NÍVEA MOREIRA VIEIRA

PHYISIOLOGICAL, GENOMIC AND METABOLIC CHARACTERIZATION OF THE OLEAGINOUS YEAST *Papiliotrema laurentii* UFV-1

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

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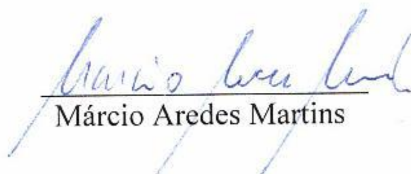

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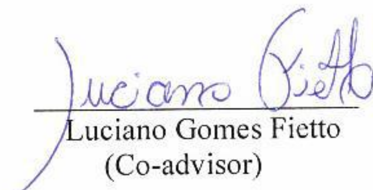

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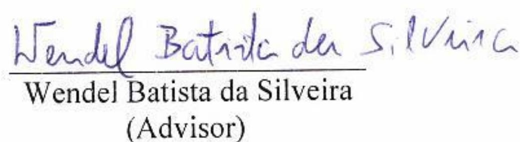

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BIOGRAFIA

NÍVEA MOREIRA VIEIRA, filha de Antônio Benevenuto Vieira e Elza Teodora Moreira Vieira, nasceu no dia 21 de junho de 1984, em Viçosa, Minas Gerais. Graduou-se em Ciências Biológicas pela Universidade Federal de Viçosa em agosto de 2007. Em agosto de 2007, iniciou o curso de mestrado no Programa de Pós-graduação em Microbiologia Agrícola da Universidade Federal de Viçosa, Viçosa, Minas Gerais, submetendo-se à defesa de dissertação no dia 17 de agosto de 2007. Em março de 2014 , iniciou o curso de doutorado neste programa, submetendo-se à defesa de tese em 28 de fevereiro de 2018.

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ABSTRACT

VIEIRA, Nívea Moreira, D.Sc., Universidade Federal de Viçosa, February, 2018. **Phyisiological, genomic and metabolic characterization of the oleaginous yeast *Papiliotrema laurentii* UFV-1**. Advisor: Wendel Batista da Silveira. Co-advisors: Luciano Gomes Fietto and Adriano Nunes Nesi.

The interest on the use of microbial oils has increased for the production of fatty acids derivate products such as biofuels. The culture of oleaginous yeasts in abundant and low-cost raw materials is a promising alternative for biodiesel production. Due to the great microbial diversity found in Brazilian soils, the present project focused on the isolation of oleaginous yeasts from Rupestrian Fields soils and the study of the most prominent oleaginous yeast. The yeast *Papiliotrema laurentii* UFV-1 presented the lipids in the culture media with glucose and xylose as carbon sources. Unlike most oleaginous yeasts, lipid accumulation by *P. laurentii* UFV-1 begins at the exponential growth phase, presentin a lipid accumulation growth associated. Furthermore, evaluation of the profile for fatty acids may be considered suitable for biodiesel production. Relevant information was obtained after genomic analysis, such as the presence of a gene encoding for the malic enzyme $NADP^+$-dependent and a single copy gene for the enzyme ATP citrate lyase, which is a key enzyme for oleaginous yeasts. We have found genes from alternative pathways for the lysine and leucine degradation, which may be alternative sources of acetyl-CoA for lipids accumulation. Analysis of expression and metabolic metabolism, including the $NADP^+$-dependent malic enzyme, are the main sources of reducing the power for high accumulation of neutral lipids. Acetyl-CoA carboxylase activity is triggered over a period of increased lipid accumulation. We have found genes from alternative pathways for degradation of the amino acids leucine and lysine, which may be alternative sources of acetyl-CoA for lipid accumulation. Profile evaluation of gene expression and metabolism identified that the malic enzyme $NADP^+$-dependent acts power reducing the great amount of neutral lipids accumulation. Interestingly, the expression profile for the *ACC1* gene, which encodes acetyl-CoA carboxylase enzyme, decreased during the period of higher lipid accumulation. Furthermore, degradation of the amino acids lysine and leucine appear to contribute to acetyl-CoA synthesis in *P. laurentii*, according to the metabolic profile analysis. In general, metabolic pathways involved nitrogenous compounds were affected, according to the analysis pathway impact, which can be explained by the directional flow from carbon to lipid synthesis.

RESUMO

VIEIRA, Nívea Moreira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Caracterização fisiológica, genômica e metabólica da levedura oleaginosa *Papiliotrema laurentii* UFV-1**. Orientador: Wendel Batista da Silveira. Coorientadores: Luciano Gomes Fietto e Adriano Nunes Nesi.

Há um crescente interesse no uso de óleos microbianos para a produção de produtos derivados de ácidos graxos, como o biodiesel. O cultivo de leveduras oleaginosas em matérias-primas abundantes e de baixo custo é uma alternativa promissora para a produção de biodiesel. Devido à grande diversidade microbiana encontrada em solos brasileiros,o presente trabalho teve como objetivos o isolamento de leveduras oleaginosas a partir de amostras de solos de Campos Rupestres e o estudo da levedura oleaginosa de maior destaque. A levedura *Papiliotrema laurentii* UFV-1 apresentou os lipídios nos meios de cultura com glicose e xilose como fontes de carbono. Ao contrário da maioria das leveduras oleaginosas, o acúmulo de lipídios por *P.laurenntii* UFV-1 iniciou na fase de crescimento exponencial. . Diferentemente da maioria das leveduras oleaginosas, o acúmulo de lipídios por *P. laurentii* UFV-1 inicia-se na fase exponencial de crescimento, apresentando um acúmulo de lipídios associado ao crescimento. Além disso, o perfil de ácidos graxos obtido nas condições avaliadas pode ser considerado adequado para a produção de biodiesel. Na análise do genoma, foram encontradas importantes informações, como a presença de um gene que codifica para a enzima málica $NADP^+$-dependente, e uma única cópia para a enzima ATP: citrato liase, enzima chave para leveduras oleaginosas. Encontramos genes de vias alternativas para a degradação da lisina e leucina, que podem ser fontes alternativas de acetil-CoA para o acúmulo de lipídios. As análises de expressão gência e perfil metabólico, permitiram-nos concluir que a enzima málica $NADP^+$-dependente é a principal fonte de poder redutor para o alto acúmulo de lipídios neutros. Surpreedentemente, o nível de expressão do gene *ACC1* que codifica a enzima acetil-CoA carboxilase caiu durante o período de maior acúmulo de lipídios. Além disso, a degradação dos aminoácidos lisina e leucina parecem contribuir para a para a síntese de acetil-CoA em *P. laurentii*, de acordo com a análise de perfil metabólico. Em geral, as vias metabólicas envolvendo compostos nitrogenados foram estatiscamente afetadas, de acordo com a análise de impactos nas vias, o que pode ser explicado pelo direcionamento do fluxo de carbono para a síntese de lipídios.

GENERAL INTRODUCTION

Over the last years, the market of bio-based products has been boosted by the environmental regulations and non-renewable resources depletion. The microbial oil use stands out as a promising alternative for the production of fatty acid-derived products such as oleochemicals and biofuels. Microorganisms such as filamentous fungi, yeasts, microalgae, bacteria and cyanobacteria can accumulate carbon stores in the form of lipids. Among the oleaginous microorganisms, yeasts have called attention due to their ability to growth in various carbon substrates, which are found in abundant and inexpensive feedstocks as lignocellulosic biomasses. In addition, metabolic engineering strategies are more easily established in yeasts than in other oleaginous microorganisms.

The understanding of how the metabolic pathways involved with the lipid metabolism are regulated in oleaginous yeasts is pivotal to improve the production of fatty-acid derived products. In spite of achieved advances over the last years, the regulation of lipid metabolism in those yeasts is far from clear. Moreover, most of the studies has been performed with *Yarrowia lipolytica*, which is considered the model oleaginous yeast. Thus, the metabolic characterization of other oleaginous yeasts can bring new insights into the regulation of lipid metabolism.

This doctoral thesis is divided into 3 chapters. The first one is a bibliographical review to introduce readers to the world market of fatty-acid derived products and the importance of using microorganisms as an oil source for their production. In this chapter, we also highlight the importance of the study and the application of oleaginous yeasts and their advantages over other oleaginous organisms. Lastly, we described the main metabolic pathways involved in in the lipid metabolism of those yeasts.

The second chapter covers the isolation of oleaginous yeasts from soil, selection and physiological characterization of the isolate *Papiliotrema laurentii* UFV-1. This yeast was selected due to its capacity of accumulation high amounts of lipids from lignocellulosic sugars. Contrary to most of the oleaginous yeasts, it showed a pattern of lipid accumulation growth-associated and fast lipid accumulation.

In the third chapter, we studied the regulation of lipid metabolism of *P. laurentti* UFV-1 under excess of carbon and nitrogen limitation. Genomics, gene expression and metabolomic analyses were used in order to better understand how the metabolic pathways are regulated during the lipid accumulation phase.

CHAPTER 1

Literature review

Bio-based oleochemicals and fatty acid bio-based biofuel

Increasingly stringent environmental regulations and non-renewable resources depletion have stimulated the market of bio-based products. The key drivers to their market growth are the high demand from consumer markets, availability of raw materials and a growing market for green chemicals. Oleochemicals are bio-based products derived from triacyclglycerols (TAGs) used in manufacturing of surfactants, personal care, soaps, detergents and food additives.

The main oleochemicals are fatty acids, fatty alcohols, and glycerin. It is expected that the oleochemicals market will be USD 25.91 billion in 2019. Fatty acids have been standing out as the main oleochemical, about 56.34% of the total consumption. Indeed, it is rising the fatty acids application in soaps, detergents and personal care industry, besides, it is expected an increase of their demand over the next eight years. The global fatty alcohols demand is also growing, is expected to exceed 3.2 million tons by 2024. Glycerin derivatives and glycerol monostearate have gained prominence in a wide range of food and beverage applications. The glycerin market segment is expected to grow at a compound annual growth rate of 7.7% to reach a net worth exceeding USD 340 million by 2024 globally. For the biodiesel market, it is expected to reach USD 54.8 billion in 2025, with an annual growth rate of 7.3%. The rising demand for the use of biodiesel as fuel for automobiles, as well as the need of reducing of greenhouse gas emissions has boosted the growth of this sector (Markets and Markets consulting 2018; Grand View Research consulting, 2018).

Despite soaps and surfactants are the main oil-derived products obtained from renewable sources, it has been observed over the last years a growing interest in the production of fatty-acid derived biofuel (Caspeta and Nielsen, 2013; Saenge et al., 2011; Tanimura et al., 2014), supplements, food additives (Dufossé et al., 2014; Hernández-Almanza et al., 2014), and fatty acids for the cosmetic industry (Kolouchová et al., 2016).

Traditionally, oils have been extracted from plants such as such as soybean, sunflower, palm and canola. Nevertheless, the requirement for high-quality arable land

competes with the agricultural sector (Chisti, 2007). An advantage of vegetable shortening is the possibility of obtaining fatty acids of purity suitable for chemical conversion and synthesis of chemically pure compounds. Plants have specialized organs for the storage of lipids such as seeds, which facilitates their extraction (Schulze, 2014). Therefore, there is a need of developing alternative processes for the oil production that are sustainable. In this context, the production of microbial oil, the single oil cell (SCO), stands out as a promising strategy.

Microbial oil

Some microorganisms including bacteria, yeasts, fungi and microalgae are capable of accumulating lipids by more than 20% of their dry weight (Ratledge, 1991). Recently, several studies have focused on developing microbial platform for fatty acid-derived biofuels. Yeast and microalgae have been used more frequently than fungi and bacteria for biodiesel production (Huang et al., 2013; Probst et al., 2015).

Microalgae display potential to be used as an oil source for the biodiesel production, however, their photosynthetic cultivation require large areas (Meng et al., 2009). In addition, photosynthetic cultivation is subjected to seasonal variations of light incidence, contamination by predatory bacteria and protozoa. In addition, high volumes of cultivation are required for the biodiesel production (Chisti, 2007). Besides microalgae, oleaginous yeasts have called attention. They have increasingly used for the production of fatty acid-derived chemicals and biofuels. The advantages of yeast cultivation over microalgae cultivation include faster growth, high density growth, lesser susceptibility to viral infection, and control of bacterial contamination by using low pH values (Sitepu et al., 2014). In addition, yeasts are more tolerant to high sugar concentrations and inhibitors. Another important feature is their capacity of assimilating a wide range of sugars, which confer them the capacity of growing from several agro-industrial by-products such as lignocellulosic biomass whey, crude glycerol, palm oil mill effluent, waste oil from chicken products fat, (Leiva-Candia et al., 2014). Among them, lignocellulosic biomasses stand out as abundant and inexpensive feedstocks. Indeed, the conversion of lignocellulosic sugars into lipids is an attractive strategy for making the fatty acid-based production feasible (Lee et al., 2017). Moreover, the possibility of metabolic engineering can improve the yield and the fatty acid composition (Gong et al., 2012; Liang et al., 2012; Zhou et al., 2014).

Most of the studies involving oleaginous yeasts have been carried out with *Yarrowia lipolytica, Lipomyces starkeyi, Rhodosporidium toruloides* and *Cryptococcus curvatus* (Feofilova et al., 2010; Hu et al., 2011; Papanikolaou and Aggelis, 2010; Thiru et al., 2011; Wild et al., 2010). *Yarrowia lipolytica* is considered the model oleaginous yeast. Those yeasts are taxonomically diverse and belongs to two of the seven fungal phyla, Ascomycota and Basidiomycota. Other species and strains belonging to the genus *Scheffersomyces, Kurtzmaniella, Myxozyma, Cuniculitrema, Filobasidium, Cryptococcus, Tremella, Trichosporon, Prototheca, Hannaella* has been also recognized as oleaginous (Sitepu et al., 2014, 2013). Several efforts have been performed over last years to understanding of the regulation of lipid metabolism in oleaginous yeasts, however, the mechanisms involved are still not clear (Zhu et al., 2012).

Lipid metabolism in yeasts

Under high nitrogen starvation, the carbon flux in oleaginous yeasts is directed towards to lipid accumulation. Besides nitrogen, the limitation of other nutrients such as phosphorus, magnesium, zinc and iron also favor the lipid accumulation (Beopoulos et al., 2011). Lipids are accumulated in organelles that have a monolayer of phospholipids, the lipid droplets or adiposomes. Adiposomes are intracellular organelles found in most cells, where they play key roles in cell metabolism. The prominent function is the storage of neutral lipids as energy reserve and substrate for components of cell membranes (Thiam et al., 2013). Nitrogen starvation leads to the decrease of the intracellular concentration of adenosine monophosphate (AMP), because it is converted to the inosine monophosphate (IMP) and NH_4^+. The NH_4^+ is used by the yeast as a nitrogen source. The AMP decrease, which is the allosteric activator of the isocitrate dehydrogenase, impairs the oxidative oxidative decarboxylation of isocitrate to α-cetoglutarate, causing the citrate accumulation. This regulation is unique for oleaginous yeasts (Adrio, 2017). When the citric acid concentration reaches critical values, it is transported form the mitochondria to the cytosol via the citrate-malate transport and cleaved by the enzyme ATP: citrate lyase to form oxaloacetate and acetyl-CoA. Acetyl-CoA is the building block for the fatty acid synthesis(Papanikolaou and Aggelis, 2011). ATP citrate lyase is found only in oleaginous organisms (Ratledge, C., Wynn, 2002). Oleaginous yeasts accumulate mainly triacylglycerols, about 90% of the lipid storage. About 44 % the triacylglycerols, contain unsaturated fatty acids. Other neutral lipids such as free fatty acids, monoglycerides, diglycerides, sterile esters, sterols and polar fractions are produced in considerable

amounts. The major fatty acids in oleaginous yeasts are myristic (C14: 0), palmitic (C16: 0), stearic (C18: 0), oleic (C18: 1), palmitolytic (C16: 1) and linoleic acid (C18:2) (Uemura, 2012).

Acetyl-CoA carboxylase (Accase) biotin-dependent is the enzyme responsible for catalyzing the irreversible carboxylation of acetyl-CoA to malonyl-CoA. It has been considered the rate-limiting step in the fatty acid synthesis. In Saccharomyces *cerevisiae*, a non-oleaginous yeast, it has been well documented is that catalyzes it is regulated at both transcriptional and post-translational levels. Similar to genes involved with the phospholipid synthesis, its transcription is regulated by the transcriptional activators Ino2 and Ino4 and by the negative regulator Opi1. The role of Opi1 seems rely on of phosphatidic acid (PA) concentration. Opi1 is a PA-binding protein that shuttles between the endoplasmic reticulum (ER) and nucleus. In the presence of inositol, the PA levels are low; thus, Opi1 translocates from ER to nucleus, where it interacts with the Ino2/Ino4p complex attenuating the transcription of genes that have UAS_{INO} elements in the promoters. Opi1 seems act into the nucleus sequestering molecules of Ino2, preventing its action (Hofbauer et al., 2014; Shi et al., 2014a; Tehlivets et al., 2007a). At the post-translational level, the action of the Accase relies on the action of the Snf1 kinase. This kinase inhibits the action of Accase by through its phosphorylating. The yeast Snf1 is a widely conserved serine/theronine kinase in eukaryotes required for cellular energy homeostasis (Kayikci and Nielsen, 2015). In high glucose concentrations*, the Snf1 kinase* is inactive (Kayikci and Nielsen, 2015; Shirra et al., 2001). For the oleaginous yeasts, the Acc1 regulation is until unclear.

Lipid synthesis requires large amount of reducing power (Adrio, 2017). The NADPH required for lipid synthesis in oleaginous yeasts can be obtained from different sources, among them the reaction catalyzed by glucose 6-P dehydrogenase, a NADP-dependent enzyme of the oxidative phase of the pentose phosphate pathway. Some studies have pointed out that this pathway is the main source of NADPH in *Yarrowia lipolytica* (Wasylenko et al., 2015). Otherwise, most of the oleaginous yeasts use the NADPH formed by the reaction catalyzed by malic enzyme (ME). In *Y. lipolytica,*, the malic enzyme $NADP^+$-dependent is absent. (Dulermo et al., 2015). Importantly, the overexpression of the gene encoding the Malic enzyme increased the lipid content in the dimorphic fungi *Mucor circinelloides* and in *Rhodotorula glutinis* (Li et al., 2013). An alternative route for NADPH generation may be the reaction catalyzed isocitrate dehydrogenase ($NADP^+$-dependent), a cytosolic enzyme (Ratledge, 2014).

