JOÃO VICTOR MARQUES GONÇALVES ASSIS

IDENTIFICATION AND GENE EXPRESSION ANALYSIS OF GENES ENCODING LACTOSE PERMEASE AND β-GALACTOSIDASE IN THE BASIDIOMYCOTA YEAST *Papiliotrema laurentii*

Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Wendel Batista da Silveira

Co-advisers: Rafaela Zandonade Ventorim
Luciano Gomes Fietto

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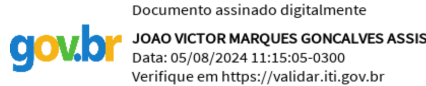

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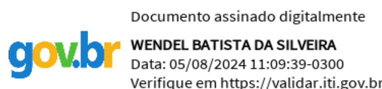

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ABSTRACT

ASSIS, João Marques Gonçalves de, M.Sc. Universidade Federal de Viçosa, February, 2024. **Identification and gene expression analysis of genes encoding lactose permease and β-galactosidase in the Basidiomycota yeast *Papiliotrema laurentii***. Adviser: Wendel Batista da Silveira. Co-advisers: Rafaela Zandonade Ventorim and Luciano Gomes Fietto.

Ricotta whey, an effluent generated by the dairy industry during the ricotta cheese manufacturing, is rich in the disaccharide lactose. Our research group recently isolated from soil the oleaginous yeast *Papiliotrema laurentii* UFV-1, which grows and produces lipids from this effluent, paving the way toward its application in third-generation biodiesel production. Nevertheless, increasing lipid production parameters is pivotal to make the bioprocess feasible. Over the last years, metabolic engineering has contributed to enhance the fermentative performance of yeasts in bioprocesses. However, improving lipid production by *P. laurentii* UFV-1 through metabolic engineering strategies is not a trivial task, as the proteins involved with lactose metabolism are so far unknown. Therefore, the identification of the genes responsible for lactose metabolism in *P. laurentii* UFV-1 is required for applying metabolic engineering strategies to increase lipid production on lactose-rich substrates, such as ricotta whey. Thus, this study aimed to identify and analyze the expression of the genes encoding putative lactose permeases and the β-galactosidase enzymes in *P. laurentii* UFV-1. By using bioinformatics tools, we identified the genes encoding lactose permease (*Papla_5974*) and β-galactosidase (*Papla_5976*). Surprisingly, we also identified a transcription factor gene (*Papla_5975*) between both genes. The three genes are on the same chromosome near each other, constituting a gene cluster. To evaluate the expression of these genes, we cultivated *P. laurentii* UFV-1 in Yeast Nitrogen Base (YNB) medium containing glucose, galactose, or lactose as the sole carbon sources. Besides, we cultivated this yeast in a YNB medium containing two carbon sources: glucose and lactose, and glucose and galactose. We quantified the gene expression via RT-qPCR at different growth stages. The lactose permease gene was induced in the presence of both lactose and galactose; otherwise, the β- galactosidase gene was induced only by lactose. For instance, it is unclear whether the transcriptional factor is involved in the activation of the genes encoding lactose permease and β-galactosidase. Notably, the expression of the transcriptional factor increased in the late stationary phase for glucose and lactose; thus, it may play a role under nutrient-limiting conditions. Furthermore, *P. laurentii* UFV-1 exhibited diauxic growth, which is consistent with the repression of the lactose permease and β-galactosidase genes by glucose in cultivations with glucose plus lactose and glucose plus

galactose. Therefore, we describe for the first time a cluster of genes related to lactose metabolism in a Basidiomycota yeast, opening perspectives to improve lipid production by lactose-based media such as ricotta whey.

Keywords: Ricotta whey. Lactose. Oleaginous yeast. Lipid production. Cluster genes.

RESUMO

ASSIS, João Marques Gonçalves de, M.Sc, Universidade Federal de Viçosa, fevereiro, 2024. **Identificação e análise da expressão de genes que codificam a permease de lactose e β-galactosidase na levedura Basidiomycota *Papiliotrema laurentii***. Orientador: Wendel Batista da Silveira. Co- orientadores: Rafaela Zandonade Ventorim e Luciano Gomes Fietto.

O soro de ricota, efluente gerado pela indústria de laticínios durante a fabricação de ricota, é rico no dissacarídeo lactose. O nosso grupo de pesquisa isolou recentemente a levedura *Papiliotrema laurentii* UFV-1, que é capaz de crescer neste efluente e produzir lipídios, abrindo perspectivas para sua aplicação na produção de biodiesel de terceira geração. No entanto, é crucial aumentar os parâmetros de produção de lipídios para tornar o bioprocesso viável. Nos últimos anos, a engenharia metabólica tem contribuído para melhorar o desempenho fermentativo de leveduras em bioprocessos. No entanto, melhorar a produção de lipídios por *P. laurentii* UFV-1 por meio de estratégias de engenharia metabólica não é trivial, uma vez que as proteínas envolvidas no metabolismo do açúcar lactose são desconhecidas até o momento. Sendo assim, faz-se necessária a identificação dos genes responsáveis pelo metabolismo desse açúcar em *P. laurentii* UFV-1 para aplicar estratégias de engenharia metabólica visando o aumento da produção de lipídios em substratos ricos em lactose, como o soro de ricota. Assim, este estudo teve como objetivo a identificação e análise da expressão dos genes que codificam a permease de lactose e a enzima β-galactosidase em *P. laurentii* UFV-1. Empregando ferramentas de bioinformática, identificamos os genes que codificam a permease de lactose (*Papla_5974*) e a β-galactosidase (*Papla_5976*). Surpreendentemente, foi identificado um gene que codifica um fator de transcrição (*Papla_5975*) entre os genes do metabolismo de lactose descritos acima. Os três genes estão no mesmo cromossomo e próximos entre si, constituindo um *cluster* gênico. Para avaliar a expressão desses genes, *P. laurentii* UFV-1 foi cultivada em meio *Yeast Nitrogen Base* (YNB) contendo glicose, galactose ou lactose como únicas fontes de carbono. Além disso, essa levedura foi cultivada em meio YNB contendo duas fontes de carbono: glicose e galactose, e glicose e lactose. A quantificação da expressão gênica foi realizada via RT-qPCR em diferentes estágios de crescimento. O gene da permease de lactose foi induzido na presença de lactose e galactose, enquanto o gene da β-galactosidase foi induzido apenas por lactose. Ademais, não está claro se o fator transcricional está envolvido na ativação dos genes que codificam a permease de lactose e a β-galactosidase. A expressão do fator transcricional aumentou notavelmente na fase estacionária tardia para os cultivos em glicose ou lactose; assim, ele pode estar envolvido com respostas relacionadas à limitação

nutricional. *P. laurentii* UFV-1 apresentou crescimento diáuxico, condizente com a repressão dos genes da permease de lactose e β-galactosidase por glicose em meios de cultivos contendo glicose com lactose, e glicose com galactose. Assim, foi descrito pela primeira vez um *cluster* de genes relacionados ao metabolismo da lactose em uma levedura Basidiomycota, abrindo perspectivas para melhorar a produção de lipídios em substratos a base de lactose, como o soro de ricota.

Palavras-chave: Soro de ricota. Lactose. Levedura oleaginosa. Produção de lipídeos. Agrupamento gênico.

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INTRODUCTION

Biofuels are alternative energy sources to mitigate the dependence on fossil fuels and reduce their impact on climate change. In 2022, approximately 6.25 million m^3 of biodiesel was produced in Brazil, predominantly (65.8%) using soybean oil as the raw material (ANP, 2023). However, the production of biofuels from food sources (first-generation biofuels) can generate social and economic problems, such as increased food prices and inequality in food access, and excessive use of arable land, water, and fertilizers (KUROWSKA et al., 2020; TOMEI; HELLIWELL, 2016). Therefore, it is crucial to develop and boost alternative technologies, such as second and third-generation biofules, which are based on lignocellulosic materials, and microbial biomass as feedstocks, respectively. In this context, oil produced by microorganisms can be used for third generation biodiesel production (ABIDEEN et al., 2014; AHMAD et al., 2011; LEIVA-CANDIA et al., 2014).

Microalgae can be used as "single-cell oil" (SCO) platforms, but they require high light intensity, controlled temperatures, and high production costs (CHOWDHURY; LOGANATHAN, 2019). Bacteria have a high specific growth rate, but few species accumulate triacylglycerols. Another drawback is that the lipids are found aggregated to their plasma membrane, impairing the extraction process. On the other hand, oleaginous yeasts, which are capable of accumulating at least 20% of their dry mass as lipids, mainly triacylglycerols, display high growth rates and capacity to assimilate carbon sources found in byproducts generated from different industries (ADRIO, 2017; LEIVA-CANDIA et al., 2014). In the context of a circular economy, which focuses on using industrial byproducts as raw materials, oil production by oleaginous yeasts is desirable. Dairy byproducts such as whey, whey permeate, and ricotta whey, which are rich in lactose, are promising feedstocks for developing bioprocesses using lactose-assimilating yeasts. For every kilogram of cheese produced, about nine liters of whey are generated (CARVALHO; PRAZERES; RIVAS, 2013; SISO, 1996). For instance, most of the whey produced worldwide is still not used in bioprocesses (MARWAHA; KENNEDY, 1988; PARASHAR et al., 2016). Thus, prospecting oleaginous yeasts capable of converting lactose into lipids is a promising alternative to combine the treatment of these effluents with the production of a bio-based product of industrial interest (LEONG et al., 2018).

