EDUARDO LUÍS MENEZES DE ALMEIDA

NEW *Papiliotrema laurentii* UFV-1 STRAINS WITH IMPROVED ACETIC ACID TOLERANCE SELECTED BY ADAPTIVE LABORATORY EVOLUTION

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

Orientador: Wendel Batista da Silveira

Coorientadora: Rafaela Zandonade Ventorim

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Assentimento:

Eduardo Luís Menezes de Almeida
Autor

Wendel Batista da Silveira
Orientador

A todos que fizeram possível esse caminho, especialmente à minha família.

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BIOGRAFIA

Eduardo Luís Menezes de Almeida, natural de Sete Lagoas – MG, é bacharel em Engenharia de Alimentos pela Universidade Federal de Viçosa. Iniciou a graduação em 2015, e ao longo do curso foi bolsista de iniciação científica do Laboratório de Processos Bioquímicos e Fermentativos em projetos de biotecnologia e microbiologia industrial, especialmente com o isolamento e caracterização de leveduras para produção de bebidas, e a otimização da produção de hidromel e outras bebidas. Se formou em 2019, ingressando no mesmo ano no mestrado pelo Programa de Pós-Graduação em Microbiologia Agrícola, da Universidade Federal de Viçosa, onde ingressou no Laboratório de Fisiologia de Microrganismos, e iniciou os estudos de fisiologia de leveduras e biologia de sistemas.

"... microbiology is important because it deals so directly with the fundamental life forces constitutive of all biological processes. Without an understanding of microbes, there can only be a limited understanding of life."

Maureen A. O'Malley

Philosophy of Microbiology

ABSTRACT

ALMEIDA, Eduardo Luís Menezes de Almeida, M.Sc., Universidade Federal de Viçosa, March, 2021. **New *Papiliotrema laurentii* UFV-1 strains with improved acetic acid tolerance selected by adaptive laboratory evolution.** Advisor: Wendel Batista da Silveira. Co-advisor: Rafaela Zandonade Ventorim.

Depletion of fossil fuels and increase in greenhouse gas emissions have boosted the development of new technologies for biodiesel production. Oil extracted from soybeans is the major source for Brazilian biodiesel production (69.8%); nevertheless, its utilization as feedstock requires arable land, water, and nutrients that could be utilized for food crops and conversion to native vegetation. These drawbacks can be circumvented by using yeast oil for biodiesel production. The oleaginous yeast *Papiliotrema laurentii* can accumulate a high amount of lipids and metabolize lignocellulose-derived sugars. Due to the recalcitrant nature of lignocellulosic biomasses, a pretreatment step is required. Nevertheless, acid pretreatment, the most used in lignocellulosic biomasses, leads to the formation of toxic compounds that can inhibit yeast growth. Among them, acetic acid is the most abundant, and in its undissociated form diffuses through the cell membrane and dissociates in the cytosol, disrupting cell homeostasis. To circumvent the inhibitor effect, detoxification processes are applied to remove or reduce their concentrations. However, the detoxification strategies applied are usually insufficient to reduce the acetic acid concentration. For this, oleaginous yeasts capable of tolerating acetic acid are of interest. Recently, our research team isolated and characterized a *P. laurentii* able to achieve the highest lipid contents from xylose as the sole carbon source. Nevertheless, we observed in this work that its growth is severely impaired by acetic acid (1.0 g/L). Therefore, we applied Adaptive Laboratory Evolution (ALE) to select strains of *P. laurentii* UFV-1 tolerant to acetic acid. We selected and characterized three Acetic acid Tolerant Strains (ATS). All strains evolved displayed the tolerance phenotype (able to grow in the presence of 1.5 g/L of acetic acid) after 398 generations being exposed to increasing concentrations of acetic acid (0.7, 0.9, and 1.5 g/L). However, different phenotypes emerged alongside. Although the acetic acid tolerance presented by ATS II was, along with ATS I, the highest observed in this work, it displayed trade-offs in the absence of the acid. as its lipid productivity, biomass and specific growth rate decreased. ATS I and III showed physiological parameters similar to the parental strain (lipid and biomass production, and sugar uptake) in stress absence. However, the ATS III, in contrast to ATS I, did not display the oleaginous

phenotype (<20% g lipids/ g DW) when challenged with 1.75 g/L of acetic acid. Therefore, ATS I was the most promising strain, showing tolerance to acetic acid and oleaginous phenotype in all conditions evaluated.

Keywords: Yeast. Oleaginous. Inhibitors. Lignocellulosic biomass.

RESUMO

ALMEIDA, Eduardo Luís Menezes de Almeida, M.Sc., Universidade Federal de Viçosa, março de 2021. **Novas linhagens de *papiliotrema laurentii* ufv-1 com maior tolerância ao ácido acético selecionadas por evolução adaptativa em laboratório.** Orientador: Wendel Batis da Silveira. Coorientadora: Rafaela Zandonade Ventorim.

A escassez de combustíveis fosseis e o aumento na emissão de gases do efeito estufa têm incentivado o desenvolvimento de novas tecnologias para a produção de biodiesel. O óleo de soja é a matéria-prima mais empregada na produção de biodiesel no Brasil (69,8%); no entanto, sua utilização requer terras cultiváveis, água e nutrientes que podem ser utilizados para culturas de alimentos ou reflorestamento. Esses problemas podem ser evitados a partir da utilização de óleos de leveduras para a produção de biodiesel. A levedura oleaginosa *Papiliotrema laurentii* pode acumular grandes quantidades de lipídios e metabolizar açúcares liberados de biomassas lignocelulósicas. Devido à natureza recalcitrante da biomassa lignocelulósica, uma etapa de pré-tratamento é aplicada. No entanto, o pré-tratamento ácido, o mais utilizado para esse tipo de biomassa, leva à formação de componentes tóxicos que podem inibir o crescimento de leveduras. Dentre eles, o ácido acético é o mais abundante, e na sua forma dissociada difunde pela membrana celular e se dissocia no citosol, comprometendo a homeostase celular. Para contornar esse efeito inibitório, processos de detoxificação são aplicados para remover ou reduzir a concentração desses compostos. Porém, as estratégias de detoxificação comumente aplicadas são insuficientes para reduzir a concentração de ácido acético. Sendo assim, a utilização de leveduras oleaginosas capazes de tolerar o ácido acético é de interesse em bioprocessos envolvendo o uso de hidrolisados hemicelulósicos. Recentemente, o nosso grupo de pesquisa isolou e caracterizou uma *P. laurentii* capaz de atingir altos conteúdos de lipídios a partir de xilose. No entanto, foi observado que seu crescimento é prejudicado pelo ácido acético. Neste estudo, a estratégia de Evolução Adaptativa em Laboratório foi utilizada para selecionar linhagens de *P. laurentii* UFV-1 tolerantes ao ácido acético. Todas as linhagens evoluídas (ATS – *Acetic acid Tolerant Strains*) apresentaram o fenótipo de tolerância, isto é, foram capazes de crescer na presença de 1,5 g/L de ácido acético após 398 gerações. Porém, diferentes fenótipos foram observados entre as três linhagens evoluídas. Embora a ATS II apresentou, junto com a ATS I, maior tolerância ao ácido acético, ela exibiu *trade-offs* na ausência do ácido porque a produtividade de lipídios, biomassa e velocidade específica de crescimento diminuíram. ATS I e III apresentaram parâmetros fisiológicos similares àqueles apresentados pela linhagem parental (produção de lipídios, biomassa e consumo de açúcares).

Apesar disso, ATS III, ao contrário da ATS I, não apresentou o fenótipo oleaginoso (< 20% g lipídios/ g massa seca) na presença de 1.75 g/L de ácido acético. Portanto, a ATS I foi considerada a mais promissora, apresentando tolerância ao ácido acético e o fenótipo oleaginoso em todas as condições avaliadas neste estudo.

Palavras-chave: Leveduras. Oleaginosas. Inibidores. Biomassa lignocelulósica.

SUMMARY

INTRODUCTION

Depletion of fossil fuels and increase in greenhouse gas emissions have boosted the development of new technologies for biodiesel production. In 2018, 5.4 million m^3 of biodiesel was produced in Brazil (ANP, 2019). Oils extracted from soybeans are the major source for Brazilian biodiesel production (69.8%); nevertheless, its utilization as feedstock requires arable land, water, and nutrients that could be utilized for food crops for human consumption and conversion to native vegetation (Fargione et al., 2008; Kumar et al., 2017). These drawbacks can be circumvented by biodiesel production from microbial oils (Spagnuolo et al., 2019). Oleaginous yeasts display the potential to accumulate triglycerides in organelles called lipid droplets. Among them, *Lipomyces starkeyi* and *Rhodosporidium toruloides* have a prominent position due to their ability to accumulate the highest lipid content, usually ranging from 20-70% (g lipid/g DW). They also metabolize a wide range of sugars, including glucose and xylose, found in lignocellulosic biomasses, and glycerol, a by-product generated from biodiesel production (Spagnuolo et al., 2019).

Over the last years, lipid production by oleaginous yeasts from lignocellulosic biomasses has been considered a promising alternative in biorefineries. Since these biomasses - constituted by cellulose, hemicellulose, and lignin - are recalcitrant, it is necessary to apply physical, chemical, and biological pretreatments to make cellulose and hemicellulose accessible to enzymatic hydrolysis. The dilute acid pretreatment has been used the most because it allows achieving high recovery of hemicellulose sugars and is easy to scale up (Haghighi Mood et al., 2013; Jin et al., 2015).

However, the acid pretreatment generates inhibitory compounds such as furfural, HMF (hydroxymethylfurfural), and acetic acid. Acetic acid, formed by hydrolysis of acetyl groups from the hemicellulose, is the most abundant and inhibitory compound. It can diffuse through the cell membrane in its undissociated form and dissociates in the cytosol, causing acidification, accumulation of anions, and dissipation of the proton motive force (Jönsson & Martín, 2016). Hence, microbial cells have their metabolism impaired by the decrease in enzymatic activities which leads to growth reduction and cell death (Palmqvist & Hahn-Hägerdal, 2000b). One strategy to overcome the inhibitory effects provoked by lignocellulose-derived inhibitors on yeast growth is the detoxification of the pretreated biomass to remove or reduce their concentration. However, the detoxification step is usually not effective to remove acetic acid (Bonturi et al., 2017 e Chandel et al., 2013; Palmqvist & Hahn-Hägerdal, 2000a); therefore,

oleaginous yeasts capable of assimilating xylose, the main hemicellulosic sugar, and tolerating acetic acid are of great interest for industrial applications.

Our research team isolated and characterized the *Papiliotrema laurentii* UFV-1 strain, an oleaginous yeast that accumulates the highest lipid contents from xylose (Vieira et al. 2020). For instance, a previous study conducted by Sitepu et al. (2014) identified the inhibitory effect of acetic acid on the growth of the *P. laurentii* UCDFST 12 strain, which indicates that the utilization of this yeast for lipid production from hemicellulosic hydrolysate can be impaired. To circumvent this inhibitory effect, adaptive laboratory evolution (ALE) can be applied. In ALE, cells are cultivated in defined media and controlled conditions during extended periods in either batch or continuous cultivations to increase the fitness of microorganisms under stress conditions (Dragosits & Mattanovich, 2013; LaCroix et al., 2017). As such, this approach has been widely applied to obtain robust microbial cells that are more tolerant to bioprocesses conditions (Sandberg et al., 2019). In this work, the main objective was to use adaptive laboratory evolution (ALE) to select a strain of *P. laurentii* UFV-1 with improved tolerance to acetic acid.

This dissertation is organized into two chapters. The first one presents a literature review covering the main topics regarding this work, such as biofuels and biorefineries, oleaginous yeast and lipid production, lignocellulosic biomass utilization as a feedstock, pretreatment of lignocellulosic biomass, formation of inhibitory compounds as well as their detoxification, and Adaptive Laboratory Evolution (ALE). In the second chapter, it is reported the selection and characterization of three acetic acid-tolerant strains (ATS) of *P. laurentii* UFV-1 by ALE. The selected strains presented improved growth in the presence of acetic acid; however different phenotypes emerged alongside. ATS II presented trade-offs in the absence of the acid, suggesting a specialized phenotype of tolerance to this acid, while ATS I and III presented phenotypes more associated with the behavior of generalists.

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CHAPTER 1 – LITERATURE REVIEW

1.1. Biofuels and biorefineries

Demand for fuels and energy has grown in the past years, however, the utilization of fossil sources has been considered unsustainable due to their depletion and elevated emission of Greenhouse Gases (GHG) such as CO_2, CH_4, and N_2O. Fossil fuel dependence can be reduced by biofuels; therefore, it is pivotal to improve existing technologies and develop new strategies for biofuel production (Cherubini, 2010; Ubando et al., 2020). In this context, the concept of biorefineries was introduced, focusing on sustainable production and processing of biomass into a wide range of marketable products and energy. As such, biorefineries aim to separate biomass resources into their constituents mainly carbohydrates, proteins, and lipids, to produce foods, feeds, biofuels, and chemicals through biochemical and thermochemical platforms (Cherubini, 2010).

Biofuels are liquid, gas, and solid fuels produced from biomasses, and they can be divided into two major classes: primary, non-processed biomass for direct combustion (firewood, landfill gas) and secondary, such as bioethanol and biodiesel, produced from processed biomasses. Currently, ethanol and biodiesel have been the most biofuel produced. They are advantageous and more environmentally friendly due to their portability, biodegradability, combustion-based on carbon-dioxide cycle, and low contents of sulfur and aromatics (Gaurav et al., 2017; Leong et al., 2018; Nigam & Singh, 2011).

Biofuels are classified as first, second, and third generations. First-generation (1^{st} G) biofuels are produced mainly from sugars, grains, or seeds requiring simple steps to be converted into fuel. Bioethanol is produced by fermentation of sugars extracted from plants and starch (corn, sugar-cane) by *Saccharomyces cerevisiae* or bacteria. Biodiesel is obtained by transesterification of triglycerides (TGA) extracted from oleaginous plants (palm, soybean, sunflower, coconut) and animal fat (Nigam & Singh, 2011). Although it has been produced in large quantities worldwide - around $3.8x10^{10}$ L in 2019 (OECD/FAO, 2020) - several issues emerge from the utilization of edible feedstocks. They require land and water resources that could be utilized for food production. According to Rulli et al. (2016), about 70 million people could be fed by the resources employed for biodiesel production in 2013. To surpass these concerns, second and third-generation biofuels have been considered promising technologies.

Second-generation biofuels, already applied on a commercial scale, are produced by two major approaches. One of them utilizes lignocellulosic biomass, which is – pretreated to provide sugars for fermentation (especially for bioethanol production), whilst the second one utilizes non-edible plant biomass (biodiesel formation). Therefore, both approaches do not compete with food production. Second-generation (2^{nd} G) biodiesel has been produced by the utilization of non-edible oils obtained by *Acrocomia aculeata* and *Jatropha curcas* (Nigam & Singh, 2011). They also tend to contribute with less GHG emissions compared to 1^{st} G biofuels, however, 2^{nd} G production requires more operations and sophisticated equipment leading to higher costs of investment and complexity for biofuel production (Cherubini & Ulgiati, 2010; Leong et al., 2018).