In *S. cerevisiae* the degradation of fatty acids occurs via the β-oxidation pathway, a multistep process. The enzyme localization is restricted to peroxisomes in *S. cerevisiae* In oleaginous yeasts, there are enzymes of this pathway acting in mitochondria (Vorapreeda et al., 2012). For *S. cerevisiae,* the fatty acid β-oxidation is the process of breaking down the long-chain acyl-CoA molecule to acetyl-CoA molecules. At the end of each β-oxidation cycle, two new molecules are formed, one acetyl-CoA and one acyl-CoA that is two carbons shorter. During β-oxidation, the reducing power formed is used by the electron transport chain to produce ATP. There are different isoforms of these enzymes of β-oxidation, which have different affinities for different fatty acid chain lengths. For example, there is a very long-chain acyl-CoA dehydrogenase, the long-chain acyl-CoA dehydrogenase, the medium-chain acyl-CoA dehydrogenase, and the short-chain acyl-CoA dehydrogenase. The β-oxidation pathway has been considered as a potential target for the engineering and increase of the accumulation of lipids (Dulermo and Nicaud, 011; Imatoukene et al., 2017; Ledesma-Amaro et al., 2015).

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CHAPTER 2

The oleaginous yeast *Papiliotrema laurentii* UFV-1 displays faster lipid accumulation after isolation and description of physiological traits

Abstract

Oleaginous yeasts have used lignocellulosic sugars to accumulate lipids. This procedure has been a promising strategy for biodiesel production, since these yeasts have been frequently isolated from soils samples. Brazilian soils have an immense microbial biodiversity. Thus, the purpose of this study was to isolate and select oleaginous yeasts from Campo Rupestre soils samples. Five out of 129 yeast isolates were classified as the best lipid producers. They were taxonomically identified as *Papiliotrema laurentii* (UFV-1 and UFV-2), *Meyerozyma caribbica, Candida sojae* and *Candida pseudointermedia.* In this study, *P. laurennti* UFV-1 had the highest measure of lipid contents after 48 h with the percentage of 43% and 30% for cell dry mass (w/w) in growth media containing glucose and xylose, respectively. On the contrary to most of the oleaginous yeasts, lipid accumulation in the yeast *P. laurentii* UFV-1 initiated during the exponential growth phase and had one of the fastest accumulation cycle known. Moreover, its fatty acid profile is suitable to be used as feedstock for biodiesel production.

Introduction

Global warming has raised some concerns in the past years due to the increase on the emission of greenhouse gases from the burning of fossil fuels. The Bloomberg New Energy Finance (BNEF) is an organization involved in the evaluation of investments on renewable energy market. BNEF presented a report in 2016 pointing out that the peak of fossil fuels consumption will be achieved in 2025. For this reason, most countries are committed to reduce the emission of greenhouse gases in the Conference of the Parties (COP21) in Paris, France. Then, it is necessary to motivate the development of alternative energy sources to the energy from the burning of fossil fuels.

From the biofuels available in the market, ethanol has a large-scale production and has been used as an alternative fuel to the use of gasoline and is also used as a blended

fuel mixed to gasoline. One biofuel used as an alternative fuel to diesel is biodiesel, which has the need to increase production in order to become it a viable fuel in the market. Currently, biodiesel is produced by a transesterification reaction using vegetable oils and methanol which is a derivate from natural gas. Nevertheless, it presents some drawbacks such as the waste production and the need of large areas as arable lands or for edible plants cultivation.

Alternatively, microbial oil can be produced quickly by cultivation of microorganisms in bioreactors without the need of using arable lands. The use of microbial oil present some advantages when comparing edible oil such as the lower emission of greenhouse gases and the most favorable energy balance for biodiesel production (Caspeta and Nielsen, 2013). This comparison considers the entire biodiesel process, which includes plant cultivation for oil obtainment. Thus, the interest in biodiesel production from microbial oils has grown over the last years. Currently, from 50 to 160 yeast species out of 1600 known species can be oleaginous (Sitepu et al., 2014). Some typical genera of yeast species are *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces* (Ageitos et al., 2011). Recently, new species and strains were recognized as oleaginous yeasts such as species from the genus *Scheffersomyces, Kurtzmaniella, Myxozyma, Cuniculitrema, Filobasidium, Cryptococcus, Tremella, Trichosporon, Prototheca, Hannaella* (Sitepu et al., 2013, 2012). Lignocellulosic biomasses can be used as a sugar source during oleaginous yeasts cultivation, which can motivate lipid production at a large scale and consequently biodiesel. In Brazil, sugarcane bagasse and sugarcane straw are abundant agroindustrial by-products that can also be used as sugar source for the same purpose of lipid or biodiesel production (Ferreira-Leitao et al., 2010).

Brazilian soils contain a great microbial diversity that has not yet been fully explored (Duarte et al., 2013). Thus, this study had the purpose to isolate oleaginous yeasts from soils of two Brazilian National Parks known as "*Caparaó*" and "*Serra dos Órgãos*" with the hability to accumulate oil from lignocellulosic sugars. The new strain *P. laurentti* UFV-1 was isolated during the experiment and displays the ability of accumulating lipids in growth media containing either glucose or xylose through a fast cycle.

Material and Methods

Sampling soil collection and yeast isolation

Yeasts were isolated from soil samples originally found in two Brazilian National Parks known as "*Caparaó"* and "*Serra dos Órgãos"*. Twelve soil samples were collected in *Caparaó,* meanwhile sixteen samples were collected in *Serra dos Órgãos*.

In order to isolate yeasts from each sample, three soil samples were collected and mixed so that the procedure for yeast isolation was carried out as follows: 1 g of each soil sample was added to a 250 mL Erlenmeyer flask containing 50 mL of glycerol-enriched growth media containing 100 g/L of glycerol, 0.5 g/L of $(NH_4)_2SO_4$ 1, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ and 0.2 g/L of yeast extract. The Erlenmeyer flasks were incubated and mantained in a rotatory shaker at 30°C and 200 rpm for 24 h. A volume of 1 mL of these pre-cultured yeasts was used to perform 10-fold serial dilutions. Aliquots of 0.1 mL from each serial dilution ranging from 10^{-1} to 10^{-5} were spread onto growth medium on plates prepared with: 20 g/L of xylose, 0.5 g/L of $(NH_4)_2SO_4$ 5, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$, 0.5 g/L of yeast extract, 20 g/L of agar, and 50 mg/L of streptomycin and incubated at 30°C for 2 to 4 days [8].

Evaluation of oleaginous yeasts

The isolated yeasts were evaluated for their lipid-producing ability by a semi-quantitative analysis using a Nile Red Fluorometric method [4]. In this step, the oleaginous yeast *Yarrowia lipolytica* CBS2072 was used as a control yeast. This yeast was obtained from the collection available at Oswaldo Cruz Foundation (Fiocruz). In order to evaluate lipid accumulation, the yeasts were cultivated in growth media (SS2) [(Tanimura et al., 2014), with the following protocol modification that is the use of 1 g/L of yeast extract (Table 1). Yeast culture was performed in 1000 ml Erlenmeyer flasks filled with 100 mL of the growth media (Table 1). These yeast cultures were incubated in a rotatory shaker for 120 h at 30°C and 200 rpm.

Cell mass density was adjusted in a new SS2 growth media to the optical density of 1.0 (OD600). For each strain, 250 µL of each culture were transferred to a 96-well black microplate in technical triplicates. Subsequently, 25 µL of a mixture containing

DMSO and SS2 in the ratio 1:1 (v:v) were added to each well. The initial fluorescence was measured in a spectrofluorometer model M5 (Molecular Devices), with excitation and emission wavelengths set to 488 and 585 nm, respectively. Subsequently, 25 µL of Nile Red at 0.05 mg/mL were mixed into the culture for the second fluorescence measurement, after 10 min of reaction, reaching the final concentration of 5 µg/mL for Nile red [(Sitepu et al., 2012).

Potentially oleaginous yeast colonies were maintained at 4°C in solid media YPD composed of 20 g/L of glucose, 10 g/L of yeast extract and 20 g/L of peptone. After evaluating yeasts, the strains had their ability to accumulate lipids evaluated in a growth media containing glucose or xylose as a carbon source.

Table 1. Composition of growth media for culturing the yeast *P. laurenti* UFV-1.

	Carbon source of media		
Reagents	**G (g/L)**	**X (g/L)**	**GX (g/L)**
Glucose	30	-	21,42
Xylose	-	30,62	9,19
$(NH_4)_2SO_4$	0,7	0,7	0,7
NaCl	0,1	0,1	0,1
$CaCl_2$	0,1	0,1	0,1
$MgSO_4$	0,5	0,5	0,5
Yeast extract	1	1	1

G is the abbreviation of glucose; X is the abbreviation of xylose; and GX is a mixture containing 70% glucose and 30% xylose.

Absolute quantification of lipids

Quantification of polar and non-polar lipids was based on the method described by Dyer and Bligh (1959) with some modifications. Lipid extraction was performed by using 50 mg of lyophilized biomass and adding 1 mL of a solution composed by methanol and chloroform solution in the ratio of 2:1 (v/v). The suspension was homogenized for 5 min using the TissueLyser II equipment (Qiagen) and a frequency of 30 shakes per second. After this procedure, centrifugation was performed at 14,000 x g for 5 min and repeated 3 times to ensure total lipids extraction from initial biomass. The supernatants were collected and stored in the same 15 mL glass centrifuge tube. Subsequently, 3 ml of chloroform were added to the supernatants in the glass centrifuge tubes and, then, the

mixture was homogenized. In order to generate a two-phase liquid system, 2 mL of saline solution at 1% (w/v) were added to the mixture and, then, the system was homogenized again. The liquid phases were separated by centrifugation for 20 min at 3000 rpm in a Sorval centrifuge RC5C. The lower phase was transferred to previously weighed microtubes. The samples were then evaporated in Speedvac (Eppendorf) at 60°C for 24 h. The lipid content was determined by a gravimetrically analysis.

Identification of oleaginous yeasts

Taxonomic identification of yeasts isolated from soil samples in the evaluation step was performed by sequencing the D1/D2 domains of the gene encoding subunit 26S from ribosomal DNA.

The universal primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') were used for D1/D2 amplification [(Lachance et al., 2003). Reactions for DNA sample were prepared using the Big Dye Kit version 3.1 (Applied Biosystems) according to protocol recommendations to be sequenced in the ABI 3730 automated sequencing system (applied Biosystems).

Nucleotide sequences obtained after DNA sequencing were analyzed and compared to the sequences deposited in the National Center for Biotechnology Information database (NCBI), using the BLASTn tool available in NCBI database as one of the Basic Local Alignment Search Tools (BLAST Tools).

Evaluation of lipid production by *Papiliotrema laurentii* UFV-1

Isolated yeasts were cultured in liquid growth media containing one type of carbon source that could be: i) glucose (G); ii) xylose (X); or iii) glucose (70%) and xylose (30%) (GX). Initial pH values for the three growth medias ranged from pH 5 to 6. Initial composition of growth media was defined to guarantee a mass ratio of C / N ~ 48, that was identical in the three growth medias. Contribution of the organic nitrogen from yeast extract was included to calculate the ratio C / N (Table 1).

Confocal Microscopy

Microscopic observation was performed using a Laser Scanning Confocal Microscope, Zeiss LSM510 META. Samples cultured for 48 h and with a maximum lipid accumulation were diluted in peptone water 0.1% (w / v) and had the cell density (OD 600nm) of 1.5. Subsequently, 1 mL of each sample was centrifuged for 5 min at 14,000 x g. The pellet was fixed with 100 µL of the fixative solution containing 700 µL NaOH 6 M, 1.36 g of KH_2 PO_4, 100 µL of $MgCl_2$ 1M and 4% paraformaldehyde. One µl of Nile Red at 0.05 mg/mL was added. The samples were stirring for 20 min in the dark at room temperature.

Consumption of ammoniacal nitrogen

Nitrogen consumption during yeast culture was measured by the method proposed by Marbach and Chaney (1961) [(Marbach and Chaney, 1961). In this method, 5 µL of the sample (previously diluted in deionized water, if necessary) were mixed with 100 µL of the solution A [5% of phenol (w/v) and 0.025% of sodium nitroprusside (w/v)] and 100 µL of the solution B [2.5% NaOH (w/v), 1% NaOCl (w/v)]. This solution was incubated and maintained at 39°C for 20 min and the absorbance was read at 630 nm on the Muitiskan GO plate reader (ThermoScientific). The standard curve was prepared using solutions with different concentrations of ammonium chloride (ranging from 0 to 12 mM).

Fatty acids profile

Samples from the yeast *Papiliotrema laurentii* UFV-1 strain were cultured in three different growth media for 24 h, which comprises the lipid accumulation phase (Table 1) and, then, they were centrifuged at 12000 × *g* and 4°C for 10 min. Subsequently, the pellet obtained after centrifugation was lyophilized. The fatty acids in yeast samples (4 - 5 mg of the dry weight) were saponified, methylated and extracted, according to the manufacturer's instructions (MIDI microbial identification system). The resulting methyl ester mixtures were separated using an Agilent 7890A gas chromatography with a flame ionization detector (Agilent Technologies) and identified using the MIDI microbial

identification system (Sherlock 6.0 microbial identification system) at Microbial ID, Inc. (Newark, DE).

Glucose repression

The yeast strain was cultured overnight at 30°C in a tube with a volume of 50 mL containing 20 mL of yeast extract at 10 g/L, 20 g/L of peptone and 20 g/L of xylose (YPX) in growth media. For glucose repression evaluation, yeast cells were diluted to the optical density (D.O. 600nm) of 1, with the use of fresh peptonized water. Then, serial dilutions were prepared up to 10^{-5} and a volume of 6 µL of each dilution was submitted aseptically to a spot assay onto agar plates. In addition, strains were streaked onto YPX agar plates for culturing with or without the addition of 2-deoxy-D-glucose (2-DOG, Sigma) at a final concentration of 200 µg/mL. Plates were incubated and maintained at 30°C for 48 to 72 hours. The yeast *Spathaspora passalidarum* NRRLY 27907 was used as a positive control for the experiment.

Results

Isolation and screening of oleaginous yeasts

Oleaginous yeasts have been isolated from different environments such as soils, wastewaters, dairy products, flowers and others habitats (Anastassiadis et al., 2003; Cloete et al., 2009; Pan et al., 2009; Pirozzi et al., 2013; Sláviková and Vadkertiová, 2003). There is a lack of studies reporting the isolation of oleaginous yeasts from soils of the two Brazilian National Parks known as *Caparaó* and *Serra dos Órgãos*. In this study, a total of 129 yeasts were isolated from soils that were sampled in these Brazilian National Parks. Their ability to accumulate lipids was evaluated by a fluorometric method that uses the Nile Re, a lipophilic stan commonly used for lipid quantification. Five isolates (SO91, SO19, SO3, SO65 and CA19) stood out by their higher total fluorescence in a growth media with high carbon/nitrogen ratio, that is, as promising lipid producers (Figure 1). Subsequently, five yeasts presented a higher lipid production, were identified taxonomically and had their lipid production ability evaluated by the gravimetric analysis.

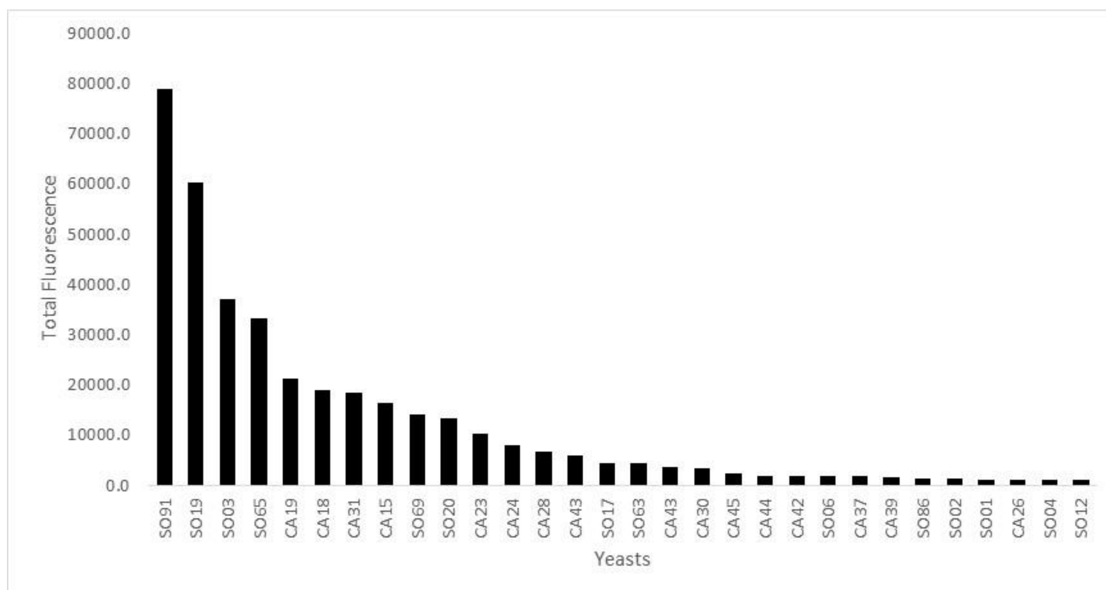

Figure 1. Total lipid fluorescence emission measured in the 30 best yeast strains that were selected during the evaluation step. This plot shows the fluorescence emitted in optical density (OD) per unit (Nile Red Fluorescence Arbitrary Units, RFU) after 120 h of culturing during the evaluation step. The following yeast isolates SO91, SO19, SO03, SO65 and CA19 had the highest lipid fluorescence emission per OD unit.

Taxonomic identification of five promising isolates and evaluation of their lipid accumulation

Five yeast isolates were taxonomically identified as *Papiliotrema laurentii, Meyerozyma caribbica, Candida sojae* and *Candida pseudointermedia* (Table 2).

Table 2. Taxonomic identification of oleaginous yeasts which displayed a higher lipid accumulation.

Code Collection	Species	Strain	Species Code	NCBI Access	Identity
SO91	*Papiliotrema laurentii*	UFV-1	CBS 139	AF075469.1	579/581(99%)
SO19	*Papiliotrema laurentii*	UFV-2	CBS 139	AF075469.1	561/562(99%)
SO3	*Candida sojae*	UFV-3	CBS 7871	KJ722420.1	510/513(99%)
SO65	*Candida pseudointermedia*	UFV-4	NRRL Y-10939	U44816.1	432/434(99%)
CA19	*Meyerozyma caribbica*	UFV-5	NRRL Y-27274	U348786.1	534/535(99%)

Yeasts from *Candida* spp. were isolated and identified in this study have been commonly found in lignocellulosic biomass. Indeed, tropical forest soils are rich of rocks but poor of organic matter and nutrients, which may favor the growth of yeasts supporting stress situations, such as nitrogen limitation and carbon accumulation in the form of lipids. The soil samples analyses from Parnaso were in supplementary data. The presence of lignocellulosic wastes favors the selection of yeasts that use glucose and xylose as carbon source in growth media. The gravimetric analysis was conducted after 120 h of growth, the yeast *C. pseudointermedia* UFV-1 accumulated 21.9% and 24.7% of lipids for growth medias containing glucose and xylose as carbon sources, respectively. Similarly, the yeast *C. sojae* UFV-1 accumulated 22% and 25.1% of lipids for growth medias with glucose and xylose as carbon sources, respectively. The yeast *Meyerozyma caribbica* UFV-1 accumulated 29.7% of lipids in its dry weight after 120 h in the growth media containing glucose as the carbon source. Similarly, other yeast strains of *Meyerozyma caribbica* were also reported as oleaginous yeasts [(Brar et al., 2017; Polburee et al., 2015).

