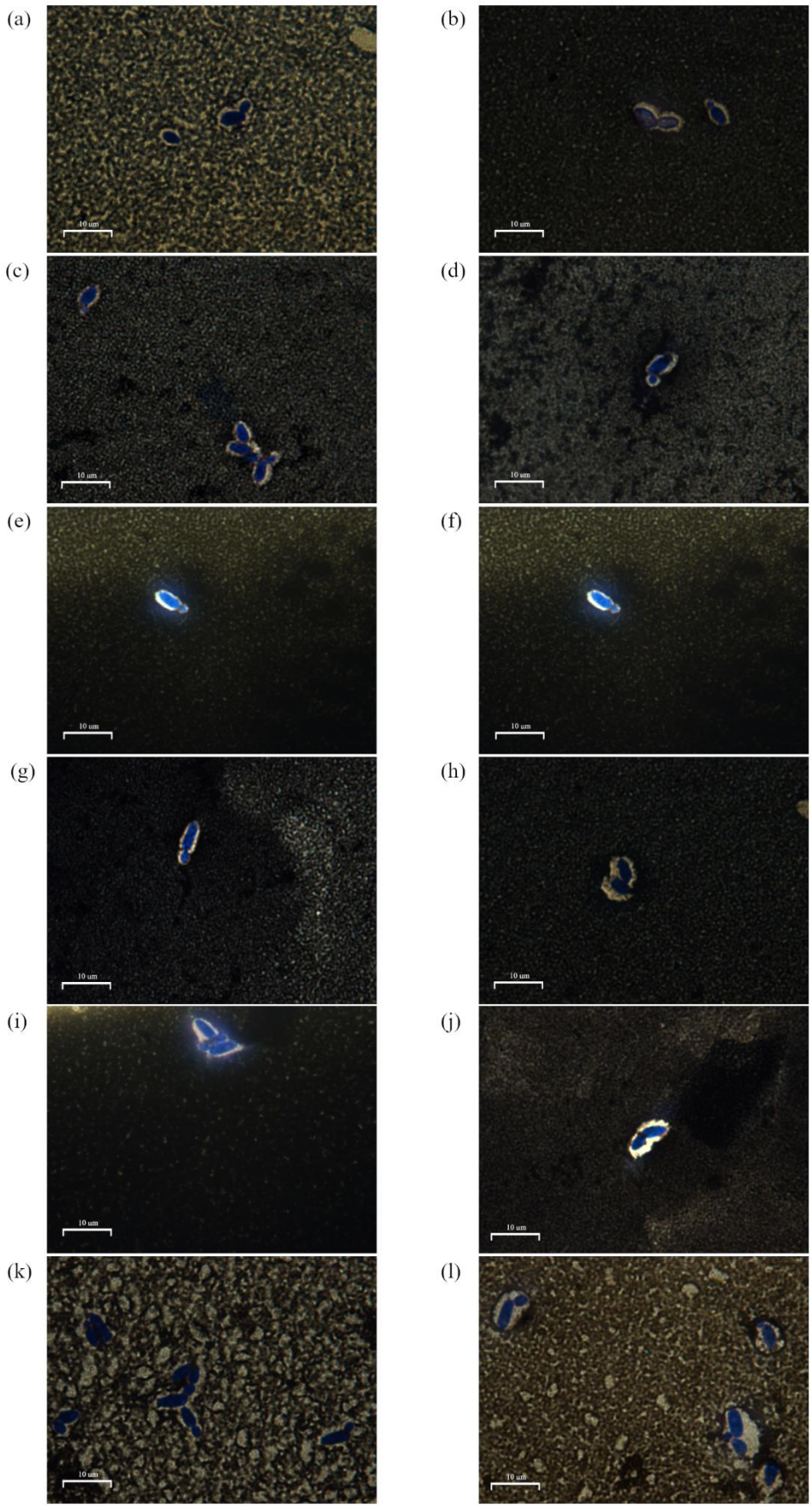

Fig. 5 Negative staining of *K. marxianus* CCT 7735 under non-stress (left column) and 2-PE stress (right column) conditions. The slides photography of yeast cells collected at 0 (a, b), 1 (c, d), 2 (e, f), 4 (g, h), 8 (i, j) and 12 h

(k, l) was taken with a high-resolution microscope (Olympus BX51) at 100x. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures. The colorless space around the cells correspond to the exopolymer

Several physiological functions are proposed for the exopolysaccharides production by microorganisms, such as protection against environmental pressures (osmotic stress, temperature, oxidants, desiccation), cell adherence to surfaces and carbon or water storage (Freitas et al. 2017). We confirmed that the extracellular compound is an exopolysaccharide using FITC conjugated lectin, which show green fluorescence when its binds to N-acetyl-β-D-glucosaminyl residues or N-acetyl-β-D-glucosamine oligomers (Online Resource 6). We also observed the difference in production of this compound between stressed and non-stressed cells through the higher fluorescence intensity under the 2-PE stress condition (Online Resource 6d).

