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**Yeasts, Advanced Liquid Biofuels, Biorefineries, and
Circular Bioeconomy**

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Yeasts, Advanced Liquid Biofuels, Biorefineries, and Circular Bioeconomy

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ABSTRACT

This talk explores the role of yeast biology and biotechnology in advancing the sustainable production of biofuels, particularly in the context of the transportation sector, a major contributor to greenhouse gas emissions and air pollution. Focusing on oleaginous yeasts, the presentation highlights their potential as microbial cell factories to produce alternative lipids, replacing vegetable oils in biorefineries. These biofuels are produced using agricultural and forest residues, as well as agro-industrial by-products, as feedstocks. The research presented includes recent findings on yeast diversity, physiology, and functional genomics aimed at identifying robust strains for efficient microbial oil production from lignocellulosic biomasses and organic residues. Additionally, the talk addresses the challenge of enhancing stress tolerance in the oleaginous yeast *Rhodotorula toruloides*, with the goal of improving its performance in lipid-based biorefinery processes. This work contributes to the development of a sustainable circular bioeconomy by promoting innovative, cleaner value chains and reducing reliance on fossil fuels.

Key words: Yeasts, *Rhodotorula toruloides*, stress responses, stress tolerance, microbial oils, liquid biofuels, yeast biorefineries, circular bioeconomy.

As Leveduras, os Biocombustíveis Líquidos Avançados, as Biorrefinarias, e a Bioeconomia Circular

RESUMO

Esta comunicação aborda o papel da biologia e biotecnologia das leveduras na produção sustentável de biocombustíveis, com particular foco no setor dos transportes, uma das principais fontes de emissões de gases com efeito de estufa e poluição atmosférica. A atenção recai sobre as leveduras oleaginosas, que têm um grande potencial como fábricas celulares microbianas para a produção de lípidos alternativos, substituindo os óleos vegetais em biorrefinarias. Estes biocombustíveis são produzidos a partir de resíduos agrícolas, florestais e subprodutos agroindustriais, como matérias-primas. A investigação apresentada inclui resultados recentes sobre a diversidade de leveduras, fisiologia e genómica funcional, com o objetivo de identificar estirpes robustas para a produção eficiente de óleos microbianos a partir de biomassa lignocelulósica e resíduos orgânicos. A palestra também aborda o desafio de melhorar a tolerância ao stress da levedura oleaginosa *Rhodotorula toruloides*, com vista a otimizar o seu desempenho nos processos de biorefinaria baseados em lípidos. Este trabalho contribui para o desenvolvimento de uma bioeconomia circular sustentável, promovendo cadeias de valor mais limpas e reduzindo a dependência dos combustíveis fósseis.

Palavras-chave: Leveduras, *Rhodotorula toruloides*, respostas a stress, tolerância a stress, óleos microbianos, biocombustíveis líquidos, biorrefinarias de leveduras, bioeconomia circular.

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CIRCULAR BIOECONOMY AND SUSTAINABILITY

The concept of Bioeconomy became recently very popular due to the urgency of ensuring a development that respects terrestrial and marine ecosystems, counteracts climate change and biodiversity reduction, and the depletion of resources. A decade ago, this new paradigm of economic and social development was an obscure concept for policymakers and the society in general. The European Union (EU) strategy for the Bioeconomy which is considered one of the main pillars for the Green Transition in Europe, the European Green Deal (EC, 2019; Mengal, 2020), covers all sectors and systems that depend on biological resources (animals, plants, microorganisms and derived biomass, including organic waste) to produce food, materials and energy. The focus is on the Circular Bioeconomy which, in contrast to the end-of-life concept of the linear economy, allows economic growth to be dissociated from the increase in resource consumption and minimizes waste generation (Sá-Correia, 2021a; Tan & Lamers, 2021). Several countries, including all the G7, have a national strategy and consistent policies for the development of the Bioeconomy (Marvik & Philip, 2020). It has also been on the agendas of the Organization for Economic Cooperation and Development (OECD) (OECD, 2018) and the EU (Bogdanski *et al.*, 2021; EC, 2019; Fritsche *et al.*, 2021; Mengal, 2020). The Food and Agriculture Organization (FAO) is also engaged in this transition, aligned with the Sustainable Development Goals of the United Nations (UN) 2030 Agenda, the Paris Agreement, and other environmental agreements. There are strong expectations that the development of a sustainable bioeconomy will contribute to cutting the current dependence on fossil fuels in favour of renewable sources of biological origin, to reduce greenhouse gas emissions and to modernize industries and create new jobs (Bogdanski *et al.*, 2021; EC, 2019; Fritsche *et al.*, 2021; Marvik & Philip, 2020; Mengal, 2020; OECD, 2018; Tan & Lamers, 2021). The Circular Bioeconomy also constitutes a business opportunity being considered a catalyst for systemic change to promote new forms of production and use of natural resources and for the modernization of industry, stimulating new cleaner value chains, innovations, technologies and industrial processes. Bioindustries are a major employer, and, due to the nature of the circular bioeconomy, it can benefit regional economies, as they depend on locally sourced biomass (Mengal, 2020; Sá-Correia, 2021a). However, it must be recognized that the implementation of the Bioeconomy has been slow and the