Two strains of the yeast *Papiliotrema laurentii* were identified with a higher ability to acummulate more lipids than the other yeasts isolated from soil samples in this study. These two strains were obtained from two different soil samples. Other studies have also reported the isolation of the yeast *Papiliotrema laurentii* from soil samples, previously classified as *Cryptococcus* [(Liu et al., 2015). The *Papiliotrema laurentii* strains UFV-1 and UFV-2 accumulated, respectively, 43 and 40 % of their dry weight as lipids after 48 h (Figure 3). Since lipid accumulation by UFV-1 strain was higher than lipid accumulation by UFV-2, further studies were carried out with the UFV-1 strain in order to evaluate its ability to be used as an oil source for fatty acid-based biofuels.

Lipid production by the yeast *Papiliotrema laurentii* UFV-1

Initially, we evaluated the growth in culturing for the yeast *Papiliotrema laurentii* UFV-1 and fluorescence emission, which is related to lipid accumulation, in minimal growth media with a high ratio of carbon/nitrogen. Carbon sources on growth media were glucose, xylose or a mixture of glucose (70%) and xylose (30%).

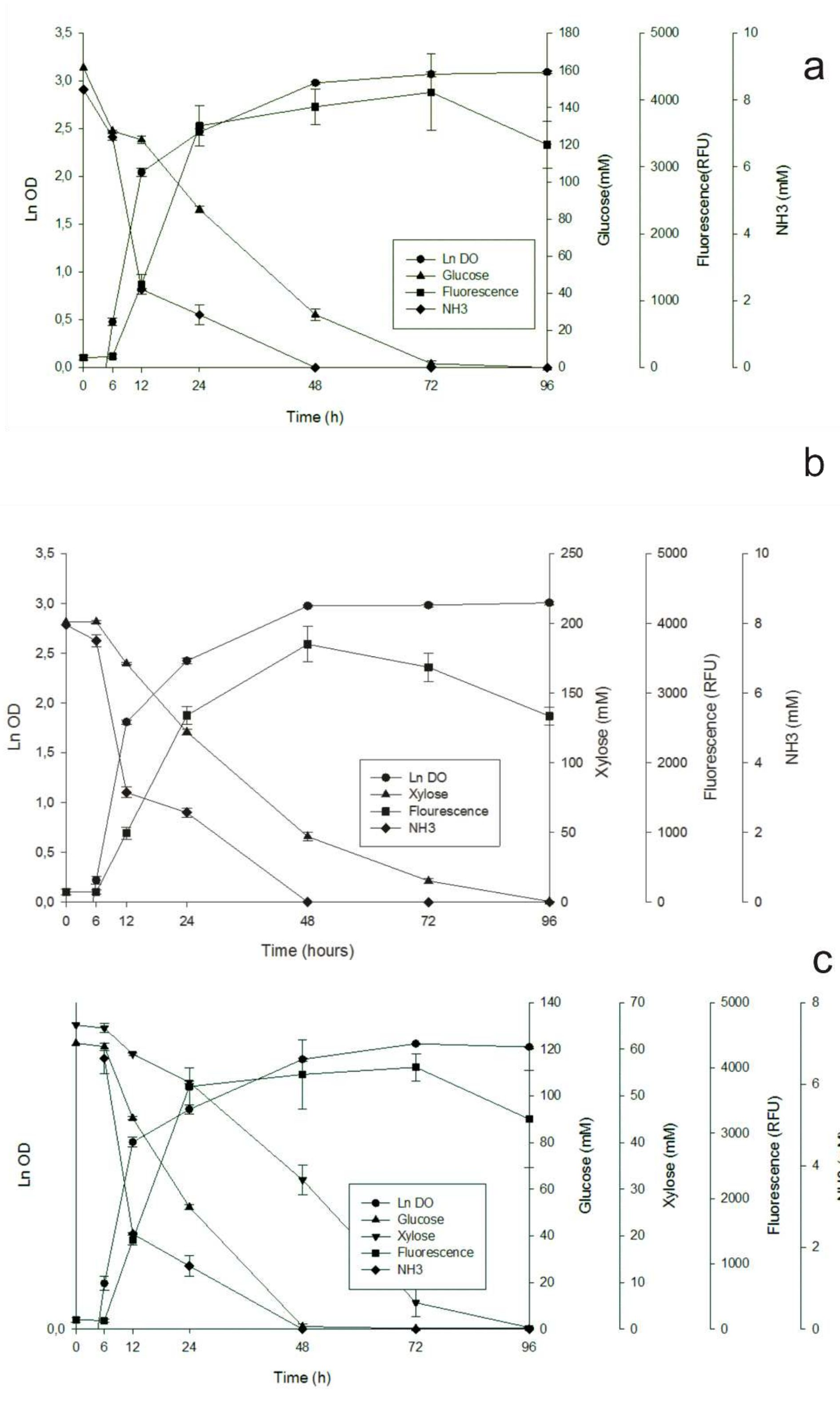

Figure 2. Cellular density (ln OD), sugar consumption (mM), ammoniacal nitrogen (mM) and estimated lipid accumulation by fluorescence emission (RFU/OD unit). The yeast *P. laurentii* UFV-1 was cultured for 96 h in growth media containing as carbon sources (a) glucose (G), (b) xylose (X) or (c) a mixture of glucose (70%) and xylose (30%) (GX).

Yeast, algae, filamentous fungi and bacteria present lipids, which are known as single cell oil (SCO). In oleaginous yeasts, carbon is converted mainly to triglycerides (TAGs) and steryl esters (SE), which are accumulated into lipid droplets (LDs) [(Athenstaedt and Daum, 2006). LDs are intracellular organelles also known as adiposomes or lipid bodies. They are typically smaller than one micron in yeast [(Thiam et al., 2013). In order to confirm the period of maximum lipid accumulation and lipid content decrease, we performed lipid quantification through gravimetric analysis after both 48 h and 96 h. The highest lipid content (43%) was obtained in growth media containing glucose. Although, lipid production has been lower (30%) in growth media containing xylose as carbon source, the yeast revealed a potential to accumulate oil from this sugar. When the growth media had both glucose and xylose as carbon sources, the yeast had 37.4% of the lipid content. Similar to the fluorescence results, gravimetric analysis in this study measured a decrease in the lipid content after 96 h with no association to the type of growth media used for yeast culture (Figure 3a). Remarkably, we observed that LDs in the yeast *P. laurentii* UFV-1 were widely spread in the cytoplasm after 48 h of culture (Figure 3b).

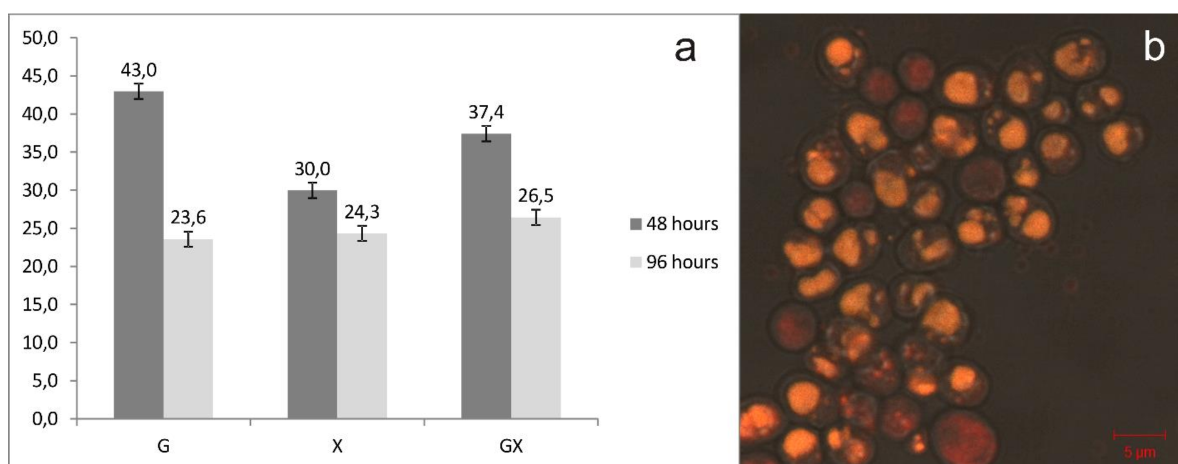

Figure 3. (a) Lipid contents in the yeast *P. laurentii* UFV-1 measured after 48 h and 96 h of culture in growth media containing as carbon sources glucose (G), xylose (X), or a mixture of glucose (70%) and xylose (30%) (GX). (b) Photomicrography from a fluorescence microscopy stained with Nile Red fluorophore after 48 h of culture in GX growth media.

We observed an increase of fluorescence emission over the log phase, which indicated the occurrence of lipid accumulation. Considering the best five isolates, this

lipid accumulation was only observed for yeasts from the genus *Papiliotrema* (Figure 2 and Figure S1). In addition, lipid accumulation in *P. laurentii* UFV-1 continued after the log phase. It should be noted that the ammonium was completely consumed after 48 h in three growth media. We observed a decrease in the fluorescence emission after 72 h of growth. Similarly, we also verified a high lipid accumulation after 48 h and a drop of lipid content after 72 h in the three growth medias. This decrease may be related to the degradation of the accumulated neutral lipids through β-oxidation (Figure 3, a). We can observe that in the GX growth media, the consumption of both glucose and xylose started after 6 h of growth. However, it was observed that the rate of glucose consumption is considerably higher than xylose consumption (Figure 2, c), and that xylose consumption rate increased after glucose consumption. These results indicate that there was glucose repression of genes involved in xylose use. To confirm that, *P. laurentii* UFV-1 and *Spathaspora passalidarum* NRRLY 27907 were cultured in a growth media containing xylose and 2-deoxyglucose, which is a glucose analogue that cannot undergo further glycolysis (Figure S3). *S. passalidarum* was used as a control yeast, because glucose does not repress xylose use by this yeast. Compared to *S. passalidarum*, the *P. laurentti* growth was considerably lower, indicating that there was glucose repression.

The use of microbial oil for biodiesel production relies on its fatty acid profile, since this profile is related to biodiesel quality. In this sense, the presence in oils of C16 and C18 fatty acids is an important feature related to biodiesel quality(Papanikolaou and Aggelis, 2011). Therefore, we evaluate whether the profile of *P. laurentii* UFV-1 is proper for biodiesel production. High proportions of the oleic acid (approximately 59%) and the palmitic acid (around 28%) were found in the profile of fatty acids regardless of the growth media used (Table 3), which indicates that the oil obtained from the yeast *P. laurentii* UFV-1 is suitable for biodiesel production.

Table 3. Fatty acid profile for the yeast *P. laurentii* UFV-1 cultured in the growth media enriched with the carbon source glucose (G), xylose (X) or the mixture containing glucose (70%) and xylose (30%) (GX). The profile of fatty acids was determined after 48 h of growth, when the yeast had the maximum lipid accumulation.

Fatty acids (%)	G	X	GX
Miristic acid	0.5±0.0	0.5±0.0	0.5±0.0
Palmitic acid	29.4±0.7	28.8±0.02	28.4±0.3
Stearic acid	9.8±0.9	11±0.8	9.5±1.4
Arachidic acid	0.8±0.0	0.7±0.0	0.7±0.1
Palmitoleyl alcohol/cis-10-Palmitoleic acid	0.2±0.0	0.2±0.0	0.2±0.0
Oleic acid	59.3±0.7	58.6±0.8	60.5±1.5

Discussion

Approximately 10% from the 1600 known yeast species are described as oleaginous yeasts (Sitepu et al., 2014). Several studies have explored the microbial community found in soils for isolating oleaginous yeasts (Aksu and Tuğba Eren, 2005; Chandran and Das, 2012; Cloete et al., 2009; Kitcha and Cheirsilp, 2011; Pan et al., 2009; Schulze et al., 2014; Sláviková and Vadkertiová, 2003; Takashima et al., 2012). Nevertheless, there are no reports about the isolation of oleaginous yeasts from soils sampled in the Brazilian National Parks *Caparaó* (CA) and *Serra dos Órgãos* (SO). Therefore, we isolate oleaginous yeasts for the first time from soils of those parks. It is important to point out that the yeasts *C. sojae* and *C. pseudointermedia* had not yet been characterized as oleaginous yeasts. Therefore, we report for the first time their potential to accumulate lipids. Other yeast strains such as *M. caribbica* (Polburee et al., 2015) and *P. laurentti* (Castanha et al., 2014; Sarkar et al., 2018a; Wei et al., 2014) had already their oleaginous phenotype demonstrated. Similarly, this phenotype was confirmed in this study.

Development and breakdown of droplets are important for cellular metabolism. Lipid accumulation in oleaginous yeasts occurs when there is limited nitrogen and an exccess of carbon (Ratledge, C., Wynn, 2002). Since yeast growth is limited in these conditions, the carbon is used in lipid synthesis, resulting in neutral lipids accumulation. WYN et al. (2002) described different patterns for lipid accumulation. The yeast *Yarrowia lipolytica* is a model organism used in studies for bio-oils production (Beopoulos et al., 2009; Sabirova et al., 2011; Wu et al., 2016; Zhao et al., 2015), it

converts carbon excess into a lipid storage when having a nutrient imbalance condition. This pattern has also been observed in other lipid-accumulating yeasts as *Rhodosporidium azoricum, Trichosporon oleaginosus* and *Lipomyces starkey* (Capusoni et al., 2017; Uzuka et al., 1985). It has been reported in the literature that lipid accumulation in oleaginous yeasts starts in the late exponential phase and continues until reaching the stationary phase (Raschke and Knorr, 2009). This same pattern of lipid accumulation is verified in the yeast *Yarrowia lipolytica* (Figure S2). Furthermore, there is another pattern for lipid accumulation observed for the yeast *Cryptococcus terricolus* that is growth-associated (Boulton, C. A. , Ratledge, 1984). These authors claimed that *C. terricolus* have a faster sugar uptake ability directing it to the cycle for lipid storage than converting sugar into cellular material.

The yeast *Papiliotrema laurentii* has been considered a promising candidate for industrial application due to its ability to assimilate several carbon sources (Sitepu et al., 2014). Indeed, it is able to grow in media containing different wastes and feedstocks such as sugars that can be obtained from lignocellulosic biomass [(de Souza et al., 2013), hydrocarbons (Chandran and Das, 2012), glycerol (Polburee et al., 2015) polyphenols/winery wastewater and lactose/ricotta cheese whey. Our data show that lipid accumulation in *P. laurentii* UFV-1 presented a similar pattern to *Cryptococcus terricolus* remarkably starting prior to the late exponential phase during the log phase.

Further studies should be performed to gain insights regarding the uncommon growth-associated lipid accumulation. The yeast *Yarrowia lipolytica* should be included in further studies since it starts lipid accumulation after the late exponential phase. Interestingly, the maximum lipid content was achieved in our study after 48 h of growth, which is considered a short period compared to other oleaginous yeasts (Table 4). The yeast *P. laurentii* UFV-1 showed a faster lipid accumulation being a desirable feature since a high volumetric productivity can be reached in bioprocesses. After 48 h of growth, the lipid content decreased. Similarly, other oleaginous yeasts also had a reduction on their lipid content after reaching the maximum accumulation of lipids. It is noteworthy that the yeasts were under a starvation condition during the growth period, with full consumption of nitrogen and a low concentration of the carbon source. Therefore, lipid degradation occurred through a β-oxidation pathway to generate energy for cell maintenance and homeostasis. It has already been proven that under nitrogen starvation, the genes involved with the beta-oxidation pathway are upregulated genes even in the exponential phase Sarkar et al. (2018). The authors claimed that lipid degradation

generate the required energy amount for a faster growth of yeasts. This might explain the short period to accumulate and decrease lipid contents in the yeast *P. laurentii.*

Table 4. Traits for several oleaginous yeasts, when they present a maximum lipid accumulation.

Yeasts	Time (h)	Lipid content (%)	Carbon source	Fermentation mode	Reference
Cryptococcus laurentii UCD 68-201	60	32.64	Ricotta cheese whey	Batch	(Carota et al., 2017)
Trichosporon oleaginosus ATCC20509	120	60	Sorghum hydrolysates	Batch	(Lee et al., 2017)
Lipomyces starkeyi ATCC 56304	120	44	Sorghum hydrolysates	Batch	(Lee et al., 2017)
Cryptococcus albidus ATCC10672	120	42	Sorghum hydrolysates	Batch	(Lee et al., 2017)
Cryptococcus vishniaccii	216	53.40	Paper mill sludge extract	Batch	(Deeba et al., 2016)
Yarrowia lipolytica ATCC20460 (engineered)	120	61.7	Glucose	Batch	(Tai and Stephanopoulos, 2013)
Trichosporon cutaneum 2.1374	60	31	Glucose	Batch	(Liu et al., 2013)
Papiliotrema laurentii UFV-1	48	43/30	Glucose/Xylose	Batch	This work

The development and breakdown of the LDs organelles are important in oleaginous yeasts. These LDs organelles interact with each other through fusions, fissions and among them. These processes are promoted by specific structural proteins (Walther and Farese, 2009). The fusion of LDs organelles occurs during organelles development, while the fission (fragmentation) of LDs organelles may be originated by stress such as thermal shock and type of the carbon source (Ta et al., 2012). The LDs fragmentation pattern observed in the yeast *P. laurentii* UFV-1 may have been caused by typical stress conditions, which are inherent to the stationary phase. Futhermore, it was also observed an acidification of the growth media (data not shown), besides the nutritional stress.

