Universidade do Minho
Escola de Ciências

Bruna Daniela Faria Oliveira

Manipulation of glucose transport for improved growth and fermentation of *Torulaspora delbrueckii*

Outubro 2022

Manipulation of glucose transport for improved growth and fermentation of *Torulaspora delbrueckii*

Bruna Daniela Faria Oliveira

UMINHO | 2022

Universidade do Minho
Escola de Ciências

Bruna Daniela Faria Oliveira

Manipulation of glucose transport for improved growth and fermentation of *Torulaspora delbrueckii*

Dissertação de Mestrado

Mestrado em Bioquímica Aplicada

Área de Especialização em Biomedicina

Trabalho efetuado sob a orientação de

Professora Doutora Maria João Marques Ferreira Sousa Moreira

Doutor Ricardo Filipe Azevedo Franco Duarte

Outubro 2022

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.

Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.

Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositoriUM da Universidade do Minho.

Atribuição-NãoComercial-SemDerivações
CC BY-NC-ND

https://creativecommons.org/licenses/by-nc-nd/4.0/

Acknowledgements

Terminar esta dissertação representa o fim da mais gratificante etapa da minha formação académica. Esta não teria sido tão enriquecedora sem o apoio e ajuda destas pessoas:

Em primeiro lugar, não posso deixar de agradecer aos meus orientadores. À professora Maria João Sousa, obrigada por todo o conhecimento que me transmitiu, pela disponibilidade e rigor que foram, sem dúvida, fundamentais ao longo deste ano. Ao doutor Ricardo Franco Duarte, pelo conhecimento partilhado, pela disponibilidade, pelo apoio e incentivo e também pelo rigor, que foram contributos indispensáveis para a realização deste trabalho.

À professora Susana Chaves, que foi parte essencial na realização deste trabalho. Agradeço pelo conhecimento transmitido, pela disponibilidade, paciência e incentivo.

Ao professor Björn Johansson, por nos ter cedido o plasmídeo construído no seu laboratório.

To Eckhard Boles (Goethe University Frankfurt, Germany) who kindly gifted the strain *S. cerevisiae* EBY.VW4000 used in this work.

Como não poderia deixar de ser, às meninas da Micro I, que tão bem me acolheram no laboratório e sempre estiveram disponíveis para me ajudar e orientar em tudo aquilo que precisei. Em especial à Filipa Mendes, por toda a ajuda, disponibilidade e paciência que teve comigo. À Leslie Amaral, pela ajuda, paciência e incentivo ao longo do ano. À Flávia Sousa, que me ajudou nos meus primeiros meses no laboratório.

À minha Carol, que me acompanha desde o primeiro dia de licenciatura, pela amizade e apoio ao longo destes anos cheios de histórias. Ao Pedro, por sempre acreditar em mim, me motivar e incentivar, pela paciência e por estar sempre presente.

Por fim, às pessoas mais importantes da minha vida, aos meus pais e irmã. Obrigada pelo apoio incansável e incondicional, pela paciência, por me incentivarem a seguir os meus sonhos e principalmente pelo amor que sempre me deram.

This work was supported by the UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) and UID/BIA/04050/2019 strategic programs and by the project PTDC/BIA-MIC/32059/2017, which is funded by national funds through the FCT- Fundação para a Ciência e Tecnologia and by the ERDF-European Regional Development Fund through the COMPETE2020–Programa Operacional Competitividade e Internacionalização (POCI) and Sistema de Apoio à Investigação Científica e Tecnológica (SAICT).

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Manipulação do transporte de glucose de forma a melhorar o crescimento e fermentação de *Torulaspora delbrueckii*

Resumo

A glucose, fonte de carbono e energia preferencial para a maioria das células eucariotas, é capaz de estimular o seu próprio transporte através da membrana plasmática. Este é um passo limitante que afeta a capacidade fermentativa das leveduras. Em *Saccharomyces cerevisiae,* tal como em *Torulaspora delbrueckii,* que emergiu como uma espécie de levedura promissora para ser implementada nas indústrias vínica e da panificação, a entrada de hexoses nas células ocorre pelo intermédio de proteínas transportadoras. Em *T. delbrueckii,* o transporte de glucose é mais sensível aos efeitos inibitórios causados pelo etanol do que em *S. cerevisiae.* Assim, a caracterização e manipulação dos transportadores de glucose de *T. delbrueckii* poderá ser uma estratégia adequada para aumentar a sua taxa de crescimento e melhorar a sua capacidade fermentativa na presença de etanol. No presente trabalho, avaliamos a taxa específica de crescimento de 41 estirpes de *T. delbrueckii* e examinamos os seus genomas de forma a caracterizar o seu repertório de transportadores de hexoses. Todas as estirpes codificam pelo menos 5 transportadores, mas a maioria codifica um ou dois transportadores adicionais. Não foi observada uma correlação entre as taxas específicas de crescimento em meio YPD, o número de transportadores e as origens das estirpes analisadas. Análises adicionais foram realizadas usando a estirpe *T. delbrueckii* PYCC 5321, onde os níveis de expressão dos seus 4 genes mais conservados foram analisados ao longo das diferentes fases de crescimento. Os resultados não indicaram variação significativa nos níveis de expressão de 3 desses genes, no entanto, um deles é significativamente mais expresso na fase estacionária. Adicionalmente, tentamos caracterizar os mesmos 4 transportadores, clonando e expressando-os numa estirpe de *S. cerevisiae hxt* null. De forma semelhante, tentamos melhorar o crescimento e capacidade fermentativa de *T. delbrueckii* através da expressão dos transportadores de *S. cerevisiae* menos sensíveis ao etanol. No entanto, as duas estratégias de clonagem ainda estão a ser otimizadas. Este trabalho revelou alguns aspetos interessantes, relacionados com o transporte de glucose e as proteínas transportadoras de *T. delbrueckii*, contribuindo assim para aprofundar o conhecimento desta espécie.

Palavras-chave: Crescimento e capacidade fermentativa, *Saccharomyces cerevisiae, Torulaspora delbrueckii,* proteínas transportadoras, transporte de glucose.

Manipulation of glucose transport for improved growth and fermentation of *Torulaspora delbrueckii*

Abstract

Glucose, the preferred carbon and energy source for most eukaryotic cells, is able to stimulate its own transport across the plasma membrane. This is a rate limiting step which, consequently, affects yeasts fermentation capacity. In *Saccharomyces cerevisiae*, as well as in *Torulaspora delbrueckii*, a yeast species that has emerged as a promising species to be implemented in wine and bread making industries, the uptake of hexoses occurs mainly through the intermediary of transporter proteins. It has been reported that *T. delbrueckii*'s glucose transport has a higher sensitivity to inhibitory effects caused by ethanol when compared to *S. cerevisiae*'s. Thus, the characterization of *T. delbrueckii*'s glucose transporters and the manipulation of its hexose transport might be a suitable strategy to increase this species growth rate and improve its fermentation capacity when ethanol is present. In the present work, we assessed the specific growth rate of 41 *T. delbrueckii* strains and examined their genome to characterize the species repertoire of hexose transporter genes. All strains encode at least five transporters, but most encode one or two additional ones. No correlation was observed between the values obtained for the specific growth rate, number of transporters encoded and the origins of the *T. delbrueckii* strains analysed in the scope of this work. Further analyses were performed using *T. delbrueckii* PYCC 5321, where the expression levels of the four most conserved transporters were analysed, throughout the different phases of its growth. Results indicated no significant variation in the expression levels of three of the transporters, however, one of them was significantly more expressed on the stationary phase. Additionally, we tried to characterize the same four transporters of this species by cloning and expressing them on a *S. cerevisiae hxt* null strain. In a similar way, we also tried to improve the growth and fermentation of *T. delbrueckii* by the expressing *S. cerevisiae*'s transporters less sensitive to ethanol. However, both cloning strategies are still being optimized. The present work revealed some interesting aspects related to *T. delbrueckii*'s glucose transport and protein transporters, contributing, then, to deepen the knowledge about this species.

Keywords: Glucose transport, growth and fermentation capacity, *Saccharomyces cerevisiae, Torulaspora delbrueckii,* transporter proteins.

Table of contents

List of abbreviations and acronyms

°C	Degrees Celcius
µg	Micrograms
µL	Microliters
Acetyl-CoA	Acetyl-Coenzyme A
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bps	Base pairs
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DSB	Double Stranded Break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
G418	Geneticin
HR	Homologous Recombination
iTOL	Interactive Tree of Life
LB	Lysogenia Broth, Luria Broth or Luria-Bertani medium
M	Molar
MEGA11	Molecular Evolutionary Genetics Analysis 11
MFS	Major Facilitator Superfamily
mL	Millilitres
mM	Milimolar
NAD^+	Nicotinamide Adenine Dinucleotide (oxidized)
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NCBI	National Center for Biotechnology Information
ng	Nanograms
NHEJ	Non-Homologous End Joining
nm	Nanometers
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Rotations per minute
RT-PCR	Real Time- PCR
SDS	Sodium dodecyl sulphate
SOC	Super Optimal broth with Catabolite repression medium
SP	Sugar Porter
TE	Tris-EDTA

TMS	Transmembrane Spanning
Tris	Tris(hydroxymethul)aminonmethane
v/v	Volume/volume
w/v	Weight/volume
WT	Wild Type
YNB	Yeast Nitrogen Base
YPD	Yeast extract Peptone Dextrose medium
YPDA	Yeast extract Peptone Dextrose Agar medium

List of Figures

List of Tables

Chapter 1. Introduction

1. Introduction

1.1. *Torulaspora delbrueckii*: An unconventional yeast

Since early in human history, yeasts have been used daily for the production of fermented products, such as bread, wine and beer (Faria-Oliveira et al., 2013). Before the biochemical process of fermentation was completely understood, these products were obtained relying on the spontaneous fermentation performed by the microorganisms naturally present in the raw materials used (Tondini et al., 2019). During the 19th century, Pasteur's experiments revealed the fundamental role of yeasts in the manufacturing of fermented products (Pech-Canul et al., 2019). Over time, some yeast species have become a fundamental component in the food and beverages fermentation processes and, consequently, have been the main group of microorganisms exploited by mankind (Benítez et al., 1996). Yeasts are essential for a wide range of industrial applications, being the major producer of biotechnological products worldwide and having a huge importance at fundamental and industrial levels (Johnson, 2013a). Among the yeasts that contribute to the production of fermented foods and beverages of commercial importance, the genus *Saccharomyces* is the most important from fundamental and applied perspectives (Johnson, 2013a; Ray & Montet, 2017). *Saccharomyces cerevisiae* stands out in this group as the best studied, best characterized and most commonly used yeast. Because of its ubiquity in fermented products, scientific research efforts have been mainly focused on this species, which has been used as a model organism to study several fundamental aspects of eukaryotic cell biology research. *S. cerevisiae*'s unique characteristics, such as its fermentation capacity and stress resistance, also grant this microorganism a great importance in various biotechnological applications (Parapouli et al., 2020; Pech-Canul et al., 2019).

The domestication of *S. cerevisiae* is considered a vital event in human history and has allowed important advancements (Johnson, 2013a) not only at a biotechnological level, but also at the fundamental research level. However, the universal use of strains belonging to this species has limited the ability to diversify the organoleptic properties of the fermented products, such as flavour and aroma. This limits the potential to improve and to create new products. Therefore, the use of other yeast species to perform fermentations usually carried

out by *S. cerevisiae* has proved useful and advantageous. For this purpose, in recent years, non-*Saccharomyces* (also referred as unconventional) yeasts have been given special attention, since they represent an enormous source of opportunities to obtain new and innovative products (Benito, 2018; Pech-Canul et al., 2019).

Non-*Saccharomyces* yeasts are being increasingly used for a variety of different applications, not only in industry, but also, for instance, in agriculture, as agents of biocontrol, bioremediation, and as indicators of environmental quality (Johnson, 2013b). Among this group, the best studied, commercialized, and utilized at the industrial level is *Torulaspora delbrueckii* (Belda et al., 2017; Benito, 2018). This species belongs to the *Torulaspora* genus, defined in 1895 by Lindner, along with five others: *T. franciscae*, *T. pretoriensis*, *T. microellipsoides*, *T. globosa* and *T. maleeae* (Benito, 2018; Kurtzman, 2011; Ramírez & Velázquez, 2018). Recently, in a study regarding the variability of *T. delbrueckii*'s genome, it was found that several strains included in this species actually belong to 3 undescribed species: *T. indica, T. nypae,* and *T. quercuum* (Silva et al., 2022). The history of this genus reflects its close relationship with *Saccharomyces* and *Zygosaccharomyces*, and because they share various morphological and physiological features, these genera have been misclassified in the past (Barnett, 1992; Oda et al., 1997). In recent study regarding the improvement of *T. delbrueckii*'s genome annotation, phylogenetic analyses were performed. In this study it was reported that the *Zygotorulaspora* and *Zygosaccharomyces* genera are the most closely related to *T. delbrueckii* and that *S. cerevisiae* strains display less sequence similarity than the aforementioned genera, together with *Kazachstania, Naumovozyma, Tetrapisispora*, and *Vanderwaltozyma* (Santiago et al., 2021).

Torulaspora cells are usually smaller than those of *S. cerevisiae*, being mostly spherical and ellipsoidal, with dimensions of approximately 2-6 x 3-7 µm, and their life cycle has not yet been elucidated. All species belonging to this genus reproduce asexually by multilateral budding and sexual reproduction may occur in sporulation media (Ramírez & Velázquez, 2018). *T. delbrueckii* (previously called *T. rosei*, *T. fermentati* or *T. vafer*) (Benito, 2018) is a ubiquitous yeast species (Pech-Canul et al., 2019), frequently found in association with human activity and food processing (Mecca et al., 2020), appearing among the 20 most commonly described foodborne yeasts (Benito, 2018). This species is widely distributed in nature, and is commonly isolated from fermented drinks (Benito, 2018) and from spontaneous bread dough

fermentations (Almeida & Pais, 1996), but has also been reported in soil, berry juices, agave juice, tea-beer and tree bark (Kurtzman, 2011). *T. delbrueckii* is a common food and beverage spoilage organism, for instance in dairy products, and appears to be of minor clinical importance (Kurtzman, 2011). For many years, *T. delbrueckii* was described as a haploid yeast, mainly because of its small cell size and the rare observation of tetrads in sporulation media. In a recent study, already mentioned above, the authors detected high levels of homozygosity in the genome of the studied *T. delbrueckii* strains, which is in accordance with a haploid genome (Silva et al., 2022). However, it has also been suggested that this species is actually diploid, the reduced size of cells being due to the fact that it only possesses 16 chromosomes in the diploid phase, instead of 32 chromosomes like *S. cerevisiae* (Albertin et al., 2014; Ramírez & Velázquez, 2018). *T. delbrueckii*, which has been considered as microbial contamination in the past, has shown several advantages over *S. cerevisiae* (Pech-Canul et al., 2019). In the production of alcoholic beverages such as wine and beer, this species has been reported to have a positive effect on the taste and aroma, exhibit a low production of undesirable metabolites and increase the levels of compounds that positively contribute to the flavour of the final product (Pacheco et al., 2020). *T. delbrueckii* has been considered of oenological interest for decades and it was one of the first non-*Saccharomyces* species to be commercially available as active dry yeast for industrial producers (Mecca et al., 2020; Pech-Canul et al., 2019). Additionally, strains isolated from bread doughs exhibit good baking ability and resistance to osmotic and freeze-thaw stresses (Almeida & Pais, 1996; Hernandez-Lopez et al., 2003; Pacheco et al., 2020), which makes this species a strong candidate to be used in the bread making industry (Alves-Araújo et al., 2004).

The unique abilities of *T. delbrueckii* are advantages to its use and can be potentially exploited in the biotechnology industry. Thus, this species is starting to be recognized as an important organism of study due to its biological features (Hernandez-Lopez et al., 2002). However, the lack of specific genetic and molecular tools for its study has been a complication in regard to the understanding of the molecular bases of *T. delbrueckii*'s physiological behaviour. For this reason, the development of tools for metabolic engineering in this microorganism is very important in view of its application to improve industrial processes (Alves-Araújo et al., 2007; Pacheco et al., 2020; Pech-Canul et al., 2019).

1.2. Yeast Sugar Metabolism

To survive and grow, yeast cells require macronutrients, such as sources of carbon, nitrogen, oxygen, and trace elements, such as calcium, zinc, and iron in their growth media. Most yeasts are able to grow in simple media, only supplying carbon, nitrogen, inorganic ions and growth factors, which are organic compounds required in low concentrations that are not used as energy sources (Walker, 2009). Like other heterotrophic organisms, yeast anabolism is coupled with catabolism, which means that the energy and carbon metabolisms operate together (Faria-Oliveira et al., 2013). As yeasts are chemoorganotrophic organisms, they obtain carbon and energy in the form of organic compounds (Walker, 2009). Organic molecules can be oxidized, resulting in chemical energy, in the form of ATP, which is used as an energy source by the cell, or used as a carbon source for biosynthesis (Faria-Oliveira et al., 2013).

Overall, yeasts display great metabolic complexity, as a result of a wide environmental distribution. These microorganisms are able to metabolize polyols, alcohols, organic acids, and amino acids. However, they preferentially metabolize sugars, such as hexoses, which include glucose, fructose, galactose, and mannose. Some are also able to metabolize pentoses, such as xylose and arabinose and disaccharides, such as maltose and sucrose. Despite being able to use multiple carbon and energy sources available in nature, yeasts preferred substrates are glucose, fructose and mannose (Boles & Hollenberg, 1997; Carlson, 1998; Faria-Oliveira et al., 2013). Besides being able to deal with a wide range of sugar concentrations, various yeast species exhibit characteristic responses to the alteration of hexose concentrations in their environment (Boles & Hollenberg, 1997). For most eukaryotic cells, glucose is the preferred carbon and energy source and has important effects on cellular functions (Özcan et al., 1996, 1998), regulating several aspects of cell growth, metabolism, and development (Özcan & Johnston, 1999) and being able to stimulate the first step of its metabolism, its transport across the plasma membrane (Özcan et al., 1996). Apart from being a metabolic substrate, glucose is also a signalling molecule that regulates physiological and pathological processes (Özcan & Johnston, 1999; Towle, 2005), which means that the maintenance of glucose homeostasis is indispensable to many organisms (Özcan et al., 1998).

Respiration and fermentation are catabolic pathways, where ATP is produced from the glucose used by yeasts (**Figure 1**) (Dzialo et al., 2017). Both processes start with glycolysis,

which operates in the same way under aerobic and anaerobic conditions, and where one molecule of glucose results in the production of two molecules of pyruvate and ATP. In most yeasts, pyruvate is then further degraded via respiration or fermentation (Escalante, 2012; García et al., 2016; Pfeiffer & Morley, 2014). In respiration, pyruvate is converted into acetyl-CoenzymeA (acetyl-CoA), which is then oxidized to CO_2 in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation and where additional ATP is produced. This is an aerobic metabolic pathway for energy production, where glucose is completely oxidized (Escalante, 2012; García et al., 2016; Pfeiffer & Morley, 2014; Walker, 2009). In the case of *S. cerevisiae,* an estimated yield of 18 moles of ATP per mole of glucose consumed are produced in this process (Pfeiffer & Morley, 2014; van Gulik & Heijnen, 1995). In fermentation, pyruvate is converted into ethanol. This pathway does not produce additional ATP besides the 2 molecules produced in glycolysis. The NADH released in glycolysis is recycled back into NAD+, which is necessary for the maintenance of the redox balance and, consequently, fermentation can occur in the absence of oxygen (García et al., 2016; Pfeiffer & Morley, 2014).

Despite the low ATP yield, alcoholic fermentation is the most common process for glucose catabolism. In this process, ethanol and CO_2 are the main final products (Faria-Oliveira et al., 2013), according to the equation:

$C_6H_{12}O_6$ (glucose) → 2 C_2H_5OH (ethanol) + 2 CO_2 (carbon dioxide) (Maicas, 2020).

From a biochemical point of view, fermentation is a process in which an organism, in an environment where oxygen is absent or present in low concentrations, converts a carbohydrate into an alcohol or an acid. As mentioned above, glucose metabolism generates pyruvate, which is then broken into ethanol and carbon dioxide. Because a low amount of energy is produced under anaerobic conditions, yeasts must consume high amounts of glucose to obtain enough energy (Escalante, 2012; Maicas, 2020). Consequently, ethanol, the main fermentation by-product that accumulates in the fermentation medium, exerts an inhibitory effect and hinders the fermentative activity of yeasts (Escalante, 2012; Maicas, 2020; Puligundla et al., 2011). From a technological point of view, the capacity of yeasts to produce ethanol is important to determine their usability in fermentative processes (Escalante, 2012). Besides ethanol, glycerol and small amounts of acetic acid are also produced. Glycerol contributes to restore the redox balance inside the cell and acetic acid,

which is the main organic acid produced during the fermentation of glucose, is responsible for the acidification and the decrease of pH of the medium (Escalante, 2012).