The yeast *Papiliotrema laurentii* UFV-1, isolated from soil, can assimilate different sugars from agro-industrial byproducts and rapidly produce lipids. For example, this yeast accumulated up to 63.5% of lipids in a cultivation with medium containing xylose as a

carbon source (VIEIRA et al., 2020). Lactose comprises about 4.8-5.0% in ricotta whey (CARVALHO; PRAZERES; RIVAS, 2013). Previously, *P. laurentii* UFV-1 presented a lipid titer of 9.3 g/L with a suitable fatty acid profile for biodiesel production when cultivated in ricotta whey (COTRIM, 2021). In another study, the strain *P. laurentii* UCD 68-201 achieved a lipid titer of 5.06 ± 0.28 g/L (CAROTA et al., 2017).

In Fungi, lactose can be assimilated and hydrolyzed inside the cell and/or hydrolyzed in the external environment (DICKSON; BARR, 1983; PARK; DE SANTI; PASTORE, 1979; SONG et al., 2010). In *Kluyveromyces lactis* and *K. marxianus*, lactose is transported via symport by proteins of the Major Facilitator Superfamily (MFS) and Sugar Porter (SP) subfamily (DA SILVEIRA et al., 2019; DONZELLA; SOUSA; MORRISSEY, 2023; VARELA et al., 2019).

In the cytosol, lactose is hydrolyzed by a glycosyl hydrolase (GH) of the GH2 family in yeasts, also known as lactase or β-galactosidase, releasing glucose and galactose (KALATHINATHAN et al., 2023; SAQIB et al., 2017). Glucose is readily assimilated in the glycolytic pathway, while galactose is converted to glucose-6-phosphate in the Leloir pathway (KALATHINATHAN et al., 2023; SELLICK; CAMPBELL; REECE, 2008).

In *K. lactis*, *K. marxianus,* and other ascomycetes that hydrolyze lactose intracellularly, the genes encoding β-galactosidase and lactose permease are located near each other. In yeasts specialized in lactose consumption, these genes are coordinately induced in the presence of lactose/galactose in a regulatory mechanism involving the transcriptional activator Gal4p, the repressor Gal80p, and a bifunctional galactokinase Gal1p, which plays a regulatory role (ANDERS et al., 2006; DA SILVEIRA et al., 2019; FEKETE et al., 2012; SCHAFFRATH; BREUNIG, 2000). In soil Fungi, such as *Trichoderma reesei* and *Aspergillus nidulans*, these genes seem to be related to the coordinated expression of cellulases for degrading oligosaccharides of plant origin (IVANOVA et al., 2013; ZHANG et al., 2019).

Therefore, in order to gain insights into the lactose metabolism and allow future metabolic engineering in *P. laurentii*, we applied herein an *in silico* strategy to identify the genes encoding lactose permease and β-galactosidase. In addition, we evaluated their expression *in vivo* via RT- qPCR under inducing and non-inducing conditions.

LITERATURE REVIEW

Biodiesel

The growing concern about climate change, which is partially driven by fossil fuel use, has boosted biodiesel's utilization to mitigate the pollution caused by petrochemical diesel (HÖÖK; TANG, 2013). Biofuels can be classified based on the feedstock used in their production. First generation-biofuels are produced by fermentation from carbohydrates derived from food crops, such as corn starch or sugarcane sucrose. These biofuels also include biogas and biodiesel. Biodiesel is a product of the transesterification of free fatty acids and triglycerides, yielding alkyl esters and long-chain fatty acids (NAIK et al., 2010; RODIONOVA et al., 2017). In Brazil, biodiesel is predominantly a first-generation biofuel as it is produced from soybean oil (ANP, 2023). From an environmental standpoint, these sources are considered renewable due to the circular carbon chain generated by capturing CO_2 from the atmosphere through fuel combustion (SOMERVILLE, 2007). However, from a sustainability and social security perspective, replacing the fossil fuel production chain with a renewable plant-derived biofuel production route is undesirable. This is due to allocating resources such as water, arable land, and fertilizers for biofuel production rather than directing them toward human and animal consumption (KUROWSKA et al., 2020; TOMEI; HELLIWELL, 2016). Nevertheless, these challenges can be mitigated by employing second and third-generation biofuels.

Second-generation ethanol is based on sources that would be discarded from the food or first- generation ethanol industries, such as lignocellulosic sources, notably sugarcane bagasse and corn straw (RODIONOVA et al., 2017). Second-generation biodiesel uses oil from non-food sources, such as *Croton megalocarpus* and *Cerbera manghas* (KAFUKU; MBARAWA, 2010; ONG et al., 2014). However, there is still a need for land area for planting, which would compete with arable land or contribute to deforestation (HAVLÍK et al., 2011). In third- generation biodiesel, the oil for transesterification is derived from oleaginous microorganisms, mainly yeasts and microalgae. Oleaginous yeasts have stood out due to their capacity to accumulate high amounts of lipids from by-products, integrating the production of a renewable energy source with the use of substrates derived from industrial waste, such as industrial wastewater or whey permeate (CHOWDHURY; LOGANATHAN, 2019; HÖÖK; TANG, 2013; LEDESMA-AMARO; NICAUD, 2016; LEE; LAVOIE, 2013; MILANO et al., 2016).

Ricotta whey

Ricotta is a dairy industry product generated by the precipitation of milk whey proteins through thermal treatment, typically between 80-90 °C, followed by the addition of salts and organic acids (SISO, 1996). The residual fluid from ricotta production, known as ricotta whey, comprises approximately 50 g/L of lactose, with a pH ranging from 3 to 6, protein concentration between 0.5 to 8.0 g/L, nitrogen around 2 g/L, and lipids between 0.5 to 8.0 g/L (CARVALHO; PRAZERES; RIVAS, 2013). Despite the nutrient availability, the quantity of dissolved salts makes it unsuitable for human consumption. Additionally, it exhibits chemical oxygen demand (COD) values between 60 and 80 g/L and biological oxygen demand (BOD) values around 30 g/L. As such, its disposal is highly polluting, potentially leading to water eutrophication and jeopardizing the entire ecosystem where it is discarded. However, its biodegradability, i.e., the ratio of BOD/COD, exceeds 0.1, making its biological degradation possible (CARVALHO; PRAZERES; RIVAS, 2013; VALDEZ CASTILLO et al., 2020).

From a circular economy point of view, effluents such as ricotta whey should be used as feedstock to generate value-added products rather than solely prepared for disposal. Its acidic pH and nutrient availability make it a suitable medium for yeast growth, such as *K. marxianus* for bioethanol production (SANSONETTI et al., 2009; ZOPPELLARI; BARDI, 2013) and can induce the oleaginous phenotype in yeasts like *C. oleaginosus* and *P. laurentii* for lipid production, which can be used as an oil source for biodiesel production (CAROTA et al., 2017; COTRIM, 2021).

Sugar transport in yeasts

Since membrane proteins mediate the absorption and extrusion of various molecules between the extracellular and intracellular environments in yeasts, their activity is a limiting step for their growth (DOES; BISSON, 1989; DONZELLA; SOUSA; MORRISSEY, 2023; NOGUEIRA et al., 2020). Increasing the expression of a sugar transporter can lead to an increase in the growth rate of yeasts but can also result in intoxication due to the accumulation of the substrate in the intracellular medium, causing cell osmotic lysis (LODI; DONNINI, 2005; NIKEREL et al., 2012; POSTMA et al., 1990).

In yeasts, the MFS is the principal family of substrate transporters, including the SP subfamily, which is the main responsible for transporting sugars across the plasma membrane (SAIER, 2006). Sugar transport occurs through facilitated diffusion when the

solute moves along a concentration gradient without the need for energy. Other transporters use a proton gradient to transport the solute coupled to a proton against a concentration gradient, i.e., with energy expenditure (SAIER, 2006). In *S. cerevisiae*, there are also MFS proteins such as Snf3 and Rgt2 that act as sensors of extracellular nutrient concentrations rather than permeases. Such sensors induce the expression of hexose transporter (HXT) genes (OZCAN et al., 1996).

MFS transporters are structurally characterized by a pair of six alpha-helices connected by a hydrophilic loop forming a unit situated in the plasma membrane (LEANDRO; FONSECA; GONÇALVES, 2009). SPs are characterized by five relatively conserved motifs crucial for both the kinetics and specificity of substrates (DE HERTOGH et al., 2006).

Sugar transporters exhibit different levels of promiscuity, thereby having a broad spectrum of affinity for sugars. There are transporters that display a high and low affinity for their substrates (DOES; BISSON, 1989; GOFFRINI; FERRERO; DONNINI, 2002; KRUCKEBERG; BISSON, 1990; WALSH et al., 1994). The hexose transporter Hxt1p has a low affinity for glucose and fructose (REIFENBERGER; BOLES; CIRIACY, 1997). Hxt2p is an example of a high-affinity transporter that is only expressed under low glucose concentration conditions and is degraded in the vacuole under high glucose concentration conditions (KRUCKEBERG; BISSON, 1990; OZCAN et al., 1996). In *K. lactis* and *K. marxianus*, the proton-coupled lactose transporter can also transport cellobiose and galactose (DA SILVEIRA et al., 2019; VARELA et al., 2019). *Cryptococcus neoformans*, a Basidiomycota yeast that can colonize mammal tissues, causing lethal meningoencephalitis, possesses a sugar transporter, Hxs1p, with a high affinity for glucose. This transporter also acts as a glucose sensor (LIU et al., 2013). As such, the activity of transporters relies on environmental conditions and ecological niches (DONZELLA; SOUSA; MORRISSEY, 2023).