Most of the studies on oleaginous yeasts are carried out with the yeast *Y. lipolytica*. Despite its ability to grow in media with unconventional carbon sources such as hydrocarbons (Beopoulos et al., 2009), this yeast is not able to use xylose as the unique carbon source. D-xylose is one of the most abundant pentose sugars found in nature [(Pan et al., 2009). In the context of biofuels, the oleochemicals production by using xylose is a promising route, since ethanol production from this sugar still finds drawbacks (Zhang et al., 2013). As mentioned previously, the yeast *P. laurentii* UFV-1 had a superior production of lipids by the use of glucose than xylose as carbon source in growth media. However, the oleaginous phenotype was also observed in the growth media containing xylose, even under unoptimized conditions. This difference seems to be related to the NADPH requirement for the xylose metabolism with the need of the enzyme xylose reductase to catalyze the first reaction. Three copies of the gene encoding this enzyme were predicted in the genome of *P. laurentti* UFV-1 (as described in Chapter 3). In addition, it was predicted that xylose reductase uses the coenzyme NADPH to carry out xylose reduction to xylitol. It is noteworthy that the lipid biosynthesis also requires NADPH and, therefore, the NADPH availability on media growth containing xylose is likely lower than on media with glucose, impairing the fatty acid synthesis. Considering that there was lipid accumulation under unoptimized conditions, further studies should be performed to optimize oil production by the yeast *P. laurentii* UFV-1 on media containing xylose.

The fatty acid composition influences the quality of the biodiesel produced. Suitable compositions of fatty acids for biodiesel production consist mainly of the palmitic (16: 0), stearic (18: 0), oleic (18: 1), linoleic (18: 2) and linolenic (18: 3) fatty acids [(Knothe, 2009). The yeast *P. laurentti* UFV-1 presented a profile of fatty acids suitable for biodiesel production, since the oleic and palmitic fatty acid were the most abundant. Thus, these results highlight *P. laurentti* UFV-1 ability to be an oil source for biodiesel industry.

Thus, in this study there is a description for the first time of the procedure for isolating oleaginous yeasts from soil samples of the Brazilian National Parks *Caparaó* and *Serra dos Órgãos*. The yeast *P. laurentii* UFV-1 is a promising isolate that can pottentially accumulate oil from lignocellulosic sugars. Further studies should be performed to optimize lipid production on growth media containing xylose as the sole carbon source. Interestingly, the yeast *P. laurentii* UFV-1 showed a remarkable phenotype of growth-associated lipid accumulation. Moreover, lipid accumulation by this

yeast was faster than those reported in the literature for other oleaginous yeasts. Therefore, the profile of fatty acids for the yeast *P. laurentii* UFV-1 indicates that this species is suitable for biodiesel production.

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Supplementary data

Table S1. Estimation of lipid accumulation by a semi-quantitative analysis using a Nile Red Fluorometric method. Measure of the Fluorescence per OD unit, Optical Density (OD 600 nm) and Total Fluorescence for 129 isolates obtained after a 120 h of culturing in growth media.

Yeast	Fluorescence 5 days	OD 5 days	Total
SO91	3316.0	23.8	78920.8
SO19	2785.0	21.7	60434.5
SO03	1265.8	29.3	37087.9
SO65	1773.4	18.8	33339.9
CA19	726.8	29.5	21440.6
CA18	680.9	28.0	19038.7
CA31	664.3	28.0	18601.6
CA15	680.9	24.2	16464.8
SO69	676.3	20.8	14066.8
SO20	1155.0	11.7	13513.6
CA23	334.0	30.9	10319.5
CA21	262.8	33.4	8778.0
CA24	882.3	9.0	7940.4
CA08	231.2	32.2	7444.4
CA28	1435.2	4.8	6888.8
CA25	251.9	24.8	6247.6
CA43	1591.5	3.7	5888.7
CA17	244.9	20.5	5016.2
SO17	285.1	15.7	4476.3
SO63	364.8	12.2	4466.8
CA43	1527.4	2.5	3818.4
CA30	1677.2	2.0	3354.4
SO68	89.5	36.0	3221.7
SO54	201.1	12.9	2594.2
SO56	190.7	13.0	2479.1
CA45	980.5	2.5	2451.4
SO45	167.3	13.4	2241.2
SO50	192.2	11.1	2142.1
SO87	111.4	18.8	2092.3
SO53	204.9	10.2	2089.7
CA44	1173.3	1.7	1994.6
CA42	1101.7	1.8	1983.0
SO06	1660.6	1.2	1971.1
CA37	880.5	2.1	1848.9
CA39	996.1	1.8	1792.9
SO76	131.7	11.7	1540.9

SO86	799.6	1.8	1455.3
SO02	1232.5	1.2	1442.0
SO01	982.3	1.2	1172.9
CA26	296.4	3.8	1126.3
SO04	1332.0	0.8	1065.6
SO12	800.6	1.3	1056.7
SO14	810.2	1.3	1029.0
CA01	480.3	1.9	926.9
SO43	156.8	5.5	862.4
SO38	163.7	5.2	851.2
CA20	801.3	1.1	841.4
SO61	531.1	1.0	548.1
SO09	487.0	1.1	529.3
SO11	692.6	0.7	505.6
SO64	533.1	0.9	489.4
SO66	216.2	2.1	449.6
SO07	558.8	0.7	402.3
SO85	173.8	2.2	384.1
SO83	152.5	2.5	381.3
SO67	1308.8	0.3	366.5
CA22	143.5	2.3	330.1
SO13	184.8	1.6	290.1
SO05	1227.5	0.2	269.3
SO36	145.5	1.8	261.9
SO10	355.3	0.7	259.4
SO25	119.9	2.1	251.8
SO82	259.3	1.0	251.5
SO08	279.9	0.8	221.1
SO48	192.3	1.1	203.9
CA10	7.5	26.5	198.7
SO22	458.2	0.4	183.3
SO58	176.0	1.0	176.0
SO29	188.2	0.9	163.8
SO28	161.6	1.0	161.6
CA34	290.2	0.5	148.4
SO60	160.8	0.9	144.7
SO40	142.4	1.0	142.4
SO71	277.0	0.5	130.2
SO24	126.7	1.0	126.7
SO39	156.1	0.8	124.9
SO75	89.2	1.2	107.0
SO33	354.3	0.3	106.3
SO34	63.0	1.6	100.8
SO47	122.3	0.8	97.8
SO37	97.3	1.0	97.3

CA36	264.8	0.4	96.2
SO74	65.0	1.4	91.0
SO27	95.1	0.9	85.6
SO42	83.4	1.0	83.4
SO32	89.4	0.9	80.5
SO84	63.8	1.2	76.6
SO44	185.1	0.4	72.2
CA04	190.0	0.4	71.4
CA35	224.6	0.3	70.4
SO16	179.1	0.4	69.8
SO57	292.9	0.2	67.4
SO79	51.7	1.3	67.2
SO35	95.2	0.7	66.6
SO30	70.1	0.9	63.1
SO73	66.3	0.9	59.7
CA02	155.0	0.3	46.5
SO78	109.3	0.4	43.7
CA06	123.6	0.3	37.1
SO59	172.1	0.2	34.4
SO72	151.3	0.2	30.3
SO88	18.1	1.4	25.3
CA32	121.5	0.2	24.3
SO23	72.7	0.3	21.8
CA03	100.4	0.2	20.1
SO70	78.8	0.2	15.8
SO18	201.2	0.1	10.1
SO55	100.3	0.1	10.0
SO49	84.0	0.1	8.4
SO31	83.8	0.1	8.4
SO52	83.7	0.1	8.4
CA13	39.2	0.2	7.8
SO15	68.4	0.1	6.8
CA29	21.1	0.3	6.3
SO41	62.1	0.1	6.2
CA16	27.9	0.2	5.6
CA09	46.5	0.1	4.7
SO51	41.5	0.1	4.2
CA05	40.7	0.1	4.1
SO17	40.0	0.1	4.0
SO81	35.7	0.1	3.6
CA07	35.5	0.1	3.6
CA14	32.1	0.1	3.2
CA11	27.7	0.1	2.8
CA33	21.1	0.1	2.1
SO80	3.9	0.2	0.8

SO62	68.8	0.0	0.0
CA12	50.8	0.0	0.0

Table S2. Chemical analysis of Parnaso National Park soil samples.

Samples	P	K	Ca2+	Mg2+	Al3+	OM	pH
	mg/dm3	mg/dm3	mg/dm3	cmolc/dmc	cmolc/dmc	dag/kg	
1	1.1	47.0	0.3	0.1	1.4	1.3	4.5
2	5.2	45.0	0.3	0.1	1.6	3.6	4.9
3	10.3	72.0	0.6	0.2	1.4	7.7	4.8
4	27.2	46.0	0.4	0.1	1.7	8.3	4.8
5	2.7	62.0	0.5	0.1	1.2	2.1	4.6
6	2.8	37.0	0.2	0.1	1.5	2.9	4.8
7	6.5	43.0	0.6	0.2	1.9	10.6	4.4
8	26.8	43.0	0.4	0.1	2.2	9.0	4.2
9	2.4	35.0	0.4	0.1	1.3	1.8	4.7
10	2.1	48.0	0.1	0.1	2.1	3.1	4.4
11	10.6	43.0	0.3	0.1	1.8	8.3	4.7
12	5.2	47.0	0.3	0.1	2.1	8.6	4.8
13	1.9	51.0	0.6	0.2	1.3	2.4	4.4
14	6.2	28.0	0.3	0.1	1.6	3.2	4.4
15	4.9	47.0	0.3	0.1	1.7	6.8	4.6
16	68.0	35.0	0.3	0.1	2.2	8.0	4.9

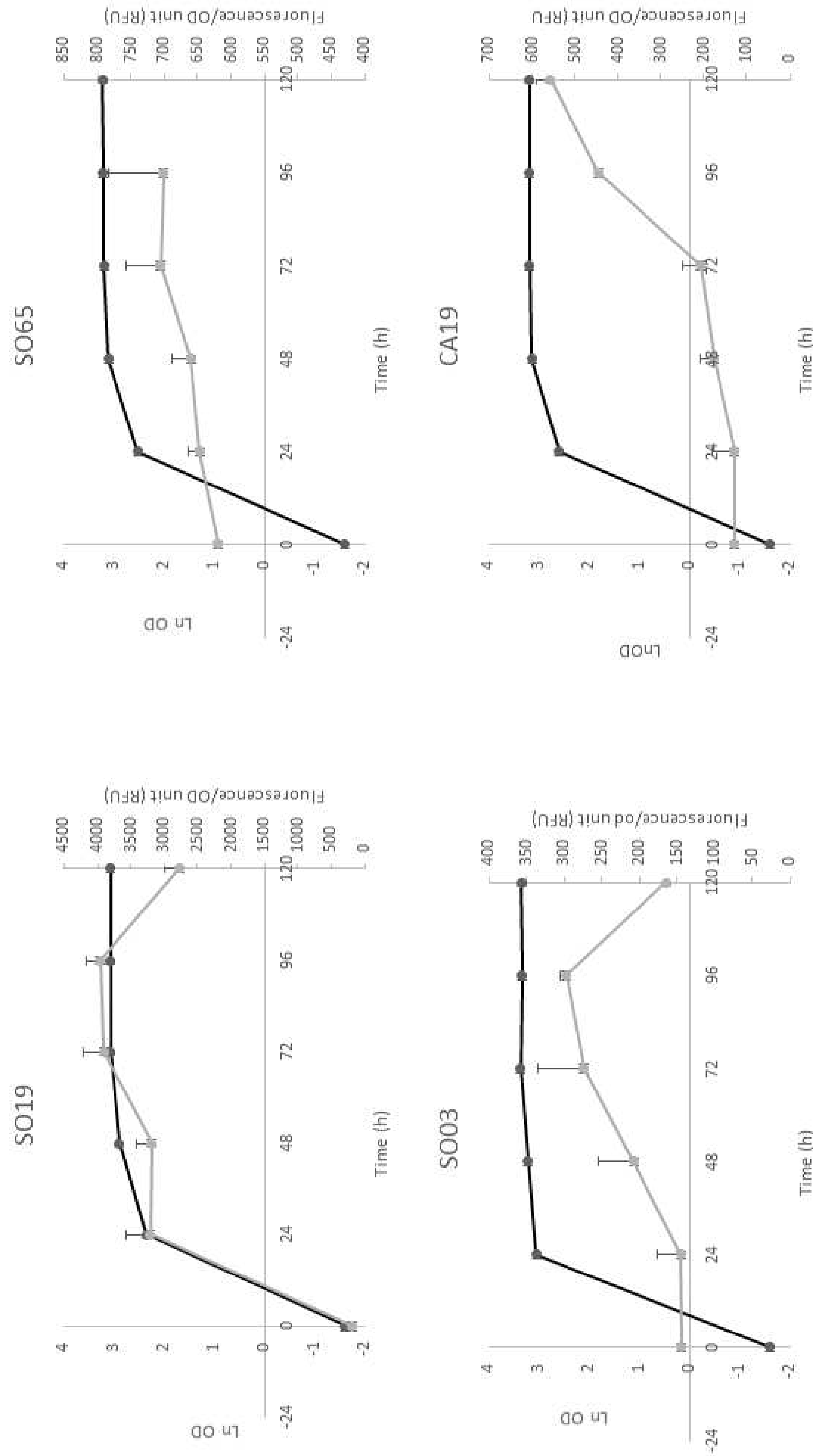

Figure S1. Cellular density (ln OD) and lipid content estimated by fluorescence emission (RFU/OD unit) during culturing of the yeast isolates SO19, SO65, SO03 and CA19 for 120 h.

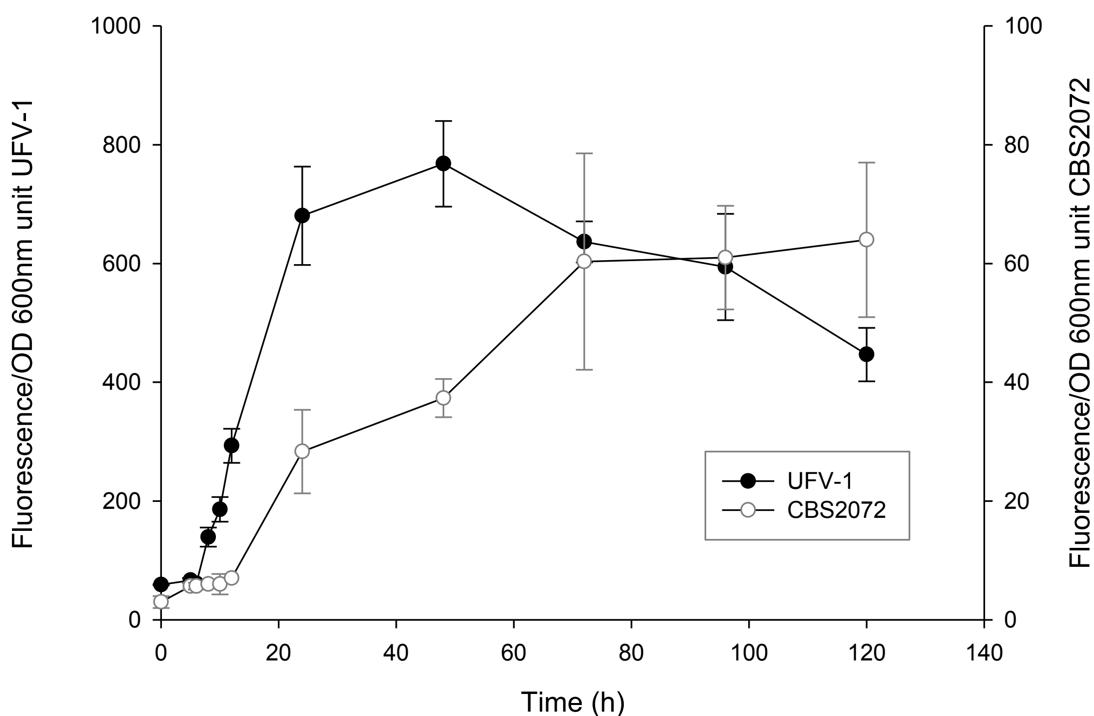

Figure S2. Cellular density (ln OD) and lipid content estimated by fluorescence emission (RFU/OD unit) during its culture for 120 h for the yeast strains *Papiliotrema laurentii* UFV-1 and *Yarrowia lipolityca* CBS2072.

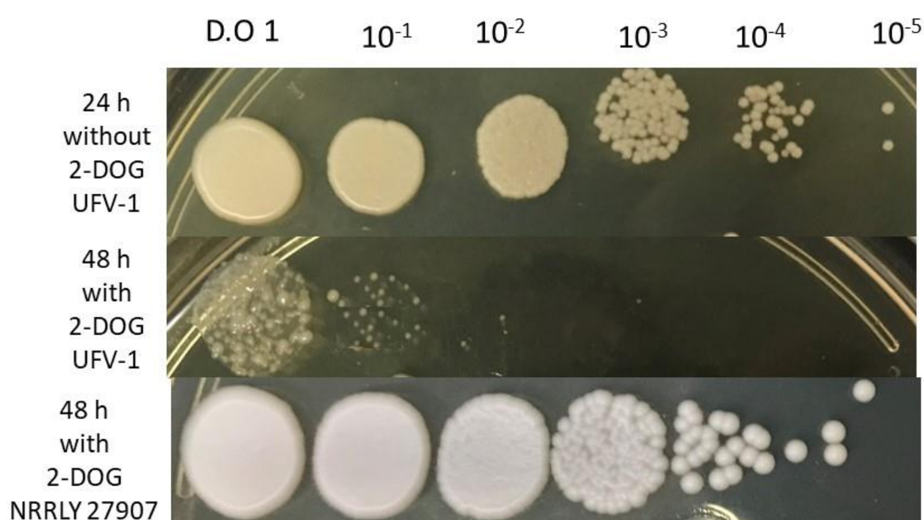

Figure S3. Glucose repression in the yeast *P. laurentii* UFV-1 and *S. passalidarum* NRRLY 27907. Yeast cultures were stained in the growth media YPX with or without the glucose analogue known as 2-deoxyglucose.

CHAPTER 3

Insights into lipid metabolism in the oleaginous yeast *Papiliotrema laurentii* revealed by genomic, gene expression and metabolomic analyses

Abstract

There is a rising interest in using microbial oil to produce both oleochemicals and fatty acid-based biofuels. The oleaginous yeast *Papiliotrema laurentii* UFV-1, isolated from a Campo Rupestre soil, is capable of accumulating lipids from lignocellulosic sugars such as glucose and xylose. In this work, genomic, gene expression and metabolomic analyses were carried out in order to better understand the regulation of lipid metabolism in this oleaginous yeast. Genomic and gene expression analyzes demonstrated that the NADP +-dependent malic enzyme is the main source of reducing fatty acid synthesis in *P. laurentii*. Interestingly, expression of the *ACC1* gene encoding acetyl-CoA carboxylase (Accase) decreased after 24h, but had a higher lipid accumulation. This result suggests a transcriptional regulation for ACC1 gene in *P. laurenttii* different from that described in the non-oleaginous yeast *Saccharomyces cerevisae*. Metabolomic analysis revealed that the degradation pathways of the amino acids lysine and leucine contributes for acetyl-CoA synthesis. The tricarboxylic acid cycle (TCA) and the pentose phosphate pathway (PPP) were less important during the of higher lipid accumulation phase. Moreover, pathways involved in nitrogen metabolism were negatively regulated, which confirms the redirection from the carbon flux to the fatty acid synthesis.