market for new bio-based products has been quite volatile as many new initiatives appear and disappear. The urgency of this transition requires more research and technological development, effective political measures, and cooperation between the scientific community, private companies, governments, and the society in general (EC, 2019; Mengal, 2020).

ADVANCED LIQUID BIOFUELS AND YEASTS

The energy transition is one of the biggest challenges we face to counteract the current dependence on fossil fuels for various economic activities. It is no longer possible to ignore the serious global crisis, best expressed in the climate crisis, and it is necessary to abandon an economy based on fossil fuels. The world today is extremely dependent on oil, coal, and gas, for mobility (aircraft, merchant and passenger ships, trucks and automobiles), industrial activity and air conditioning, responsible for most global gas emissions. greenhouse effect. As electrification is a slow process and other alternatives, such as hydrogen, are still in their infancy, the increased incorporation of liquid biofuels is a fundamental complement to accelerate the energy transition in the transportation sector, one of the main sources of greenhouse gas emissions and air pollution in cities, especially the aviation sector.

The most common liquid biofuels currently in use are bioethanol and biodiesel, both of which strongly depend on first-generation (1G) technology, as they are produced from food crops. The transition to advanced biofuels (2G) with the consequent valorization of agricultural, forestry, and industrial wastes, will make it possible to meet sustainability criteria and benefit rural economies (Sá-Correia, 2021a). The production of biodiesel based on the use of virgin edible vegetable oils (palm, soybean, canola and others) was first considered a green alternative to diesel derived from petroleum refining (Sá-Correia, 2024). However, the use of this 1st generation biodiesel led to the necessary expansion of plantations with changes in land use and deforestation of tropical forests with a devastating impact on biodiversity (Sá-Correia, 2024). It is now very clear that the use of food crops for biofuels must be severely restricted and gradually eliminated from renewable targets, by 2030 at the latest, through European regulation. The recycling of edible vegetable oils widely used in frying is an alternative but of a very

complex and inefficient collection logistics. The transition to advanced biofuels, produced by microorganisms/microbial cell factories from lignocellulosic biomass and agricultural, forestry and agro-industrial residues, will allow sustainability criteria to be met, reducing the amount of waste and valuing it (Mengal, 2020; Sá-Correia, 2021a, 2021b). However, the success of the sustainable bioeconomy strongly depends on scientific development and disruptive technologies in biorefining, this justifying the recent increase in researchers' interest in the topic to make advanced biorefineries a sustainable alternative to oil refineries, mitigate environmental problems and contribute to achieving decarbonization Goals (Banerjee & Singh, 2024).

The most widely used biofuel worldwide is bioethanol as a blending agent with gasoline. It is produced by fermentation of sugars by effective strains of *Saccharomyces cerevisiae*, the baker's or wine yeast, given their natural capacity for efficient alcoholic fermentation and accessibility to genetic manipulation, although the spectrum of sugars that can be used is limited (Sá-Correia, 2021a, 2021b, 2024). The 1G bioethanol industry has already demonstrated its viability based on the use of starchy food crops (corn, wheat, etc.) and sugar crops (sugar cane, beet) and has a well-developed market. The largest producer in the world is the USA, mainly from corn, followed relatively closely by Brazil and by a long way by the EU (Sá-Correia, 2021b). To diversify the raw materials to be fermented and avoid competition with food production, attention has been focused on 2G bioethanol, which continues to face difficulties in becoming economically viable (Sá-Correia, 2021b). This requires the release of fermentable sugars from the natural polymers present in lignocellulosic biomass, and during this deconstruction, toxic compounds are generated that inhibit the metabolism of yeast, which adds another challenge to the need to ferment, at least, another type of sugar (xylose) (Figure 1) and to the typical challenges of 1G ethanol production (ethanol toxicity, among others) (Cunha *et al.*, 2019; Palma *et al.*, 2018). To make cellulosic ethanol production economically viable, the trend is towards the coproduction of bioethanol and other bioproducts by superior yeast strains from a wide range of biomasses, within the concept of an efficient and integrated biorefinery (Banerjee & Singh, 2024; Sá-Correia, 2021a, 2021b).