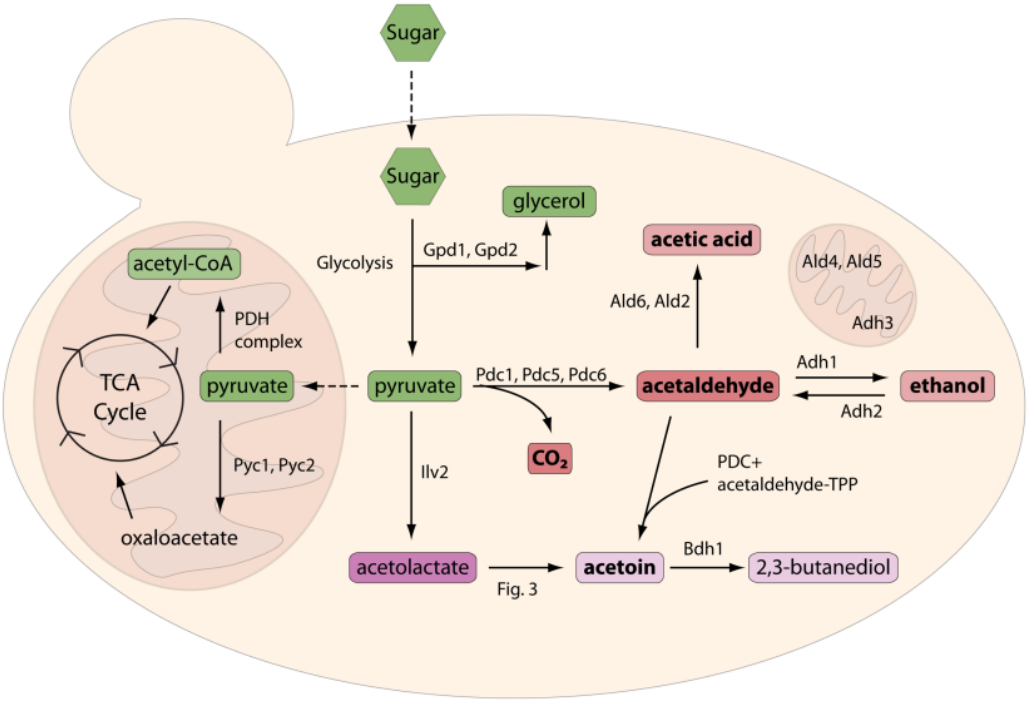


Figure 1. Dissimilation pathways of respiration and fermentation. Respiration is represented on the left side of the image and fermentation on the right. In yeasts, sugars are converted into pyruvate or glycerol through the process of glycolysis. Pyruvate can then take two directions: in aerobic conditions (respiration), pyruvate is converted into CO_2 and acetyl-CoA which then integrates the TCA cycle; in anaerobic conditions (fermentation), pyruvate is ultimately converted into ethanol and CO_2 is released. Adapted from (Dzialo et al., 2017).

To be able to metabolize glucose, yeasts need to sense its presence in the environment and then transport it across the plasma membrane (Faria-Oliveira et al., 2013). The presence of glucose in growth media induces the expression of a set of genes encoding glycolytic enzymes, glucose transporters, ribosomal proteins and other proteins and represses the expression of another set of genes involved in the use of alternative carbon sources, gluconeogenesis, TCA cycle, respiration, peroxisomal functions and others. This is called "glucose repression". When glucose is limiting, glucose repression stops and this last set of genes is expressed (Carlson, 1998). A coordinated combination of different signals and metabolic interactions for regulation of gene expression and other cellular processes is behind the effect of glucose on yeast metabolism (Kayikci & Nielsen, 2015). The availability of glucose and oxygen regulates respiration and fermentation in yeast cells, and it is linked to the

expression of the Pasteur effect (activation of sugar metabolism by anaerobiosis) and the Crabtree effect (ethanol production in aerobic conditions in the presence of glucose) (Walker, 2009). Usually, fermentation happens when oxygen is not present, but this is not always the case. Even in the presence of high levels of oxygen, yeasts can perform fermentation instead of respiration. This phenomenon (Crabtree effect) occurs when the glucose concentration is sufficiently high and the aerobic metabolism is inhibited both in the presence or absence of oxygen (Crabtree, 1929; Faria-Oliveira et al., 2013). When glucose is accessible, even in aerobic conditions, *S. cerevisiae* displays a fermentative metabolism and represses respiration, the use of alternative carbon sources and gluconeogenesis. This glucose repression is transmitted to the cellular machinery by interlinked regulatory interactions and signalling pathways. Thus, sensing intra- and extracellular glucose concentrations, to which yeast cells must adjust their cellular activities, is fundamental for the coordination of their carbon metabolism (Kayikci & Nielsen, 2015).

The metabolism of carbon shares common pathways both in *Saccharomyces* and non-*Saccharomyces* yeasts (Escalante, 2012). Despite that, the mechanisms involved in the regulation of respiration/fermentation metabolism can differ significantly among yeasts (Flores et al., 2000). *T. delbrueckii* is an important case study among the non-*Saccharomyces* yeasts, and several physiological and biochemical studies have been carried out for a better evaluation of the potential of this yeast (Pacheco et al., 2012). Among the non-*Saccharomyces* yeasts, *T. delbrueckii* is one of the most similar to *S. cerevisiae* when it comes to the features required for industrial alcoholic fermentation. When considered as options for different industrial applications, the small differences in the physiological characteristics of the two yeasts are relevant and affect the choice for the process in question (Ramírez & Velázquez, 2018). Even though *T. delbrueckii* displays a clear fermentative metabolism, it was found that there is a higher contribution of respiration in this yeast when compared to *S. cerevisiae,* being the rates of CO_2 production and O_2 consumption lower in the latter. However, the sugar utilization and regulation is similar in both species (Alves-Araújo et al., 2007; Ramírez & Velázquez, 2018).

1.3. Glucose Transport

The utilization of sugars, both via respiration or fermentation, plays a fundamental role in biological systems as those are the processes through which cells obtain carbon and energy. In either pathway, sugars must be first transported across the plasma membrane into the cells. This is the first, obligatory and essential step of sugar metabolism (Bisson et al., 1993; Boles & Hollenberg, 1997). As biological membranes have a restricted permeability, sugars and other cell nutrients must enter the cells through specific transporter systems. In yeasts, hexose transport can be mediated by two mechanisms: i) energy-independent facilitated diffusion, mediated by carriers, where the substrates are transported down a concentration gradient; and ii) energy-dependent active proton symport systems, where the uptake of the sugar is coupled to the uptake of protons, accumulating solutes in the cell against a concentration gradient, which is important during growth at low extracellular sugar concentrations (Boles & Hollenberg, 1997; Lagunas, 1993).

The amount of sugar taken up by cells and the rate at which it happens depends on the physiological and environmental circumstances. The mechanisms that control the uptake and the catabolism of sugars reflect cellular needs for carbon and/or energy (Bisson et al., 1993). It has been proposed that the uptake of glucose exerts a high control of glycolytic flux and, therefore, it is a rate-limiting step of sugar metabolism and glycolysis (Boles & Hollenberg, 1997). It is believed that the transport of glucose into the cells has a direct role in the sensing of glucose and in signal transduction (Bisson et al., 1993; Boles & Hollenberg, 1997; Özcan et al., 1996; Thevelein & Hohmann, 1995; Walsh et al., 1996). While the mechanisms of glucose transport and its transporters are well studied and characterized in *S. cerevisiae,* there is still a lack of knowledge about those aspects in *T. delbrueckii* and further studies are needed to deepen the knowledge about its sugars uptake and characterize its sugar transporters (Pacheco et al., 2020).

1.3.1. Glucose Transporters in *S. cerevisiae*

In *S. cerevisiae*, the uptake of hexoses occurs exclusively through facilitated diffusion mediated by transporter proteins, and it is an essential step in their utilization. However, this yeast also possesses proton symport systems for the uptake of disaccharides (Boles &

Hollenberg, 1997; Lagunas, 1993). Since transport of substrates through facilitated diffusion systems is most effective under constant levels of that substrate, *S. cerevisiae* has developed a diversity of hexose transporter proteins with specific individual properties and kinetics whose expression is tightly regulated by the presence and concentration of their substrates in the environment (Boles & Hollenberg, 1997). The large number of hexose transporters present in *S. cerevisiae* makes it able to deal with a broad range of sugar concentrations and reflects its adaptation to the variety of environmental conditions to which yeast cells are exposed (Boles & Hollenberg, 1997; Pacheco, 2008).

The Major Facilitator Superfamily (MFS) is a functionally diverse superfamily of membrane transporter proteins. One of the largest families included in this group is the sugar porter (SP) family (Saier, 2000; Saier et al., 1999). Hexose transporters form a subfamily in this group, which, besides the sugar transporters, also includes transporters of other carbon compounds that share a common ancestral origin (André, 1995; Boles & Hollenberg, 1997). Proteins included in the MFS family exhibit structural conservation, but they may share little sequence similarity (Vardy et al., 2004). Members of this family usually consist of a single integral membrane protein with twelve putative transmembrane segments, comprising two sets of six hydrophobic transmembrane-spanning (TMS) α-helices connected by a hydrophilic loop, whose N- and C-terminal regions are on the cytoplasm (Kruckeberg, 1996; Saier, 2000).

Analysis of *S. cerevisiae*‘s genome led to the discovery of the HXT family of homologous genes encoding hexose transporters (Diderich et al., 1999). This family, which has been defined based on sequence similarity and related function, consists of 20 members, including 18 genes encoding hexose transporters (*HXT1-17* and *GAL2*, which encodes a galactose transporter) and 2 genes encoding hexose sensors (*SNF3* and *RGT2*, encoding putative sensors of high and low glucose concentrations, respectively) (Boles & Hollenberg, 1997; Diderich et al., 1999; Faria-Oliveira et al., 2013; Kruckeberg, 1996; Özcan & Johnston, 1999). These proteins, sensors and transporters, are fundamental interveners on sugar metabolism (Faria-Oliveira et al., 2013). Sequence alignment of these 20 proteins revealed conservation over a portion of the sequence comprising 12 putative membrane-spanning regions with conserved spacing between them and several common amino acid motifs (Boles & Hollenberg, 1997; Hellborg & Piškur, 2009; Kruckeberg, 1996). The amino- and carboxyl-terminal regions, which are predicted to be on the cytosolic side of the membrane, are considerably different in their length and sequence (Boles & Hollenberg, 1997). The 20 members of this family are

distributed along 10 of the 16 chromosomes present in *S. cerevisiae*, some occupying internal positions and others being in or adjacent to subtelomeric regions (Kruckeberg, 1996).

Mutants deficient in any of the *HXT* genes do not show a detectable growth phenotype (Boles & Hollenberg, 1997). When the genes *HXT1-4* and *HXT6-7* are expressed individually as single chromosomal copies in an *hxt* null strain, cells are able to utilize glucose. However, the growth of these single-HXT strains differs in media with different concentrations of glucose (Boles & Hollenberg, 1997; Diderich et al., 1999). This indicates that the proteins encoded by these six genes are the main glucose transporters in *S. cerevisiae,* having different affinities and being differently regulated in response to this sugar (Boles & Hollenberg, 1997; Maier et al., 2002; Reifenberger et al., 1995). Genes *HXT8–HXT17* are phenotypically silent and may not be expressed under normal physiological conditions (Diderich et al., 1999; Pacheco, 2008). The regulation of the various *HXT* genes in response to the extracellular glucose and inactivation of Hxt proteins under certain conditions, allow *S. cerevisiae* cells to only express the glucose transporters appropriate for the amount of glucose available (Kim et al., 2013; Özcan & Johnston, 1999). For instance, Hxt1, a low affinity glucose transporter, is only expressed at high levels of glucose; Hxt2, a high-affinity glucose transporter, and Hxt4 are expressed at low levels of glucose; Hxt3 is expressed both at low and high glucose concentrations. Hxt6 and Hxt7, high affinity glucose transporters, are expressed at low glucose concentrations or in the presence of non-fermentable carbon sources, but repressed by high concentrations of glucose (Kim et al., 2013).

1.3.1.1. *SNF3* and *RGT2*

Snf3 and Rgt2 are sensors of glucose, glucose receptors, that generate an intracellular signal for induction of *HTX* gene expression in yeasts, in response to the availability and concentration of glucose in the growth medium (Özcan et al., 1996, 1998). The *SNF3* gene encodes a protein with 884 amino acids, homologous to sugar transporter proteins from various organisms (Kruckeberg, 1996). Besides the 12 putative membrane-spanning regions, Snf3 contains additional cytoplasmatic long sequences both at the N- and C-termini (Boles & Hollenberg, 1997). The *RGT2* gene encodes a protein with 763 amino acids, similar to Snf3, located 100 kb downstream of *SNF3*, on chromosome IV (Boles & Hollenberg, 1997). Both proteins have long C-terminal extensions, which distinguish them from the Hxt proteins of

yeasts and glucose transporters of many other organisms. The sequences of these tails are similar only in a 25 amino acid repeat, that is important for the Snf3 and Rgt2 signalling function (Özcan et al., 1998). These proteins do not transport glucose since they do not enable a mutant deficient in glucose transport to grow on glucose. However, mutations in these proteins cause hexose transport phenotypes and that is why they are included in the hexose transporter family (Boles & Hollenberg, 1997). These two proteins have different affinities for glucose. While Rgt2 is a low-affinity receptor, Snf3 is a high-affinity one. Low concentrations of glucose stimulate Snf3, which mediates the transcriptional induction of the genes *HXT2-4*, *6-7*, and *SUC2*, which encodes invertase. High concentrations of glucose activate Rgt2, which mediates the induction of *HXT1* (Özcan et al., 1998).

1.3.1.2. *HXT* family

As mentioned before, the *HXT* family is composed of 18 genes, *HXT1-17* and *GAL2*. The main aspects of each of the main glucose transporters in *S. cerevisiae* are summarized in **Table 1.**

Hxt1 consists of 570 amino acids, and *HXT1*, the gene encoding this protein, is located on the right arm of chromosome VIII. Hxt1 has an extremely low affinity for glucose, fructose and mannose. Expression of *HXT1* in *hxt1-7* null strains can restore cell growth on high concentrations of glucose, fructose and mannose. Over-expression of *HXT1* increases the glucose uptake activity of the cells, indicating that Hxt1 is directly involved in sugar transport (Boles & Hollenberg, 1997).

The protein Hxt2 consists of 541 amino acids and *HXT2* is located on the right arm of chromosome XIII. The protein is a high-affinity transporter for glucose, fructose and mannose. *HXT2* is induced only by low levels of glucose and is repressed in the presence of high concentrations of glucose or in its absence, being both positively and negatively regulated by the availability of this sugar (Boles & Hollenberg, 1997).

Hxt3 consists of 567 amino acids; the gene is located on the right arm of chromosome IV and, such as its closest relative, Hxt1, it is a low-affinity hexose transporter. An *hxt1-7* null strain expressing only *HXT3* only grows at glucose concentrations higher than 5 mM (Boles & Hollenberg, 1997).

Hxt4 consists of 576 amino acids, and the gene encoding this protein is located on the right arm of chromosome VIII, downstream of *HXT1*. Its closest relatives are Hxt6 and Hxt7. Hxt4 has a moderately low affinity for glucose, and it could only restore growth of the *hxt1-7* null strain at glucose concentrations higher than 5 mM. The *HXT4* gene is induced by low levels and completely repressed at high levels of glucose (Boles & Hollenberg, 1997).

Hxt5 consists of 592 amino acids and the gene is located on the right arm of chromosome VIII, upstream of *HXT1* and *HXT4* (Boles & Hollenberg, 1997). These three genes (*HXT1*, *HXT4* and *HXT5*) are organized in tandem, forming a cluster in this chromosome (Özcan & Johnston, 1999; Reifenberger et al., 1995). *HTX5* levels of transcripts are low in wild-type cells, and Hxt5 does not contribute significantly to catabolic glucose transport. However, overproduction of this protein partially restored growth on glucose, fructose and mannose in a *hxt1-7* null strain. This indicates that if present in sufficient amounts, Htx5 is able to transport these hexoses (Boles & Hollenberg, 1997).

Hxt6 and Hxt7 are highly related, both consisting of 570 amino acids and differing in only two amino acid residues (Boles & Hollenberg, 1997). The genes encoding these proteins are located in tandem, on the right arm of chromosome IV, downstream of *HXT3*, forming a three gene cluster (Boles & Hollenberg, 1997; Reifenberger et al., 1995). Some *S. cerevisiae* strains may contain only a *HXT6/7* chimeric gene instead of distinct *HXT6* and *HXT7* loci. Both proteins are high-affinity glucose transporters and expression of each of them in the *hxt1-7* null strain supported growth on 5 mM glucose. (Boles & Hollenberg, 1997).

Table 1. Size, coding gene localization and glucose affinity of the main *S. cerevisiae* hexose transporter proteins (Boles & Hollenberg, 1997).

Hxt protein	Number of aminoacidic residues	Localization of the gene on *S. cerevisiae*'s genome	Affinity for glucose
Hxt1	570	Right arm of chromosome VIII	Extremely low
Hxt2	541	Right arm of chromosome XIII	High
Hxt3	567	Right arm of chromosome IV	Low
Hxt4	576	Right arm of chromosome VIII (downstream of *HXT1)*	Moderately low
Hxt5	592	Right arm of chromosome VIII (upstream of *HXT1* and *HXT4*)	Does not contribute significantly to transport of glucose used in catabolic pathways
Hxt6 and Hxt7	Both 570	Right arm of chromosome IV (downstream of *HXT3*)	High

1.3.1.3. *GAL2*

Like glucose transport, the transport of galactose occurs through facilitated diffusion, mediated by the Gal2 protein. Gal2-deficient mutants do not grow well on media containing galactose as the only carbon source. *GAL2* is located on the right arm of chromosome XII, the protein it encodes consists of 574 amino acids and it is closely related to the hexose transporter family of yeasts, being Hxt6 and Hxt7 its closest relatives. Galactose uptake kinetics in wild-type cells growing on galactose or *hxt1-7* null strain cells is biphasic. It has high- and low-affinity components, suggesting that galactose uptake by the Gal2 is also modulated or influenced by other proteins. Furthermore, Gal2 is also able to transport glucose with high affinity. *GAL2* expression is induced by galactose and repressed by glucose (Boles & Hollenberg, 1997).

1.3.2. Glucose Transporters in *T. delbrueckii*

Even though *T. delbrueckii* has been receiving increasing attention from the biotechnology industry, little is known about its sugar transporters and sugar transport capacity (Pacheco et al., 2020). Being a ubiquitous yeast, *T. delbrueckii* can be found in a wide variety of habitats, where different sugars are present at different concentrations which are constantly changing. Because of that, the sugar transport activity and the sugar transporters need to be responsive to these dynamic conditions. In a similar way to what happens in *S. cerevisiae*, it is likely that

T. delbrueckii has a several sugar transporter genes encoding proteins with different affinities for glucose, allowing this species to respond to the constantly changing conditions of its natural environments (Pacheco, 2008). In fact, a study by Pacheco and colleagues (2020) reported the existence, in the *T. delbrueckii* type strain, of a cluster of 4 genes displaying high homologies to *HXT* genes of *S. cerevisiae.* However, only two of those glucose transporters have been characterized, Lgt1 and Igt1 (Alves-Araújo et al., 2005; Pacheco et al., 2020).

Alves-Araújo et al. (2005) identified the first hexose transporter in *T. delbrueckii*, encoded by *LGT1*. This protein is a low affinity glucose transporter which is also able to mediate the transport of fructose (Pacheco et al., 2020). Deletion of *LGT1* resulted in a small decrease in glucose uptake rates, only evident when this sugar is present in high concentrations. Together with the fact that a *lgt1* null strain did not present growth defects when grown in glucose media, these results indicate that this transporter has a low affinity for glucose and imply the existence of other relevant hexose transporters. Furthermore, the overexpression of *LGT1* did not change glucose uptake rates, suggesting that expression of this gene does not limit glucose transport (Pacheco et al., 2020).

According to the results described in the study mentioned above, other relevant glucose transporters in *T. delbrueckii* were thought to exist. Because of that, in another study, Pacheco et al. (2020) characterized a new glucose transporter in this species. From a genomic library of *T. delbrueckii* PYCC 5321, four *S. cerevisiae* EBY.vW4000 (*hxt* null strain) transformants were selected for their ability to grow on glucose as a sole carbon source. Sequencing of the plasmid insert from one of them revealed the presence of an open reading frame (ORF), which was named *IGT1,* with homology to known yeast hexose transporters and 70% total identity with *LGT1* at the nucleotide level. *IGT1* encodes an intermediate-affinity glucose transporter and displays a high similarity to *LGT1* and to other yeast glucose transporter genes (Pacheco et al., 2020). The introduction of *IGT1* into a *S. cerevisiae hxt* null strain was sufficient to allow it to grow on glucose, fructose, and mannose (2%, w/v). Furthermore, growth was also observed at various concentrations of glucose, suggesting that Igt1 is expressed and enables transport at both high and low glucose concentrations. This is a similar behaviour to that of Hxt2 in *S. cerevisiae* (Pacheco et al., 2020).

IGT1 and *LGT1* are adjacent in *T. delbrueckii* PYCC 5321's genome, the first being located upstream of the latter. It is likely that both genes are part of a cluster, located in chromosome 5, along with two other potential transporters, TDEL_0E02280 and TDEL_0E02290 (Pacheco

et al., 2020). *LGT1* and *IGT1* encode proteins with 567 and 569 amino acids, respectively. Comparison of the amino acid sequence of both proteins showed that differences between them occur mainly in two regions at their N-terminal end (Pacheco et al., 2020).