Third-generation (3^{rd} G) biofuels are produced by microbial biomass. The 3^{rd} G biodiesel is produced from microbial oil of oleaginous microorganisms (Leong et al., 2018). After the lipid accumulation phase, microbial cells are lysed by solvent, mechanical, and/or enzymatic methods, separated from cell fraction, and then neutral lipids are used for biodiesel production. Overall, this is performed by either acid or basic hydrolysis in the presence of alcohol. Compared to vegetable oils, microbial oil can be produced across the year, its production is not seasonal-dependent, besides agricultural lands are not required.

Microalgae oil has some intrinsic limitations such as sunlight availability, low growth rate, and culture contamination (bacteria and protozoa) in traditional open tanks. Otherwise, yeast oil can be grown in tightly controlled and closed bioreactors and can achieve high cell density and lipid contents. Moreover, yeasts are less susceptible to viral infection, and contamination can be reduced by cultivation in low pH values (Sitepu et al., 2014). For instance, yeast 3^{rd} G biofuels are hampered by their elevated costs (growth media and purification steps) and are not applied in commercial scales. Lignocellulosic biomass can be utilized as a cheap carbon source for oil production, especially by non-conventional oleaginous yeast able to assimilate glucose and xylose, the most abundant sugar released after its hydrolysis. However, most of the oleaginous yeasts cannot utilize xylose for growth and lipid biosynthesis (Sreeharsha & Mohan, 2020). Therefore, it is important to select strains able to assimilate and accumulate high contents of lipids from both glucose and xylose.

1.2. Oleaginous yeasts and lipid production

Oleaginous yeasts are capable of accumulating at least 20% of their dry weight (DW) as lipids (Ratledge, 1991). Currently, there are around 70 known species of oleaginous yeasts. Ascomycete yeasts include *Y. lipolytica,* a model organism for lipid production studies, and important genera such as *Myxozyma, Lipomyces, Candida.* Among basidiomycete yeasts, species of *Rhodotorula, Papiliotrema,* and *Cryptococcus* have been characterized by their great diversity in nutritional requirements and lipid production (Sreeharsha & Mohan, 2020; Yaguchi et al., 2017).

Studies about microbial lipids date to the final of the 19th century, led by German scientists until the mid of the 20th century. For example, they reported the capacity of lipid accumulation of *Metschnikowia gruessii* strains in culture media containing industrial wastes such as whey and bran (Ratledge & Wynn, 2002; Sitepu et al., 2014; Woodbine, 1959). However, in that same period, the world passed through an agricultural revolution that made the price of vegetable oils such as soy and canola, cheaper than microbial, discouraging microbial oil production. In the 1970s, Ratldge (1976) defined Single Cell Oil (SCO) as edible oils obtained from microbial single cells, and since the 1980s SCO became an alternative source of nutraceuticals for adults and infants, especially represented by polyunsaturated fatty acids (PUFAs) (Ratledge, 2004).

Better utilization of oleaginous yeasts for lipid production relies on understanding how they regulate the fatty acids synthesis, as well as accumulate high amounts of lipids (Figure 1.1). The oleaginous phenotype in yeast is related to an efficient supply of acetyl-CoA (the basic building block of fatty acids), malonyl-CoA (elongation unit), and NADPH (reducing power) for fatty acid synthesis. Acetyl-CoA can be supplied from three major pathways: glycolysis and pyruvate break down in the mitochondria, pyruvate-acetaldehyde-acetate in the cytosol, and citrate conversion to acetyl-CoA and oxaloacetate in the cytosol. Non-oleaginous yeasts obtain acetyl-CoA from two major pathways: glycolysis and pyruvate breakdown in mitochondria; pyruvate-acetaldehyde-acetate pathway in the cytosol.

Each step of carbon chain elongation by FAS (Fatty Acid Synthase) requires two NADPH, which can be formed by two pathways: reaction catalyzed by malic enzyme and oxidative pentose phosphate pathway, in the reaction catalyzed by glucose 6-phosphate dehydrogenase (Beopoulos et al., 2011).

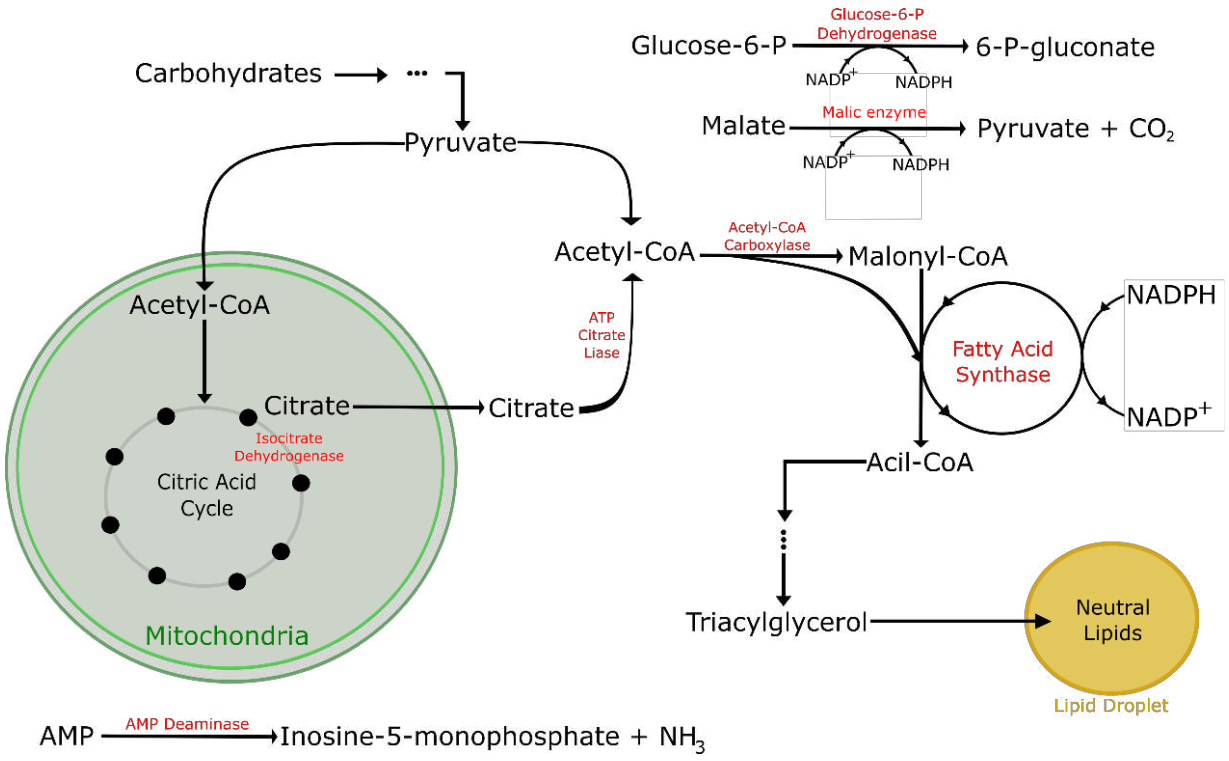

Figure 1.1 – Schematic representation of oleaginous yeast lipid accumulation metabolism. Acetyl-CoA derived from sugar catabolism can be channeled directly to lipid metabolism or the citric acid cycle in mitochondria. In nitrogen depletion conditions, AMP is deaminated to provide NH_3, leading to a reduction of the isocitrate dehydrogenase activity. This decrease promotes the accumulation of citrate in the mitochondria which is transported to the cytosol by the malate/citrate shuttle. In the cytosol, ATP citrate lyase catalyzes citrate conversion to acetyl-CoA. The pool of acetyl-CoA is utilized for lipid biosynthesis. Fatty Acid Synthase catalysis the formation of acyl chains, requiring NADPH as reducing power. The NADPH pool is supplied by the activity of glucose-6-P dehydrogenase and the malic enzyme.

Oleaginous yeasts have an additional source of acetyl-CoA due to the presence of ATP:citrate lyase (ACL), an enzyme characteristic of the oleaginous phenotype. The citrate accumulated from Tricarboxylic Acid Cycle (TCA) is transported from mitochondria to the cytosol by malate/citrate shuttle. In the cytosol, ACL catalyzes the cleavage of citrate, expending one ATP and Coenzyme A (CoA), to form acetyl-CoA and oxaloacetate. ACL is formed by two subunits, ACL1 and ACL2 encoded by two different genes activated by ammonium ions (Beopoulos et al., 2011).

Adenosine monophosphate (AMP) plays an important role in citrate availability in oleaginous yeasts whose oleaginous phenotype is well characterized. This takes place as the

activity of isocitrate dehydrogenase, a TCA enzyme that converts citrate in α-ketoglutarate and CO_2, is dependent on AMP concentration. The AMP concentration decreases under nitrogen limiting conditions because AMP deaminase catalyzes the conversion of AMP to inosine-5-monophosphate and NH_3 (Ratledge, 2004). As such, the isocitrate dehydrogenase activity is reduced leading to citrate accumulation (Beopoulos et al., 2011).

FA synthesis requires a supply of malonyl-CoA for the enzymatic complex FAS. The FAS complex in yeast is formed by two subunits [β (Fas1) and α (Fas2)] organized in a hexameric format (six α and six β). Malonyl-CoA is formed in the reaction catalyzed by acetyl-CoA carboxylase (ACC), in which acetyl-CoA condenses with bicarbonate ion (HCO_3) (Beopoulos et al., 2011).

In oleaginous yeasts, FAS, ACL, and the malic enzyme can form a complex to facilitate fatty acid synthesis and elongation. The major end products of FAS are saturated fatty acids between 14 and 18 carbons. Lately, elongation and desaturation of these FAs take place in the endoplasmic reticulum (ER), by specifics elongases and/or desaturases (Beopoulos et al., 2011; Ratledge, 2004). Desaturases are proteins that utilize acyl-CoA or acyl residues of phospholipids as substrates to catalyze the formation of double bonds in saturated fatty acids. It requires at least three different functions: cytochrome b5 reductase; cytochrome b5 oxidase; dehydrogenase. Elongases also require several catalytic subunits. They act on 16:0 to form 18:0, on 18:0 to form 20:0, and on 20:0 to form 22:0. A sequence of four linked reactions is required: condensation of an activated carbon chain with malonyl-CoA, forming ketoacyl and CO_2; reduction of ketoacyl to γ-hidroxiacyl; dehydration to form an enoyl; final reduction to form a two-carbon elongated fatty acid (Ratledge & Wynn, 2002).

Fatty acids synthesized or incorporated from culture media are further esterified in a glycerol backbone or a sterol to form triacylglycerols (TAGs) and steryl esters, respectively (Ageitos et al., 2011). Consecutive acylations of glycerol-3-phosphate (G-3-P), catalyzed by three acyltransferases, lead to TAGs formation, known as Kennedy's pathway. In the first step, G-3-P is acylated by G-3-P acyltransferase, generating lysophosphatidic acid (LPA). Furthermore, LPA is acylated by lysophosphatidic acyltransferase, leading to phosphatidic acid (PA) formation. Afterward, the phosphate group is removed by PA phosphohydrolase, resulting in diacylglycerol (DAG) (Beopoulos et al., 2009). Finally, DAG can be acylated in the third carbon remaining from glycerol by two pathways: acyl-CoA dependent and acyl-CoA independent. In the acyl-CoA independent pathway, the reaction is catalyzed by an enzyme, the phospholipid:diacylglycerol acyltransferase (Lro1p), localized in ER and on lipid droplets

surface. The acyl-CoA-dependent pathway is catalyzed by three enzymes: diacylglycerol transferase (Dgap) and two steryl ester synthetases (Are1p e Are2p). In oleaginous yeast, along with Lro1p, Dgap is localized on the surface of lipid droplets (Beopoulos et al., 2011).

The neutral lipids produced, mainly triacylglycerols, are accumulated in the lipid droplets in the yeast cells. Lipid droplets (LDs) are cellular organelles covered by a phospholipid monolayer that stores neutral lipids (mainly TAGs), sterols, and/or steryl esters. These compartments are derived from ER, where most enzymes related to neutral lipids formation are localized (Radulovic et al., 2013). The surface of LDs is also covered by some of these enzymes, along with other proteins related to signalization. LDs from yeasts are usually spherical structures that can have a diameter ranging from 300 nm (exponential phase) to 1 µm (stationary phase) (Kohlwein et al., 2013).

Importantly, the lipids accumulated in yeast's LDs and the FA profile of oleaginous yeasts are generally appropriated for biodiesel (18:0, 18:1, 18:2, and 16:0) and can be produced with strains able to utilize carbon sources from low-cost feedstocks (Patel et al., 2016).

1.2.1. Papiliotrema laurentii

Papiliotrema laurentii, previously known as *Cryptococcus laurentii,* is a non-conventional oleaginous yeast belonging to the Basidiomycota phylum, Tremellomycetes class, and Tremellales order (Liu et al., 2015). It is non-motile, encapsulated, and dimorphic yeast (Kurtzman, 1973). It is capable of assimilating different sugars as carbon sources: glucose, xylose, arabinose, cellobiose, mannose, galactose, rhamnose, sucrose, and galacturonic acid) (Sitepu et al., 2014). This yeast is distributed in many ecological niches: bird excrete (Brito et al., 2019); wheat and corn kernels surfaces (Kurtzman, 1973); kombucha tea (Chakraborty et al., 2016); vineyard (Wang et al., 2018); *Populus tremuloides* exudate (Sitepu et al., 2013); *Solanum torvum* leaf surface (Sitepu et al., 2014); aircraft internal surfaces (Hung et al., 2019); blue lupin rhizosphere (Moller et al., 2016); sugarcane bagasse (Gebbie et al. 2020); palm oil (Polburee et al., 2015); hydrocarbon-contaminated soil (Chandran & Das, 2012); and rupestrian field soil (Vieira et al., 2020). *P. laurentii* is also isolated from blood, cerebrospinal fluid, skin, and lungs, of immunocompromised patients, mainly in hospitals, as an opportunistic pathogen (Ferreira-Paim et al. 2014).

This yeast is promising in different biotechnological fields and applications. *P. laurentii* is broadly used for biocontrol of phytopathogenic fungi and contribute to quality maintenance

in post and preharvest fruits, like pears, sweet cherry, table grapes, and strawberry (Wei et al., 2014). In blue lupin rhizosphere, improve mycorrhizal colonization, nitrogen nutrition and plant growth (Moller et al., 2016). It is important in the degradation of industrial polymers, such as polyester (Barlow et al., 2020; Hung et al., 2019) and diesel (Chandran & Das, 2012). Also, it has potential in the bioremediation of heavy metals, like Plumb (II), Arsenium (III), and Chromium (IV) (Sarkar et al., 2019).