Introduction

Over the last years, oleaginous yeasts have called attention due to their potential use as an oil source for production of both oleochemicals and biofuels. Their physiology is remarkably different from non-oleaginous yeasts and, so far, most of the studies have focused on the model oleaginous yeast *Yarrowia lipolytica* (Beopoulos et al., 2009; Sabirova et al., 2011; Sestric et al., 2014). Lipid synthesis has also been studied in other oleaginous yeasts such as *Lipomyces starkey*, *Rhodosporidium toruloides*, *Rhodotorula* sp. (Amaretti et al., 2010; Calvey et al., 2016; Huang et al., 2016; Probst, 2014; Suzuki et al., 1973). Nevertheless, regulation of lipid metabolism in oleaginous yeasts is not well understood. Studies with other promising oleaginous yeasts improve the understanding

about lipid metabolism, highlighting potential targets to improve lipid synthesis. The yeast *Papiliotrema laurentii* is a basidiomycete with the ability to accumulate lipids having as source lignocelullosic sugars, which is needed to improve lipid production from abundant and inexpensive feedstocks. In the literature, there is only one work available reporting gene expression under nitrogen starvation (Sarkar et al., 2018) for *P. laurentii*. In this work, transcriptomic analysis revealed upregulated genes involved with transmembrane transporters, lipid homeostasis and fatty acid beta-oxidation pathways. Nevertheless, regulation of the lipid metabolism in *P. laurentii* remains to be elucidated.

Acetyl-CoA and NADPH are the building block and reducing power for fatty acid synthesis. Adenosine monophosphate (AMP) plays an central role in the regulation of lipid metabolism in oleaginous yeasts, because it is the allosteric activator of the mitochondrial enzyme isocitrate dehydrogenase NAD^+ -dependent. The enzyme ATP citrate lyase catalyses the reaction of citrate when it is transported from the mitochondria to the cytosol and is converted to acetyl- CoA and oxaloacetate (Ratledge, 2004). Recently, functional comparative genomic analyses revealed new pathways involved in acetyl-CoA synthesis in the oleaginous fungi *Yarrowia lipolytica, Rhizopus oryzae, Aspergillus oryzae* and *Mucor circinelloides* (Vorapreeda, 2012; Kerkhoven, 2017). These pathways are involved in the amino acids leucine and lysine degradation.

The substrate Acetyl-CoA is used by the enzyme Acetyl-CoA carboxylase (Accase). Accase enzyme plays a central role in fatty acid metabolism, since this enzyme catalyzes the first committed and rate-limiting step in fatty acid synthesis. The expression of the *ACC1* gene seems to be controlled by the transcriptional repressor Opi1 and by the transcriptional activators Ino2-Ino4. Indeed, a functional UAS_{INO} site was identified in the promoter of the *ACC1* gene (Tehlivets et al., 2007). At the post-translational level, the ACC enzyme has its activity inhibited by the phosphorylation catalyzed by the Snf1 kinase (Shi et al., 2014).

As aforementioned above, NADPH decreases fatty acid synthesis, since it is involved in two reduction steps during the elongation phase. The reaction catalyzed by the malic enzyme, which is a cytosolic $NADP^+$- dependent enzyme, has been considered an important NADPH source in the oleaginous yeast (Ratledge, 2014). On the other hand, there is an absence of the cytosolic malic enzyme in *Yarrowia lipolytica*. In this yeast, glucose-6-phosphate dehydrogenase catalyses the reaction of the pentose phosphate pathway, which has been pointed out as the main source of NADPH (Ratledge, C., Wynn, 2002). Furthermore, the reaction catalyzed by the cytosolic isocitrate dehydrogenase

NADP^{+-} dependent may contribute for NADPH synthesis (Ratledge, C., Wynn, 2002; Ratledge, 2004). Although, its role has not yet been confirmed experimentally.

The aim of this study was to evaluate lipid metabolism in the oleaginous yeast *P. laurentii* UFV-1. Its genome was sequenced and some genes involved in lipid metabolism was annotated in silico. Futhermore, gene expression was performed by quantitative real time PCR (qRT-PCR) under limited nitrogen condition. In addition, we also evaluated the metabolic profile for this strain under the same condition. Overall, our results provide relevant information about the regulation of lipid metabolism in *P. laurentii* UFV-1.

Material and Methods

Microorganism

The yeast *Papiliotrema laurentti* UFV-1 used in this work belongs to the culture collection of the Microorganisms Physiology Laboratory, Department of Microbiology (DMB), Federal University of Viçosa (UFV).

Growth media

The growth media utilized in this work are shown in the Table 1. The initial pH values of these growth media ranged from 5 to 6. The initial composition of each media was defined to guarantee a mass ratio of C / N = 48. The contribution of organic nitrogen from the yeast extract was included to calculate the C / N ratio.

Table 1

Composition of growth media used in this work UFV-1 yeast

Components	G (g/L)	X (g/L)	GX (g/L)
Glucose	30	-	21,42
Xylose	-	30,62	9,19
$(NH_4)_2SO_4$	0,7	0,7	0,7
NaCl	0,1	0,01	0,1
$CaCl_2$	0,1	0,1	0,1
$MgSO_4$	0,5	0,5	0,5
Yeast extract	1	1	1

Genome sequencing of *Papiliotrema laurentti* UFV-1

DNA extraction

The yeast *P. laurentii* UFV-1 in 5 mL of YPD (glucose 20 g/l, petone 20 g/L, yeast extract 10 g/L) growth media in 50 mL Erlenmeyer flasks. The strain was incubated at 30 °C under agitation at 200 rpm for 18 h. After incubation, 1.5 ml of the culture was

transferred to microcentrifuge tubes and centrifuged at 12,000 rpm for 5 min at room temperature. The obtained cell pellet was washed in 0.01% (w / v) peptone water twice. Subsequently, the pellet formed was resuspended in 500 μL extraction buffer [2 mL Triton 100x (v / v), 10 mL 10% (v / v) SDS, 10 mL 1M NaCl, 10 mL 1M Tris pH 8, and 0.5M EDTA pH 8.0 (v/v)] and 0.3 g of glass beads (0.5 mm diameter) were added. The suspension was homogenized using the Tissuelyser II equipment (Qiagen) for 2 min and a frequency of 30 shakes per second. After this procedure, centrifugation was performed at 14,000 x g for 5 min at 4 °C. The supernatant was collected and 800 μL ice cold isopropanol and 120 μL NaCl (5 mM). The mix was centrifuged at 14,000 x g for 15 min at 4 °C. The supernatant was discarded and resuspend in 400 uL in TE (Tris-HCl 10 mM, 1M EDTA, pH 8.0). After, was added 400 μl of PCI (Phenol Chloroform: isoamyl alcohol, pH 8.0). The sample was vortexing for 5 seconds to resuspend the pellet and was awaited 5 min. The sample was vortexed again and centrifuged at 13000 rpm, for 5 min at 4 °C. The upper phase was collected. And 4 μl of RNase (10 mg/mL) and incubate for 2 h at 37 °C. Add 80 μL of sodium acetate (3 M, pH 5.2) and 800 μL of 100% ethanol. The sample was incubated on ice for 20 to 30 min. The sample was centrifuged at peed 14000 x g, fo 5 min at 4 °C. The supernatant was discarded and washed 2 times with 500 μl of 70% ethanol. The pellet was dried at room temperature for 2 h and after resuspended in 30 μL of ultra-pure water.

Preparation genomic library

The genomic DNA was quantified by fluorescence method established by Quant-iTTM kit PicoGreen® dsDNA Kit (InvitrogenTM). Around 100 ng of DNA 100 submitted to the genomic library preparation protocol of the Nextera DNA library Preparation kit (Illumina®). The library quality was with a High Sensitivity DNA kit (Agilent Technologies) in Agilent 2100 Bioanalyser. These steps were performed according to the manufacturers' guidelines.

Genome Sequencing

The *Papiliotrema laurentii* UFV-1 genome was sequenced with a 2x100-pb paired-end library using Illumina HiSeq 2500, yielding 19.97 million of reads. Sequence adapters were removed using Cutadapt version 1.15 (Martin, 2011) and the reads were further trimmed for quality (Q20 score) and filtered for length (25 nt) using Trimmomatic version 0.36 (Bolger et al., 2014), producing a data set containing 18.65 million of reads. The *P. laurentii* UFV-1 genome was de novo assembled using SPAdes Genome

Assembler version 3.10.1 (Bankevich et al., 2012) by testing all k-mers with odd lengths ranging from 21 to 99. Protein coding genes were predicted from the assembled contigs using Augustus version 3.3 (Stanke et al., 2006; Stanke and Waack, 2003), selecting *Cryptococcus neoformans* as species model. Ribosomal genes were predicted using Barnap version 0.4.2 and tRNA genes were predicted using tRNAscan-SE (Lowe and Eddy, 1996). The completeness of genome assembly was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) version 3.0.1 (Simão et al., 2015) and a codon usage table was calculated using the cusp tool of EMBOSS version 6.6.0 (Rice et al., 2000). The proteins encoded by the predicted genes were annotated through similarity searches (e-value threshold=1E-50) using BLAST+version 2.7.1(Camacho et al., 2009). The proteins were aligned against sequences available at Swiss-Prot and UniProt Knowledgebase (UniProtKB) databases using BLASTp tool and against EuKaryotic Orthologous Groups (KOG) database using RPSBLAST tool. For the prediction of protein domains InterPro software (Finn et al., 2017) and the Pfam proteins database (Finn et al., 2016). The gene sequences from *Yarrowia lipolytica* CLIB 122, *Lipomyces starkeyi* NRRL Y-11557, *Rhodosporidium toruloides* NP11, *Cryptococcus neoformans* ATCC 208821 and *Kwoniella mangroviensis* CBS 10435, For the determination of the presence of the UAS_{ino} sequence in the promoter region of the *ACC1* gene, a curated repository Yeastract on-line platform was used (Teixeira et al., 2017).

Real-time PCR (qPCR)

Real-time PCR Primer Design

The real-time PCR primers were designed with GenScript®. They were tested in order to identify target sequences with e-PCR (NCBI).

Table 2

Primes used in this work.

Enzyme	Gene	Primers	Tm °C
ATP:citrate lyase	ACL	5'′-CGAGGGTGTTGCCATTGGTG-3'	58.95
		3'-CCTGCTTGACGGCCTCGATA-5'	59.08
Isocitrate dehydrogenase NAD+-dependent	ICDH-NAD	5'-CCTTGTTCGCCAACGTCAGG-3'	58.97
		3'- CTCGCTCGGAAGCCTCGTAA-5'	59.1
Isocitrate dehydrogenase NADP+-dependent	ICDH-NADP	5'-AGTGCCGAGGCGATCAAGAA-3'	58.93
		3'-GCTCTCGGAAGACGGTTCCA-5'	58.94
Malic enzyme	ME	5'-CCTCCCACCCTTCCATCGAC-3'	58.99
		3'-GTCCTCCCGTCGGATCTTGG-5'	59.08
Acetyl-CoA Carboxylase	ACC	5'-GGCCAACTCGAGCAGATCCT-3'	59.00
		3'-GGTGGCGAGGACATCGAAGA-5'	59.01
Glucose-6P Dehydrogenase	G6PDH	5'-CGCGTCTGGTGACAAACCTG-3'	58.97
		3'- CGTCATGGCCGCATAAGTCG-5'	58.98
Actin	ACTIN	5'-GTGTCCCGAGGCTCTCTTCC-3'	59.01
		3'- CCTGATGTCCAGGTCGCACT-5'	58.93
Glyceraldehyde-3 P dehydrogenase	G3PDH	5'-GTTCGTCTGCGGTGTCAACC-3'	58.98
		3'-CGTGGACGGTGGTCATGAGA-5'	58.94

RNA isolation

The RNA isolation was performed with TRIzol™ (Thermo Scientific). Posteriorly, 3 µg of each sample was treated with deoxiribonuclease I (Sigma). These steps were carried out according to the manufacturer's information.

cDNA synthesis

The cDNA of each sample was synthesized with ImProm-II™ Reverse Transcription System (Promega), according to the manufacturer's information.

q-PCR

Gene expression was assessed using a StepOnePlus ™ Real-Time PCR System (Thermo Fisher Scientific) and Power SYBR Green Master Mix (Thermo Fisher Scientific). Cycling conditions were as follows: 15 secs at 95 ° C, 40 cycles of 95 ° C for 3 secs; 30 secs at 60 ° C, and final denaturation at 95 ° C for 20 secs, followed by a melting curve.

Data were normalized using expression values of Actin (ACT) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as reference gene (Vandesompele et al., 2002). A total of three biological replicates and two technical replicates were performed for each gene.

The results were submitted to analysis of variance (ANOVA) and Bonferroni test using the Graphpad Prism 5 Software®. A $p < 0.05$ was statistically significant.

Metabolic profile

Metabolic Quenching

Cell samples were collected during both log phase (6 h of growth) and the lipid accumulation (24 h of growth). Aliquots of these samples were quickly mixed with a 1: 5 methanol solution and frozen in liquid nitrogen (Canelas et al., 2008). Posteriorly, the samples were centrifuged, and the supernatants were discarded. Cell pellets were kept in ultra-freezer at -80 °C until extraction. The metabolic quenching was performed in triplicate.

Extraction

Initially, 700 µL of cold methanol 100 % (v/v) were added to suspend the cell pellets. Posteriorly, 30 µL of ribitol (0.2 mg/mL in ultra-pure water), the internal standard, was added to each sample, followed by vortex homogenization and stirring for 15 min at 70 °C and 950 rpm in a Thermomixer (Bioer). Afterwards, centrifugation was carried out at 14,000 x g for 10 min. The supernatants were collected and transferred to new microtubes. The supernatants were partitioned with 375 µL of chloroform and 750 µL ultra-pure water, followed by vortexing for 15 secs and centrifugation at 14000 x g for 15 min. The polar (upper) phase was collected and aliquoted into new microtubes and then evaporated under vacuum (Speedvac, Eppendorf). After, the samples were stored at -80°C or derivatized as described in the next topic.

Derivatization

The samples were derivatized by the addition of 40 µL of pyridine (Sigma Aldrich), containing methoxyamine hydrochloride (Sigma Aldrich) at 20 mg/mL, to the microtube containing the completely dried samples. The samples were shaken at 950 rpm in Thermomixer (Bioer) at 37 °C for 2 h. Afterwards, 70 µL of the MSTFA solution

(Sigma Aldrich) were added with FAMEs standards for the generation of the retention index according to Lisec et al.(2006). Samples were kept at 37 °C for 30 min with shaking at 950 rpm. Finally, 100 µL of this aliquot were transferred to automatic sampler glass flasks for subsequent gas chromatographic analysis.

GC-MS

The samples were analyzed using a GC-MS TruTOF system, Agilent Chromatograph, Technologies 7890A and TruTOF® HT TOFMS Spectrometer, Leco, equipped with a 30 m capillary column (DB-35 MS, Agilent Technologies) according to Lisec et al. (2006). One µl of eacher sample was injected in the splitless mode at 230 °C, carried by the helium gas, continuous flow of 2 mL/min. The oven temperature was initially kept constant at 80 °C and then increased 15 °C/min up to reach 330 °C, at which was maintained for 6 min. Chromatograms had the baseline corrected and their deconvolution performed through the software, ChromaTOF (LECO). The peaks were assigned using the deconvoluted spectra obtained using the TagSearch Software (Cuadros-Inostroza et al., 2009) and spectral mass libraries of trimethylsilyl derivative compounds (TMS) obtained from the Max Planck Institute of Plant Molecular Physiology (http://csbdb.mpimp-golm.mpg.de/csbdb). Chromatographic peak areas for previously fragmented ions were verified and normalized by the peak area corresponding to ribitol and corrected for cell mass.

Statistical and metabolic pathways analysis

For metabolites selection the data obtained were analyzed using the Pearson's correlation, p-value of 0.05. Only metabolites with a negative correlation were selected. In this analyzis, the data set were filtered by the standard deviation and transformed to the log scale and staggered under Pareto scale. Uncorrelated metabolites were analyzed in the Pathway Analyses module. The pathway topological analysis generated both probability and impact data on metabolic pathways. The pathway impact is the cumulative percentage from the matched metabolite nodes generated by the topological analysis. The data of Saccharomyces cerevisiae were used as reference for this analysis. Moreover, Principal component analysis (PCA) was performed to evaluate an overall analysis of the data. The average values of the triplicates were used to construct the heatmap. These analyses were performed by using the Metaboanalyst Software (Xia et al., 2015, 2012).

Targeted metabolomics

Cell samples were collected at 6, 8, 24 and 48 h of growth in the growth media with glucose. Aliquots of 1 mL of these samples were quickly mixed with a 1: 5 methanol solution and frozen in liquid nitrogen (Canelas et al., 2008). Posteriorly, the samples were centrifuged, and the supernatants were discarded. Cell pellets were kept in ultra-freezer at -80 °C until extraction. The metabolic quenching was performed in triplicate. The extraction was carried out as described in item 2.4.2. The liquid chromatography mass spectrometry analyses were performed the liquid chromatography instrument UHPLC Agilent Technologies 1290 Infinity coupled with an Agilent triple quadruple 6430 mass spectrometer with electrospray ionization (Agilent Technologies). Chromatographic separation was carried out using the Eclipse Plus C18 column (50 × 2.1 mm) (Agilent Technologies). The separation was separated using binary gradient elution, which mobile phase A was water containing 0.01% acetic acid, and phase B was acetonitrile containing 0.02% acetic acid. The MS/MS parameters for targeted metabolites analyzed are representing in the Table 2. The results were submitted to analysis of variance (ANOVA) and Tukey's test using the Graphpad Prism 5 Software®. A $p < 0.05$ was statistically significant.

Table 3

MS/MS parameters for compound standards

Metabolite	Precursor Ion	Product Ion	Ionization Mode
AMP	348	136	+
Panthothenate	220	90	+
Mannitol	183	69	+
Erythrose 4P	201	99	+

The results are submitted to analysis of variance (ANOVA) and Tukey's test using the Graphpad Prism 5 Software®. A $p < 0.05$ was statistically significant.

