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Membrane stress caused by short chain fatty acids in *Saccharomyces cerevisiae*

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Abstract

Renewable biomass-based processes are expected to contribute substantially to the future supply of fuels, chemicals and materials due to the prospects of environmental friendliness and reducing the use of petrochemicals. The use of microorganisms to convert lignocellulosic biomass into desired products is a promising opportunity. The diverse nature of the biomass feedstocks, together with the biodiversity of microorganisms, has the potential to lead to the production of a wide array of molecules of industrial interest. Nonetheless, many microbial production processes fail to reach the titers, yield and productivity required for process economy due to cellular inhibition. Inhibitory compounds released from the feedstock pretreatment or produced during fermentation can negatively interfere with the microbial cell factory metabolism and compromise the overall fermentation process. Organic acids often represent a major hurdle in industrial bio-based microbial processes. They can be released from lignocellulosic feedstocks pretreatment, inhibiting the fermentative processes, and, at the same time, can be the desirable products obtained by microbial fermentation with applications in different industrial sectors. However, when high titers are reached the product itself becomes an additional stress factor for the cells.

The aim of the research described in this thesis was to evaluate the possibility of modifying the membrane lipid composition of *Saccharomyces cerevisiae* to obtain mutants with increased organic acid tolerance and also with improved organic acid production capacity. We focused on ergosterol, the major sterol of yeast plasma membrane. Ergosterol is an essential component of the plasma membrane and contributes to membrane integrity, fluidity and permeability, as well as to the sorting of lipid rafts,

which are sterols and sphingolipids-enriched clusters that house several membrane proteins, among which the proton pump H^+-ATPase Pma1. By combining *in vitro* and *in vivo* complementary assays, we demonstrated that modulation of ergosterol content is crucial for *S. cerevisiae* to gain tolerance towards organic acids. In the *in vitro* assay, we prepared synthetic lipid vesicles, from commercially available yeast lipids, enriched, or not, with different amounts of ergosterol. Liposomes were filled with a pH-responsive fluorescent dye, allowing to monitor pH and pH changes inside the liposomes upon exposure to organic acids. This assay, indirectly allowed to investigate the diffusion of organic acids through the liposomal membranes, independent of yeast metabolism. We observed changes in organic acids diffusion through the membrane as a function of ergosterol content. Following the positive indications observed, we extended our approach to *in vivo* using *S. cerevisiae*. Given the ability of transcription factors to regulate multiple genes, they can be recruited to modulate entire metabolic pathways, as in the case of ergosterol biosynthesis in *S. cerevisiae*. We focused on the transcription factor *ECM22*, acting as the main regulator of ergosterol biosynthesis in *S. cerevisiae*. The overexpression of *ECM22* increased the ergosterol content of *S. cerevisiae* which, in turn, revealed to be crucial for increased yeast tolerance towards lactic acid stress. Importantly, we observed that ergosterol's effect on yeast resistance towards organic acid stress changes depending on the organic acid.

The plasma membrane lipid composition is equally crucial when organic acids are being produced by *S. cerevisiae*, both for the proper export of the acids and also to avoid their re-entrance in the cell. In an attempt to increase lactic acid production of two *S. cerevisiae* lactic acid

homofermentative production strains, the transcription factor *ECM22* was overexpressed in these strains. Phenotypic characterization of the obtained engineered strains revealed that *ECM22* overexpression resulted in increased tolerance to lactic acid stress. However, the production of lactic acid was unaffected, both in terms of titer and yield. Despite these findings, this research revealed that there is still much to learn about the role of plasma membrane composition in the production of organic acids, specifically lactic acid.

Riassunto

I processi basati sulla biomassa rinnovabile potranno contribuire alla futura richiesta di combustibili, prodotti chimici e materiali date le prospettive di compatibilità ambientale e la riduzione dell'uso di prodotti petrolchimici. L'uso di microrganismi per convertire la biomassa lignocellulosica nei prodotti desiderati è un'opportunità promettente. La combinazione della diversa natura delle biomasse di scarto, insieme alla biodiversità dei microrganismi, rappresentano un'ottimo punto di partenza per la produzione di un'ampia gamma di molecole di interesse industriale. Tuttavia, molti processi di produzione microbica non riescono a soddisfare i requisiti dei processi industriali a causa dell'inibizione della crescita cellulare. Composti inibitori, come gli acidi organici, rilasciati dal pretrattamento della materia prima, come le biomasse lignocellulosiche, oppure prodotti durante la fermentazione interferiscono negativamente con il metabolismo dei microrganismi, compromettendo il processo fermentativo. Lo scopo della ricerca descritta e´ quello di valutare la possibilità di modificare la composizione lipidica della membrana di *Saccharomyces cerevisiae* per ottenere mutanti con una maggiore tolleranza agli acidi organici ed anche una migliore capacità di produzione di questi ultimi. Ci siamo concentrati sull'ergosterolo, un componente essenziale della membrana che contribuisce alla sua integrità, fluidità e permeabilità, nonché allo smistamento dei *rafts* lipidici, dei *cluster* arricchiti di steroli e sfingolipidi che ospitano diverse proteine di membrana, tra cui la pompa protonica ATPasica (Pma1). Dalla combinazione di saggi complementari *in vitro* e *in vivo*, abbiamo visto che la modulazione del contenuto di ergosterolo è fondamentale in *S. cerevisiae* per ottenere una maggiore tolleranza verso gli acidi organici. Nel saggio *in vitro*, abbiamo

preparato vescicole lipidiche sintetiche contenenti diverse quantità di ergosterolo. I liposomi sono stati riempiti con un colorante fluorescente sensibile al pH, che consente di monitorarne i cambiamenti all'interno dei liposomi dopo l'esposizione agli acidi organici. Questo saggio, indirettamente, permette di indagare la diffusione di acidi organici attraverso le membrane liposomiali, indipendentemente dal metabolismo del lievito. Abbiamo osservato cambiamenti nella diffusione degli acidi organici attraverso la membrana, in funzione del contenuto di ergosterolo. Successivamente, abbiamo esteso il nostro approccio *in vivo* utilizzando *S. cerevisiae*. I fattori di trascrizione possono essere reclutati per modulare intere vie metaboliche, come nel caso della biosintesi dell'ergosterolo in *S. cerevisiae*. Ci siamo concentrati sul fattore di trascrizione *ECM*22, che funge da principale regolatore della biosintesi dell'ergosterolo. L'overespressione di *ECM*22 ha aumentato il contenuto di ergosterolo in *S. cerevisiae*, conducendo maggiore tolleranza allo stress da acido lattico. La composizione lipidica della membrana plasmatica influenza anche la produzione di acidi organici in *S. cerevisiae*. Il fattore di trascrizione *ECM*22 è stato quindi overespresso in due differenti ceppi di *S. cerevisiae* che producono acido lattico. La caratterizzazione fenotipica dei ceppi ottenuti ha rivelato che l'overespressione di *ECM*22 ha portato ad una maggiore tolleranza allo stress da acido lattico. Tuttavia, la produzione di acido lattico non è stata influenzata. Nonostante questi risultati, tale studio ha rivelato che c'è ancora molto da imparare sul ruolo della composizione della membrana plasmatica nella produzione di acidi organici.

Introduction

Circular economy, Biorefineries and renewable biomass

On the 11th of March 2020, the world health organization declared Covid-19 a global pandemic. In just a few months, the Covid-19 pandemic swept across the world restricting the movement of millions of people, impacting lives and jobs, disrupting international supply chains, and negatively impacting global economies. Covid-19 has highlighted the environmental unsustainability of 'take, make, dispose' linear economical model of materials and energy flows (Meyer, Walter, & Seuring, 2021). With this economic model, it is estimated that around 90% of the raw materials used in manufacturing become waste before the final product leaves the production plant and around 80% of products manufactured are disposed of within the first 6 months of their life (Martins & Castro, 2020). This unlimited use of natural resources with no concern for sustainability jeopardizes the future of the planet's resource supply. Circular economy emerges as a valid alternative to the current economic model by adopting a strategy of closed-loop value chains, where renewable biomass is transformed into value added products (**Figure 1**). Biomass means the biodegradable fraction of products, waste and residues from biological origin from agriculture, including vegetal and animal substances, from forestry and related industries, including fisheries and aquaculture, as well as the biodegradable fraction of waste, including industrial and municipal waste of biological origin (European Union, 2018).

In order to maximize the value of biomass feedstocks, the concept of biorefineries has emerged. Biorefineries are facilities that optimize the sustainable production of food, feed, materials, chemicals, fuels, power and heat from renewable biomass (Ree & Zeeland, 2014). Therefore, biorefineries represent an ideal alternative to petroleum-based refineries,

reducing the dependence on fossil-fuel resources and contributing to the decarbonization and sustainable growth of the society and are considered one of the key technologies in a circular bioeconomy context. Nonetheless, achieving responsible innovation is at the same time crucial and challenging, and can only be effectively faced by merging diverse expertise and visions (Lange et al., 2021).

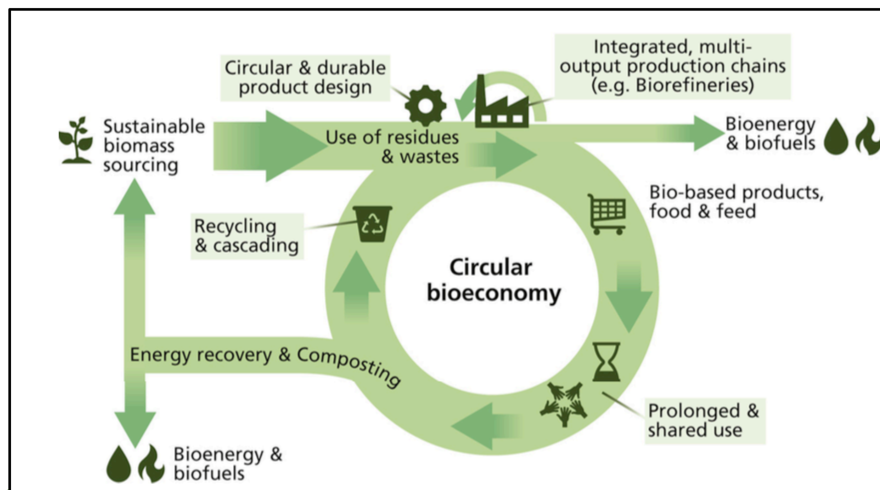

Figure 1. The circular bioeconomy and its elements, from Stegmann, Londo & Junginger (2020).

Among the diverse renewable feedstocks, lignocellulosic biomass (LCB) is one of the most attractive and studied, due to its large abundance and universal availability. Examples of LCB are barley straw, coconut husk, corn stover, empty fruit bunch, rice, sugarcane bagasse straw, sorghum stalks, wheat, and wood. Annually, about 1.3 billion tons of LCB are generated all around the world but only a small fraction is exploited to produce biochemical, bioenergy, and non-food related bio-products (Baruah et al., 2018). LCB relates to second-generation biomass feedstocks and presents an alternative to first-generation biomass feedstocks that compete with food crops for land (Naik, Goud, Rout, & Dalai, 2010). LCB is mainly composed of three polymers: 35-50% cellulose, 20-35% hemicellulose and

10-25% lignin together with small amounts of other components like acetyl groups, mineral and phenolic substituents (**Figure 2**) (Isikgor & Becer, 2015). Cellulose, the most abundant LCB polymer, is composed of ß-D-glucose units linked by ß-(1,4) glycosidic bonds, with cellobiose as the fundamental repeating dimeric unit. Around 500-1400 glucose molecules compose the cellulose chains forming the microfibrils that are embedded in the lignocellulosic matrix. Hemicellulose is the second most abundant polymer. Unlike cellulose, hemicellulose has a random and amorphous structure, and is composed of several heteropolymers including xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan. The heteropolymers of hemicellulose are composed of different 5 and 6 carbon monosaccharide units: pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. Hemicelluloses are embedded in the plant cell walls to form a complex network of bonds that provide structural strength by linking cellulose fibers into microfibrils and cross-linking with lignin. Finally, lignin is a three-dimensional polymer of phenylpropanoid units. It functions as the cellular glue which provides compressive strength to the plant tissue and the individual fibers, stiffness to the cell wall and resistance against insects and pathogens. Its high polyphenolic content makes it the ideal natural source of aromatic compounds and (poly-)phenols (Ma et al., 2019). These three polymers, cellulose, hemicellulose and lignin, are interlinked resulting in a complex and rigid three-dimensional structure that hinders its industrial utilization. Therefore, the lignocellulose conversion into value-added products requires multistep processing including chemical, physical, or biological pretreatments to access the desired fraction, followed sometimes by an enzymatic or chemical depolymerization to release the

monomers that will then be converted into the product of interest (Romaní, Rocha, Michelin, Domingues, & Teixeira, 2020).

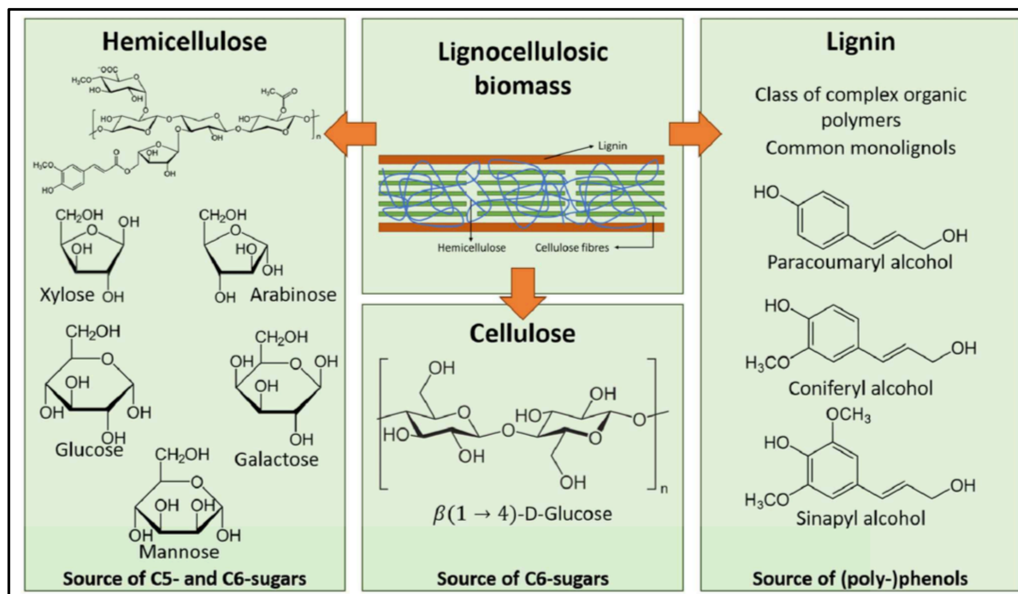

Figure 2. Composition of lignocellulosic biomass, from Isikgor & Becer (2015).

Depending on the feedstock and pretreatment process, different hydrolysates can be obtained. Therefore, the pretreatment step is one of the most important steps in the entire process as both the efficiency of the conversion and downstream processing steps depend on it (Bertacchi, Jayaprakash, Morrissey, & Branduardi, 2022).

Among the diverse methods of biomass valorization, microbial fermentations offer many possibilities for obtaining the desired products. The diverse nature of the biomass feedstocks, together with the biodiversity of microorganisms, has the potential to lead to the production of a diverse array molecules of industrial interest (Branduardi, 2021).

Microbial cell-factories

Microorganisms have successfully colonized almost every niche on Earth. Their survival and reproduction depends on their ability to produce appropriate responses to the environmental conditions in which they live (Lund et al., 2020). Therefore, the physiological diversity of microorganisms, isolated from the most different habitats, results in a fascinating capacity to utilize a variety of substrates and produce a diverse array of value added compounds (Pham et al., 2019). The use of microorganisms in traditional fermented products such as bread, cheese, wine, and beer is traditionally marked as the first step of biotechnology, dating back several millennia (Sicard & Legras, 2011). More recently, with advances in genomics, metabolic engineering, systems and synthetic biology, microorganisms are being used for the production of biopharmaceuticals (hormones, enzymes, antibiotics, vitamins), polymers, monomers, high-value chemicals and other bioproducts (Adegboye, Ojuederie, Talia, & Babalola, 2021). By unlocking the potential of microorganisms into industrial processes, we might turn worldwide problems (waste, pollution and lack of resources) into solutions (products, materials, chemicals).

Nonetheless, the commercialization of important compounds produced by microbial fermentation is still a challenge. This is because there is still a large difference in crucial performance metrics of titer (g/L product), yield (g product per g substrate), and productivity (g/L h^{-1}) obtained in the research laboratories and the requirement for an economically attractive bioprocess for industrial production, making it difficult to attract financing for further development (Fletcher, Krivoruchko, & Nielsen, 2016; Van Dien, 2013).

The economic feasibility of microbial bio-based industrial processes is based on three major points: the renewable carbon source (substrate costs), the bioconversion process (titer, yield and productivity), and the purification of the product (downstream processing costs). Microorganisms, performing as cell factories, are the central player of these processes and therefore, choosing the ideal microbial host for specific bio-processes is of major importance (Sauer & Mattanovich, 2012). Fletcher and co-workers (2016) summarized the key characteristics of an ideal microbial cell factory: 1) easy to engineer, 2) tolerant to industrial stresses, 3) capable of expressing complex heterologous metabolic pathways and 4) efficient producer (high metrics on titer, yield and productivity).

Yeasts have been the elected microorganism in many industrial applications, not only because they have a long history of application within the brewing, wine and bread industry, but also because they fit the characteristics mentioned above quite well and yeast cannot be contaminated by phages unlike bacteria (Kavšček, Stražar, Curk, Natter, & Petrovič, 2015; Seo, Park, Jung, Ryu, & Kim, 2020). Traditionally, “yeast” denotes *Saccharomyces cerevisiae* and its close relatives, however, more than 1500 yeast species have been identified. Non-*Saccharomyces* yeasts are usually denoted as “non-conventional” yeasts, *Pichia pastoris* (syn. *Komagataella phaffii*), *Hansenula polymorpha* (syn. *Ogataea parapolymorpha*), *Yarrowia lipolytica*, *Pichia stipitis* (syn. *Scheffersomyces stipitis*), or *Kluyveromyces marxianus* are examples of yeast species with major interest in biotechnology (Mattanovich, Sauer, & Gasser, 2014).

Nonetheless, *S. cerevisiae* is one of the most used workhorses in many biological processes with successful applications in the production of both bulk and fine chemicals (**Figure 3**) (Parapouli, Vasileiadis, Afendra, &

Hatziloukas, 2020). The sequencing of the complete genome of *S. cerevisiae* has allowed the development of an extensive source of genetic engineering toolboxes for this yeast (Nevoigt, 2008). Additionally, *S. cerevisiae* is very robust in large-scale fermentations, is very osmo-tolerant, can grow on cheap media, is tolerant to low pH, thus, making it one of the best suitable cell factories for many bio-based industrial processes (Kavšček et al., 2015). *S. cerevisiae* is also generally recognized as safe (GRAS) by the American Food and Drug Administration, as it does not produce harmful toxins for humans. For all these reasons, *S. cerevisiae* was the microbial cell-factory used in the research carried out during this PhD thesis. One disadvantage of *S. cerevisiae*, from a perspective of bio-based industrial processes, is its inability to utilize alternative carbon sources abundant in the lignocellulosic biomass feedstocks, such as xylose and arabinose; however, this feature can be genetically engineered (Moysés, Reis, de Almeida, de Moraes, & Torres, 2016). Adding to this limitation, during bio-based industrial processes, microorganisms are also subjected to different kinds of stress associated with industrial process conditions, such as non-optimal temperature, inhomogeneous mixing, unfavorable pH, high osmotic pressure, oxygenation levels, presence of inhibitory compounds and final product toxicity (Wehrs et al., 2019). These stress factors might inhibit cellular metabolism and compromise the performance of the fermentative process. For this reason, large investments have been made to create efficient cell factories characterized by high rates of production and, at the same time, improved stress tolerance.

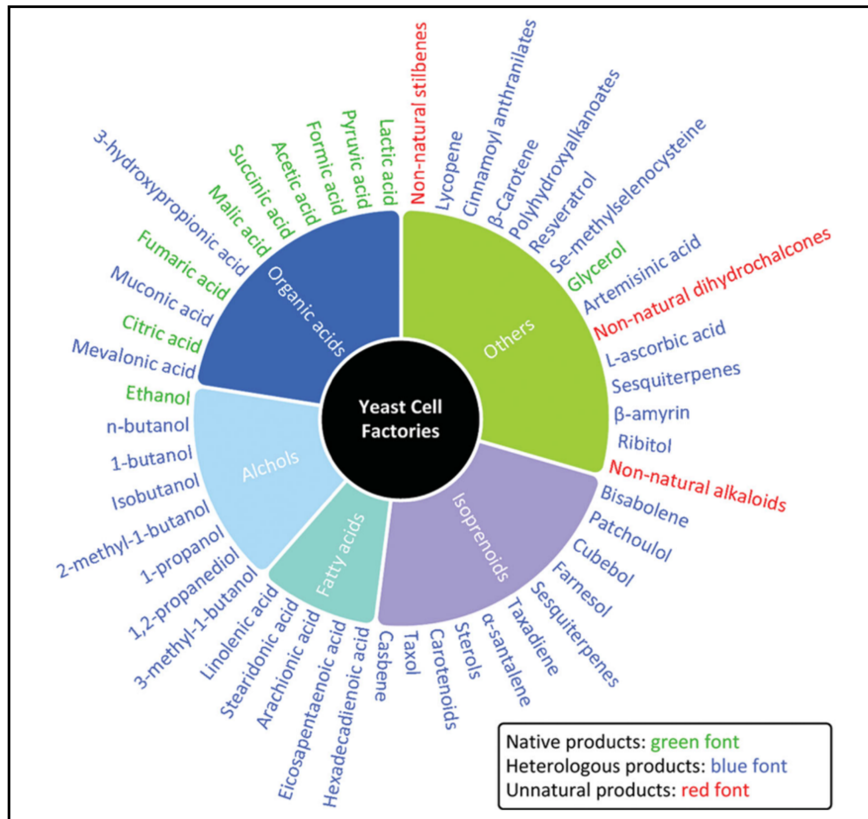

Figure 3. Variety of chemicals that can be obtained using yeast cell factories, from Li & Borodina (2015).