1.4. Ethanol Inhibition of Glucose Transport

As mentioned above, ethanol is the main product of yeast fermentative metabolism. The accumulation of high concentrations of ethanol in the fermentation medium has an inhibitory effect on the fermentative activity of the yeast (Escalante, 2012; Maicas, 2020; Puligundla et al., 2011). This is a common problem in wine fermentation, where accumulation of ethanol as well as other factors can cause incomplete fermentations (Santos et al., 2008). Indeed, ethanol damages the cell membrane of yeasts (Barnett & Robinow, 2002; You et al., 2003) and inactivates their transport systems, which results in an inability to metabolize nutrients that can lead to cell death (Santos et al., 2008). Accumulation of high concentrations of acetic acid in the fermentation medium further exacerbates the effect of ethanol (Cardoso & Leão, 1992). In fact, in a study by Santos et al (2008), the authors evaluated the resistance (expressed as the capacity to remain viable) of several commercial *S. cerevisiae* strains (PYCC 5792, PYCC 5793, PYCC 5794, PYCC 5795, PYCC 4072, and Bio-PF), as well as *Z. bailii* ISA 1307 and *T. delbrueckii* PYCC 5321 and Bio-J32, to high ethanol (18 %, v/v) and acetic acid (0.06 %, v/v) concentrations. While *T. delbrueckii* and *Z. bailii* strains displayed approximately 100% of survival after 2 hours of incubation, the studied *S. cerevisiae* strains, displayed 10% or less survival after the same period. However, the resistance to ethanol and acetic acid displayed by these strains did not reflect a better capacity to consume fructose (the main sugar present at the end of fermentation), since *S. cerevisiae* strains PYCC 5792 and PYCC 5793 consumed fructose faster. To try to understand what determines the sugar consumption capacity, the authors also studied the transport of glucose across the plasma membrane, by determination of labelled glucose uptake rates in the presence and absence of ethanol and acetic acid. For all studied strains, an inverse correlation between the rate of fructose consumption and sugar transport inhibition by ethanol was observed. Yeast strains where ethanol inhibited less than 50% of glucose transport displayed higher capacity to consume fructose. In the other hand, strains where ethanol inhibited more than 50% of glucose transport showed a lower capacity of fructose consumption. Furthermore, the authors examined the effect of ethanol on glucose

transport kinetics of exponential- and stationary-phase *S. cerevisiae* cells expressing glucose transporters Hxt1-7 individually. It was found that all these glucose transporters display different sensitivities to ethanol. While cells individually expressing the proteins Hxt1 and Hxt3 showed lower levels of inhibition of glucose transport by ethanol, cells expressing Hxt6 and Hxt7 displayed high sensitivity and stationary-phase cells expressing only Htx5 were the most resistant. Almeida & Pais (1996) and Alves-Araújo et al. (2004) described *T. delbrueckii* strains as being highly freeze tolerant, a property related to their capacity of preserving their membrane integrity. This property can be one reason why this yeast has a higher resistance to cell death induced by ethanol and acetic acid.

Chapter 2. Aims

2. Aims

Torulaspora delbrueckii is a non-conventional yeast with unique characteristics and great potential to be implemented in the biotechnological industry, mainly in bread and wine making. However, since the knowledge about this yeast is still limited, further investigation about this yeast's potential interesting features is required. Genetic manipulation and construction and analysis of mutant strains is, then, a crucial step for the characterization of this yeast. In contrast to *Saccharomyces cerevisiae*, in *T. delbrueckii* little is known about its glucose transporters and glucose transport capacity. However, it has been reported that *T. delbrueckii's* glucose transport is more sensitive to the inhibitory effects caused by ethanol than commercial strains of *S. cerevisiae.* This disadvantage limits its capacity to grow in media containing high concentrations of ethanol, one of the main end products of alcoholic fermentation. Thus, the present work aimed to characterize the repertoire of hexose transporters in *T. delbrueckii,* to manipulate its hexose transport and to evaluate the effect of ethanol in its glucose transport. In particular, the specific aims of this work were:

I. To identify the relevant transporters of *T. delbrueckii* during fermentation.

II. To manipulate the expression of glucose transporters in *T. delbrueckii*, in order to increase its growth rate and its fermentation capacity when in the presence of higher ethanol concentrations.

Chapter 3. Materials and Methods

3. Materials and Methods

3.1. Yeast Strains

Strains of *T. delbrueckii* used in this work are listed in **Table 2**. *S. cerevisiae* strains used were BY 4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*; EUROSCARF) EBY.vW 4000 (CEN.PK2-1C *hxt13*Δ *hxt15*Δ *hxt16*Δ *hxt14*Δ *hxt12*Δ *hxt9*Δ *hxt11*Δ *hxt10*Δ *hxt8*Δ *hxt514*Δ *hxt2*Δ *hxt367*Δ *gal2*Δ *snf3*Δ *stl1*Δ *agt1*Δ *ydl247w*Δ *yjr160c*Δ; (Wieczorke et al., 1999)), RE601A (*MATa hxt1 Δ::HIS3::Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::Δhxt6 hxt7::HIS3 ura3-52::Ylp5-HXT1*; (Reifenberger et al., 1995)) and RE603A (*MATa hxt1Δ::HIS3:: Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::Δhxt6 hxt7::HIS3 ura3-52::Ylp5-HXT3*; (Reifenberger et al., 1995)).

3.2. Bioinformatics Analysis

A protein BLAST analysis was conducted using, as query, the aminoacidic sequence of Lgt1 transporter protein (from *Torulaspora delbrueckii;* Accession number: AY598344.1), obtained from NCBI, against the genome of each of the 41 *T. delbrueckii* strains (deposited on NCBI) to find other similar proteins. BLAST was also performed to identify genes from other yeast species, as well as genes from *T. delbrueckii* CBS 1146 (type strain), having higher identity to each of the hexose transporter genes identified on the 41-strain set (**Table 2**). Furthermore, gene sequences of the hexose transporter genes and of the genes from other yeast species with high identity percentage were aligned using the MEGA11 software (Tamura et al., 2021) and cladograms were constructed using iTOL (Letunic & Bork, 2021).

Table 2. *T. delbrueckii* strain set available at CBMA. The 41 strains in the collection are categorized according to their origin: wine (11 strains), other beverages (2 strains), bread (4 strains), food (6 strains), water (3 strains), arboreal/soil (11 strains), clinical (1 strain) and unknown (3 strains).

Strain Code	Other Codes	Substrate of Isolation	Technological group
T01	ISA 1229	Wine fermentation, José Maria da Fonseca	Wine
T02	PYCC 3209	Potato starch factory	Unknown
T03	PYCC 2916	Rhagi	Other beverages
T04	PYCC 2477	Unknown	Unknown
T05	ISA 1549	Aestuary water from Guadiana River	Water
T08	PYCC 5323	Homemade corn and rye bread dough	Bread
T09	PYCC 5321	Homemade corn and rye bread dough	Bread
T11	V187/ TB193	Green beans	Food
T13	V393/ TB509	Artichoke	Food
T14	V405/TB522	Strawberry	Food
T15	MTF 1142	Grape berries	Wine
T19	MTF 3799	Fermenting grape juice (Sauvignon)	Wine
T22	MTF 3985	bakery	Bread
T23	MTF 3987	bakery	Bread
T26	MTF 4301	Green oak bark	Arboreal / Soil
T27	MTF 4303	Pedunculated oak bark	Arboreal / Soil
T28	MTF 4307	Pedunculated oak bark	Arboreal / Soil
T30	Zymaflore® Alpha	Wine	Wine
T34	EVN 1129	Grape must of portuguese wine Castelão	Wine
T35	EVN 1141	Grape must of portuguese wine Castelão	Wine
T36	EVN 1155	Grape must of portuguese wine Castelão	Wine
T38	NS-G-9	Grape must of Prieto Picu	Wine
T39	NS-G-62	Grape must of Prieto Picu	Wine
T40	NS-G-72	Grape must of Prieto Picu	Wine
T41	NS-PDC-169	Grape must of Prieto Picu	Wine
T42	PYCC 2478	Souring milk	Food
T43	PYCC 2713	Unknown	Unknown
T44	PYCC 2844	Skin lesion on 3-month-old girl	Clinical
T45	PYCC 2913	Sorghum brandy (kaoliang-chui)	Other beverages
T46	PYCC 2999	Coastal sea water (Florida, near Miami)	Water
T47	PYCC 4739	Coastal sea water (shore near Lisbon)	Water
T49	PYCC 6792	Cheese	Food
T50	PYCC 6819	Soil	Arboreal / Soil
T51	PYCC 7193	Fallen leaf from olive tree	Arboreal / Soil
T56	PYCC 8309	Olives washing water	Food
T57	PYCC 8413	Bark of *Quercus rubra*	Arboreal / Soil
T58	PYCC 8414	Bark of *Quercus acutissima*	Arboreal / Soil
T59	PYCC 8415	Bark of *Quercus velutina*	Arboreal / Soil
T60	PYCC 8416	Soil	Arboreal / Soil
T63	PYCC 8419	Soil	Arboreal / Soil
T64	PYCC 8420	Soil underneath *Quercus petraea*	Arboreal / Soil

3.3. Growth Conditions and Media Composition

Yeast strains were grown in liquid YPD medium [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] and maintained on solid YPDA plates [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose, 2% (w/v) agar] until use. For the construction of growth curves, cells were grown overnight in 10 mL of YPD medium, until reaching an OD_{640nm} = 0.8 and then diluted to an OD_{640nm} = 0.1 in 10 mL of fresh medium without or with 8% (v/v) ethanol in conical tubes. Cell growth was monitored for up to 30 hours. Yeast strains containing the pTA6 plasmid backbone were selected and grown in liquid and solid YPD medium containing 200 µg/mL of geneticin (G418) (Formedium™). Strains containing the pRS316 plasmid backbone were selected and grown in selective medium lacking uracil [0.5% (w/v) $(NH_4)_2SO_4$, 0.17 % (w/v) YNB without amino acids, 0.14% (w/v) drop-out, 2% (w/v) glucose, 0.04% (w/v) leucine, 0.008% (w/v) histidine, 0.008% (w/v) tryptophan, 0.1% (w/v) proline, 2% (w/v) agar]. Growth in liquid media of all yeast strains was performed at 30 °C and 200 rpm in an orbital shaker. Competent *E. coli* XL-21 Blue cells and competent *E. coli* NZY5α cells (NZYtech) were grown in liquid or solid LB medium [0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl, 2% (w/v) agar] supplemented with 100 µg/mL of ampicillin (Formedium™). Growth in liquid media of both bacterial strains was performed at 37 °C and 200 rpm.

3.4. Plasmids

Genes encoding the *S. cerevisiae* glucose transporters Hxt1 and Hxt3 (*ScHXT1* and *ScHXT3*) were cloned in plasmid pTA6_TEF1_linker_yEGFP_RPL22A, provided by Prof. Björn Johansson. Genes coding the *T. delbrueckii* PYCC 5321 glucose transporters Lgt1, Igt1, TDEL_0E02280 and TDEL_0E022890 (*TdLGT1*, *TdIGT1*, *Td2238*, *Td2239*) were cloned in plasmid pRS316-*GFP*, constructed from pRS316 in this work.

3.4.1. Construction of pRS316-*GFP*

The pRS316 plasmid (Lorenz et al., 1995) was linearized using HindIII (New England BioLabs) and XhoI (New England BioLabs) restriction enzymes. The digestion was performed,

first overnight, at 37 °C with HindIII, and then for 1 hour at 37 °C with XhoI. *GFP* was amplified by PCR from the plasmid pTA6_TEF1_ linker_yEGFP_RPL22A with NZYProof 2x Green Master Mix (NZYtech), using the primers 1 and 2 listed in **Table 3**.

S. cerevisiae BY 4741 cells were transformed with 5 ng pRS316 plasmid and 15 ng of the PCR product according to the protocol described in Section 3.5. Cells were plated on selective medium lacking uracil and incubated for 2 days. Positive transformants were selected by colony PCR, using primers 2 and 3 listed in **Table 3** and DreamTaq DNA polymerase (ThermoFisher Scientific).

Plasmid DNA from a positive clone was extracted with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions, preceded by a step of yeast cell wall disruption using glass beads, and then quantified using the Nanodrop (ND-1000 Spectrophotometer). 1 ng of the positive clone selected was transformed in *E. coli* NZY5α competent cells (NZYTech) by heat shock, following the manufacturer's protocol. Positive transformants were again selected by colony PCR, using primers 2 and 3, listed on **Table 3** and DreamTaq DNA polymerase (ThermoFisher Scientific). Plasmid DNA was then extracted with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions.

3.4.2. Cloning of Transporter Genes

3.4.2.1. Genomic DNA Template Preparation

S. cerevisiae BY 4741 cells or *T. delbrueckii* PYCC 5321 cells were grown overnight to a 1 < OD_{640nm} < 2. Cells were harvested, washed twice, and resuspended in 1 M sorbitol supplemented with 100 mM EDTA pH 7.5. For cell wall digestion, 20 mg/mL of Zymolyase (Grisp Research Solutions) was added. The suspension was then incubated at 37 °C for 20 minutes, Tris-HCl containing 20 mM EDTA pH 7.4, 10% SDS was added to the spheroplasts, and the suspension was incubated at 65 °C for 5 minutes. The cells were then collected by centrifugation at maximum speed for 30 minutes at 4 °C, the resulting supernatant was transferred to new Eppendorf tubes and 1 volume of isopropanol was added. The suspension was incubated at room temperature for 5 minutes, then centrifuged at maximum speed for 15 minutes at 4 °C and washed twice with 70% (v/v) ethanol and once with 100% (v/v) ethanol.

Finally, the pellets were dried at room temperature and resuspended in TE. The genomic DNA was then quantified on the Nanodrop (ND 1000 Spectrophotometer) and stored at -20 °C.

3.4.2.2. Amplification of Genes Encoding Transporter Proteins

Sc*HXT1* and Sc*HXT3* were amplified from *S. cerevisiae* BY 4741 genomic DNA and *TdLGT1*, *TdIGT1*, *Td2238*, *Td2239* from *T. delbrueckii* genomic DNA by Polymerase Chain Reaction, using primer pairs 4/5, 6/7, 8/9, 10/11, 12/13, and 14/15, respectively (**Table 3**) with Supreme NZYProof 2x Green Master Mix (NZYtech).

Table 3. Primers used for gene amplification and colony PCR confirmations. All primers were designed in the scope of this work.

Number	Name	Sequence (5' → 3')
1	pRS316_linker_Fw	CTGCAGGAATTCGATATCAAGCTTGAAGGTAAATCTTCTGGTTCTGGTTCTG
2	pRS316_Tterm_Rv	GGGTACCGGGCCCCCCCTCGAGTGTTTCTTAATCTGTTGTTTTGGTGGTTTA
3	T7 fw	GAAATTAATACGACTCACTATAGG
4	HXT1_PTA6_Fw	ACTTTCTCACTAGTGACCTGCAGCCGACAATGAATTCAACTCCCGATCTAATATCTCC
5	HXT1_PTA6_Rv	TTCAGAACCAGAACCAGAAGATTTACCTTCGACGTCCCCGGGTTTCCTGCTAAACAAACTCTTGTAAAATG
6	HXT3_PTA6_Fw	CTTTCTCACTAGTGACCTGCAGCCGACAATGAATTCAACTCCAGATTTAATATCTCCAC
7	HXT3_PTA6_Rv	TTCAGAACCAGAACCAGAAGATTTACCTTCGACGTCCCCGGGTTTCTTGCCGAACATTTTCTTGTAGAAT
8	pRS316_LGT1_Fw	AGCTCCACCGCGGTGGCGGCCGCGGGATGGTTGTTGGATATTATTCACG
9	pRS316_LGT1_linker_Rv	CAGAAGATTTACCTTCAAGCTTTTTGGAGAAAAATCTCTTGTACATTGGC
10	pRS316_IGT1_Fw	AGCTCCACCGCGGTGGCGGCCGCCTGCAAGAACTGCCTCTCCAAG
11	pRS316_IGT1_linker_Rv	AGAAGATTTACCTTCAAGCTTGTTTCTGGAGAAAAGTCTCTTGTACATTG
12	pRS316_2238_Fw	TTGGAGCTCCACCGCGGTGGCGGCCGCATTTGTTACGCCCAAGTCCTACG
13	pRS316_2238_linker_Rv	CAGAAGATTTACCTTCAAGCTTATTTCTACGCAGCATTCTTTTGTAGAAG
14	pRS316_2239_Fw	AGCTCCACCGCGGTGGCGGCCGCAAACTCAAAATCGCAAGATCTAGTAGG
15	pRS316_2239_linker_Rv	GAAGATTTACCTTCAAGCTTGTTCTTACCAAACATTCTCTTGTAAAATGG

3.4.3. Vector Preparation

Plasmid pTA6_TEF1_ScCYC1_lnk_yEGFP_RPL22A was linearized with SmaI (New England BioLabs) for 15 minutes at 25 °C. pRS316_*GFP* was linearized, first overnight with HindIII (New England BioLabs), and then with NotI (New England BioLabs) for 1 hour.

3.4.4. Gap Repair

S. cerevisiae BY 4741 cells were transformed with linearized pRS316_*GFP* and PCR products from the amplification of each the 4 transporters and plated on selective medium lacking uracil. *S. cerevisiae* BY 4741 and *S. cerevisiae* EBY.vW 4000 cells were transformed with linearized pTA6_TEF1_linker_yEGFP_RPL22A and PCR products from the amplification of *HXT1* and *HXT3* genes and plated on YPD plates containing geneticin. Transformants were then visualized by epifluorescence microscopy to select positive clones. DNA from cells expressing pTA6_HXT1 and pTA6_HXT3 was purified and transformed into 100 µL of NZY5α *E. coli* cells for amplification, but no colonies were obtained.

3.5. Yeast Transformation

A single yeast colony was grown overnight, diluted in fresh 2x glucose YPD medium [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 4% (w/v) glucose] to an OD_{640nm} = 0.2 and grown in the same conditions until reaching an $OD_{640\ nm}$ = 0.6 – 0.8. The cells were collected, centrifuged, washed twice, and resuspended in sterile water. The cell suspension was distributed into one Eppendorf for each transformation, centrifuged, resuspended in transformation mix (as presented in **Table 4**), and incubated in the water bath for 40 minutes at 42 °C. Cells were then pelleted at maximum speed for 1 minute, resuspended in YPD medium, incubated for approximately 3 hours, plated, and incubated at 30 °C for 2 days. Controls without DNA, with the empty non-linearized vector, and with the linear vector but without insert were performed in each experiment.

Table 4. Transformation mix.

Reagents	Negative control (w/o plasmid and PCR products) (µL)	Control 1 and Control 2 * (µL)	Transporters (µL)
PEG 3350 (50%)	250	250	250
Lithium Acetate (LiAc) (1M)	36	36	36
Boiled ssDNA (carrier 10 mg/ml)	50	50	50
H_2O	34	29	14
Plasmid DNA	-	5	5
PCR product	-	-	15

* Control 1 (w/ non-linearized plasmid and w/o PCR products) and Control 2 (w/ linearized plasmid and w/o PCR products.

3.6. Bacterial Transformation

100 to 200 ng of plasmid DNA was added to *E. coli* competent cells and incubated on ice for 30 minutes, followed by a heat shock in a water bath (42 °C for 45 seconds), and another incubation on ice for 10 minutes. SOC medium [2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ 20 mM glucose] was added, and the cells were incubated for 1 to 3 hours. Cells were then collected by centrifugation, part of the supernatant was discarded, cells were resuspended in the remaining supernatant. Finally, cells were plated and incubated overnight. A negative control without plasmid DNA, and a control with uncut plasmid were performed.

3.7. Fluorescence Microscopy

Cells were visualized with a Leica Microsystems DM-5000B microscope with a 100x oil immersion objective. Images were obtained with a Leica DFC350 FX Digital Camera and processed with LAS X Microsystems software.

3.8. Expression Analysis

The expression levels of *LGT1, IGT1, TDEL_0E02280* and *TDEL_0E02290* were assessed by real-time PCR (RT-PCR). RNA from *T. delbrueckii* PYCC 5321 was extracted using TRIzol/chloroform and RNA Clean & Concentrator-5 Kit (Zymo Research). Cells were grown overnight in YPD medium until an OD_{640nm} = 0.8, the culture was diluted in fresh medium to an OD_{640nm} = 1, and cells collected after 1, 7, 12 and 24 hours and stored at -80 °C. Cells (equivalent to 5 mL at an OD_{640nm} = 1) were then pelleted by centrifugation, resuspended in 0.75 mL of TRIzol, and incubated for 5 minutes. 0.2 mL of Chloroform was added, and the suspension was incubated at room temperature for another 5 minutes. The samples were centrifuged at 12000 xg, 4 °C for 15 minutes, after which the mixture separates into 3 phases. The aqueous phase containing the RNA was transferred to new Eppendorf tubes and purified using the RNA Clean & Concentrator-5 Kit (Zymo Research), following the manufacturer's instructions. RNA was then quantified and stored at -80 °C. cDNA was synthesized by reverse transcription from 500 ng of RNA, using iScript™ cDNA Synthesis Kit (Bio-Rad laboratories), according to the manufacturer's instructions. The RT-PCR reactions were performed using the primers 1—10 listed on **Table 5** and KAPA SYBR ® FAST qPCR Master Mix (2x) Kit (Sigma-Aldrich), following the manufacturer's instructions, in an CFX96TM Real-Time System C100TM Thermal Cycler (Bio-Rad laboratories). Quantification was performed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and expression of the genes was normalized to *ACT1*, encoding actin (a structural protein in cytoskeleton).

Table 5. Primers used for Real Time-PCR reactions. All primers were designed in the scope of this work.