Effect of 2-PE on FAME's profile and ergosterol content

We investigated changes in membrane fluidity through the FAME's profile analysis (Fig. 6; Online Resource 7). We observed no alterations in the percentage of total saturated and unsaturated fatty acids under non-stressed and 2-PE stress condition over time. However, the oleic acid (18:1n-9) level increased mainly during the first 2 h and between 8 and 12 h of growth under stress condition, resulting in a total increase of 8.73%; and the palmitoleic acid (16:1n-7) level decreased 4.44% in the first hours of stress which was maintained throughout the cultivation. As regards the non-stressed cells, we observed no statistic difference in the oleic acid level at 12 h. Apart from this, the palmitoleic acid level increased 11.47% during the same period of cultivation. Remarkably, the percentages of both monounsaturated and polyunsaturated fatty acids remained unaltered under the non-stressed condition, but they were affected by exposure to 2-PE (Fig. 6; Online Resource 7). At 12 h of cultivation, the polyunsaturated fatty acids percentage increased in stressed cells, whilst the monounsaturated fatty acids percentage decreased after the same time.

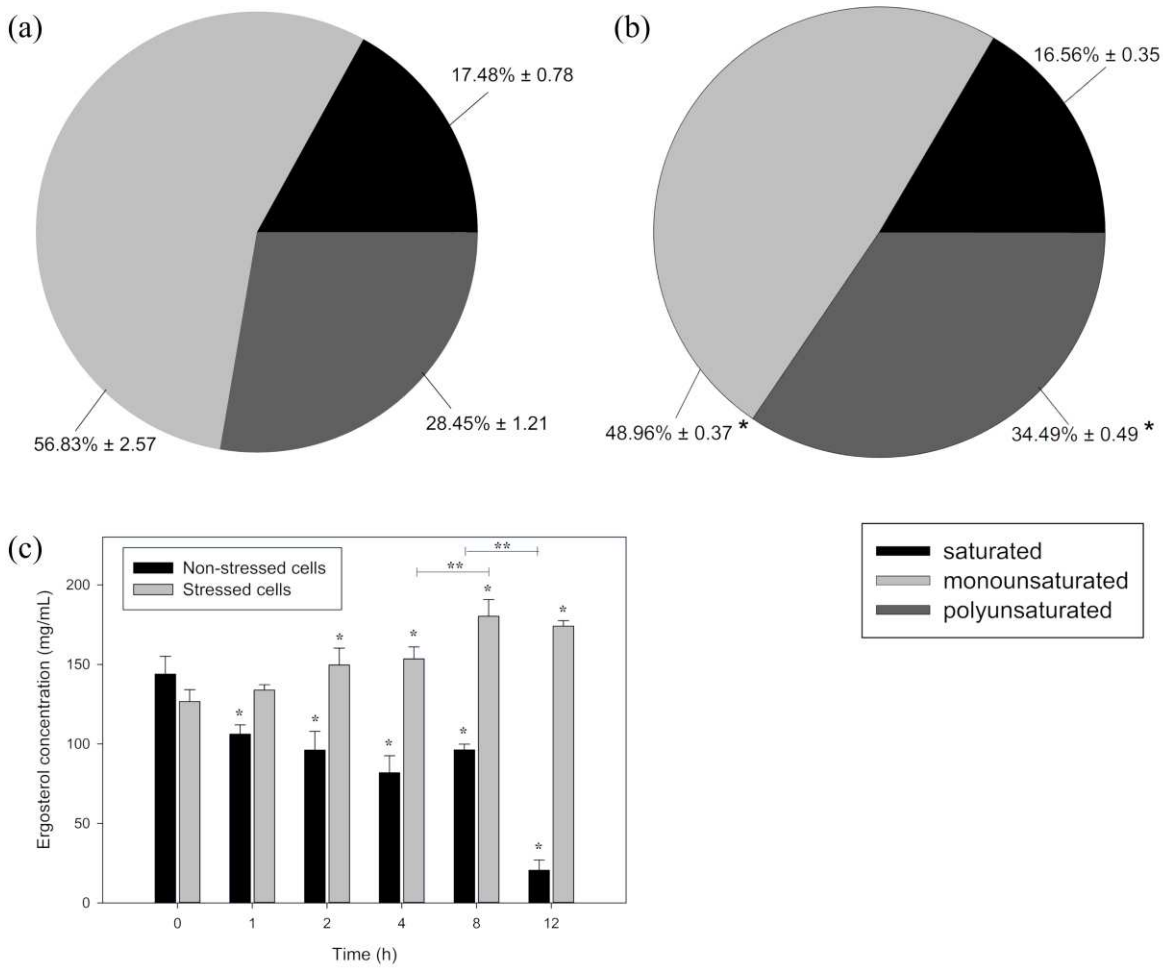

Fig. 6 FAME's profile and ergosterol concentration of *K. marxianus* CCT 7735. FAMES's profile of (a) non-stressed cells (0 h) and (b) 2-PE stressed cells after 12 hours of growth. (c) ergosterol concentration between 0 and 12 h of non-stressed and stressed cells. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures. (*) means significant difference between FAME's profile or ergosterol concentration in relation to 0 h and (**) between other times, according to Student's t-test ($p = 0.05$)

Ergosterol acts in plasma membrane stabilization due to the reduction in lipid interdigitations on phospholipid bilayers (Vanegas et al. 2012). We analyzed the influence of 2-PE on ergosterol concentration over 12 h of cultivation under both non-stressed and 2-PE stressed conditions (Fig. 6c). In stressed cells, ergosterol concentration increased between 4 and 8 h of cultivation. In contrast, the ergosterol concentration was reduced in non-stressed cells during the first hour and between 8 and 12 h of cultivation, periods during which these cells were in stationary growth phase.