Hurdles of bio-based industrial processes

As mentioned before, lignocellulosic biomass is one of the most attractive and studied renewable biomass, however, it is also a challenging substrate for microbial utilization. A pretreatment step, involving harsh conditions such as high temperatures and the addition of acids/bases, is required to release the fermentable sugars from lignin-cellulose-hemicellulose complexes (**Figure 4**). This pretreatment process destroys the lignin barrier and partially converts cellulose and hemicelluloses into fermentable sugars such as glucose, xylose, arabinose galactose, and mannose. A subsequent enzymatic hydrolysis process transforms any remaining polymers into fermentable sugars. However, these processes generally lead to the formation of inhibitory by-products to the microbial cell factories. Depending on the type of pretreatment and process parameters, different inhibitory compounds may be released such as: phenolic compounds, furfural, hydroxymethylfurfural (HMF) and different organic acids such as, acetic, levulinic, ferulic, and glucuronic (Chandel, Garlapati, Singh, Antunes, & da Silva, 2018; Jönsson & Martín, 2016; Palmqvist & Hahn-Hägerdal, 2000). The amount and type of inhibitory compounds in the hydrolysates is also dependent on lignocellulosic source (Ko, Um, Park, Seo, & Kim, 2015). The presence of these inhibitory by-products in the fermentation medium can inhibit microbial activity, compromising the feasibility of the processes and thus, making these processes economically unviable (Almeida et al., 2007).

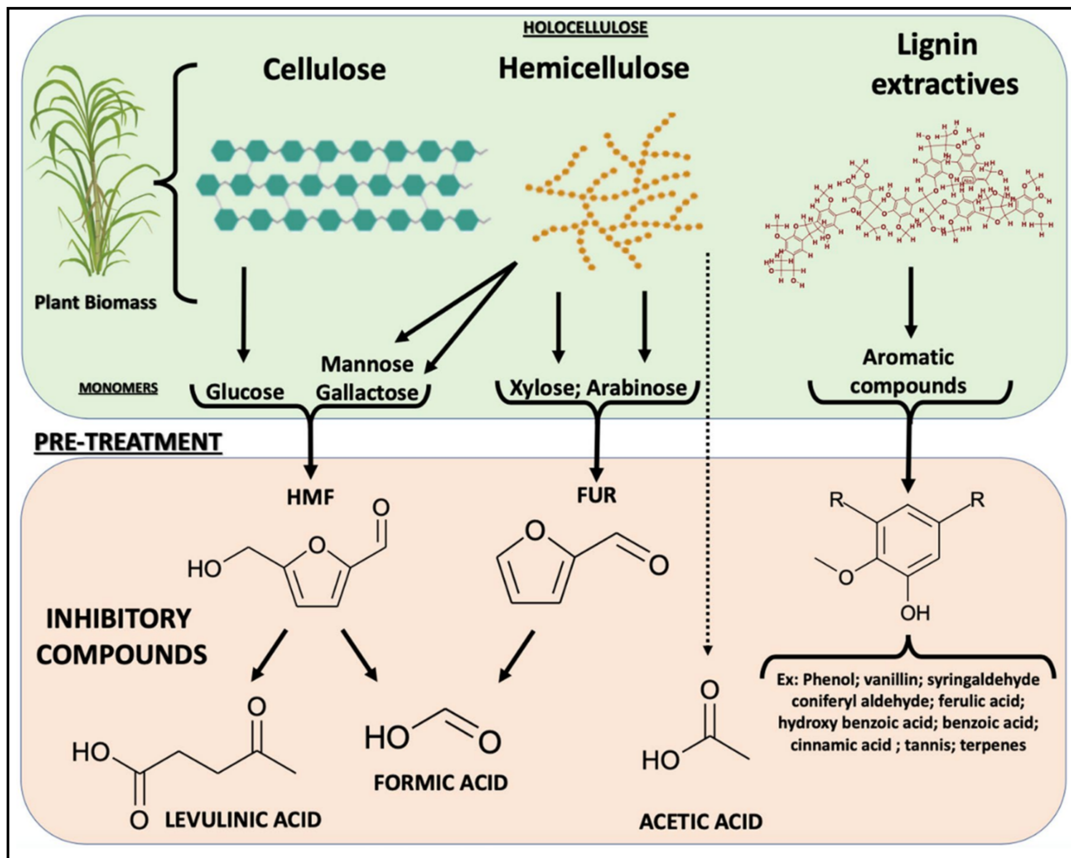

Figure 4: Lignocellulosic biomass structure and types of inhibitory by-products formed after pretreatment, from Tramontina et al. (2020).

To overcome this issue, innovation in biomass feedstock enhancement has been the subject of many studies. Using genetically modified crops to increase amenability for bio- processing or the exploration of existing plant materials to allow multiproduct production from a single crop have been considered for this purpose (Chapotin & Wolt 2007).

In this work, we were particularly interested in studying the stress caused by organic acids and on the development *S. cerevisiae* strains with increased tolerance towards this stress factor. Organic acids represent a double edge sword in bio-based processes since they can be released from the pretreated biomass, inhibiting the fermentative process but, at the same time, they can be desirable products obtained by microbial fermentation as valuable building block chemicals with many different

industrial applications (Sauer, Porro, Mattanovich, & Branduardi, 2008). Indeed, the major inhibitory compound released from the pretreatment of lignocellulosic biomass is acetic acid, which is released from acetyl groups on the hemicellulose polymer, even under relatively mild pretreatment conditions (Jönsson & Martín, 2016).

Yeasts inhibition by organic acids is exacerbated when the medium pH is below the acid’s pKa. Under this condition, the undissociated form of acid prevails, enters yeast cells by simple diffusion through the plasma membrane lipid bilayer and dissociates in the near-neutral cytosol leading to the accumulation of protons (H^+) and counter anion in the cell interior (Mira, Teixeira, & Sá-Correia, 2010). The accumulation of both species inside the cell has detrimental effects for the cell. Protons accumulation lead to intracellular acidification and to the consequent decrease of DNA and RNA synthesis, inhibition of metabolic activities and disruption of the proton gradient maintained across the plasma membrane. To maintain the intracellular pH within physiological levels, yeast cells activate the plasma membrane H^+ ATPase Pma1, that couples ATP hydrolysis to proton extrusion (Ferreira, Mason, & Slayman, 2001). On the other hand, the accumulation of the counter anion may cause an increased turgor pressure, oxidative stress, protein aggregation, lipid peroxidation, inhibiting of membrane trafficking and perturbation of plasma and vacuolar membrane spatial organization. Combined, these effects can lead to decreased cellular performance and eventually to cell death (Ludovico, Sousa, Silva, Leão, & Côrte-Real, 2001; Mira et al., 2010; Piper, 2011). Inconveniently, low pH is a preferable condition in yeast bio-based industrial processes as it is crucial to avoid unwanted bacterial contamination (Lund et al., 2020).

On top of these stress factors, the target product itself will, in most of the cases, negatively affect yeast cell metabolism. Organic acids are an essential group of platform chemicals that can be produced by microbes (Di Lorenzo, Serra, Porro, & Branduardi, 2022). Chemical synthesis from petroleum derivatives is currently the most common method of producing these chemicals. Nonetheless, some organic acids, such as succinic, lactic, citric, gluconic, and acetic acid, are already produced industrially using microbial cell factories (Alonso, Rendueles, & Díaz, 2015). Lactic acid production is one example. It is used extensively as a food additive and can also be used as a precursor in the production of biodegradable polyesters (Sauer, Porro, Mattanovich, & Branduardi, 2010). When organic acids are the product of interest, maintaining the culture pH as low as possible helps to simplify the purification of the acid considerably, and thereby lowers the production costs (Sauer et al., 2008). In the case of lactic acid, Lactic acid bacteria (LAB) are the best natural producers: the major disadvantage is that their growth decreases at pH values lower than 5, but a pH value lower than this is desirable for purification. Many industrial approaches focus, therefore, on engineered yeast strains, such as *S. cerevisiae,* which are typically more acid tolerant than LAB (Lund et al., 2020). The development of tolerant microorganisms is crucial for the sustainable production of fuels, chemicals and materials, from renewable biomasses, at high yields, titer and productivity.

Improvement of microbial traits for bio-based industrial processes

Microorganisms can be engineered to improve their tolerance to specific stress factors, to increase fermentation yields of desirable products and also to broaden substrate utilization (Adrio & Demain, 2006).

If information is available on the molecular mechanisms involved in obtaining a specific phenotype, targeted strain engineering approaches can be applied (Liu, Zhang, & Nielsen, 2019). On the other hand, if the molecular mechanisms behind a desirable phenotype are yet to be disclosed, or if a desired phenotype depends on multiple factors, random engineering approaches may be used (Dragosits & Mattanovich, 2013). If properly evaluated, random approaches also have the potential to provide physiological information on the cellular response to a specific stress factor. Evolutionary engineering, also known as adaptive laboratory evolution, is a random engineering method to evolve and then select cells towards desirable phenotypes (Dragosits & Mattanovich, 2013). Using this approach, cells are cultivated in either batch or continuous cultures for several hundred generations under increasing selective pressure in order to enrich for desired variants. This is a simple method and very often allowed to obtain improved strains, with the desired phenotypes. Nonetheless, the selective pressure and experimental conditions must be carefully designed so that other physiological traits are not lost (Hahn-Hägerdal et al., 2005). Thanks to the increased access to whole-genome sequencing, and techniques for the characterization and understanding of the molecular mechanisms behind tolerant phenotypes, in addition to obtaining improved strains, it is now possible to elucidate the mechanisms behind specific phenotypes (Caspeta et al., 2014; González-Ramos et al., 2016).

On the other hand, when information is available on the molecular mechanisms behind specific phenotypes, target engineering approaches may be applied. In this sense, over the past decades, synthetic biology has emerged as a discipline that combines biological and engineering concepts (Cheng & Lu, 2012). Synthetic Biology offers innovative approaches for engineering new biological systems or re-designing existing ones to address global health, food and energy challenges and drive industrial transformation (Shapira, Kwon, & Youtie, 2017). Synthetic biology can be used to express synthetic pathways for the production of fuels, bio pharmaceuticals, fragrances, and food flavors in diverse microbial cell factories. Nonetheless, directing fluxes through these synthetic pathways towards the desired product can be demanding due to complex regulatory processes or poor gene expression (Fletcher et al., 2016). Synthetic biology can also be used to render microbial cell factories tolerant to specific stress factors. From a perspective of lignocellulosic biomass valorization, microorganisms have been metabolic engineered in order to withstand lignocellulosic process–derived stress factors (Cunha, Romaní, Costa, Sá-Correia, & Domingues, 2019).

Last but not least, microbial diversity can also be used for purposes other than natural evolution, such as designing synthetic microbial communities that meet industrial needs. This reaffirms the idea that making the transition to sustainable bio-based production requires a nature-inspired design (Branduardi, 2021).

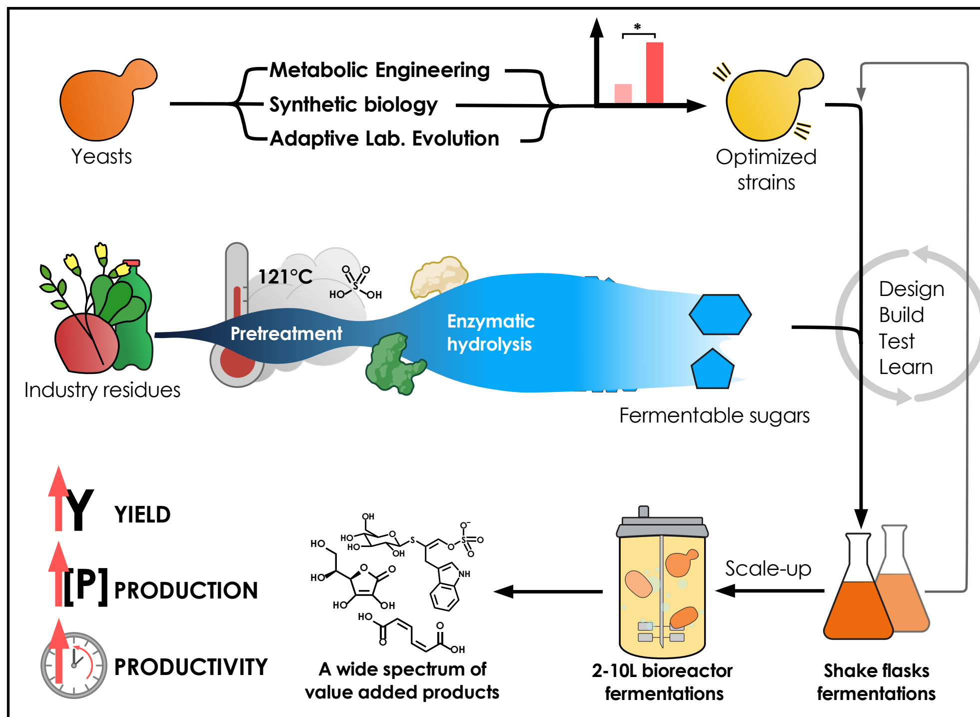

Figure 5: Schematic representation of bio-based microbial processes, involving the tailoring microbial cell factories for the production of value-added products by valorizing industrial residues, from https://indbiotechlab.btbs.unimib.it/.

As previously mentioned, we were interested in studying stress caused by organic acids. So far, strategies to improve yeast tolerance to organic acid stress have focused on reducing the damage they cause inside the cell. Some of these strategies focused on counteracting the decrease in intracellular pH, removing the inhibitory molecules from the cell by efflux or consumption, by the conversion into less toxic compounds or by increasing the production of protective metabolites (Ding et al., 2015; Grant, 2001; Rodrigues et al., 2012; Wei, Quarterman, Kim, Cate, & Jin, 2013). Differently, improved tolerance to organic acid stress can be targeted by preventing the damage from occurring. In the case of organic acid stress, this might be achieved by preventing its entry into the cell or reducing the diffusion rate of the acids. In this regard, the plasma membrane plays a key role since it is a physical barrier that separates the

extracellular environment and intracellular components, controls the exchange of compounds into and out of the cell, is responsible for maintaining the correct ion homeostasis and is the sensor of the overall cellular environment, rearranging its composition in response to different stimuli (Stewart, 2017). A complete description of the yeast plasma membrane structure is provided in Chapter 1, "*The Plasma Membrane at the Cornerstone Between Flexibility and Adaptability: Implications for Saccharomyces cerevisiae as a Cell Factory* ".

Different strategies can be adopted to modify the plasma membrane. The biodiversity of microorganisms present in nature, possessing desirable phenotypes according to the environmental conditions in which they can be found, may be used as inspiration and serve as guidelines for potential membrane engineering strategies. Naturally tolerant organisms can be studied to identify novel lipid species with interesting properties that are not present in the targeted microbial cell factory. In this case, membrane engineering will focus on introducing the new biosynthetic pathways for this heterologous lipid species. Differently, a tolerant organism can also be studied to identify changes in membrane lipid abundance, in this case, the intrinsic lipid biosynthetic pathways can be engineered to obtain the desirable phenotype. Nevertheless, it is difficult to predict which membrane composition will lead to a specific emerging property.

Differently, when microorganisms are exploited for the production of molecules of interest, the export of the product into the medium is preferable, mainly for limiting the downstream processing costs. Researchers have been focusing on maximizing the export of the compounds of interest by overexpressing specific membrane transporters, engineering transporters for improved efficiency or even introducing

heterologous transporters in the cell factory (Boyarskiy & Tullman-Ercek, 2015; Erian, Egermeier, Rassinger, Marx, & Sauer, 2020; Kell, Swainston, Pir, & Oliver, 2015; Soares-Silva et al., 2020). However, many times, the simple overexpression of a transporter does not result in the intended increased export rates. This is because, membrane lipid composition is crucial for the proper display and functioning of membrane transporters. Furthermore, the plasma membrane lipid composition also determines physiochemical properties such as fluidity, permeability and elasticity, which can facilitate, or not, the export of products of interest (Royce et al., 2015).

Overall, despite its importance, the yeast plasma membrane has been subjected to surprisingly few studies over the last years. Plasma membrane engineering is still a highly complex approach as membrane lipids and membrane homeostasis are vital for many cellular functions, making it difficult to predict the outcomes of altering membrane elements in the whole membrane system. Furthermore, the analysis of the plasma membrane composition is still challenging, which hampers a detailed association between specific composition and physicochemical properties of the plasma membrane. In this work, we also investigated if changes in the ergosterol composition of *S. cerevisiae* could influence the producing levels of lactic acid in engineered strains.

Thesis scope and outline

The aim of this work was to develop *S. cerevisiae* strains engineered in the plasma membrane composition to manifest increased tolerance towards organic acids, either as external stress factors or as products of interest formed by yeast.

In the first chapter of this work, "*The Plasma Membrane at the Cornerstone Between Flexibility and Adaptability: Implications for Saccharomyces cerevisiae as a Cell Factory* ", we reviewed *S. cerevisiae* plasma membrane composition, namely lipids and proteins, as well as, complementary research techniques that can be used to study the plasma membrane. Furthermore, we looked into different membrane engineering strategies applied to *S. cerevisiae* to enhance its fitness under industrially relevant conditions as well as strategies to increase microbial production of the metabolites of interest.

In the second chapter, "*The impact of ergosterol content on Saccharomyces cerevisiae tolerance towards acetic and lactic acid stress*", we investigated the impact of ergosterol on the *S. cerevisiae* tolerance against organic acid stress by coupling complementary *in vitro* and *in vivo* assays. Ergosterol, the major yeast sterol, is essential for the correct structure of the plasma membrane, modulating its thickness, fluidity and permeability. Ergosterol also houses several membrane proteins, among which is the proton pump H^+-ATPase Pma1. Additionally, sterols increase membrane rigidity by ordering the fatty acyl chains, allowing a tighter lipid packing. Therefore, a more rigid and less permeable membrane is likely to reduce the diffusion of acids into the cell and, likely to increase resistance towards the acids. In the *in vitro* assay, synthetic lipid vesicles, containing different concentrations of ergosterol, were prepared. We observed changes in

organic acids diffusion through the membrane as a function of ergosterol content. Then, we extended our approach *in vivo*, engineering *S. cerevisiae* to change the ergosterol content of the plasma membrane. We focused on *ECM22*, an important transcription-factor, involved in the regulation of ergosterol biosynthesis. The overexpression of *ECM22* was sufficient to increase ergosterol levels in *S. cerevisiae*, resulting in an enhanced tolerance towards organic acid stress. This complementary approach allowed a deeper understanding of the ergosterol impact on acid tolerance. The *in vitro* assay provided indications of the structural role of ergosterol, independently of yeast metabolism, whereas, in the *in vivo* assay, yeast metabolism can balance the increased ergosterol levels caused by *ECM22* overexpression by multiple mechanisms.

Finally, in the third and final chapter, “*Effect of ECM22 overexpression on Saccharomyces cerevisiae lactic acid production strains*”, with the goal of increasing lactic acid tolerance and consequently the lactic acid production capacity of two engineered homofermentative strains, we investigated the effect of *ECM22* overexpression. Phenotypic characterization of the obtained engineered strains revealed increased tolerance to lactic acid stress upon *ECM22* overexpression, but this did not result in higher lactic acid titer or yield during the fermentation process of production.

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The plasma membrane at the cornerstone between flexibility and adaptability: implications for *Saccharomyces cerevisiae* as a cell factory

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Abstract

In the last decade, microbial-based biotechnological processes are paving the way towards sustainability as they implemented the use of renewable feedstocks. Nonetheless, the viability and competitiveness of these processes are often limited due to harsh conditions such as: the presence of feedstock-derived inhibitors including weak acids, non-uniform nature of the substrates, osmotic pressure, high temperature, extreme pH. These factors are detrimental for microbial cell factories as a whole, but more specifically the impact on the cell's membrane is often overlooked. The plasma membrane is a complex system involved in major biological processes, including establishing and maintaining transmembrane gradients, controlling uptake and secretion, intercellular and intracellular communication, cell to cell recognition and cell's physical protection. Therefore, when designing strategies for the development of versatile, robust and efficient cell factories ready to tackle the harshness of industrial processes while delivering high values of yield, titer and productivity, the plasma membrane has to be considered. Plasma membrane composition comprises diverse macromolecules and it is not constant, as cells adapt it according to the surrounding environment. Remarkably, membrane-specific traits are emerging properties of the system and therefore it is not trivial to predict which membrane composition is advantageous under certain conditions. This review includes an overview of membrane engineering strategies applied to *Saccharomyces cerevisiae* to enhance its fitness under industrially relevant conditions as well as strategies to increase microbial production of the metabolites of interest.