There are few studies reporting lipid production by *P. laurentii* with contents ranging from 26.6 to 63.5% (Wang et al., 2018; Polburee et al., 2015; Castanha et al., 2014; Carota et al., 2017; Sitepu et al., 2013; Sitepu et al., 2014; Vieira et al., 2020) (Table 1.1). It is noteworthy that its FA profile is suitable for biodiesel production (Carota et al., 2017; Castanha et al., 2014; Vieira, 2018; Wang et al., 2018). In addition, biodiesel produced from *P. laurentii* oil was in agreement with the standards of quality (regarding European and Brazillian regulation agencies) (Wang et al., 2018; Vieira et al., 2020). As mentioned previously, *P. laurentii* assimilates sugar constituents of lignocellulosic biomass; thus, it is a promising feedstock for oil production. Furthermore, this yeast can tolerate furfural and hydroxymethylfurfural (HMF), two inhibitors formed during the pretreatment of lignocellulosic biomasses (Sitepu et al., 2014). Nevertheless, the growth of *P. laurentii* is strongly impaired by acetic acid, another inhibitor generated in the pretreatment step of those biomasses (Sitepu et al., 2014).

Table 1.1 – Lipid production by different strains of *Papiliotrema laurentii.*

Strain	Growth media	Cultivation mode	Time (h)	Lipidid content [% (g lipid/ g DW)]	Reference
AM113	Supplemented inulin hydrolisate	Batch (Shake flask)	72	48.7	(Wang et al., 2018)
AM113	Supplemented inulin hydrolisate	Fed-Batch (Bioreactor)	132	54.6	(Wang et al., 2018)
DMKU AmC14	Synthetic Media (Glycerol)	Batch (Shake flask)	120	28.4	(Polburee et al., 2015)
UCD 68-201	Diluted ricotta cheese whey plus $(NH_4)_2SO_4$	Batch (Bioreactor)	96	62.6	(Carota et al., 2017)
UCDFST 12-803	Alkaline pretreated corn stover	Batch (Shake flask)	168	26.6	(Sitepu et al., 2014)
UCDFST 68-684.1	Synthetic media (Medium A+ Glucose)	Batch (Shake flask)	72	31.3	(Sitepu et al., 2013)
UFV -1	Modified SS2 (Glucose)	Batch (Shake flask)	48	43.0	(Vieira et al., 2020)
UFV -1	Modified SS2 (Xylose)	Batch (Shake flask)	48	30.0	(Vieira et al., 2020)
UFV -1	Modified SS2 (Glucose + Xylose)	Batch (Shake flask)	48	37.4	(Vieira et al., 2020)
UFV -1	Modified SS2 (Xylose)(Optimized conditions)	Batch (Shake flask)	48	63.5	(Vieira et al., 2020)
UNESP 11	Cheese whey	Batch (Shake flask)	240	27.8	(Castanha et al., 2014)

1.3. Lignocellulosic biomass as a feedstock for lipid production

The development of modern biorefineries is of great interest since they can process biomasses into a wide range of marketable products, energy, and biofuels (Cherubini, 2010). Lignocellulosic biomass is constituted mostly by cellulose (25-53%), hemicellulose (12-36%), and lignin (6-36%). It is the most abundant and renewable material available on the planet, comprehending many sources such as herbaceous plants, grains (rice, wheat, corn), cotton, and sugarcane from agricultural activities, willow, eucalyptus, wood blocks, wood chips, and barks from the forest sector; husk, bagasse, cob, and sawdust, from the industry (Cai et al., 2017). In Brazil, it is mostly derived as byproducts from the sugar and alcohol industries, especially from sugarcane (stem and straw), since the country is the biggest producer of this feedstock in the world (in 2018/19 harvest 620.4 million tons were produced) (Conab, 2019). Taking into account that each ton produced generates an average of 275 kg of bagasse and 140 kg of straw (Canilha et al., 2012), in the harvest of 2018/19, around 257.5 million tons of lignocellulosic biomass were generated from sugarcane. Nowadays part of that is utilized for 2nd G bioethanol production or combustion.

Cellulose is a linear polymer of glucose with β(1-4)-glycosidic bonds forming cellobiose, which is repeated several times along the chain, forming fibers intra- and interconnected by hydrogen bonds. Hemicellulose is a heteropolysaccharide composed of pentoses (D-xylose forming xylan, L-arabinose) for the most part, hexoses (D-glucose, D-galactose, D-mannose), linked to D-glucuronic acid and 4-O-methyl-D-glucuronic acid. The structure of hemicellulose is amorphous and branched, different from cellulose, which makes it easier to hydrolyze. Lignin is a macromolecule formed by three hidroxycinamyl alcohol monomers (syringyl, guaiacyl, and p-hydroxy phenol) with different degrees of methoxylation (Figure 1.2)(Canilha et al., 2012; Haghighi Mood et al., 2013). In their native form, these components are not assimilable by most microorganisms, requiring three major steps to become available for fermentation: pretreatment, hydrolysis, and detoxification (Figure 1.3) (Kumar et al., 2017).

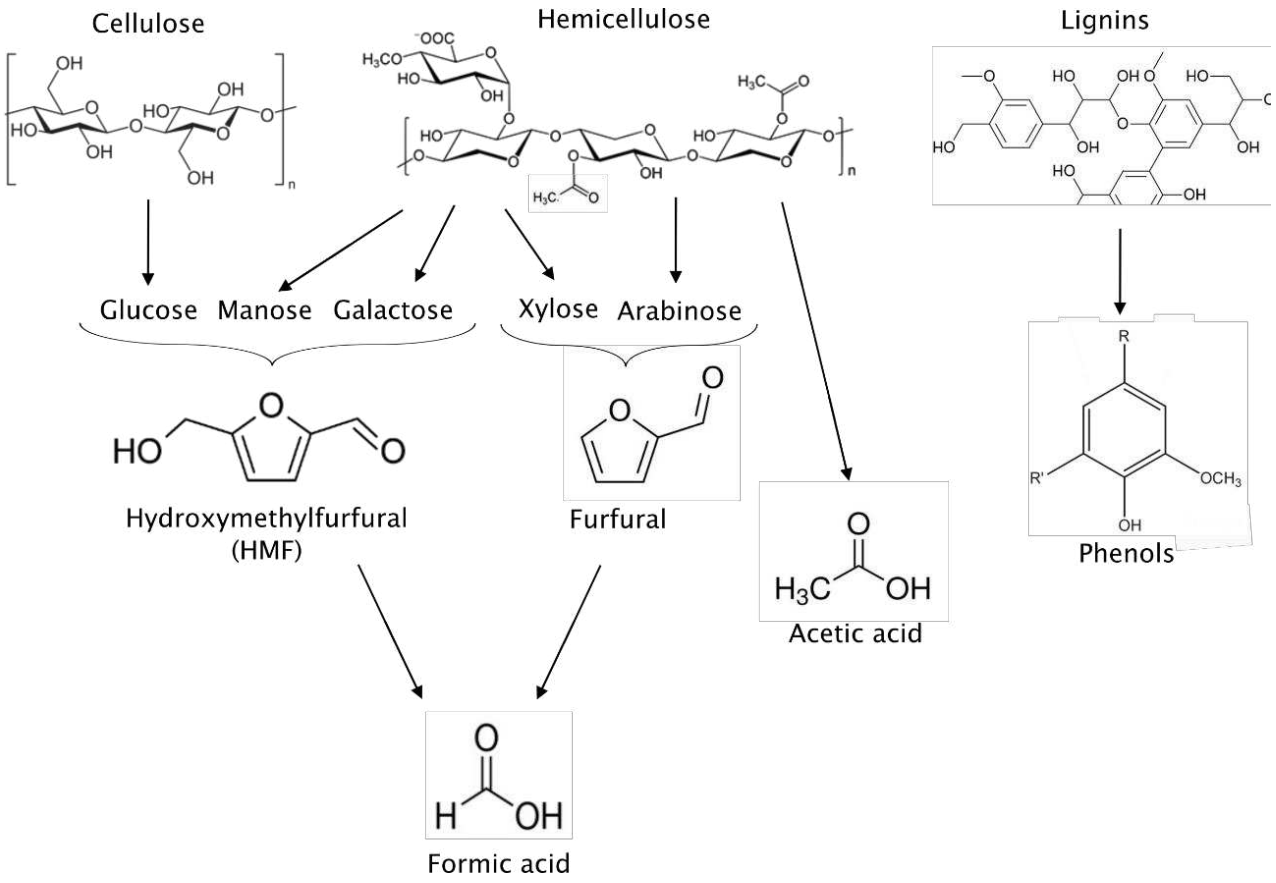

Figure 1.2 – Schematic representation of lignocellulosic biomass components (cellulose, hemicellulose, and lignin) breakdown, major sugars obtained (glucose, mannose, galactose, xylose, and arabinose), and most abundant inhibitors formed (HMF, furfural, formic acid, acetic acid, and phenols).

Lignocellulosic biomass is recalcitrant, as such its pretreatment is an indispensable step to separate its components by lignin removal, hemicellulose separation, and reduction of cellulose crystallinity (Kucharska et al., 2018). It involves the application of physical, chemical, and/or biological processes to disrupt the structure. The objective of physical pretreatments (like milling, chipping, grinding) is to increase surface area, reduce the particle size, and the degree of polymerization and crystallization. Among the chemical methods, dilute acid pretreatment is the most reported and utilized to solubilize hemicellulose and lignin and increase accessibility to cellulose. Usually, sulfuric acid is utilized in the concentration range of 0.2 – 2.5%, mixed with biomass, heated to a temperature between 100-210 °C, preferentially with stirring (Kumar et al., 2017).

Although biological methods take advantage of the degradation mechanisms used by saprophytic fungi and are more environmentally friendly, their use are less common, because they are time-consuming (at least one weak) and the scale-up is not a trivial task. Among all pretreatment methods, dilute acid pretreatment has been the most utilized (Kumar et al., 2017; Jin et al., 2015).

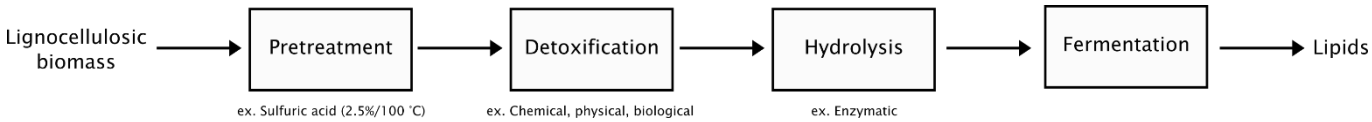

Figure 1.3 – Major steps required by lignocellulosic biomass (pretreatment, detoxification, and hydrolysis) to become suitable for lipid production.

The pretreatment of lignocellulosic biomasses leads to the formation of toxic compounds that can inhibit microbial growth such as weak acids (e.g. acetic acid), furans (e.g. furfural and HMF), and phenolics (e.g. vanillin) (Zha et al., 2014). Bond breaks in lignin result in a high number of phenolics compounds and acids, like 4-hydroxybenzoic acid, 4-hidroxybenzaldehyde, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, syringic acid, Hibbert's ketones, p-coumaric, and ferulic acid (Jönsson & Martín, 2016). During pretreatment, the hemicellulose is hydrolyzed, releasing sugars. However, degradation also can occur causing the liberation of its side chains, composed of aliphatic acids (acetic acid, formic acid, and levulinic acid). Pentose (e.g., xylose) dehydration led to 2-furaldehyde (furfural) formation, and hexose (e.g. glucose, mannose, galactose) dehydration lead to 5-hydroxymethyl-2-furaldehyde (HMF) formation. Under more severe conditions furfural and HMF can be further degraded to formic and levulinic acids (Figure 1.2). Phenolics compounds can inhibit growth and reduce product yield interfering in cell membrane function and structure (Jönsson et al., 2013). Furans (furfural and HMF) are usually less toxic to cells, since can be oxidized in the cytosol to their respective alcohols (furfuryl and 5-hydroxymethyl furfuryl) and acids (furoic), but can still alter membrane permeability, induce oxidative stress, inhibit glycolytic enzymes, and cause damages in DNA and cellular structures in concentrations up to 25 mM (Ask et al., 2013; Chandel et al., 2013; Allen et al., 2010; Taherzadeh et al., 2000).

Weak acids, such as acetic acid, are more toxic since their undissociated form can diffuse across the cell membrane and dissociate in the cytosol, leading to the dissipation of the proton motive force and intracellular anion concentration. These changes provoked by organic acids can lead to growth arrest, a decrease in product yield, sugar uptake rate, or even cell death, due to the increase in ATP requirement and decrease in enzyme activity (Palmqvist & Hahn-Hägerdal, 2000b).

Detoxification processes have been applied to remove or reduce the concentration of inhibitors and prevent or avoid these inhibitory effects. Evaporation and membrane-mediated (e.g. adsorption) are the most common physical methods of detoxification. Chemical methods have a broad spectrum of action including precipitation and removal of toxic compounds, mainly furans and lignin-derived molecules. Biological methods can result in high yields, but they tend to be prolonged and lead to loss of fermentable sugars (Canilha et al., 2012; Jönsson et al., 2013; Jönsson & Martín, 2016; Lee et al., 2014; Palmqvist & Hahn-Hägerdal, 2000a; Zhang et al., 2010). Even though a detoxification step has been commonly used, it presents drawbacks such as the addition of one step in the bioprocess and incomplete removal of some inhibitors (Jin et al., 2015; Jönsson & Martín, 2016). Most of the detoxification methods are more efficient to reduce furans and phenolic compounds, therefore high concentrations of acetic acid can be found in the hemicellulosic hydrolysate after the detoxification step. Frequently, those methods present none (Yu et al., 2011) or insufficient decreases (only 40-50%) (Bonturi et al., 2017; Lee et al., 2014) of that compound.

After pretreatment and detoxification, hydrolysis of cellulose and hemicellulose is performed mainly by either enzymes or acids. Although acid hydrolysis can reach a high yield in relatively short times, it causes degradation of sugars, generation of a higher number of inhibitors, and corrosion. As such, enzymatic hydrolysis has been more applied as it is conducted at low temperatures (40-60 °C) and slightly low pH ranges (4-6), compared to acid hydrolysis ($pH \leq 2$). Besides, it is not corrosive, under optimized conditions high yields can be achieved and enzymes can be partially or completely recycled. Cellulose hydrolysis requires enzymes called cellulases, that comprehend 15 families and a few sub-families. The enzymes most utilized are endo-glucanases, exo-glucanases, and β-glucosidases. Endo-glucanases attack amorphous parts (low crystallinity) and leave free chain-ends, which are attacked by exo-glucanases, releasing cellobiose (glucose dimers), which in turn is converted to glucose by β-glucosidases. It is important to point out that high cellobiose concentrations have inhibitory effects on cellulases, as high concentrations of glucose inhibit the activity of β-glucosidases.