Discussion

K. marxianus CCT 7735 subjected to 2-PE stress (3.0 g/L) displayed changes in terms of growth, glucose uptake, membrane permeability, cell viability, metabolism, membrane fatty acid composition, ergosterol content, exopolysaccharides production and morphology. A number of studies have shown similar responses to stress caused by ethanol and 2-PE in yeast, inhibiting growth and reducing cell viability (Fabre et al. 1998; Eshkol et al. 2009; Stanley et al. 2010). It is interesting to note that these alterations in *K. marxianus* CCT 7735 were time dependent, that is, yeast cells underwent an immediate impact followed by an adaptive response. In agreement with this observation, the same yeast strain exposed to ethanol also presented changes depending on the time involved (Alvim et al. 2019).

In the first 2 h of exposure to 2-PE, neither growth nor glucose consumption were observed. However, the cells still maintained viability. In the range between 2 and 4 hours under 2-PE stress, both growth and glucose consumption were hindered and cell viability decreased.

Importantly, *K. marxianus* grew and the glucose consumption increased after 4 h of stress. However, cell viability continued to drop gradually and membrane permeability increased, indicating the cell membrane was an important target of 2-PE. Similarly, Alvim et al. (2019) showed that the membrane permeability of *K. marxianus* CCT 7735 was affected by ethanol exposure. In *S. cerevisiae* under 2-PE stress, the decrease in membrane proton-pumping capacity took place concomitantly with a loss of cell viability (Seward et al. 1996). Other studies have also commented on the increase in cell membrane permeability caused by 2-PE in *Neurospora crassa* and *Escherichia coli* (Lester 1965; Silver and Wendt 1967). In *S. cerevisiae* exposed to 2-PE, ionic channels related to osmoregulation and the membrane potential balance were up-regulated (Jin et al. 2018).

It is interesting to note that the increase in glucose uptake occurred during the same period where the membrane permeability remarkably increased. This is likely related to the fact that the glucose transport system is a passive transport system (da Silveira et al. 2019), therefore it is not affected by the proton motive force disruption. In contrast, lactose was not transported in *K. marxianus* CCT 7735 stressed with ethanol (Alvim et al. 2019), because the lactose transporter system is a proton symport (da Silveira et al. 2019).

Notably, the roughness of *K. marxianus* CCT 7735 cells was higher in the first two hours of 2-PE stress. Alterations in roughness and permeability were also observed in both *S. cerevisiae* and *Schizosaccharomyces pombe* under ethanol stress; nevertheless, both were more affected in *S. pombe* (Canetta et al. 2006). Consistent with the AFM results, the flow cytometer analyses also indicated that the 2-PE exposure caused the increase in *K. marxianus* cells. On the other hand, the *S. cerevisiae* morphology did not change under stress condition established by endogenous 2-PE (Stark et al. 2003). For instance, morphological changes caused by stress conditions such as heat, osmotic, oxidative and hypoxia have also been observed in *S. cerevisiae* (Meaden et al. 1999; Pratt et al. 2003; Aon et al. 2018; Altenburg et al. 2019).

We also speculate that the morphological changes might be associated with the adaption of *K. marxianus* to the 2-PE. Han et al. (2013) showed *Candida albicans* hyphal formation suppression under 2-PE concentrations between 5 and 15 mM. In this yeast, the 2-PE acted as a signalizing molecule to environment stress. In contrast, the exposure of the yeast *Kloeckera apiculata* to 1.5 μL/mL of 2-PE induced the hyphal formation, allowing it to enhance its adherence and biofilm formation (Liu et al. 2014).

In our work, the stressed cells showed a notable increase in exopolysaccharide production over cultivation. It is important to point out that the formation of capsules has been related to the mechanisms that enable microorganisms to concentrate nutrients when grown

in low nutrient environments and to reserve water to resist the desiccation (Yurkov 2018; Sarkar et al. 2019). In *Cryptococcus neoformans*, the capsule conferred protection on ROS (Zaragoza et al. 2008). In *Papiliotrema laurentii,* the capsule formation occurred under nitrogen deprivation and might be related to cell protection (Sarkar et al. 2019). Based on this, we suggest that increased exopolysaccharide production in *K. marxianus* is associated with a mechanism of protection to the 2-PE. To the best our knowledge, this is the first work that showed the effect of 2-PE on yeast cell roughness and exopolysaccharide formation in *K. marxianus*.