Introduction

The urge for the production of goods and services for a growing population, adopting the principles of linear economy, has led to massive consumption of natural resources, resulting in an unbalance between the request and the supply of these resources. Biorefineries are intended to address this urgency by utilizing and converting residual and renewable biomasses into a spectrum of marketable products such as biofuels, materials, chemicals, feed and food (Branduardi, 2021). Among the diverse methods of valorization of biomass, microbial fermentations offer many possibilities for obtaining the desired products. The diverse nature of the feedstocks, together with the biodiversity of microorganisms, has the potential to lead to the production of many classes of products (Hong & Nielsen, 2012). Yeasts are among the most prominent microorganisms used in industrial biotechnology because they unify the advantage of unicellular organisms with eukaryotic nature. *Saccharomyces cerevisiae*, in particular, plays a major role, mainly thanks to its ancient history of domestication by humans (Branduardi & Porro, 2012). Moreover, microbial biotechnology applications have increased in the last decades, with the constant evolution of genomics, metabolic engineering, systems and synthetic biology. This has enabled the production of numerous valuable products of primary and secondary metabolism, enzymes and biopharmaceutical proteins, which are of high demand in various industrial sectors. Once more, *S. cerevisiae* is still scoring positive returns as a cell factory (Li & Borodina, 2015).

Nonetheless, harsh industrial conditions put microbial cell factories in very stressful environments. The development of tolerant strains, able to handle the requirements of industrial processes is therefore highly desirable. During industrial processes, microorganisms can be subjected to many and

different kinds of stresses: high metabolite concentration, substrate variety, high osmotic pressure and ion toxicity, high temperature, extreme pH, high concentrations of weak acids, among others that compromise cell metabolism (Deparis, Claes, Foulquié-Moreno & Thevelein, 2017). In this regard, the plasma membrane plays a key role since it is a physical barrier that separates the extracellular environment and intracellular components, is responsible for maintaining the correct ion homeostasis and is the sensor of the overall cellular environment, rearranging its composition in response to different stimuli (Stewart, 2017). Moreover, increased production of metabolites of interest by the microbial cell factory also puts augmented pressure on the plasma membrane. It is therefore evident that the plasma membrane is crucial for the successful development of many bioprocesses (Jezierska & Van Bogaert, 2017).

Thus, the plasma membrane has to be considered when designing strategies for the development of versatile, robust and efficient cell factories ready to tackle the harshness of industrial processes while delivering high yield, titer and productivity. In this sense, the concept of membrane engineering has emerged. The plasma membrane is a complex and dynamic system, whose behavior is challenging to predict due to the connections, competitions, dependencies, or other types of interactions between its components. Furthermore, the behavior of a system is also influenced by its surrounding environment. This is precisely what happens at the membrane level, where lipids and proteins interact and influence each other. For this reason, it is not trivial to predict which element(s) should be changed to trigger a specific rewiring of the overall system.

When employing membrane engineering strategies, researchers have mainly been focusing on alterations in single elements of the plasma

membrane rather than on the membrane as a whole system. Changes in single elements might trigger an overall response of the membrane resulting in a global reshaping of the system. On the other hand, being a highly interconnected network, the plasma membrane might not be affected by single modifications as it can counteract minor alterations by different regulation mechanisms (Sandoval & Papoutsakis, 2016; Jezierska & Van Bogaert, 2017).

This review aims to describe the most recent efforts to engineer the plasma membrane of microbial cell factories, with particular emphasis on *S. cerevisiae*, to increase its fitness and performance in biotechnological processes.

Yeast Plasma Membrane

All cells are surrounded by a plasma membrane that defines the boundary between the cell itself and the environment. In *S. cerevisiae*, the cell envelope alone (plasma membrane and cell wall) occupies about 15% of the total cell volume (Stillwell, 2016).

In bacteria and eukaryotic cells, the plasma membrane is structured as a bilayer mainly composed of a mixture of (phospho)lipids and proteins which, by their interactions, govern the structure of the membrane and determine its physicochemical properties.

Lipids represent around 50% of the membrane composition. The major lipid classes present in the *S. cerevisiae* membrane are glycerophospholipids (about 70%), sphingolipids (about 15%) and sterols (about 15%) (**Table 1**) (Klose et al., 2012). Glycerophospholipids have a glycerol backbone connected to two fatty acyl chains by an ester-linkage and can be further divided according to their head group, attached to glycerol through phosphate, into: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidic acid (PA) (Klug & Daum, 2014). The fatty acyl chains of glycerophospholipids are usually C_{16}-C_{18}. Sphingolipids contain a sphingoid backbone connected by an amide link to a fatty acyl chain and are classified by their head group into inositol phosphoryl ceramide (IPC), mannosyl-inositol phosphoryl ceramide (MIPC) and mannosyl-di-inositol phosphoryl ceramide (MIP_2C). Sphingolipids have long-chain bases (LCB) originating from C_{16}-C_{18} fatty acids combined with very-long-chain fatty acid (VLCFA), usually C_{24}-C_{26}. Sphingolipids acyl chains are completely saturated, differently, the acyl chains of glycerophospholipids can be unsaturated (Megyeri et al., 2016). Saturated fatty acyl chains increase lipid packing and

membrane thickness due to their straight conformation, in comparison to unsaturated fatty acyl chains, which have a bent conformation. Furthermore, long fatty acyl chains increase membrane rigidity by increasing the membrane thickness and lipid packaging (Slotte, 2016). Ergosterol is the main sterol in yeast: its general structure comprises a hydroxyl head group, an alkenyl side chain and a four-ring nucleus (Klug & Daum, 2014). Sterols increase membrane rigidity by ordering fatty acyl chains allowing tighter lipid packing (Caron, Mark, & Poger, 2014). Nonetheless, portraying the impact of lipid species on the membrane physicochemical properties is an oversimplification and the reality is not as straightforward. The lipid composition of membranes is not constant. In *S. cerevisiae*, the ratios of lipids may differ not only among different strains but also depending on the carbon source and cultivation conditions (Patton & Lester, 1991). Changes in the lipid composition can have profound effects on cellular functions, including signal transduction, membrane elasticity and membrane trafficking (Santos & Preta, 2018). Moreover, the biosynthesis pathways leading to different lipid species are tightly connected with complex cross-talks (Henry, Kohlwein, & Carman, 2012).

Table 1: Main classes and subclasses of lipids in *S. cerevisiae*.

Lipid classes	Lipid subclasses
Glycerophospholipids	Phosphatidylcholine (PC) Phosphatidylethanolamine (PE) Phosphatidylinositol (PI) Phosphatidylserine (PS) Phosphatidylglycerol (PG) Phosphatidic acid (PA)
Sphingolipids	Ceramide (CER) Inositol phosphoryl ceramide (IPC) Mannosyl-inositol phosphoryl ceramide (MIPC) Mannosyl-di-inositol phosphoryl ceramide (MIP_2C)
Sterols	Ergosterol

Another major component of the plasma membrane is proteins, representing around 40% of the membrane composition. A total of 1000 different proteins are estimated to be located in the yeast plasma membrane (Stewart, 2017). Not all of these proteins are present at the same time and the amount and type of proteins changes according to different cellular stimuli, which means that the actual number of functional membrane proteins is much smaller. The majority are transport proteins while other membrane proteins are involved in cell wall synthesis, signal transduction or take part in the definition of the cytoskeleton. Remarkably, the activity and stability of membrane proteins is dependent on the lipids that surround them (Coskun & Simons, 2011). Membrane proteins require specific lipids, as cofactors for their functions or as "co-structures" for their

correct folding and stability. Therefore, the composition of the lipid bilayer must be optimal for obtaining the correct activity or the desired biological function of the membrane proteins (Lee, 2004). This awareness is very important (and far from being trivial) when introducing heterologous proteins in different microorganisms (Opekarová & Tanner, 2003). Also, the physicochemical properties of the plasma membrane, such as thickness, viscosity, tension and permeability are affected by the interactions between lipids and proteins (Lee, 2004). Lipid rafts are a good example of the interaction between lipids and proteins (Klose et al., 2010). Lipid rafts are dynamic nanoscale ergosterol and sphingolipid-enriched clusters with higher order than the surrounding membrane areas. It has been suggested that the bulky sterol rings pack better next to saturated acyl chains of sphingolipids and are shielded from the aqueous environment by the large sphingolipids head groups. Membrane rafts house several membrane proteins as some concentrate in these specific areas, among which, the proton pump H^+ -ATPase Pma1 (Ferreira, Mason, & Slayman, 2001). This is the major proton pump present in the yeast plasma membrane and represents 15% of all plasma membrane proteins. The importance of this proton pump for yeast cells under different stress conditions has been highlighted (see for a recent example Lee et al. (2017)). The correct positioning and activity of Pma1 into plasma membrane lipid rafts has been correlated with the presence of very long chain fatty acids and ergosterol (Eisenkolb, Zenzmaier, Leitner, & Schneiter, 2002; Gaigg, Toulmay, & Schneiter, 2006). These studies highlighted the importance of membrane lipid composition for the correct integration and functioning of proteins in the plasma membrane.

Exploring microbial membranes

It becomes evident that either for understanding biological functions or for exploiting cellular systems in microbial-based processes, biochemical and physical understanding of plasma membranes is essential. Nevertheless, it is difficult to predict which membrane composition will lead to a specific emerging property. However, it is possible to use and integrate the data deriving from different research technologies (**Table 2**) to describe the plasma membrane not as a sum of elements, but as a whole system.

Lipids are highly complex and dynamic molecules with thousands of species dynamically changing to support variations in physiological and environmental conditions. For this reason, the identification and quantification of lipids over time is very difficult (Yang & Han, 2016). Lipidomics is the technology that aims to analyze and quantify lipid species in a cell, organism or context (Klose & Tarasov, 2016). Lipidomics methods reveal the lipid status of a cellular phenotype at a particular time point and therefore allow researchers to correlate a specific membrane lipid composition with specific conditions. However, it does not allow researchers to understand the cellular mechanism which led to a specific membrane composition.

To obtain further information related to the structure and the dynamics of the plasma membrane molecular dynamics (MD) simulations are used (Pluhackova & Böckmann, 2015). MD simulation studies can be used to investigate the physicochemical properties of the membrane, the interdependent influence of proteins and lipids and also the formation of membrane nanodomains (Lindahl et al., 2016, 2018). MD simulations may also be used as predictive, to provide strategies for membrane engineering.

However, most MD simulations are performed using very simple membrane models which limits the biological relevance of these studies.

Atomic Force Microscopy (AFM) is another valuable tool used in membrane research. AFM has emerged as a powerful tool to investigate microbial cells at the nanoscale level and to measure the nanomechanical properties of cell surface topology such as stiffness, elasticity or roughness (Dague et al., 2010; Shi et al., 2018). AFM provides three-dimensional views of biological structures in real-time, on living cells and also under biologically relevant conditions (Alsteens, Müller, & Dufrêne, 2017). AFM can provide complementary information on how cells can cope with a certain type of stress, which can be relevant in the design of novel strategies for the development of improved microbial cell factories. Using AFM, Niu and collaborators (2016) studied the effect of ethanol stress on *S. cerevisiae* plasma membrane elasticity and fluidity. Under ethanol stress, the integrity of the plasma membrane was reduced which led an increased membrane permeability and fluidity. Furthermore, an increase on the plasma membrane elasticity was also observed. For a more detailed information on the application of AFM to explore yeast cells under stress conditions the reader should look at the following manuscripts (Dufrêne, 2002; Francois et al., 2013; Schiavone et al., 2016).

In vitro membrane models represent another way to study the physicochemical properties of lipid bilayers (Lopes et al., 2017). However, these models rely on commercially available lipids. There is still a lack of internal standards for some lipids classes and therefore, these types of studies are usually done using very simple membrane models consisting of only some classes of lipids. For these reasons, the physiological relevance of these studies is often limited.

Genome-scale metabolic models (GEMs) can also be a valuable tool for the design and optimization of microbial cell factories. The reproducibility of the simulations in *“in vivo”* data is, however, dependent on the quality of the GEM (Garcia-Albornoz & Nielsen, 2013). Recently, Tsouka & Hatzimanikatis (2020) developed a metabolic model called “Reduced lipids-centric model” (redLips), which focused on the lipid metabolism of *S. cerevisiae.* “RedLips” was constructed through the integration of detailed lipid metabolic pathways into already existing genome-scale metabolic models. Overall, this model can be used as a scaffold for integrating lipidomics data to improve predictions in studies of lipid-related biological functions (Tsouka & Hatzimanikatis, 2020).

Table 2: Overview of research techniques used to characterize different parameters of the plasma membrane.

Technique	Strengths	Limitations	Relevant works cited within this review
Lipidomics	Identification and quantification of lipids	Accuracy	(Klose & Tarasov, 2016)
Molecular dynamic simulations	Physiochemical characterization of the plasma membrane	Simplicity of the models available	(Pluhackova & Böckmann, 2015)
Atomic force microcopy	Working on living cells under physiological relevant conditions, Investigation of cell surface nanomechanical properties	Sample preparation, Scanning speed, Low scan image size	(Dufrêne, 2002; Francois et al., 2013; Shi et al., 2018)
***In vitro* membrane models**	Enables the prediction of physiochemical properties of lipid bilayers	Limited to the classes of lipids commercially available	(Lopes et al., 2017)
Genomic scale metabolic models (GEMs)	Helpful to predict phenotypes based on genotype manipulation.	Reproducibility of simulations *“in vivo”* is dependent on the quality of the GEM	(Garcia-Albornoz & Nielsen, 2013)

Membrane engineering in microbial workhorses

Overall, the different technologies that allow researchers to study the plasma membrane are still incomplete in describing what is occurring at the membrane level at a certain time point, and even more in the fluctuating conditions of industrial processes. Notably, several approaches involving the plasma membrane have been used to ameliorate yeast cell factories. In this section, we provide an overview of membrane engineering strategies applied to *S. cerevisiae* to improve its robustness towards different stress conditions present at industrial levels, strategies focusing on improving *S. cerevisiae* productivity, yield and production will also be described (**Figure 1).**

Improving the robustness of microbial cell factories

Robustness is an important beneficial trait for any microorganism to acquire improved fitness, which in some cases correlates with an efficient biosynthesis of desirable molecules (Nicolaou et al., 2010). During biotechnological processes, yeasts encounter different kinds of stresses that are generally associated with the growth conditions (temperature, pH, oxygenation, …), the starting substrates and the final products or byproducts (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). Because the economic viability of bioprocesses is often limited by damage to the microbial plasma membrane, assessing changes due to stressors becomes essential to design counteractions.

Ethanol

The effects of ethanol on *S. cerevisiae* membrane permeability were firstly described by Leão & Van Uden (1984), who reported an increased

permeability of the plasma membrane to protons in the presence of ethanol. Later it was shown that ethanol also increases the diffusion of acetic acid in *S. cerevisiae* (Casal, Cardoso & Leão, 1998). More recently, a similar effect was also reported by Niu et al. (2016), who showed that the exposure of yeast cells to ethanol resulted in increased membrane permeability (characterized by relative electric conductivity) together with an increase in cell swelling rate, indicating that the plasma membrane integrity was reduced in the presence of ethanol. Furthermore, using AFM, Schiavone et al. (2016) demonstrated that ethanol stress caused a reduction of the plasma membrane thickness.

The relationship between ethanol tolerance and lipid composition of the plasma membrane is strongly dependent on the experimental conditions that are used. For this reason, a clear connection between plasma membrane composition and ethanol tolerance remains to be elucidated (Henderson & Block, 2014).

A common strategy to improved *S. cerevisiae* robustness to ethanol has been the development of strains with an increased fraction of glycerophospholipids containing oleic acid ($C_{18:1}$). Oleic acid has unique properties: on one side its 18 carbon chain length contributes to increasing membrane thickness, but on the other side its degree of unsaturation contributes to an increase of membrane fluidity and decreases membrane thickness. The effect of increased amounts of oleic acid in the plasma membrane is difficult to predict and must be verified experimentally case by case, considering the overall changes in fatty acyl chain length and saturation, and the experimental conditions. Kajiwara et al. (2000) reported a 33% increase in oleic acid content at the expense of palmitic acid ($C_{16:0}$) when *OLE1,* encoding for a stearoyl-CoA 9-desaturase which catalyzes the

transformation of saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) to unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$), was overexpressed in *S. cerevisiae*, overall measuring a 7% increase in unsaturated fatty acids. The engineered strain showed improved growth and ethanol production at low temperatures (10 °C). Since lowering of the temperature leads to a more ordered membrane structure and hence a reduction in fluidity (Shinitzky & Henkart, 1979) which is largely determined by the packing of oleic acid ($C_{18:1}$) and palmitoleic acid ($C_{16:1}$), the impact of the observed increase in oleic acid in membrane fluidity could potentially underlay the improved performance of the strain.

Differently, in other study, conducted at 30 °C, the overexpression of *OLE1* did not result in changes in the content of oleic acid (Zheng et al., 2013). This discrepancy might be because cells increase their membrane unsaturation at lower temperatures (Aguilera, Randez-Gil, & Prieto, 2007).

S. cerevisiae membrane engineering can also be based on the introduction of heterologous genes or lipid species. Yazawa et al. (2011) expressed the rat elongase 1 gene (*rELO1*) in *S. cerevisiae*, obtaining vaccenic acid instead of oleic acid, but with no effect on ethanol tolerance. The desired effect was reached by the introduction of rat elongase 2 (*rELO2*), which increased oleic acid content by 18%. Importantly, under these conditions the unsaturation index was very similar to that of the control, meaning that the increase of oleic acid was determinant for ethanol tolerance. Furthermore, *rELO2* overexpression also conferred tolerance to n-butanol, n-propanol, and 2-propanol.

Despite the positive results obtained, it is not clear if the advantage derives from the oleic acid content or other alterations affecting the membrane

properties, as they were not measured. Indeed, other attempts seem to suggest that we are still exploring trial and error strategies.

Organic acids

The effects of weak organic acids on *S. cerevisiae* have been generally ascribed to acidification of the cytosol by protons released and/or accumulation of the anionic form of the acid. These, in turn, can cause several cell alterations such as the disruption of the proton gradient across the plasma membrane, increased turgor pressure, oxidative stress, protein aggregation, lipid peroxidation, inhibition of membrane trafficking, and perturbation of plasma and vacuolar membranes spatial organization (Piper, 2011).

Guo and co-workers (2018) performed a comparative analysis of lipids in *S. cerevisiae* grown in the presence of four different weak acids, which are known to be detrimental for yeast growth: acetic, cinnamic, formic and levulinic acids.

Yeast cells counteract the stress caused by organic acids by modulating the plasma membrane lipid composition and the modifications differ depending on the molecular conformations of the acids. Formic, levulinic and acetic acids did not affect the levels of PA in comparison to the control, while in cells exposed to cinnamic acid there was a decrease of 30% in PA content. Furthermore, the contents of PE, PC and PS decreased in yeast cells under any acid condition compared to the control. Differently, the content of PI increased in yeast cells subjected to acid stress during growth on glucose, but decreased during the adaptation phase on ethanol, compared to the control. Concerning the fatty acids (profile FAs), in yeast cells under acid stress, the amount of myristic acid ($C_{14:0}$) was around 5% of the total

fAs, similar to that of the control. However, a decrease in palmitic acid ($C_{16:0}$) and palmitoleic acid ($C_{16:1}$) and an increase in elaidic acid ($C_{18:1}$) and stearic acid ($C_{18:0}$) were observed for all the acid-stressed cells. While the unsaturation index in cells exposed to cinnamic acid was unaffected, exposure to acetic, formic and levulinic acid resulted in an increase in the saturation index of fAs in comparison to the control. Exposure of the cells to acids led to a continuous increase of the sterol content during the different phases of growth, while the control showed a gradual decrease in ergosterol from the exponential phase to the stationary phase. Ergosterol, the major yeast sterol, is essential for the structure of the plasma membrane, modulating its thickness, fluidity and permeability and regulating the activity of membrane-associated transporters (Abe & Hiraki, 2009; Kodedová & Sychrová, 2015). Therefore, ergosterol plays a key role in the resistance towards inhibitory compounds.

The work developed by Guo and collaborators (2018) has the important merit to describe how the plasma membrane lipid composition adapt to different stress factors and could be used as a guide for membrane engineering strategies, despite it did not take into account the membrane physicochemical properties as well as the rearrangement of membrane proteins.

Lindberg et al. (2013) explored a comparative approach: in an attempt to increase *S. cerevisiae* tolerance to acetic acid, the authors studied the lipid profile of *Zygosaccharomyces bailii*, a food spoilage yeast well known for its resistance to weak organic acids (Fleet, 1992). After exposure to acetic acid, *Z. bailii* revealed large lipidomic changes while smaller changes were observed in *S. cerevisiae*. A higher degree of saturation of the glycerophospholipids and increased amounts of complex sphingolipids at

the expense of glycerophospholipids were the most noticeable changes in the adaption of *Z. bailii* to acetic acid. These results are consistent with the previously described role of sphingolipids in cell death induced by acetic acid in *S. cerevisiae*, where the deletion of *ISC1*, coding for the inositol phosphosphingolipid phospholipase C, responsible for the hydrolysis of complex sphingolipids, lead to increased resistance to acetic acid (Rego et al., 2012).

