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Characterization of sugar transporters of biotechnological interest of S. cerevisiae strains

End of Degree Project

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BACHELOR'S DEGREE IN BIOTECHNOLOGY

Characterization of Sugar Transporters of Biotechnological Interest of *S. cerevisiae* Strains

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Caracterización de transportadores de azúcares de interés biotecnológico en cepas de *S. cerevisiae*

Resumen:

En la elaboración del vino, hay diversos genes que tienen una función en el transporte de azúcares, los genes de transportadores de hexosas (*HXT)*, que son una extensa familia multigénica. La hipótesis que se demuestra en este proyecto es que, en una cepa ancestral, posteriormente a la duplicación del genoma, algunas *HXT* se duplicaron y evolucionaron independientemente.

Sin embargo, debido a la gran similitud de su secuencia, pudieron ocurrir recombinaciones de una cepa a otra. Estas cepas incluyen tanto a *S. cerevisiae* (que es una de las cepas más importantes en la elaboración del vino) como a otras especies del género *Saccharomyces*, cada una con recombinaciones distintas. Concretamente, este trabajo se enfoca en la recombinación entre *HXT6* y *HXT7.*

Es interesante estudiar estas recombinaciones porque el tener un diferente transporte de azúcares puede afectar a las rutas metabólicas. Esto se demuestra experimentalmente en este proyecto. En otros casos en los que hay cambios en las rutas metabólicas no solo cambia el porcentaje de alcohol final, sino que también pueden cambiar los aromas, los cuales son otros metabolitos como los alcoholes superiores, el glicerol, y los ésteres de interés en la calidad final del vino.

Todo esto está relacionado con el balance redox NADH/NAD+, ya que en las levaduras las rutas metabólicas han evolucionado para regular y mantener la homeostasis redox, que se manifiesta principalmente por los niveles relativos de las parejas de cofactores NADH/NAD+ y NADPH/NADP+. Estos forman parte de unas doscientas reacciones en *S. cerevisiae* (Minebois *et al.,* 2020).

En consecuencia, las distintas características genómicas afectan a las diversas rutas metabólicas produciendo metabolitos diferentes, y, por lo tanto, características del vino como el aroma se verán afectadas. Por todo esto, durante este proyecto se utilizan diversas técnicas para demostrar el papel de los transportadores que presentan recombinación entre *HXT6* y *HXT7*.

Este estudio demuestra que los transportadores que muestran recombinaciones entre los genes *HXT6* y *HXT7,* presentan diferencias significativas en el consumo de glucosa y fructosa. Los resultados obtenidos han ayudado a ampliar el conocimiento en la caracterización molecular del transporte de azúcares, y su impacto en la velocidad de la fermentación vínica al crecer en diferentes fuentes de azúcares y concentraciones de estos.

Este trabajo se relaciona con los siguientes Objetivos de Desarrollo Sostenible (ODS) de la Agenda 2030: Trabajo decente y crecimiento económico y Producción y consumo responsables.

Palabras claves: *Saccharomyces cerevisiae,* transportadores de azúcares, fermentación, edición génica, factores de transcripción, vino.

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Characterization of sugar transporters of biotechnological interest of *S. cerevisiae* strains

Abstract:

In wine production, there are diverse genes that play a role in sugar transport, hexose transporter genes (*HXT)*, which are a wide multigenic family. The hypothesis that is demonstrated in this project is that, in an ancestral strain, after the genome duplication, some *HXT* were duplicated and evolved independently.

However, due to the high similarity in their sequence, recombinations from one strain to another one could have occurred. These strains include not only *S. cerevisiae* (which is one of the most important strains in wine production), but also other species of the genus *Saccharomyces*, each one with different recombinations. Concretely, this project is focused on the recombination between *HXT6* and *HXT7*.

It is interesting to study these recombinations because having a different sugar transport can affect metabolic pathways. This is experimentally demonstrated in this project. In other cases, in which changes in the metabolic pathways have happened, not only the final percentage of alcohol is changed, but also the aromas can change. These are other metabolites such as the superior alcohols, glycerol, and esters of interest in the final wine quality.

All this is related to the redox balance NADH/NAD+. In yeasts, metabolic pathways have evolved to tightly regulate and conserve redox homeostasis. It is exposed by the relative levels of pairs of cofactors NADH/NAD+ and NADPH/NADP+, which are electron carriers in about two hundred reactions in *S. cerevisiae* (Minebois *et al.,* 2020)*.*

In consequence, the different genomic characteristics affect the diverse metabolic pathways producing different metabolites, and therefore, wine characteristics such as the aroma can be affected. Due to all this, in this project several techniques are used to demonstrate the role of transporters that present the recombination between *HXT6* and *HXT7*.