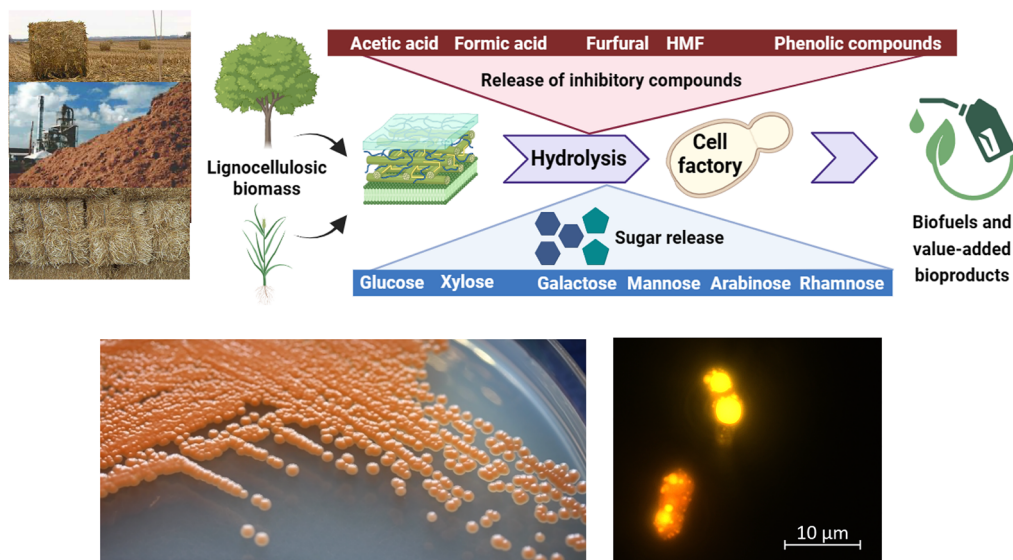
Oleaginous yeasts are now seen as promising micro factories to produce

alternative lipids for a more sustainable biodiesel industry (Sá-Correia, 2024). This is a topic of intense current research and innovation because they grow quickly, do not require large amounts of arable land, are easy to produce biotechnologically, are independent of seasonal or climatic effects, can be used for a wide range of renewable carbon sources/raw materials and have a high lipid content under optimized conditions (Martins *et al.*, 2020; Martins *et al.*, 2021; Mota *et al.*, 2022; Sá-Correia, 2024). However, there are economic bottlenecks (Banerjee & Singh, 2024).

YEAST DIVERSITY, YEAST IMPROVEMENT, AND BIOREFINERIES

Although *S. cerevisiae* is an essential experimental model yeast widely employed in biotechnology, natural strains lack many desired traits to be useful for a circular bioeconomy (Martins *et al.*, 2020; Mota *et al.*, 2022). They lack the capacity to use a wide range of carbon (C) sources, specialized metabolic potential, and high multi-stress tolerance. Therefore, the exploration of yeast diversity for the sustainable production of biofuels, in particular biodiesel, has been gaining momentum in recent years (Martins *et al.*, 2020; Mota *et al.*, 2022; Sá-Correia, 2024) (Figure 2). The so-called oleaginous yeasts (accumulate more than 20% of their dry weight in lipids and may reach 70%-80%) (Figure 1 and Figure 2) are the most promising microorganisms for the synthesis of oils comparable to vegetable oils for biodiesel (Mota *et al.*, 2022; Sá-Correia, 2024). They are cell factories widely explored in the biotechnology industry, having the capacity to assimilate different sources of carbon and nitrogen with high cell yields in short growth cycles. Furthermore, controlled production in bioreactors is possible, without requiring large cultivation areas or being limited by climate. However, sustainable and economically viable bioprocesses require yeast strains exhibiting: (i) high tolerance to multiple bioprocess-related stresses, including the various chemical inhibitors present in hydrolysates from lignocellulosic biomass and residues; (ii) the ability to efficiently consume all the major carbon sources present; (iii) the capacity to produce lipids with adequate composition in high yields (Figure 1). Hundreds of non-conventional (non-Saccharomyces) yeast species have been described as oleaginous, but only a much smaller group of yeasts are relatively well studied showing the potential to produce oils for biodiesel and other promising coproducts utilizing agroforestry and industrial organic residues