Even though acidic conditions during pretreatment are capable of disrupting part of the heteropolymeric structure of hemicellulose, some enzymes can be applied to enhance the liberation of sugar monomers. Endo-β-1,4-xylanase produces xylooligosaccharides from xylan, β-xylosidase produces monomers of xylose, which comprehends the main hemicellulases applied (Kumar et al., 2017). Together, cellulases and hemicellulases, form the most common enzymatic cocktails in lignocellulosic hydrolysis. They can be utilized separately from fermentation or simultaneously. Simultaneous saccharification and fermentation (SSF) reduce contamination risks and costs for lipid production. Another process that can be used is the consolidated bioprocess (CBP), in which the microorganism used in the fermentation step also produces the hydrolytic enzymes required for both cellulose and hemicellulose hydrolysis. The CBP is promising to make the lipid production from lignocellulosic biomass feasible (Jin et al., 2015).

Lipids produced by oleaginous yeasts from lignocellulosic biomasses can be used for biodiesel production and other bio-based products that have applications in both food and chemical industries within a biorefinery concept. Some works evaluated the lipid production by oleaginous yeasts from different lignocellulosic biomasses. *Rhodosporidium toruloides* Y4 achieved 36.4% (g lipid/g DW) in corn stover hydrolysate (Xie et al., 2012). Lipid content of 40.0% (g lipid/g DW) was reached by *Trichosporon fermentans* in rice straw hydrolysate (Huang et al., 2009). Lipid contents as high as 27.1% (g lipid/g DW), 24.6% (g lipid/g DW), 20.7% (g lipid/g DW), 29.1% (g lipid/g DW) were obtained using *Cryptococcus curvatus*, *Rhodosporidium glutinis*, *R. toruloides*, and *Lipomyces starkeyi* in detoxified wheat straw, respectively (Yu et al., 2011). An engineered *Yarrowia lipolytica* Po1g achieved 48.0% (g lipid/g DW) and 58.5% (g lipid/g DW) in rice bran, and sugarcane bagasse hydrolysates, respectively (Tsigie et al., 2011; Tsigie et al., 2012). *R. graminis*, *Papiliotrema laurentii* UCDFST 12, and *C. humicola* UCDFST 10-1004 achieved lipid contents of 34% (g lipid/g DW), 26.6% (g lipid/g DW), and 43.1% (g lipid/g DW) in corn stover hydrolysates, respectively (Galafassi et al., 2012; Sitepu et al., 2014).

1.4. Adaptive Laboratory Evolution (ALE)

Adaptive Laboratory Evolution (ALE), also called experimental evolution, consists of the cultivation of cells for prolonged periods, in a controlled environment to improve the overall fitness of the population through natural selection (LaCroix et al., 2017; Sandberg et al., 2019). The first study that employed this methodology backs to Darwin's contemporary (XIX century), William Dallinger, that showed increased temperature selection over time for protozoa (Dallinger, 1878). The field remained dormant until the second half of the XX century, and the major example is the long-term evolution experiment with 12 populations of *Escherichia coli* conducted by Richard Lenski's research team since 1988, and that already passed 74,000 generations (Lenski et al., 1991; Lenski, 2017). The emergence of ALE studies in recent years has been driven by the increasing availability of new-generation sequencing technologies, bioinformatics tools, omics analysis, genome-scale metabolic models, and flux balance analysis. Those techniques allow a better understanding of the molecular and genomic bases of evolution, the relationship between genotype and phenotype, fitness characterization, and mutation dynamics in populations. The majority of mutations detected in ALE are single-nucleotide variations (61%) followed by deletions (29%), insertions (7%), and insertion sequence (IS) movements (3%) (Conrad et al., 2011).

The utilization of microorganisms such as bacteria, yeast, and viruses, present major advantages for ALE experiments: they are usually easy to propagate (simple nutrient requirements) and count; have short generation times (< 10h); large populations can be contained in relatively small volumes; cell from different times of evolution can be frozen and stored for recover of the experiment in case of lost and/or contamination, and further genetic and phenotypic analyses (Elena & Lenski, 2003). ALE experiments allow the gain of insights into a series of characteristics of evolution dynamics, such as genetic bases of increase in fitness, implications of historical contingency, second-order effects, the relation between population size and evolution, clonal interference, and epistasis (Dragosits & Mattanovich, 2013).

ALE experiments are performed either in batch or continuous cultures. Batch cultivations are usually performed in shake flasks with serial transfers at regular intervals. An aliquot of the culture (e.g., 1%) is transferred to a flask with a fresh medium for a new round of growth. To decrease complexity and facilitate the determination of adaptation pathways, the microbial population can be maintained in one growth phase, like exponential, which makes fitness directly related to the growth rate (Dragosits & Mattanovich, 2013; LaCroix et al., 2017). The serial transfer is the most popular setup, due to its simplicity and requirement of cheaper equipment, making it able to be performed in almost any microbiology laboratory. However, conditions are not always constant since populations alternates between high and low population densities, due to dilution rate, which also amplifies the chances of genetic drift events (Van den Bergh et al., 2018; LaCroix et al., 2017). ALE experiments can be conducted in continuous culture, usually in chemostats or turbidostats. In chemostats, fresh medium is continuously added to the culture at a defined rate in the same amount that culture is removed from the reactor. The feed media present a nutrient in limiting concentrations (carbon, nitrogen, phosphorus, sulfur) and dictates the steady-state condition. This setup was first utilized by Jacques Monod, in 1950, and further named and characterized by Novick and Szilard (Gresham & Hong, 2015; Novick & Szilard 1950). When the steady-state is reached and all conditions remain constant (pH, dissolved oxygen, cellular density, temperature) the growth rate of the culture can be controlled by modulating the culture dilution rate. The turbidostat works similar to a chemostat but without cells experiencing a nutrient limitation, which makes the steady-state condition more similar to the mid-log growth phase (Gresham & Dunham, 2014). Setting up a chemostat can be changeling due to its more complex equipment requirements and costs. However, continuous cultures present some advantages for ALE experiments compared to traditional batch serial transfer such as the tight control of nutrient supply and environmental conditions, which ensures sustained and invariant selective pressure. Another advantage is the maintenance of culture with high cell densities (10^7-10^9), which increases the chances of selecting beneficial mutations in addition to reducing genetic drift (Gresham & Dunham, 2014). It is noteworthy that to reduce struggles commonly present in ALE experiments (ex. difficulties in continuous replications, risk of contamination due to manipulation), and increase the number of parallel populations in the same study, automated platforms for microbial evolution are emerging, such as eVOLVER (Wong et al., 2018), and the system developed by Horinouchi et al. (2014).

The eVOLVER is a framework that allows automated culture growth with temperature, culture density, medium composition control. It can continuously control multiple (ex. hundreds) of individual cultures, collecting, measuring, and recording data in real-time. Algorithmic culture routines can be programmed, coupling the status of the culture (ex. optical density) to automated manipulation (ex. dilution into fresh media). Its noteworthy, that this system can be designed to function with continuous or batch regimes (Wong et al., 2018). Horinouchi et al. (2014) developed an automated culture system with serial transfer using a 96-well microplate. The system is placed in a clean booth and connected to a plate reader and incubator. It allows automated maintenance of hundreds of independent culture series in a specific growth phase (ex. exponential) in a batch regime.

ALE can be also useful for biotechnological applications such as activation of latent pathways, utilization of non-native substrates or production of non-native products, optimization of substrate utilization, increase of production rate or titer, improvement of the growth rate, increase of tolerance to a specific compound, environmental condition or stress commonly present in industrial processes (Portnoy et al., 2011; Sandberg et al, 2019).

In industrial applications microorganisms are frequently challenged by harsh or stressful conditions, such as fluctuations in pH, dissolved oxygen, temperature, pressure (atmospheric and hydrostatic), nutrient availability, and/or presence of compounds that could inhibit growth. ALE can be successfully applied to overcome such limitations, emerging both specialists and generalists (Dragosits & Mattanovich, 2013). A specialist is adapted to thrive in a specific environment but will struggle in other situations (environments different from those that applied in the evolution). Adaptation to constant conditions, or when stress is progressively increased, can favor the emergence of such populations, presenting phenotypes trade-offs, also called negative correlated responses or costs of adaptation. It is worth mentioning that trade-offs can also contribute to the evolution of diversity. Trade-offs can result from an accumulation of neutral mutations in the experimental conditions but can become deleterious in other situations or for characteristics other than selective pressure (Van den Bergh et al., 2018; Maddamsetti et al., 2015). Generalists can thrive in a wide range of environments and often arise when the population is exposed to different conditions during the experiment (Sandberg et al., 2017). This can constrain the evolutionary process, and result in lower fitness when compared to a specialist in a determined condition (Van den Bergh et al., 2018).

The emergence of stressful conditions during biofuel production can be also addressed by ALE. Studies involving ALE and stress tolerance are efficacious due to the complexity of stress response, which frequently employs global regulatory and physiological responses. Conditions such as high-temperatures (Caspeta et al., 2014), hyperosmotic (Tilloy et al., 2014), different ranges of pH (Fletcher et al., 2017; Narayanan et al., 2016), high ethanol concentration (Avrahami-Moyal et al., 2012; Stanley et al., 2010; Da Silveira et al., 2020) and oxidative (Cakar et al., 2005), organic acids (Aarnio et al., 1991; Fletcher et al., 2017) have been used to obtain microbial strains with improved tolerance. Specifically, the inhibitors and conditions during lignocellulosic biomass pretreatment and hydrolysis can also be targeted by ALE: copper released from equipment in acidic conditions (Adamo et al., 2012), HMF toxicity (Sehnem et al., 2013), corn stover hydrolysate (Almario et al., 2013; Wang et al., 2018), spruce hydrolysate (Koppram et al., 2012), bamboo hydrolysate (Qin et al., 2016), inhibitors cocktail (acetic acid, furfural, HMF, and vanillin) (Narayanan et al. 2016), acetic acid toxicity (Wright et al., 2011), the combination of inhibitors and temperature (Wallace-Salinas & Gorwa-Grauslund, 2013). A common feature in ALE experiments involving stress tolerance is cross-protection. In this case, microorganisms exposed to a stressor improves their fitness to other stress conditions. Some examples of cross-protection are: long-term adaptation to oxidative stress in *S. cerevisiae* also improved its tolerance to salt stress (Dhar et al., 2013); continuous exposure to cobalt led to improved tolerance to other metals, and pulsed exposure to thermal and oxidative stress in *S. cerevisiae* led to an improvement in both phenotypes (Çakar et al., 2009); *n*-butanol adaptation led to increasing hyper-osmotic, oxidative, acidic and osmotic stress tolerance (Dragosits et al., 2013).

Only six works reported the use of ALE to select oleaginous yeast strains (Díaz et al., 2018; Daskalaki et al., 2019; Walker et al., 2019; Hicks et al., 2020; Wang et al., 2021). Four of them successfully selected strains of *R. toruloides* (Díaz et al., 2018; Liu et al 2021), *Metschnikowia pulcherrima* (Hicks et al., 2020), and *Yarrowia lipolytica* (Wang et al., 2021) more tolerant to those inhibitors by applying a serial passage experimental design, with formic acid (Hicks et al., 2020), ferulic acid (Wang et al., 2021), non-detoxified lignocellulosic biomass (Díaz et al., 2018; Liu et al 2021), and a cocktail of inhibitors (furfural, HMF, formic and acetic acid; Hicks et al., 2020).

Therefore, ALE is a powerful technique to investigate evolutionary processes and improve metabolic engineering strategies. ALE can complement, or replace, rational strain designs (e.g. pathways insertion/deletions and induction/repression of promotor sites) and random mutagenesis (e.g. U.V exposure) approaches, being of great utility to Design-Built-Test (DBT) cycle employed in strain construction, especially in design and build steps (Sandberg et al., 2019; Shepelin et al., 2018).

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CHAPTER 2 - NEW *Papiliotrema laurentii* UFV-1 STRAINS WITH IMPROVED ACETIC ACID TOLERANCE SELECTED BY ADAPTIVE LABORATORY EVOLUTION

2.1. Introduction

The increasing emissions of greenhouse gases and depletion of fossil fuels have boosted the development of new technologies for biodiesel production. There are concerns regarding the utilization of vegetable oils for biodiesel production as they require arable land, water, and nutrients, which could be utilized to grow crops for human consumption and native vegetation (Kumar et al., 2017). Microbial oils produced mainly by microalgae and yeast are a promising oil source for biodiesel production. Non-conventional oleaginous yeast such as *Lipomyces starkeyi, Rhodosporidium toruloides,* and *Papiliotrema laurentii* can accumulate high amounts of lipids (20-70% g lipid/g DW) and metabolize lignocellulose-derived sugars (Spagnuolo et al., 2019; Viera et al., 2020).

Due to the recalcitrant nature of lignocellulosic biomasses, a pretreatment step is applied to separate hemicellulose and cellulose as well as exposure them to hydrolytic enzymes. The most employed pretreatment [sulfuric acid 0.2-2.5% combined with heat (100-210 °C)] leads to the formation of toxic compounds that can inhibit yeast growth (Kumar et al., 2017; Jin et al., 2015). Hemicellulose degradation results in a release of its ramifications, composed of aliphatic acids such as acetic and formic. Pentoses and hexoses dehydrations lead to furfural and HMF (hydroxymethylfurfural) formation, respectively. Moreover, furfural and HMF can be transformed into formic acid at elevated temperatures and pressures (Zha et al., 2014).

Acetic acid is the most abundant inhibitor after the pretreatment step, ranging from 0.5 – 4.0 g/L. Its undissociated form diffuses through the cell membrane and dissociates in the cytosol, causing acidification, anion concentration, and dissipation of the proton motive force. This disturbs cell homeostasis and energy requirements, leading to growth reduction, cell death, and drops in productivity (Palmqvist & Hahn-Hägerdal, 2000; Pampulha & Loureiro-dias 2000; Jönsson & Martín, 2016).

In *Saccharomyces cerevisiae,* the acetic acid uptake takes place by diffusion in the cell membrane. In addition, its uptake can be facilitated by the aquaglyceroporin Fps1 (Mollapour & Piper, 2007). Once inside the cytosol, where the pH is close to the neutral, it dissociates. Acetate can be used as a carbon source for biomass formation. The assimilation of acetate

requires the activity of acetyl-CoA synthase, which converts acetate to acetyl-CoA, with ATP consumption. Acetyl-CoA can be incorporated into different biomass components. It can be directed to lipid biosynthesis through acetyl-CoA carboxylase activity – converts acetyl-CoA in malonyl-CoA – or to the formation of amino acid precursors. In *S. cerevisiae,* this occurs through the glyoxylate shunt requiring malate synthase and isocitrate lyase activity, whilst in *Zygosaccharomyces bailii* it is metabolized in the citric acid cycle (Chen et al., 2012; Ludovico et al., 2012; Palma et al., 2018). The utilization of acetic acid as a carbon source can occur along with sugar consumption as reported for *Z. bailii* (Ludovico et al., 2012). For instance, the transport and metabolism of acetic acid in *P. laurentii* are unknown.