This study demonstrates that the transporters that show recombinations between *HXT6* and *HXT7* genes present significant differences in glucose and fructose consumption. The obtained results have helped to broaden the knowledge in the molecular characterization of sugar transport and its impact in the wine fermentation rate, due to the growth in different sugar sources and their concentrations.

This project is related with the following Sustainable Development Goals (SDG) of 2030 Agenda: Decent work and economic growth and Responsible consumption and production.

Key words: *Saccharomyces cerevisiae,* sugar transporters, fermentation, genetic editing, transcription factors, wine.

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Nomenclatures and abbreviations

- **AUC:** area under the curve
- **Cfu:** colony-forming unit
- **DMSO:** dimethyl sulfoxide
- **FSY1:** fructose symporter yeast 1 gene
- **Fsy1p:** fructose symporter yeast 1 protein
- **GAL2:** galactose permease gene
- **GCAT:** growth curve analysis tool
- **gDNA:** genomic DNA
- **GFP:** green fluorescent protein
- **GPY:** glucose-yeast-peptone medium
- **HPLC:** high performance liquid chromatography
- **Hsps:** heat shock proteins
- ***HXT:*** hexose transporter gene
- ***HXT*-ampl:** hexose transporter amplifying primer
- **Hxtp:** hexose transporter protein
- ***HXT*-Seq:** hexose transporter sequencing primer
- **K_m:** Michaelis-Menten constant
- **LiAc:** lithium acetate
- ***MAL-R:*** maltose regulatory gene
- ***MAL-S*:** maltase gene
- ***MAL-T*:** maltose transport gene
- **MQ water:** milli-q water
- **NaOH:** sodium hydroxide
- **OD:** optical density
- **ODS:** Objetivos de Desarrollo Sostenible (Sustainable Development Goals)
- **ONT:** Oxford Nanopore Technology
- **PacBio:** Pacific Biosciences
- **PCR:** polymerase chain reaction
- **PEG-4000:** polyethylene glycol-4000
- **Rpm:** revolutions per minute
- **RGT2:** glucose transport restorer gene
- **SDG:** Sustainable Development Goals
- **SDS:** sodium dodecyl sulphate
- **SNF3:** sucrose non-fermenting protein gene
- **Snf3p:** sucrose non-fermenting protein
- **ssssDNA:** single-strand salmon sperm DNA
- **Tm:** melting temperature
- **UV/VIS:** UV–Visible spectrophotometer
- **YNB:** yeast nitrogen base

1. Introduction

1.1 *S. cerevisiae*

S. cerevisiae is a microorganism that has been considered for many years a biologic model for eukaryotic organisms. It has been used since the ancient times for industrial applications such as bread making or the alcoholic fermentation in the production of wine (Kessi-Pérez *et al*., 2020). The function and cellular organization of *S. cerevisiae* resemble the ones of more complex organisms. More than half of its genes are homologous to the human ones.

Generation time is the mass duplication per unit of time, and it is influenced by the growth media, de temperature and the strain used (Rn *et al.,* 2016). This yeast has a short generation time (between one and a half and three hours), a reproducible and genetically stable growth, and easy genetic manipulation. All this contributes to this yeast being used as a model organism for multiple studies of biological importance at a molecular level (Smith & Li, 2006).

Yeast's biotechnology has incorporated during the last decades new disciplines, such as functional genomics, metabolomics, metabolic engineering, and system biology, which offers us the opportunity to explore concrete properties in yeast with an important impact in the industry (Mattanovich *et al*., 2014).

The complete genome sequencing of S288c *S. cerevisiae* strain was achieved in 1996, by an international programme in which many scientists took part. This gave importance to *S. cerevisiae* being used as a model organism (Dolinski & Botstein, 2005). Eukaryotic organisms, including the yeast *S cerevisiae,* have genomes with very repetitive sequences in the chromosomes. As the second-generation sequencing techniques make very short lectures, they are not the best ones for the sequencing of these genomes (Jenjaroenpun *et al*., 2018).

The *de novo* assembly technology does very long lectures, what gives more capacity. These long lectures can be created using third-generation sequencing technologies, such as Oxford Nanopore Technology (ONT) or Pacific Biosciences (PacBio). For the error correction, short lectures obtained with Illumina can be used. With the assembly of the lectures obtained by these three techniques, complete sequences for all the yeast chromosomes are obtained, including the mitochondrial chromosome. Moreover, all this is done only in one step (Jenjaroenpun *et al.,* 2018).