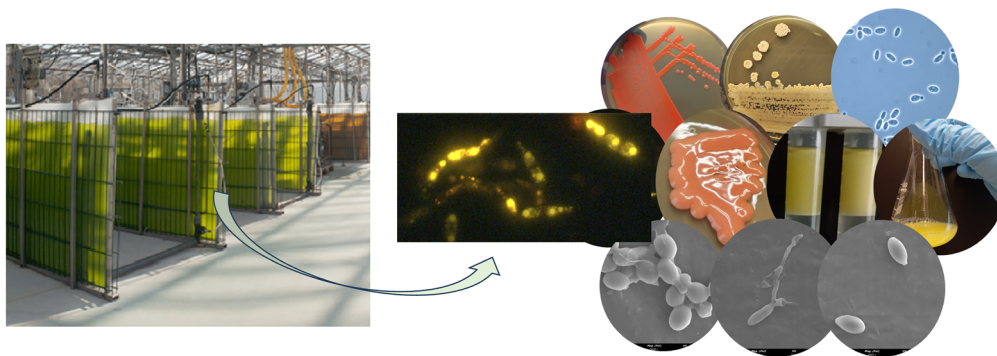
(Mota *et al.*, 2022; Sá-Correia, 2024).



and carotenoid pigments based on lignocellulosic biomasses. The promising *Rhodotorula toruloides* species capable of using a very wide range of carbon-sources to efficiently produce lipids and carotenoids is highlighted by showing the characteristic red yeast colonies due to carotenoid production and the intracellular fluorescent lipid droplets stained with Nile Red.

The development of biofuel production by yeasts can be fueled by the exploitation of recent advances in biosciences and biotechnologies based on functional and comparative genomics, techniques to alter genes and genomes more quickly and precisely, and the development of new tailor-made bioprocesses supported by synthetic biology and metabolic engineering that promise to optimize regulatory pathways and endogenous metabolism, redirect metabolic pathways, (re)construct metabolisms to produce desired products in cellular micro factories (Godinho & Sá-Correia, 2019; Palma *et al.*, 2018; Perpelea *et al.*, 2022). These developments, leveraged by computer science, automation and artificial intelligence, are now fueling a new wave of innovation and are available for the development of biofuel production by yeast (Banerjee & Singh, 2024; Godinho *et al.*, 2021; Monteiro *et al.*, 2020; Oliveira *et al.*, 2021). They provide answers to major challenges that require continuous innovation, better tools (databases with coupled bioinformatics tools to better explore them)

(Godinho *et al.*, 2021; Monteiro *et al.*, 2020; Oliveira *et al.*, 2021), the exploitation of biodiversity, and the development of superior yeast strains (Mota *et al.*, 2022; Perpelea *et al.*, 2022) (Figure 2). This superiority includes the extension of usable raw materials through more efficient use of sugars and other carbon sources, the reprogramming of cellular metabolism, higher levels of tolerance to high concentrations of sugar, ethanol, high temperatures, and other stresses present in lignocellulosic biomass hydrolysates-based processes, and a lower production of by-products. Indeed, the availability of the right yeast strain to be used and the appropriate physiological and bioprocess conditions for each specific bioethanol or lipid production process from a given substrate directly influence its productivity and economy. The message is: any effort with strain improvement is more deserving if applied to innate robust strains with suitable catabolic and biosynthetic potential.



is instrumental to identify innate robust strains with relevant catabolic and biosynthetic potential, suitable for further genetic improvement. In this case, marine yeasts with biotechnological potential, in particular promising strains of different species were isolated in association with industrial cultures of microalgae. They can produce microbial oils for sustainable biodiesel production and efficiently use of a wide range of sugars and other carbon sources for growth. These yeast isolates are deposited in the IST-Yeast Culture Collection <https://blueyeastsc.tecnico.ulisboa.pt>.

MICROBIAL OILS: AN ALTERNATIVE TO ACCELERATE THE ENERGY TRANSITION AND WASTE RECOVERY?