To circumvent the inhibitor effect of the compounds aforementioned, detoxification of lignocellulosic hydrolysates is applied to remove or reduce their concentrations. However, detoxification is usually insufficient to remove acetic acid in these hydrolysates (Bonturi et al., 2017; Chandel et al., 2013). Therefore, oleaginous yeasts capable of tolerating acetic acid are of interest in bioprocesses based on the use of hemicellulose acid hydrolysates.

Recently, our research team isolated and characterized a *Papiliotrema laurentii* strain able to achieve high lipid contents from a minimal medium containing xylose as the sole carbon source (Vieira et al. 2020). Nevertheless, its capacity to grow in hemicellulosic hydrolysates was not evaluated. Previously, Sitepu (2014) showed that the *P. laurentii* UCDFST 12 strain growth was severely impaired by acetic acid.

Adaptive Laboratory Evolution (ALE) has been successfully used to select microbial strains with improved tolerance to inhibitor compounds. In ALE, microbial cells are cultivated in defined culture media under controlled conditions during extensive periods in either batch or continuous cultures. This allows increasing the overall fitness of microorganisms by natural selection (Dragosits & Mattanovich, 2013; LaCroix et al., 2017). As such, it has been widely applied to obtain robust microbial cells more tolerant to bioprocesses conditions and to understand their response mechanisms. Regarding the environmental stresses caused by pretreated biomass inhibitors, several works involving ALE have been performed, mainly with strains of *Saccharomyces cerevisiae* (Avrahami-Moyal et al., 2012; Caspeta et al., 2014; Fletcher et al., 2017; Narayanan et al., 2016; Stanley et al., 2010; Tilloy et al., 2014; Sandberg et al., 2019). To the best of our knowledge, only six works have reported the use of ALE to select more robust oleaginous yeast strains (Díaz et al., 2018; Daskalaki et al., 2019; Walker et al., 2019; Hicks et al., 2020; Wang et al., 2021). Four of them successfully selected strains of *Rhodosporidium toruloides* (Díaz et al., 2018; Liu et al., 2021), *Metschnikowia pulcherrima*

(Hicks et al., 2020), and *Yarrowia lipolytica* (Wang et al., 2021) more tolerant to inhibitors derived from lignocellulosic processing. They applied a serial passage experimental design, with formic acid (Hicks et al., 2020), ferulic acid (Wang et al., 2021), non-detoxified (Díaz et al., 2018; Liu et al., 2021), or detoxified (Liu et al., 2021), lignocellulosic biomass and a cocktail of inhibitors (furfural, HMF, formic and acetic acid; Hicks et al., 2020). Therefore, ALE can be successfully applied to select oleaginous yeast strains with enhanced tolerance to inhibitory compounds found in hemicellulosic hydrolysates, paving the way to use them in lignocellulosic-based biorefineries.

In our study, we selected and characterized three acetic acid-tolerant strains (ATS) of *P. laurentii* UFV-1 by ALE. The selected strains presented improved growth in the presence of acetic acid. However, different phenotypes emerged alongside. The ATS II, presented trade-offs in the absence of the acetic acid, suggesting that it displays a specialized phenotype of tolerance to this acid. On the other hand, ATS I and III presented phenotypes more associated with the behavior of generalists.

2.2. Materials and Methods

2.2.1. Yeast strain and maintenance

The *Papiliotrema laurentii* UFV-1 parental strain belongs to the culture collection of Microbial Physiology Laboratory of Microbiology Department at Federal University of Viçosa (UFV). The tolerant strains obtained by Adaptive Laboratory Evolution (ALE) were denominated **A**cetic acid **T**olerant **S**trains (**ATS**). The yeast cultures were maintained frozen (-80 °C) in YP medium [yeast extract 1% (w/v), peptone (1% w/v)] containing 30% (v/v) glycerol.

2.2.2. Effect of lignocellulose-derived inhibitors on the growth of Papiliotrema laurentii UFV-1

For pre-inoculum preparation, a single colony of *P. laurentii* UFV-1 grown on YPD agar medium [yeast extract 1% (w/v), peptone 1% (w/v), glucose 2% (w/v), agar 1.5% (w/v)] for 72 h and 30 °C was transferred to 250 mL Erlenmeyer's flask containing 50 ml of Yeast Nitrogen Base (YNB) medium without amino acids (Sigma Chemical CO., St. Louis, MO, USA) 6.7 g/L with xylose 20 g/L as carbon source and cultivated at 30 °C and 200 rpm for 16h. After this period, the yeast culture was centrifuged at 4 °C and 10,000 *g* for 10 min, washed twice with peptone water (0.1% w/v) and diluted to an Optical Density at 600 nm (OD_{600}) of

0.1. *Papiliotrema laurentii* UFV-1 was inoculated in 250 mL Erlenmeyer's flask containing 100 mL YNB medium containing xylose (20 g/L) and separately added with lignocellulose-derived inhibitors (acetic acid, HMF, furfural, and formic acid) at concentrations ranging from 0.1 to 2.0 g/L and control (0 g/L). Growth was monitored by measuring the OD_{600} using a spectrophotometer (BECKMAN DU series 600). The experiments were conducted as single biological replicates to investigate more concentrations in the screening step.

2.2.3. Adaptive Laboratory Evolution (ALE)

For pre-inoculum preparation, a single colony of *P. laurentii* UFV-1 grown on YPD agar medium for 72 h at 30 °Cwas transferred to 50 ml of SS2 nitrogen-rich medium (referred as medium A) [$(NH_4)_2SO_4$ (5 g/L), NaCl (0.1 g/L), $CaCl_2$ (0.1 g/L), $MgSO_4$ (0.5 g/L), yeast extract (0.1 g/L)] with xylose (5 g/L) in 250 mL Erlenmeyer flask, incubated at 30 °C and 200 rpm for 16 h (pH ~ 5.5). After incubation, the yeast culture was centrifuged at 4 °C and 10,000 *g* for 10 min and washed twice with peptone water (0.1% w/v). *Papiliotrema laurentii* UFV-1 was inoculated in 50 mL medium A with xylose (5.0 g/L) and acetic acid (0.7 g/L) in a 125 mL Erlenmeyer flask to obtain an initial optical density at 600 nm (OD_{600}) of approximately 0.1. The ALE was performed in serial passages of 1% (v/v) of the precedent batch (late exponential/stationary phase) to the next fresh medium. When the yeast population showed evidence of adaptation, that is, higher growth rate and shorter lag phase, the concentration of acetic acid was increased initially to 0.9 g/L (after 87 generations or 22 accumulated batches) and then to 1.5 g/L (after 247 generations or 50 accumulated batches). The whole ALE experiment consisted of 82 serial batches, around 398 generations (Figure 1). Three individual populations were simultaneously evolved and every 50 generations, samples were taken to verify the purity of evolved strains, store the evolved strains and evaluate the growth of the evolved strains in SS2 medium with acetic acid. The number of generations per batch (~4.85 generations) was estimated considering the parental strain exponential phase growing in media containing 0.7 g/L of acetic acid.

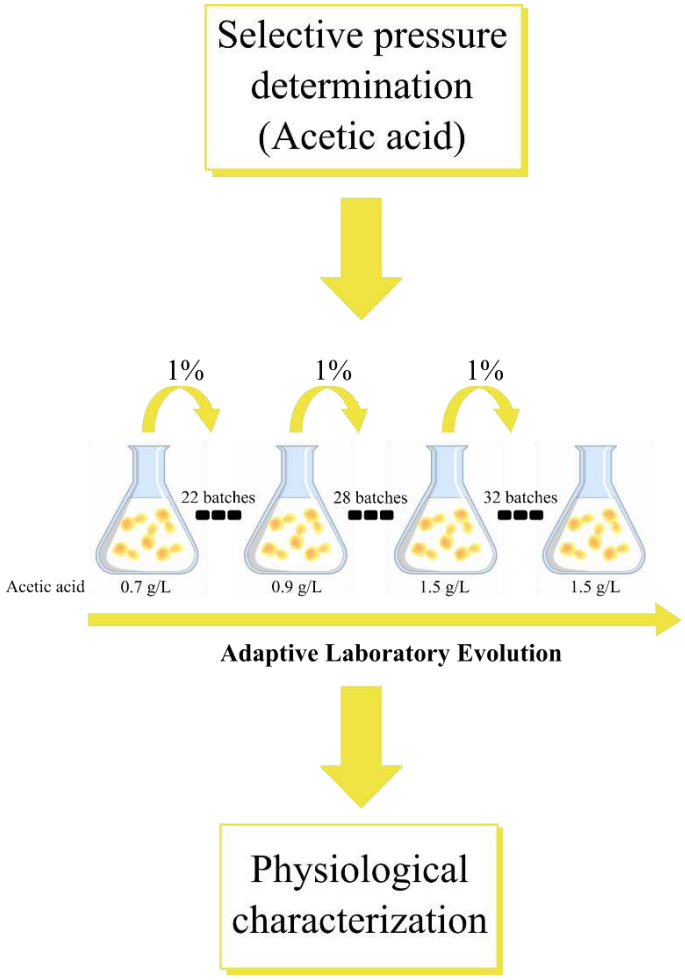

Figure 2.2.1 - Workflow of the applied Adaptive Laboratory Evolution (ALE). Firstly, the inhibitory effect of the acetic acid was evaluated to determine the concentration to be used as the selective pressure during the ALE. Adaptive Laboratory Evolution was performed in serial passages of 1% (v/v) of the precedent batch to the next fresh medium. The concentration of acetic acid was increased twice: firstly, to 0.9 g/L (after 22 batches); secondly, to 1.5 g/L (after 50 batches). After ending the ALE, the evolved strains were characterized regarding the oleaginous phenotype and tolerance to acetic acid.

2.2.4. Physiological characterization

The physiological characterization of *P. laurentii* ATS and parental strains were performed by determining kinetic and fermentative parameters, in different batch cultures. A single colony of *P. laurentii* UFV-1 cultivated in YPD agar medium for 72 h at 30 °C was transferred to 50 ml of medium A with xylose (5 g/L) in 250 mL Erlenmeyer flask, incubated at 30 °C and 200 rpm for 16 h. After incubation, yeast cultures were centrifuged at 4 °C and 10,000 *g* for 10 min, washed twice with peptone water (0.1% w/v). For batch cultivations (n = 2 or 3), *P. laurentii* UFV-1 strains were inoculated (to reach an initial OD_{600} of approximately 0.1) in 250 mL Erlenmeyer flasks with 100 ml of medium A with xylose (2.5 g/L) in the absence

or presence of acetic acid (0.7, 0.9, 1.5, 1.75, 2.0 g/L) and in 100 ml of SS2 medium with C:N ratio of 100:1 (w:w) (referred as medium B) [xylose (30 g/L) and $(NH_4)_2SO_4$ (0.09 g/L)] in the absence or presence of acetic acid (1.5 and 1.75 g/L). Yeast cultures were incubated at 30 °C and 200 rpm. Culture Medium pH was at 5.5 without acetic acid, and at 3.0-3.6 with the acid at the beginning of cultivations. In all cases, at the end of all cultivations, the pH was around 2.0.

Aliquots of 2 mL were withdrawn to measure the OD_{600} and centrifuged at 4 °C 10,000 *g* for 10 min. Then supernatants were filtered (pore size 0.22 µm) to quantify xylose and acetic acid concentrations. Samples were also collected to analyze lipid content and dry weight.

2.2.5. Determination of specific growth rate and dry weight

Cell growth was monitored by OD_{600}. Biomass was determined by establishing a calibration curve of cell dry weight (DW) versus optical density (DO_{600}). Overnight yeast cultures grown in 50 mL SS2 medium with xylose (20 g/L) (30 °C and 200 rpm) in 250 mL Erlenmeyer's flasks were centrifuged at 10,000 *g* at 4°C for 10 min. The pellet was resuspended in 6 mL of distilled water. Four aliquots of 1 mL were harvested and dried at 105 °C/24 h to determine cell dry weight. In parallel, 1 mL aliquots of cell suspension were diluted (1×10^{-2}, 2×10^{-2}, 3×10^{-2}, 4×10^{-2}, 5×10^{-2}, 6×10^{-2}) and the OD_{600} was measured. The calibration curve was obtained from linear regression between absorbance (OD_{600}) and DW (mg/mL).

Specific growth rate (µ) was determined by linear regression between values of OD_{600} and time (h) in the exponential growth phase.

2.2.6 Determination of biomass yield and parameters of lipid production

$$Biomass\ Yield\ (Y_{x/s}) = \frac{X_f - X_0}{S_0 - S_f} \qquad \text{(Eq. 1)}$$

Where: $X_f \equiv$ final biomass (g/L); $X_0 \equiv$ initial biomass (g/L); $S_f \equiv$ final xylose concentration (g/L); $S_0 \equiv$ initial xylose concentration (g/L).

$$Lipid\ \%\ (w/w) = \left(\frac{P}{DW}\right) \times 100 \qquad \text{(Eq. 2)}$$

Where: P ≡ final lipids (mg) (see 2.2.7); DW ≡ dry weight (mg).

$$Lipid\ Titer\ (g/L)\ = Lipid\ \%\ (\mathrm{w/w})\ \times\ \mathrm{X}_{f} \qquad \text{(Eq. 3)}$$

$$Lipid\ \text{Yield}\ (\mathrm{Y_{P/s}})\ = \frac{Lipid\ Titer\ (g/L)}{\mathrm{S_0 - S_f}} \qquad \text{(Eq. 4)}$$

Where: S_f ≡ final xylose concentration (g/L); S_0 ≡ initial xylose concentration (g/L).

$$Lipid\ \text{Productivity}\ (\mathrm{g/L\ h})\ = \frac{Lipid\ Titer\ (g/L)}{\mathrm{t}} \qquad \text{(Eq. 5)}$$

Where: t ≡ total time of cultivation (h).

2.2.7. Xylose and acetic acid quantification

Xylose and acetic acid concentration were determined by High-Performance Liquid Chromatography (HPLC) – LC-20AT (Shimadzu, Japan) - coupled to refractive index detector RID-20A (Shimadzu, Japan), and an Aminex HPX-87H ion exchange column (300 × 7.8 mm, 9 μm, Bio-Rad, Munich, Germany) column. The mobile phase was H_2SO_4 5 mM, with a flow rate of 0.7 mL/min, at 45 °C. The concentration of the compounds was calculated using xylose (concentrations ranging from 1.5 to 150 mM) and acetic acid (concentrations ranging from 0.2 to 30 mM) as external standards.

2.2.8. Lipid quantification

Total lipid quantification was carried out according to the procedure described by Bligh & Dyer (1959) with modifications described by Vieira et al. (2020). Fifty mg of lyophilized biomass was used to extract the lipid fraction. One mL of a methanol:chloroform solution [2:1, (v/v)] was added. The suspension was homogenized using Tissuelyser II (30 shakes per second for 5 min) (Qiagen, Germany). Then the solution was centrifuged at 12,000 × g for 10 min. This procedure was repeated 3 times to ensure the total extraction of lipids from the initial biomass. The supernatants were collected and stored in 15-mL centrifuge glass tubes. Next, 3 mL of 100% chloroform were added and the mixture homogenized. Two mL of NaCl 1% (w/v) was added to obtain a two-phase liquid system. The phases were separated by centrifugation at 1464 × g for 20 min. The lower phase was transferred to previously dried and weighed microtubes. The samples were then evaporated at 60 °C for 24 h, and the lipid content was determined gravimetrically.