In addition, with the direct sequencing of the RNA using the ONT technique, transcripts of complete length can be obtained. This can be used to identify transcriptional landscapes (Jenjaroenpun *et al.,* 2018). These techniques have allowed to distinguish hexose transporter genes (*HXT*) sequences from different species, which are very similar among them.

1.2 *A*lcoholic fermentation

Yeasts take part in the production of most biotechnological products around the world, as they create compounds used in a wide variety of products, such as drinks, human and animal feed, chemicals, and recombinant proteins (REPORTLINKER, 2018). The production and economic benefits derived from these are higher than the ones produced by any other industrial microorganism (REPORTLINKER, 2018). Some strains of *Saccharomyces cerevisiae* have acquired characteristics beneficial for specific industrial processes, due to the use of the capacity of this yeast to perform the alcoholic fermentation for the obtention of fermented products (Gallone *et al*., 2019). Therefore, the environmental pressure has an important role in the new functions' acquisition by yeasts (Galeote *et al*., 2010).

Concretely, these traits are derived from adaptative genomic changes, known as footprints of the domestication process (Minebois *et al*., 2021). They include chromosome (mis)segregation, aneuploidy, genome hybridization, horizontal gene transfer and chromosomal rearrangement (De Vries *et al*., 2019). One example of these new characteristics is a chromosomal rearrangement that occurred when a reciprocal translocation between chromosomes VII and XVI took place. This increased sulphite resistance, that is only found in *S. cerevisiae* wine strains (Pérez-Ortín *et al*., 2002).

Another acquired trait was a result of the genes duplicated in tandem *FOT1-2* and fructose symporter yeast 1 gene (*FSY1)*. The first one encodes an oligopeptide transporter, while the expression of the second one results in a high-affinity fructose/H+ symporter. Both give an advantage to wine strains during fermentation. These genes were found in region C, which is a result from the horizontal gene transfer from *Torulaspora microellipsoides* to *S. cerevisiae* wine yeasts (Minebois *et al*., 2021).

Grape must have a big quantity of fructose and glucose. *S. cerevisiae* prefers glucose over fructose (Guillaume *et al*., 2007). Therefore, glucose is the first component of the media to ran out during fermentation. An elevated fructose/glucose ratio could cause slow or stuck fermentations, what leads to the finished wines having an elevated fructose content, what is a big issue in wine industry (Galeote *et al*., 2010).

Ohmic technologies have allowed the discovery of a few molecular bases that lead to the particular characteristics of *S. cerevisiae* strains (Minebois *et al*., 2021). As a case in point, transcriptomic research was done to suggest that *S. cerevisiae* wine strains have a better performance at low temperatures due to the sulphur assimilation pathway and glutathione biosynthesis' genes up-regulation (García-Ríos *et al.,* 2014). Another example is that many metabolomic studies, by using phenotypic criteria, have defined the range of metabolic diversity in *S. cerevisiae* (Spor *et al*., 2009). However, more research about the wide metabolic diversity of *S. cerevisiae* is needed, overall, the existing one in wine production strains.

In alcoholic fermentation, most sugars are metabolized by yeasts, to obtain ethanol and carbon dioxide. Even if oxygen is present, this process takes place through alcoholic fermentation (Wunderlich & Back, 2009). It has been

used by humans for miles of years for wine and beer fermentation, as well as for baking (Galeote *et al*., 2010).

The sugars that are not degraded, are used for biomass synthesis, as well as several additional metabolites. These include organic acids, secondary metabolites that activate the taste and aroma, esters, and alcohols of sugars such as glycerol, which is the most important subproduct after carbon dioxide and ethanol (Minebois *et al.,* 2020). This is why the individual contribution of the carbon metabolism of yeasts to the global profile of the wine is very important for oenologists, as they search for wine production with balanced characteristics (acidity, alcohol, aroma) (Minebois *et al.,* 2020).

Yeasts can degrade sugars through two metabolic pathways, which are the fermentative and the oxidative ones. The glycolysis is common in both processes, and in it, sugars are metabolized into pyruvate. In the fermentative pathway, pyruvate is decarboxylated into acetaldehyde, which is reduced to ethanol. However, in the respiratory pathway, in presence of coenzyme A in the mitochondria, pyruvate suffers an oxidative decarboxylation (González-Siso *et al*., 2009).