Microbial oils, produced by different species of bacteria, yeast, and algae, are increasingly being explored as a sustainable potential source of biofuels. They can be converted into biodiesel and other biofuels, offering several advantages: i) sustainability (can be produced from renewable resources and

waste materials, reducing dependence on fossil fuels); ii) diversity of feedstocks which may help in addressing environmental issues; iii) high yield (certain microorganisms can produce oils in high quantities), iv) tailored properties: (microbial genetic engineering can be used to optimize oil yield and properties for specific fuel applications); v) lower carbon footprint. Microbial oils have diverse compositions that can vary significantly based on the microbial strain and the cultivation conditions. The primary component of microbial oils are fatty acids that can be saturated, monounsaturated, or polyunsaturated (e.g., palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1) linoleic acid (C18:2), alpha-linolenic acid (C18:3)) (Fernandes *et al.*, 2023; Mota *et al.*, 2022). In microbial cells, the main storage form of fats are triglycerides (esters formed from glycerol and fatty acids) (Fernandes *et al.*, 2023; Mota *et al.*, 2022). Microbial oils hold significant potential as a sustainable alternative for fuels for aviation, marine, and road transportation (cars and trucks) (Banerjee & Singh, 2024; Mota *et al.*, 2022). They can be processed (hydroprocessing and transesterification) to produce bio jet fuels, which are renewable alternatives to conventional aviation fuels. Microbial oils can be converted into biodiesel (by transesterification), which can be used in diesel engines for ships and boats and in road vehicles.

Although microbial oils produced by oleaginous yeasts are promising substitutes for vegetable oils for conversion into biodiesel and bio jet fuels, this bio-fuel production has been limited by the high cost of raw materials, which can represent a very significant part of global expenditure (Banerjee, S. & Singh, 2024; Sá-Correia, 2024). Reducing production costs is therefore essential. Several studies indicate that the recovery and valorization of agro-industrial waste, agricultural and forestry waste and other industrial organic wastes, such as raw glycerol, can lead to a viable and sustainable biofuel production technology. This is the case of abundant raw materials such as lignocellulosic waste, despite the technical limitations of its deconstruction processes with the generation of several compounds that inhibit yeast growth and metabolism (Cunha *et al.*, 2019; Sá-Correia, 2021b). Therefore, the development of more robust yeasts is part of the challenges. The exploration of a multi-product biorefinery strategy, where several co-products are generated together with lipids, is recommended to ensure the overall economic viability of biofuel production (Sá-Correia, 2021a, 2021b). The

metabolism of oleaginous yeasts allows the coproduction of, for example, carotenoids, organic acids, proteins (Mota *et al.*, 2022; Sá-Correia, 2024).

In Figure 1, it is possible to see drops of the oil accumulated during the multiplication of the yeast species *Rhodotorula toruloides* in sugar beet pulp hydrolysates, a by-product of the sugar industry (Martins *et al.*, 2020; Martins *et al.*, 2021). This yeast species also produces carotenoids (natural pigments) that give their colonies a reddish color (“red yeast”), being important co-products. After extracting the lipids, the resulting microbial biomass can further be used for animal feed or subjected to further processing to obtain amino acids or peptides. Ongoing research and technological advancements (cost-effective production methods, efficient extraction processes, and the scalability of production systems) will continue to improve the viability of microbial oils as a key player in the biofuels sector (Mota *et al.*, 2022). Although the economic feasibility of large-scale production of yeast lipids remains a challenge (Banerjee & Singh, 2024), consistent legislation, policies and regulations as well as support for the expansion of bio-based businesses, are additional factors in boosting this bio-industry.

IMPROVING YEAST STRESS TOLERANCE TO BOOST MICROBIAL OIL PRODUCTION FROM LIGNOCELLULOSIC HYDROLYSATES: LESSONS FROM OUR CURRENT RESEARCH

Adaptive laboratory evolution (ALE) to enhance nonconventional yeast tolerance to bioprocess-related multiple stresses

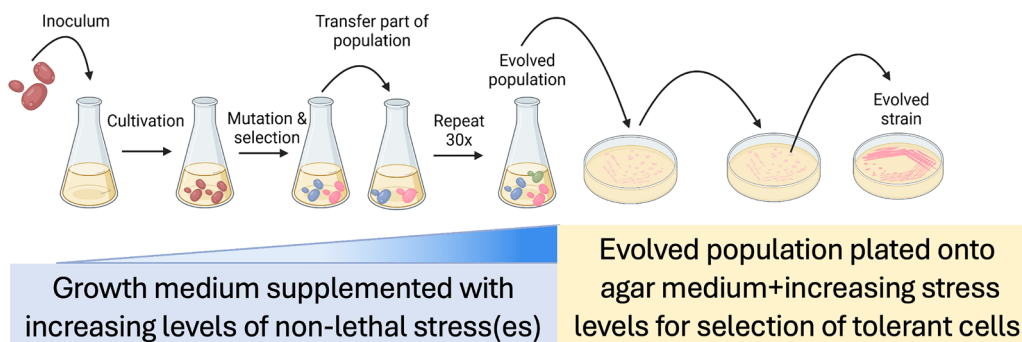
Among the main barriers affecting the efficiency of lignocellulose-based lipid production for biofuels, is the presence of toxic compounds in lignocellulosic hydrolysates (LCH) hindering the content and productivity of intracellular lipids by oleaginous yeasts as the result of the inhibition of important cellular processes and the diversion of energy into cellular adaptive responses to their deleterious effects (Cunha *et al.*, 2019; Fernandes *et al.*, 2023; Palma *et al.*, 2018). The diverse growth and metabolism inhibitors present belong to three major groups: weak acids, furan derivatives, and phenolic compounds arising from pretreatment, hydrolysis, and fermentation of lignocellulosic biomasses (Cunha *et al.*, 2019; Fernandes *et al.*, 2023) (Figure 1). The inhibitory compounds concentration,