2.2.8. Statistical analysis

Physiological characterization experiments were performed considering a completely randomized design (n = 2 or 3). All data were analyzed with the aid of the OriginPro 2016® software (OriginLab Corporation, Northampton, MA, USA). An analysis of variance was carried out between the samples using the F test at a 5% probability level. When differences were detected, the Tukey test was conducted, at the same probability level, for the comparison between treatments.

2.2.9. Accessibility

All colored figures were optimized to be accessible to color-blind individuals, as described by Wong (2011).

2.3. Results

2.3.1. Effect of lignocellulose-derived inhibitors on the growth of Papiliotrema laurentii UFV-1 strain

Papiliotrema laurentii UFV-1 strain displayed different inhibitory profiles in culture media containing compounds derived from lignocellulosic biomass (acetic acid, formic acid, furfural, and HMF) (Figure 2.2). Formic acid severely impaired yeast growth even in concentrations as low as 0.1 g/L (2.2 mM). Acetic acid and furfural also showed a considerable inhibitory effect, and it should be noted that *P. laurentii* UFV-1 did not grow in concentrations from 1.0 g/L (16.7 and 10.4 mM, respectively). The inhibitor effect of HMF was lower than other inhibitors evaluated herein. Although has been observed an inhibitory effect of HMF at concentrations ranging from 0.5 to 2.0 g/L (4 to 15.9 mM), the yeast was still able to grow. Even though the formic acid was the most inhibitory compound and the furfural also severely reduced the *P. laurentii* UFV-1 growth, acetic acid was chosen to be used as a selection pressure in the ALE experiment due to its toxicity and the fact of the detoxification step does not reduce the acetic acid concentration efficiently in hemicellulosic hydrolysates (see item 2.4. Discussion).

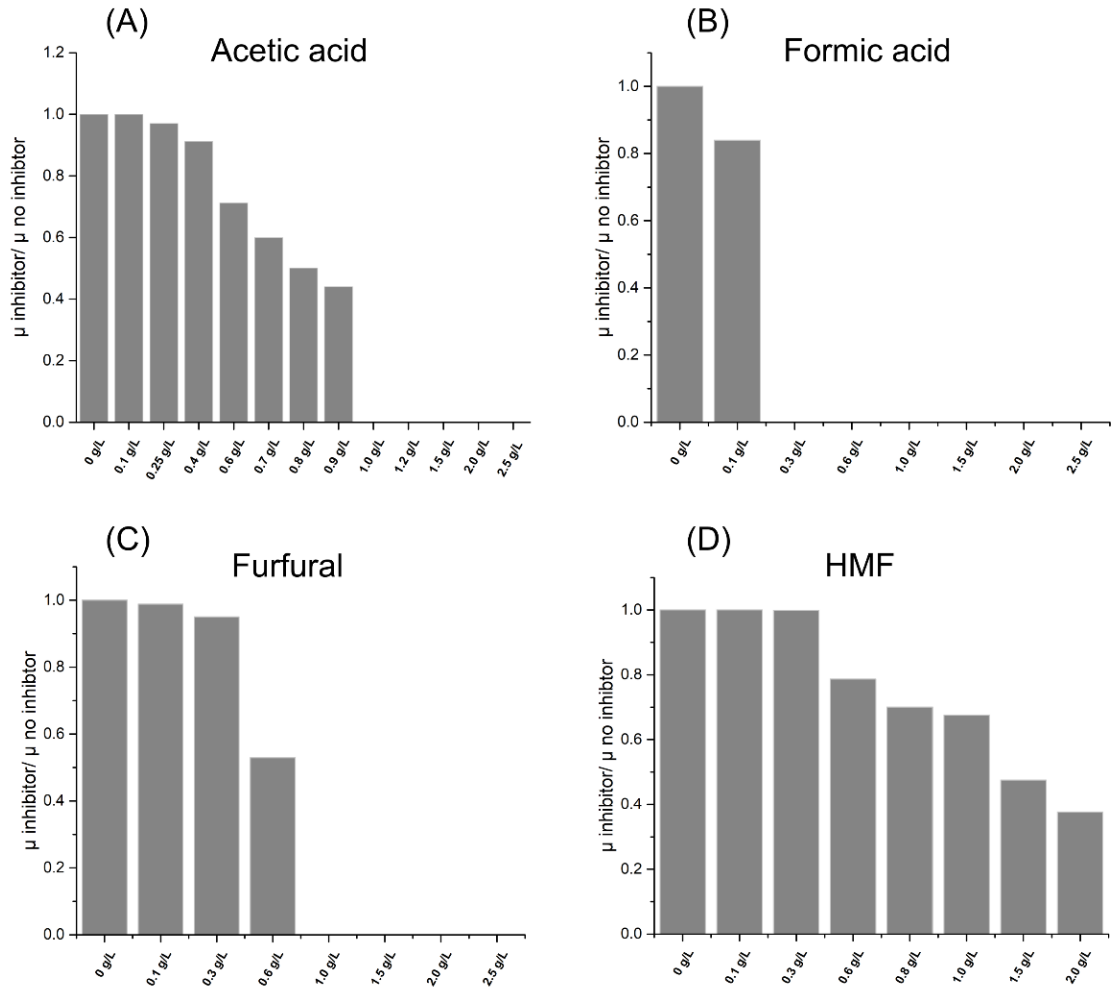

Figure 2.2.2 –Effect of lignocellulose-derived compounds [(A) acetic acid; (B) formic acid; (C) furfural; (D) HMF – hydroxymethylfurfural] on *Papiliotrema laurentii* UFV-1 growth, where: $\mu_{inhibitor} \equiv$ *P. laurentii* UFV-1 parental strain growth rate (h^{-1}) in the presence of the inhibitor; $\mu_{no\ inhibitor} \equiv$ *P. laurentii* UFV-1 parental strain growth rate (h^{-1}) in the absence of the inhibitor.

2.3.2. Adaptive Laboratory Evolution (ALE)

The ALE experiment was performed with three independent populations (batches) in increasing acetic acid concentrations 0.7, 0.9, and 1.5 g/L, which correspond to 11.7, 15, and 25 mM, respectively, for 97 days, corresponding to 82 serial passages (approximately 398 accumulated generations) (Figure 2.3). The passages were performed when de OD_{600} was > 1.0. The evolved strains are referred to as ATS I, II, and III (Acetic acid Tolerant Strains). The three populations displayed an adaptation period at the beginning of the experiment (0.7 g/L), as well as when the acetic acid concentration was raised to 1.5 g/L. After the adaptation period, the ATSs presented a steadier growth profile.

Although the three strains reached a population density sufficient to be passed to a fresh culture medium in the presence of 1.5 g/L of acetic acid, the OD_{600} values remained lower until the end of the experiment. It should be noted that the steady growth during almost 150 generations in culture media containing 1.5 g/L of acetic acid indicates that more tolerant populations of *P. laurentii* UFV-1 were successfully selected during this ALE experiment since the parental strain could not grow in concentrations above 1.0 g/L (Figure 2.2).

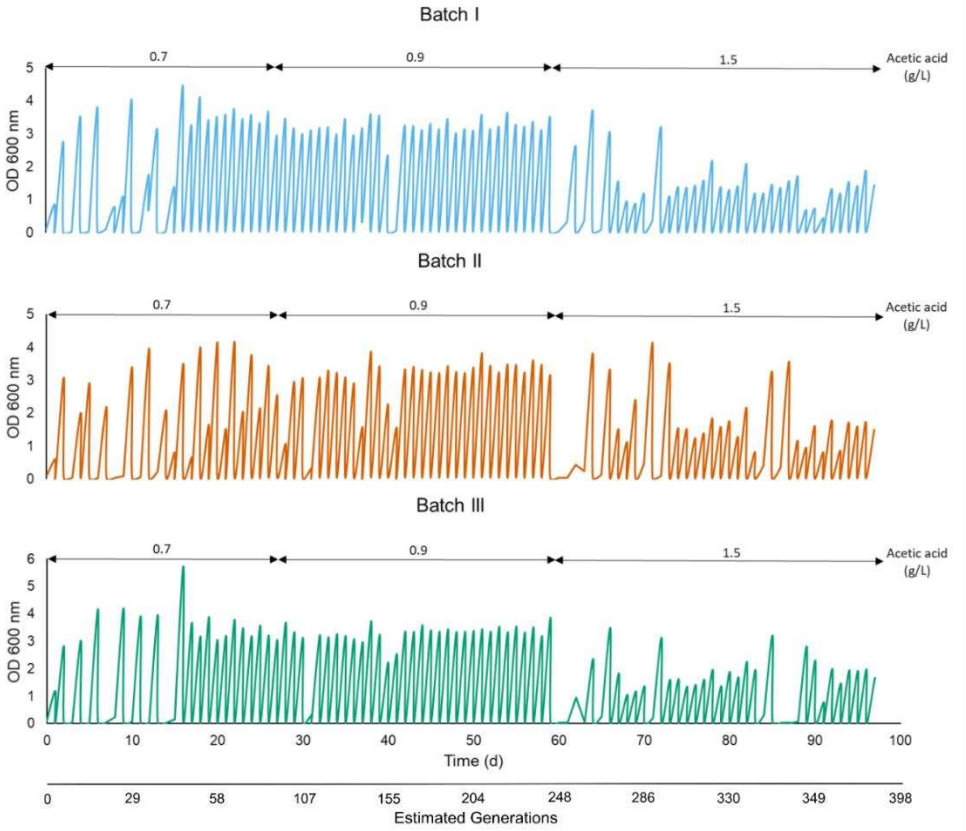

Figure 2.3 – Optical density (OD_{600}) of the three independent batches in the ALE experiment, before and after consecutive serial passages. The peaks represent the OD_{600} of the precedent culture before the passage, and the valleys represent the initial OD_{600} in the new passage.

2.3.3. Physiological characterization

To confirm if steady growth in different acid concentrations during the ALE represented the selection of yeast strains more tolerant to acetic acid, we evaluated their growth in the presence of this inhibitor, which allowed us to determine the lag phase period and specific growth rate (Figures 2.4 and 2.5, respectively).

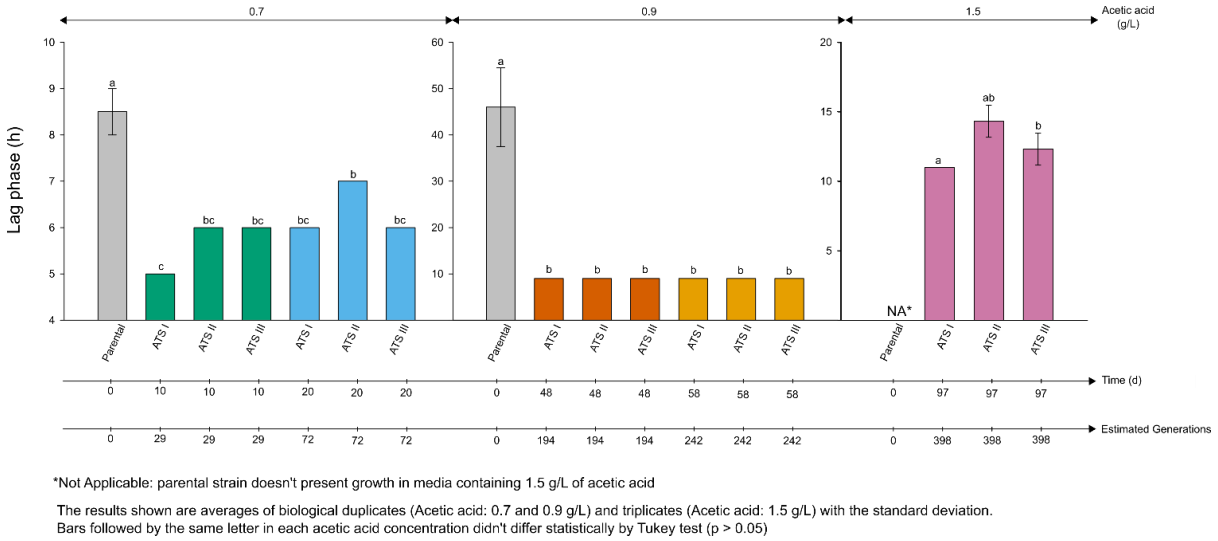

Figure 2.4 – Lag phase period (h) of the parental *P. laurentii* UFV-1 and ATS strains in cultivations carried out in the presence of three different concentrations of acetic acid (0.7, 0.9, 1.5 g/L)**.**

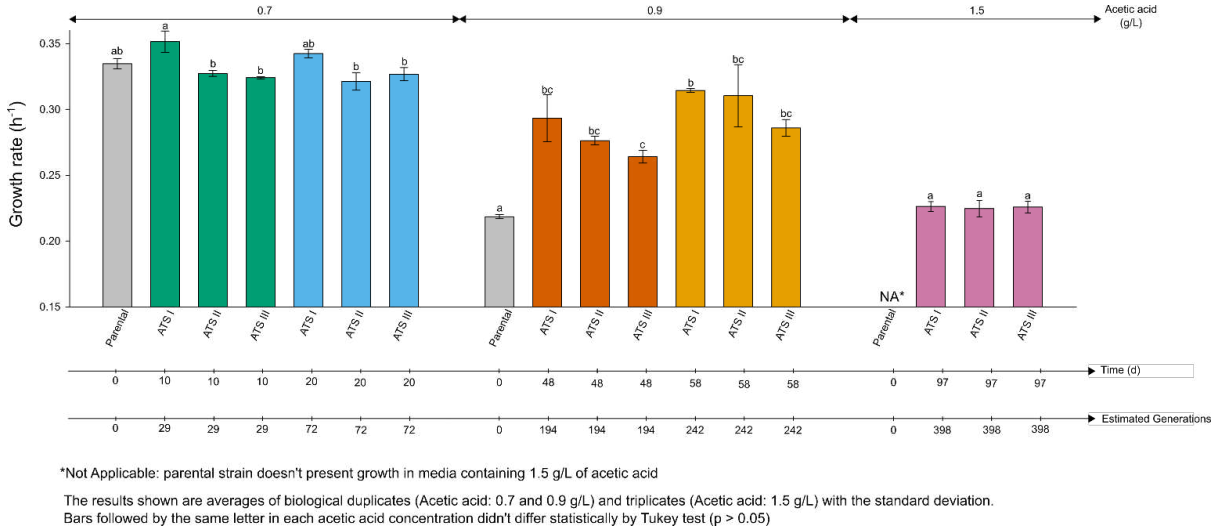

Figure 2.5 – Specific growth rates (h^{-1}) of the parental *P. laurentii* UFV-1 parental and ATS strains in cultivations carried out in the presence of three different concentrations of acetic acid (0.7, 0.9, 1.5 g/L).