Lactose metabolism in Fungi

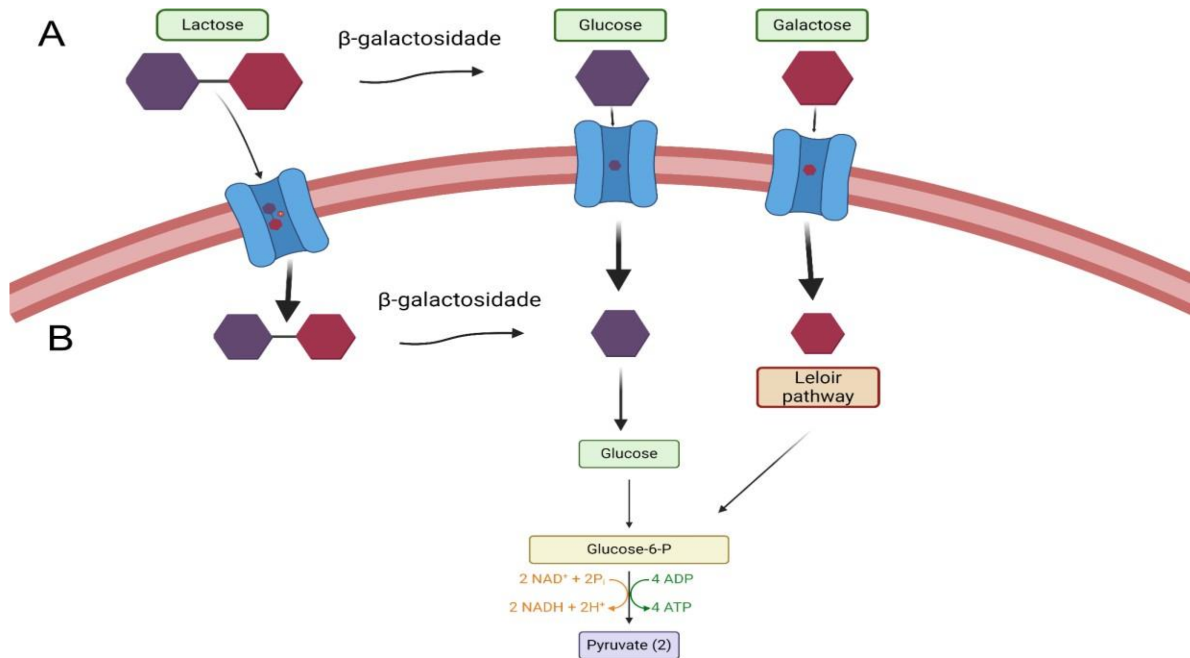
The disaccharide lactose (β-D-galactopyranosyl-(1-4)-α-D-glucopyranose) is the most abundant sugar in milk. This sugar undergoes the action of glycosyl hydrolase (GH), predominantly classified in the GH2, GH35, and GH42 families and known as β-galactosidase or lactase. The enzymes described in bacteria and yeasts are commonly classified in the GH2 family (FEKETE et al., 2012; KALATHINATHAN et al., 2023).

These enzymes catalyze the hydrolysis or transgalactosylation of lactose, generating glucose and galactose or galacto- oligosaccharides, respectively (KALATHINATHAN et al., 2023). The two reactions compete with each other, and lactose concentration is the determinant factor for one reaction's predominance (PANESAR et al., 2018).

Filamentous fungi not related to mammals and commonly isolated from soil and plants, such as *A. nidulans*, *T. reesei*, *Penicillium echinulatum* and *Acremonium cellulolyticus,* can also consume lactose For these fungi, lactose metabolism seems to be related to a coordinated response to common sugars in the soil, as their growth on lactose-based media also induces the production of various enzymes related to the consumption of oligosaccharides of plant origin (FANG et al., 2008; FEKETE et al., 2012; SEHNEM et al., 2006; SEIBOTH; HEROLD; KUBICEK, 2012). On the other hand, some yeasts specialized in metabolizing lactose, such as *K. lactis*, *K. marxianus*, and *Candida pseudotropicalis*, have been isolated from dairy products (INCHAURRONDO; YAUTORNO; VOGET, 1994; VARELA et al., 2019).

Fungi use lactose in three distinct ways: (i) β-galactosidase is secreted, and hydrolysis occurs in the extracellular medium, followed by the uptake of the resulting monomers; (ii) lactose is imported to the cytosol by an MSF-type sugar transporter, then hydrolyzed through a GH; (iii) both external and internal hydrolysis occur (DICKSON; BARR, 1983; PARK; DE SANTI; PASTORE, 1979; SONG et al., 2010). Overall, the secreted β-galactosidase has an optimal acidic pH due to acidification caused by fungi, while intracellular β-galactosidase has an optimal pH close to 7 (FEKETE et al., 2012; KALATHINATHAN et al., 2023). The resulting glucose enters the glycolytic pathway, whilst galactose is converted to glucose-6-phosphate through the Leloir pathway (Fig 1) (SELLICK; CAMPBELL; REECE, 2008).

Figure 1. **Schematic representation of lactose consumption and hydrolysis in fungi**. A: Lactose is hydrolyzed by extracellular β-galactosidase to glucose and galactose (monosaccharides), which are transported by a transmembrane protein. B: Lactose transported is hydrolyzed by an intracellular β- galactosidase to glucose and galactose. The galactose is converted into glucose-6-phosphate by the Leloir pathway to be used in central metabolism.

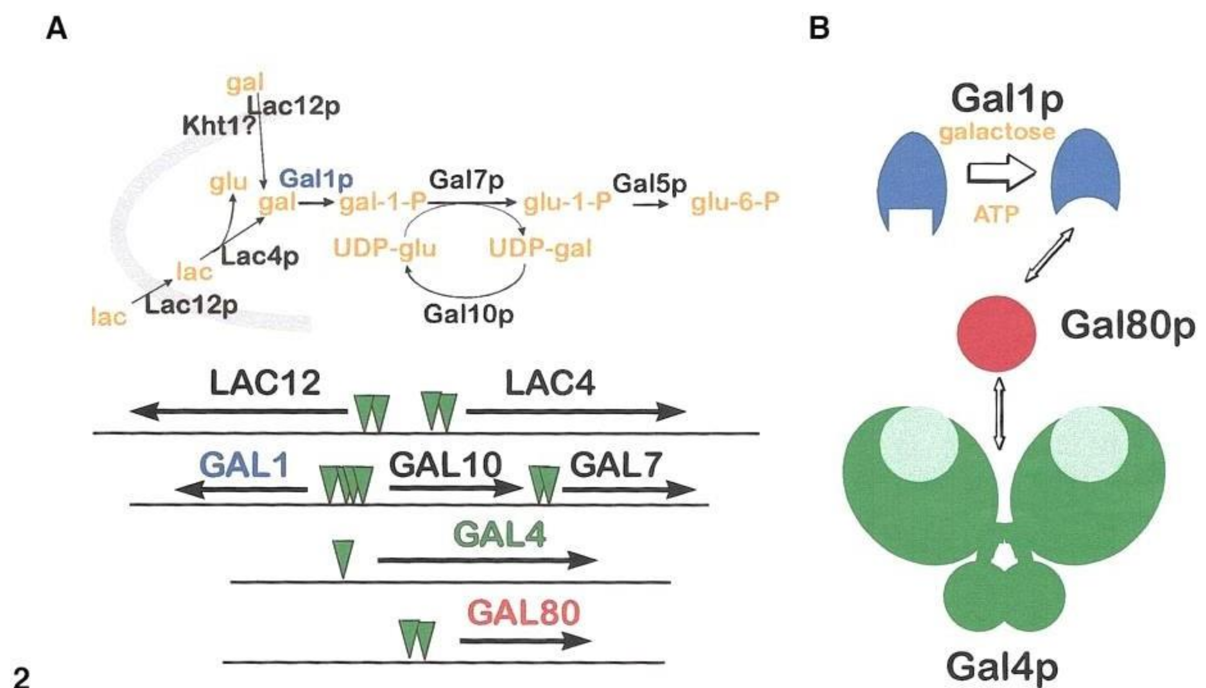


Notably, genes responsible for lactose utilization in yeasts exhibit synteny. FEKET et al., (2012) demonstrated that in 15 ascomycetes, the β-galactosidase gene was close to a gene encoding an MFS protein putatively identified as a lactose permease. In fungi, it is common to find genes responsible for the metabolism of alternative carbon sources or specific synthesis pathways genetically linked, unlike other eukaryotes. This may facilitate regulatory mechanisms and prevent the accumulation of toxic intermediates in the cell if only one gene of the pathway is lost after a deletion and/or mutation event (ROKAS; WISECAVER; LIND, 2018).

The lactose/galactose cluster in *K. lactis*, which is specialized for lactose and galactose metabolism, also ensures cellobiose consumption. The lactose permease (Lac12p) in this yeast can transport both lactose, cellobiose, and galactose (VARELA et al., 2019). This transporter is expressed in coordination with β-galactosidase (Lac4p) and the other genes responsible for galactose metabolism. The regulation of the cluster is governed by the proteins Gal4p (transcriptional activator), Gal80p (repressor protein), and the enzyme galactokinase Gal1p, which plays both catalytic and regulatory roles. Gal4p forms a homodimer and binds to the regulatory regions of *LAC/GAL* genes, including itself, which

characterizes an autoregulation mechanism (Figure 2A). In the absence of galactose or lactose, the Gal80p binds to Gal4p, sterically blocking its interaction with the transcription machinery (Fig 2B). Therefore, the induction of these genes requires alleviating the repression caused by Gal80p, which relies on the intracellular availability of galactose. The increase of galactose concentration takes place via intracellular hydrolysis of lactose, or galactose uptake. The protein responsible for releasing the inhibitory complex is Gal1p, which binds to Gal80p in the nucleus, forming a tetramer (Figures 2A, B) (ANDERS et al., 2006; SCHAFFRATH; BREUNIG, 2000).