Respiration can take place at low sugar levels and when oxygen is present. However, at high sugar concentrations, yeast metabolize sugars in addition by the fermentative pathway even if there is oxygen (Liu *et al*., 2017). According to the Crabtree effect in *S. cerevisiae*, at high sugar levels, cells change from a respiration that is energy-efficient, to a fermentation that is energy-inefficient. The reason of this shift is still not clear. This spilling of energy is also present in other unicellular organisms that live in high substrate concentrations (De Alteriis *et al*., 2018).

S. cerevisiae has two types of Crabtree effects, which are the short-term and the long-term ones. The short-term Crabtree effect is the start of the alcoholic fermentation when too much sugar is added to yeast cultures that have a sugar-limited respiration. However, the long-term Crabtree effect is the aerobic alcoholic fermentation in steady-state conditions at high glucose levels and high growth rates (De Alteriis *et al.,* 2018).

Yeast growth is the result of cellular division and the cell cycle progression. Under adequate growth conditions, yeast kinetic growth is like the typical growth curve of microbes, with four principal phases: latent (lag), exponential, diauxic and stationary (Stahl *et al.,* 2004). During the lag phase yeasts adapt to their environment synthetising enzymes to achieve a high growth rate. Then the exponential phase begins, in which DNA replication and cell division with the highest rate take place. In the diauxic phase, a slow growth takes place because of the change from a fermentative to a respiratory metabolism due to the lack of sugars in the media. Ethanol becomes the main carbon source (Stahl *et al*., 2004).

When the media runs out of nutrients, or other environmental factors, such as toxic compounds accumulation or high temperatures take place, cell division stops, and the stationary phase begins. Cells can survive in this phase for long periods of time, but if optimal environmental conditions aren’t established, cells would end dying (Werner-Washburne *et al*., 1993).

1.2.1 Role of hexose transporters in alcoholic fermentation

The first essential step in hexose sugars utilization is their absorption by yeasts. In these microorganisms there are two types of sugar transporters. The first type are the transporters of facilitated diffusion, that use a mechanism that is independent of energy, which mediates the sugar transport down their concentration gradient (Galeote *et al*., 2010). The second type are the symporters of sugar and protons, that are active and dependent on energy. They only act when there is a low sugar concentration, and they couple the sugar absorption to the proton one. This is relevant when yeast growth is at low sugar concentrations in the extracellular media (Galeote *et al*., 2010).

Sugar transporters are very important in the alcoholic fermentation, as it is based on the conversion of sugar into alcohol (Luyten, 2002). This is why the characterisation of these molecules in the model organism *S. cerevisiae* is the main aim of this project.

1.3 Hexose transport in *S. cerevisiae*

Glucose is not only the main source of both energy and carbon in most cells, but also the monosaccharide most present in nature (Özcan & Johnston, 1999). Its metabolism starts with glucose going through the plasma membrane, what limits the rate. Glucose can regulate the activity of glucose transporters, as well as their number and types. It achieves this because it becomes a stimulus in the environment that takes part in the regulation of these transporters at both transcriptional and posttranslational levels, making possible a glucose efficient metabolism (Özcan & Johnston, 1999).

This happens overall in the yeast *Saccharomyces cerevisiae,* where hexose transport takes place only by facilitated diffusion transporters. Twenty genes are translated into hexose transporters. These genes include *HXT1-HXT17*, galactose permease gene (*GAL2)*, sucrose non-fermenting protein gene (*SNF3)*, and glucose transport restorer gene (*RGT2),* which belong to the major facilitator superfamily and have twelve transmembrane domains (Henricsson *et al*., 2005), (Diderich *et al*., 1999).

The affinity of these proteins for glucose is different among them, but their expression and function are regulated by different levels of glucose. Therefore, when the substrate concentration increases, transporters are either negatively or positively regulated. At high glucose levels, *HXT1* transporter is expressed, while at low glucose concentrations the main transporters are *HXT2* and *HXT4* (Ozcan et al., 1998), (Ozcan and Johnston, 1995). The main molecules that mediate glucose, fructose and mannose transport are Hxt1-4p, Hxt6p and Hxt7p. Moreover, Hxt5, Hxt8-11p, Hxt13-17p, Gal2p and some maltose transporters can transport glucose and therefore, when they are overproduced, can support growth in a strain without any other transporter (Özcan & Johnston, 1999).