By combining lipidomic analysis with molecular dynamic simulations, in a multidisciplinary work, Lindahl et al. (2016) reported that membranes with high content of sphingolipids are thicker and denser than control and membrane permeability decreases. Taking this into account, Lindahl et al. (2017) tried to increase the fraction of complex sphingolipids in the plasma membrane of *S. cerevisiae* by altering the expression of genes associated with the production of long-chain base (LCB) and very-long-chain fatty acids (VLCFA) (C_{24-26}), and with the conversion of ceramides into complex sphingolipids. The authors overexpressed *ELO3*, involved in fatty acid elongation and *AUR1*, encoding an enzyme that catalyzes the formation of complex sphingolipids, and deleted *ORM1* and *ORM2*, encoding negative regulators of sphingolipids biosynthesis. However, neither the overexpression of *ELO3* or *AUR1* influenced the lipid metabolism. The deletion of *ORM1* and *ORM2* lead to a decrease in both complex sphingolipids and phosphatidylinositol, which diminished cell viability. When combined, the reduction in growth caused by the *orm1/2* deletion was alleviated by the overexpression of *ELO3* and *AUR1*, which also determined an increase in the fatty acyl chain length. Overall, the authors were not successful in the attempt to increase levels of complex sphingolipids in *S. cerevisiae*.

Increasing the length of fatty acids has been a common strategy employed to increase microorganisms' fitness towards organic acids. Zheng et al. (2013) overexpressed *ELO1*, encoding a fatty acid elongase, in *S. cerevisiae* to improve cellular tolerance to acetic acid. These authors observed an 18% increase in the cellular content of oleic acid, which resulted in a 44% increase in survival after acetic acid exposure, but the molecular system was not completely described.

Godinho et al. (2018) unveiled the relation between the yeast ABC transporter Pdr18 and ergosterol levels in yeast adaptation and tolerance to acetic acid stress. Pdr18 has been proposed to mediate the incorporation of ergosterol in the plasma membrane. The authors reported a coordinated activation of the transcription of *PDR18* and several ergosterol biosynthesis pathway genes during the period of adaptation to acetic acid. Therefore, Pdr18 has been suggested to be essential to keep maximum ergosterol content in the plasma membrane in the presence of acetic acid, thus maintaining the plasma membrane order, electrochemical potential and permeability in the presence of acetic acid. The role of Pdr18 in the maintenance of the plasma membrane physicochemical properties in the presence of acetic acid is crucial for the adequate functioning of the membrane.

The expression of heterologous genes in *S. cerevisiae* to increase its robustness to organic acids has also been attempted. Cyclopropane ring formation on fatty acyl chains occurs in both bacteria and archaea and it has been associated with an increase in the plasma membrane rigidity (Oger & Cario, 2013). Liu et al. (2013), expressed *Escherichia coli cfa* gene in *S. cerevisiae* and successfully converted 10% of fatty acids into

cyclopropanated fatty acids. However, mutants failed to show octanoic acid resistance and no further stress conditions were tested.

Thermotolerance

Thermotolerance is a desirable trait in microbial cell factories: it can result in reduced cooling costs, and contamination risks and can boost enzyme activity during simultaneous saccharification and fermentation (Abdel-Banat et al., 2010). However, heat is also a stress factor, known to disturb protein stability, cell membrane order, and cytoskeleton structures, with consequences such as protein dysfunction, metabolic imbalances and loss of metabolic activity (Verghese et al., 2012).

Caspeta et al. (2014) set up an adaptive laboratory evolution (ALE) experiment to select yeast strains with improved growth and ethanol production at temperatures higher than 40 ºC. Sequencing of the evolved strain, capable to grow at high temperatures, revealed, among other findings, a point mutation in the *ERG3* gene, encoding a structural enzyme in the ergosterol biosynthesis pathway. In this strain, fecosterol, a sterol precursor, became the major sterol in the plasma membrane rather than ergosterol. The authors reported that the substitution of the “flat” ergosterol by the “bended” fecosterol in the evolved *S. cerevisiae* strain seems to be responsible for the maintenance of optimal membrane fluidity at high temperatures and therefore crucial for the thermotolerant phenotype (Caspeta et al., 2014).

More recently, García-Ríos et al. (2021) aimed to generate a robust *S. cerevisiae* strain to be used in cocoa fermentation. ALE was conducted in a defined medium at 40 ºC for 150 generations. The evolved strain exhibited a significantly increased growth rate in comparison to the parental strain. Lipidomic analysis revealed that, at 40 ºC, the evolved strain exhibited a

higher ergosterol/zymosterol ratio compared to the parental strain. Authors claim that this difference could be responsible for the adaptation of the evolved strain to higher temperatures. Differently from the higher levels of fecosterol reported by Caspeta et al. (2014), in the obtained evolved strain García-Ríos et al. (2021) observed the accumulation of episterol, which is the next intermediate in the sterol biosynthesis pathway. Furthermore, no significant differences were found in terms of the fatty acid profile between the two strains.

These results highlight that the adaptation of the yeast plasma membrane to heat stress is not straightforward and membrane lipid composition changes differently according to the background of the strain used and also with the conditions in which the ALE experiments are carried out.

In another work, Liu et al. (2017) compared the thermotolerance of *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, *erg3Δerg4Δ*, *erg3Δerg5Δ* and *erg4Δerg5Δ S. cerevisiae* strains. The mutants lacking either of the four enzymes are viable, with intermediate sterols instead of ergosterol accumulated in the membrane. All mutant strains displayed a higher growth rate than wild type at 39.5 ºC. The *erg3Δerg5Δ* strain, in particular, exhibited and 2.24-fold increase in growth rate relative to wild type at this temperature (Liu et al., 2017). Modifications of the sterol composition directly affect the fluidity and permeability of the plasma membrane, as well as the localization and activity of membrane proteins (Kodedová & Sychrová, 2015). This work highlighted the importance of the sterol composition on yeast response to high temperatures and can be used as a guide for future membrane engineering approaches.

Different from these approaches, the study of the membrane composition of thermotolerant yeasts such as *Kluyveromyces marxianus* can be valuable

to provide guidelines and ideas to engineering the plasma membrane of *S. cerevisiae*.

Temperature is also one of the most important parameters affecting wine fermentation. Low fermentation temperature improves the characteristic taste and aroma of wines. However, low temperature fermentations result in increased lag phases and lower growth rates for yeasts, causing fermentation to stop (Bisson, 1999). As mentioned above, low temperatures also affect the plasma membrane, leading to a decrease in membrane fluidity (Shinitzky & Henkart, 1979). Metabolic profiling done by López-Malo et al. (2013b) revealed that the main metabolic differences between *S. cerevisiae* growing at 12 ºC (common fermentation temperature for wine) and 28 ºC were related to lipid metabolism.

Genes involved in the phospholipid, sphingolipid and ergosterol metabolism were identified as those causing the most significant effects on yeast growth at low temperatures (López-Malo et al. (2013a)). *OLE1* was one of the genes identified and its overexpression lead to an improved fermentation ability at lower temperatures (12 ºC) in synthetic must. Furthermore, the wine produced from this strain revealed a specific aroma profile (López-Malo et al., 2014). As previously mentioned, *OLE1* encodes a stearoyl-CoA 9-desaturase which catalyzes the transformation of saturated fatty acids (C16:0 and C18:0) to unsaturated fatty acids (C16:1 and C18:1). The presence of unsaturated fatty acids contributes to increased membrane fluidity, which is advantageous for the cells at low temperatures (Aguilera et al., 2007).

In a different approach, López-Malo et al. (2015) performed an ALE in synthetic must to obtain a wine yeast strain able to ferment at low temperatures. The evolved strain exhibited improved growth and higher

fermentation performance at low-temperature in comparison to the parental strain. Genome sequencing of the evolved strain revealed the presence of a single nucleotide polymorphism (SNP) in the *GAA1* gene, which encodes a subunit of the glycosylphosphatidylinositol (GPI) transamidase complex. This complex adds GPI, required for inositol synthesis, to newly synthesized proteins, including mannoproteins. Inositol is an essential phospholipid precursor in yeast cells and could be incorporated into phosphatidylinositol (PI), sphingolipids and glycosylphosphatidylinositol anchors. Using a reverse engineering strategy, a site-directed mutation ($GAA1^{Thr108}$) was introduced in the parental strain, which resulted in improved fermentation performances. This result reveals a higher inositol requirement for *S. cerevisiae* cells grown at low temperatures.

Overall, these works highlighted the importance of the plasma membrane lipid composition in yeast response to sub-optimal temperatures.

Osmotic stress

Osmoregulation is fundamental for living cells and is particularly relevant for industrial biotechnology. Yeast adaptation to osmotic stress is an active process based on sensing and counterbalancing osmotic changes. Morphologic changes are key towards osmotic stress as yeast cells change their volume in response to osmotic challenges, decreasing volume in response to hypertonic stress and increasing volume in the presence of hypotonic stresses. Therefore, the ability to tolerate osmotic stress is strongly influenced by plasma membrane permeability (Gonzalez et al., 2016).

Through an adaptive laboratory evolution experiment, Zhu et al. (2020) were able to isolate a strain with improved tolerance to osmotic stress.

Transcriptome sequencing (RNA-seq) suggested that mRNA levels of *ELO2* were differentially upregulated in the isolated strain. Using a reverse engineering strategy, *ELO2* was overexpressed in a wild-type strain, resulting in enhanced very long fatty acids content (the contents of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ were increased by 52.3%, 94.1% and 14.4%, respectively). Furthermore, the levels of complex sphingolipids were increased. These modifications have been reported to promote a thicker and less permeable membrane. Flow cytometry analysis of cells stained with SYTOX green revealed that the *ELO2* overexpressing strain exhibited a 24.4% higher membrane integrity than the wild-type strain, resulting in an enhanced osmotic tolerance.

Similarly, Yin et al. (2020) were able to increase the tolerance of *S. cerevisiae* to salt stress by significantly improving the yeast membrane potential and integrity. Using a nitroguanidine mutagenesis strategy, authors identified *CDS1,* encoding a phosphatidate cytidylyltransferase, and *CHO1*, encoding a phosphatidylserine (PS) synthase, as key factors to yeast tolerance towards salt stress. The combined overexpression of *CDS1* and *CHO1* resulted in a redistribution of membrane phospholipids and a decreased anionic-to-zwitterionic phospholipid ratio. In *S. cerevisiae*, anionic phospholipids are mainly PA, PI, and PS, while zwitterionic phospholipids are PE and PC. These results indicate that a higher presence of zwitterionic phospholipids may be beneficial to deal with salt stress (Yin et al., 2020).

These works highlight that different membrane compositions can be advantageous towards the same stress agent. In both works, Yin et al. (2020) and Zhu et al. (2020), the levels of PS and PE were increased, 35.5% and 15%, 25.2% and 18.9%, respectively. However, when *ELO2* was

overexpressed the levels of PC, PA, and PI did not change. On the other hand, the double overexpression of *CDS1* and *CHO1* led to a 28.6% increase in PC and to a decrease in the levels of PA and PI of 14.6% and 39.8%, respectively.

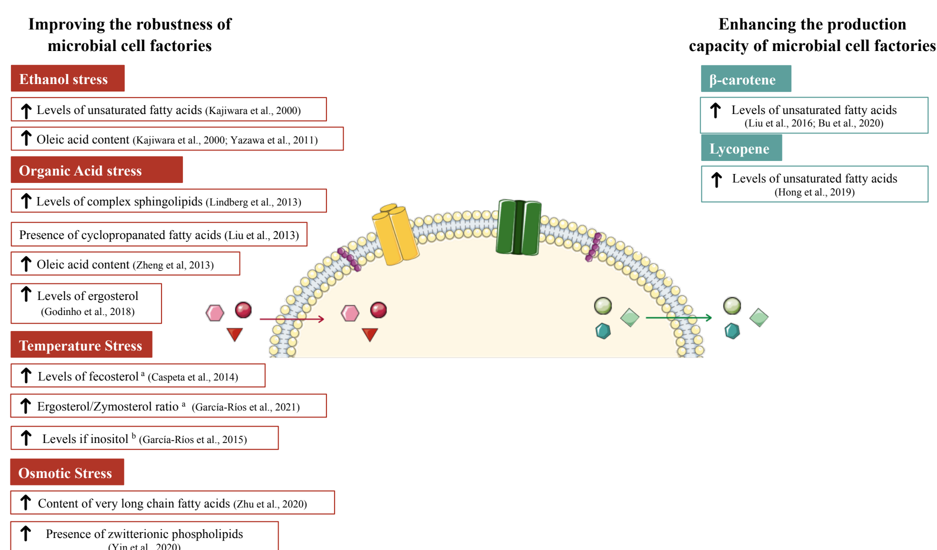

Figure 1: Plasma membrane modifications in *S. cerevisiae* aiming to improve yeast fitness on industrial bio-processes. On the left side are represented modifications to increase yeast robustness toward stress conditions (ethanol, organic acids, temperature, and osmotic stress). Plasma membrane modifications on the right side were done to enhance the production capacity of microbial cell factories. Red arrows indicate the entrance of the stress agents. Green arrows indicate the exit of metabolites of interest. [a] and [b] stand for works performed at high and low temperatures, respectively. The figure was produced using the vector image of Servier Medical Art (http://smart.servier.com/).

Enhancing the production capacity of microbial cell factories: when membrane composition can contribute to increasing product export

Microorganisms can be exploited for the production of several compounds with applications in a wide range of industrial sectors, and whenever possible the export of the product into the medium is preferable, mainly for limiting the downstream processing costs, but very often also for increasing the flux towards the product itself (Chung et al., 2015; Tsuge et al., 2016; Porro & Branduardi, 2017). Therefore, a proper export of the products of interest is often indispensable for a profitable and efficient cell factory. Researchers have been focusing on maximizing the export of the compounds of interest by overexpressing specific membrane transporters, engineering transporters for improved efficiency or even introducing heterologous transporters in the cell factory (Boyarskiy & Tullman-Ercek, 2015; Kell et al., 2015; Erian et al., 2020; Soares-Silva et al., 2020). For more detailed information on the engineering of membrane transporters for industrial biotechnology applications, the reader should look the works mentioned above.

However, many times, the simple overexpression of a transporter does not result in the intended increased export rates. This is why in this review we wanted to focus on and highlight strategies where the plasma membrane composition was changed, and in turn influencing the properties of the entire structure, considered as a system. Indeed, the plasma membrane composition is very crucial and must be considered since it determines physiochemical properties such as fluidity, permeability and elasticity, which can facilitate the export of the products of interest (Royce et al., 2015). So far there are not many examples of membrane engineering strategies focusing on increasing metabolites secretion in yeast and in

particular in *S. cerevisiae*. However, there are many works performed in *E. coli* (Tan et al., 2016, 2017; Wu et al., 2017; Kanonenberg et al., 2019) which may serve as guidance for future work in yeast.

One interesting example in *S. cerevisiae* relates to the work of Liu et al. (2016) who reported a decrease in the fluidity of the plasma membrane caused by the decrease of unsaturated fatty acids in *S. cerevis*iae strain producing β-carotenes. Carotenoids accumulate in the cell membrane and therefore, high production levels of carotenoids can cause membrane stress (Gruszecki & Strzałka, 2005).

In this strain, carotenoid biosynthesis shares the precursors acetyl-CoA and farnesyl pyrophosphate (FPP) with unsaturated fatty acids and ergosterol, respectively. Therefore, heterologous carotenoid biosynthesis could decrease the content of unsaturated fatty acids and ergosterol due to competition for these precursors. Given the importance of plasma membrane fluidity in cellular metabolism and physiology (such as facilitating the absorption of essential substances), authors sought to restore it. The addition of linoleic acid ($C_{18:2}$) to the culture media restored the plasma membrane fluidity by the incorporation of unsaturated fatty acids in the membrane. This resulted in a 24.3% increase in the production of β -carotene (Liu et al., 2016).

A different approach was used by Bu and collaborators (2020). The authors were able to counteract the decrease of membrane fluidity caused by the accumulation of β-carotenes by overexpressing *OLE1*. Overexpression of *OLE1* could improve the fatty acid unsaturation and membrane flexibility, which conferred cells a high tolerance to various types of stress, as reported above (Fang et al., 2017; Nasution et al., 2017). Indeed, the overexpression of *OLE1* promoted cell membrane fluidity (measured by fluorescence

anisotropy) and resulted in an improved β-carotene secretion (Bu et al., 2020). The same strategy was also used by Hong and collaborators (2019) for the production of lycopene, a red carotenoid pigment. *OLE1* overexpression led to improved lycopene production suggesting that an increase in unsaturated fatty acids content in the cell membrane might relieve the carotenoid toxicity (Hong et al., 2019).

Another example of how the membrane composition affects the export of molecules was reported by Wang et al. (2013). The addition of surfactants, such as Triton X-100, led to increased permeability and fluidity of the plasma membrane in the yeast *Monascus purpureus*, which resulted in a 56.8% higher production of pigments. The addition of Triton X-100 led to an increased degree of unsaturation in the membrane lipids. According to the authors, these changes, facilitated the secretion of intracellular pigment to the broth thus alleviated the product feedback inhibition and enhanced pigment production (Wang et al., 2013). These results suggested that Triton X-100 could markedly affect the fatty acid composition of *M. purpureus* H1102 by significantly increasing the degree of unsaturation of the cell membrane lipids, thus improving the fluidity and permeability of the cell membrane.

Conclusion

Overall, despite its great potential, membrane engineering is still a highly complex approach as membrane lipids and membrane homeostasis are vital for many cellular functions. Moreover, it is difficult to predict the outcomes of altering membrane elements in the whole membrane system. The understanding of membranes and their structure has changed enormously over the last years. The availability and development of high-throughput methods have allowed researchers to deepen their knowledge on the plasma membrane conformation and dynamics. However, the analysis of the plasma membrane composition is still challenging which hampers a detailed association between specific composition and physicochemical properties of the plasma membrane. The interdependency between membrane lipids and proteins cannot be neglected. In the future, a combination of efforts between researchers from different areas of study, such as lipidomics, molecular dynamics simulations and membrane biophysics will be crucial to gain a better understanding of the plasma membrane and therefore plan strategies to tailor it at a systems level.

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The impact of ergosterol content on *Saccharomyces cerevisiae* tolerance towards acetic and lactic acid stress

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Abstract

Organic acid stress often represents a major hurdle in industrial bio-based microbial processes. Organic acids can be released from lignocellulosic feedstocks pretreatment and can also be desirable products obtained by microbial fermentation with applications in different industrial sectors. Yeasts are prominent cell factories. However, the presence of organic acids can compromise yeast metabolism, impairing fermentation performances and limiting the economic feasibility of the processes. Plasma membrane remodeling is deeply involved in yeast tolerance to organic acids, but the detailed mechanisms and potentials of this phenomenon remain largely to be studied and exploited. We investigated the impact of ergosterol on the *Saccharomyces cerevisiae* tolerance against organic acid stress by coupling *in vitro* and *in vivo* assays. In the *in vitro* assay, synthetic lipid vesicles were prepared containing different concentrations of ergosterol. We observed changes in organic acids diffusion through the membrane as a function of ergosterol content. Then, we extended our approach *in vivo*, engineering *S. cerevisiae* with the aim to change the ergosterol content of cells. We focused on *ECM22*, an important transcription factor, involved in the regulation of ergosterol biosynthesis. The overexpression of *ECM22* was sufficient to increase ergosterol levels in *S. cerevisiae*, resulting in an enhanced tolerance towards lactic acid stress of the engineered strain.

Introduction

The yeast *Saccharomyces cerevisiae* has been one of the earliest domesticated living organisms, used since ancient times for baking and brewing (Hittinger, Steele, & Ryder, 2018). Since the era of r-DNA it also became a widely used workhorse for recombinant production processes, and it is among the most frequently used microorganisms in biotechnology with successful applications in the production of both bulk and fine chemicals from renewable biomasses, such as starch or sugar or lignocellulosic biomass (Hong & Nielsen, 2012). However, during bio-based industrial processes yeasts are subjected to different kinds of stress associated with industrial process conditions, such as non-optimal temperature, inhomogeneous mixing, unfavorable pH, high osmotic pressure. These stress factors might inhibit cellular metabolism and compromise the performance of the fermentative process (Deparis, Claes, Foulquié-Moreno, & Thevelein, 2017; Piper, 2011). Among the different stresses, organic acids represent a double edge sword in bio-based processes. Organic acids can be released from the pretreatment of biomass, inhibiting the fermentative process but, at the same time, they can be desirable products obtained by microbial fermentation as valuable building block chemicals with many different industrial applications (Sauer, Porro, Mattanovich, & Branduardi, 2008). For some of these applications, as for obtaining biopolymers, the undissociated form of organic acids is preferable, therefore it is preferable to run the fermentations at low pH (below the pKa of the acid). Low pH is also critical to avoid unwanted bacterial contamination. However, the desired undissociated form can cross the plasma membrane by simple diffusion (Mira, Teixeira, & Sá-Correia, 2010). Once inside the cell, in a higher pH environment, the acid

dissociates leading to the release of protons and the respective counter ion. The accumulation of both species inside the cell has detrimental effects ranging from intracellular acidification, inhibition of metabolic activities, inducing oxidative stress, interfering with lipid organization, plasma membrane permeability and integrity, leading to decreased cellular performance and eventually to cell death (Mira et al., 2010; Piper, 2011). Moreover, when organic acids are produced by yeast and high titers are reached, the interaction of the accumulating products with the plasma membrane might become an additional stress factor (Jezierska & Van Bogaert 2017). The plasma membrane plays a key role in these processes, acting as a selective gate, controlling the flux of compounds into and out of the cell (Ferraz, Sauer, Sousa, & Branduardi, 2021). As such, the plasma membrane has been proven to be an important target for stress adaption when designing strategies for the development of versatile, robust and efficient cell factories ready to tackle the harshness of industrial processes while delivering high yield, titer and productivity (Russell et al., 1995). Lipid composition, in particular, is fundamental to determining the biophysical characteristics of the plasma membrane: changes in the lipid composition can have profound effects on cellular functions, including signal transduction, membrane elasticity, and membrane trafficking (Santos & Preta, 2018). Remarkably, the activity and stability of membrane proteins are also dependent on the lipids that surround them (Coskun & Simons, 2011). Membrane proteins require specific lipids, as cofactors for their functions or as “co-structures” for their correct folding and stability. Therefore, the composition of the lipid bilayer must be optimal for obtaining the correct activity or the desired biological function of the membrane proteins (Lee, 2004). Certain plasma membrane lipid

compositions can be more advantageous than others under specific stress conditions. For this reason, cells are able to adapt their plasma membrane according to the surrounding environment. (Guo, Khoomrung, Nielsen, & Olsson, 2018; Klose et al., 2012). Sterols are an essential component of the plasma membrane of eukaryotic cells and the major sterol present in yeast is ergosterol. Sterols contribute to membrane integrity, fluidity and permeability as well as to the sorting of lipid rafts, which are sterols and sphingolipids-enriched clusters that house several membrane proteins, among them the proton pump H^+-ATPase Pma1 (Dufourc, 2008; Ferreira, Mason, & Slayman, 2001). Additionally, sterols increase membrane rigidity by ordering the fatty acyl chains and thus allowing a tighter lipid packing (Caron, Mark, & Poger, 2014). The accumulation of different ratios, amounts and structures of sterols in the plasma membrane can lead to different membrane properties (Dufourc, 2008).