Number	Name	Sequence (5' → 3')
1	lgt1_fw	CACGTTACCTGGTCGAGCA
2	lgt1_rv	CACCAGTCAATTGTTGTAGAGATTGAAG
3	igt1_fw	CACGTTACTTAGTTGAAGTTGGTAACTTC
4	igt1_rv	TCTTGGTAGAGAACAATTCACCGAT
5	2238_fw	GTTTCTTCACTCCTTTCATCACATCC
6	2238_rv	CAAGGCAAGACACGTTCTTCAT
7	2239_fw	CCAGCTATGTGCAGACGTACTATC
8	2239_rv	CTTGTCAACAGTGTAAAGAGACAAAGC
9	ACT1_fw	TGGTTACTCCTTCTCCACTACT
10	ACT1_rv	GCAGCGGTTTGCATTTCTT

Chapter 4. Results

4. Results

4.1. Evaluation of the Growth Rate of *T. delbrueckii* strains

The specific growth rate of a microorganism is the rate at which a cell population increases its biomass. Therefore, in the biotechnological industries where the goal is to obtain cell biomass, such as in the industrial production of baker's yeast, it is important to use strains with high values of specific growth rates. It has been reported that different *T. delbrueckii* strains display variable values of specific growth rates when growing in YPD medium. For this reason, we constructed growth curves, in YPD medium, for each one of the 41 strains in the *T. delbrueckii* collection (**Table 2**). **Appendix 1 – Figure 1** illustrates the exponential growth phase of each strain, based on which the growth rate was calculated. **Table 6** lists the specific growth rates of the 41 strains, ordered from the lowest to the highest values. There is a variability in the specific growth rates, with the lowest being 0.22 h^{-1} (T49) and the highest 0.45 h^{-1} (T58, T44, T59), which in general don't seem to be related with the origin of the strains (also represented on **Table 6**). In general, strains from Arboreal/Soil origins have higher specific growth rates. Wine strains have very similar growth rates (0.37 – 0.38 h^{-1}), except for 4 strains whose specific growth rates are higher. Strains from other origins have very variable specific growth rate values.

Table 6. Specific growth rate of the 41 *T. delbrueckii* strains grown on YPD medium. Strains origins are represented in the first column: Pink – Wine; Black – Unknown; Blue – Other Beverages; Turquoise – Water; Yellow – Bread; Orange – Food; Green – Arboreal/Soil; Red – Clinical.

Strain/Origin	Specific Growth Rate (h^{-1})	Strain/Origin	Specific Growth Rate (h^{-1})
T49	0.22 ± 0.05	**T28**	0.38 ± 0.06
T45	0.23 ± 0.07	**T60**	0.39 ± 0.02
T42	0.28 ± 0.05	**T11**	0.39 ± 0.08
T22	0.29 ± 0.07	**T14**	0.39 ± 0.08
T47	0.31 ± 0.08	**T30**	0.40 ± 0.03
T04	0.31 ± 0.08	**T43**	0.40 ± 0.04
T02	0.31 ± 0.06	**T01**	0.41 ± 0.05
T46	0.32 ± 0.06	**T63**	0.42 ± 0.04
T56	0.33 ± 0.02	**T50**	0.42 ± 0.05
T09	0.35 ± 0.05	**T03**	0.42 ± 0.04
T23	0.36 ± 0.03	**T05**	0.43 ± 0.04
T13	0.36 ± 0.04	**T40**	0.43 ± 0.03
T15	0.37 ± 0.04	**T34**	0.43 ± 0.03
T57	0.37 ± 0.03	**T08**	0.43 ± 0.04
T39	0.37 ± 0.04	**T64**	0.44 ± 0.01
T41	0.37 ± 0.04	**T26**	0.44 ± 0.04
T38	0.38 ± 0.08	**T27**	0.44 ± 0.05
T35	0.38 ± 0.07	**T58**	0.45 ± 0.06
T51	0.38 ± 0.03	**T44**	0.45 ± 0.03
T36	0.38 ± 0.08	**T59**	0.45 ± 0.01
T19	0.38 ± 0.04		

4.2. Characterization of the Repertoire of Hexose Transporters in *T. delbrueckii*

In *S. cerevisiae*, there are 20 characterized genes related to hexose transport, which encode proteins whose expression is regulated by the presence and concentration of their substrate in the environment. This set of transporters with different affinities allows *S. cerevisiae* cells to respond and adapt to changes that occur in their natural environment. Taking this into consideration, we aimed to understand if *T. delbrueckii*'s genome, like *S. cerevisiae*'s, possesses several encoded transporters with different properties. For this purpose, the aminoacidic sequence of the Lgt1 protein, enconding a glucose transporter from *T. delbrueckii*, obtained from NCBI, was used as a query to perform a protein BLAST against

the translated genome of each strain in the *T. delbrueckii* collection, deposited on NCBI. This analysis indicated that most strains encode 6 (24 strains), but there were also strains encoding 5 (7 strains) and 7 (10 strains) possible hexose transporters, as illustrated in **Figure 2**. It is to be noted that 10 out of the 11 Arboreal/Soil strains encode 6 transporters. However, in general, the number of transporters encoded by each strain does not seem to be related to their origin, since it varies between strains belonging to the same biotechnological group (**Appendix 1 – Table 1**).

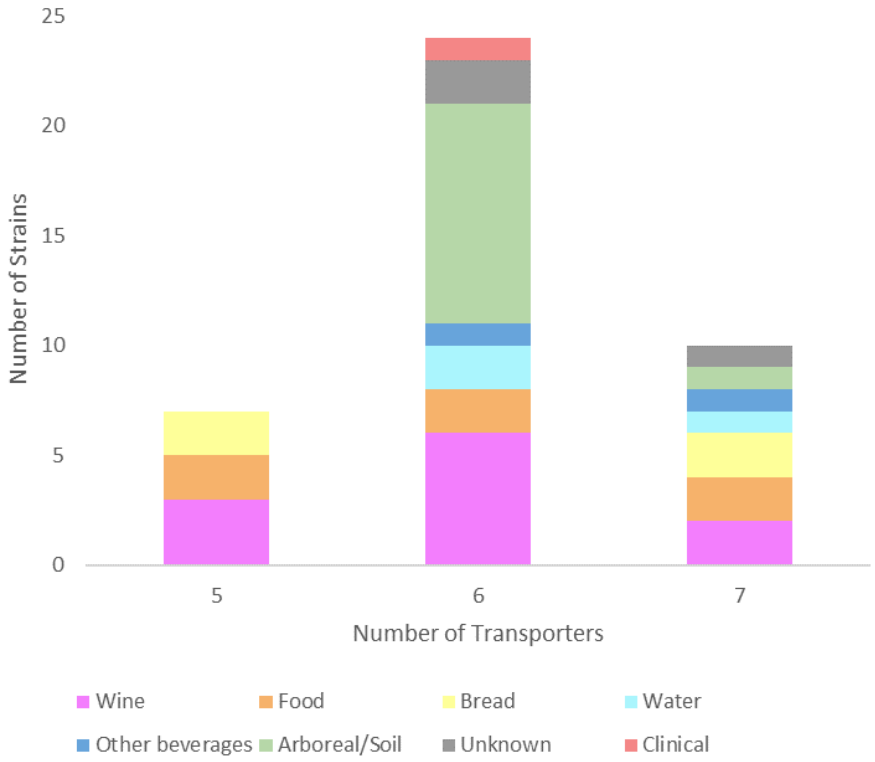


Figure 2. Number and origin of strains encoding 5, 6 and 7 transporters and respective origins. BLAST analysis, using Lgt1 aminoacidic sequence as a query, was performed to find out how many transporters are encoded in the genome of each of the 41 strains of *T. delbrueckii* in the collection.

In order to ascertain their similarity, the aminoacidic sequences of the different transporters encoded by all the studied *T. delbrueckii* strains were aligned and a cladogram was contructed. Based on the organization of the genes in the cladogram branches, groups were formed with different colours attributed, as illustrated in **Figure 3**. It is important to refer that one of the proteins encoded by *T. delbrueckii* PYCC 2913 (T45), identified as 4982 in this

strain, does not appear in the cladogram. The reason for this is the fact that the aminoacidic sequence of this gene didn't align with one of the other proteins. However, another cladogram (data not shown) was constructed substituting these proteins, which allowed to include 4982 from T45 in the *LGT1* branch.

Complementarily, a BLAST analysis was performed with the goal of finding the proteins from *T. delbrueckii* CBS 1146 (type strain) with higher identity percentage to all the proteins identified previously in all the strains of the *T. delbrueckii* collection. These proteins are represented on **Figure 3**, as well as in **Appendix 1 – Figure 2**. Proteins belonging to group represented in Green/*IGT1* have the highest identity percentage to TDEL_0E02300, in Red/*LGT1* to TDEL_0E02310, yellow to TDEL_0E02290, blue to TDEL_0E02280, orange and purple to TDEL_0A04250, pink and turquoise to TDEL_0C00150; brown to TDEL_0A00420, and, finally, grey to TDEL_0E00200. Furthermore, a second BLAST analysis was performed to find which of the *S. cerevisiae* transporters have higher identity percentage to the all the proteins identified on the *T. delbrueckii* strains. Proteins identified in this analysis are illustrated in **Figure 3.**

For each group of proteins formed based on sequence similarity (**Figure 3**) a cladogram was constructed with the goal of understanding if strains belonging to the same biotechnological group have similar aminoacidic sequences. **Appendix 1 – Figure 3** represents the cladograms resulting from this analysis. In general, proteins encoded by strains with the same origin don't seem to have a particularly close similarity. However, an exception are Arboreal/Soil and Wine strains, which appear in close positions in the trees.

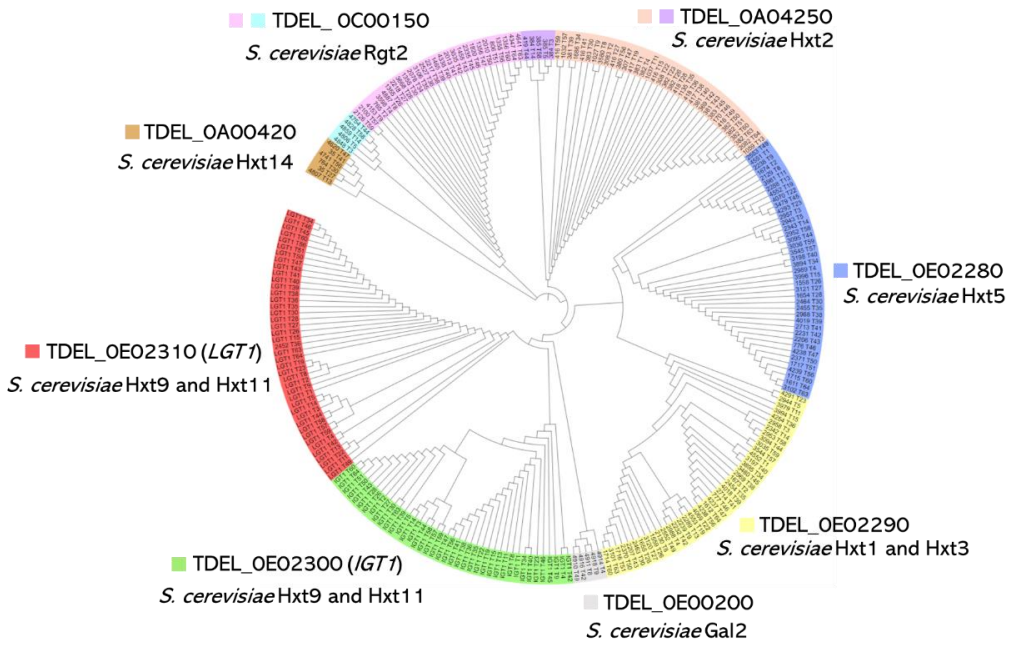


Figure 3. Cladogram representing the similarities between the aminoacidic sequences of the transporters from the 41 strains in the *T. delbrueckii* collection. After the alignment of the sequences from proteins identified as possible transporters in the different *T. delbrueckii* strains, a cladogram was constructed. Groups were formed taking into consideration the distribution of the proteins in the main branches of the cladogram, i.e., according to their sequence similarity. Groups are represented by different colours. Proteins from *T. delbrueckii* CBS 1146 and *S. cerevisiae* with highest identity percentage to the proteins from all strains of the *T. delbrueckii* collection.

4.3. Analysis of the Transporters encoded by *T. delbrueckii* PYCC 5321

4.3.1. Bioinformatics Analysis of *T. delbrueckii* PYCC 5321 transporters

LGT1 and *IGT1* were first characterized in *T. delbrueckii* PYCC 5321. Thus, this strain was selected to be further analysed in relation to its hexose transporters. Aiming to find out which are the proteins with the highest sequence similarity to each other, the aminoacidic sequences from the 7 putative transporters encoded by this strain were aligned and a cladogram was constructed. As illustrated on **Figure 4,** *IGT1* and *LGT1* as well as 2238 (TDEL_0E02280) and 2239 (TDEL_0E02290) are the most similar to each other, respectively. Together, these are the 4 most conserved hexose transporters in this strain. Furthermore, BLAST analysis allowed to identify the transporters from *S.*

cerevisiae with highest identity percentage to each gene from *T. delbrueckii* PYCC 5321, which are also represented in **Figure 4.**

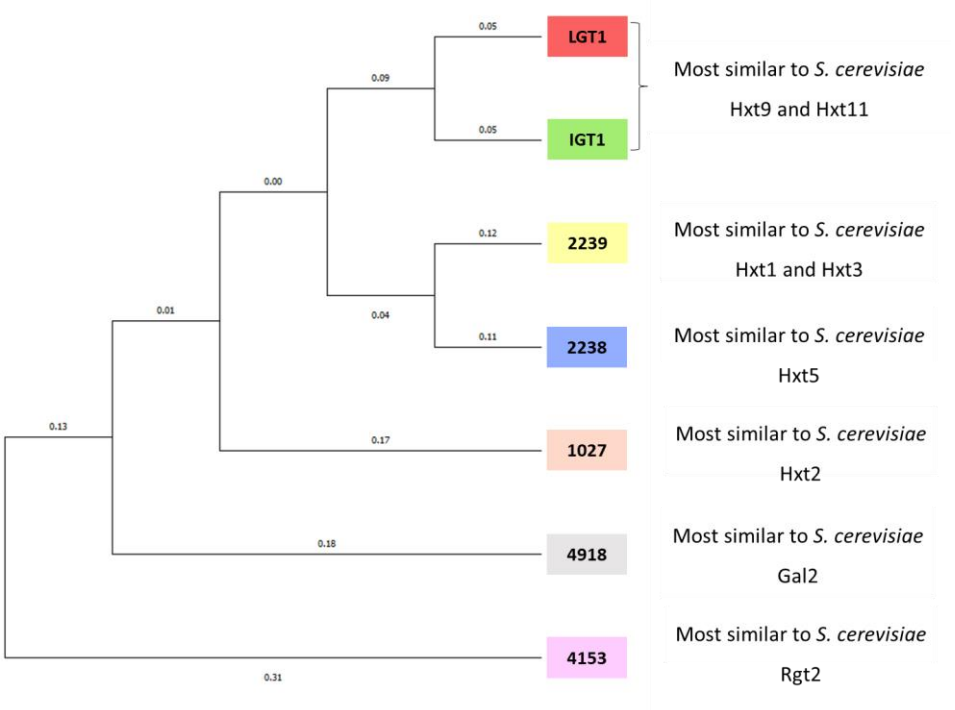


Figure 4. Cladogram representing *T. delbrueckii* PYCC 5321 transporters and the corresponding *S. cerevisiae* transporters with the highest similarity.

4.3.2. Characterization of the 4 Most Conserved Transporters in *T. delbrueckii* PYCC 5321

From the 4 most conserved hexose transporters in PYCC 5321, only *LGT1* and *IGT1* were previously characterized. Therefore, the next step was to determine whether the predicted transporters TDEL_0E02280 (2238, on **Figure 4**) and TDEL_0E02290 (2239, on **Figure 4**) are functional. First, the expression levels of all 4 transporters were assessed by real-time PCR (RT-PCR), after 1, 12 and 24 hours of growth in YPD medium, corresponding to the lag, log and stationary growth phases. Preliminary results of this analysis are represented in **Figure 5.** Since *LGT1* expression was always detected and levels did not vary significantly in relation to the 1h time point (not shown), expression of the other genes are shown relative to *LGT1*, all previously normalized to *ACT1*.

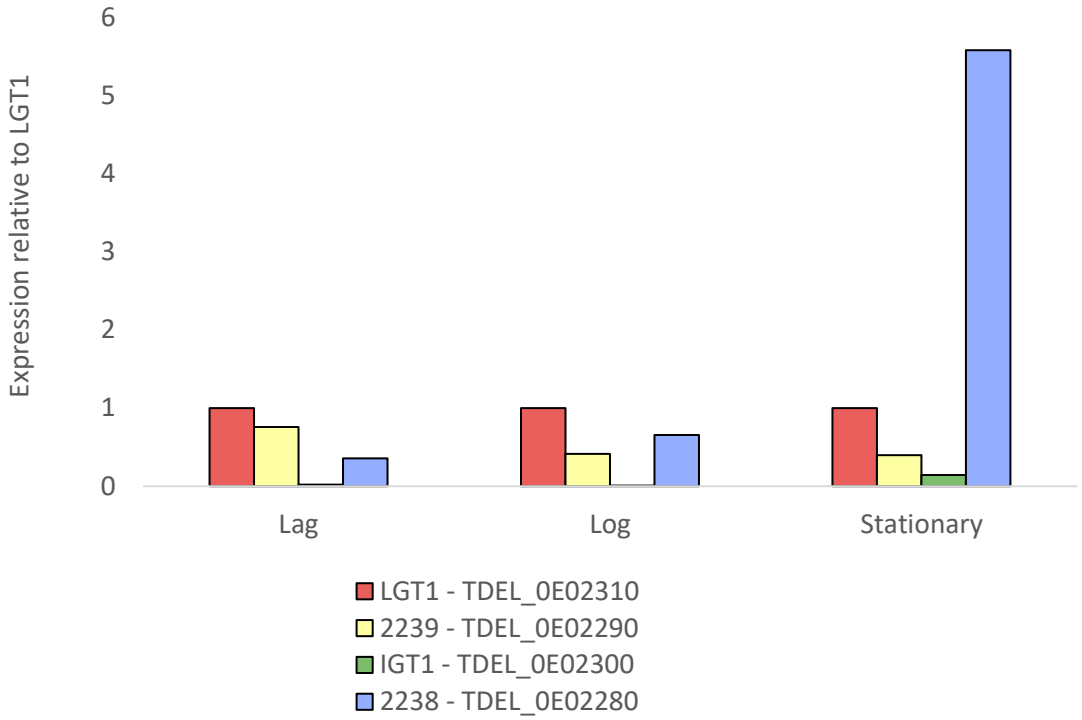


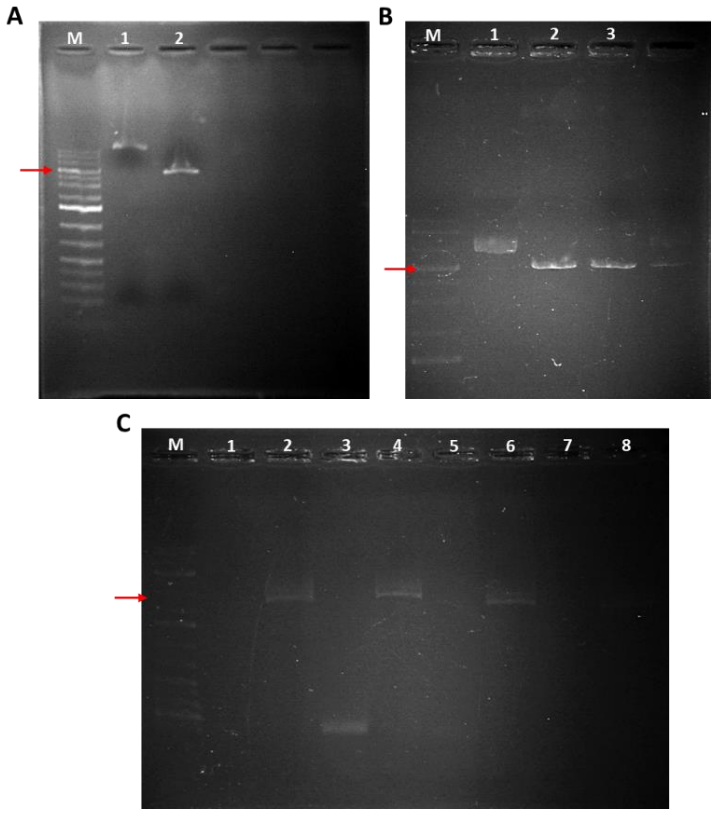
Figure 5. Expression levels of *LGT1, IGT1*, TDEL_0E02280 and TDEL_0E02290 in the lag, log and exponential phases of growth. Cells from this strain were cultured overnight in Erlenmeyer flasks, in YPD medium, until reaching a OD_{640nm} = 0.8 and then diluted to a OD_{640nm} = 0.1. RNA was extracted after 1, 12 and 24 hours of growth, corresponding to the lag, log and exponential growth phases. cDNA was then synthesized by reverse transcription and the RT-PCR reactions were carried out for the indicated genes and *ACT1* and normalized to *LGT1.*

All genes were expressed, with *LGT1* and TDEL_0E02290 showing similar expression patterns with low variation. In contrast, expression of *IGT1* and TDEL_0E02280 increased in stationary phase, with the latter increasing significantly.