Figure 2. **Genes responsible for lactose/galactose metabolism in *K. lactis***. A: Lactose/galactose assimilation pathway; Leloir pathway; Schematic representation of regulatory sites in lactose/galactose metabolism genes. B: Schematic representation of the interaction between regulatory proteins in *K. lactis*. Source: (SCHAFFRATH; BREUNIG, 2000).



Recently, a new unique cluster was discovered in the *C. intermedia* genome. This cluster contains a duplicated transcriptional factor (*LAC9*) co-localized with the genes *GAL1*, *GAL10* (required for galactose metabolism) and *XYL1*, which encodes an aldose reductase. This new cluster is pivotal for yeast growth on lactose/galactose-based media (PERI et al., 2023). Even though lactose metabolism is common in Basidiomycota, the regulatory mechanisms underlying this cluster remain elusive.

Oleaginous yeasts and lipid accumulation

Yeasts capable of accumulating at least 20% of their dry mass in lipids are considered oleaginous (SALVADOR LÓPEZ; VANDEPUTTE; VAN BOGAERT, 2022). Overall, under high carbon-to-nitrogen (C/N) ratio conditions, the oleaginous phenotype is induced in these yeasts, leading to lipid accumulation, mainly triacylglycerols (VASCONCELOS et al., 2019). The oleaginous phenotype is distributed across the Fungi kingdom in different clades, including both Basidiomycota, such as *Rhodotorula toruloides* and *Papiliotrema laurentii* (ALMEIDA et al., 2023; ZHANG et al., 2021), and Ascomycota, such as *Yarrowia lipolytica* (GROENEWALD et al., 2014).

The oleaginous phenotype occurs under nitrogen-limiting conditions, in which the concentration of adenosine monophosphate (AMP) decreases due to the activity of AMP deaminase, leading to the inhibition of the enzyme isocitrate dehydrogenase (ICDH), which is AMP-dependent. Then, the conversion of isocitrate to α-ketoglutarate decreases, resulting in an excess of citrate in the mitochondria. The citrate is then exported to the cytosol where it is converted into acetyl-CoA and oxaloacetate by the enzyme ATP-citrate lyase, a key enzyme for lipid accumulation in oleaginous yeasts. Acetyl-CoA is carboxylated to malonyl-CoA by the enzyme acetyl-CoA carboxylase, which is considered the rate-limiting step of fatty acid synthesis. Malonyl-CoA is the building block for fatty acid synthesis by the fatty acid synthase complex. The products of this complex are transported to the endoplasmic reticulum, where they will be used to produce triacylglycerols (ADRIO, 2017; DE ALMEIDA et al., 2022).

In a circular economy context, the industrial process of lipid production by yeasts relies on their ability to use industrial byproducts such as whey, whey permeate, lignocellulosic hydrolysates, and glycerol. Their capacity to withstand the inhibitors found in these by-products is also important (ABELN; CHUCK, 2021).

The first attempt to develop a bioprocess of lipid production in whey was with the yeast *Cutaneotrichosporon oleaginosus* aiming at a substitute for cocoa butter. However, using this yeast on an industrial scale became unfeasible due to the fall in the price of cocoa butter (ABELN; CHUCK, 2021). This yeast is still the most widely used for this purpose because it can produce lipids at 1 g/(L h) (YKEMA et al., 1988) from whey permeate. This yeast can consume oligosaccharides (SCHULZE et al., 2014), xylose (YU et al., 2014b) and lactose (DAVIES; HOLDSWORTH; READER, 1990), as well as displays tolerance to

growth inhibitors (YU et al., 2014a). In addition, *C. oleaginous* can secret lipids when grown in acetate (HUANG et al., 2018).

Yarrowia lipolytica has been extensively studied for lipid production in recent decades (NICAUD, 2012). It is usually isolated in environments rich in fat and oil. It is, therefore, cultivated in glycerol for lipid production (WORKMAN; HOLT; THYKAER, 2013). Another advantage is the wide availability of genetic tools for metabolic engineering purposes. On the other hand, wild isolates of this yeast do not grow in culture media containing xylose (QUARTERMAN et al., 2017) or cellulose (GUO et al., 2017), a major drawback for its application in lignocellulosic biorefineries.

Papiliotrema laurentii

Previously classified as *Cryptococcus laurentii, P. laurentii* is a dimorphic, encapsulated yeast with a saprophytic lifestyle. This yeast can assimilate a broad spectrum of carbon sources such as pentoses, hexoses, and oligosaccharides, as well as unconventional carbon sources like biodiesel, polyester with polyurethane, and polyethylene succinate (DE ALMEIDA et al., 2022). Additionally, *P. laurentii* exhibits certain tolerance to inhibitors commonly found in hydrolysates of lignocellulosic materials such as hydroxymethylfurfural; however, its growth is inhibited by acetic acid, which is also present in these hydrolysates (ALMEIDA et al., 2023).

Our research team isolated the strain *P. laurentii* UFV-1 from the soil samples collected. This yeast accumulated up to 63.5% of its biomass as lipids when cultivated under optimal conditions in a culture medium containing xylose within 48 h (VIEIRA et al., 2020). Furthermore, our research team reconstructed the first genome-scale metabolic model of *P. laurentii*, providing valuable insights into metabolism to optimize culture media and guide metabolic and genetic engineering efforts (VENTORIM et al., 2022). A recent laboratory adaptive evolution aimed to improve acetic acid tolerance, an inhibitor present in lignocellulosic hydrolysates. The best strain, ATS I, tolerated a concentration of 20 g/L of acetic acid, while the parental strain did not grow under the same condition (ALMEIDA et al., 2023).

P. laurentii UFV-1 also displays the potential to produce high amounts of lipids from lactose- based media. When it was cultivated in ricotta whey under optimized conditions, that is, initial OD_{600} 1.0, pH equal to 5, ratio of medium volume/flask volume 50/500, this yeast consumed 98.88% of the lactose present in the medium, recording a lipid titer of 9.3 (g/L).

Its fatty acid profile is suitable for biodiesel production (COTRIM, 2021).

HYPOTHESIS

The genes associated with the lactose metabolism in *Papiliotrema laurentii* can be identified by combining *in silico* analyses and *in vivo* gene expression.

MAIN OBJECTIVE

Identify and analyze the expression of genes encoding putative lactose permeases and β- galactosidase enzymes in *Papiliotrema laurentii*.

SPECIFIC OBJECTIVES

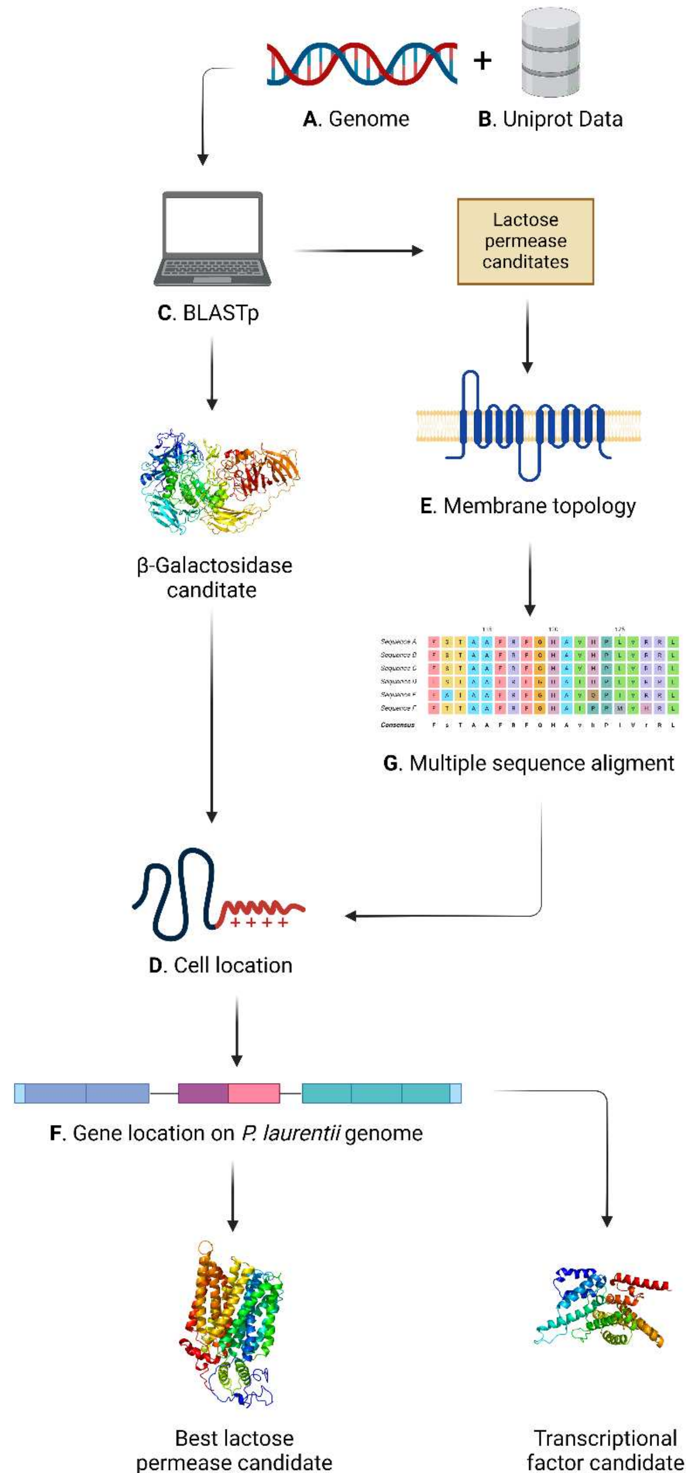
- Identify the genes encoding proteins responsible for lactose uptake and hydrolysis in
  Papiliotrema laurentii;
- Evaluate the expression of genes encoding putative proteins involved in lactose metabolism throughout cultivation in different carbon sources (glucose, galactose, or lactose);
- Evaluate the expression of genes encoding putative proteins involved in lactose metabolism in culture media containing two carbon sources: glucose and lactose, and glucose and galactose.