In this work, we are particularly interested in organic acid stress of yeast cells and how membrane composition, in terms of ergosterol content, relates to stress tolerance. The impact of individual lipid species on the plasma membrane physicochemical properties is difficult to predict. (Ferraz et al., 2021). Nevertheless, a more rigid and less permeable membrane is likely to reduce the diffusion of acids into the cell and, therefore, likely to increase resistance towards the acids. In this work, we used complementary *in vitro* and *in vivo* approaches to study the impact of ergosterol on yeast plasma membrane tolerance against organic acid stress. Acetic acid was selected for this study, as it is the major inhibitory compound released from the pretreatment of lignocellulosic biomass (Jönsson & Martín, 2016). We also studied lactic acid - a chemical platform molecule with an ample spectrum of industrial applications, including food preservation, and use as

pharmaceutical additive, also having the potential to be used for bioplastic production from a renewable source (Porro & Branduardi, 2017).

The *in vitro* assay, based on synthetic lipid vesicles, prepared from commercially available yeast lipids, enriched, or not, with different amounts of ergosterol, takes advantage of the use of a pH-responsive fluorescent dye, loaded into the vesicles. This assay allows to monitor pH and pH changes inside the liposomes and, indirectly, investigate the diffusion of organic acids through the liposomal membranes, independent of yeast metabolism. We observed that, at low pH, in the presence of acetic acid higher levels of ergosterol decrease the acid diffusion into the liposomes. Differently, in the presence of lactic acid, we observed that increasing ergosterol abundance is not directly proportional with decreased diffusion into the liposomes. Given the promising indications observed, we extended our approach to an *in vivo* approach, taking advantage of *S. cerevisiae* cells engineered to modulate the quantity of ergosterol by overexpressing *ECM22,* encoding the transcription factor described as the main regulator of ergosterol biosynthesis (Vik & Rine, 2002). The overexpression of *ECM22* revealed to be crucial for increased yeast tolerance towards lactic acid stress. Moreover, and coherently in *in vitro* and *in vivo* assays, we observed that the impact of ergosterol depends on the organic acid under investigation.

Results and Discussion

Impact of ergosterol content of the lipid membrane of artificial liposomes on organic acids diffusion

To study the influence of ergosterol on the diffusion of acetic and lactic acid through the plasma membrane, an indirect *in vitro* technique based on a liposomal fluorescent assay was designed. Liposomes incorporated 10 mM Na-HEPES buffer solution with pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt, HPTS), a ratiometric fluorophore, at pH 7.4. Pyranine is a water-soluble, membrane-impermeable fluorophore, having fluorescent excitation and emission spectra that are highly dependent on the pH. Therefore, it is used as pH sensitive fluorescent probe to monitor pH and, indirectly, can allow evaluating the membrane diffusion to organic acids. Liposomes were prepared starting from Yeast Lipid Total extract, acquired from Avanti Polar lipids, and were enriched, or not, with different concentrations of ergosterol. Five different liposome compositions were used in this study. As a reference, in *S. cerevisiae* ergosterol concentration is about 10 mol% of the total lipids (Klose et al., 2012). The presence of different amounts of ergosterol did not significantly affect the size of the liposomes (**Table 1**).

Table 1: Ergosterol composition of different liposome preparations, liposome average size (nm) and polydispersity index (PdI). Standard deviations were calculated from 3 independent replicates.

Ergosterol concentration (Mol%)	Average size (nm)	PdI
Standard	148 ± 0.3	0.141 ± 0.011
+ 3	143.6 ± 1.5	0.152 ± 0.013
+ 10	141.2 ± 3.6	0.258 ± 0.025
+ 20	151.7 ± 5.6	0.163 ± 0.012
+ 30	146.6 ± 6.1	0.134 ± 0.02

Liposomes were exposed for 5 minutes to 65 mM of acetic or lactic acid solutions at different pH values, and then the final pH values inside the different liposomes were calculated through the correlation with the measured fluorescence. The results are shown in **Figure 1**. We observed that both acetic and lactic acid caused pH drops in all the liposomes under all conditions tested. Upon the exposure to acetic and lactic acid at pH 7 and pH 5, no differences were observed in the final internal pH among the different liposomes (**Figure 1 A and B**). Nonetheless, at these pH values, acetic acid caused larger pH drops than lactic acid. Differently, at pH 3, (**Figure 1 C**) differences were observed between the different liposomes. In the presence of acetic acid at pH 3, liposomes enriched with 30 mol% ergosterol were able to maintain a higher internal pH when compared with the reference composition. The pH difference between the control and the 30 mol% ergosterol enriched liposomes was about 0.41 pH units (p-value 0.0316). Also, under this condition, we observe a trend where increasing amounts of ergosterol lead to smaller decrease in the liposomes internal pH. Differently, in the presence of lactic acid at pH 3, liposomes enriched

with 10 mol% ergosterol were able to sustain a higher internal pH than the other liposomes. The pH difference between the control and the 10 mol% ergosterol liposomes was about 0.25 pH units (p-value 0.0335). Also, under this condition, a significant drop in the internal liposomal pH was observed between liposomes enriched with 10 and 20 mol% ergosterol (p-value 0.0027). This pH drop was unexpected and further testing will be needed to clarify this difference. Control condition where water was added instead of organic acids can be found in the supplementary material.

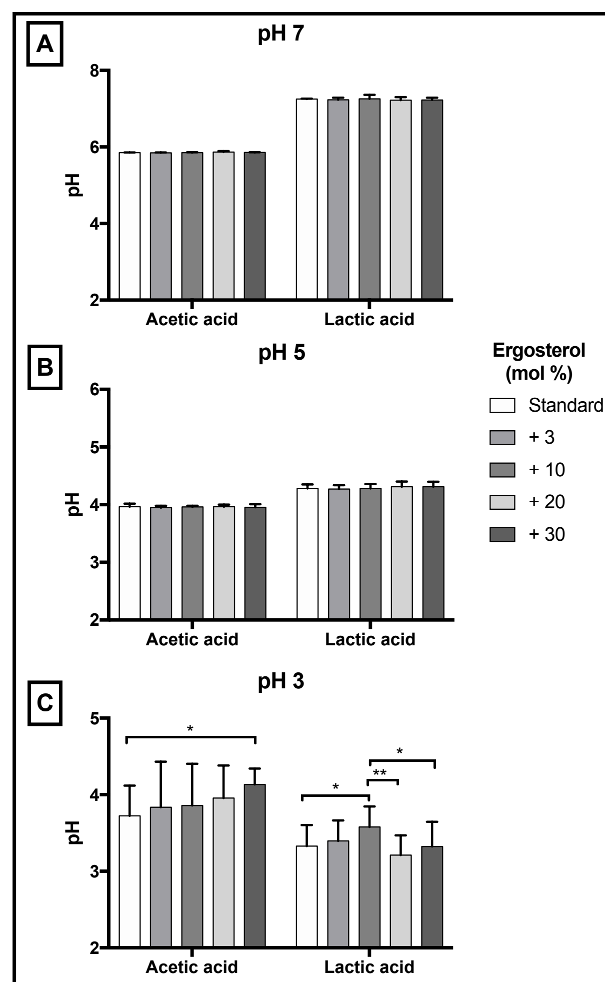

Figure 1: Internal pH (pH_i) of the different liposomes after exposure to 65 mM of Acetic acid or Lactic acid solutions, at different pH values A) pH 7, B) pH 5 and C) pH 3. The initial pH inside the liposomes was 7.4. *$p<0.05$ and **$p<0.01$. Please note that y axes are different.

In solution, organic acids exist in a pH-dependent equilibrium between dissociated and undissociated states, as described by the Henderson-Hasselbalch equation: pH = pK_a + log $[A^-]/[HA]$, where A^- and HA are the dissociated and undissociated species, respectively. This equation indicates that at pH values above the acid pK_a value (acetic acid pK_a = 4.74, lactic acid pK_a = 3.86), more than 50% of the acid is dissociated and that the concentration of the undissociated acid increases exponentially as the pH declines below this threshold. Undissociated weak acids diffuse passively through the plasma membrane until an equilibrium is established. As the undissociated acid enters the liposome lumen, it dissociates at the higher internal pH (pH_i). This results in the formation of charged anions and protons, which cause a reduction of the internal pH once the buffering capacity is exceeded. The concentration of these molecules differs based on the molar concentration of the acids in the medium and the pH_e and pH_i values.

Our results showed that at pH_e=3, the pH drop caused by lactic acid was higher than acetic acid, which may be related with the lower the pK_a of lactic acid. Organic acids with a lower pK_a have a higher tendency to dissociate and therefore have a greater ability to donate protons and collapse the pH, when going from a medium at a pH where they are mostly undissociated to an environment with a pH higher than their pK_a.

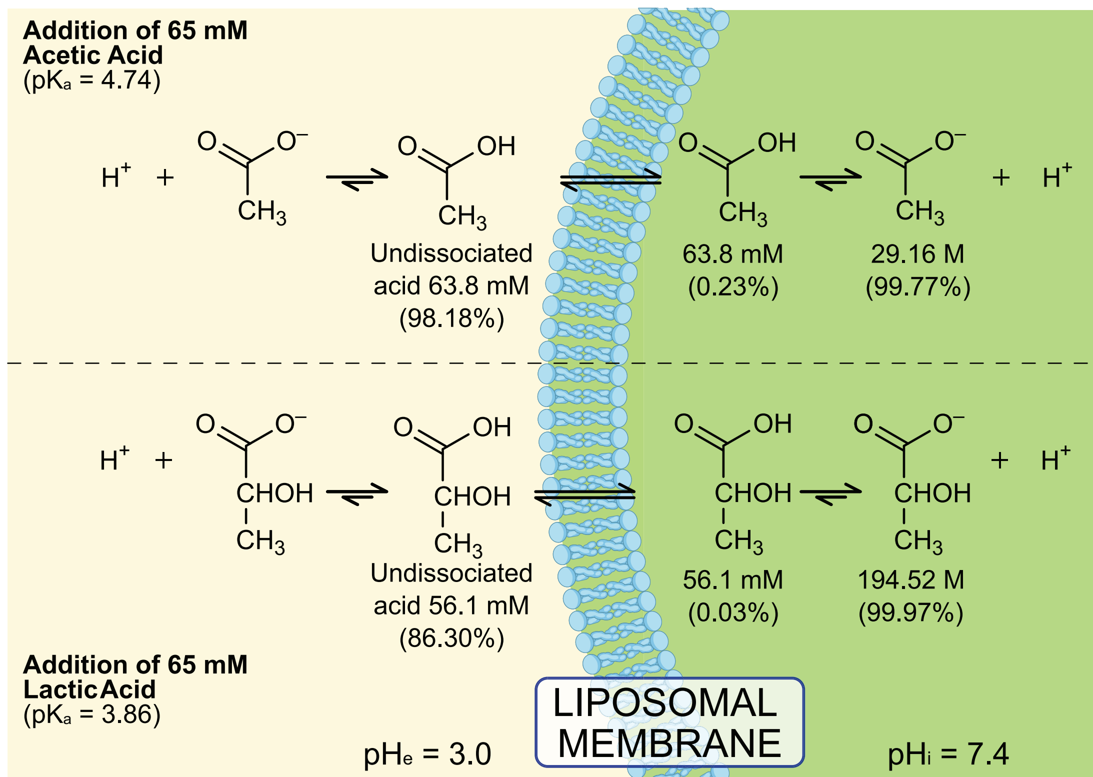

Figure 2: Illustration of the concentrations of anions and undissociated acids that would be present in the medium and inside the liposomes (based on the pH_e and pH_i) when 65 mM of either acetic acid or lactic acid is present. The concentrations were calculated based on the Henderson-Hasselbalch equation and assuming the initial pH values do not change. The theoretical concentration of dissociated acids (anions) would reach 29.16 M acetic acid and 194.35 M lactic acid under the stated conditions, illustrating the higher capacity of lactic acid to release protons. In most situations, decreases in pH_i also influence the values shown.

Maintaining cytoplasmic pH is essential for optimal cellular metabolism, growth and proliferation (Casey, Grinstein, & Orlowski, 2010). Several studies have shown that the ability to maintain pH_i is crucial for acid stress resistance (Arneborg, Jespersen, & Jakobsen, 2000; Halm, Hornbæk, Arneborg, Sefa-Dedeh, & Jespersen, 2004). The pH differences obtained by the modulation of the ergosterol composition of liposomal membranes (0.41 and 0.25 pH units in the presence of acetic and lactic acid, respectively, for the best performing setting) have the potential to be

essential for an efficacious cellular response to organic acid stress. Srivastava and co-workers (2007) reported that changes of pH_i within a range of 0.2 to 0.3 units function as a signaling mechanism to regulate a number of cellular processes such as cell cycle progression, proliferation/apoptosis and differentiation.

Ergosterol is essential for maintaining the correct structure of the plasma membrane. The accumulation of ergosterol may protect the cell membrane against acid stress (Vanegas, Contreras, Faller, & Longo, 2012). Importantly, we observed *in vitro* that the effect of ergosterol changes with the organic acid in question. An enrichment with 30 mol% of ergosterol was required to maintain a higher pH_i in the presence of acetic acid, whereas 10 mol% was enough to maintain a higher pH_i in the presence of lactic acid. Interestingly, a larger quantity did not show the same effect. As there are no similar studies *in vitro* as comparison, it is not trivial to comment our findings. One possibility could be that the quantity of ergosterol needed to have a protective effect against acid diffusion is higher in the case of acetic acid. This might be in agreement with an *in vivo* studies where the authors demonstrated that at low pH the permeability of the yeast membrane to acetic acid is much higher than to lactic acid (Casal, Cardoso, & Leão, 1996). Therefore, it is rational and consistent that a higher amount of ergosterol is needed to decrease the permeation of acetic acid through the liposomal membrane than lactic acid. Nonetheless, further studies are necessary to better describe these finding, also because it is not clear why the diffusion of lactic acid is minimal with an addition of 10 mol% of ergosterol, but increases again with 30 mol%. This indicates that an increasing ergosterol abundance is not straight forward proportional with a decreased diffusion

of organic acids in general, but also that the effect depends on the type of organic acid.

From *in vitro* to *in vivo*: overexpression of *ECM22* in yeast cells changes the ergosterol content

Given the significant differences, correlating with different incorporation of ergosterol in the liposomal membrane, we attempted to modulate the ergosterol composition of a *S. cerevisiae* strain to test the effects *in vivo*. The plasma membrane is a complex and dynamic system, whose behavior is challenging to predict due to the connections, competitions, dependencies, or other types of interactions between its components. For this reason, it is not trivial to predict which element(s) should be changed to trigger a specific rewiring of the overall system. Multiple genetic modifications are often required to unlock phenotypes of interest. Therefore, engineering a desired phenotype would be facilitated by simultaneous multiple gene modifications. In the case of ergosterol, the overexpression of a few enzymes has revealed to be insufficient to modulate the ergosterol metabolism (He, Guo, Liu, & Zhang, 2007; He, Zhang, & Tan, 2003; Polakowski, Bastl, Stahl, & Lang, 1999). Given the ability of transcription factors to regulate multiple genes, they can be recruited to modulate entire metabolic pathways, such as the sterol biosynthesis in *S. cerevisiae*. We focused on the transcription factor encoded by the *ECM22* gene. Ecm22 acts as the main regulator of ergosterol biosynthesis by binding to sterol regulatory elements in the promoter of ergosterol biosynthesis (ERG) genes (Vik & Rine, 2002). Hypothesizing a pivotal role of Ecm22 in the overall ergosterol composition of *S. cerevisiae*, we

overexpressed *ECM22* and investigated the ergosterol levels of the two different strains: CEN.PK 102-5B WT and CEN.PK 102-5B *ECM22*.

The mRNA levels of the *ECM22* gene, measured in exponentially growing cells **(Figure 3 A**), were almost five-fold higher in the overexpressing strain, CEN.PK 102-5B *ECM22*, compared to the wild-type (WT) strain.

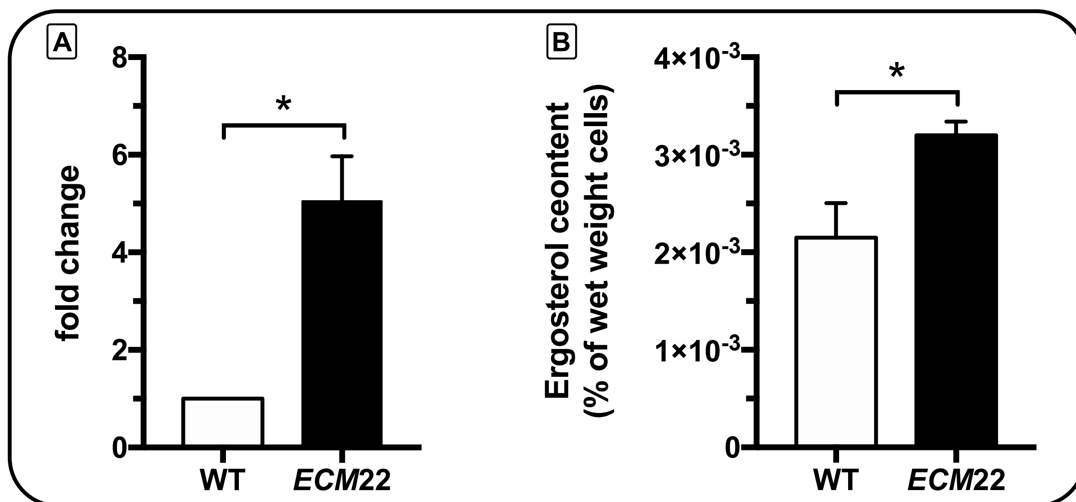

Figure 3: A) Expression levels of *ECM22* in the CEN.PK 102-5B WT (white bar) and CEN.PK 102-5B *ECM22* (black bar) strains analysis during exponential growth phase. B). Ergosterol content in CEN.PK 102-5B WT (white bar) and CEN.PK 102-5B *ECM22* (black bar) strains. (*) *$p<0.05$.

The ergosterol analysis (**Figure 3 B**) revealed differences between the WT and CEN.PK 102-5B *ECM22* strain, the latter revealing an increased ergosterol content of almost 50% (**Figure 3 B**). The increased levels of ergosterol caused by the overexpression of *ECM22* have also been evidenced indirectly by Wang and collaborators (2018).

Effect of the overexpression of *ECM22* on organic acids tolerance

Sterols play a crucial role in the architecture of the plasma membrane, namely by ordering fatty acyl chains allowing tighter lipid packing and thus reducing membrane permeability. A less permeable plasma membrane might be able to reduce the permeation of organic acids into the cells, contributing to other counteraction mechanisms, and therefore resulting in more tolerant strains. The different ergosterol content observed between the strains opened the question of a possible effect of these changes on the cellular response to organic acids stress, and about the possibility to compare the data with these obtained with the *in vitro* assay. We investigated the effect of *ECM22* overexpression on the growth of cells challenged with acetic or lactic acid, at low pH, and in particular at pH 3 as this value revealed significant differences in the *in vitro* assay. Firstly, strains were used for a drop test on solid minimal media with 2% w/v glucose at pH 3 in the presence or absence of organic acids (**Figure 4**). Acetic acid is more toxic to yeast cells than lactic acid (Narendranath, Thomas, & Ingledew, 2001). Therefore, to impair yeast growth, the concentration of lactic acid used the in the following studies was increased, similar to the work of Berterame and co-authors (2016)**.**

Strains	Minimal synthetic medium	Minimal synthetic medium + 65 mM acetic acid	Minimal synthetic medium + 555 mM lactic acid
Dilution factor	10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4}
CEN.PK102-5B WT			
CEN.PK102-5B *ECM22*			

Figure 4: Spot assay of CEN.PK 102-5B WT and CEN.PK 102-5B *ECM22* cells in the absence and presence of acetic (65 mM) or lactic (555 mM) acids, pH 3 at 30 ºC. The images were taken after 72 h of growth.