Results and discussion

Genome annotation

The genome size of the yeast *Papiliotrema laurentii* UFV-1 is 19.28 Mb (Table 4). The genome contains 7,218 protein coding genes, 6 rRNA genes and 97 tRNA genes. Total gene length was up to 11.8 Mb, with a ratio of 57.6% for Gene/Genome. The assessment of genome completeness using 1,335 datasets from the BUSCO group of Basidiomycota identified that the genome is 83.2% complete (C) (0.2% duplicated [D]), 10.2% fragmented (F), and 6.6% missed (M). These results indicate that the majority of expected genes for Basidiomycota were covered by the assembled genome.

Table 4 . Assembly statistics for the *Papiliotrema laurentii* UFV-1 genome.

Total size (nt)	19,281,337
GC content	57.69 %
Number of contigs	1,275
Largest contig (nt)	198,684
N50 (n)	34,392 (170)
Average size of contigs (nt)	15,122.62
Coverage	200 x
N count	0
Gaps	0

In silico and transcriptional analysis of genes involved in lipid metabolism

In order to analyze the domains of a key protein for the accumulation of lipids in oleaginous yeasts, we compared the predicted ATP citrate lyase sequences from the *P. laurentii* genome with those of *Y. lipoltytica*, *L. starkeyi, R. toruloides, C. neofarmans* and *K. mangroviensus* (Figure 1). Interestingly, we observed differences for the ATP citrate lyase, which is found only in oleaginous yeasts. This enzyme is responsible for citrate conversion into acetyl-CoA (Ratledge, 2002), the building block for lipid synthesis.

```
Y. Lipolytica     MSAKSIHEADGKALLAHFLSKAPVWAE-QQPINTFEMGTPKLASLTFEDGVAP--EQIFAAAEKTYPWLLESGAKFVAKPDQLIKRRGKAGLLVLNKSWEECKPWIAERAAKPINVEGID
L. Starkeyi       MSAKSIHEADGKALLSYFLPRSPLLTK-EGTSTEFVPAPPRLASLTFPDDSPATVKAVLDAAESTYGWLLAPGAKFVAKPDQLIKRRGKSGLLSLNVTWQQARDWITVRAGKKLVVEGIP
R. toruloides     MSAKPIREYDAKLLLAYHLARAPTAGSKAVARDGFQSPEVKVAQVSW-DPETNQVT----PDAALPHWVFTE--KLVVKPDQLIKRRGKAGLLALNKTWAEGKQWIAERAGKQVQVEKTT
C. neoformans     MSTKAIREYDAKQLVSYWLNRSPTP---IPCKTDTSFTAVKVAQVQW-DPATKQLSPPIRPGQGLPDWVFSS--KLVGKPDQLIKRRGKAGLLCLNKGWEETGAWIEERAGKPVTVEKTT
K. mangroviensus  MSTKAIREFDAKQLVSYWLNRSPTP---IPTKSESSLSAAQVAQVEW-DPVTKQLSPPIKPGQGLPDWVFTS--KLVGKPDQLIKRRGKAGLLCLNKGWEETGAWIEQRAGKPVTVESTT
P. laurentii      MSTKAIREYDAKQLVSYWLNRSPTP---IPTVTSTSAAAVRVAQVEW-DPVTKQLTPPIR

Y. Lipolytica     GVLRTFLVEPFVPHDQKHEYYINIHSVREGDWILFYHEGGVDVGDVDAKAAKILIPVDIENEYPSNATLTKELLAHVPED-QHQTLLDFINRLYAVYVDLQFTYLEINPLVVIPTAQG--
L. Starkeyi       GYLRTFLVEPFVPHPQETEYYININSVREGDWILFYHEGGVDVGDVDSKASKLLIPVDLDKEYPTNATIISTLLSKVPEA-QHATLVDFINRLYAVYVDLQFTYLEINPLVVIPTASG--
R. toruloides     GTLNNFIVEPFCPHPSDAEYYICINSVREGDVILFTHEGGVDVGDVDAKALTLLVPVG--GELPSRDEIRSQLLKHVTGAERQEALIDYIIRLYSVYVDLHFAYLEINPLVAVENPSTGK
C. neoformans     GTLNTFIIEPFCPHPAETEYYVCINSAREGDWILFTHEGGVDVGDVDAKALKLLIAVG--EEFPTRETIISTLLANVAPA-KQDVLCDFLIRLYGVYVDLHFAYLEINPLVCLDAVDGKP
K. mangroviensus  GTLNTFIIEPFCPHPAETEYYVCINSTREGDWILFTHEGGVDVGDVDAKALKLLLPVG--EEFPTRENLISTLLANVAPA-KQDVLADFLIRLYAVYVDLHFAYLEINPLVVLDAAEGKP
P. laurentii      GTLNTFIIEPFTPHPANTEYYVCINSAREGDWILFTHEGGVEVGDVDAKALKLLVKVN--EPFPSREELVSTLLTHVPEA-KKDVLADFLIRLYGVYVDLHFAYLEINPLVVLDSVDGKP

Y. Lipolytica     VEVHYLDLAGKLDQTAEFECGPKWAAARSPAALGQVVTIDAGSTKVSIDAGPAMVFPAPFGRELSKEEAYIAELDSKTGASLKLTVLNAKGRIWTLVAGGGASVVYADAIASAGFADELA
L. starkeyi       VEVHYLDLAAKLDQTAEFECGAKWAGARAPTALG--ITPAKNGASINIDAGPPMVFPAPFGRELSDEEAYIAELDAKTGASLKLTVLNPLGRVWTLVAGGGASVVYADAIASAGYANDIA
R. toruloides     TDIFYLDMAAKLDQTAEYVVGPKWAIARDPSIINPAAAPMSNG-KISADKGPPMFWPPPFGRDLTKEEAYIAKLDGSTGASLKLTVLNAEGRIWTMVAGGGASVVYSDAIAAHGFAHELA
C. neoformans     AEIQYLDMAAKLDQTADFLCGPMWAVARDVS------STTSGATGIKADRGPPMVWPAPFGRDLTKEEAYIQKLDASTGASLKLTVLNSEGRVWTMVAGGGASVVYSDAIAAAGFAHELA
K. mangroviensus  SEIHYLDMAAKLDQTADFLCGPKWAVARDTV------TPSAGSSAIKADRGPPMVWPAPFGRDLTKEEAYIQKLDASTGASLKLTVLNQAGRVWTMVAGGGASVVYSDAIAAAGFAHELA
P. laurentii      CEIHYLDMAAKLDQTADFLCGPKWAIARDIS------GPANGAS-IKADRGPPMVWPAPFGRDLTKEEAYIQKLDASTGASLKLTVLNPTGRVWTMVAGGGASVVYSDAIAAAGFAHELA

Y. lipolytica     NYGEYSGAPNETQTYEYAKTVLDLMTRGDAHPEGKVLFIGGGIANFTQVGSTFKGIIRAFRDYQSSLHNHKVKIYVRRGGPNWQEGLRLIKSAGDELNLPMEIYGPDMHVSGIVPLALLG
L. starkeyi       NYGEYSGAPTETQTYEYAKTVLDLMTRGTPVEGGKVLFIGGGIANFTQVGSTFKGIIRAFKDYQSQLHLHGVKMYVRRGGPNWQEGLRLIKSCGEELSIPMEVYGPDMHVSGIVPLALLK
R. toruloides     NYGEYSGAPTQTQTYEYAKTILDLMTRGTPNPQGKLLFIGGGIANFTNVAATFKGIITALKEYQHRLQEHKVRIFVRRGGPNYQEGLKAMRLLGETLGVEIQVFGPETHITSIVPLGLGL
C. neoformans     NYGEYSGAPTEGQTYEYAKTIIDLMTRGTPHPEGKVLIIGGGAANFSDVAATFKGIIRALKEYKEGLLRHNVKIWVRRAGPNYQEGLKAMRLCGESLGVFMKVYGPESPITAIVPMALGI
K. mangroviensus  NYGEYSGAPTEGQTYEYAKTIIDLIVRGEPHPEGKVLIIGGGAANFSDVAATFKGIIRALKQFKEGLLRHKVSIWVRRAGPNYQEGLKAMRLCGESIGVPMKVYGPESPITAIVPMALGI
P. Laurentii      NYGEYSGAPTEGQTYEYAKTIIDLIVRGEPHPEGKVLIIGGGAANFSDVAATFKGIIRALKQYQEGLQRHKVSIWVRRAGPNYQEGLKAMRLCGESIGVPMKVYGPESPITAIVPMALGI

Y. lipolytica     ------------KRPK-----NVKPFGTGPST------------------EASTP----------------------------------------------------------------
L. starkeyi       ------------KRPA-----GIYPFGSSASS------------------SAVSV----------------------------------------------------------------
R. toruloides     IKSVDDALKVPGARAAADATGTLTPVPGSPKSRAAQLPT-----------GASTP--SRQQPQDNIVSFSDKVHAPD-SGRPWYRPFDETTRSIVYGLQPRAIQGMLDFDFACGRETPSV
C. neoformans     ------------ERPPTAITRDVTPIPSAPVTPPNGAASGI--ANMANNVGAVKADGSREQPNDQIVRFDTE---PITGSRPWFRPFDEHTRSFVFGLQPRAIQGMLDFDFSCGRKVPSV
K. mangroviensus  ------------QRPASAATRDVTPIASSPASPSANSPAPLPGSGEPSTVGSVKPDGSREQPNDHIVRFETE---KL-GSRPWFRPFDEHTRSFVFGLQPRAIQGMLDFDFSCGRKTPSV
P. laurentii      ------------TKPASAATREITPVHTGNAQTEGANGA--NGSAAQASVGTIKPDGSREQPNDHIVRFQAE---TP-GSRPWFRPFDEHTRSFVFGLQPRAIQGMLDFDFSSGRKTPSV

Y. lipolytica     -----------------------------------------------------------------------------------------------------------------------
L. starkeyi       -----------------------------------------------------------------------------------------------------------------------
R. toruloides     AAMVYPFGGHHVQKFYWGTKETLLPVFTSMKEAVAKCPDADVVVNFASSRSVYQSTLEALEFPQIKAIALIAEGVPERHAREILHLAKKKEVIIIGPATVGGIKPGCFRIGNTGGMNENI
C. neoformans     AAMIYPFGGHHIQKFYWGTKETLLPVYTSVGEAIKKHPDVDVVVNFASSRSVYASTLEILSFPQIKAIGIIAEGVPERHARELLHLALKKQVIIIGPATVGGIKPGCFRIGNTGGMMDNL
K. mangroviensus  AAMIYPFGGHHIQKFYWGTKETLLPVYTSVGEAIKKHPDVDVVVNFSSSRSVYASTLDILTYPQIKAIGIIAEGVPERHARELLHLAVKKEVIIIGPATVGGIKPGCFRIGNTGGMMDNL
P. Laurentii      AAMIYPFGGHHIQKFYWGTKETLLPVYTSVAEAIKKHPDVDVVVNFSSSRSVYASTLDILNFPQIKAIGIIAEGVPERHARELLHLAQAKKVIIIGPATVGGIKPGCFRIGNTGGMMDNL

Y. Lipolytica     -----------------------------------------------------------------------------------------------------------------------
L. Starkeyi       -----------------------------------------------------------------------------------------------------------------------
R. toruloides     LSSKLYRAGSVGYVSKSGGMSNELNNILSLTTDGAYEGIAIGGDRYPGTTFIDHLLRYEADPNCKMLVLLGEVGGVEEYRVIEAVKSGQIKKPIVAWAIGTCAKMFATDVQFGHAGSMAN
C. neoformans     IACKLYRPGSVGYVSKSGGMSNELNNILSYTTNGVYEGVAIGGDRYPGTSFIDHLLRYEADPECRMLLLLGEVGGTEEYRVIEAVKQGIIKKPIVAWAIGTCAKMFTSEVQFGHAGSMAN
K. Mangroviensus  IACKLYRPGSVGYVSKSGGMSNELNNILSYTTNGVYEGVAIGGDRYPGTSFIDHLLRYEADPECKLLLLLGEVGGTEEYRVIEAVKKGIIKKPIVAWAIGTCAKMFTSEVQFGHAGSMAN
P. laurentii      IACKLYRPGSVGYVSKSGGMSNELNNILSYTTNGVYEGVAIGGDRYPGTSFIDHLLRYEADPECKLLLLLGEVGGTEEYRVIEAVKQGIIKKPIVAWAIGTCAKMFTSEVQFGHAGSMAN

Y. lipolytica     -----------------------------------------------------------LGV---------------------------------------------------------
L. starkeyi       -----------------------------------------------------------L-----------------------------------------------------------
R. toruloides     SDLETAEAKNNAMRAAGFIVPPTFEELPQVLAETYQKLVGDGTIQPKPEVPPPQIPMDYNWAQTLGMVRKPAAFISTISDERGQELLYAGMPISKVFEEDIGIGGVVSLLWFKRRLPAYA
C. neoformans     SDMETADAKNRAMRAAGFIVPDTFEDLPDTLKAVYNQLVTKGAIVPKAEIEPPQIPMDYQWASKLGLIRKPAAFISTISDERGQELMYAGMRISDVFKEEIGIGGVISLLWFKRRLPAYA
K. mangroviensus  SDLETADAKNRAMKAAGFIVPDTFEDLPEVLKATYQKLVSAGAIVPKAEIEPPQIPMDYQWASKLGLIRKPAAFISTISDERGQELMYAGMRISDVFKEELGIGGVISLLWFKRRLPAFA
P. laurentii      SDLETADAKNRAMKAAGFIVPDTFEDLPEVLKATYQQLVANGTIVPKPEVEPPAIPMDYQWAAKLGLVRKPAAFISTISDERGQELMYSGMRISDVFKEELGIGGVISLLWFKRRLPPFA

Y. lipolytica     -----------------------------------------------------------------------------------------------------------------------
L. starkeyi       -----------------------------------------------------------------------------------------------------------------------
R. toruloides     TKFLEMVLMLTADHGPAVSGAMTTVITTRAGKDLVSSLVAGLLTIGDRFGGALDGAAQEFTRAFEAGLTPREFVDSMRKANKLIPGIGHKVKSKANPDKRVELVKNYVFKHFPSAKLLEY
C. neoformans     CKFIEMVLQLTADHGPAVSGAMNTIITARAGKDLISSLVAGLLTIGDRFGGALDGAAAEFSNGLTSGQTPREFVDSMRKANRLIPGIGHKIKSKTNPDLRVVLVVDFVRKHFPNHKTLDF
K. mangroviensus  CKFIEMVLQLTADHGPAVSGAMNTIITARAGKDLISSLVAGLLTIGDRFGGALDGAAAEFSRGVSSGLTPREFVDQNRKANKLIPGIGHKIKSKTNPDLRVTLVVDYVKKHFPSHKTLDF
P. laurentii      AKFIEMVLQLTADHGPAVSGAMNTIVTARAGKDLISSIVSGLLTIGDRFGGALDGAAAEFSRGANSGLTPREFVDSMRKANKLIPGIGHKIKSKTNPDLRVTLVVDYVKKHFPQHKTLDY

Y. lipolytica     -----------------------------------------------------------------------------------------------------
L. starkeyi       -----------------------------------------------------------------------------------------------------
R. toruloides     ALAVEDVTSAKKDTLILNQSRFLS---------SGAFTAEEAAEYMKIGTLNGLFVLGRSIGFIAHHLDQKRLKQPLYRHPADDIFIQPFNTDRILVQQRQ--
C. neoformans     ALAVEDVTTQKSGSLILNVDGAIAASFCDLVSGCGAFTEEEAQEYLKNGTLNGLFVLGRSIGFIGHYLDQRLLKQPLYRHPHDDIFYP--SAERVVIQPGKKA
K. mangroviensus  ALAVEDVTTQKANTLILNVDGAIAASFCDLLSGSGAFTEEEAAEYLKSGTLNGLFVLGRSIGFIGHYLDQRLLKQPLYRHPADDIFIN--MQERVVFQPGSA-
P. Laurentii      ALAVEEVTTQKSGTLILNVDGAIAASFVDLLSGTGAFSEEEAAEYLKIGTLNGLFVLGRSIGFIGHYLDQKQLKQPLYRHPADDIHIN--MQDRTLVTPGSF-
```

Figure 1. Sequence alignment for the gene ATP citrate lyase from *Y. lipolytica, L. starkeyi, R. toruloides, C. neoformans* and *P. laurentii* UFV-1. The domains ATP-grasp (red) and Citrate Bind (green) are present in *Y. lipolytica* and *L. lipomyces*. In *R. toruloides* beyond the domains ATP-grasp and Citrate Bind, there are also the domains CoA-binding, Ligase- CoA (yellow) and Citrate Synthase (pink). In *C. neoformans* and *P. laurentii* UFV-1 the four domains Citrate Bind, CoA-binding, Ligase-CoA and Citrate Synthase are present.

In *L. starkevi* and in the model oleaginous yeast *Y. lipolytica,* there are only the two domains ATP grasp and Citrate bind. In *R. toruloides* beyond the domains ATP-grasp and Citrate Bind, there are also the domains CoA-binding, Ligase-CoA and Citrate Synthase. In *C. neofarmans, K. mangroviensus* and *P. laurentii* UFV-1, there are the four domains in the gene ATP citrate lyase: Citrate Bind, CoA bind, Ligase CoA and Citrate Synthase. Due to these differences between these fungi, it is possible that the enzyme

regulation is different in these organisms. Additional studies are necessary to determine regulation for the ATP: citrate lyase gene in *P. laurentii* UFV-1.

Acetyl-CoA carboxylase (Accase) plays a central role in lipid metabolism regulation, because this enzyme catalyzes the first and rate-limiting step of the fatty acid synthesis. We identified that the Accase in *P. laurentii* has five domains: Biotin carboxylation, ATP-grasp, Lipoyl-binding, CoAcarboxyltransferase N-terminal and CoA carboxyltransferase C-terminal. These same domains were present in the Accase enzyme from *Y. lipolytica* and *S. cerevisae*. It has been reported for the non-oleaginous yeast *S. cerevisiae* that there is a transcriptional regulation for *ACC1* gene by the complex Ino2-Ino4. When the metabolites inositol and choline are in low concentrations, the Ino2-Ino4 complex acts as a positive regulator for *ACC1* gene expression (Krivoruchko et al., 2015; Tehlivets et al., 2007). We detected only one copy of the *ACC1* gene in *P. laurentii*. Futhermore, we identified the upstream activator sequence UAS_{ino} in the promoter of *ACC1* gene of *P. laurentii* (Figure 2), suggesting that this complex may also regulate gene expression (Figure 2).