MATERIAL AND METHODS

Identification of putative genes involved in both uptake and hydrolysis of lactose

Initially, we retrieved protein sequences deposited in UniProt (THE UNIPROT CONSORTIUM et al., 2023) as "lactose permease" and "β-galactosidase" for the Fungi kingdom and performed a BLASTp against the *Papiliotrema laurentii* UFV-1 predicted proteome (ALMEIDA et al., 2023). We used DeepLoc 2 (THUMULURI et al., 2022) to predict the cellular localization of the candidate genes. In addition, we used CCTOP (DOBSON; REMÉNYI; TUSNÁDY, 2015) to predict the membrane topology of lactose permease candidates and verify if they belong to MFS. Next, we carried out the alignment using MUSCLE (EDGAR, 2004) within the software MEGA X (KUMAR et al., 2018) to identify conserved domains in the amino acid sequences of lactose permease candidates (LEANDRO; FONSECA; GONÇALVES, 2009). We screened for [RK]XGR[RK], RX3-GX3-GX6- PXYX2-E-X6-RGX6-QX5-G, PESPRXL, [LI]-Q-X2-Q-Q-X-[ST]-[GN]-

Figure 3. Summary of the *in silico* strategy adopted to identify the genes encoding proteins related to both lactose uptake and hydrolysis. A: Protein selection from gene annotation; B: Creation of a database for target proteins; C: BLASTp search for best hits on the genome. D: Prediction of the cell location for the best β-galactosidase candidate; E: Prediction of the membrane topology for MFS; F: Alignment for characterized MFS as SP. G: Search for β-galactosidase locus and near genes. The 3D structures of *Papla_5976*, *Papla_5974*, and *Papla_5975* were predicted using Phyre2.



X3-Y-Y-F, and PETKGXXXE conservative domains that are normally found in protein sequences described as a Sugar Porter (SP) (LEANDRO; FONSECA; GONÇALVES, 2009). To select the best lactose permease candidate, we searched within the genome for the β-galactosidase gene and subsequently identified the co-localized MFS candidate using the Artemis software (CARVER et al., 2012). Finally, we predicted the functional domains of the two closest genes of β- galactosidase using InterPro (HUNTER et al., 2009). A summary of the *in silico* approaches is presented in Figure 3.

Yeast strain

Papiliotrema laurentii UFV-1 belongs to the culture collection of the Microbial Physiology Laboratory of the Microbiology Department at Universidade Federal de Viçosa. Yeast cultures were stored in cryogenic tubes frozen at -80 °C in YP medium [1% (w/v) yeast extract, 1% (w/v) peptone] supplemented with 30% (w/v) glycerol.

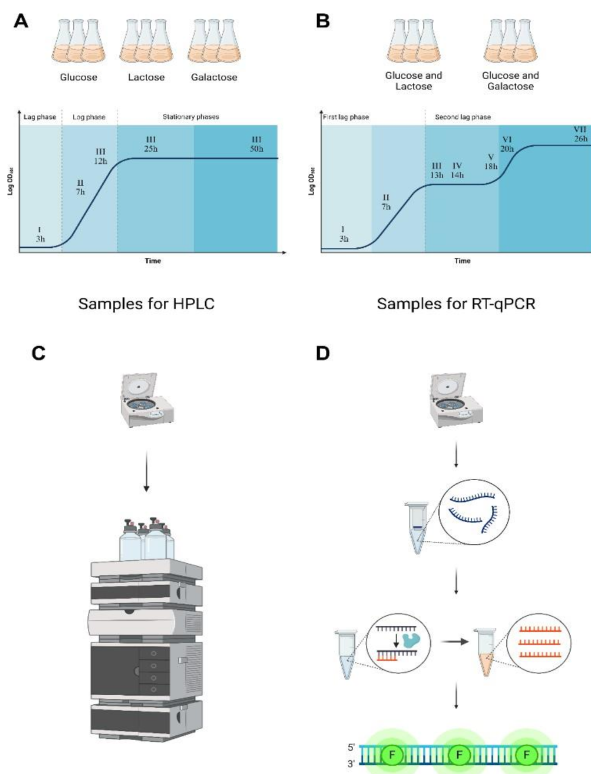
Cultivation conditions

We used a single colony of *P. laurentii* UFV-1, previously cultivated on YPD agar during 72 h at 30 °C, to prepare the pre-inoculum in Yeast Nitrogen Base medium (YNB medium) 6.7 g/L without amino acids and with ammonium sulfate and glucose 10 g/L, as the sole carbon source. Yeast cultures were cultivated in 50 mL of culture media in 250 mL Erlenmeyer flasks with a stirring rate of 200 rpm at 30 °C for 16 h. Next, we centrifuged the yeast cells at 5,000 *g* for 15 min at 4 °C. Then, we washed the pellet twice with a saline solution (NaCl 8,5 g/L). The cultivation started with an optical density at 600 nm (OD_{600}) of about 0.1. Initially, we cultivated

P. laurentii UFV-1 in YNB media containing glucose, galactose, or lactose at 5.0 g/L as carbon sources in 500 mL Erlenmeyer flasks containing 200 mL of medium with a stirring rate of 200 rpm at 30 °C in triplicates. We collected samples of 1 mL every hour until 12 h, then at 26 and 50 h (Fig 4A). Subsequently, we cultivated *P. laurentii* UFV-1 in YNB media containing two carbon sources: glucose (2.5 g/L) plus galactose (2.5 g/L) and glucose (2.5 g/L) plus lactose (2.5 g/L) in the same conditions described for sole carbon sources. We collected samples of 1 mL every hour until 20 h, then at 26 h (Fig 4B) to determine the sugar concentration by High- Performance Liquid Chromatography (HPLC) (Fig 4C) and growth by the OD_{600}. For the qPCR analysis, we collected samples of 1 mL from cultivations carried out in YNB media with only one carbon source at the following period in h: 3 (lag phase), 7 (exponential phase), 12 (late exponential phase), 25 (early stationary), and 50 (late

stationary). For cultivations in YNB media containing two carbon sources, we collected samples at 3 (lag phase), 7 (first exponential phase), 13 and 14 (transition phase), 18, 20, and 26 h (second growth phase) (Figure 4D). We immediately frozen samples in liquid nitrogen and stored at -80 °C until RNA extraction. A summary of the *in vivo* methodologies used in this work is depicted in Figure 4.

Figure 4. **Summary of the experimental design to evaluate the growth parameters and gene expression**. A: Cultivation in minimal medium (YNB) containing glucose, lactose, or galactose as the sole carbon sources in triplicate. The samples for sugar quantification and optical density measure were collected each hour until 12 h; next, the samples were collected at 25 and 50 h. The samples for measuring the gene expression were collected in 3, 7, 12, 25 and 50 h, representing the lag, exponential, late exponential, early stationary and late stationary phases. B: Cultivation in minimal medium (YNB) with two carbon sources: glucose plus lactose and glucose plus galactose, were also conducted in triplicate. The samples for sugar quantification and optical density measure were collected each hour until 20 h; afterward, the sample was collected in 26 h. The samples for measuring the gene expression were collected in 3, 7, 13, 14, 18, 20 and 26 h. C: All samples collected for sugar consumption were centrifuged, and the supernatant was recovered for sugar consumption analysis by HPLC. D: All samples collected for measuring the gene expression were frozen in liquid nitrogen, after that, the samples were treated with DNAse, followed by reverse transcription and quantification by quantitative PCR.



Sugar quantification

To determine the sugar consumption during the cultivation, we first centrifuged the samples at 12,000 *g*/4 °C for 10 min. Then, we filtered (0.22 µm) the supernatant and determined the concentration of lactose, glucose, or galactose using HPLC: LC-20AT (Shimadzu, Japan) - coupled to the RID-20A refractive index detector (Shimadzu, Japan) and an Aminex HPX-87H ion exchange column (300 x 7.8mm, 9µm, Bio-Rad, Munich, Germany); mobile phase: 5 mM H_2SO_4 with a 0.7 mL/min flow rate. We calculated the carbon source concentrations using glucose, galactose, or lactose as external standards ranging from 0.04 to 7 g/L.

Determination of growth parameters

We determined the specific growth rate (µ) in the exponential growth phase by linear regression between the Ln of OD_{600} values and time. We calculated the Biomass Yield ($Y_{x/s}$) using the linear regression for biomass concentration (x – g/L) against the sugar concentration (s – g/L). The specific sugar consumption rates *q* (g/g DW h) is represented as the µ by $Y_{x/s}$.

RNA extraction, reverse transcription, and quantitative polymerase chain reaction (qPCR)

First, we ground the samples collected from each cultivation with liquid nitrogen using pistils for microtubes. Then, we extracted the total RNA using the TRI Reagent® (Sigma-Aldrich) following the manufacturer's instructions. We assessed the quality and quantity of RNA by NanoDrop (Thermo Fisher Scientific Inc.). We assessed RNA integrity by 1% (w/w) agarose gel electrophoresis.