In the absence of organic acids, no differences between the parental and the mutant strain were observed. Similarly, despite the addition of acetic acid had a negative effect on the growth of both strains, there are no significant differences among them. The addition of lactic acid also impaired the growth of both strains. However, under this condition, differences in the growth between the strains were observed. The strain CEN.PK 102-5B *ECM22*, with a higher content of ergosterol, demonstrated an increased growth capacity in comparison with the CEN.PK 102-5B WT strain.

To further characterize the consequences of the stress imposed by the organic acids, we performed growth kinetics experiments in shake flasks (**Figure 5**). In the absence of stress, no differences were found between the parental and the mutant strain, none of the strains exhibited lag phase and the maximum specific growth rate was 0.15 and 0.16 h^{-1} from the wt and the overexpressing strain, respectively (**Figure 5 A**). Consistently with drop test results, the addition of acetic acid had a negative impact on growth, leading to an increased lag phase. However, after approximately 20 hours both strains were able to resume growth in a similar way, the maximum specific growth rate was 0.04 h^{-1} for both the strains (**Figure 5 B**). Lactic acid had a strong negative effect on the growth of both strains. The CEN.PK 102-5B WT was not able to resume growth for the entire period of observation, while the strain overexpressing *ECM22* was able to resume growth after a long lag phase (**Figure 5 C**). These results indicate that the increased levels of ergosterol revealed to be crucial to improve *S. cerevisiae* tolerance towards lactic acid stress.

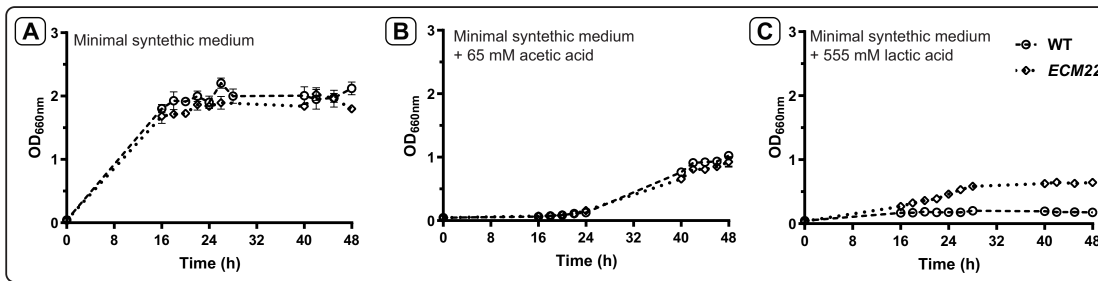

Figure 5: Growth of CEN.PK 102-5B WT and CEN.PK 102-5B *ECM22* cells in the absence and presence of acetic or lactic acid. Yeast cells were grown in shake flasks in minimal synthetic media with 2% w/v glucose at pH 3, without (panel A) or with (panel B) 65 mM of acetic acid or (panel C) 555 mM of lactic acid. Growth was assessed by measuring the OD at 660 nm. CEN.PK 102-5B WT (open circles -○-) and CEN.PK 102-5B *ECM22* strains (open diamonds -◇-). Values are the mean of three independent experiments. Standard deviations are included. For some points, the error bars are shorter than the height of the symbol, in these cases, the error bars are not visible.

One important mechanism for yeast cells to counteract stress caused by organic acids is by modulating the plasma membrane lipid composition (Mira et al., 2010). Recently, Guo and collaborators (2018) performed a comparative analysis of lipids in *S. cerevisiae* grown in the presence of four different organic acids, acetic, formic, cinnamic and levulinic acid. The authors observed that *S. cerevisiae* adapts its lipid composition differently according to the organic acid present in the media. In particular, during growth on glucose in the presence of the organic acids studied, levels of ergosterol increased to different extents according to the organic acid in question, revealing a specific response.

The importance of ergosterol has also been associated with the remarkable resilience against acetic acid stress of the yeast *Zygosaccharomyces bailii*. *Z. bailii* is a food spoilage yeast that is typically isolated as a contaminant during wine fermentation, as well as from many acidic, high-sugar and canned foods (Kuanyshev, Adamo, Porro, & Branduardi, 2017). Lindberg

and co-authors (2013) investigated the lipidomic response of *Z. bailii* to acetic acid stress. Differently from *S. cerevisiae*, this yeast is capable of maintaining its intracellular pH and plasma membrane integrity upon acetic acid stress (Fleet, 1992). Lipid analysis revealed *Z. bailii*'s ability to maintain high levels of ergosterol, upon acetic acid exposure, as one of the main reasons for its high tolerance. Our data suggest that the increased level of ergosterol obtained is enough to boost *S. cerevisiae*'s tolerance to lactic acid stress but not to acetic acid. In fact, the results obtained with the liposomal membranes (**Figure 1**) suggest that higher levels of ergosterol are required to decrease the diffusion of acetic acid than lactic acid. We speculate that the ergosterol levels obtained in the *ECM22* overexpressing strain were high enough to diminish the lactic acid diffusion into the cell, but had no effect on acetic acid.

Plasma membrane response to lactic acid stress

Given the growth differences observed, we tried to understand, at the plasma membrane level, if there were differences that could explain the increased tolerance to lactic acid of the overexpressing strain. Lipid peroxidation is another of the reported effects of the organic acids counter-anions on *S. cerevisiae* cells (Mira et al., 2010). Lipid peroxidation is a sudden molecular rearrangement that starts with the attack of a radical Reactive Oxygen Species (ROS) to a double bond of a polyunsaturated fatty acid, resulting in the formation of radical polyunsaturated fatty acids. These species, due to their high reactivity, can lead to the formation of several products, including malondialdehyde (MDA), which can be used as an index of lipid peroxidation level. Here, we were interested to determine if lipid peroxidation can occur after sudden exposure to organic acids, and how

wild-type and engineered strains could react. We observed that lactic acid stress correlates with a significant increase in peroxidized lipid content when compared with the control condition (**Figure 6 A**), with no significant differences between the two strains. So far, it remains to be elucidated how membrane remodeling influences the fatty acid composition and degree of saturation and unsaturation. Literature analysis seems to indicate that the relative ratio of the degree of unsaturation of fatty acids varies widely among species, between strains of the same species, with environmental conditions and in respect to the different organic acid tested. Guo and collaborators (2018) reported that the exposure of *S. cerevisiae* cells to the acetic, formic and levulinic acids leads to a continuous increase in the unsaturation index of fatty acids. Differently, authors reported that the exposure to cinnamic acid did not affect the saturation index. Berterame and co-authors (2016), using a different *S. cerevisiae* background, observed a reduction of lipid peroxidation after cells exposure to lactic acid. Authors speculated that unsaturated membrane lipids decreased in favor of saturated ones after acid treatment.

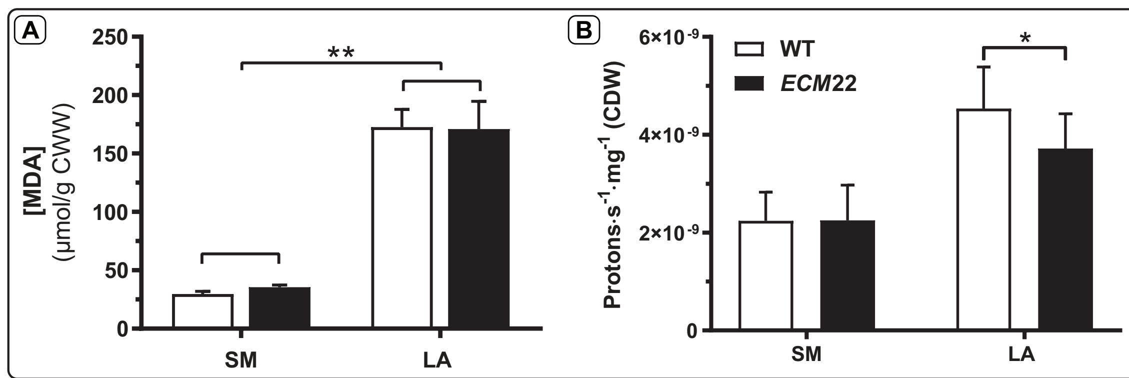

Figure 6: Effect of lactic acid pulse on A) lipid peroxidation, measured as MDA levels, and B) rates of the net proton efflux. Cells were grown in minimal synthetic media (pH 3) until the exponential phase and then were treated (LA) or not (SM) with a 30 minutes pulse of lactic acid 555 mM at pH 3. CEN.PK 102-5B WT (white bars), CEN.PK 102-5B *ECM22* (black bars). (*) *p<0.05 and **p<0.01.

Plasma membrane proteins represent around 40% of the membrane composition. A total of 1000 different proteins are estimated to be located in the yeast plasma membrane (Stewart, 2017). The abundance and activity of membrane proteins changes according to different cellular stimuli. Remarkably, the activity and stability of membrane proteins is dependent on the lipids that surround them (Coskun & Simons, 2011). Membrane proteins require specific lipids, as cofactors for their functions or as "co-structures" for their correct folding and stability. Therefore, the composition of the lipid bilayer must be optimal for obtaining the correct activity or the desired biological function of the membrane proteins (Lee, 2004). In this work, we are particularly interested in understanding how the plasma membrane ergosterol composition can influence *S. cerevisiae* robustness towards acetic and lactic acids. Nonetheless, intracellular acidification is considered one of the major causes of growth inhibition for *S. cerevisiae* cells upon organic acid stress (Ullah, Orij, Brul, & Smits, 2012) and, to counteract this acidification, cells strongly rely on the proper

functioning of the proton pump H^+-ATPase to maintain the intracellular pH close to neutral (Ferreira et al., 2001). Pma1, the major proton pump present in the yeast plasma membrane, is localized at plasma membrane lipid rafts. Lipid rafts are heterogeneous, highly dynamic, ergosterol and sphingolipid enriched domains that compartmentalize cellular processes and house several membrane proteins (Ferreira et al., 2001; Pike, 2006). The importance of this proton pump for yeast cells under different stress conditions has been highlighted in several studies (Lee et al., 2015, 2017; Serrano, Kielland-Brandt, & Fink, 1986). Eisenkolb and collaborators (2002) suggested that the correct positioning and activity of Pma1 into plasma membrane lipid rafts has been correlated with the presence of sterols. When we measured the effect of lactic acid stress on proton extrusion by assessing the extracellular acidification rate, we observed an increased flux of protons in both strains, with respect to the control condition (**Figure 6 B)**. Our results show that, under lactic acid stress, the proton efflux rate was higher in the CEN.PK 102-5B WT strain than in the CEN.PK 102-5B *ECM22*. Maintenance of pH_i homeostasis can be energetically expensive, resulting in the membrane H^+-ATPase consuming between 40 and 60% of the total cellular ATP (Holyoak et al., 1996). Therefore, the maintenance of pH_i homeostasis in the presence of organic acids may deplete cellular ATP levels significantly. Based on our observation, it is possible to speculate that the higher levels of proton efflux observed in the wild-type strain, depleting ATP, consequently preventing growth. These results could justify the growth resumption shown by the CEN.PK 102-5B *ECM22* strain in the presence of lactic acid (**Figure 5 C**).

Conclusion

In this work, using *in vitro* and *in vivo* complementary approaches, we demonstrate that a modulation of the ergosterol content is crucial for *S. cerevisiae* to gain tolerance towards organic acids. Synthetic lipid vesicles were used to characterize the role of ergosterol on organic acid diffusion through different membrane compositions. This allowed us to further comprehend the structural role of ergosterol in the yeast plasma membrane, independently of yeast metabolism. Given the promising indications observed, we evaluated the response to acetic and lactic acid of *S. cerevisiae* mutant strain with different ergosterol compositions. Increased ergosterol levels in a *S. cerevisiae* mutant strain resulted in an increased tolerance towards lactic acid stress, but not acetic acid stress. This is in line with *in vitro* observation that a moderate increase of the ergosterol content decreases lactic acid diffusion through the membrane, but not acetic acid diffusion. To decrease acetic acid diffusion, a large increase of the ergosterol content was required, which was interestingly not suitable to inhibit lactic acid diffusion. Therefore, both the *in vitro* and *in vivo assay* indicated that the same membrane composition led to different outcomes in the presence of different organic acids. Membrane engineering strategies must be specific for different kinds of acids.

Materials and methods

Liposomes preparation

Liposomes were prepared by an adapted DRV (Dried reconstituted vesicle) method (Suleiman et al., (2019), initially developed by Kirby & Gregoriadis (1984). Yeast lipid total extract was purchased from Avanti Polar Lipids and ergosterol from Sigma-Aldrich. The standard amount of ergosterol present in Yeast Lipid Total extract is not reported by the company. The desired amount of yeast lipid extract and ergosterol were transferred into a rotating round bottomed flask and the organic solvents were evaporated, at 60 ºC in a water bath at reduced pressure until a dried lipid film was generated. Liposomes also incorporate Biotin-X-DHPE(N-((6-(Biotinoyl)amino)hexanoyl)-1,2-dihexadecanoylsn-glycero-3-phosphoethanolamine, triethylammonium salt), to allow binding to streptavidin coated plates, used to study the diffusion of organic acids into the liposomes. The lipid film was rehydrated with 2 mL of 10 mM Na-HEPES buffer solution (pH 7.4) containing 500 µM of Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt, HPTS). Rehydration of the lipid film with buffer, as well as, liposome size reduction by extrusion was performed at 60 ºC. The final lipid suspension (5 mg/mL) was extruded on a LIPEX™ Extruder (Northern Lipids Inc., Burnaby, BC, Canada) equipped with Whatman® Nuclepore track-etc0hed membranes. Up to ten extrusion cycles with 800, 400 and 200 nm membranes were performed, in order to obtain homogeneous suspension. Five different liposome formulations were prepared, consisting of either pure yeast lipid total extract (100 mol%) or enriched with different concentrations of ergosterol: 3, 10, 20 or 30 mol %. The resulting suspension was stored at 4 °C. The liposome mean diameter and the homogeneity were measured by DLS (dynamic light

scattering) with a Zetasizer Nano-ZS with software version 7.03 (Malvern Instruments, Malvern, UK) at 25 °C. Liposomal suspensions were diluted 100-fold with double distilled water prior the measurement. Each suspension was measured 3 times, and each consisted of 15 repetitions. Size distribution was calculated by the intensity report. The homogeneity of the liposomes was determined by the polydispersity index (PdI).

Pyranine pH calibration

A pH calibration curve was established for the ratiometric fluorophore pyranine. Pyranine solutions (500 µM) were prepared in 10 mM Na-HEPES buffer in the pH range from 3 to 8. The fluorescence intensity upon excitation at both 405 and 453 nm, emission ratio at 510 nm, was recorded using the Infinite M200 plate reader (Tecan). The ratio 453/405 nm was calculated for each pH. Later, equation (1) ($y = 2 \times 10^{-5} e^{1.485\,x}$) was used to convert the measured ratio to pH values. The pH determination using pyranine is based on ratiometric measurements between two fluorescence excitations: one at 453 nm, which is pH dependent, and the other at 405 nm, which is a pH-independent isosbestic point. Therefore, the total amount of the fluorophore does not influence the pH calculation.

Liposomes exposure to acetic and lactic acids

Streptavidin-coated 96-well plates (Thermo Scientific) were used to study the impact of organic acids on liposomes internal pH. Wells were washed with 10 mM Na-HEPES buffer (pH 7.4). After, 50 µL of biotinylated liposomes were added to the wells and incubated at room temperature for 30 minutes, with shaking. After the binding step, wells were washed with 10 mM Na-HEPES buffer (pH 7.4), to remove unbound liposomes and traces of pyranine. The fluorescence (emission at 510 nm) of the bound liposomes

was measured in the M200 plate reader (Tecan) and the ratio between excitation 453nm and 405 nm was converted to pH using equation (1). Next, organic acid solutions, 65 mM at different pH values, were added to the wells containing the liposomes. The pH of the organic acids solutions was adjusted to the desired pH by the addition of KOH or HCl 1 M. Incubation with organic acids was done at room temperature for 5 min, with shaking. Finally, M200 plate reader (Tecan) was used to monitor the fluorescence intensity changes caused by the addition of organic acids to the wells. Final pH values inside the liposomes were obtained using equation (1).

Yeast strains, transformation and growth conditions

The *S. cerevisiae* strain CEN.PK 102-5B [*MATa his3Δ1, ura 3–52, leu2-3,112 MAL2–8c SUC2*] was transformed with the integrative plasmids pYX022 and pYX042. This strain was used in this study for the overexpression *ECM22*, encoding the transcription factor *ECM22* (**Table 2**). Yeast transformation was performed according to the LiAc/PEG/ss-DNA protocol (Gietz, 2014). For each set of transformation, at least three independent transformants were tested, showing no significant differences among them. All the strains used and created in this work are listed in **Table 2**. Yeast cultures were grown in minimal synthetic media (0.67% w/v YNB media with ammonium sulphate; cat. No. 919-15, Difco Laboratories, Detroit, MI, USA) with 2% w/v D-glucose as carbon source. Uracil and the required amino acids were added to a final concentration of 50 mg/L. For the kinetics of growth, acetic and lactic acid stress was imposed by adding the desired amount of the organic acids (Sigma Aldrich) to the culture media. The final media were prepared starting from different stock solutions, 1.54 mol of acetic acid, 2.22 mol of lactic acid and synthetic minimal media 2X. The pH of the organic acids stock solutions and of the culture media was adjusted to 3 by

the addition of KOH or HCl 1 M. Cell growth was monitored by measuring the OD at 660 nm at regular time intervals and cells were inoculated at an initial OD of 0.05. All cultures were incubated in shake flasks at 30 °C degrees and 160 r.p.m.

Gene amplification and plasmid construction

All the primers used in this work are listed in **Table 3**. Endogenous *ECM22* was deleted by the insertion of a *KanMX* cassette that integrates into the genome by homologous recombination, precisely replacing the target coding sequence. The *KanMX* resistance cassette was amplified from pUG6 (Güldener, Heck, Fiedler, Beinhauer, & Hegemann, 1996) with the primers Kan_fw and Kan_rv, design with specific flanks complementary to the upstream and downstream regions of the *ECM22* gene to allow homologous recombination and used to transform the strain CEN.PK 102-5B. Transformed clones were selected in yeast extract/peptone/dextrose (YPD) agar plates supplemented with the antibiotic G418 (Merck Millipore, Billerica, Massachusetts, USA) at the final concentration of 0.5 mg/mL. Correct integration of the cassette into the genome was confirmed by PCR analysis with the primers Del_fw and Del_rv (**Table 3**). After, *ECM22* gene sequence was amplified by PCR, with primers Ecm_fw and Ecm_rv (**Table 3**), using genomic DNA from CEN.PK strain as template, extracted by standard methods (Maniatis, Fritsch, & Sambrook, 1988). The obtained fragment was digested with *Sma*I and *Xho*I and then ligated to YCpLac33 (Gietz & Akio, 1988) previously digested with *Sma*I and *Xho*I and dephosphorylated. Clones were confirmed by enzymatic digestion. The obtained plasmid, in which the *ECM22* gene is under the control of the *TPI* promotor (TPIp), was named YCplac33_*ECM22*. All the restriction enzymes utilized are from NEB (New England Bio-labs, UK). The plasmid

YCplac33_*ECM22* was transformed in the CEN.PK 102-5B, deleted for the endogenous *ECM22*, to obtain the strain CEN.PK 102-5B *ECM22*. The starting strain, CEN.PK 102-5B, was transformed with the empty YCpLac33 plasmid, named CEN.PK 102-5B WT and used as control. Both CEN.PK 102-5B WT and CEN.PK 102-5B *ECM22* carry the plasmids pYX022 and pYX042 for auxotrophic complementation.

Table 2: List of strains and plasmids used in this work.

Parental strain		
Strains	**Genotype**	**Source**
CEN.PK 102-5B	*MATa*, *ura 3-52*, *his3-11*, *leu2-3/112*, *TRP1*, *MAL2-8c*, *SUC2*	(Entian & Kötter, 1998)
Transformed strains		
Strains	**Plasmids**	**Source**
CEN.PK 102-5B WT	pYX022, pYX042, YCplac33	This work
CEN.PK 102-5B *ECM22*	pYX022, pYX042, YCplac33_*ECM22*, YLR228C::*kanMX*	This work
Plasmids	**Segregation**	**Markers**
pYX022 (R&D Systems, Wiesbaden, Germany)	Integrative	*HIS3*
pYX042 (R&D Systems, Wiesbaden, Germany)	Integrative	*LEU2*
YCplac33 (GenBank accession numbers X75456, L26352)	Centromeric	*URA3*
YCplac33_*ECM22* This work	Centromeric	*URA3*

Table 3: List of primers used in this work.