Importantly, the exposure of *K. marxianus* CCT 7735 to 2-PE caused a notably sudden increase in the ROS levels, which might be related to alterations in mitochondria. There are protein complexes in the mitochondria responsible for the transfer of high-energy electrons from NADH and $FADH_2$ to oxygen in order to generate ATP. However, electrons may react with oxygen in non-enzymatic reactions, resulting in the production of ROS (Pan 2011). Consistent with our assumption, the exposure of *Penicillium italicum* to 2-PE provoked alterations in mitochondria such as degraded and disorganized cristae, leakage of the outer mitochondrial membrane and massive mitochondrial vacuolation (Liu et al. 2014). Furthermore, the increase in ROS level is consistent with other stress conditions that led to ROS formation (Machida et al. 1998; Dirmeier et al. 2002; Pérez-Gallardo et al. 2013; Mejía-Barajas et al. 2017; Jing et al. 2018). It is worth mentioning that the ROS level reduced over the period of 2-PE exposure, suggesting that *K. marxianus* CCT 7735 was able to develop an adaptive response. Indeed, it has been reported that yeast presents both early and late responses to the oxidative stress (Farrugia and Balzan 2012). Interestingly, *K. marxianus* CCT 7735 exposed to ethanol was also capable of coping with oxidative stress. Under this condition, there was an increase in the abundance of proteins involved with responses to oxidative stress (Alvim et al. 2019).

Notably, the ergosterol content was enhanced in response to 2-PE. We believe that this occurred to counteract the damages caused by 2-PE in plasma membrane. Our assumption is due to the role played by ergosterol in terms of ethanol tolerance in *S. cerevisiae*. Indeed, various works have shown the increase in ergosterol synthesis in *S. cerevisiae* exposed to ethanol (Walker-Caprioglio et al. 1990; Lahtvee et al. 2016). For ethanol stress, increases in both ergosterol and unsaturated fatty acids contents are important responses, because they act by preventing membrane interdigitations and maintain an optimal membrane thickness (Vanegas et al. 2012). The ergosterol accumulation in *S. cerevisiae* under organic acid stress has also been considered as an adaptive response (Guo et al. 2018).

Changes in membrane composition take place as an adaptive response to stressful conditions. Although the percentage of total saturated and unsaturated fatty acids has not been changed, oleic acid and polyunsaturated fatty acids had their levels increased. In *S. cerevisiae* the 2-PE stress also caused the increase of oleic acid (Mameeva and Podgorsky 2013). The increase in oleic acid level leads to the increase of membrane fluidity, which seems to be pivotal for yeasts counteracting the damages caused by ethanol (You et al. 2003; Ding et al. 2009; Lahtvee et al. 2016) and organic acids, such as acetic, formic and levulinic acids (Guo et al. 2018). Interestingly, Kajiwara et al. (1996) showed that the overexpression of the *FAD*2 gene encoding the desaturase enzyme improved the *S. cerevisiae* ethanol tolerance. Therefore, higher levels of both oleic and polyunsaturated fatty acids in *K. marxianus* exposed to 2-PE stress appear to be related to its adaptation to this stress condition.

To conclude, our results helped to better understand the 2-PE effects on *K. marxianus* and its adaptive responses. 2-PE stress impaired *K. marxianus* CCT 7735 growth, glucose uptake and cell membrane composition. Moreover, 2-PE stress reduced cell viability and provoked morphological changes. Plasma membrane is an important target of 2-PE in *K. marxianus*. Remarkably, the cells displayed adaptive responses to the 2-PE such as changes

in membrane fatty-acids composition, ergosterol content, exopolysaccharide production and reduction in ROS levels. To the best of our knowledge, we showed for the first time the effect of 2-PE on roughness yeast cells and exopolysaccharide formation. Further work should be undertaken to address how exopolysaccharide production is associated with adaptive responses to the 2-PE.

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Author Contribution Statement

WBS and TRB conceived or designed research. TRB and LLO conducted experiments or contributed analytical tools. TRB, FAS, RZV, AGN, LLO, WBS worked in data analyses and wrote the manuscript. All authors have read and approved the manuscript.