For reverse transcription, we added DNase I (Sigma-Aldrich) for 2 h min at 25 °C to total RNA samples, then stopped the reaction using 1 µL of RQ1 DNase Stop Solution (Sigma-Aldrich) for 10 min at 65 °C. We verified the absence of DNA using PCR with a primer for the constitutive gene encoding β-actin in *P. laurentii* UFV-1 (Table 1) followed by agarose gel electrophoresis 1.5% (w/w). Next, we used the ImProm-II™ Reverse Transcription System (Promega) for reverse transcription. We also verified the presence of cDNA using PCR with a glyceraldehyde-3-phosphate dehydrogenase, as described above (Table 1). We analyzed the gene expression using the qPCR StepOnePlus™ (Thermo Fisher Scientific) and Power SYBR Green Master Mix (Thermo Fisher Scientific). We used the

standard curve method followed by normalization using a housekeeping endogenous gene, β-actin, to normalize the expression of the putative genes encoding β-galactosidase (*Papla_5976*), lactose permease (*Papla_5974*), and transcriptional factor (*Papla_5975*). Besides the normalization using β-actin and standard curve, the fluorescence values were normalized considering the samples extracted in the lag phase of cultivations in i) medium containing glucose as the sole carbon source or ii) medium containing glucose and galactose as carbon sources. We used these fluorescence values as references for relative expression.

Table 1. Oligonucleotides used for amplification.

Gene	Sequence	TM (°C)	Reference
β-Actin	5′-GTGTCCCGAGGCTCTCTTCC-3′ 3′-CCTGATGTCCAGGTCGCACT-5′	59.01 58.93	(VIEIRA et al., 2020)
Glyceraldehyde -3-phosphate dehydrogenase	5′-GTTCGTCTGCGGTGTCAACC-3′ 3′-CGTGGACGGTGGTCATGAGA-5′	58.98 58.94	(VIEIRA et al., 2020)
Papla_5974	5’-CACGATTGCTCAGTTCACCG-3’ 3’-ACTTGACACCTACCAAGCCA-5’	59.56 59.24	This study
Papla_5975	5'-GCAGAAACAGGTGTACGAG -3' 3’-GATAAGAAGCCGCACCTAC-5’	59.6 59.6	This study
Papla_5976	5’-TACACTGCCACCGAAGACTC-3’ 3’-CACAGCCTCACTCTAACCCA-5’	59.4 59.39	This study

RESULTS

Prediction of genes related to lactose metabolism

We were able to predict the putative gene encoding β-galactosidase on the genome of *P. laurentii* UFV-1 using BLASTp. The most promising gene (*Papla_5976*) had a unique copy in the entire genome. Additionally, we used BLASTp to search putative genes encoding lactose permease. We selected a total of 10 candidates (Table 2).

Table 2. Top 10 lactose permease best hits from *P. laurentii* UFV-1 vs. Uni-Prot lactose permease.

Protein	Hits	Predicted function
Papla_1298	238	Sugar Porter
Papla_147	511	Sugar Porter
Papla_2941	421	Sugar Porter
Papla_3771	537	Sugar Porter
Papla_4959	83	Sugar Porter
Papla_5647	238	Sugar Porter
Papla_5974	622	Sugar Porter
Papla_6447	660	Sugar Porter
Papla_7330	474	Sugar Porter
Papla_7626	814	Sugar Porter
Papla_764	1	Sugar Porter

In order to characterize these candidates, we used CCTOP to predict the transmembrane domains. All candidates display 12 transmembrane domains with cytosolic C-terminal and N- terminal ends (Fig 5), indicating that they belong to the MFS family (SAIER, 2006). We also aligned their amino acid sequences to search for the typical conserved domains of the SP subfamily (Fig 6). We found the following domains: [RK]XGR[RK] (Fig 6A); RX3-GX3- GX6-PXYX2-E-X6-RGX6-QX5-G (Fig 6B), PESPRXL (Fig 6C), [LI]-Q-X_2-Q-Q-X-[ST]-[GN]-X_3-Y-Y-F (Fig 6D), and PETKGXXXE (Fig 6E). As such, we characterized the 10 MFS candidates as SPs (LEANDRO; FONSECA; GONÇALVES, 2009).

Figure 5. **Topology of membrane for lactose permease candidates**.

Transmembrane Inside Outside Re-entrant loop

Papla_4959
Papla_2941
Papla_147
Papla_3771
Papla_1298
Papla_7330
Papla_5647
Papla_7626
Papla_6447
Papla_5974

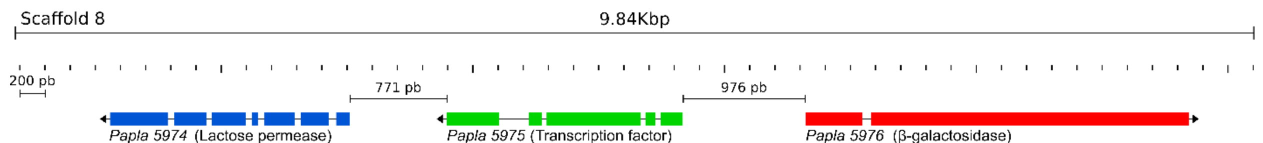
Figure 6. **Characterization of MSF as SP**. Top: Illustration of the position of the domains in the proteins represented below. A: Domain: RXGRR; B Domain: RX3-GX3-GX6-PXYX2-E-X6-RGX6-QX5-G; C: Domain: PESPRXL; D: Domain: [LI]-Q-X_2-Q-Q-X-[ST]-[GN]-X_3-Y-Y-F; E: Domain: PETKGXXXE.

Outside
Cytoplasm
N-Terminus
C-Terminus

Papla_4959
Papla_2941
Papla_147
Papla_3771
Papla_1298
Papla_7330
Papla_5647
Papla_7626
Papla_6447
Papla_5974
LAC12 K. lactis
LAC12 K. marxianus
HXT1 S. cerevisiae
HXT2 S. cerevisiae
Consensus

Since it is not possible to identify lactose permeases based only on sequence and structure of SPs, we searched for such transporters closest to the β-galactosidase gene (*Papla_5976*) on the *P. laurentii* UFV-1 genome. Our strategy was based on the fact that in fungi such as *K. lactis, K. marxianus,* and *A. nidulans* the genes encoding lactose permease and β-galactosidase are clustered (DICKSON; BARR, 1983; FEKETE et al., 2012)**.** The closest MFS gene to the β- galactosidase gene (*Papla_5976)* was *Papla_5974*, one of the 10 candidates predicted in the BLASTp search (Table 2); thus, it was selected as the promising candidate for lactose transporter. Interestingly, we found the gene *Papla_5975* between the genes *Papla_5976* and *Papla_5974*, with physical distances equal to 976 bp and 771 bp, respectively (Fig 7). Surprisingly, we identified this gene as a possible transcriptional factor due to its Zinc finger domain (InterPro entry: IPR007219). The topology of the cluster comprising these three genes is depicted in Figure 7. Subsequently, we predicted the cellular location of the three proteins using DeepLoc 2. The β-galactosidase candidate was predicted to be located in the cytosol, the lactose permease candidate in the cell membrane, and the transcriptional factor candidate in the nucleus.

Figure 7. **Location of the genes related to lactose metabolism in the genome of the yeast *Papiliotrema laurentii* UFV-1.** The genes are located on *Scaffold 8*.



Analysis of expression patterns of putative genes encoding proteins involved in the lactose metabolism

In order to evaluate the expression of the putative genes encoding lactose permease (*Papla_5974*), β-galactosidase (*Papla_5976*) and transcriptional factor (*Papla_5975*), we cultivated *P. laurentii* UFV-1 for 50 h in minimal media (YNB) containing lactose, galactose or glucose as the sole carbon sources. *P. laurentii* UFV-1 presented the highest specific growth rate in glucose, followed by lactose and galactose, and the lowest biomass yield for galactose and lactose (Table 3). For the specific sugar consumption rates, *P. laurentii* UFV-1 had the lowest value for the cultivation with glucose. In addition, we did not observe statistical differences between the cultivations with lactose and galactose (Table 3).