Name	Sequence (5'-3')
Kan_fw	ATGACATCCGATGATGGGAATGCTGGACAAGAAAGAGAGAAGGATGCTGAGACATGGAGGCCCAG
Kan_rv	TTACATAAAAGCTGAAAAGTTTGTAGTGGTCATAGAAGGTAACCCACCTCCAGTATAGCGACCAGCATT
Del_fw	CCTCGTTCTTGTCGGAAAA
Del_rv	TTGATGCTCGATGAGTTTTTCTAA
Ecm_fw	AGATTCCCGGGATCTAACATAACATGACATC
Ecm_rv	AGGCTCTCGAGTTACATAAAAGCTGAAAAGT
RtEcm_fw	CCAGGGAATTCGCCATTGAG
RtEcm_rv	CTGCTGCAATCCATTCGTCA
RtAct_fw	CATTGCCGACAGAATGCAGA
RtAct_rv	ACGGAGTACTTACGCTCAGG

Reverse transcription quantitative PCR

Total RNA was extracted from the transformant strain–CEN.PK 102-5B *ECM22* and from the wild-type strain, CEN.PK 102-5B WT, using the kit ZR Fungal/Bacterial RNA Miniprep (Zymoresearch/The epigenitics company). Strains were grown until exponential growth phase in minimal synthetic media with 2% w/v glucose. The retrotranscription to obtain cDNA was performed using the kit iScriptTM cDNA Synthesis (Bio-Rad Laboratories, Inc.). The obtained cDNA was used to perform a reverse transcription quantitative PCR using SsoFastTM EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories,Inc.) with the specific primers RtEcm_fw and RTEcm_rev (**Table 3**), according to the manufacturer's instructions. Expression levels of *ECM22* were normalized to the expression of the Actin housekeeping gene. Relative expression was calculated using the DDCt method.

Ergosterol quantification in yeast cells

Total sterols were extracted as described before **(**Arthington-Skaggs, Jradi, Desai, & Morrison, 1999**)** with slight modifications. Briefly, 2 mL overnight culture from single colonies were used to inoculate 50 mL of minimal synthetic media with 2% w/v glucose, overnight. Exponential-phase cells were collected by centrifugation at 2700 r.p.m for 10 minutes and washed twice with distilled water. Cell densities were measured based on OD_{660nm} of the cultures and equal amounts of cells were processed for sterol extraction. 5 mL of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 mL of sterile distilled water, brought to 100 mL with 96% ethanol) was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to glass tubes and were incubated in a 90 °C water bath for 2 hours. Following incubation, the tubes were allowed to cool to room temperature. Sterols were then extracted by addition of 5 mL of n-hexane followed by centrifugation at 2700 r.p.m for 5 minutes to facilitate phase separation. The hexane layer was transferred to a clean tube and scanned spectrophotometrically between 240 and 300 nm against a hexane blank with a V-770 UV-Visible/NIR spectrophotometer. The presence of ergosterol in the extracted sample resulted in a characteristic four-peak curve. The ergosterol content was determined using equations previously reported by (Demuyser, Van Dyck, Timmermans, & Van Dijck, 2019).

Spot assay

Spot assays were performed by cultivating yeast cells in minimal synthetic media with 2% w/v glucose. Cells were cultivated until mid-exponential phase and then diluted to an OD_{660nm} of 0.5. Four 1:10 serial dilutions were made and spotted on solid minimal synthetic media containing agar (2% w/v) and 2% w/v glucose, supplemented with acetic or lactic acid. The pH

of the organic acids stock solutions and of the culture media was adjusted to 3 by the addition of KOH or HCl 1M. Plates were incubated for 72 hours at 30 °C.

Evaluation of lipid peroxidation

Cells were grown until exponential phase in minimal synthetic media (pH 3) with 2% w/v glucose, collected and transferred to flasks containing lactic acid (555 mM), adjusted to pH 3. Cells were incubated at 30 °C and 160 r.p.m. for 30 minutes. An estimation of lipid peroxidation was based on the level of malondialdehyde formed after incubation in minimal synthetic media with or without lactic acid, as described by Fernandes and collaborators (2000). Briefly, after treatment with or without lactic acid, cells were collected, resuspended in 100 mM Tris pH 7.8 and broken by glass beads. After centrifugation, the supernatant was collected and 250 µL of the extract were mixed with 500 µL of the mix TBARS (15% w/v trichloroacetic acid, 0.375 % w/v thiobarbituric acid, 0.25N hydrochloric acid). The solution was heated for 1 hour in a thermomixer. The absorbance of the sample was determined at 535 nm against a blank that contained all the reagents, except the extract. Results are expressed as micromoles of malondialdehyde per gram of wet weight biomass.

Proton movements assays

S. cerevisiae cells were grown until exponential-phase in minimal synthetic media with 2% w/v glucose, at pH 3. Cells were harvested and inoculated in minimal synthetic media with 2% w/v glucose containing 555 mM of lactic acid (pH 3) for 30 minutes. Cells were again harvested, washed with distilled water and resuspended in distilled water with a final concentration of 50 mg cell dry weight mL^{-1} and kept on ice. Proton movements were measured,

at room temperature, by recording the pH of cell suspensions with a standard pH meter (PHM 82; Radiometer) connected to a potentiometer recorder (BBC-GOERZ METRAWATT, SE460). The pH electrode was immersed in a water-jacketed chamber of 10 mL capacity with magnetic stirring. To the chamber, 4.5 mL water and 0.5 mL yeast suspension were added (Leão & Van Uden, 1984). For the measurement of proton efflux, the initial pH was adjusted to 5 using HCl (100 mM) and a baseline was established. The addition of 2% w/v glucose triggered H^+ efflux, leading to an acidification of the extracellular environment. When glucose is added to *S. cerevisiae* cells, protons are extruded after a delay of 10–30 seconds and the external pH drops, the rate constants were calculated from the maximum instant slope of the records. The rate of acidification, calibrated with 10 mM HCl, was taken as a measure of proton extrusion activity.

Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism 7. In all the assays, means and standard deviations were determined based on 3 independent experiments (n = 3). Results were compared using t-test (Real Time quantitative PCR; Evaluation of lipid peroxidation; Proton movements assays) and one-way ANOVA, with Turkey's multiple comparison statistical test (Liposomes exposure to organic acids). All tests were performed with a confidence level of 95%.

Supplementary Material

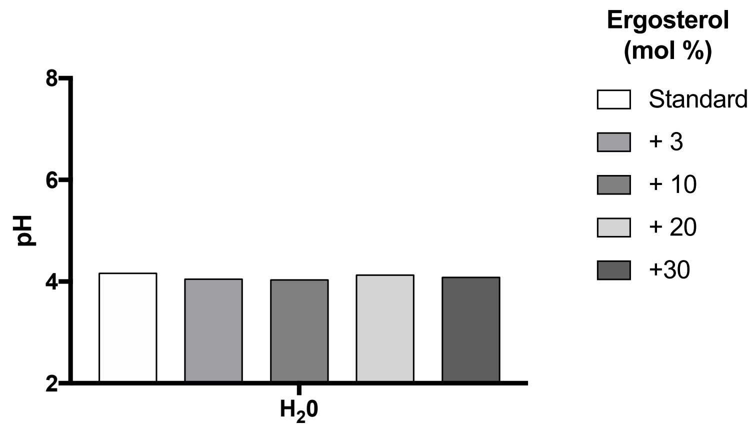

Figure 1: Internal pH (pH_i) of the different liposomes after exposure to water at pH 3. The initial pH inside the liposomes was 7.4.

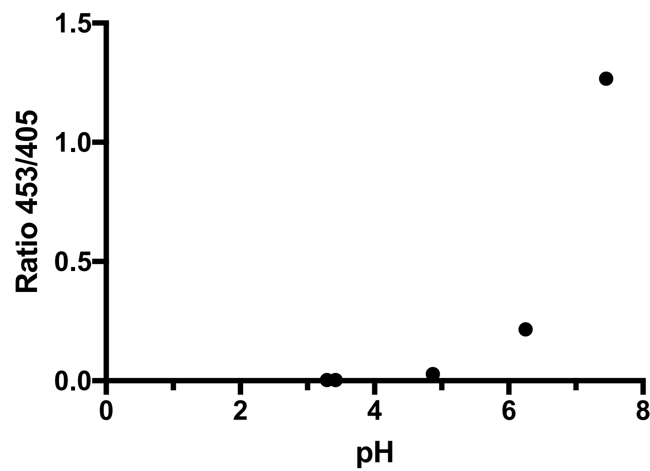

Figure 2: pH calibration curve established for the ratiometric fluorophore pyranine.

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Effect of *ECM22* overexpression on *Saccharomyces cerevisiae* lactic acid production strains

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Abstract

The use of polylactic acid (PLA) for the creation of biodegradable plastics has sparked a lot of interest in the production of highly pure lactic acid enantiomers. Despite not being natural producers, yeasts can be engineered and used as an alternative to lactic acid bacteria for lactic acid synthesis since they can handle acidic environments better. Nonetheless, the accumulation of high concentration of lactic acid is harmful for the cells and therefore a limiting step in the fermentative process. Given the importance of plasma membrane in organic acid tolerance, we investigated the possibility of changing the plasma membrane lipid composition to increase yeast tolerance to lactic acid and consequently increase the performances of lactic acid production. To do so, we focused on ergosterol, the major sterol present in yeast, and crucial for maintaining plasma membrane integrity, fluidity and permeability. The *ECM22* transcription factor, acting as the main regulator of ergosterol biosynthesis, was overexpressed to increase the ergosterol content in two homolactic producing strains optimized for lactic acid production at high yield and titer. Phenotypic characterization, by spot assays, revealed that *ECM22* overexpression resulted in increased tolerance to lactic acid stress. However, the production of lactic acid was unaffected, both in terms of titer and yield.

Introduction

Lactic acid (LA) is a chemical platform with a wide range of industrial applications such as in the food, pharmaceutical, cosmetics or textile sectors. The production of highly pure lactic acid enantiomers has received a lot of attention because of the application of the derived polymer, polylactic acid (PLA) for the production of biodegradable plastics (Castro-Aguirre, Iñiguez-Franco, Samsudin, Fang, & Auras, 2016). LA can be produced either by chemical synthesis or by microbial fermentation, being the second of interest because of environmental friendliness and of using renewable resources instead of petrochemicals (Ajala, Olonade, Ajala, & Akinpelu, 2020). Furthermore, microbial fermentation is very attractive for lactic acid production since it allows to obtain pure isomers (L(+)- or D(−)-LA) instead of racemic mixture (DL-LA), usually obtained by chemical synthesis (Di Lorenzo, Serra, Porro, & Branduardi, 2022).

Currently, industrial scale lactic acid fermentations are performed mainly using lactic acid bacteria (LAB) (Rawoof et al., 2021). However, pH plays a critical role in the lactic acid production as the undissociated form of the acid is the preferred one, especially for its use as a monomer for PLA production. Therefore, the final pH of the medium must be below the pK_a of the acid, which is 3.78. Importantly, at low pH, most LABs show irreversible damage to their metabolic functions, compromising the fermentation process (Okano, Tanaka, Ogino, Fukuda, & Kondo, 2010). In order to maintain the pH of culture broth constant (around 5), large amounts of $CaCO_3$ are added during the fermentation: this, in turn, requires additional purification steps to obtain the undissociated form of the acid (Alves De Oliveira et al., 2020).

Along with LAB, other microorganisms have been evaluated as potential lactic acid producers. Yeasts in particular have attracted a lot of attention due to their intrinsic ability to tolerate low pH. In 1994, Dequin and Barre described the first metabolically engineered *Saccharomyces cerevisiae* strain expressing a heterologous L-lactate dehydrogenase, obtaining a hetero-fermentative strain producing both ethanol and lactic acid (Dequin & Barre, 1994). Then, many strategies to improve lactic acid production in *S. cerevisiae* have been employed, among which: a) the deletion of pyruvate decarboxylase gene(s), to avoid ethanol production and consequently increase production and yield of lactic acid (Adachi, Torigoe, Sugiyama, Nikawa, & Shimizu, 1998; Ishida et al., 2005; Porro et al., 1999); b) the use of *S. cerevisiae* strains from different genetic backgrounds and different sources of heterologous L-lactate dehydrogenases (Branduardi et al., 2006); c) the of overexpression of the hexose transporters for higher glucose uptake and consequently increased lactic acid production (Rossi, Sauer, Porro, & Branduardi, 2010) and d) the improvement of cellular tolerance traits towards the fermentation process, such as the maintenance of higher intracellular pH (Liu, 2005; Valli et al., 2006). The latter strategy, in particular, is of major importance because, even though *S. cerevisiae* has a high tolerance towards low pH, the accumulation of lactic acid in the fermentation medium, at low pH, exerts a high levels of stress in the cells, representing one of the major bottlenecks in lactic acid production using microorganisms (Halm, Hornbæk, Arneborg, Sefa-Dedeh, & Jespersen, 2004). Valli and co-workers (2006) demonstrated that *S. cerevisiae* strains capable of maintaining a higher intracellular pH are able to produce higher amounts of lactic acid, as they can consume more glucose as a consequence of a higher viability.

Indeed, intracellular pH homeostasis is a critical element in cell viability. In *S. cerevisiae* intracellular pH homeostasis is tightly regulated and among the effectors of this regulation the proton pump ATPase Pma1, present in the plasma membrane, plays a prominent role. When intracellular acidification occurs due to weak acid stress, yeast cells rely primarily on the activity of the H^+ ATPase Pma1 to pump protons out of the cytosol (Mira, Teixeira, & Sá-Correia, 2010). The active expulsion of lactic acid from the cell requires energy, and it is also a futile effort if the undissociated acid re-enters at low external pH. Therefore, cells have developed mechanisms to restrict the amount of passive diffusion and consequently the re-entrance of undissociated lactic acid (Martínez-Muñoz & Kane, 2008). In line with this, the lipid composition of the plasma membrane changes upon weak organic acid stress.

Among the strategy to reduce the diffusion of undissociated acid through the membrane during organic acid stress, *S. cerevisiae* naturally increases ergosterol levels to increase membrane rigidity and reduce membrane permeability (Abe & Hiraki, 2009). Previously, we demonstrated that the overexpression of *ECM22,* encoding a transcription factor described as the main regulator of ergosterol biosynthesis, results in an increase of the ergosterol content of *S. cerevisiae* cells. The obtained mutant, with increased levels of ergosterol, exhibited a higher tolerance towards lactic acid stress, at low pH (Manuscript under revision).

Having this in mind, and with the goal of increasing lactic acid tolerance and consequently the lactic acid production capacity of two engineered homofermentative strains, we investigated the effect of *ECM22* overexpression. Phenotypic characterization of the obtained engineered strains revealed increased tolerance to lactic acid stress upon *ECM22*

overexpression, but this did not result in higher lactic acid titer or yield during the fermentation process of production.

Results and Discussion

Effect of *ECM22* overexpression on ergosterol content and lactic acid tolerance

The strains used in this work are homolactic fermentative cell factories (Liu, 2005). The strain CEN.PK 876, expressing a *Lactobacillus plantarum* lactate dehydrogenase, is deleted in all the *pyruvate decarboxylase* genes (*PDC*) genes, therefore converting the natural alcoholic fermentation into lactic fermentation of pyruvate. The recombinant CEN.PK m850 deriving from the CEN.PK 876, underwent selection, following an adaptive laboratory evolution (ALE), and possesses an improved capacity of lactic acid production (Liu, 2005). However, the underlying molecular changes of this evolved strain were never identified, as this strain was not genome sequenced.

To improve lactic acid tolerance of the lactic acid producing strains CEN.PK 867 and CEN.PK m850, the transcription factor *ECM22* was overexpressed, resulting in the strains CEN.PK 867 *ECM22* and CEN.PK m850 *ECM22*. Ecm22 acts as the main regulator of ergosterol biosynthesis by binding to sterol regulatory elements in the promoter of ergosterol biosynthesis (ERG) genes (Vik & Rine, 2002). Ergosterol, the most abundant sterol in yeast, is an essential component of eukaryotic cells' plasma membrane. Sterols help to maintain membrane integrity, fluidity, and permeability (Dufourc, 2008; Ferreira, Mason, & Slayman, 2001). Additionally, sterols also improve membrane rigidity by arranging fatty acyl chains, allowing for tighter lipid packing (Caron, Mark, & Poger, 2014). A less permeable plasma membrane might be able to limit organic acid influx into the cells and thus, resulting in more tolerant strains. The overexpressing of *ECM22* in *S. cerevisiae* has been previously demonstrated to increase ergosterol levels (Wang, Wang,

Liu, Deng, & Wang, 2018; Ferraz et al., 2022 (manuscript under submission)).

To investigate the effect of *ECM22* overexpression on the two mutant strains, we tested their susceptibility to the antifungal agent nystatin. Nystatin, a polyene macrolide derived from the mold *Streptomyces noursei*, targets ergosterol in the fungal plasma membrane (Ivanov, Ćirić, & Stojković, 2022), destabilizing the whole structure. Strains with reduced ergosterol content have been previously shown to have resistance to nystatin (Ghannoum & Rice, 1999), as the target of the drug is less represented. As example, a strain overexpressing *ERG6* with increased levels of ergosterol, has been reported to be hypersusceptible to nystatin (Zhang et al., 2009).

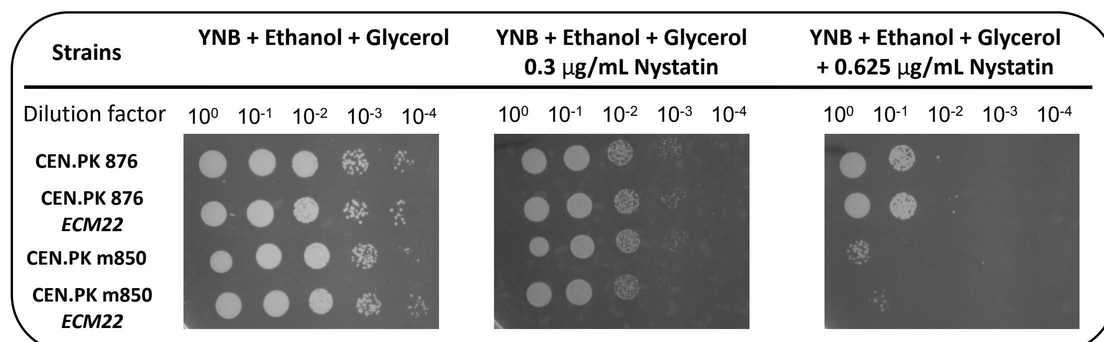

Figure 1: Spot assay of CEN.PK 867, CEN.PK 876 *ECM22*, CEN.PK m850, CEN.PK m850 *ECM22* cells in the absence and presence of Nystatin at 30 ºC. The images were taken after 3 days of growth.

Figure 1 shows the results of a drop test for strains CEN.PK 867, CEN.PK m850, CEN.PK 867 *ECM22* and CEN.PK m850 *ECM22* exposed to nystatin. We observed that in the absence of nystatin, all the strains exhibited the same growth. The same result was observed when a low concentration of nystatin was present in the medium. Differently, in the presence of a higher concentration of nystatin, different phenotypes were observed. The strains

CEN.PK 867 and CEN.PK 867 *ECM22* exhibited the same growth. This result was unexpected as *ECM22* overexpression has been reported to increase the ergosterol levels of the cells. Further characterization will be needed to better elucidate the lipid profile of these 2 strains. However, differences were observed between CEN.PK 867 and CEN.PK m850 strains, and between the strains CEN.PK m850 and CEN.PK m850 *ECM22*.

First of all, the growth of CEN.PK m850 strain is only slightly appreciable if compared to the growth of CEN.PK 867 strains. This might suggest that among the differences that the evolved strain acquired, there could be modification in plasma membrane composition, possibly also comprising higher ergosterol content. Moreover, the growth of the strain overexpressing *ECM22* was lower than that of its parental strains. This result seems to indicate that in the evolved strains it is possible to further increase ergosterol content thanks to *ECM22* overexpression. Nonetheless, also in this case a detailed lipidomic analysis would be required to fully understand the differences between these strains.

Starting from this evidence, in order to investigate the tolerance to lactic acid stress, strains were spotted on solid medium at pH3 in the absence or presence of different concentrations of lactic acid. In **Figure 2,** we observed that in the absence of lactic acid all the strains exhibited the same growth. Differently, the presence of lactic acid significantly impaired the growth of all the strains. In the presence of 25 g/L of acetic, we observed that the 2 *ECM22* overexpressing strains exhibit a higher growth than the two parental strains. The same phenotypes were observed in the presence of 30 g/L of lactic acid. Under these conditions only the *ECM22* overexpressing strains were able to resume growth.

Despite no differences were observed in presence of nystatin (**Figure 1**) between the strains CEN.PK 867 and CEN.PK 876 *ECM22*, significant differences were observed in the tolerance towards lactic acid stress. A detailed lipidomic analysis would be required to explain the different phenotype observed. As for the strains CEN.PK m850 and CEN.PK m850 *ECM22*, consistent with what we observed in the previous paragraph, higher levels of ergosterol resulted in an increase tolerance to lactic acid stress.