Compliance with Ethical Standards

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

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

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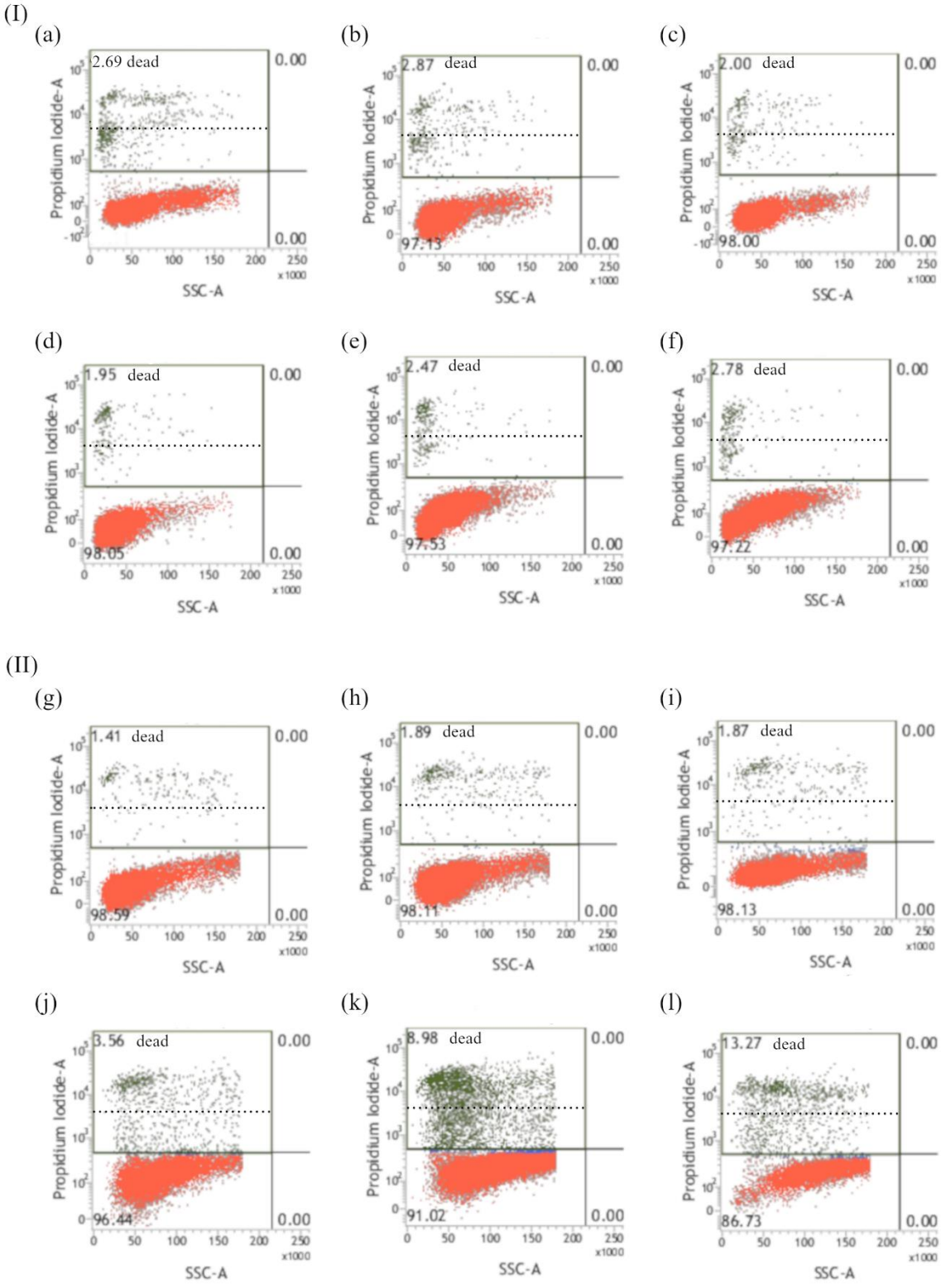
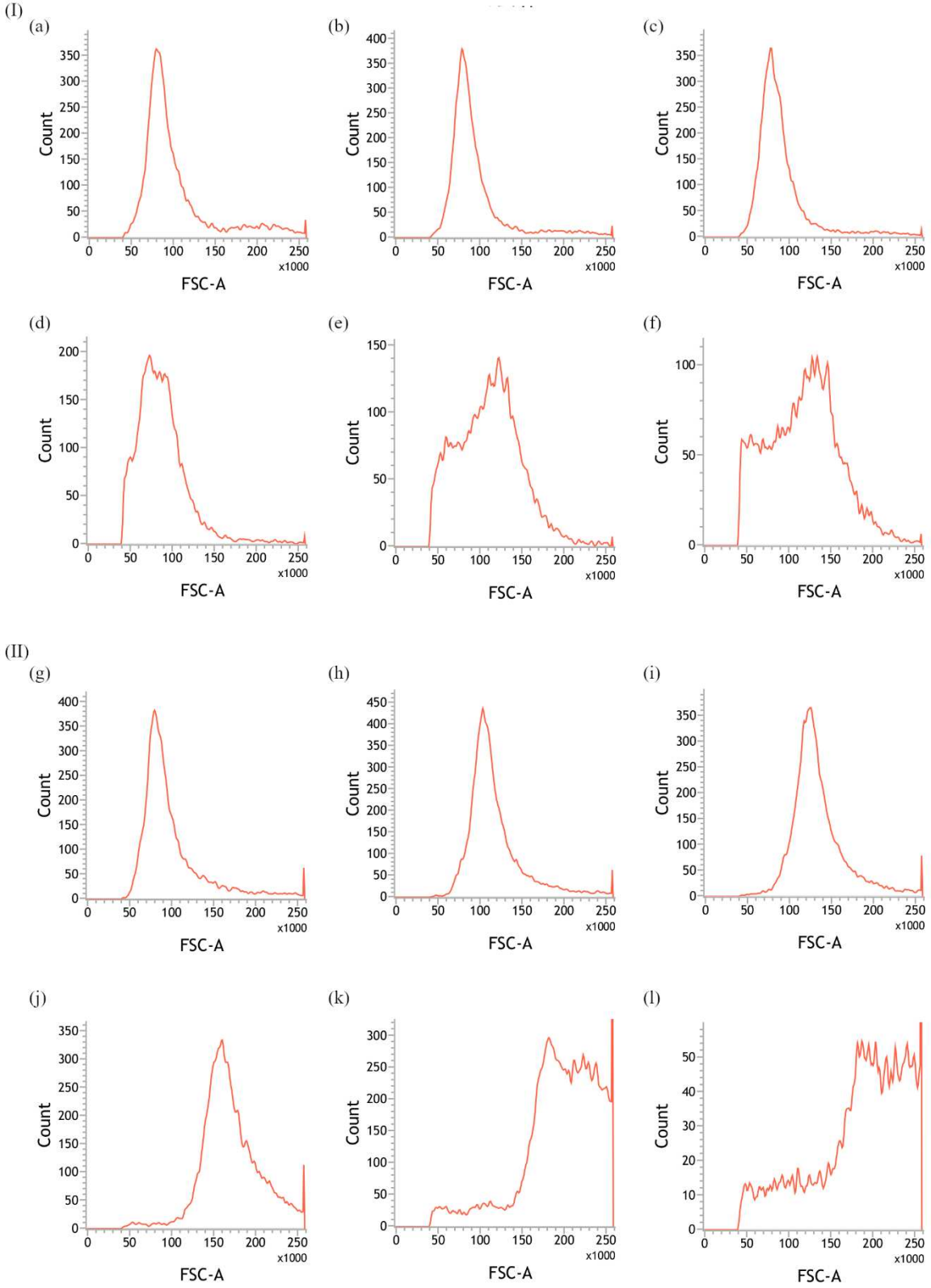
Supplementary Material