4.3.3. Cloning of Transporter Genes

After confirming the expression of all the 4 most conserved transporters in *T. delbrueckii* PYCC 5321, we aimed to express them individually in a *S. cerevisiae hxt* null strain in frame with *GFP.* For that purpose, *GFP* was first inserted in the pRS316 centromeric plasmid by gap repair. pRS316 was initially linearized using HindIII and XhoI restriction enzymes, and digestion was confirmed (**Figure 6. A**). The plasmid pTA6_TEF1_linker_yEGFP_RPL22A was then used as template for the amplification of *GFP* flanked by pRS316 homology sequences by PCR. *S. cerevisiae* BY 4741 cells were then

transformed with both the digested plasmid and the PCR product. Cells were able to grow in selective medium lacking uracil. The plasmid DNA was then extracted from positive clones. pRS316-*GFP* was then linearized with HindIII and NotI, and digestion was confirmed (**Figure 6. B**). Genes encoding the 4 *T. delbrueckii* transporters (coding sequence plus upstream endogenous promoter) were amplified by PCR reaction from genomic DNA of PYCC 5321 (**Figure 6. C**). The digested pRS316-*GFP* plasmid and the PCR products resulting from the amplification of the 4 transporters were used to transform *S. cerevisiae* BY 4741 for cloning purposes. However, resulting colonies able to grow on media lacking uracil did not display any fluorescence above background. Cloning of the coding sequences under the control of the *S. cerevisiae TPI* promoter is thus ongoing.



F**igure 6.** Cloning of *LGT1, IGT1*, 2238 and 2239. **A.** Digestion of pRS316 with HindIII and XhoI, visualized on an 1% agarose gel under UV light. M – Molecular marker: GeneRuler 1kb Plus DNA Ladder (ThermoFisher Scientific); 1 – Control digestion reaction, without restriction enzymes; 2 – Digested plasmid. **B.** Digestion of pRS316-*GFP* with HindIII and NotI, visualized on an 1% agarose gel under UV light. M – Molecular marker: GeneRuler 1kb Plus DNA Ladder (ThermoFisher Scientific); 1 – Control digestion reaction, without restriction enzymes; 2 and 3 – Digested plasmid. **C.** PCR product of the amplification of *IGT1, LGT1*, 2238 (TDEL_0E02280) and 2239 (TDEL_0E02290) from *T. delbrueckii* PYCC 5321 genomic DNA, visualized on an 1% agarose gel under UV light. M – Molecular marker: GeneRuler 1kb Plus DNA Ladder (ThermoFisher Scientific); 1, 3, 5 and 7 – Control PCR reactions, without the primers for amplification of *IGT1, LGT1*, 2238 (TDEL_0E02280) and 2239 (TDEL_0E02290), respectively; 2, 4, 6 and 8 – Amplified *IGT1, LGT1*, 2238 (TDEL_0E02280) and 2239 (TDEL_0E02290), respectively.

4.4. Manipulation of glucose transport in *T. delbrueckii*: Cloning of *S. cerevisiae*'s *Hxt1* and *Hxt3* transporter proteins

To test if altering transporter levels could alter the ability *T. delbrueckii* PYCC 5321 to grow in media containing ethanol, the growth of PYCC 5321, a mutant strain not expressing Lgt1 or overexpressing the same protein was assessed in medium containing ethanol and their specific growth rate was calculated (**Figure 7 and Table 7**). Specific growth rates of strains growing in the presence of ethanol were significantly lower than those of the ones growing in medium without ethanol, but no differences were observed between the strains.

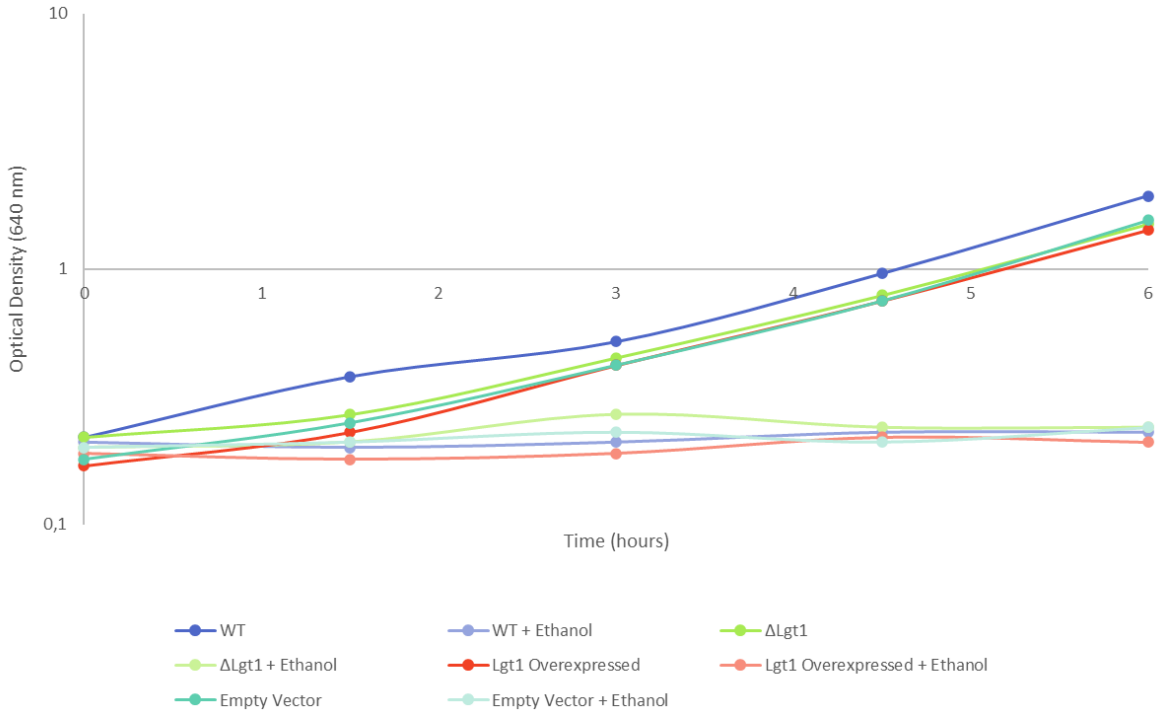


Figure 7. Exponential phase of growth curves of *T. delbrueckii* PYCC 5321 (WT), *T. delbrueckii* PYCC 5321 not expressing *LGT1* (*lgt1*Δ), and overexpressing the same gene, as well as the same strain transformed with the same empty vector in YPD medium and YPD medium containing 8% (v/v) ethanol. Strains were grown overnight in 10 mL of YPD to a OD_{640nm} = 0.8, and then diluted to a OD_{640nm} = 0.1 in 10 mL of YPD without or with ethanol. The growth of each strain was assessed in timepoints of 1.5 hours, for approximately 30 hours, and their growth curves were constructed.

Table 7. Specific growth rates of *T. delbrueckii* PYCC 5321 (WT), *T. delbrueckii* PYCC 5321 not expressing *LGT1* (Δ*lgt1*), and overexpressing the same gene, as well as the same strain transformed with the same empty vector in YPD medium and YPD medium containing 8% (v/v) ethanol.

Strain	Specific Growth Rate (h^{-1})	
	Absence of Ethanol	Presence of 8% (v/v) Ethanol
WT	0.35	0.02
Δ*lgt1*	0.33	0.03
***LGT1* Overexpressed**	0.36	0.03
Empty Vector	0.36	0.02

To test if *T. delbrueckii* PYCC 5321 could grow better in medium containing ethanol, if it expressed high levels of the *S. cerevisiae* transporters most resistant to ethanol, the next step was to clone *HXT1* or *HXT3*, in frame with *GFP*, under the control of a strong promoter. First, *HXT1* and *HXT3* were amplified from the genomic DNA of *S. cerevisiae* BY 4741 flanked by homology regions to pTA6-yEGFP (**Figure 8. A)**, and plasmid pTA6_yEGFP was linearized with SmaI restriction enzyme **(Figure 8. B)**. *S. cerevisiae* BY 4741 and EBY.vW4000 (*hxt* null) cells were then transformed both with the amplified transporter genes and the digested plasmid. Both strains were able to growth in plates with YPD containing geneticin, and positive clones were selected by fluorescence microscopy. **Figures 8. C** and **8. D** illustrate positive clones of *S. cerevisiae* BY 4741 and *hxt* null, respectively. Membrane localization of the genes tagged with *GFP* can be observed.

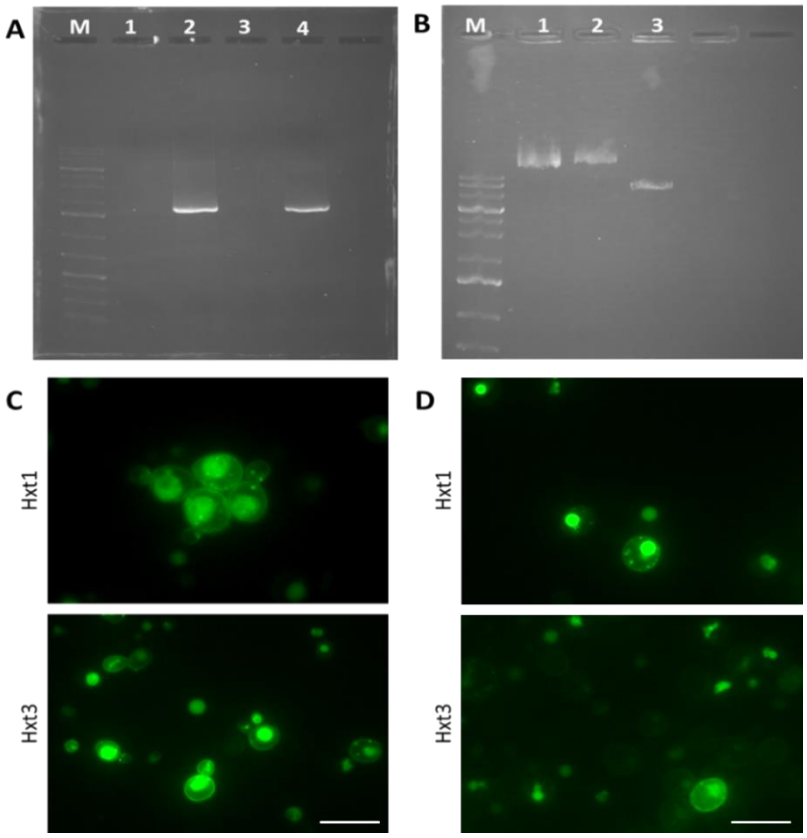


Figure 8. Cloning of *HXT1* and *HXT3*. **A.** PCR product from *HXT1* and *HXT3* amplification ran on an 1% agarose gel visualized under UV light. M – Molecular marker: GeneRuler 1kb Plus DNA Ladder (ThermoFisher Scientific); 1 and 3– Control PCR reaction without primers to amplify *HXT1* and *HXT3*, respectively; 2 and 4 – *HXT1* and *HXT3* amplified with the respective primers. **B.** Digestion of pTA6_yEGFP with SmaI, visualized on an 1% agarose gel under UV light. M – Molecular marker: GeneRuler 1kb Plus DNA Ladder (ThermoFisher Scientific); 1 – Undigested plasmid; 2 – Control digestion reaction, without restriction enzyme; 3 – Digested plasmid. **C.** *S. cerevisiae* BY 4741 cells expressing *HXT1* and *HXT3* genes tagged with *GFP*. **D.** *S. cerevisiae hxt* null cells expressing *HXT1* and *HXT3* genes tagged with *GFP*. Bar, 5 µm.

Both *Hxt1-GFP* and *Hxt3-GFP* were expressed at high levels, from the strong TPI promoter in pTA6. Moreover, a membrane localization was clearly observed, as expected. The functionality of *Hxt1-GFP* and Hxt3-*GFP* was then confirmed. For that purpose, randomly selected positive clones of the *hxt* null strain expressing *HXT1-GFP* or *HXT3-GFP* were grown liquid YPD medium with 2% (w/v) glucose and their growth was followed. For comparison, mutant strains *S. cerevisiae* RE601A and RE603A, which express only Hxt1 or Hxt3 (respectively), from the Hxt1-7 transporters, were also grown in the same conditions. Specific growth rates were calculated based on the exponential growth phase of each strain. For strains transformed with *HXT1-GFP* and *HXT3-GFP*, specific growth rates were approximately 0.31 h^{-1} and 0.32 h^{-1}, respectively. For strains RE601A and RE603A, growth rates were slightly

higher, both approximately 0.36 h^{-1}. The strain transformed with the empty vector was not able to grow in this medium.

Plasmid DNA from positive clones *of S. cerevisiae* BY 4741 transformed with both transporter genes was extracted, transformed in competent *E. coli* cells, but no colonies were obtained in several attempts. Another method for plasmid recovery is currently under optimization.

Chapter 5. Discussion

5. Discussion

The quality of yeasts used in industrial processes is determined by several parameters that make them adequate for different applications, such as storage stability, osmotolerance, freeze-thaw resistance, among others (Van Hoek et al., 1998). High fermentative capacity and specific growth rates are particularly important (Van Hoek et al., 1998), being essential parameters to consider when choosing a yeast strain for an industrial process. In fact, they impact the duration and the yield of the production processes, especially when considering growth-associated products (Srivastava & Gupta, 2011). *T. delbrueckii* strains have been getting increasing attention in the biotechnological industry, mainly for their unique features that make them appealing candidates to use in wine and bread making. It has been reported that strains belonging to this species present different capacities to ferment and assimilate carbon compounds (Kurtzman, 2011). Thus, the assessment of the variability of the specific growth rates of *T. delbrueckii* strains is important to understand the biotechnological potential of this species. For this purpose, the specific growth rates of 41 *T. delbrueckii* strains were calculated and **Table 6** summarizes the results obtained. It is possible to observe a wide range of values for this parameter among the tested strains, being the lowest 0.22 h^{-1} and the highest 0.45 h^{-1}. In general, the specific growth rate does not seem to be related to the origin, since strains from the same biotechnological group present variable values for this parameter. Nevertheless, the 11 strains belonging to the Arboreal/Soil group showed the highest average specific growth rate (0.42 h^{-1}) when compared to the other groups. The Clinical isolate group only includes one strain (since this species is not commonly isolated from clinical environments), which presents a specific growth rate of 0.45 h^{-1}. Although the two strains from the group Other Beverages display the lowest average specific growth rate (0.32 h^{-1}), they individually present very different values for this parameter: from this group, strain T45 has a specific growth rate of 0.23 h^{-1}, and strain T03 of 0.42 h^{-1}. Wine strains have the most similar values of specific growth rate within the group, varying from 0.37 h^{-1} (T15) to 0.43 h^{-1} (T34). Strains from the other biotechnological groups (Food, Bread, Water and Unknown) show a wide range of values of specific growth rates among strains of each group. According to Boender et al. (2009), in chemostat cultures, the steady state specific growth rate of *S. cerevisiae* usually ranges from 0.03 h^{-1} to 0.4 h^{-1}, values that are relevant for several industrial

applications. Strains of *T. delbrueckii* studied in the present work present specific growth rates within the values observed for *S. cerevisiae*.

Glucose is the preferred carbon and energy source for most eukaryotic cells, and it is responsible for the regulation of their growth, metabolism, and development (Özcan et al., 1996, 1998; Özcan & Johnston, 1999). The transport of glucose across the plasma membrane is, then, of critical importance for the proper functioning of cells. In yeasts, the uptake of glucose occurs through differently regulated transporter proteins with different affinities for this sugar (Boles & Hollenberg, 1997; Maier et al., 2002; Reifenberger et al., 1995). While 20 genes related to the transport of hexoses have been identified and extensively studied in *S. cerevisiae*, only 2 have been characterized in *T. delbrueckii*. However, more transporters are known to exist in this species (Pacheco et al., 2020). In the study of Pacheco et al., (2020), the authors found in Chromosome 5 of the type strain, *T. delbrueckii* CBS 1146 (T04), a cluster with 3 genes annotated as putative Hxt orthologs (TDEL_0E02310, TDEL_0E02300 and TDEL_0E02280) and another gene (TDEL_0E02290) which also has high identity percentage to *HXT* genes. TDEL_0E02310 and TDEL_0E02300 have high identity percentage (99%) to the 2 characterized transporters, *LGT1* and *IGT1*, respectively. It is known that the hexose transporter proteins of *S. cerevisiae* are involved and influence the specific growth rate and fermentation behaviour of strains, so it is likely that the same happens in *T. delbrueckii*. Because of this, it is of extreme importance to better study and characterize the transporter proteins encoded by the different strains of this species. With this purpose, in the present work, several bioinformatic analysis were carried out. Initially, a BLAST using the aminoacidic sequence of *LGT1* from *T. delbrueckii* PYCC 5321 (the first characterized transporter in *T. delbrueckii*) as a query against the translated genome of the same 41 strains studied earlier in this work (**Table 2**), allowed to identify the number of transporters encoded by each strain. All strains encoded at least 5 Hxt-like transporters, but there were some where one or two additional transporters were present. The number of transporter proteins encoded by the strains does not seem to be related to their origin. **Figure 2** illustrates the number of strains encoding 5, 6 and 7 transporters, as well as their origins. Seven strains encode only 5 transporters, and their origins include Wine, Food and Bread. Most strains (24 strains), from all biotechnological groups, encode 6 transporters. From the 11 Arboreal/Soil strains covered in the collection, 10 are included in this group. Strains encoding 7 transporters (10 strains) also

belong to various biotechnological groups. The number of transporters does not seem to be related to the specific growth rate of the studied strains grown in YPD medium. Strains encoding the same number of transporter proteins show very different specific growth rate values. Further analysis allowed to group the transporters encoded by each strain according to their sequence similarity and identify the corresponding hypothetical proteins from the *T. delbrueckii* type strain (the only strain in the collection with a published assembled genome at the time of this analysis) with higher identity percentage (**Figure 3**). According to the BLAST performed for this purpose, *LGT1* (represented in red) and *IGT1* (represented in green) correspond to the protein TDEL_0E02310 and TDEL_0E02300, respectively, which form a cluster present in chromosome V with TDEL_0E02290 with higher similarity to the group represented in yellow and with TDEL_0E02280 with higher similarity to the group represented in blue. The group represented in orange and purple is most similar to TDEL_0A04250, in pink and turquoise to TDEL_0C00150, in grey to TDEL_0E00200, and finally, in brown to TDEL_0A00420, these transporters appearing in different positions in the type strain chromosomes. It is important to mention that, even though groups represented in orange and purple and pink and turquoise are most similar to the same proteins, they were separated in a preliminary analysis because the sequences seemed sufficiently different, a fact that is corroborated by their separation in different branches of the cladogram. For each group, individual cladograms were constructed. In the images resulting from this analysis (**Appendix 1 – Figure 3**), it is not possible to observe any relevant pattern of sequence similarity in strains of the same origin. In general, strain belonging to the same biotechnological group appear in separate branches of the cladograms. Exceptions to this are strains from Wine and Arboreal/Soil, which mostly appear in close positions on the cladogram branches in most groups.

Further analysis allowed to identify the transporters from *S. cerevisiae* with higher identity to *T. delbrueckii*'s. Genes corresponding to TDEL_0E02310 (*LGT1*/red) and TDEL_0E02300 (*IGT1*/green) are most similar to *S. cerevisiae* Hxt9 and Hxt11, TDEL_0E02290 (yellow) to Hxt1 and Hxt3, TDEL_0E02280 (blue) to Hxt5, TDEL0A04250 (orange and purple) to Hxt2, TDEL_0C00150 (pink and turquoise) to Rgt2, TDEL_0E00200 (grey) to Gal2 and finally TDEL_0A00420 (brown) to Hxt14 (**Figure 3)**.

LGT1 and *IGT1* were characterized in *T. delbrueckii* PYCC 5321 (T09), and because of that, within the scope of this work, the same strain was used to further analyse other possible hexose transporters. The cladogram representing the transporters encoded by this strain (**Figure 4**) shows that the genes with greater sequence similarity to each other are *LGT1* and *IGT1* as well as TDEL_0E02280 and TDEL_0E02290. The results are in accordance to what was found in the study of Pacheco et al., (2020): TDEL_0E02310 (*LGT1*), TDEL_0E02300 (*IGT1*), TDEL_0E02280 and TDEL_0E02290 are the most conserved transporters and are located in adjacent positions in this strain's genome, forming a cluster as referred above. It is reported in the same work that this cluster, like the *HXT3*, *HXT6* and *HXT7* cluster from *S. cerevisiae*, is adjacent to the genes *SVF1* and *MSR1*, which suggests a common ancestral origin for the *HXT* and *T. delbrueckii* genes. It is likely that *LGT1* and *IGT1* arose from a duplication of the ancestral of *HXT7* gene in *T. delbrueckii*. A gene duplication of the ancestor of *HXT3* is also likely to have happened, giving rise to TDEL_0E02280 and TDEL_0E02290. These are the most conserved transporter genes in *T. delbrueckii*, since the 4 of them are encoded by all strains studied in this work. Exception to this is strain T36, which encodes 2 genes with high identity percentage to *LGT1* and does not encode a gene with high identity percentage with TDEL_0E02280. Another exception is the case of T45, which encodes 2 genes with high identity percentage to *IGT1* and of T49, which, in contrast, does not encode any transporter with high identity percentage with *IGT1*. The presence of 2 genes with high identity percentage to *LGT1* in T36 and *IGT1* in T45 could be a result of a duplication of the genes.