The specific growth rates presented by the parental and ATS strains in the presence of 0.7 g/L of acetic acid were not statistically different (Figure 2.5). Nevertheless, the lag phase period of the three ATS decreased significantly compared to the parental strain, indicating the acquisition of the tolerance phenotype to acetic acid (Figure 2.4). This phenotype was more evident when the growth of the ATS evolved after 194 and 242 generations were evaluated in a culture medium containing 0.9 g/L of acetic acid. The specific growth rates of the ATS were about 60% higher than of the parental strain. Similar growth rates were observed between the parental and intermediary evolved strains in 0.7 g/L of acetic acid (Figure 2.5). The lag phase

was also shorter in ATS than in parental. Note that the y-axes in Figure 2.4 are in different ranges according to the acetic acid concentrations.

The duration of the lag phase of ATSs (398 generations) grown in media containing 1.5 g/L of acetic acid was similar to the parental strain in media with 0.7 or 0.9 g/L of acetic acid. These results are consistent with the tolerance phenotype and steady behavior in the serial passages (Figure 2.2). A similar profile was observed for the specific growth rate (Figures 2.4 and 2.5). There were no statistical differences between ATS I, II, and III in 1.5 g/L of acetic acid.

The ATS I, II, and III were characterized in different cultivation media in terms of physiological and kinetic parameters. To verify if trade-offs evolved alongside the tolerance to the acetic acid, the first characterization was performed in an altered SS2 medium (C:N – 100:1) without acetic acid to favor lipid accumulation (Figure 2.6 and Table 2.1).

ATS II, compared to the parental strain, had a reduction in most parameters evaluated (50% of the final biomass, 62% of specific growth rate, 66% of xylose consumption, 58% of lipid yield, 86% of lipid productivity, and titer), except $Y_{x/s}$. Moreover, ATS II did not display the oleaginous phenotype [only 0.13 (g Lipid/ g DW)], which agrees with the lower values of both $Y_{p/s}$ and lipid productivity. Therefore, the ATS II showed important trade-offs regarding the oleaginous phenotype and growth in the absence of acetic acid (Figure 2.6 and Table 2.1).

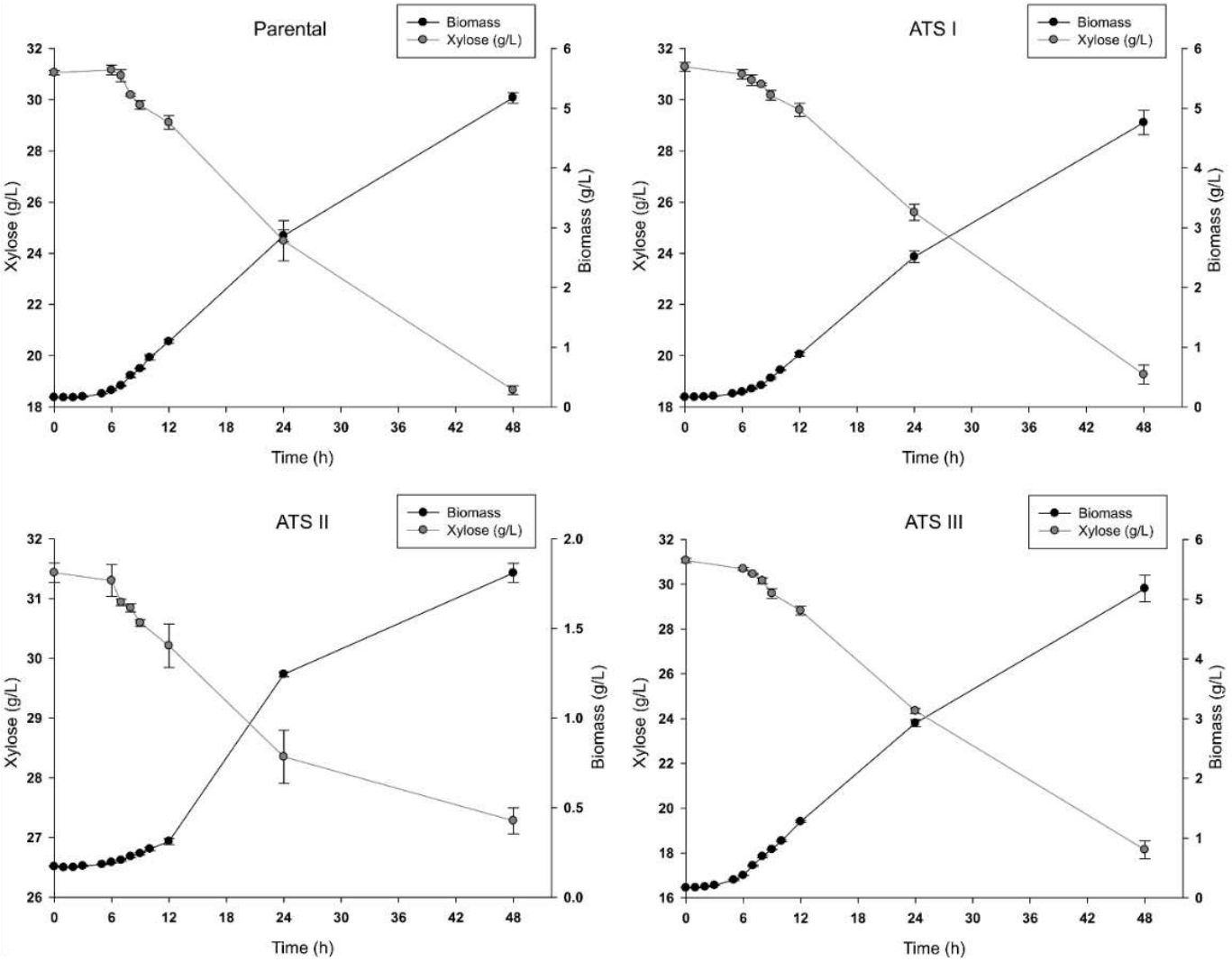

Figure 2.6 - Biomass (g/L) and Xylose (g/L) during cultivation of *Papiliotrema laurentii* UFV-1 Parental and Acetic acid Tolerant Strains (ATS) in xylose media (C:N – 100:1)(medium B) without acetic acid. The results shown are means of three replicates with the standard deviation.

Table 2.1 – Kinect and physiological parameters of *Papiliotrema laurentii* UFV-1 Parental and Acetic acid Tolerant Strains (ATS) after 48h of cultivation in xylose media (C:N – 100:1) (medium B) without acetic acid.

	Parental	**ATS I**	**ATS II**	**ATS III**
Growth Rate (h^{-1})	0.419 ± 0.015 a	0.317 ± 0.011 c	0.160 ± 0.004 d	0.375 ± 0.001 b
Final Biomass (g/L)	5.175 ± 0.087 a	4.761 ± 0.201 b	1.810 ± 0.054 c	5.178 ± 0.221 a
Xylose Consumed (g/L)	12.40 ± 0.22 ab	12.02 ± 0.20 b	4.15 ± 0.18 c	12.92 ± 0.45 a
Biomass Yield ($Y_{x/s}$)	0.417 ± 0.014 a	0.396 ± 0.010 a	0.437 ± 0.032 a	0.401 ± 0.003 a
Lipid Titer (g/L)	1.620 ± 0.091 a	1.509 ± 0.130 ab	0.235 ± 0.033 b	1.723 ± 0.061 c
Lipid % (w/w)	31.30 ± 1.57 a	31.51 ± 2.68 a	13.05 ± 1.81 b	33.30 ± 1.25 a
Lipid Yield ($Y_{p/s}$)	0.131 ± 0.006 a	0.127 ± 0.009 a	0.057 ± 0.005 b	0.133 ± 0.005 a
Lipid Productivity (g/L h)	0.034 ± 0.002 ab	0.031 ± 0.003 b	0.005 ± 0.001 c	0.036 ± 0.001 a

The results shown are means of three replicates followed by the standard deviation. Means in the same line with the same letter did not differ by Tukey test ($p > 0.05$).

Even though the ATS I and III showed a reduction in the specific growth rate, parameters related to the oleaginous phenotype such as $Y_{p/s}$; lipid productivity; lipid content % (w/w), and lipid titer were similar to those displayed by the parental strain. Although similar, some parameters such as final biomass and specific growth rate were statistically different and presented a reduction comparing the parental and the ATS I. The xylose consumption, final biomass, and $Y_{x/s}$ were similar between ATS I, ATS III, and the parental strain.

To address whether the evolved strains (ATS I, II, and III) acquired tolerance to higher concentrations of acetic acid, which are similar to those found in hemicellulosic hydrolysates upon detoxification step, they were cultivated in culture media containing 1.5, 1.75, and 2.0 g/L of acetic acid. Remarkably, ATS II presented a specific growth rate higher (1.5 g/L) or similar (1.75 g/L) when growing in the absence of acetic acid. ATS I and II, also grew in media containing 2.0 g/L after 182 and 151 hours, respectively. Although ATS III presented similar growth in the presence of 1.5 and 1.75 g/L of acetic acid, it did not grow with 2.0 g/L, even after more than 240 hours (10 days) (Table 2.2 and 2.3).

Table 2.2 – Specific growth rate (h^{-1}) of *Papiliotrema laurentii* UFV-1 Parental and Acetic acid Tolerant Strains (ATS) cultivated in culture media A containing xylose (5.0 g/L) and different acetic acid concentrations.

	Acetic acid (g/L)		
	1.5	**1.75**	**2.0**
Parental	NA*	NA*	NA*
ATS I	0.221 ± 0.005 a	0.155 ± 0.003 a	0.079 ± 0.012 a
ATS II	0.230 ± 0.002 a	0.165 ± 0.038 a	0.099 ± 0.028 a
ATS III	0.221 ± 0.011 a	0.162 ± 0.005 a	NA*

*NA: Not Applicable – Strain did not grow in the evaluated acetic acid concentration.
The results shown are means of two replicates followed by the standard deviation. Means in the same column with the same letter did not differ by Tukey test ($p > 0.05$).

Table 2.3 – Lag phase (h) of *Papiliotrema laurentii* UFV-1 Parental and Acetic acid Tolerant Strains (ATS) in culture media A containing xylose (5.0 g/L) with different acetic acid concentrations.

	Acetic acid (g/L)		
	1.5	**1.75**	**2.0**
Parental	NA*	NA*	NA*
ATS I	14.5 ± 0.7 b	56.5 ± 2.1 a	181.5 ± 6.4 a
ATS II	19.0 ± 1.4 ab	56.5 ± 2.1 a	150.5 ± 13.4 a
ATS III	23.0 ± 1.4 a	60.5 ± 3.5 a	NA*

*NA: Not Applicable – Strain did not grow in the evaluated acetic acid concentration.
The results shown are means of two replicates followed by the standard deviation. Means in the same column with the same letter did not differ by Tukey test ($p > 0.05$).

Based on the results, ATS I and III displayed fewer trade-offs compared to parental when cultivated in the absence of acetic acid, and, thus, we considered these strains as evolved strains. In addition, ATS I and III were evaluated in altered SS2 media with a high carbon:nitrogen ratio (C:N – 100:1), which favors the lipid accumulation in the presence of 1.5 and 1.75 g/L acetic acid (Table 2.4 and Figure 2.7).

Table 2.4 – Productivity parameters of *Papiliotrema laurentii* UFV-1 strains tolerant to acetic acid - Acetic Acid Tolerant Strains (ATS I and III) after 60 h of growth beginning in xylose (30 g/L) media B (C:N – 100:1) containing acetic acid (1.5 and 1.75 g/L).

	1.5		**1.75**	
	ATS I	**ATS III**	**ATS I**	**ATS III**
Final Biomass (g/L)	1.521 ± 0.020 a	1.569 ± 0.018 a	1.102 ± 0.021 b	1.081 ± 0.066 b
Xylose Consumed (g/L)	2.95 ± 0.07 a	3.14 ± 0.43 a	2.87 ± 0.31 a	3.00 ± 0.71 a
Biomass Yield ($Y_{x/s}$)	0.516 ± 0.007 a	0.386 ± 0.039 ab	0.507 ± 0.069 ab	0.370 ± 0.062 b
Lipid Titer (g/L)	0.31 ± 0.03 a	0.31 ± 0.03 a	0.20 ± 0.01 b	0.15 ± 0.02 c
Lipid % (w/w)	20.21 ± 1.80 a	19.97 ± 0.88 a	18.50 ± 0.82 a	13.65 ± 0.85 b
Lipid Yield ($Y_{p/s}$)	0.104 ± 0.005 a	0.066 ± 0.010 b	0.072 ± 0.006 bc	0.050 ± 0.006 c
Lipid Productivity (g/L h)	0.004 ± 0.000 a	0.004 ± 0.000 a	0.002 ± 0.000 b	0.001 ± 0.000 c

The results shown are means of three replicates followed by the standard deviation. Means in the same line with the same letter did not differ by Tukey test ($p > 0.05$).

Growth of ATS I and ATS III in acetic acid concentrations of 1.5 and 1.75 g/L seem to be related to acetic acid detoxification. Although both evolved strains have completely consumed the acetic acid present in the culture media (Figure 2.7), the biomass production, xylose consumption, and lipid production decreased in comparison with the cultivations performed without acetic acid (Table 1 and 4). ATS I showed the oleaginous phenotype in all conditions tested, along with higher lipid yield, productivity, and titer. The ATS III was more impacted by the presence of the acid and did not display the desired phenotype in the presence of 1.75 g/L of acid. For both evolved strains, the specific growth was impaired by higher acid concentrations. It should be noted that biomass yield was higher in the presence of 1.5 g/L when compared to conditions without acetic acid, for both strains, even with lower xylose uptake. This indicates that another carbon source, likely acetic acid, was utilized for biomass formation along with xylose. Indeed, the acetic acid consumption coincided with the beginning of growth (Figure 2.7).