Table 3 - Physiological parameters for cultivation in minimal medium.*

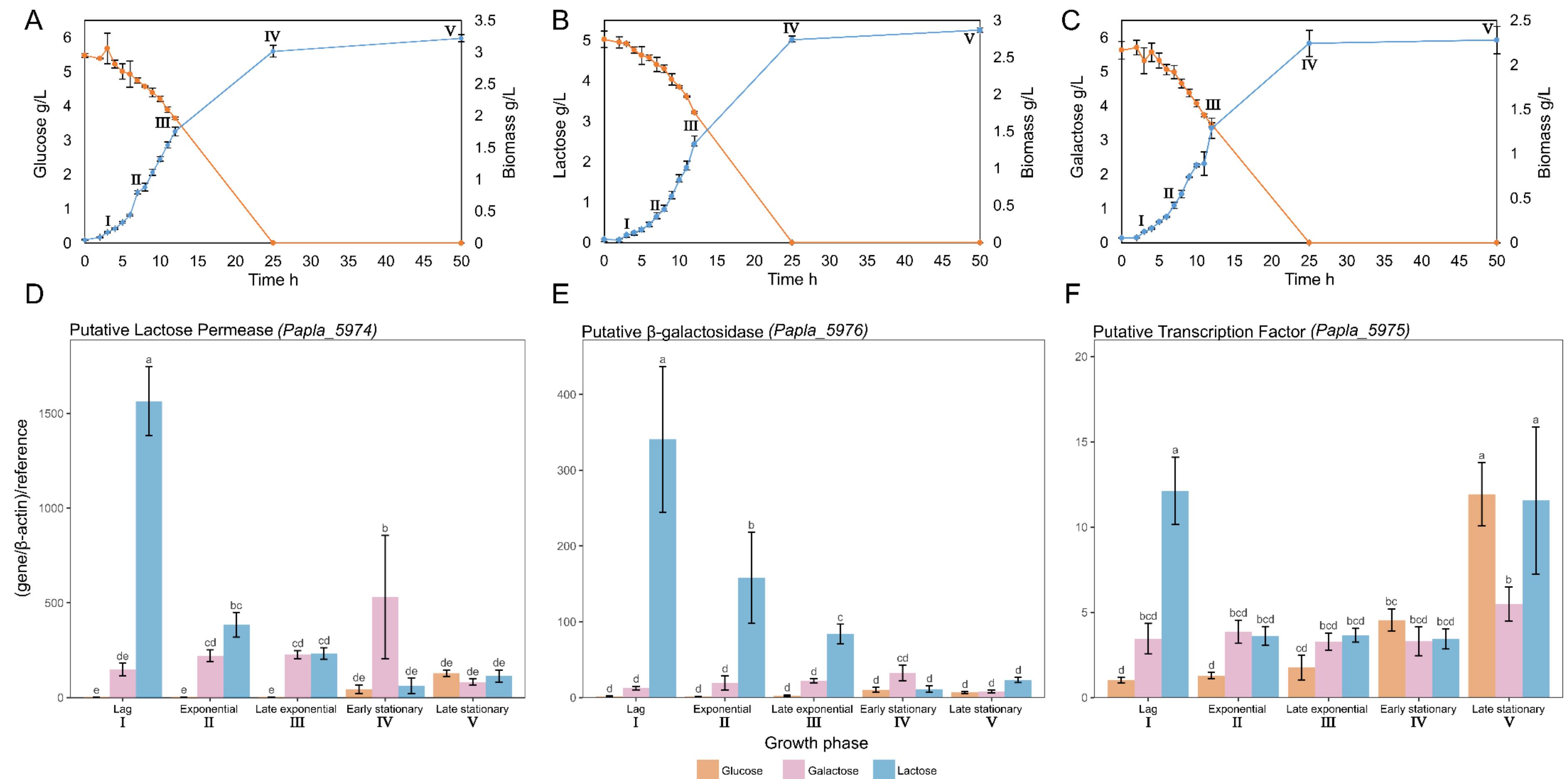
Sugar	μ (h^{-1})*	$Y_{x/s}$ (g_{dw}/g)*	*q* (g/g DW h)*
Glucose	0.308 ± 0.004 a	0.905 ± 0.060 a	0.341 ± 0.068 b
Lactose	0.290 ± 0.005 b	0.450 ± 0.024 b	0.694 ± 0.020 a
Galactose	0.290 ± 0.008 b	0.373 ± 0.006 b	0.753 ± 0.021 a

*μ: specific growth rate (h^{-1}); $Y_{X/S}$: Biomass yield (g_{dw}/g); *q:* specific sugar consumption rate (g/g DW h). Values followed by the same letter do not show significant differences ($p > 0.05$, Tuckey's test)

It should be noted that the sugars were completely consumed in the early stationary phase. The lactose permease (*Papla_5974*) and β-galactosidase (*Papla_5976*) candidate genes had their expression induced in culture media with lactose. On the other hand, the expression of the β- galactosidase gene did not increase in medium with galactose. Expression of these genes was repressed by cultivation in glucose (Fig 7D and E). Moreover, the expression of the genes encoding the lactose permease (*Papla_5974*) and β-galactosidase (*Papla_5976*) reduced from the early stationary phase due to sugar depletion (Fig 7D and E).

We detected the expression of the gene encoding the putative transcriptional factor (*Papla_5975*) in cultivations with glucose, galactose or lactose (Fig 7F). For galactose, the expression of the gene encoding the putative transcriptional factor was similar over cultivation. For the cultivation in medium with lactose, the gene expression was similar in the exponential, late exponential and early stationary phases and increased in the late stationary phase. Otherwise, its expression increased over time in the culture medium with glucose, peaking at the late stationary phase. Altogether, these results indicate that the expression of the putative transcriptional factor increases under starvation conditions (late stationary phase) for cultivations in minimal media containing glucose or lactose as the sole carbon sources.

Figure 7. **Growth and gene expression on sole glucose, galactose or lactose.** Upper part of the figure: Growth and sugar consumption profile in minimal medium (YNB). A: Glucose; B: Lactose; C: Galactose. I, II, III, IV, and V represent the lag, exponential, late exponential, early stationary, and late stationary phases, respectively. Lower part of the figure: The housekeeping gene used was β-actin, and the normalization was made by the average expression to the glucose lag phase. D: Putative lactose permease (*Papla_5974*); E: Putative β-galactosidase (*Papla_5976*); F: Putative Transcription Factor (*Papla_5975*). Means followed by the same letter are not significantly different ($p > 0.05$, Tuckey's test).

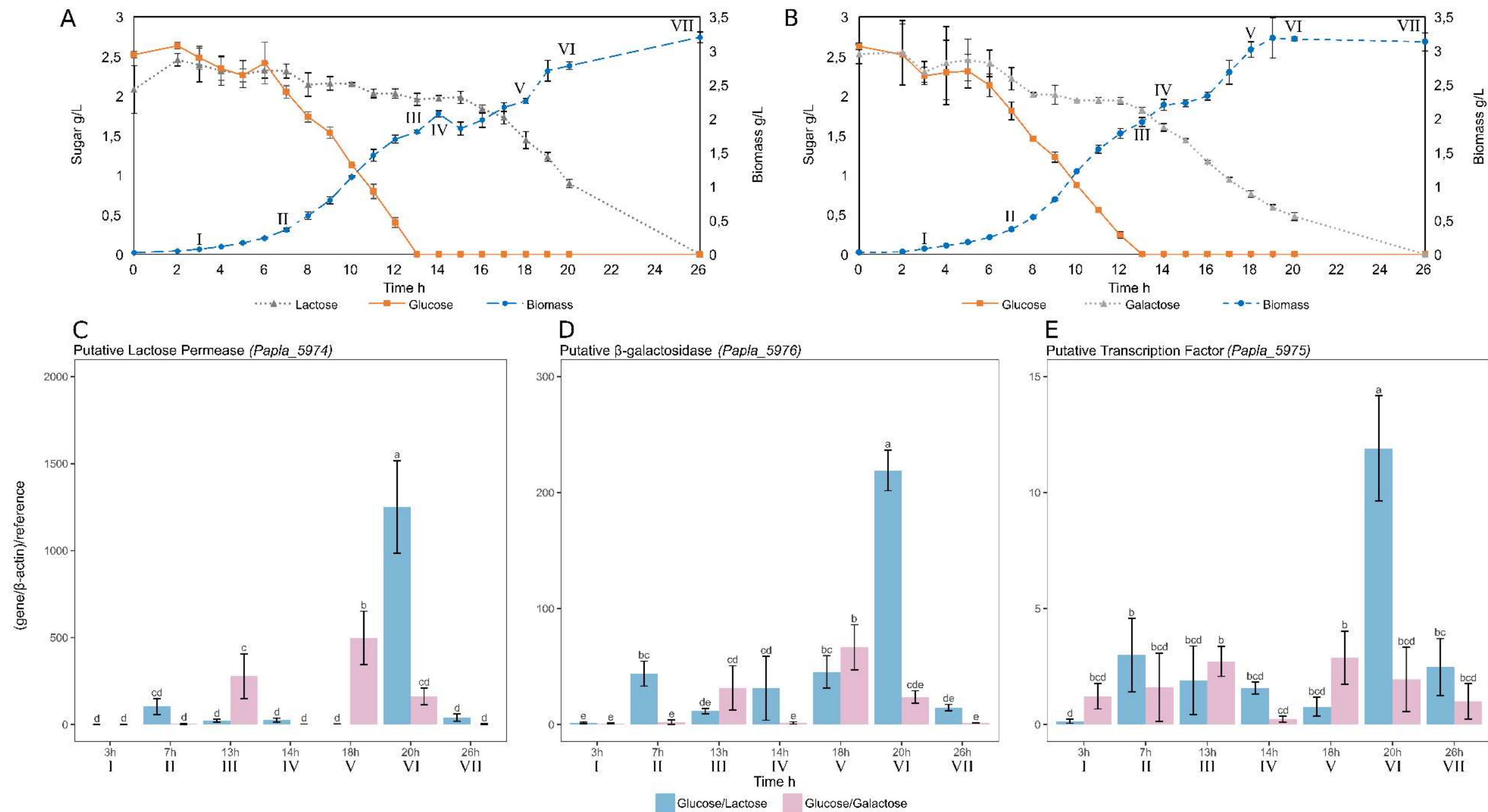


Effects of glucose on expression patterns of genes encoding putative proteins involved in the lactose metabolism

To evaluate the effects of glucose on gene expression, *P. laurentii* UFV-1 was cultivated in YNB media containing two carbon sources: glucose plus lactose, and glucose plus galactose (Fig 8A and B). *P. laurentii* UFV-1 displayed a diauxic growth on glucose plus lactose, and glucose plus galactose. It should be noted that glucose was consumed prior to lactose or galactose. Nevertheless, we verified that after the complete glucose consumption (13 h of cultivation), galactose was totally consumed earlier than lactose (Fig 8A and B). Consistent with this, the growth in the medium containing glucose plus galactose as carbon sources was resumed before the growth with glucose plus lactose, achieving the highest biomass in a period of 20 h. In contrast, the highest biomass on glucose plus lactose as carbon sources was reached at 26 h. In agreement with the growth parameters, the lactose permease candidate (*Papla_5974*) was expressed only when glucose was completely consumed (Fig 8C). Overall, the expression of the putative lactose transporter (*Papla_5974)* in the medium with glucose plus lactose seems to be repressed by glucose. This effect is less pronounced in the medium with glucose plus galactose. It is noteworthy that the expression of the lactose permease gene in the medium with glucose plus galactose (at 13 and 18 h of cultivation) occurred earlier than in the medium containing glucose and lactose (at 20 h). These results are consistent with the fact that the galactose consumption took place prior to the lactose consumption (Figure 8C). The expression of the putative gene encoding β-galactosidase was higher in the medium containing glucose and lactose (at 20 h) than in the medium containing glucose and galactose. This result is likely associated with the relevance of the β-galactosidase enzyme in culture media containing lactose as a carbon source.