Growth curves constructed for strains *T. delbrueckii* PYCC 5321 not expressing Lgt1 and a *S. cerevisiae hxt* null strain containing a plasmid overexpressing the same protein in the presence and absence of ethanol and their calculated growth rates indicated that the glucose uptake was affected in the strains growing in the presence of ethanol (**Figure 7**). In accordance with what has been previously reported regarding the growth of these strains (Pacheco et al., 2020), the results indicated that the absence and the overexpression of Lgt1 in those strains did not affect the glucose uptake rate, as evident through the comparison of the strains specific growth rates (**Table 7**). These results indicate that other glucose transporters must be expressed. Indeed, the 4 most conserved genes in the species were expressed in *T. delbrueckii* PYCC 5321, the same strain where both *LGT1* and *IGT1* were characterized, although with different patterns. **Figure 5** represents the results obtained from the RT-PCR analysis

performed. While the expression levels of 2239 slightly decreased from lag to log and stationary phases, *IGT1* was almost not expressed in the lag and log phases but its expression level value augmented very slightly in the stationary phase. The most noticeable variation in the expression levels of the 4 analysed genes was that of 2238, which significantly increased in the stationary phase of growth, a phase where glucose is present in low concentrations in the growth medium. This indicates that 2238 probably encodes a high affinity glucose transporter, maybe higher than the intermediate-affinity Igt1.

As reported by several authors, *T. delbrueckii* has unique features that make it stand out from the more traditionally used *S. cerevisiae*. Its physiological and metabolic features make it a promising species to be exploited for industrial applications and enable the enhancement and diversification of the final products (Fernandes et al., 2021). Despite its numerous advantageous properties, the use and exploitation of *T. delbrueckii* (and other non-conventional yeasts) in industry is still challenging. In the last decades, to expand the potential further commercialization and implementation of non-conventional yeasts and, particularly, of *T. delbrueckii* in diverse research areas, there has been an effort to characterize potential interesting strains for industrial use at the physiological and biochemical levels and to find strains with improved traits and phenotypes (Fernandes et al., 2021; Pacheco et al., 2012). In the past few years, the number of studies related to *T. delbrueckii* has increased. However, there are still limitations on the metabolic engineering of this yeast due to the lack of specific genome editing tools and incomplete knowledge about its genetics, metabolism and cellular physiology (Fernandes et al., 2021; Löbs et al., 2017). To further investigate the unique characteristics of *T. delbrueckii*, the construction and analysis of mutant strains is of great importance (Pacheco et al., 2009). Gene sequence modification technologies and gene targeting techniques make it possible to edit genomes in a simple and efficient way (Ding et al., 2019). In recent past, several studies reporting the successful experiments of genetic manipulation in *T. delbrueckii* have been published (Hernandez-Lopez et al., 2002, 2003; Pacheco et al., 2009, 2020; Watanabe et al., 1996), which lead the way to the possibility of improving and developing genetic tools specific to *T. delbrueckii*. Thus, with the purpose of better understanding and characterizing *T. delbrueckii*'s transporters, within the scope of this work, it was attempted to manipulate the glucose transport of *T. delbrueckii* PYCC 5321 using traditional cloning techniques.

For the first cloning strategy, where the final goal was to transform the *hxt* null strain with the most conserved transporters from *T. delbrueckii* PYCC 5321, we first constructed a pRS316 plasmid containing a *GFP* sequence and successfully amplified it in *E. coli* cells. However, transformation of the *hxt* null strain with this constructed plasmid and the different *T. delbrueckii* transporter genes was not successful. In another cloning strategy, where the final goal was to clone the genes *HXT1* and *HXT3* in *T. delbrueckii*, these transporters were initially cloned in *S. cerevisiae* BY 4741. The introduction of double stranded breaks (DSB) in the genome is a crucial step for its editing. There are two ways of repairing these breaks: non-homologous end joining (NHEJ), or homologous recombination (HR) (Cai et al., 2019; Van Dyck et al., 1999). In genetic manipulation experiments, it is preferable that DSB's are repaired via HR, which repairs breaks in a more accurate way than NHEJ pathway, which is an error prone process. It has been reported that *T. delbrueckii* exhibits a low efficiency of HR (Alves-Araújo et al., 2004; Pacheco et al., 2009) and because of this, an initial step in the cloning strategy developed within the scope of this work was the cloning of *HXT1* and *HXT3* in *S. cerevisiae*, where the dominant repair mechanism for DSB's is HR. This makes genome engineering in this species easier and more efficient (Löbs et al., 2017). From this initial step of the cloning strategy, clones of *S. cerevisiae* BY 4741 and *hxt* null strains expressing both genes were obtained. Images obtained by fluorescence microscopy (**Figure 6. C** and **6. D**) illustrate *S. cerevisiae* BY 4741 and *hxt* null transformed with a plasmid expressing *HXT1* and *HXT3* genes tagged with *GFP*. Even though there is some fluorescence in the cell's vacuole, it is possible to see that the transporter proteins are also localized at the plasma membrane. Functionality of the transporters was confirmed by following the recombinant strains growth. Furthermore, their specific growth rate was calculated and compared with the specific growth rate of strains *S. cerevisiae* RE601A and RE603A, which are mutant strains that only express transporters Hxt1 and Hxt8-17 and Hxt3 and Hxt8-17, respectively (Maier et al., 2002). The strains constructed in this work were able to grow in YPD medium with 2% (w/v) glucose. The calculated growth rates were approximately, 0.31 h^{-1} and 0.32 h^{-1}, for strains RE601A and RE603A, respectively. The growth of a strain transformed with the empty vector was not able to grow in the same medium. These results indicate that the Hxt proteins expressed in these strains are functional. Specific growth rate of both *S. cerevisiae* RE601A and RE603A was 0.36 h^{-1}. Even though, the strains constructed in this work have a slightly lower growth rate, the difference of values is not significative, which implies that the glucose uptake rate is not

affected. Positive transformants of both *S. cerevisiae* BY 4741 and *hxt* null strains were selected and their plasmid DNA was extracted. After obtaining positive clones, expressing the *HXT* genes, the next step in the cloning strategy was to amplify the amount of recombinant plasmid DNA. Thus, positive clones were selected, their plasmid DNA was extracted and then transformed in competent *E. coli* cells. However, bacterial cells transformed with the recombinant plasmid were not able to grow in plates with their selective marker. This might be due to the low concentrations of recombinant plasmid DNA obtained from the extraction of yeast cells. Several attempts and protocols were performed to try to increase the concentration of DNA obtained. First, a bacterial plasmid DNA extraction kit was used together with a previous step of cell wall disruption with glass beads, which resulted in concentrations of plasmid DNA of less than 20 ng/µL for both transporters. Then the same kit was used, but additional steps were performed (incubation with DTT and addition of zymolyase). This protocol resulted in slightly higher concentrations of plasmid DNA, but they were never higher than 50 ng/µL. The volume of cell cultures used in the initial step of these protocols was also increased, but this increase in the volume was not reflected in an increase of plasmid DNA concentration, since the values were similar to those obtained for smaller volumes. Transformation of *E. coli* cells with plasmid DNA obtained from all these protocols was never successful, even when different concentrations of DNA were used. Another attempt was the extraction of DNA using the genomic DNA extraction protocol, which obviously resulted in higher concentrations of DNA, but transformation of bacterial cells was again not successful. Another reason why these transformations might have not worked was the competent cells used. Transformations were initially performed with competent *E. coli* XL-21 Blue, prepared using the Rubidium Chloride ($RbCl_2$) protocol. Even though these cells were efficient when used in other transformations, they might not be sufficiently efficient for transformations with this plasmid. NZY5α competent *E. coli* cells, which have higher efficiency, were then used for transformation experiments. However, all the attempts of transformations using these cells were still not successful. Another reason for the low transformation efficiency might be related to the size of the plasmid that was used. The original plasmid contains approximately 8000 bps and the *HXT* genes both contain around 1700 bps. This makes the recombinant plasmid almost 10000 bps long, which can be a factor impairing the transformation efficiency. Thus, changing the plasmid used in this cloning strategy might also be a good solution. Experiments to try to obtain *hxt* null strains expressing *T. delbrueckii*'s glucose transporters as

well as *T. delbrueckii* strains expressing Hxt transporters from *S. cerevisiae* are still ongoing and optimizations are being performed.

Chapter 6. Concluding Remarks and Future Perspectives

6. Concluding Remarks and Future Perspectives

The unique properties of *T. delbrueckii* make it stand out as a promising non-conventional yeast to be explored for biotechnological applications, mainly in wine and bread making. Despite the numerous advantageous properties that would enable to enhance and diversify the industrial final products, the exploitation of *T. delbrueckii* in these contexts is challenging. In recent years, the number of studies regarding this and other non-conventional yeasts has increased, but there is still a lack of information about its genetics, metabolism and cellular physiology (Fernandes et al., 2021). The characterization and further investigation of *T. delbrueckii* is a crucial step to understand the basis behind its unique and interesting features. To achieve this, the genetic manipulation and construction and analysis of mutant strains is essential (Pacheco et al., 2009).

In contrast to *S. cerevisiae,* there is still limited knowledge about the glucose transporters encoded by *T. delbrueckii*. The results obtained in the scope of this work allowed to obtain further information regarding the number of transporters and their sequence similarities in the different studied strains. The differences observed regarding the glucose transporters might be related to the different behaviours displayed by strains belonging to this species. Information regarding the specific growth rate of these strains might be useful for choosing a strain to be used in industry. Additionally, information about the transporters encoded by the different *T. delbrueckii* strains is important to deepen the knowledge about the metabolism of sugars of this species. Furthermore, two cloning strategies were designed with the goal of characterizing and manipulating the glucose transport capacity of *T. delbrueckii,* as a way to increase its growth and fermentation capacity in the presence of high concentrations of ethanol. In spite of not having been able to obtain the mutant strains needed to better characterize the glucose transporters encoded by this species, optimizations to the protocols are still ongoing. When we are able to obtain the *hxt* null strain transformed with a plasmid expressing the individual *T. delbrueckii* transporters and the *T. delbrueckii* strain transformed with the plasmid expressing the Hxt proteins, the next step is to characterize their growth in the presence and absence of ethanol.

Chapter 7. References

7. References

Albertin, W., Chasseriaud, L., Comte, G., Panfili, A., Delcamp, A., Salin, F., Marullo, P., & Bely, M. (2014). Winemaking and bioprocesses strongly shaped the genetic diversity of the ubiquitous yeast *Torulaspora delbrueckii*. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0094246

Almeida, M. J., & Pais, C. S. (1996). Characterization of the yeast population from traditional corn and rye bread doughs. *Letters in Applied Microbiology*, *23*(3), 154–158. https://doi.org/10.1111/j.1472-765x.1996.tb00053.x

Alves-Araújo, C., Almeida, M. J., Sousa, M. J., & Leão, C. (2004). Freeze tolerance of the yeast *Torulaspora delbrueckii*: Cellular and biochemical basis. *FEMS Microbiology Letters*, *240*(1), 7–14. https://doi.org/10.1016/j.femsle.2004.09.008

Alves-Araújo, C., Hernandez-Lopez, M. J., Prieto, J. A., Randez-Gil, F., & Sousa, M. J. (2005). Isolation and characterization of the *LGT1* gene encoding a low-affinity glucose transporter from *Torulaspora delbrueckii. Yeast*, *22*(3), 165–175. https://doi.org/10.1002/yea.1192

Alves-Araújo, C., Pacheco, A., Almeida, M. J., Spencer-Martins, I., Leão, C., & Sousa, M. J. (2007). Sugar utilization patterns and respiro-fermentative metabolism in the baker's yeast *Torulaspora delbrueckii*. *Microbiology*, *153*(3), 898–904. https://doi.org/10.1099/mic.0.2006/003475-0

André, B. (1995). An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast*, *11*(16), 1575–1611. https://doi.org/10.1002/yea.320111605

Barnett, J. A. (1992). The taxonomy of the genus *Saccharomyces* meyen ex reess: A short review for non-taxonomists. *Yeast*. https://doi.org/10.1002/yea.320080102

Barnett, James A., & Robinow, C. F. (2002). A history of research on yeasts 4: Cytology part I, 1890-1950. *Yeast*, *19*(2), 151–182. https://doi.org/10.1002/yea.813

Belda, I., Ruiz, J., Beisert, B., Navascués, E., Marquina, D., Calderón, F., Rauhut, D., Benito, S., & Santos, A. (2017). Influence of *Torulaspora delbrueckii* in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations. *International Journal of Food Microbiology*, *257*(June), 183–191. https://doi.org/10.1016/j.ijfoodmicro.2017.06.028

Benítez, T., Gasent-Ramírez, J. M., Castrejón, F., & Codón, A. C. (1996). Development of new strains for the food industry. *Biotechnology Progress*, *12*(2), 149–163. https://doi.org/10.1021/bp960001o

Benito, S. (2018). The impact of *Torulaspora delbrueckii* yeast in winemaking. *Applied Microbiology and Biotechnology*, *102*(7), 3081–3094. https://doi.org/10.1007/s00253-018-8849-0

Bisson, L. F., Coons, D. M., Kruckeberg, A. L., & Lewis, D. A. (1993). Yeast sugar transporters. *Critical Reviews in Biochemistry and Molecular Biology*, *28*(4), 259–308. https://doi.org/10.3109/10409239309078437

Boender, L. G. M., De Hulster, E. A. F., Van Maris, A. J. A., Daran-Lapujade, P. A. S., & Pronk, J.

T. (2009). Quantitative physiology of *Saccharomyces cerevisiae* at near-zero specific growth rates. *Applied and Environmental Microbiology*, *75*(17), 5607–5614. https://doi.org/10.1128/AEM.00429-09

Boles, E., & Hollenberg, C. P. (1997). The molecular genetics of hexose transport in yeasts. *FEMS Microbiology Reviews*, *21*(1), 85–111. https://doi.org/10.1016/S0168-6445(97)00052-1

Cai, P., Gao, J., & Zhou, Y. (2019). CRISPR-mediated genome editing in non-conventional yeasts for biotechnological applications. *Microbial Cell Factories*, *18*(1), 1–12. https://doi.org/10.1186/s12934-019-1112-2

Cardoso, H., & Leão, C. (1992). Mechanisms underlying the low and high enthalphy death induced by short-chain monocarboxylic acids and ethanol in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *38*(3), 388–392. https://doi.org/10.1007/BF00170091

Carlson, M. (1998). *Regulation of glucose utilization in yeast*. 560–564.

Diderich, J. A., Schepper, M., Van Hoek, P., Luttik, M. A. H., Van Dijken, J. P., Pronk, J. T., Klaassen, P., Boelens, H. F. M., De Mattos, M. J. T., Van Dam, K., & Kruckeberg, A. L. (1999). Glucose uptake kinetics and transcription of *HXT* genes in chemostat cultures of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *274*(22), 15350–15359. https://doi.org/10.1074/jbc.274.22.15350

Ding, Y., Wang, K. F., Wang, W. J., Ma, Y. R., Shi, T. Q., Huang, H., & Ji, X. J. (2019). Increasing the homologous recombination efficiency of eukaryotic microorganisms for enhanced genome engineering. *Applied Microbiology and Biotechnology*, *103*(11), 4313–4324. https://doi.org/10.1007/s00253-019-09802-2

Dzialo, M. C., Park, R., Steensels, J., Lievens, B., & Verstrepen, K. J. (2017). Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiology Reviews*, *41*(October), S95–S128. https://doi.org/10.1093/femsre/fux031

Escalante, W. D. E. (2012). Perspectives and Uses of Non-*Saccharomyces* Yeasts in Fermented Beverages. *Intech*, 13. http://dx.doi.org/10.1039/C7RA00172J%0Ahttps://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics%0Ahttp://dx.doi.org/10.1016/j.colsurfa.2011.12.014

Faria-Oliveira, F., Puga, S., & Ferreir, C. (2013). Yeast: World's Finest Chef. *Food Industry*, *May 2014*. https://doi.org/10.5772/53156

Fernandes, T., Silva-Sousa, F., Pereira, F., Rito, T., Soares, P., Franco-Duarte, R., & Sousa, M. J. (2021). Biotechnological importance of *Torulaspora delbrueckii*: From the obscurity to the spotlight. *Journal of Fungi*, *7*(9). https://doi.org/10.3390/jof7090712

Flores, C.-L., Rodrígez, C., Petit, T., & Gancedo, C. (2000). Carbohydrate and energy-yielding metabolism in non-conventional yeasts. *FEMS Microbiology Reviews*, *24*, 507–529.

García, M., Esteve-Zarzoso, B., & Arroyo, T. (2016). Non-*Saccharomyces* Yeasts: Biotechnological Role for Wine Production. *Grape and Wine Biotechnology*, *October*. https://doi.org/10.5772/64957

Hellborg, L., & Piškur, J. (2009). Yeast diversity in the brewing industry. *Beer in Health and Disease Prevention*, 77–88. https://doi.org/10.1016/b978-0-12-373891-2.00007-9

Hernandez-Lopez, M. J., Blasco, A., Prieto, J. A., & Randez-Gil, F. (2003). Ura- host strains for genetic manipulation and heterologous expression of *Torulaspora delbrueckii*. *International Journal of Food Microbiology*. https://doi.org/10.1016/S0168-1605(03)00249-6

Hernandez-Lopez, M. J., Prieto, J. A., & Randez-Gil, F. (2002). Isolation and characterization of the gene URA3 encoding the orotidine-5'-phosphate decarboxylase from *Torulaspora delbrueckii*. *Yeast*, *19*(16), 1431–1435. https://doi.org/10.1002/yea.929

Johnson, E. A. (2013a). Biotechnology of non-*Saccharomyces* yeasts-the basidiomycetes. *Applied Microbiology and Biotechnology*, *97*(17), 7563–7577. https://doi.org/10.1007/s00253-013-5046-z

Johnson, E. A. (2013b). Biotechnology of non-*Saccharomyces* yeasts-the basidiomycetes. *Applied Microbiology and Biotechnology*, *97*(17), 7563–7577. https://doi.org/10.1007/s00253-013-5046-z

Kayikci, Ö., & Nielsen, J. (2015). Glucose repression in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *15*(6), 1–8. https://doi.org/10.1093/femsyr/fov068

Kim, J. H., Roy, A., Jouandot, D., & Cho, K. H. (2013). The glucose signaling network in yeast. *Biochimica et Biophysica Acta - General Subjects*, *1830*(11), 5204–5210. https://doi.org/10.1016/j.bbagen.2013.07.025

Kruckeberg, A. L. (1996). The hexose transporter family of *Saccharomyces cerevisiae*. *Archives of Microbiology*, *166*(5), 283–292. https://doi.org/10.1007/s002030050385

Kurtzman, C. P. (2011). *Torulaspora*Lindner (1904). In *The Yeasts* (Vol. 2, Issue 1904). Elsevier B.V. https://doi.org/10.1016/B978-0-444-52149-1.00075-6

Lagunas, R. (1993). Sugar transport in *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, *104*(3–4), 229–242. https://doi.org/10.1016/0378-1097(93)90598-V

Letunic, I., & Bork, P. (2021). Interactive tree of life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research*, *49*(W1), W293–W296. https://doi.org/10.1093/nar/gkab301

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Löbs, A. K., Schwartz, C., & Wheeldon, I. (2017). Genome and metabolic engineering in non-conventional yeasts: Current advances and applications. *Synthetic and Systems Biotechnology*, *2*(3), 198–207. https://doi.org/10.1016/j.synbio.2017.08.002

Lorenz, M. C., Muir, R. S., Lim, E., McElver, J., Weber, S. C., & Heitman, J. (1995). Gene disruption with PCR products in *Saccharomyces cerevisiae*. *Gene*, *158*(1), 113–117. https://doi.org/10.1016/0378-1119(95)00144-U

Maicas, S. (2020). The role of yeasts in fermentation processes. *Microorganisms*, *8*(8), 1–8. https://doi.org/10.3390/microorganisms8081142

Maier, A., Völker, B., Boles, E., & Fuhrmann, G. F. (2002). Characterisation of glucose transport in *Saccharomyces cerevisiae* with plasma membrane vesicles (countertransport) and intact cells (initial uptake) with single Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7 or Gal2 transporters. *FEMS Yeast Research*, *2*(4), 539–550. https://doi.org/10.1016/S1567-1356(02)00140-X

Mecca, D., Benito, S., Beisert, B., Brezina, S., Fritsch, S., Semmler, H., & Rauhut, D. (2020). Influence of nutrient supplementation on *Torulaspora delbrueckii* in wine fermentation aroma. *Fermentation*, *6*(1), 1–14. https://doi.org/10.3390/FERMENTATION6010035

Oda, Y., Yabuki, M., Tonomura, K., & Fukunaga, M. (1997). Reexamination of yeast strains classified as *Torulaspora delbrueckii* (Lindner). *International Journal of Systematic Bacteriology*, *47*(4), 1102–1106. https://doi.org/10.1099/00207713-47-4-1102

Özcan, S., Dover, J., & Johnston, M. (1998). Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. *EMBO Journal*, *17*(9), 2566–2573. https://doi.org/10.1093/emboj/17.9.2566

Özcan, S., Dover, J., Rosenwald, A. G., Wölfl, S., & Johnston, M. (1996). Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(22), 12428–12432. https://doi.org/10.1073/pnas.93.22.12428

Özcan, S., & Johnston, M. (1999). Function and Regulation of Yeast Hexose Transporters. *Microbiology and Molecular Biology Reviews*, *63*(3), 554–569. https://doi.org/10.1128/mmbr.63.3.554-569.1999

Pacheco, A. (2008). *Baker's yeasts for use in frozen-dough technology: sugar utilization in freeze tolerant Torulaspora delbrueckii strains and elucidation of cyro-resistance mechanisms*. 1–154.