derived from diverse raw materials and pretreatments, is quite variable (Cunha *et al.*, 2019) and their combination may pose a greater challenge to yeast cell factories than the diverse compounds individually. The detoxification of the hydrolysates involves expensive methods that also can reduce sugar content while the isolation and development of superior strains, with reduced susceptibility to the effects of LCH inhibitors, is an economically favorable strategy (Cunha *et al.*, 2019; Fernandes *et al.*, 2023). In this context, a robust oleaginous red yeast *Rhodotorula toruloides* strain was previously selected in our laboratory for sugar beet pulp valorization through the production of lipids and carotenoids by the complete catabolism of the major carbon (C)-sources present in these hydrolysates (Martins *et al.*, 2021). Although sugar beet waste is not currently significant in Portugal as it is in Europe, this research was carried out in the context of the EraNet (a European network) YEASTPEC project. Moreover, these studies can be extended to other pectin-rich wastes, such as those from the processing of citrus fruits and other fruits.

The basidiomycetous yeast species *R. toruloides* is attracting great interest also due to its ability to use the main C-sources present in lignocellulosic hydrolysates (glucose, xylose, and acetic acid) and to efficiently produce lipids and carotenoids (Fernandes *et al.*, 2023; Martins *et al.*, 2020; Martins *et al.*, 2021). Moreover, this species can also efficiently use other less usual and difficult-to-catabolize carbon sources, as is the case of the acid sugar D-galacturonic acid and the neutral sugar L-arabinose, present in pectin-rich agro-industrial residues, (Martins *et al.*, 2021). However, the metabolism of this promising strain IST536 can be limited by the presence of acetic acid in the hydrolysates since this weak acid has a dual role in the bioprocess: it can be easily metabolized as carbon and energy source but, depending on the concentration and medium pH, can also be a metabolism inhibitor (Fernandes *et al.*, 2023; Martins *et al.*, 2021; Palma *et al.*, 2018).

The performance of *R. toruloides* IST536 in bioprocesses based on lignocellulosic hydrolysates was improved by exploring an adaptive laboratory evolution (ALE) strategy (Fernandes *et al.*, 2023) (Figure 3) given that, despite the remarkable progress made recently, the available tools for *R. toruloides* genome engineering are still not highly efficient. The evolved strain, IST536MM15, was found to be more tolerant to acetic and formic acids, furfural and 5-hydroxymethylfurfural (HMF), the four major inhibitors expected to be present in lignocellulosic biomass

hydrolysates (Fernandes *et al.*, 2023). The method of evolutionary engineering explored is simple and effective in improving yeast tolerance to increasing levels of selective pressure during extended cultivation for several generations (Figure 3). In this case, the selective pressure was posed by inhibitory concentrations of methanol in a growth medium with 5% (v/v) glycerol as the only carbon source that imposed an additional osmotic stress (Fernandes *et al.*, 2023). The selected evolved strain was found to exhibit the reported increased tolerance to methanol and to the above referred multiple stresses, underscoring the potential of ALE in enhancing the robustness of industrial yeast strains. The better performance of this evolved multi-tolerant strain for lipid productivity from lignocellulosic hydrolysate was confirmed (Fernandes *et al.*, 2023).



tolerant to specific stress(es), based on yeast cultivation during several generations under increasing concentrations of non-lethal selective stress(es), eventually following random mutagenesis. The ALE strategy is particularly important when used with non-conventional yeasts for which genome engineering tools are still not highly efficient.