Online Resource 1 Physiological parameters of the *K. marxianus* CCT 7735 growth under non-stress (N) and 2-PE stress (S) conditions. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

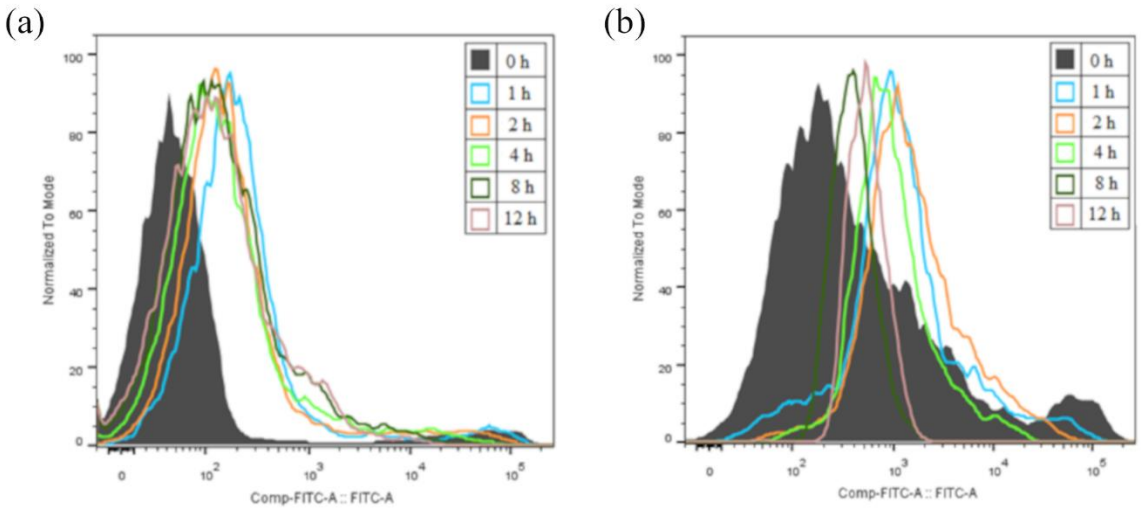
	Non-stressed cells	Stressed cells	Interval of growth (h)
Glucose consumption (%)	99.3	68.4	0-12
Biomass yield ($Y_{X/S}$, g/g)	0.0012	0.00033	0-8 (N)/0-12 (S)
Maximum biomass production (mg/L)	14.2	6.5	0-12
Ethanol concentration (g/L)	1.3	5.1	0-8



Online Resource 2 Cell viability of *K. marxianus* CCT 7735 under (I) non-stress and (II) 2-PE stress conditions. (a) and (g) represent 0 h; (b) and (h) 1 h; (c) and (i) 2 h; (d) and (j) 4 h; (e) and (k) 8 h; (f) and (l) 12 h of growth. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures. The dead cells were detected through propidium iodide, a probe that entered into cells when the membrane integrity was affected. The transition range is bounded by continuous and dotted lines. The side scatter-Area (SSC-A) measure the intracellular complexity



Online Resource 3 Cell size of *K. marxianus* CCT 7735 under (I) non-stress and (II) 2-PE stress conditions measure through forward scatter-Area (FSC-A). (a) and (g) represent 0 h; (b) and (h) 1 h; (c) and (i) 2 h; (d) and (j) 4 h; (e) and (k) 8 h; (f) and (l) 12 h of growth. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