Pacheco, A., Almeida, M. J., & Sousa, M. J. (2009). Improved gene disruption method for *Torulaspora delbrueckii. FEMS Yeast Research*, *9*(1), 158–160. https://doi.org/10.1111/j.1567-1364.2008.00452.x

Pacheco, A., Donzella, L., Hernandez-Lopez, M. J., Almeida, M. J., Prieto, J. A., Randez-Gil, F., Morrissey, J. P., & Sousa, M. J. (2020). Hexose transport in *Torulaspora delbrueckii:* Identification of Igt1, a new dual-affinity transporter. *FEMS Yeast Research*, *20*(1), 1–10. https://doi.org/10.1093/femsyr/foaa004

Pacheco, A., Santos, J., Chaves, S., Almeida, J., Leo, C., & Joo, M. (2012). The Emerging Role of the Yeast *Torulaspora delbrueckii* in Bread and Wine Production: Using Genetic Manipulation to Study Molecular Basis of Physiological Responses. *Structure and Function of Food Engineering*. https://doi.org/10.5772/46024

Parapouli, M., Vasileiadis, A., Afendra, A. S., & Hatziloukas, E. (2020). *Saccharomyces cerevisiae* and its industrial applications. In *AIMS Microbiology* (Vol. 6, Issue 1). https://doi.org/10.3934/microbiol.2020001

Pech-Canul, Á. D. L. C., Ortega, D., Garcia-Triana, A., & Solís-Oviedo, R. L. (2019). *Torulaspora delbrueckii:* Towards Innovating in the Legendary Baking and Brewing Industries.

Frontiers and New Trends in the Science of Fermented Food and Beverages, *February*. https://doi.org/10.5772/intechopen.83522

Pfeiffer, T., & Morley, A. (2014). An evolutionary perspective on the Crabtree effect. *Frontiers in Molecular Biosciences*, *1*(OCT), 1–6. https://doi.org/10.3389/fmolb.2014.00017

Puligundla, P., Smogrovicova, D., Obulam, V. S. R., & Ko, S. (2011). Very high gravity (VHG) ethanolic brewing and fermentation: A research update. *Journal of Industrial Microbiology and Biotechnology*, *38*(9), 1133–1144. https://doi.org/10.1007/s10295-011-0999-3

Ramírez, M., & Velázquez, R. (2018). The yeast *Torulaspora delbrueckii*: An interesting but difficult-to-use tool for winemaking. *Fermentation*, *4*(4). https://doi.org/10.3390/fermentation4040094

Ray, R. C., & Montet, D. (2017). Technological Innovations in Processing of Fermented Foods An Overview. *Fermented Foods: Part II: Technological Interventions*, *May*, 1–513. https://doi.org/10.1201/9781315205359

Reifenberger, E., Freidel, K., & Ciriacy, M. (1995). Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on qlycolytic flux. *Molecular Microbiology*, *16*(1), 157–167. https://doi.org/10.1111/j.1365-2958.1995.tb02400.x

Saier, M. H. (2000). Families of transmembrane sugar transport proteins. *Molecular Microbiology*, *35*(4), 699–710. https://doi.org/10.1046/j.1365-2958.2000.01759.x

Saier, M. H., Beatty, J. T., Goffeau, A., Harley, K. T., Heijne, W. H. M., Huang, S. C., Jack, D. L., Jähn, P. S., Lew, K., Liu, J., Pao, S. S., Paulsen, I. T., Tseng, T. T., & Virk, P. S. (1999). Major Facilitator Superfamily. *Journal of Molecular Microbiology and Biotechnology*, *1*(2), 257–279.

Santiago, C., Rito, T., Vieira, D., Fernandes, T., Pais, C., Sousa, M. J., Soares, P., & Franco-Duarte, R. (2021). Improvement of *Torulaspora delbrueckii* genome annotation: Towards the exploitation of genomic features of a biotechnologically relevant yeast. *Journal of Fungi*, *7*(4). https://doi.org/10.3390/jof7040287

Santos, J., Sousa, M. J., Cardoso, H., Inácio, J., Silva, S., Spencer-Martins, I., & Leão, C. (2008). Ethanol tolerance of sugar transport, and the rectification of stuck wine fermentations. *Microbiology*, *154*(2), 422–430. https://doi.org/10.1099/mic.0.2007/011445-0

Silva, M., Pontes, A., Franco-Duarte, R., Soares, P., Sampaio, J. P., Sousa, M. J., & Brito, P. H. (2022). A glimpse at an early stage of microbe domestication revealed in the variable genome of *Torulaspora delbrueckii*, an emergent industrial yeast. *Molecular Ecology*, *01*, 1–17. https://doi.org/10.1111/mec.16428

Srivastava, A. K., & Gupta, S. (2011). Fed-Batch Fermentation - Design Strategies. In *Comprehensive Biotechnology, Second Edition* (Second Edi, Vol. 2). Elsevier B.V. https://doi.org/10.1016/B978-0-08-088504-9.00112-4

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, *38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Thevelein, J. M., & Hohmann, S. (1995). Trehalose synthase: guard to the gate of glycolysis in yeast? *Trends in Biochemical Sciences*, *20*(1), 3–10. https://doi.org/10.1016/S0968-0004(00)88938-0

Tondini, F., Lang, T., Chen, L., Herderich, M., & Jiranek, V. (2019). Linking gene expression and oenological traits: Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains. *International Journal of Food Microbiology*, *294*(February), 42–49. https://doi.org/10.1016/j.ijfoodmicro.2019.01.014

Towle, H. C. (2005). Glucose as a regulator of eukaryotic gene transcription. *Trends in Endocrinology and Metabolism*, *16*(10), 489–494. https://doi.org/10.1016/j.tem.2005.10.003

Van Dyck, E., Stasiak, A. Z., Stasiak, A., & West, S. C. (1999). Binding of double-strand breaks in DNA by human Rad52 protein. *Nature*, *401*(September), 371–375.

van Gulik, W. M., & Heijnen, J. J. (1995). A metabolic network stoichiometry analysis of microbial growth and product formation. *Biotechnology and Bioengineering*, *48*(6), 681–698. https://doi.org/10.1002/bit.260480617

Van Hoek, P., Van Dijken, J. P., & Pronk, J. T. (1998). Effect of specific growth rate on fermentative capacity of baker's yeast. *Applied and Environmental Microbiology*, *64*(11), 4226–4233. https://doi.org/10.1128/aem.64.11.4226-4233.1998

Vardy, E., Arkin, I. T., Gottschalk, K. E., Kaback, H. R., & Schuldiner, S. (2004). Structural conservation in the major facilitator superfamily as revealed by comparative modeling. *Protein Science*, *13*(7), 1832–1840. https://doi.org/10.1110/ps.04657704

Walker, G. M. (2009). Yeasts. *Cell*, 478–491.

Walsh, M. C., Scholte, M., Valkier, J., Smits, H. P., & Van Dam, K. (1996). Glucose sensing and signalling properties in *Saccharomyces cerevisiae* require the presence of at least two members of the glucose transporter family. *Journal of Bacteriology*, *178*(9), 2593–2597. https://doi.org/10.1128/jb.178.9.2593-2597.1996

Watanabe, Y., Imai, K., Oishi, H., & Tamai, Y. (1996). Disruption of phospholipase B gene, PLB1, increases the survival of baker's yeast *Torulaspora delbrueckii*. *FEMS Microbiology Letters*, *145*(3), 415–420. https://doi.org/10.1016/S0378-1097(96)00442-9

Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C. P., & Boles, E. (1999). Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Letters*, *464*(3), 123–128. https://doi.org/10.1016/S0014-5793(99)01698-1

You, K. M., Rosenfield, C. L., & Knipple, D. C. (2003). Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. *Applied and Environmental Microbiology*, *69*(3), 1499–1503. https://doi.org/10.1128/AEM.69.3.1499-1503.2003

Chapter 8. Appendix

8. Appendix

Appendix 1 – Assessment of the specific growth rates of 41 *T. delbrueckii* strains and bioinformatic analysis of their glucose transporter proteins.

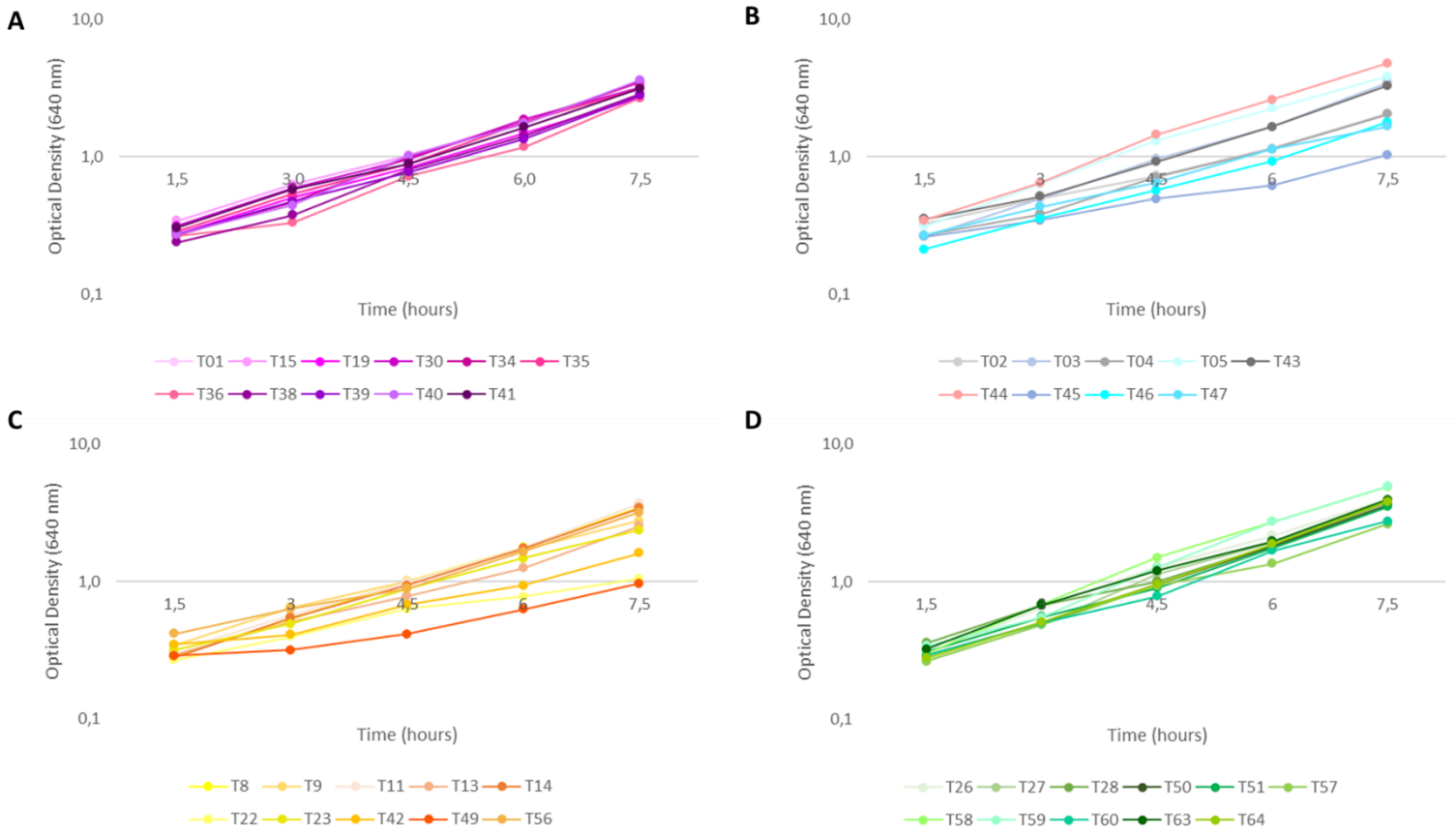


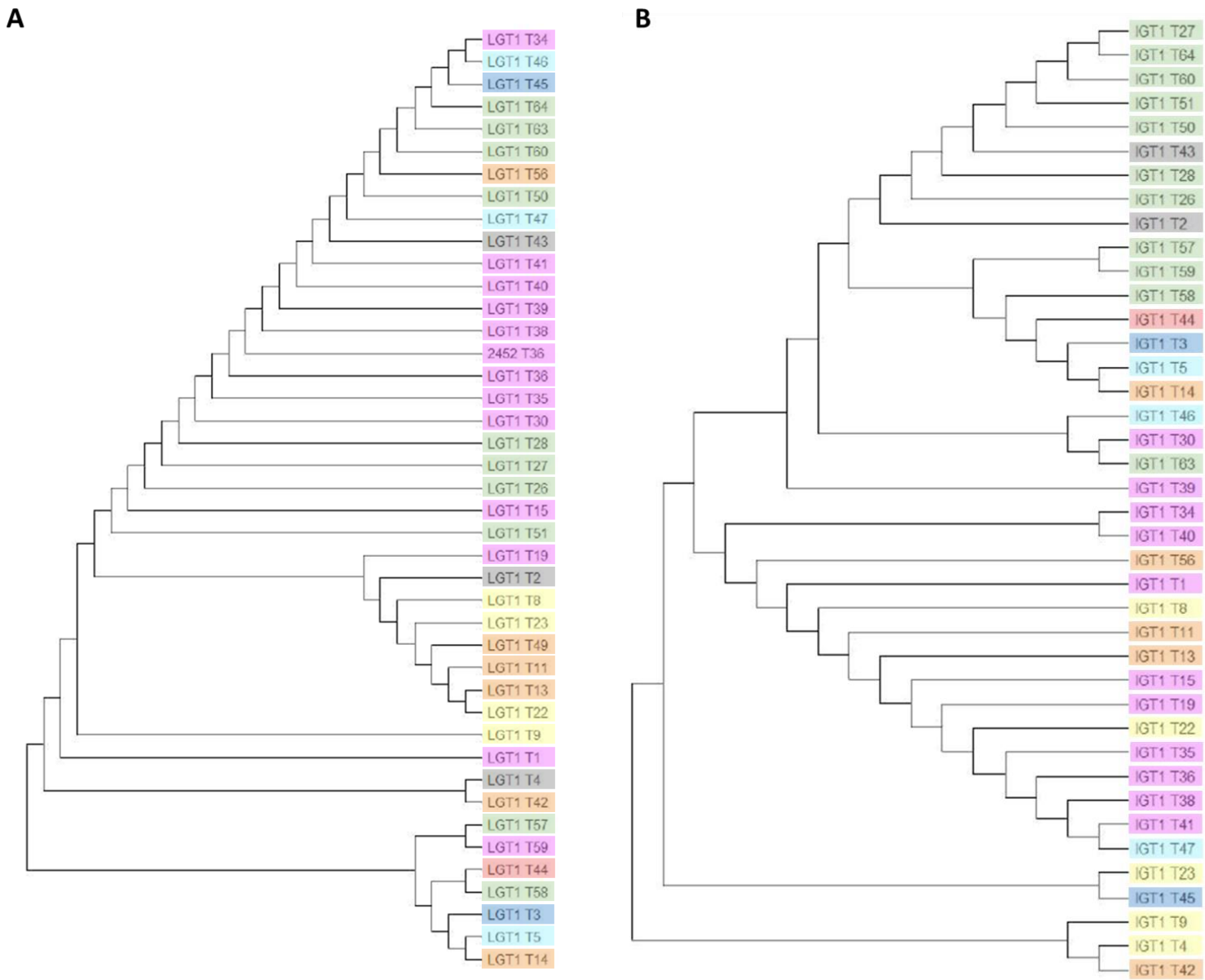
Figure A1. 1. Exponential phase (1.5 to 7.5 hours) of growth curves of the 41 *T. delbrueckii* strain set in YPD medium. Strains in the *T. delbrueckii* collection were grown overnight in 10 mL of YPD medium to a OD_{640nm} = 0.8, which was then diluted to a OD_{640nm} = 0.1 in 10 mL of fresh medium. The growth of each strain was assessed in timepoints of 1.5 hours, for approximately 30 hours, and their growth curves were constructed. At least 3 replicates were performed for each strain. Strains from **A.** Wine (T01, T15, T19, T30, T34, T35, T36, T38, T39, T40 and T41); **B.** Unknown (T02, T04 and T43), Other Beverages (T04 and T45), Water (T05, T46 and T47), and Clinical (T44); **C.** Bread (T08, T09, T22 and T23), and Food (T11, T13, T14, T42, T49, and T56); **D.** Arboreal/Soil (T26, T27, T28, T50, T51, T57, T58, T59, T60, T63, and T64).

Table A1. 1. Number of possible hexose transporters in the 41-strain set of *T. delbrueckii*. Biotechnological origins are represented in the first column: Pink – Wine; Black – Unknown; Blue – Other Beverages; Turquoise – Water; Yellow – Bread; Orange – Food; Green – Arboreal/Soil; Red – Clinical.

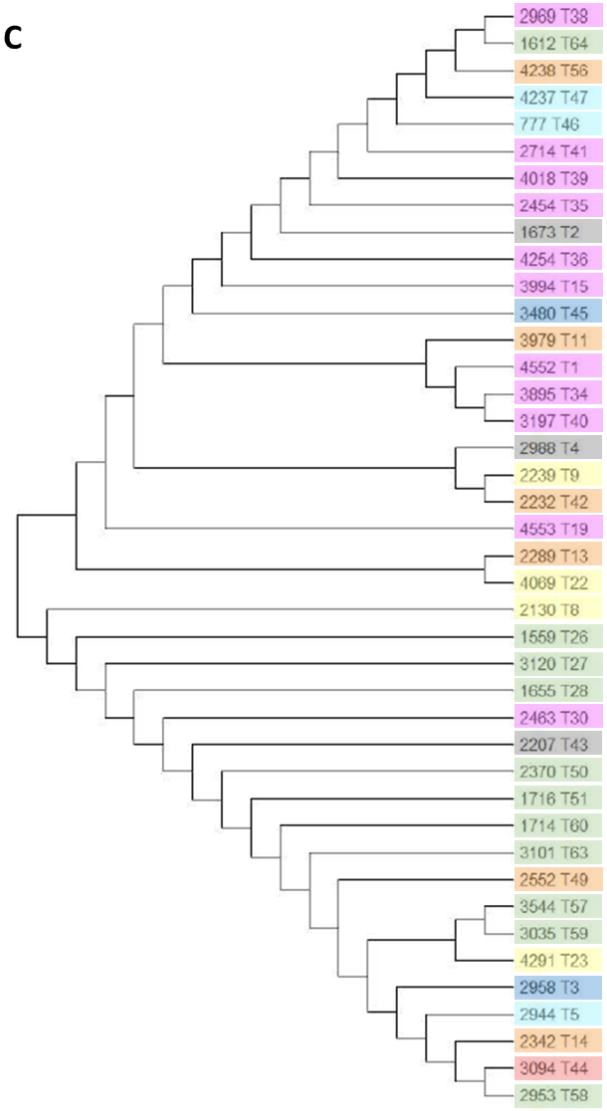
Strain/Origin	Number of Transporters	Strain/Origin	Number of Transporters
T01	5	**T38**	6
T02	6	**T39**	6
T03	6	**T40**	6
T04	7	**T41**	7
T05	6	**T42**	7
T08	7	**T43**	6
T09	7	**T44**	6
T11	5	**T45**	7
T13	6	**T46**	6
T14	6	**T47**	7
T15	5	**T49**	5
T19	5	**T50**	6
T22	5	**T51**	6
T23	5	**T56**	7
T26	6	**T57**	6
T27	7	**T58**	6
T28	6	**T59**	6
T30	7	**T60**	6
T34	6	**T63**	6
T35	6	**T64**	6
T36	6		