Functional and Comparative Genomics to uncover molecular mechanisms underlying multi-stress tolerance

The highly interesting traits of the evolved strain *R. toruloides* IST536MM15 provide the opportunity to explore it not only for biotechnological applications but also to get clues into the multi-stress tolerance phenomenon and underlying molecular mechanisms. These were obtained through comparative and functional genomic analyses of the original strain versus its evolved counterpart (Antunes *et al.*, 2024a). These strains had been preliminary characterized in our laboratory and results indicated that the evolved multi-stress tolerant strain has

a cell wall less susceptible to zymolyase activity and a decreased permeability, based on the intracellular accumulation of propidium iodide fluorescent probe (Fernandes et al., 2023). Later, the identification of key genes, at a genome-wide scale, and of global mechanisms responsible for the multiple stress tolerance features of IST536MM15 was achieved by comparing their genome sequences and the transcriptomes (Antunes et al., 2024a). Comparative genomic analysis suggested the reduction of the original strain ploidy from triploid to diploid, the occurrence of 21,489 mutations, and 242 genes displaying copy number variants in the evolved strain (Antunes et al., 2024a). Transcriptomic analysis identified genes associated with cell surface biogenesis, integrity, and remodeling and those involved in stress-responsive pathways as exhibiting the most substantial alterations at the genome and transcriptome levels (Antunes et al., 2024a). Remarkably, guided by the suggested global stress response pathways registered, the multi-stress tolerance of the evolved strain phenotype was extended to osmotic, salt, ethanol, oxidative, genotoxic, and medium-chain fatty acid-induced stresses (Antunes et al., 2024a).

The key role of the cell envelope in yeast adaptation and tolerance to multiple stresses.

The yeast cell wall is essential to maintain cell shape and integrity and is the first line of defense against diverse environmental stress factors (Ribeiro et al., 2022a). To sense, survive, and adapt to different stresses, yeasts rely on a network of signaling pathways to modulate the global transcriptional response and elicit coordinated changes in the cell (Ribeiro et al., 2022a). These pathways cooperate and tightly regulate the composition, organization and biophysical properties of the cell wall (Ribeiro et al., 2022a; Ribeiro et al., 2022c). Among these mechanisms is the alteration of the molecular composition and biophysical properties of the cell envelope, acting as the first barrier of protection against challenging environmental conditions. In the case of weak acids acetic and formic acids, this alteration may also limit the futile cycle associated with the re-entry of the acid form after the active expulsion of the counter-ion acetate from cell interior (Ribeiro et al., 2022c). However, the involvement of cell wall in the adaptation and tolerance of yeasts to multiple stresses of biotechnological relevance has not received

the deserved attention (Ribeiro et al., 2022a).

A substantial amount of information is available regarding the structure, biosynthesis, regulation, and composition of the cell wall in the model yeast, *S. cerevisiae* (Ribeiro et al., 2022a), but this information is still limited for non-conventional yeasts, particularly *R. toruloides* (Antunes et al., 2024). Recent studies from our Lab either in the model yeast or in *R. toruloides* demonstrate the major role of cell wall in the response and tolerance to stresses of biotechnological relevance in yeasts (Antunes et al., 2024a; Antunes et al., 2024b; Fernandes et al., 2023; Ribeiro et al., 2022b; Ribeiro et al., 2022c). The crosstalk between yeast cell plasma membrane ergosterol content and cell wall stiffness under acetic acid stress, involving Pdr18 was also uncovered. Pdr18 is a plasma membrane ATP-binding cassette (ABC) transporter of the pleiotropic drug resistance family plasma membrane efflux pumps related with ergosterol transport at the plasma membrane (Ribeiro et al., 2022b). Other plasma membrane efflux pumps of the Major Facilitator Superfamily MFS (Drug: H⁺ antiporters) or the ABC superfamily have also been examined and their biological function characterized and explored for the development of superior industrial yeasts (Godinho & Sá-Correia, 2019; Sá-Correia & Godinho, 2021c).