It has been shown in *S. cerevisiae* that the repressor Opi1 protein contains a phosphatidic acid (PA) binding domain. At low concentrations of PA, Opi1 has an important role in the nucleus, where it interacts with Ino2, preventing its complexation with Ino4, thus *ACC1* gene activation by the Ino2-Ino4 complex is impaired. In low inositol concentration, there is an increase in PA concentration, which is a precursor of the phosphatidylinositol. Subsequently, *P. laurentii* in contrast to *S. cerevisiae* when having a higher concentration of PA might cause the translocation of Op1p to the nucleus. Since PA is also a precursor for triacylglycerol (TAG), we hypothesize that the increase in the concentration of PA is the signal leading to the decrease of fatty acid synthesis, which is consistent with the lower *ACC1* gene expression at 24 h (Figure 3). Similarly, ACC1 gene expression was strongly downregulated after 24 h. This hypothesis can be confirmed by using lipidomic analysis, which will allow to measure concentrations of both PA and TAG.

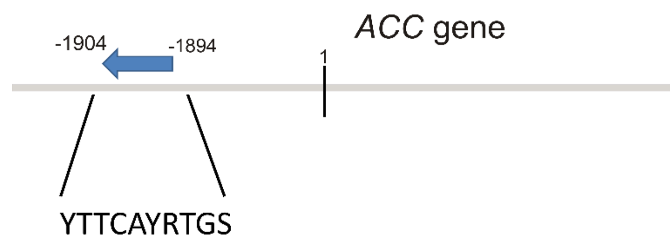

Figure 2. Gene structure for *ACC1* gene from *P. laurentii* UFV-1. In the promoter region, the UAS_{ino} sequence is located between the nucleotides -1894 and -1904.

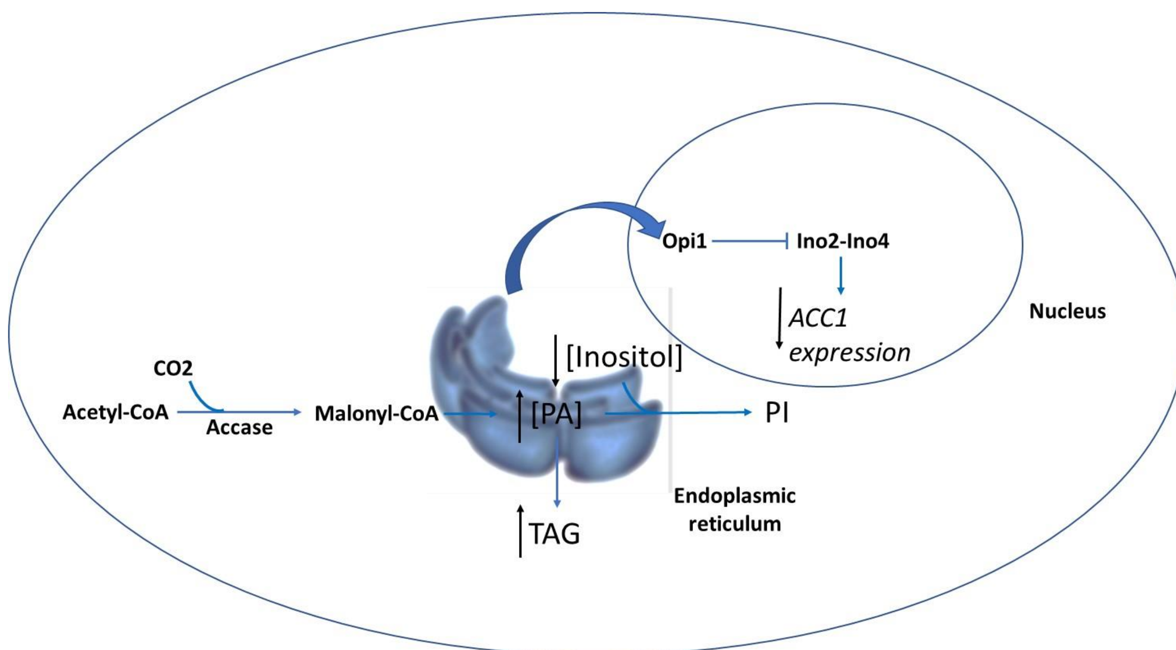

Figure 3. Proposed mechanism for the transcriptional regulation for *ACC1* gene in the *Pl laurentii* UFV-1. High concentrations of PA lead to the translocation of Opi1 from the endoplasmic reticulum (ER) to the nucleus, where it sequesters the activator Ino2. Thus, the expression for the *ACC1* gene is negatively regulated.

Based on genome analysis, it has been pointed out that acetyl-CoA can be synthesized from pathways involved in degradation of amino acids in oleaginous fungi (Vorapreeda et al., 2012). The following orthologous genes related to a possible degradation route for leucine were characterized: branched-chain α-keto acid dehydrogenase E, isovaleryl-CoA dehydrogenase, methylcrotonoyl-CoA carboxylase and hydroxymethylglutaryl-CoA lyase. The pathway for degradation of lisyne is triggered by the saccharopine synthesis and then by the by the sequential formation of of 2-aminoadipate-6-semialdehyde, 2-aminoadipate, glutaryl-dihydro-lipoamide and glutaryl-CoA. The modification of glutaryl-CoA can be performed through a sequence of Acyl-CoA intermediates in order to obtain acetyl-CoA, which is the final product. In this work,

we verified that genes encoding enzymes involved in the degradation pathway of the amino acids leucine and lysine are also present in *P. laurentii* genome (Figure 4).

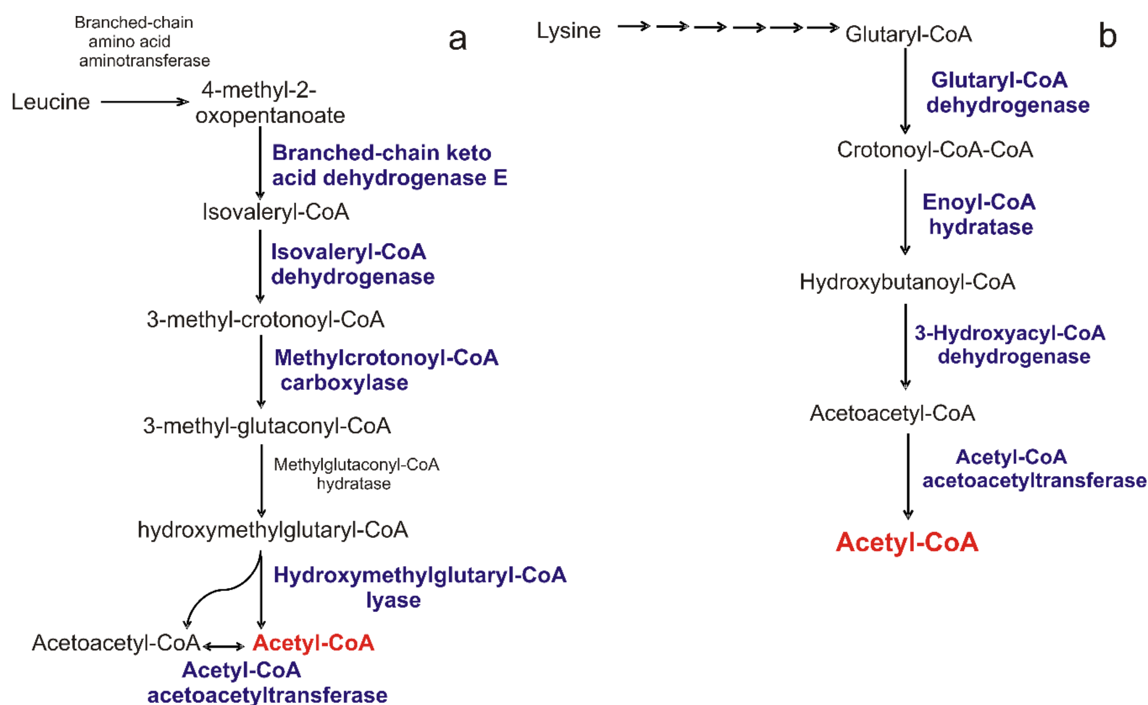

Figure 3. Degradation pathways of amino acids that leads to the synthesis of acetyl-CoA in *P. laurentii.* The additional degradation route for leucine (a) and lysine (b) involves the enzyme (blue color), whose genes were only found in genomes from oleaginous fungi (Vorapreeda et al., 2012).

Two proteins were annotated as malic enzymes in *P. laurentii* UFV-1. These proteins are important enzymes for lipid synthesis. One protein identified as a malic enzyme was predicted to be $NADP^+$ -dependent and the other protein as NAD^+ -dependent. The predicted enzyme $NADP^+$ - dependent has been considered as the primary source for downregulation of triacylglycerol biosynthesis in most of the oleaginous yeasts (Liu et al., 2013; Ratledge, 2014; Zhang et al., 2007). Protein database analyses revealed that they have two domains. Both enzymes have an active domain, responsible for the oxidative decarboxylation of malate to form pyruvate. As a result of the reducing power, NADPH or NADH is produced. On the other hand, *Y. lipolytica* has only one malic enzyme that is NAD^+ dependent.

Considering the oleaginous phenotype of *P. laurentii* UFV-1, we focused on the analysis of genes involved with lipid metabolism regulation. We observed in *P. laurentii* UFV-1 that the gene encoding ICDH-NAD^+ had a constant expression over the period

analyzed, which indicates a post-translational regulation (Figure 5, a). Furthermore, we also verified that the gene encoding ICDH-$NADP^+$ had a constant expression with no transcriptional regulation altering its gene expression (Figure 5, b).

For the enzyme ATP citrate lyase, its transcriptional regulation was evaluated in *P. laurentii* for the first time in our work (Figure 5, c). We verify that the expression of the gene encoding ATP citrate lyase was constant during the period of lipid accumulation. These results emphasize that ATP citrate lyase from *P. laurentii* play a central role in lipid synthesis even in the beginning of lipid accumulation after 8 h. It is important to mention that intracellular concentrations of citrate were neglected (data not shown), since there is an indicative that it was readily converted to acetyl-CoA and oxaloacetate.

We observed a lower expression of *ACC1* gene after 24h than after 6h and 8h (Figure 5, d). This is unexpected result, since lipid content after 24 h was higher than at other periods (data not shown). It is worth mentioning that we observed a reduction on inositol concentration after 24h (Figure 7), which may be a signal of Accase regulation in *P. laurentii,* as shown in the diagram in Figure 3.

The malic enzyme $NADP^+$ - dependent seems to be the main source of NADPH in *P. laurentii* UFV-1, since the expression of the gene encoding this enzyme was significantly higher after 24h. At this same period, there was also a higher lipid content than in the early stage of lipid accumulation after 8h (Figure 5, e). Similarly, there was no statistical differences in the expression among the periods evaluated for glucose-6-phosphate dehydrogenase (Figure 5, f). It is noteworthy that NADPH synthesis in the reaction catalyzed by this enzyme in the oxidative phase of the Pentose Phosphate Pathway has been proposed as the major source in *Y. lipolytica*.

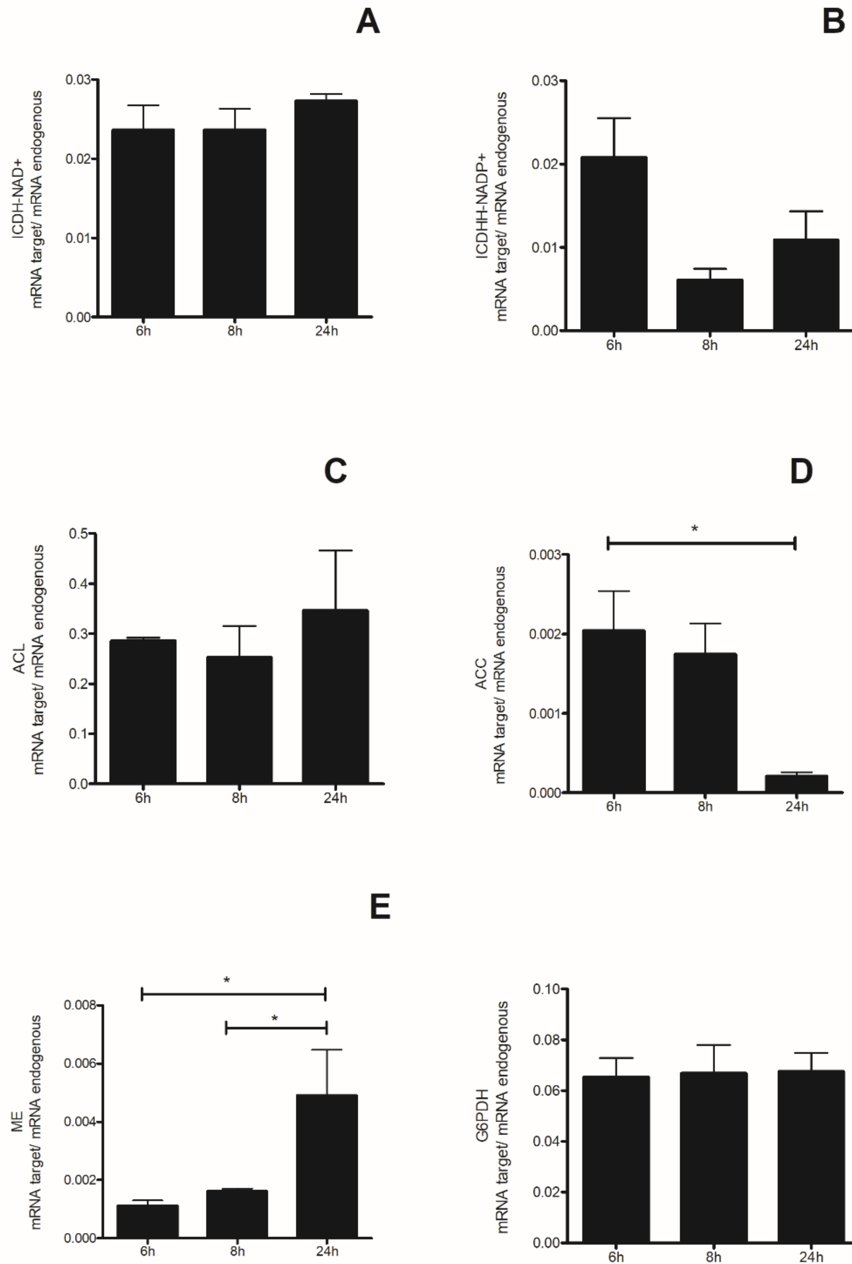

Figure 5. Relative quantification for measuring gene expression in *P. laurentii* UFV-1 using quantitative real time PCR (qRT-PCR). The endogenous genes were the actin and G3PDH genes and the target genes with their expression evaluated were: (a) isocitrate dehydrogenase NAD^+ - dependent, (b) isocitrate dehydrogenase $NADP^+$ - dependent, (c) ATP citrate lyase, (d) Acetyl-CoA carboxylase, (e) malic enzyme $NADP^+$ - dependent and (f) glucose-6-phosphate dehydrogenase.

Metabolic analysis

Lipid accumulation in *P. laurentti* UFV-1 is associated to its growth. In order to gain insights regarding this pattern of lipid accumulation during its growth, we evaluated the metabolic profile comparing samples collected after 6h (control time) and after 24h when there is lipid accumulation (target time). The analysis of Principal Component Analysis (PCA) for the three growth medias containing as carbon sources glucose (G), xylose (X) and glucose and xylose (GX) revealed a clear separation between the results from periods 6 and 24 h (Figure S1), evidencing the great change in this yeast metabolism from the log phase to a high lipid accumulation phase.

It has been reported that regulation of the enzyme ICDH-NAD^+, which is AMP dependent, is a key event for lipid accumulation in oleaginous yeasts (Adrio, 2017). Under nitrogen starvation, the allosteric activator AMP is converted to IMP and NH_4^+. Thus, oleaginous yeasts use NH_4^+ as nitrogen source. As a result of the reaction described above for production of IMP and NH_4^+, the ICDH activity decreases causing citrate accumulation, which is transported from the mitochondria to the cytosol. In order to verify if ICDH-NAD^+, also has a transcriptional regulation, we evaluated its expression in a high ratio of carbon/nitrogen.

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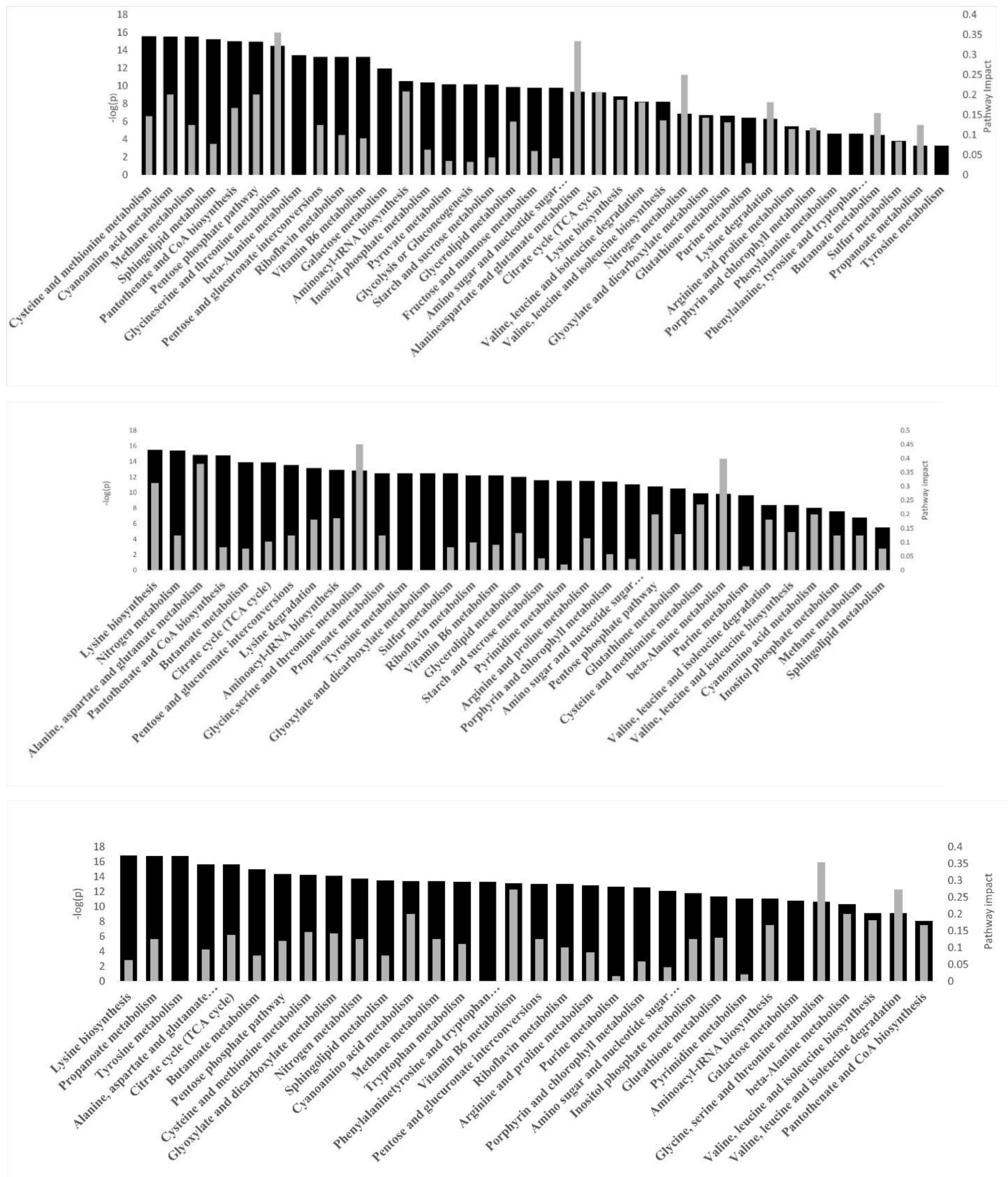

Figure 6. Pathway impact and statistical probabilities for metabolic pathways for *P. laurentii* UFV-1 were statistically affected during lipid accumulation in G (up), X (middle) and G (bottom) growth media. The -log (p) (black) are values of statistical probability found for each metabolic pathway. The Impact Pathway value (gray) indicates about the impact reached in each pathway when comparing the results from the periods of 6h (control time) and 24h (target time), which maximum value is 1.