Strain/Origin	IGT1 (TDEL_0E02300)		LGT1 (TDEL_0E02310)		TDEL_0E02290	TDEL_0E02280	TDEL_0A04250	TDEL_0C00150	TDEL_0A04250	TDEL_0C00150	TDEL_0E00200	TDEL_0A00420
T1	3775 95%		3774 99%		4552 64%	4551 65%	383 65%					
T2	1671 94%		1670 99%		1673 64%	1674 65%	3083 65%	765 36%				
T3	2959 94%		2960 98%		2958 65%	2957 64%			384 65%	4848 36%		
T4	2987 74%		2986 86%		2988 65%	2989 65%	382 65%	3598 36%			4914 66%	
T5	2945 94%		2946 98%		2944 65%	2943 64%			384 65%	4806 36%		
T8	2131 95%		2132 99%		2130 65%	2129 65%	3580 65%	4887 36%			4911 63%	
T9	2240 95%		2241 100%		2239 64%	2238 65%	1027 65%	4153 36%			4918 63%	
T11	3367 95%		3366 99%		3979 69%	3981 65%	1037 65%					
T13	2290 95%		2291 99%		2289 65%	2288 65%	1059 64%					4807 35%
T14	2341 94%		2340 98%		2342 65%	2343 64%			384 65%	4859 36%		
T15	3406 95%		3405 99%		3994 68%	3996 65%	416 65%					
T19	3684 95%		3685 99%		4553 65%	4552 65%	417 64%					
T22	3389 95%		3390 99%		4069 65%	4070 65%	308 65%					
T23	4011 94%		4010 99%		4291 69%	4293 65%	382 65%					
T26	1560 94%		1561 99%		1559 65%	1558 65%	382 65%	1355 36%				
T27	3119 94%		3118 99%		3120 65%	3121 65%	416 65%	2218 36%				35 35%
T28	1656 94%		1657 99%		1655 65%	1654 65%	418 65%	3668 36%				
T30	2462 94%		2461 99%		2463 65%	2464 65%	383 65%	1355 36%				764 35%
T34	4560 95%		4559 99%		3895 64%	3894 65%	1686 64%	2078 36%				
T35	2453 95%		2452 99%		2454 64%	2455 65%	1135 65%	3106 36%				
T36	2991 95%		2992 99%	2452 65%	4254 68%		418 65%	2527 36%				
T38	2970 95%		2971 99%		2969 64%	2968 65%	417 65%	2217 36%				
T39	4403 95%		4404 99%		4018 64%	4019 65%	381 64%	1465 36%				
T40	3196 95%		3195 99%		3197 64%	3198 65%	381 65%	4338 36%				
T41	2715 95%		2716 99%		2714 64%	2713 65%	416 64%	2013 36%				35 35%
T42	2233 74%		2234 86%		2232 65%	2231 65%	369 65	3035 36%			4916 66%	
T43	2208 94%		2209 99%		2207 65%	2206 65%	381 65%	1458 36%				
T44	3093 94%		3092 98%		3094 65%	3095 64%			419 65%	4754 36%		
T45	3481 90%	4982 99%	4963 81%		3480 64%	3479 68%	383 65%	2335 36%				
T46	778 93%		779 99%		777 64%	776 65%	170 65%	1820 36%				
T47	2266 95%		2267 99%		4237 64%	4238 65%	307 64%	1356 36%				4620 35%
T49			2550 93%		2552 69%	2553 69%	416 65%				4910 67%	
T50	2369 94%		2368 99%		2370 65%	2371 65%	382 65%	2010 36%				
T51	1715 94%		1714 99%		1716 65%	1717 65%	382 65%	806 36%				
T56	3148 95%		3147 99%		4238 64%	4239 65%	380 64%	1355 36%				4741 35%
T57	3543 94%		3542 99%		3544 64%	3545 65%	1032 64%	2100 36%				
T58	2954 94%		2955 98%		2953 65%	2952 64%			385 65%	4828 36%		
T59	3034 94%		3033 99%		3035 64%	3036 65%	416 64%	2126 36%				
T60	1713 94%		1712 99%		1714 65%	1715 65%	382 65%	1356 36%				
T63	3100 94%		3099 99%		3101 65%	3102 65%	382 65%	4641 36%				
T64	1613 94%		1614 99%		1612 64%	1611 65%	2082 65%	4347 36%				

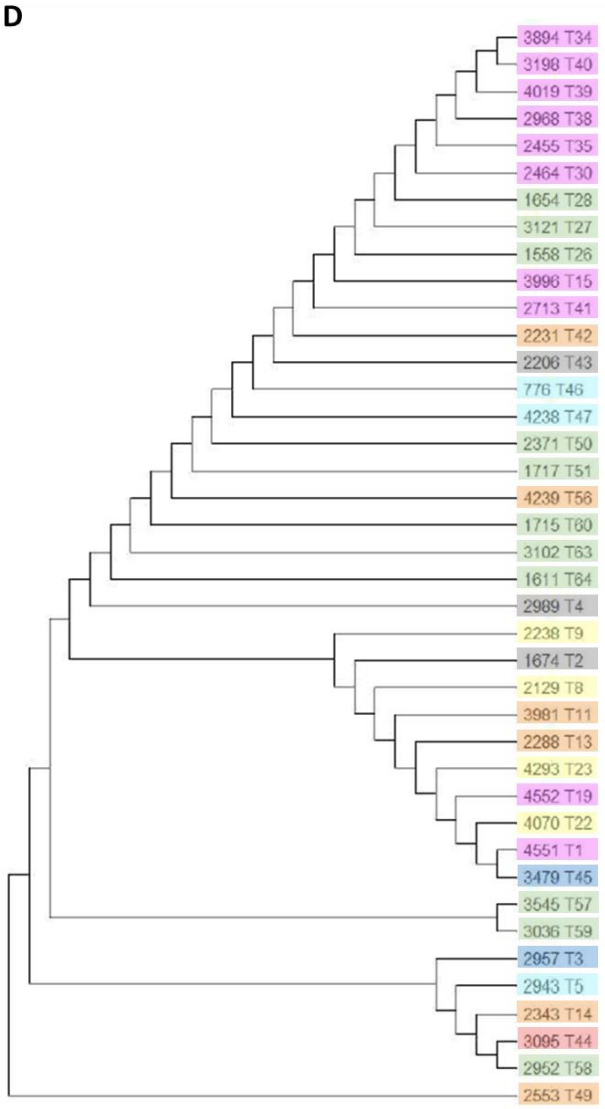
Figure A1. 2. Groups of proteins formed according to their sequence similarity and *T. delbrueckii* CBS 1146 hypothetical proteins with highest identity percentage to all the proteins in each of the 10 groups formed. Green/IGT1 - TDEL_0E02300; Red/LGT1 - TDEL_0E02310; Yellow - TDEL_0E02290; Blue - TDEL_0E02280; Orange and Purple - TDEL_0A04250; Pink and Turquoise - TDEL_0C00150; Brown - TDEL_0A00420; Grey - TDEL_0E00200. Strain origins are represented in the first column: Pink – Wine; Black – unknown; Blue – Other Beverages; Turquoise – Water; Yellow – Bread; Orange – Food; Green – Arboreal/soil; Red – Clinical.

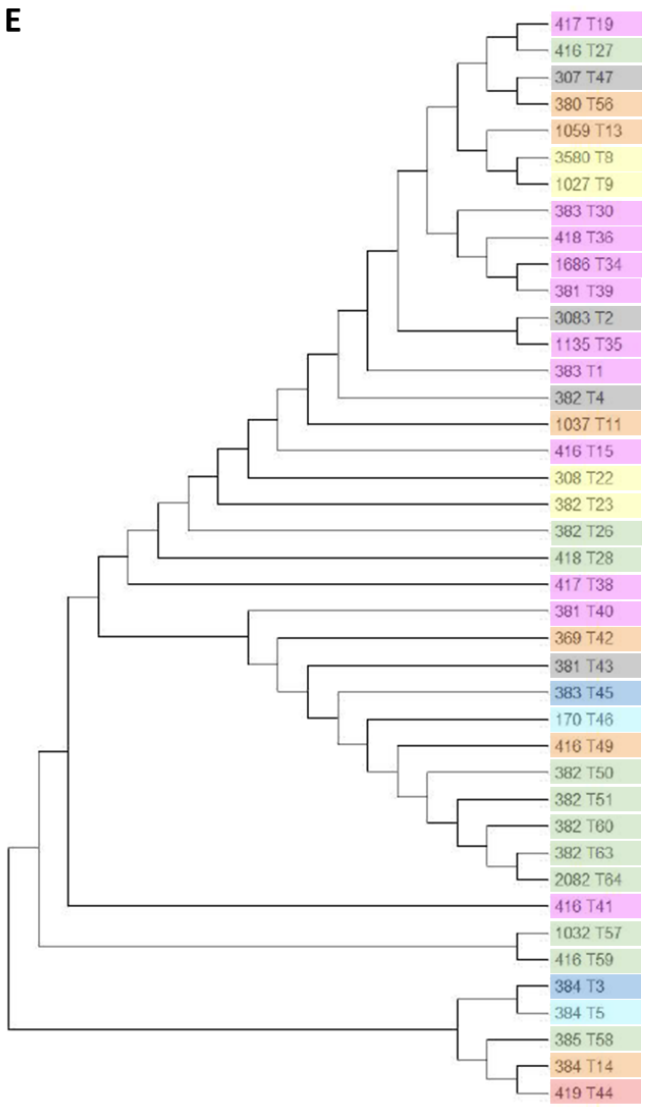

A
LGT1 T34
LGT1 T46
LGT1 T45
LGT1 T64
LGT1 T63
LGT1 T60
LGT1 T56
LGT1 T50
LGT1 T47
LGT1 T43
LGT1 T41
LGT1 T40
LGT1 T39
LGT1 T38
2452 T36
LGT1 T36
LGT1 T35
LGT1 T30
LGT1 T28
LGT1 T27
LGT1 T26
LGT1 T15
LGT1 T51
LGT1 T19
LGT1 T2
LGT1 T8
LGT1 T23
LGT1 T49
LGT1 T11
LGT1 T13
LGT1 T22
LGT1 T9
LGT1 T1
LGT1 T4
LGT1 T42
LGT1 T57
LGT1 T59
LGT1 T44
LGT1 T58
LGT1 T3
LGT1 T5
LGT1 T14
B
IGT1 T27
IGT1 T64
IGT1 T60
IGT1 T51
IGT1 T50
IGT1 T43
IGT1 T28
IGT1 T26
IGT1 T2
IGT1 T57
IGT1 T59
IGT1 T58
IGT1 T44
IGT1 T3
IGT1 T5
IGT1 T14
IGT1 T46
IGT1 T30
IGT1 T63
IGT1 T39
IGT1 T34
IGT1 T40
IGT1 T56
IGT1 T1
IGT1 T8
IGT1 T11
IGT1 T13
IGT1 T15
IGT1 T19
IGT1 T22
IGT1 T35
IGT1 T36
IGT1 T38
IGT1 T41
IGT1 T47
IGT1 T23
IGT1 T45
IGT1 T9
IGT1 T4
IGT1 T42

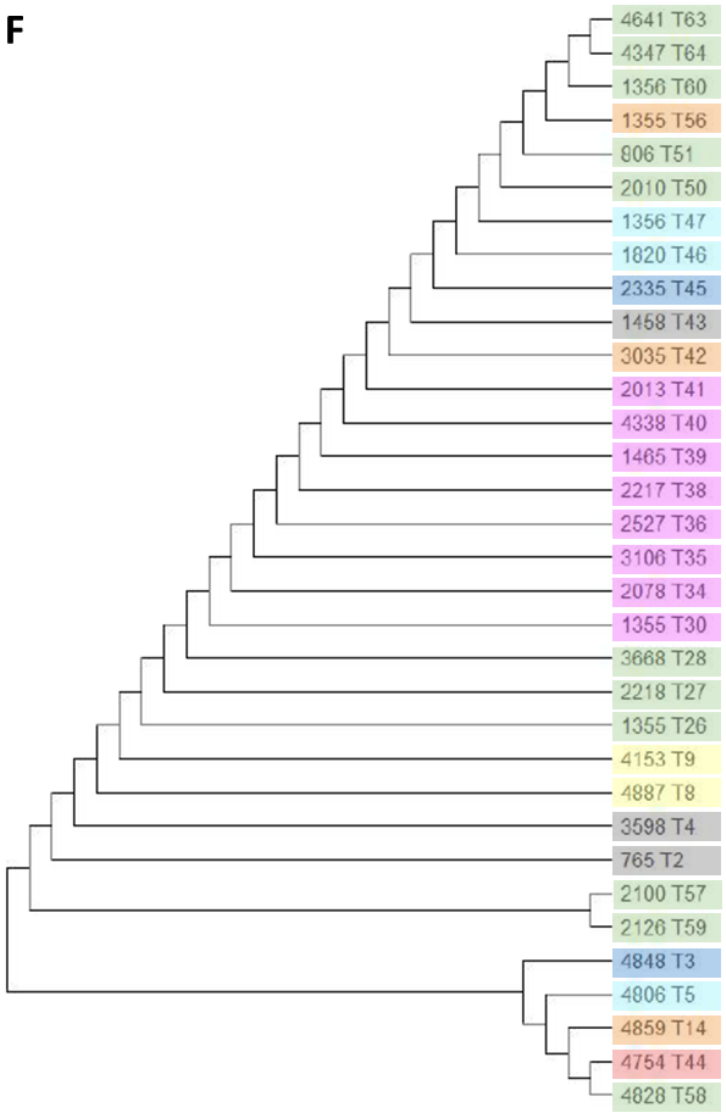
C


2969 T38
1612 T64
4238 T56
4237 T47
777 T46
2714 T41
4018 T39
2454 T35
1673 T2
4254 T36
3994 T15
3480 T45
3979 T11
4552 T1
3895 T34
3197 T40
2988 T4
2239 T9
2232 T42
4553 T19
2289 T13
4069 T22
2130 T8
1559 T26
3120 T27
1655 T28
2463 T30
2207 T43
2370 T50
1716 T51
1714 T60
3101 T63
2552 T49
3544 T57
3035 T59
4291 T23
2958 T3
2944 T5
2342 T14
3094 T44
2953 T58

D


3894 T34
3198 T40
4019 T39
2968 T38
2455 T35
2464 T30
1654 T28
3121 T27
1558 T26
3996 T15
2713 T41
2231 T42
2206 T43
776 T46
4238 T47
2371 T50
1717 T51
4239 T56
1715 T60
3102 T63
1611 T64
2989 T4
2238 T9
1674 T2
2129 T8
3981 T11
2288 T13
4293 T23
4552 T19
4070 T22
4551 T1
3479 T45
3545 T57
3036 T59
2957 T3
2943 T5
2343 T14
3095 T44
2952 T58
2553 T49


E
417 T19
416 T27
307 T47
380 T56
1059 T13
3580 T8
1027 T9
383 T30
418 T36
1688 T34
381 T39
3083 T2
1135 T35
383 T1
382 T4
1037 T11
416 T15
308 T22
382 T23
382 T26
418 T28
417 T38
381 T40
369 T42
381 T43
383 T45
170 T46
416 T49
382 T50
382 T51
382 T60
382 T63
2082 T64
416 T41
1032 T57
416 T59
384 T3
384 T5
385 T58
384 T14
419 T44


F
4641 T63
4347 T64
1356 T60
1355 T56
806 T51
2010 T50
1356 T47
1820 T46
2335 T45
1458 T43
3035 T42
2013 T41
4338 T40
1465 T39
2217 T38
2527 T36
3106 T35
2078 T34
1355 T30
3668 T28
2218 T27
1355 T26
4153 T9
4887 T8
3598 T4
765 T2
2100 T57
2126 T59
4848 T3
4806 T5
4859 T14
4754 T44
4828 T58

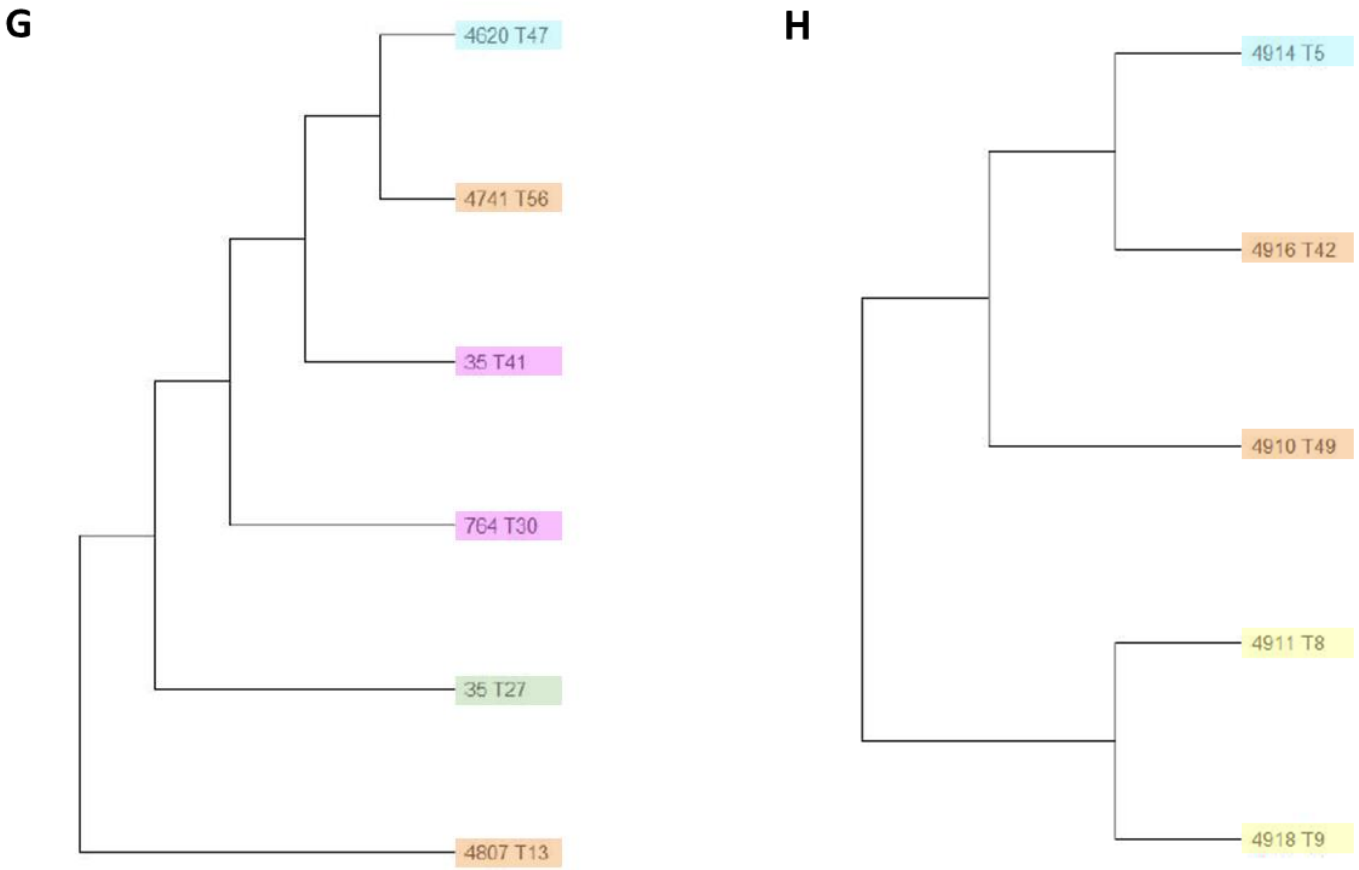


Figure A1. 3. Cladogram representing the genes belonging to each group, formed according to their sequence similarity. **A.** *LGT1;* **B.** *IGT1;* **C.** yellow group (TDEL_0E02290); **D.** blue group (TDEL_0E02280); **E.** orange and purple group (TDEL_0A04250); **F.** pink and turquoise group (TDEL_0C00150); **G.** brown group (TDEL_0A00420); **H.** grey group (TDEL_0E00200). All branch lengths were omitted, since they were all bellow 0.021. Different colours represent strains origins: Pink – Wine; Black – Unknown; Blue – Other Beverages; Turquoise – Water; Yellow – Bread; Orange – Food; Green – Arboreal/Soil; Red – Clinical.

Appendix 2 – Assessment of growth of strains expressing the *S. cerevisiae* Hxt1 and Hxt3 transporters.

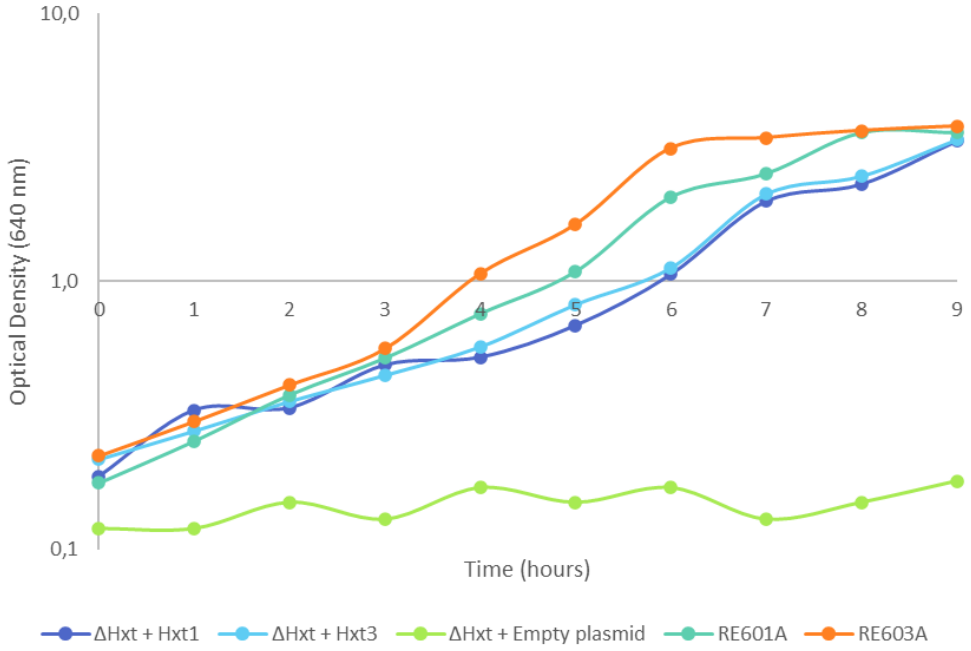


Figure A2. 1. Growth curves of *S. cerevisiae hxt* null transformed with an empty plasmid and a plasmid expressing *HXT1* and *HXT3,* and mutant strains of *S. cerevisiae* RE601A and RE603A, which only express the glucose transporters *HXT1* and *HXT3.* Strains were grown overnight in 10 mL of YPD medium and diluted to an OD_{640nm} = 0.1 in 10 mL of fresh YPD medium. Growth was followed up to 30 hours, but only the exponential phase of growth (up to 9 hours) is represented.