Understanding the composition of the cell wall and the alterations occurring at this level under stress can provide mechanistic insights to improve tolerance to industrially relevant stress conditions aiming for high productivity and product yields. The suggested occurrence of significant modifications in the cell wall of the multi-stress tolerant evolved strain led us to characterize cell wall integrity and carbohydrate composition to gain insights into the physicochemical changes associated to its remarkable multi-stress tolerance phenotype (Antunes et al., 2024b). Compared to the original strain, the evolved strain exhibited a higher proportion of glucomannans, fucogalactomannans, and chitin relative to (1→4)-linked glucans, and an increased presence of glycoproteins with short glucosamine derived oligosaccharides (Antunes et al., 2024b), which have been found to be associated to ethanol stress tolerance and physical strength of the cell wall. Interestingly, the evolved strain cells were found to be significantly smaller than the original strain and more resistant to thermal and mechanical disruption, consistent with higher proportion of beta-linked polymers instead of glycogen, conferring a more rigid and robust cell wall. These findings provided further

insights into the cell wall composition of this basidiomycetous red yeast species and into the alterations occurring in the multi-stress tolerant evolved strain. This new information is useful to guide yeast genome engineering towards more robust strains for successful biorefinery processes.

RESEARCH AND TECHNOLOGICAL ADVANCEMENTS TO IMPROVE THE ECONOMIC VIABILITY OF MICROBIAL OILS AS A KEY PLAYER IN THE BIOFUELS SECTOR

The advances made in biological sciences and biotechnology towards the sustainable bioconversion of renewable biological resources, also economically, are among the main drivers of a sustainable bioeconomy, energy transition depending on innovation (Fritsche et al., 2021; Markiv & Philip, 2020; Sá-Correia, 2021b). In recent years, international research on oleaginous yeasts has been extremely active, leading to significant advances towards the substitution of vegetable oils for conversion into biodiesel and bio jet fuels (Mota et al., 2022; Sá-Correia, 2024). Ongoing research and technological advancements (cost-effective production methods, efficient extraction processes, and the scalability of production systems) continue to improve the viability of microbial oils as a key player in the biofuels sector. Moreover, the increase of tolerance to relevant inhibitors present in lignocellulosic biomass hydrolysates and to other bioprocess related stresses, represents an advantage for the efficient and economic exploitation of a wide-range of forest and agro-industrial residues as low-price sustainable feedstocks for industrial bioprocesses (Cunha et al., 2019; Fernandes et al., 2023; Sá-Correia, 2021c; Sá-Correia, 2024). The major economic bottlenecks are highlighted and the perspectives to assist the expansion of microbial lipid-derived biofuels at an industrial scale are provided in (Banerjee & Singh, 2024).

An assessment of the agri-food, forestry and marine sectors (algae, aquaculture and fisheries) was carried out by the Bio-based Industry consortium (BIC) and published, in 2021, an updated report that identifies opportunities for a transition to the bioeconomy and sustainable development in Portugal (BIC, 2021). The generous conclusions of the study highlight the existence of a robust innovation ecosystem and the growing influence of the Biotechnology sector, the marine economy, and the opportunities for transforming organic resources and

waste into value-added products (BIC, 2021). Also, according to the National Plan for the Promotion of Biorefineries and the COTEC Portugal Report on Circular and Digital Bioeconomy Opportunities in the Portuguese Economy and Industry, 2020, there are, to explore, resources and residues of biomass generated by the forest, agricultural activities and food processing and other industrial sectors.

Around the large Bioeconomy area, there is in Portugal a substantial installed capacity in terms of R&D Units and Associate Laboratories as well as a well-established network of technology parks and business incubators close to universities, as well as Collaborative Laboratories (CoLAB). These CoLAB, still under consolidation, have the mission of bringing academia and the industrial sector closer together. Among them, the CoLAB BIOREF, Collaborative Laboratory for Biorefineries [<https://www.bioref-colab.pt/pt>] is one of the 18 co-promoters of the MOVE2LOWC project — Bio-Based Fuels [<https://www.move2lowc.com/>]. This project, financed by COMPETE 2020, mobilized Universities, R&D Institutes, SMEs and large companies, to use aquatic biomass (microalgae) and residual forestry and industrial effluents in the production of biofuels for the air and road transportation sectors, in a Circular Economy perspective. For historical reasons, Portugal has a critical mass and is internationally competitive in yeast research in academic research units and in biotechnology start-ups. If properly exploited, particularly in the context of dedicated collaborative laboratories and appropriate financial resources for academic partners, it could leverage the innovation, development and implementation of advanced liquid biofuels by those cellular micro factories. Although the economic feasibility of large-scale production of yeast lipids remains a challenge (Banerjee & Singh, 2024), consistent legislation, policies and regulations, as well as support for the expansion of bio-based businesses, are additional factors in boosting this bio-industry.

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COMUNICAÇÃO APRESENTADA À CLASSE DE CIÊNCIAS
NA SESSÃO DE 2 DE NOVEMBRO DE 2023

COMUNICAÇÃO RECEBIDA A 21 DE NOVEMBRO DE 2024

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