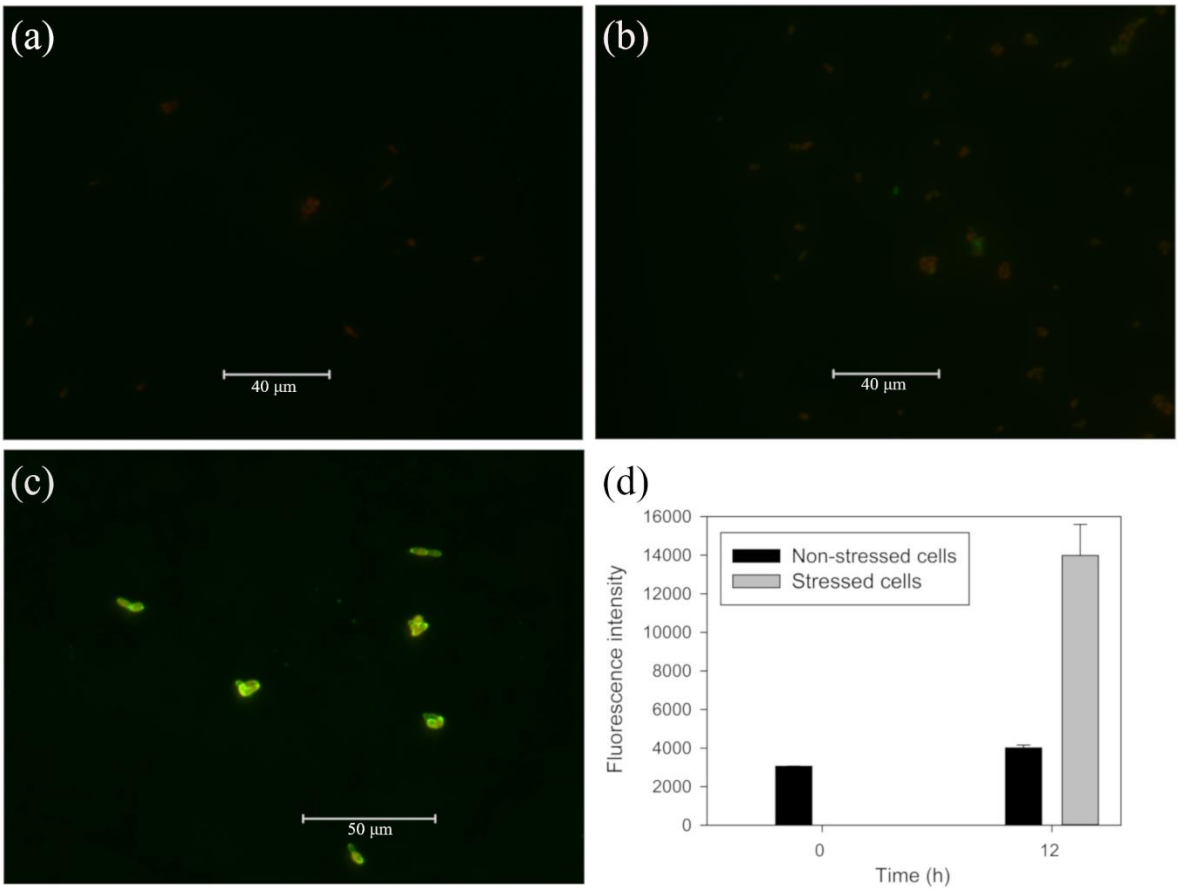


Online Resource 4 ROS production of *K. marxianus* CCT 7735 under (a) non-stress and (b) 2-PE stress conditions measured through fluorescence intensity of 2′,7′-dichlorofluorescein diacetate (DCFDA) probe. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

Online Resource 5 Percentage of exopolymer area on cell surface of *K. marxianus* CCT 7735 under non-stress and 2-PE stress conditions. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

Time (h)	Non-stressed cells	Stressed cells
0	34.06 ± 7.82	36.65 ± 0.54
1	48.42 ± 6.76	43.08 ± 2.99*
2	42.08 ± 2.73	56.86 ± 6.77*
4	47.24 ± 7.52	55.87 ± 2.25*
8	51.01 ± 3.82	48.4 ± 6.86*
12	38.97 ± 0.40	57.42 ± 7.11*

(*) means significant difference between percentage of exopolymer area in relation to 0 h according to Student's t-test ($p = 0.05$)



Online Resource 6 Fluorescence microscopy of *K. marxianus* CCT 7735. The slides photography of yeast cells collected at (a) 0 h, (b) 12 h under non-stress condition and (c) 12 h under 2-PE stress was taken with a M5000 EVOS fluorescence microscope (Thermo Fisher, China). Exopolysaccharide was detected when FITC conjugated lectin appear fluorescent green color, (d) FITC conjugated lectin fluorescence intensity. Propidium iodide were used to stain nucleic acids of cells (red). The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

Online Resource 7 FAME's profile of *K. marxianus* CCT 7735 under (a) non-stress and (b) 2-PE stress conditions. The stress condition was established by adding 3.0 g/L of 2-PE to exponentially growing yeast batch cultures

(a)