In *P. laurentii*, the majority of metabolic pathways were affected under nitrogen starvation and were related to nitrogenous compounds such as aminoacyl-RNA synthesis, pyrimidine metabolism, purine metabolism, cyanoamino acid metabolism, amino sugar and nucleotide sugar metabolism (Figure 6), which indicates that amino acid synthesis is reduced. These results are in agreement with the redirection of carbon flux from amino acids to lipids. Similar results were observed in *Y. lipolytica* (Kerkhoven et al., 2016*)*. Lysine degradation leads to acetyl-CoA production and in this study it was observed a decrease in lysine relative concentration in the growth media G and X. Relative concentration of other amino acids such as proline, glycine, valine, leucine and isoleucine were also reduced (Figure 6). Therefore, lysine degradation may provide acetyl-CoA for either lysine *de novo* synthesis or lipid synthesis (Vorapreeda et al., 2012).

The pathways for synthesis and degradation of the amino acids valine, leucine and isoleucine were present in three growth media (G, X and GX). It has been shown that both synthesis and degradation of leucine are related to high lipid accumulation in oleaginous yeasts. In the oleaginous fungi *Mucor circinelloides, leucine metabolism produces acetyl-CoA, which is required for fatty acid synthesis (Rodríguez-Frómeta et al., 2013).* In the engineered strain *Yarrowia lipolityca* grown in a media with limited nitrogen source*,* the upregulated expression of the gene encoding diacylglycerol acyltransferase (DGA1), resulted in lipid accumulation and decrease in leucine synthesis (Kerkhoven et al., 2017). The presence of those pathways is consistent with the results obtained from metabolomic analysis (Figure 7). Concentrations of leucine and lysine after 24h, which is the period of lipid accumulation, were lower than at 6 h, which is the control period with no lipid accumulation. These results indicate that these amino acids are possible sources for the production of Acetyl-CoA.

Metabolic pathways involving Pantothenate and Coenzyme A synthesis, Vitamin B6 and Riboflavin metabolism were also affected by the impact analysis of. Coenzyme A is an essential metabolite for fatty acid synthesis and for confirming this fact, the pantothenic acid concentration was measured by target analysis. The pantothenic acid is the precursor substance for Coenzyme A, an important acyl-carrier (Tehlivets et al., 2007). For *P. laurentti*, the pantothenate concentration was higher after 24 h, which is the period of lipid accumulation (Figure 8, d).

The TCA cycle was affected. Relative concentrations for the metabolites malic acid and succinic acid decreased after 24h (Figure 6). The flux through the TCA cycle in *Y. lipolytica* was approximately 20% lower under nitrogen limited condition than under

nitrogen-rich condition. Under nitrogen limitation, the decrease observed in AMP concentration (Figure 7, a) causes a reduction of the enzyme isocitrate dehydrogenase and subsequently in the concentration of organic acids of the TCA cycle. This finding is consistent with early biochemical studies on the oleaginous fungi *Mortierella alpina* and *Mucor circinelloides*, in which there are found the information about nitrogen limitation leading to a decrease in the enzyme activity from the TCA cycle and in citrate accumulation (Ratledge, C., Wynn, 2002).

We observed in *P. laurentii,* a drop in the concentration of the following three metabolites of the pentose phosphate pathway (PPP) after 24h: ribose-5-phosphate, ribulose-5-phosphate and erytrose-4-phosphate (Figure 8). Probably, this occurs because PPP is not the major source of NADPH in *P. laurentii*. Otherwise, metabolic flux analysis in *Y. lipoltyca* showed that the flux through pentose phosphate pathway was not significantly regulated by nitrogen concentration (Wu et al., 2016). Moreover, in the engineered strain *Y. lipolytica* for overproducing lipids, the PPP had an upregulated metabolism compared to the wide type. Indeed, the PPP is the primary source of NADPH for lipid accumulation in *Y. lipolytica.*

It should be noted that the following pathways were affected after 24h: pathways of sphingolipid metabolism in the growth media containing as carbon sources G, X and GX and pathways of glycerolipid metabolism in growth media G and X. These results are in agreement with the higher synthesis of TAG, which is a neutral lipid.

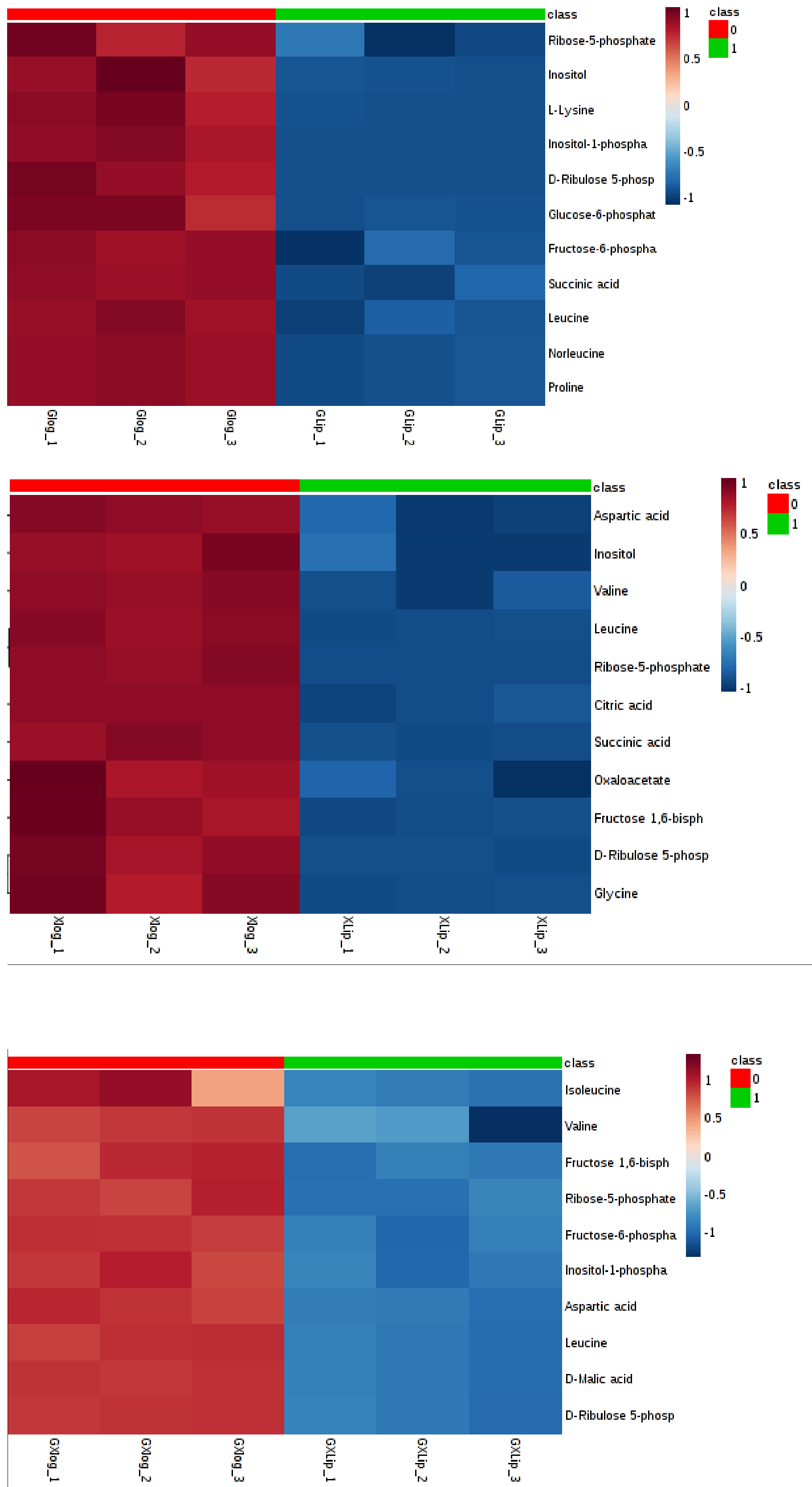

Figure 7. Relative concentrations for metabolites in *P. laurentii* UFV-1 during lipid accumulation in growth media G (up), X (middle) and G (bottom).

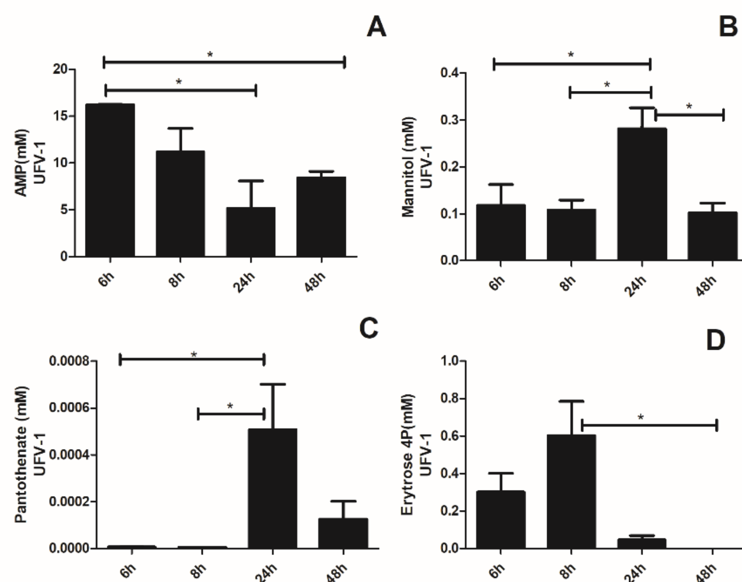

Figure 8. Target analysis of metabolites in *P. laurentii* UFV-1 cultured in growth media containing as carbon source glucose: (a) AMP, (b) mannitol, (c) pantothenate and (d) erytrose-4-phosphate

Similarly, to the constant expression of NAD-dependent isocitrate dehydrogenase enzyme, we observed that AMP concentration reduced after 24h, which is a period with an increase in lipid content (Figure 8, a). It was evidenced that the control of this enzyme occurs at the post translational level. This is one of the major enzymes from the metabolic pathway identified to be affected in the pathway impact analysis (Figure 6).

Mannitol synthesis in *Y. lipolytica* requires NADPH and there is a competition for NADPH use with the fatty acid synthesis (Dulermo et al., 2015). In *P. laurentii*, it was observed an increase in the concentration of lipid and mannitol after 24h, suggesting that mannitol synthesis in *P. laurentii* does not require NADPH.

Conclusions

In summary, this study demonstrated that the genome of *P. laurentii* UFV-1 has relevant characteristics that classifies it as an oleaginous yeast such as: the presence of a gene encoding the malic enzyme $NADP^+$ - dependent and a single copy for the enzyme ATP citrate lyase, which is a key enzyme for oleaginous yeasts; the presence of genes for alternative routes fordegradation of the amino acids leucine and lysine, which may be

alternative sources of acetyl-CoA used during lipid accumulation. As demonstrated for lipid accumulation and metabolism in *P. laurentii,* the malic enzyme $NADP^{+}$-dependent is the main source of NADPH. There is a decrease in the expression of the *ACC1* gene during the lipid accumulation phase. In addition, degradation pathways of the amino acids lysine and leucine contributes to the production of extra acetyl-CoA for high lipids accumulation. The TCA and PPP central metabolic pathways were less active during the lipid accumulation phase. Pathways related to nitrogen metabolism were negatively affected, which is consistent with the redirection of the carbon flux towards fatty acid synthesis.

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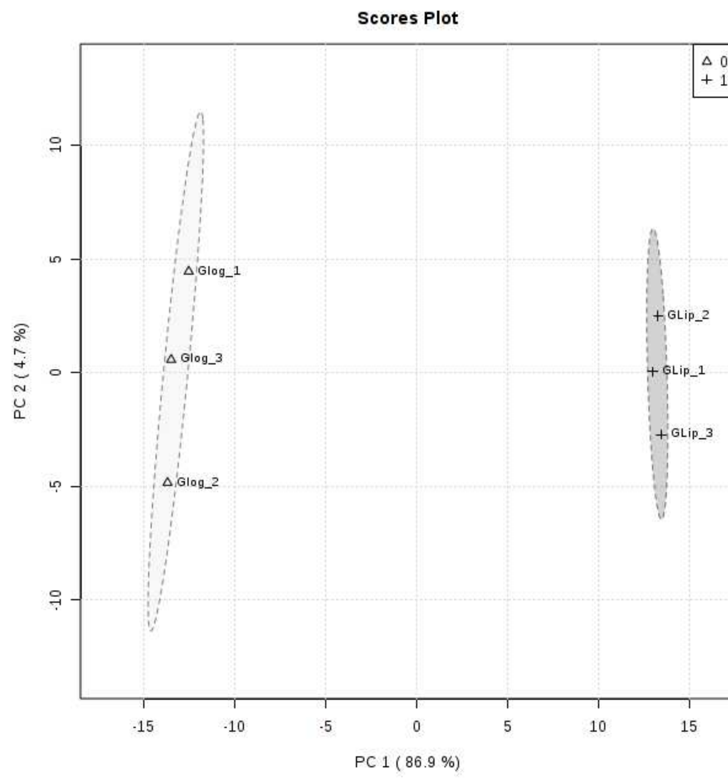
Supplementary data

a



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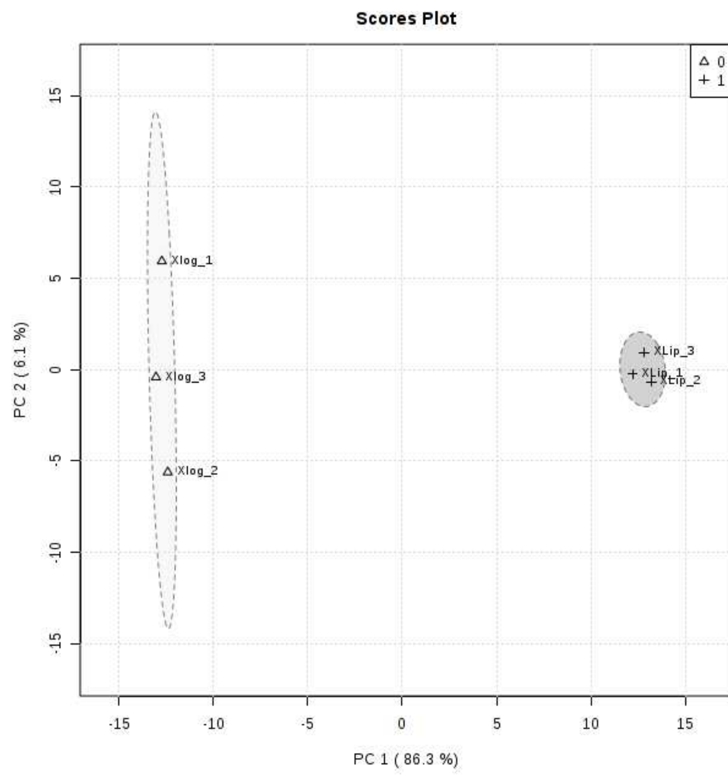

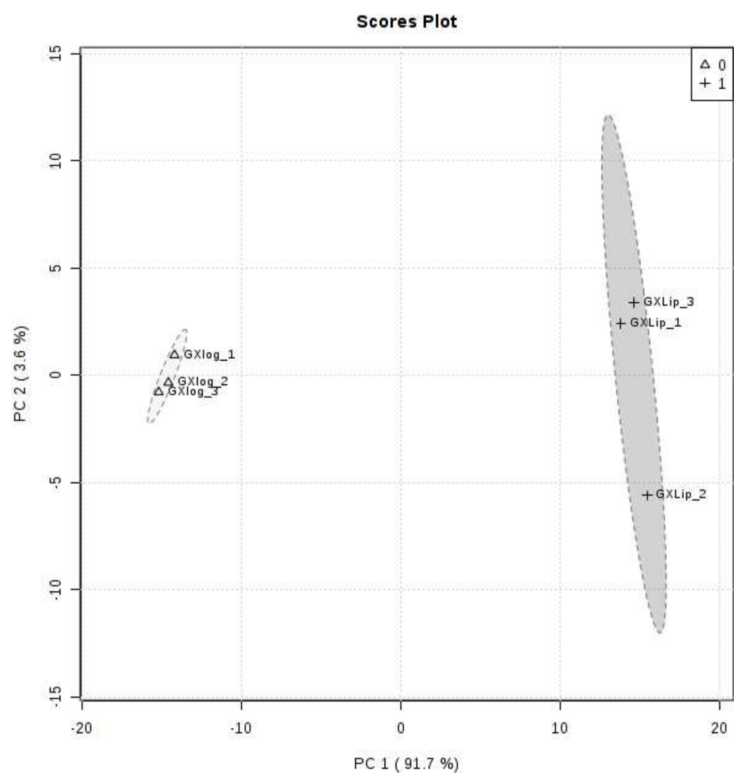

c

Fig. S1. Two-dimensional projections of principal component analyses of metabolites obtained from *P. laurentii* UFV-1 at 6 h (exponencial phase) and 24 h (lipid accumulation) the culture yeast in the following growth media: glucose (G growth media) (a), xylose (X growth media) (b) and a mixture of glucose and xylose (70/30%) (GX growth media) (c).