Glucose did not repress the expression of the gene encoding the putative transcriptional regulator (*Papla_5975*) (Fig 8E). Its expression was higher in the medium containing glucose and lactose at 20 h. This result is according to the increase of its expression when lactose was the sole carbon source (Fig 7F).

Figure 8. **Growth and gene expression on two carbon sources.** Upper part of the figure: Growth and sugar consumption in minimum medium (YNB). A: Glucose plus lactose; B: Glucose plus galactose. I, II, III, IV, V, VI, and VII representing 3, 7, 13, 14, 18, 20, and 26 h, respectively. Lower part of the figure: The gene expression normalized by β-actin expression and expression relative to the glucose plus galactose (A) lag phase (3 h). D: Putative lactose permease (*Papla_5974*); E: Putative β-galactosidase (*Papla_5976*); F: Putative Transcription Factor (*Papla_5975*). Means followed by the same letter are not significantly different ($p > 0.05$, Tuckey's test).



DISCUSSION

Papiliotrema laurentii UFV-1 can accumulate lipids from ricotta whey (Cotrim et al., 2021), opening perspectives for developing a bioprocess aligned with principles of circular economy. In this sense, identifying the genes responsible for lactose metabolism in *P. laurentii* UFV-1 is pivotal for applying metabolic engineering strategies to improve lipid production on lactose- rich substrates, such as ricotta whey. We predicted the genes encoding lactose permease from the genome of *P. laurentii* UFV-1 using BLASTp and characterized these proteins based on their sequences, domains, membrane topology, and location due to the highly conserved structures of SPs. Using this pipeline, the 10 proteins were classified as SP. However, these transporters are promiscuous and display variations of amino acids in positions that interact with sugars, making the identification of the type of sugar transported difficult (DONZELLA; SOUSA; MORRISSEY, 2023). Therefore, we cannot ensure whether the 10 proteins identified can uptake lactose based solely on sequence or structure. Conversely, the β-galactosidase candidate was identified using the BLASTp. This enzyme, found as a unique copy in the *P. laurentii* genome, has a conservative domain, and is predicted to act intracellularly. In agreement with this prediction, we did not detect glucose or galactose extracellular over the cultivation in culture media containing lactose as the sole carbon source, indicating that its hydrolysis takes place intracellularly.

In Ascomycota, lactose permease and β-galactosidase genes are found in gene clusters, which are usually divergently expressed, for example, in *K. lactis* and *A. nidulans* (FEKETE et al., 2012; SCHAFFRATH; BREUNIG, 2000). Based on that, we search for a putative lactose permease gene located close to the putative β-galactosidase gene. We identified the putative genes encoding β-galactosidase (*Papla_5976*) and lactose permease (*Papla_5974*), which are near each other on the same chromosome. The co-localization of these genes is in line with gene clusters found in Ascomycota (FEKETE et al., 2012). For instance, it has been hypothesized that copies of both β-galactosidase and lactose permease genes seem to have originated from genes belonging to this cluster (FEKETE et al., 2012; VARELA et al., 2019). An intriguing issue is the presence of lactose cluster genes in yeasts isolated from environments where lactose is unavailable. Apparently, the lactose cluster in some fungi did not evolve to metabolize this disaccharide. In *T. reesei*, *A. nidulans*, *A. cellulolyticus* and *P. chinulatum*, lactose is used to induce the coordinate expression of several cellulolytic enzymes, governed by specific transcription factors such as Xyr1p and Ace3p (FANG et al., 2008; IVANOVA et al., 2013; SEHNEM et al., 2006; SEIBOTH et

al., 2007; SEIBOTH; HEROLD; KUBICEK, 2012). Ace3p binds to the *CRT1* lactose sensor/transporter promoter in *T. ressei* and appears to induce β-galactosidase indirectly. At the same time, Ace3p binds to the *XYR1* promoter and induces the expression of cellulolytic enzymes (ZHANG et al., 2019). These soil and plant- associated fungi present low growth on lactose, which reinforces the non-lactose-adapted origin of this cluster. In *K. lactis*, yeast found in lactose-rich niches, the lactose permease can also transport cellobiose (VARELA et al., 2019). Although *P. laurentii* UFV-1 has been isolated from the soil, it grows well in ricotta whey. Hence, we hypothesize that the lactose cluster gene may have originated before separating the Basidiomycota and Ascomycota phyla or by evolutionary convergence.

Herein, the expression of putative genes encoding lactose permease and β-galactosidase was induced in a YNB medium containing lactose as the sole carbon source, indicating that these proteins are responsible for lactose uptake and its intracellular hydrolysis. Otherwise, only the putative gene encoding lactose permease had its expression induced in YNB medium containing galactose as the sole carbon source. This result suggests that the permease encoded by the *Papla_5974* gene can also transport galactose. We believe that other transporters can also uptake galactose in *P. laurentii*, similar to *K. lactis* (VARELA et al., 2019). In contrast to lactose, the expression of the β-galactosidase gene was not induced in the YNB medium containing galactose as the sole carbon source. This result is consistent with the fact that galactose is a monosaccharide; thus, the hydrolysis activity of β-galactosidase is not required. Even though both lactose permease and β-galactosidase genes are found on the same gene cluster, their regulation at the transcriptional level seems to be carbon source-dependent.

Contrary to Ascomycota, we surprisingly found a putative transcriptional factor (*Papla_5975*) between the β-galactosidase (*Papla_5976*) and lactose permease (*Papla_5974*) genes. These genes were coordinately expressed only in lactose. To the best of our knowledge, we show, for the first time, a transcriptional factor containing the Zn(II)2Cys6 motif typically involved in zinc-dependent DNA binding, co-localized with these genes. It is noteworthy that transcription factors containing the aforementioned motif are the most common in fungi genomes, including the transcriptional activator Gal4p in *S. cerevisiae* and its homolog in *K. lactis*, Lac9p (ANDERS et al., 2006). With regard to topology, there is a similar cluster gene in *A. nidulan*s; however, the central gene in this cluster encodes a GH2 glycosyl hydrolase with low expression in the presence of lactose (FEKETE et al., 2012). In the current study, the expression of the putative transcriptional

factor occurred regardless of the carbon source. As such, it is reasonable to admit that it does not act exclusively on the expression of β-galactosidase and lactose permease genes. It is important to point out that its expression increased in response to sugar depletion, indicating that it may play an important role under nutrient-limiting conditions.

Diauxic growth was evidenced herein in *P. laurentii* UFV-1. In agreement with this growth pattern, the expression of genes encoding β-galactosidase and lactose permease was repressed by glucose. Notably, the lactose-containing medium had a longer second lag phase than the galactose-containing medium. This difference could be due to a better capacity to metabolize galactose than lactose upon glucose depletion. As mentioned previously, *P. laurentii* UFV-1 was isolated from soil, where hemicellulose and derived β-galactosides are commonly found. These polymers are characterized by heterogeneity, being constituted by xylose, arabinose, mannose and galactose. As such, it is reasonable to assume that an efficient galactose metabolism is an important feature of yeast soil such as *P. laurentii*.

CONCLUSION

We identified the genes encoding β-galactosidase (*Papla_5976*) and lactose permease (*Papla_5974*) in *P. laurentii* UFV-1. The expression of the lactose permease (*Papla_5974*) gene is induced by both lactose and galactose; otherwise, only the lactose permease gene had its expression induced by galactose. Their expression was repressed by glucose, which is consistent with the diauxic growth observed in the presence of glucose. To the best of our knowledge, this study describes for the first time the presence of a transcriptional factor gene (*Papla_5975*) between the lactose permease (*Papla_5974*) and β-galactosidase (*Papla_5976*) genes in fungi; however, its role remains elusive.

Challenges and future perspectives

The study of Basidiomycota yeasts remains largely unexploited. There is little information regarding the organization of cluster genes within this phylum, making comparisons difficult. Therefore, conducting comparative studies related to cluster genes in other species, mainly lactose/galactose cluster genes, is required to support the hypothesis of the cluster's emergence with ancestry prior to the separation between Basidiomycota and Ascomycota. The expression of the genes encoding β-galactosidase (*Papla_5976*) and the lactose permease (*Papla_5974*) in an *S. cerevisiae* strain harboring only the maltose permease will allow us to evaluate whether the lactose permease is able to transport

galactose, lactose and other sugars. Additionally, further studies focusing on measuring the β-galactosidase activity and analyzing the gene expression of enzymes involved in the Leloir pathway under the cultivation conditions carried out in the current work will contribute to gain insights into the regulatory mechanisms of lactose/galactose metabolism in *P. laurentii*.

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