	Fatty acid /Time (h)	0	1	2	4	8	12
Saturated	12:0	0.3 ± 0.00	0.27 ± 0.13	0.33 ± 0.01	0.31 ± 0.06	0.33 ± 0.10	0.35 ± 0.11
	14:0	1.10 ± 0.37	0.85 ± 0.32	0.78 ± 0.11	0.79 ± 0.17	0.94 ± 0.21	1.10 ± 0.26
	16:0	13.55 ± 0.14	14.68 ± 0.96	13.38 ± 0.66	13.81 ± 0.06	14.55 ± 0.01	13.54 ± 0.78
	17:0	-	-	-	-	-	-
	18:0	1.89 ± 0.50	1.71 ± 0.47	1.12 ± 0.30	1.33 ± 0.41	0.8 ± 0.12	0.86 ± 0.28
	20:0	0.42 ± 0.04	0.43 ± 0.06	0.56 ± 0.24	0.52 ± 0.15	0.48 ± 0.28	0.73 ± 0.54
Unsaturated	16:1n-7 or 16:1n-6	17.96 ± 0.37	17.68 ± 0.41	16.45 ± 0.57	18.36 ± 1.56	24.48 ± 0.81	29.43 ± 1.68
	17:1n-8	-	-	-	0.21 ± 0.10	0.29 ± 0.18	-
	18:1n-6 or 18:1n-7	20.01 ± 0.76	15.61 ± 4.08	22.52 ± 3.10	18.38 ± 1.48	10.04 ± 1.44	9.74 ± 0.40
	18:1n-9	18.20 ± 0.88	17.55 ± 2.96	26.48 ± 3.34	16.06 ± 0.94	14.64 ± 1.03	21.82 ± 1.78
	18:2n-6 or 18:0 ante	25.15 ± 2.28	26.33 ± 1.14	27.19 ± 1.46	25.66 ± 1.10	28.71 ± 1.10	27.83 ± 0.62
	20:1n-9	-	-	-	-	-	-
	20:2n-6	0.58 ± 0.06	0.54 ± 0.08	0.72 ± 0.31	0.89 ± 0.18	0.65 ± 0.12	1.32 ± 1.04
Total	saturated	17.25 ± 2.74	17.94 ± 1.68	16.16 ± 1.69	16.7 ± 0.11	17.10 ± 0.72	16.57 ± 1.01
	monounsaturated	56.17 ± 0.70	50.84 ± 0.78	65.44 ± 9.46	53.01 ± 2.60	49.44 ± 3.21	60.98 ± 1.94
	polyunsaturated	25.72 ± 3.42	26.87 ± 0.92	27.90 ± 1.58	26.54 ± 2.96	29.36 ± 2.38	29.14 ± 0.78

(b)

	Fatty acid /Time (h)	0	1	2	4	8	12
Saturated	12:0	0.26 ± 0.08	0.20 ± 0.02	0.15 ± 0.02	0.14 ± 0.04	0.17 ± 0.06	0.15 ± 0.01
	14:0	0.77 ± 0.05	0.62 ± 0.05	0.56 ± 0.07	0.49 ± 0.05	0.49 ± 0.03	0.56 ± 0.01
	16:0	14.35 ± 0.37	13.90 ± 0.30	15.44 ± 0.46	15.25 ± 0.25	14.31 ± 0.36	13.69 ± 0.02
	17:0	-	-	-	0.13 ± 0.04	0.21 ± 0.05	0.23 ± 0.07
	18:0	1.73 ± 0.17	1.58 ± 0.33	1.66 ± 0.32	1.52 ± 0.23	1.50 ± 0.30	1.43 ± 0.17
	20:0	0.38 ± 0.05	0.52 ± 0.13	0.42 ± 0.05	0.37 ± 0.02	0.46 ± 0.02	0.50 ± 0.01
Unsaturated	16:1n-7 or 16:1n-6	16.48 ± 0.30	14.64 ± 0.14	12.04 ± 0.30	12.50 ± 0.88	11.11 ± 0.82	12.51 ± 0.57
	17:1n-8	-	0.19 ± 0.05	0.21 ± 0.01	0.36 ± 0.11	0.54 ± 0.21	0.65 ± 0.26
	18:1n-6 or 18:1n-7	20.95 ±1.39	15.88 ± 1.29	14.06 ± 0.65	10.61 ± 0.13	7.93 ± 0.57	7.49 ± 0.75
	18:1n-9	19.40 ± 0.04	20.82 ± 1.30	23.58 ± 0.65	25.86 ± 2.14	28.69 ± 2.79	28.12 ± 0.66
	18:2n-6 or 18:0 ante	27.99 ± 0.74	31.32 ± 0.74	30.51 ± 0.29	31.26 ± 0.19	31.09 ± 0.96	33.92 ± 0.56
	20:1n-9	-	-	-	0.19 ± 0.10	0.25 ± 0.01	0.19 ± 0.05
	20:2n-6	0.46 ± 0.13	0.54 ± 0.16	0.483 ± 0.11	0.38 ± 0.02	0.47 ± 0.05	0.57 ± 0.05
Total	saturated	17.48 ± 0.78	16.81 ± 0.78	18.22 ± 0.35	17.89 ± 0.04	17.13 ± 0.35	16.56 ± 0.35
	monounsaturated	56.83 ± 2.57	51.53 ± 1.47	49.89 ± 0.30	49.52 ± 2.88	48.51 ± 2.98	48.96 ± 0.37
	polyunsaturated	28.45 ± 1.21	31.86 ± 0.67	30.99 ± 0.88	31.63 ± 2.75	31.56 ± 2.55	34.49 ± 0.49

GENERAL CONCLUSION

- The *K. marxianus* CCT7735 growth, glucose uptake, fermentative metabolism, membrane permeability and cell viability were impaired by 2-PE stress.
- *K. marxianus* exposed to the 2-PE displayed changes in membrane fatty-acids profile, ergosterol content, exopolymer production and reduction of the ROS level, which are likely adaptive responses.
- Our work provided insights that can be used for improving the tolerance to 2-PE in *K. marxianus*, which in turn its production.