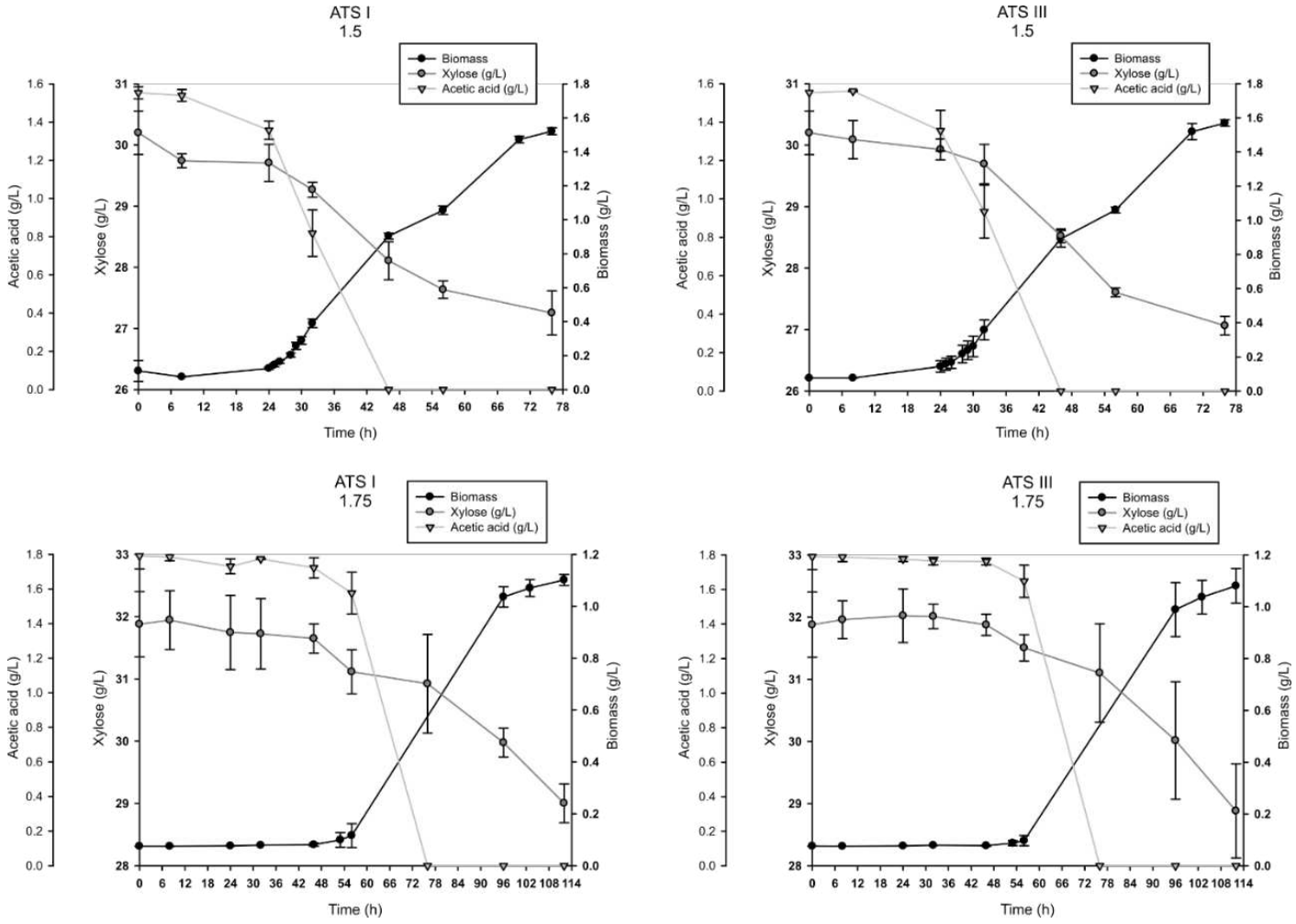

Figure 2.7 - Biomass (g/L), Xylose (g/L) and Acetic acid (g/L) during cultivation of *Papiliotrema laurentii* UFV-1 Acetic acid Tolerant Strains (ATS) I and III in xylose (30 g/L) media B (C:N – 100:1) containing acetic acid (1.5 and 1.75 g/L). The results shown are means of three replicates with the standard deviation.

2.4. Discussion

The oil production by oleaginous yeasts from hemicellulose acid hydrolysates requires the selection of strains tolerant to inhibitor compounds formed during the acid pretreatment of lignocellulosic biomasses. Initially, we evaluated the effect of the main inhibitors found in hemicellulosic hydrolysates on the *P. laurentii* UFV-1 growth. Although formic acid has displayed the highest inhibitory effect, its formation takes place only under drastic pretreatment conditions; therefore, it is frequently found in low concentrations (< 0.1 g/L) in the hemicellulosic hydrolysate. The inhibitory effect caused by HMF was lower compared to acetic acid and furfural. Likewise, Sitepu et al. (2014) also reported the inhibitory effect of the acetic acid, furfural, and HMF in *P. laurentii* UCDFST 12-803. The authors assessed growth in a qualitative manner (relative turbidity after 10 days) and indicated that the growth of *P. laurentii* reduced in the presence of 0.5 g/L of furfural and was completely inhibited at a concentration of 1.0 g/L. At 2.5 g/L of acetic acid, the *P. laurentii* UCDFST 12-803 strain did not grow. On the other hand, this strain grew well in the presence of 0.5, 1.0, and 2.0 g/L of HMF. Even though the furfural and HMF are toxic to yeast cells, organic acids such as acetic and formic tend to be more detrimental. This occurs because undissociated organic acids easily diffuse across the cell membrane and dissociate in the cytosol, leading to an increase in the intracellular anion concentration, and dissipation of the proton motive force. These impacts in cell homeostasis can arrest cell growth or even cause cell death (Palmqvist & Hahn-Hägerdal, 2000).

To circumvent the inhibitory effect of these compounds, detoxification processes are applied to reduce their concentration. Nevertheless, most detoxification methods are more efficient to decrease the concentration of furans (furfural and HMF) and phenolics compounds than organic acids. Therefore, even with the application of a detoxification step, the concentration of weak acids in hemicellulosic hydrolysate can be inhibitory to yeast growth (Yu et al., 2011; Bonturi et al., 2017; Lee et al., 2014). As such, we used Adaptive Laboratory Evolution (ALE) to select *P. laurentii* UFV-1 strains with enhanced tolerance to acetic acid.

ALE is a powerful tool for strain development aiming at applications in bioprocess. ALE can be applied to select strains with increased or optimized substrate utilization, increased productivity, and enhanced resistance to toxic compounds, such as those present in lignocellulosic biomass (Sandberg et al, 2019). Most studies using ALE to improve stress resistance focused on *Saccharomyces cerevisiae*. There are only a few reports involving

oleaginous yeasts (Díaz et al. 2018; Daskalaki et al. 2019; Walker et al., 2019; Hicks et al., 2020; Wang et al. 2021, Liu et al., 2021). Here, we described the first utilization of ALE to obtain a *Papiliotrema laurentii* strain tolerant to acetic acid. In our study, we selected and characterized three acetic acid-tolerant strains (ATS I, II, and III) of *P. laurentii* obtained by Adaptive Laboratory Evolution (ALE). ATS II presented a trade-off when it was cultivated in the absence of the acid. ATS I presented the oleaginous phenotype in all conditions tested, higher tolerance to acetic acid than the ATS III, and similar productivity parameters compared to the parental strain. As such, the ATS I is more promising for industrial applications.

The three strains evolved acquired higher tolerance to acetic acid (> 1.5 g/L) after 82 serial passages, and approximately 398 accumulated generations. Liu et al. (2021) and Díaz et al. (2018) evolved the *R. toruloides* strains (NRRL Y-1091 and 0013-09, respectively) in increasing concentrations of wheat straw hydrolysate to improve yeast resistance to a mix of inhibitors. In both cases, the evolved strains grew better in the presence of the hydrolysate (detoxified or not), or its inhibitors. Hicks et al. (2020) utilized two approaches to obtain tolerant strains of *M. pulcherrima*. Firstly, they performed 18 serial passages in media containing an inhibitor cocktail (0.7 g/L of furfural and acetic acid, and 0.35 g/L formic acid and HMF). They started five parallel cultures, but only one was evolved. This culture was expanded to five flasks. Secondly, they evolved another five parallel cultures in culture media containing increasing formic acid concentrations (0.6 g/L to 1.2 g/L). They performed 22 serial passages to select the tolerant strain. When strains are subjected to multiple inhibitors (Díaz et al., 2018; Hicks et al., 2020; Liu et al., 2021), growth can be impaired, and some populations can be lost, as observed by Hicks et al. (2020) and Liu et al. (2021). Also, it is difficult to correlate the observed phenotype with genomics data and to reintroduce the mutations acquired during evolution in the parental strains Otherwise, Wang et al. (2021) utilized a similar approach applied in our study to select an engineered strain of *Y. lipolytica* resistant to ferulic acid. These authors increased ferulic acid concentration periodically (0.5-1.5 g/L), throughout 43 serial passages, around 174 generations. When only one inhibitor is used as the selective pressure, the understanding of the acquired phenotype is easier, including the relations between genomic and physiological data. It is worth mentioning that both strategies (utilization of one inhibitor, or multiple inhibitors – synthetic mix or hydrolysates) are suitable for the selection of more robust strains of oleaginous yeasts, as demonstrated by our work, and those aforementioned.

ATS I, II, and III were replicate populations, that is, they evolved from the same parental strain, with the same culture media composition and experimental design. Importantly, the

evolved strains appear to have passed through different evolutionary pathways toward the same fitness (steady growth in culture media containing 1.5 g/L of acetic acid – Figure 2.2). Consistent with this assumption, ATS I and II displayed tolerance to acetic acid higher than ATS III (grew in 2.0 g/L of acetic acid – Table 2 and 3). However, most of the physiological parameters presented by ATS II were impaired in the absence of acetic acid, whilst the ATS III showed better results for final biomass, xylose consumption, lipid titer, and lipid productivity (Table 1). Besides, in contrast to ATS III, the ATS I preserved the oleaginous phenotype even in the presence of 1.75 g/L of acetic acid (Table 4). Taken together these results indicate that the three evolved strains accumulated different mutations related or not to the tolerant phenotype during the evolution experiment.

Evolution experiments kept under constant conditions, or conditions in which the stress levels are progressively increased, often favor the selection of specialists – a strain specialized to a specific environment – that show trade-offs in a novel or different environment (Van den Bergh et al., 2018). ATS II seems to have developed such behavior as it presented trade-offs when growing in the absence of the selective pressure (acetic acid) and grew better in the presence of the acid (Tables 1, 2, and 3). The specialist phenotype can be related to the accumulation of both neutral and deleterious mutations in the selective environment; or an antagonistic pleiotropy, where adaptive mutations under a certain condition are maladaptive in other conditions. It is noteworthy that those two mechanisms are not mutually exclusive. Although ATS I and III presented a trade-off related to specific growth rates compared to the parental strain (Table 1), the other physiological parameters were similar. This could be more related to a generalist behavior – a strain able to thrive in a wider range of environments – especially for ATS III, that showed a specific growth rate closer to the parental strain compared to ATS I under unstressed conditions (Table 1). Moreover, the ATS III, contrary to ATS I and II, did not grow in culture media containing 2 g/L of acetic acid (Tables 2 and 3). Since the ATS I presented tolerance to 2 g/L and a similar phenotype to the parental strain in the absence of the acid, it appears to be a superior generalist. This would imply that the fitness under separate conditions (absence and presence of acetic acid) is comparable to the specialist (ATS II) under the specific selective pressure (growth in the presence of acetic acid) (Van den Bergh et al., 2018).

Compared to the parental strain, ATS I, II, and III presented higher specific growth rates and shorter lag phases in the presence of acetic acid throughout the entire evolution. Hicks et al. (2020) described that strains evolving in the presence of inhibitors cocktail (furfural, HMF,

acetic and formic acid) showed an increase in lag phase duration at the beginning of the ALE, which decreased towards the end of the adaptation. All evolved strains displayed higher growth rates in the presence of the inhibitor, similar to the observations reported by Díaz et al. (2018) and Wang et al. (2021). Even though the evolved strains have been selected to grow in the presence of the inhibitors [*R. toruloides* in non-detoxified biomass (Díaz et al., 2018); *Y. lipolytica* with ferulic acid (Wang et al. 2021); *M. pulcherrima* with formic acid (Hicks et al. 2020)], their biomass formation and lipid production were affected under stressful conditions.

In our study, none of the ATSs showed improvement of the oleaginous phenotype compared to the parental strain. This also took place with *R. toruloides* that were evolved in the presence of non-detoxified biomass (Díaz et al., 2018), *Y. lipolytica* evolved with ferulic acid (Wang et al., 2021), and *M. pulcherrima* evolved in the presence of formic acid (Hicks et al., 2020). On the other hand, four evolved strains of *M. pulcherrima* reached higher lipid contents compared to parental when cultivated in the presence and absence of a mixture of inhibitors derived from lignocellulosic pretreatment (Hicks et al., 2020). The strains of *R. toruloides* selected by Liu et al. (2021) also showed higher lipid yields (and carotenoids production) when growing in hydrolysates, compared to the parental.

Both ATS I and III presented higher biomass yields in the presence of 1.5 g/L of acetic acid and similar in 1.75 g/L when compared to the growth in the absence of the acid (Tables 1 and 4). It is important to point out that this occurred in cultivations in which the xylose consumption was lower (around 3 g/L consumed) than in cultivations in the absence of acetic acid (around 12 g/L consumed). Considering that acetic acid consumption coincided with the beginning of growth, it was probably utilized as a carbon source for biomass formation, through the glyoxylate shunt (Chen et al., 2012; Palma et al., 2018), or the citric acid cycle (Ludovico et al., 2012). It is noteworthy that Ludovico et al., (2012) demonstrated, applying [U-^{14}C] acetate, that this carbon source could also be directly utilized in lipid biosynthesis (31.5%) in *Z. bailii*. This could be an indication that adaptations to the presence and consumption of acetic acid could contribute not only to biomass formation but also to lipid production.

2.5. Conclusion

Here, we demonstrated that ALE is a suitable approach to select strains of *P. laurentii* tolerant to stressful conditions encountered in pretreated lignocellulosic biomasses, such as those imposed by acetic acid. Three ATSs showing different phenotypes were selected, suggesting that they passed through distinct evolutionary pathways. ATS II seems to be a specialist, showing better growth in the presence of acetic acid while displaying important trade-offs when growing in its absence. In contrast, ATS I and III are likely generalists as they acquired tolerance to acetic acid and kept the lipid production capacity similar to the parental strain even in the absence of acetic acid. ATS I was considered the most promising evolved strain selected under the conditions reported in this study. ATS I displayed tolerance similar to the specialist strain (ATS II) and preserved the oleaginous phenotype, contrary to ATS III, in all conditions evaluated. Future works can explore the mechanisms behind the tolerant phenotype both with systemic and target approaches, evaluate the application of evolved strains in hydrolysates, and even employ the selected strains in a new round of ALE.

2.6. References

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GENERAL CONCLUSIONS

Acetic Acid Tolerant Strains (ATS) of *P. laurentii* UFV-1 were selected and characterized by Adaptive Laboratory Evolution (ALE). All strains evolved the tolerant phenotype after around 400 generations of exposure to increasing concentrations of acetic acid. ATS II displayed significant trade-offs in the absence of the acid, affecting its lipid productivity, oleaginous phenotype, and growth. However, its tolerance to acetic acid was the highest, along with ATS I. ATS I and III showed physiological parameters like the parental when growing in media lacking acetic acid. However, ATS III did not display the oleaginous phenotype when challenged with 1.75 g/L of acetic acid, different from ATS I. Therefore, ATS I is the most promising strain for future studies, since showed tolerance to acetic acid in all the conditions tested, as well as physiological parameters like the parental strain, and maintenance of the oleaginous phenotype in all conditions tested.