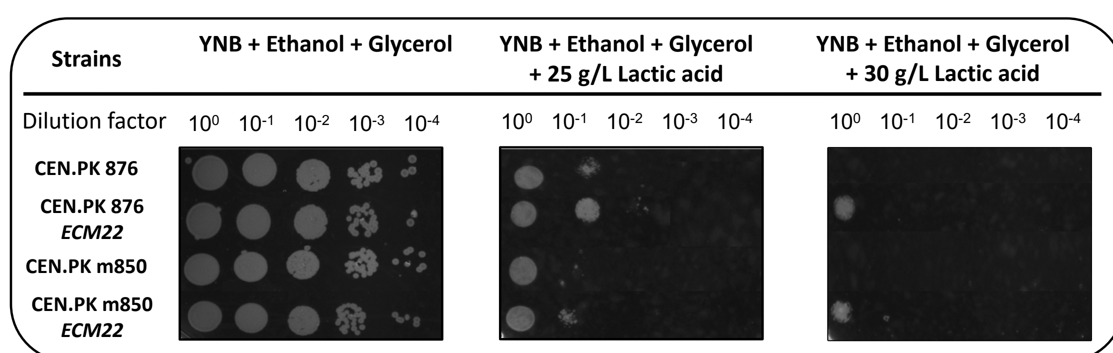

Figure 2: Spot assay of CEN.PK 867, CEN.PK 876 *ECM22*, CEN.PK m850, CEN.PK m850 *ECM22* cells in the absence and presence of lactic acid, pH 3 at 30 °C. The images were taken after 5 days of growth.

The importance of ergosterol in yeast robustness towards lactic acid has been reported before. Berterame and co-workers (2016) previously used Fourier transform infrared (FTIR), a non-invasive technique, to access the biochemical fingerprint of the *S. cerevisiae* cells under lactic acid stress. This technique provides information on the content and structure of cell's major biomolecules, including lipids, proteins, carbohydrates and nucleic acids (Ami, Natalello, & Doglia, 2012). Using this approached, authors observed that *S. cerevisiae* cells under lactic acid stress rearrange the membrane composition towards a more less permeable membrane. Among the changes observed, in the molecular fingerprint of yeast cells, authors found

a direct correlation between *S. cerevisiae* exposure to lactic acid and increased levels of ergosterol, the major sterol present in yeast. Using the same technique, Kuanyshev and co-workers evaluated the physiological and macromolecular changes of *Zygosaccharomyces bailii* upon exposure to lactic acid stress (Kuanyshev et al., 2016). This non-conventional yeast is well known to be responsible for major spoilage losses in the food and beverage industry due to its remarkable ability to grow in harsh conditions, namely in the presence of high concentrations of weak organic acids (Fleet, 2007). Results obtained by Kuanyshev and collaborators (2016) showed that this yeast undergoes larger membrane rearrangements than *S. cerevisiae* upon lactic acid stress. In particular, in later growth stages, an increase in ergosterol composition is also observed, explained as an attempt to make the membrane more compact, therefore reducing membrane permeability.

Lactic acid production by the *ECM22* overexpressing strains

Since the final goal of our studies is to find conditions that can bring advantages to lactic acid production, we tested the effects of *ECM22* overexpression in terms of lactic acid production. It is important to underline that the drop tests were done with cells taken from non-productive conditions. The recombinant strain CEN.PK m850 is a homolactic fermenting cell factory able to produce over 60 g/L lactic acid starting from 80 g/L of glucose at pH values lower than 2.5. CEN.PK m850 parental strain, CEN.PK 876, is only able to produce around 40 to 45 g/L of lactic acid under the same conditions.

For the production of lactic acid, cells were first pre-cultivated for 24 hours in minimal medium with 10 g/L ethanol and 0.5 g/L glucose, containing 0.31 g/L of $CaCO_3$ and 1.5 g/L urea, to obtain biomass. Then, cells were

transferred to a fresh medium containing 5 g/L ethanol, 75 g/L glucose, 4.5 g/L $CaCO_3$ and 1 g/L urea, for the production phase. **Figure 3** reports the fermentation parameters monitored over time: cellular growth, measured as OD_{600nm}, residual glucose and lactic acid, measured by HPLC.

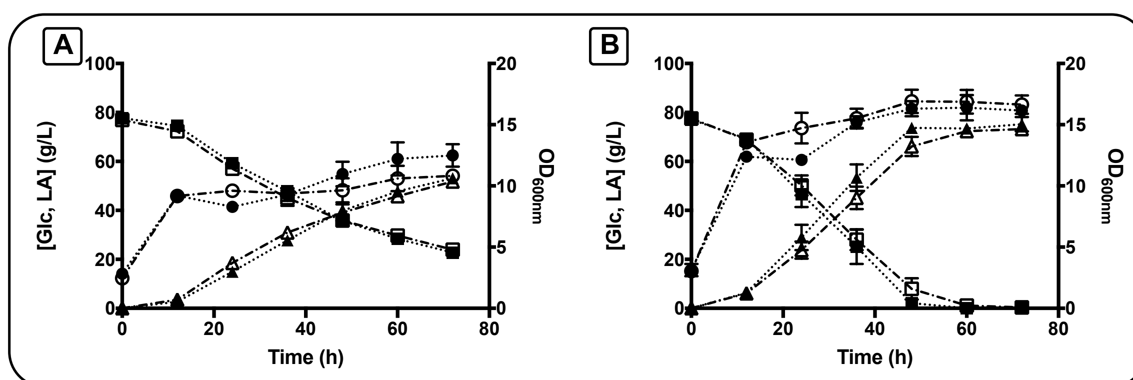

Figure 3: Batch lactic acid production in CEN.PK 876, CEN.PK m850, CEN.PK 876 *ECM22* and CEN.PK m850 *ECM22* strains. (A) Fermentation profiles of CEN.PK 876 (filled symbols) and CEN.PK 876 *ECM22* (open symbols), residual glucose (squares), produced lactic acid (triangles) and Biomass formation (OD_{600nm}) (circles). (B) Fermentation profiles of CEN.PK m850 (filled symbols) and CEN.PK m850 *ECM22* (open symbols), residual glucose (squares), produced lactic acid (triangles) and Biomass formation (OD_{600nm}) (circles).

In the panel A of **Figure 3**, we observed that the strain CEN.PK 876 produces around 50 g/L of lactic acid and was not able to consume all the glucose present in the medium. These results are similar to the results obtained from Valli and co-workers (2006). The overexpression of *ECM22* in the CEN.PK 876 strain had no positive effect on lactic acid production. Looking at **Figure 3 B**, we observed that, differently from CEN.PK 876 and CEN.PK 876 *ECM22*, the CEN.PK m850 and CEN.PK m850 *ECM22* strains are able to consume all the glucose present in the medium after 60 hours of fermentation. This result is also in accordance with previous works. In terms

of lactic acid production, the CEN.PK m850 strain was able to produce around 70 g/L of lactic. However, no differences in lactic acid production were observed in the CEN.PK m850 strain overexpressing *ECM22*. We observed that the final pH of all the fermentation media was around 2.5.

Attempts to improve the lactic acid production of CEN.PK m850 have already been reported. Dato and co-workers (2014) were able to achieve a 5.4% increase in lactic acid production by deleting *SAM2*, a gene involved in the production of S-adenosyl methionine (SAM), an important cofactor involved in phospholipid biosynthesis. In this work, we hypothesize that *ECM22* overexpression could be beneficial to increase the lactic acid production in these strains for two reasons. Firstly, because ergosterol has been identified as crucial for correct membrane permeability and fluidity, as so, an increase in ergosterol content could decrease the flux rate of lactic acid re-entering the cells. Secondly, given the importance of ergosterol for the correct integration and activity of *PMA1* in the plasma membrane, a higher activity of this proton pump could confer the cells an advantage by pumping out protons during lactic acid production. However, even though an increase tolerance to lactic acid for the *ECM22* overexpressing strains was observed in the spot assays, the lactic acid production was not augmented in the fermentation process.

It needs to be highlighted that the concentrations of lactic acid used for the spot assays were very inferior to the ones being produced by these strains. Indeed, when spot assays were performed with lactic acid concentrations similar to the ones being produced (approximately 50 g/L), none of the strains was able to resume growth (data not shown).

Conclusion

Changing the plasma membrane lipid composition of microbial cell factories is a challenging strategy, nonetheless it can be used to unlock desirable phenotypes. In this work, we attempted to improve the lactic acid tolerance and also the lactic acid production of two homolactic fermentative strains by overexpressing *ECM22*, previously proved to cause changes in the ergosterol content. The overexpression conferred to the cells an increased tolerance towards lactic acid stress. However, the lactic acid production levels remained unaltered in the newly constructed mutant strains. Together, these data confirms that yeast response to lactic acid stress as an external stress factor or as a product of yeast fermentation follow different mechanisms and further characterization needs to be done to elucidate these differences. Nonetheless, the study pointed out potential differences at membrane level for strains CEN.PK 867 and CEN.PK m850, and this novelty deserves further studies.

Materials and Methods

Strains and growth conditions

The *S. cerevisiae* strains used in this study derive from strain CEN.PK RWB 837 (*MAT*a *pdc1*::*loxP pdc5*::*loxP pdc6*::*loxP ura3-52*) (Van Maris, Winkler, Porro, Van Dijken, & Pronk, 2004). The strain CEN.PK 876 is strain CEN.PK RWB 837 transformed with the multicopy plasmid YEpLpLDH. In this plasmid, the *Lactobacillus plantarum* lactic dehydrogenase gene was expressed under control of the *S. cerevisiae TPI1* promoter. Strain CEN.PK m850 is a mutant selected from strain CEN.PK 876 (Liu, 2005). The strains CEN.PK 876 and CEN.PK m850 were transformed with the integration cassette Chr X 236336::pPGK1-ECM22-tCYC1:: Chr X 237310 originating the strains CEN.PK 876 *ECM22* and CEN.PK m850 *ECM22*. All the strains used in this work are present in **Table 1**. The strains were propagated on agar plates containing 1.7 g/L yeast nitrogen base (YNB) without amino acids, 10 g/L ethanol, and 10 g/L glycerol. Two different liquid media were used to cultivate the strains: a pre-inoculum medium containing 0.31 g/L $CaCO_3$, 1.7 g/L YNB without amino acids and without (NH4)2SO4, 1.5 g/L urea, 10 g/L ethanol, and 0.5 g/L glucose and a fermentation medium containing 4.5 g/L $CaCO_3$, 1.7 g/L YNB without amino acids and without $(NH_4)_2SO_4$, 1 g/L urea, 5 g/L ethanol, and 75 g/L glucose.

Batch culture was performed, unless otherwise stated, at 28°C in 250-ml quadruple baffled shake flasks. The cells were harvested from fresh cultures grown on agar plates and used to inoculate 100 ml of pre-inoculum medium at an optical density at 600 nm (OD_{600nm}) of 0.3. After 24 h these cells were harvested and used to inoculate 100 ml of fermentation medium at an OD_{600nm} of 3.0. Batch cultures were monitored for 72 h.

Plasmids and strains construction

To overexpress *ECM22* in the two homolactic fermentative strains, CEN.PK 876 and CEN.PK m850, we exploited the Easy-MISE toolkit (Maestroni et al., 2022, manuscript in preparation).

Briefly, *ECM22* gene sequence was amplified by PCR, with primers: ECM22_GH_Fw; ECM22_mut1_Fw; ECM22_mut1_Rv; ECM22_mut2_Fw; ECM22_mut2_Rv and ECM22_GH_Rv, using genomic DNA from CEN.PK strain as template, extracted by standard methods (Maniatis, Fritsch, & Sambrook, 1988). All the primers used in this work are present in **Table 2**.

The ORF sequence presented recognition sites for *Bsa*I and *Nhe*I restriction enzymes, we deleted those thanks to site-directed mutagenesis during pEM_*ECM22* construction.

ECM22 fragment was inserted in pGA-Blue plasmid exploiting Golden Gate Assembly strategy with T4 ligase and *Esp*3I as Type IIS restriction enzyme, originating pEM_*ECM22*. The correct insertion of *ECM22* was confirmed by sequencing using primers Seq_pGABlue_Fw and Seq_pGABlue_Rv.

To build pGA_*ECM22*, we exploited NEB®Golden Gate Assembly Kit (*Bsa*I-HF®v2) (New England Biolabs® Inc.), with T4 ligase and *Bsa*I as Type IIS restriction enzyme. pGA_*ECM22* was obtained using pGA-red plasmid as destination plasmid. The pEM plasmids listed in **Table 3** were used as donation plasmids for the final plasmid assemble. The correct assemble of the plasmid was check by colony PCR with primers PGK1_prm_FG_GG_Fw and CYC1_ter_IL_GG_Rv.

Yeast transformants were obtained exploiting the Easy Clone-Marker Free toolkit and manual (Jessop-Fabre et al. 2016). Plasmids with integration constructs were linearized with *Nhe*I, the integration fragment was gel-purified and transformed along with a gRNA helper vector (pCfB3042), into

yeast previously transformed with Cas9 plasmid (pCfB2312). The gRNA helper vector and the Cas9 plasmid pCfB2312 come from Easy Clone-Marker Free vector set, provided from Jessop-Fabre and collaborators (2016) (Addgene kit #1000000098). Correct integration of the vector into the genome was verified by colony PCR using primers X4_DW_ctr_integr and tCYC1_ctr_integr.

Q5® High-Fidelity DNA Polymerase was used on a ProFlex PCR System (Life technologies). All restriction enzymes utilized are from NEB (New England Biolabs® Inc.). All PCR fragments were sequenced thanks to Mix2Seq kit, Eurofins Genomics.

Table 1: List of strains used in this work.

Strain	Relevant genotype	Plasmids	Reference
CEN.PK 876	*MATa, pdc1(-6,-2)::loxP, pdc5(-6,-2)::loxP, pdc6 (-6,-2)::loxP, ura3-52*	YEpLpLDH (ScTPI, LpLDH, URA3)	(Liu, 2005)
CEN.PK m850	*MATa, pdc1(-6,-2)::loxP, pdc5(-6,-2)::loxP, pdc6 (-6,-2)::loxP, ura3-52, acid tolerant*	YEpLpLDH (ScTPI, LpLDH, URA3)	(Liu, 2005)
CEN.PK 876_*ECM22*	*MATa, pdc1(-6,-2)::loxP, pdc5(-6,-2)::loxP, pdc6 (-6,-2)::loxP, ura3-52* Chr X 236336::pPGK1-ECM22-tCYC1:: Chr X 237310	YEpLpLDH (ScTPI, LpLDH, URA3)	This work
CEN.PK m850_*ECM22*	*MATa, pdc1(-6,-2)::loxP, pdc5(-6,-2)::loxP, pdc6 (-6,-2)::loxP, ura3-52, acid tolerant* Chr X 236336::pPGK1-ECM22-tCYC1:: Chr X 237310	YEpLpLDH (ScTPI, LpLDH, URA3)	This work

Table 2: List of primers used in this work.

Primers	Sequence (5'-3')
ECM22_GH_Fw	TGCCAACGTCTCATGGTCTCCATTCATGACATCCGATGATGGG
ECM22_mut1_Fw	TGCCAACGTCTCACTTCGACCAGTTAGCGCAATTG
ECM22_mut1_Rv	TGCCAACGTCTCAGAAGGGAATATTGCTGAA
ECM22_mut2_Fw	TGCCAACGTCTCACAATACCGGACTCACAATCAGTC
ECM22_mut2_Rv	TGCCAACGTCTCAATTGTCGATTATCAAGTTTTTGTTATGAG
ECM22_GH_Rv	TCGATCCGTCTCAGGTCTCCCGGTCATAAAAGCTGAAAAGTTTGTAGTGG
Seq_pGABlue_Fw	GAAGCGGAAGAGCGCCCAAT
Seq_pGABlue_Rv	AAAAGGATCTTCACCTAGATCC
PGK1_prm_FG_GG_Fw	TGCCAAGGTACCGGTCTCCAGGACCTCATACTATTATCAGGGC
CYC1_ter_IL_GG_Rv	TCGATCGCTAGCGGTCTCCGTAACTTCGAGCGTCCCAAAACC
X4_DW_ctr_integr	GACGGTACGTTGACCAGAG
tCYC1_ctr_integr	TTTCTGTACAGACGCGTG

Table 3: List of plasmids used in this work.

Plasmids	Description	Reference
pGA-Blue	Destination plasmid_level 0, Kanamycin resistance cassette	Maestroni et al., 2022
pGA-Red	Destination plasmid_level 1, Ampicillin resistance cassette	Maestroni et al., 2022
pEM.H04L	Homology region locus X-4	Maestroni et al., 2022
pEM.A02L	Adaptor linker	Maestroni et al., 2022
pEM.P03R	*PGK1* promotor	Maestroni et al., 2022
pEM.ECM22	*ECM22* ORF	This work
pEM.A01R	Adaptor linker	Maestroni et al., 2022
pEM.T02R	*CYC1* terminator	Maestroni et al., 2022
pEM.H04R	Homology region locus X-4	Maestroni et al., 2022
pGA_ECM22	*ECM22* integration cassette	This work
pCfB2312	Cas9 plasmid	Maestroni et al., 2022
pCfB3042	gRNA helper vector locus X-4	Maestroni et al., 2022

Extracellular metabolites and pH determination

Samples at different times were centrifuged at 1400 rpm for 10 min. The supernatants were filtered (0.22µm filter), then glucose and lactic acid were determined by HPLC using a Resex ROA-Organic Acid (Phenomenex). The eluent was 0.005 N H_2SO_4 pumped at 0.5 ml/ min and column temperature was 40°C. Separated components were detected by a refractive-index detector and peaks were identified by comparing with known standards (Sigma-Aldrich, St Louis, MO, USA). The pH of the medium was measured with a pH- meter on fresh media or culture supernatants, after cells removal by centrifugation.

Spot assay

Spot assays were performed by cultivating yeast cells in 2 g/L glucose, 10 g/L ethanol and 1.7 g/L yeast nitrogen base (YNB) without amino acids. Cells were cultivated until mid-exponential phase and then diluted to an OD_{600nm} of 1. Five 1:10 serial dilutions were made and spotted agar plates containing, 1.7 g/L yeast nitrogen base (YNB) without amino acids, 10 g/L ethanol, and 10 g/L glycerol supplemented with different concentrations of lactic acid. The pH of the lactic acid stock solution was adjusted to 3 by the addition of KOH 4M. Plates were incubated for 5 days at 30 °C. The same procedure was followed for the spot assays in nystatin.

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Thesis Outlook

Modifying the plasma membrane composition of microbial cell factories is a challenging strategy to unlock desirable complex phenotypes. Indeed, it is a non-trivial approach since membrane lipids and membrane homeostasis are vital for many cellular functions. Furthermore, the analysis of the plasma membrane composition is still very complex, time and cost consuming, which hampers a detailed association between specific composition and physicochemical properties.

In the first chapter of this thesis, we reviewed some of the most recent efforts to engineer the plasma membrane of microbial cell factories, with particular emphasis on *S. cerevisiae*. Strategies to improve *S. cerevisiae* robustness toward different industrial stress conditions as well as strategies focusing on improving *S. cerevisiae* productivity, yield and production were described. These works provide valuable insights and can serve as guidelines for future membrane engineering strategies. I believe that membrane engineering can become a consolidated approach in microbial cell factory design over the next years. However, so far, only a few attempts have proven to be successful in increasing the yeast tolerance towards inhibitory compounds.

I envision that a combination of efforts between researchers from different areas of study, such as lipidomics, molecular dynamics simulations, membrane transporters and membrane biophysics will be crucial to gain a better understanding of the plasma membrane and therefore plan strategies to tailor it at a systems level. Integrating data deriving from different research fields is fundamental to describe the plasma membrane as an entire interconnected system and not as a sum of isolated elements.

In this sense, in the second chapter of this thesis, by combining *in vitro* and *in vivo* complementary assays, we have investigated the possibility of modifying the lipid composition of *S. cerevisiae*, in terms of ergosterol content, to obtain mutants with increased organic acid tolerance. We demonstrated that by modulating ergosterol content we can modify tolerance of *S. cerevisiae* towards organic acids. This opens the next question, which is to investigate the reasons for this change. The *in vitro* assay, based on synthetic lipid vesicles, represents a fast and reliable approach to understand the structural role of lipid species, independently of cellular metabolism, and can provide important guidelines for future membrane engineering works. We observed that in the presence of acetic acid higher levels of ergosterol decrease the acid diffusion into the liposomes. Differently, in the presence of lactic acid, we observed that ergosterol abundance is not directly proportional with decreased diffusion into the liposomes. Given the promising indications observed, we extended our approach *in vivo*, taking advantage of *S. cerevisiae* cells engineered to modulate the quantity of ergosterol by overexpressing *ECM22,* encoding the transcription factor described as the main regulator of ergosterol biosynthesis. The overexpression of *ECM22* revealed to be crucial for yeast tolerance towards lactic acid stress. Coherently in *in vitro* and *in vivo* assays, we observed that the positive impact of ergosterol depends on the organic acid under investigation. The results obtained in this work can be used as background for subsequent research on this topic.

Similarly, when microorganisms are exploited for the production of molecules of interest, such as organic acids, the plasma membrane composition is crucial for their efficacious export into the medium. In the last chapter of this thesis, *ECM22* was overexpressed in homolactic

fermentative strains (obtained by *PDC* deletion and subsequent ALE experiment for lactic acid tolerance, respectively), with the aim to increase the lactic acid production. The created strains exhibited higher tolerance towards lactic acid stress. However, we were unable to increase the production of lactic acid in these strains. Despite these results, this study demonstrates that much remains to be unraveled about the influence of plasma membrane composition in the production of organic acids, and this case lactic acid. In the future, it would be important to perform a full lipidomic analysis of these strains to have a complete picture of their lipid composition.

Overall, I believe that a deeper understanding of lipid metabolism and its regulation will allow to design the plasma membrane composition to improve microbial cell factories. Over the last years, progress has been made in the field and the understanding of membranes and their structure has changed enormously The availability and continuous development of high-throughput methods will allow researchers to deepen their knowledge on the plasma membrane conformation and dynamics.