There are different kinetic properties in the proteins coded by the seven hexose transporter genes. Their value of the Michaelis-Menten constant (K_m) is variable. Moreover, they have different methods of regulation (Galeote *et*

al., 2010). The discrepancy in the glucose and fructose utilization by *S. cerevisiae* is a step that cannot be omitted in wine fermentation. In oenological conditions, sugar transport is very relevant. Therefore, *HXT1*, *HXT2*, *HXT3*, *HXT6* and *HXT7* are needed for the optimal fermentation of sugar in grape must (Luyten, 2002).

The role of *HXTs* in a *S. cerevisiae* strain, called V5, was studied. The genes of sugar transporters *HXT1-HXT7* were eliminated from this strain. Then the *HXT* genes were cloned in V5, to find out their function in different conditions of the alcoholic fermentation. There was neither growth nor fermentation of V5 in wine production conditions (Luyten, 2002). In addition, the only transporters that when expressed alone led to a complete sugar fermentation were *HXT1* and *HXT3*. Therefore, they have an important function in wine fermentation. Nevertheless, they have different roles: *HXT3* has a predominant role, due to the fact that it is the only transporter that gives a normal fermentation profile when it is produced alone. However, *HXT1* acts only at the beginning of the fermentation (Luyten, 2002).

HXT2, *HXT6* and *HXT7* transporters have more affinity and are needed for normal fermentation (Table 1). They have also different roles: *HXT2* acts at the beginning of the growth, while *HXT6* and *HXT7* take part at the end of the alcoholic fermentation. At least four hexose transporters are necessary for this fermentation being successful. Each of them has a function in a different stage of the fermentation (Luyten, 2002).

In this way, transporters involved in the catabolic hexose consumption can be classified in three groups: *HXT6* and *HXT7* as high affinity transporters, *HXT2* and *HXT4* as medium affinity transporters and *HXT3* and *HXT1* as low affinity transporters (Perez *et al*., 2005) (Table 1).

*Table 1 Characteristics of the main HXT in yeasts. HXT: hexose transporter genes. Adapted from (*König *et al., 2009).*

HXT	**Affinity for glucose**	**Regulation by glucose in laboratory strains**	**Expression during fermentation in wine strains**
HXT1	Low	Induced by high glucose concentration	Fermentation start
HXT2	Moderate	Induced by low glucose concentration Repressed by high glucose concentration	Lag phase
HXT3	Low	Induced by high and low glucose concentration	During fermentation
HXT4	Moderate	Induced by low glucose concentration Repressed by high glucose concentration	Induced by the growth phase
HXT5	Moderate High	No regulated by glucose Regulated by the growth speed	Not inducible
HXT6	High	Induced by low glucose concentration Repressed by high glucose concentration	Induced in stationary phase
HXT7	High	Induced by low glucose concentration Repressed by high glucose concentration	Induced in stationary phase

All the hexose transporters of *S. cerevisiae* can also transport fructose. However, they have a high Michaelis constant for this sugar, so glucose is the preferred sugar for *HXT* transporters (Perez *et al*., 2005). Furthermore, in *S.*

cerevisiae no symporters of fructose/H+ can be found, not as in filamentous fungi or other hemiascomycetes yeasts. Until now in this yeast only hexose uniporters have been identified. One exception is fructose symporter yeast 1 protein (Fsy1p), a fructose transporter specific for *S. cerevisiae* (Galeote *et al*., 2010).

To efficiently use small hexose concentrations, *S. cerevisiae* needs a hexose transport with high affinity (Km, 1 to 2 mM) (Ye *et al*., 2001). The transcription of *HXT7* hexose transporter is usually repressed at high glucose concentrations, as it has a high capacity for glucose transport. In these conditions, the yeast ferments glucose to produce ethanol, regardless of the oxygen presence (Ye *et al*., 2001).

However, when the glucose is consumed and only little amounts remain in the media, the expression of this transporter increases. In this case, *S. cerevisiae* uses the respiratory catabolism (Henricsson *et al*., 2005). Moreover, if a strain only has *HXT7* as hexose transporter, its expression is essential during the whole growth phase in the glucose media, and it is higher when the glucose concentration decreases (Ye *et al*., 2001).

In addition, when the media where the yeast is growing runs out of glucose, *HXT7* mRNA in *S. cerevisiae* decreases, independently if the yeast only has the *HXT7* transporter, or it has more. Nevertheless, after glucose depletion, the *HXT7* protein remains stable (Ye *et al.,* 2001). Regarding the subcellular localization of the *HXT7* transporter, in the presence of glucose (either low or high quantities), *HXT7* is in the plasma membrane. However, after glucose depletion, *HXT7* is not needed anymore, so it is endocytosed and destroyed in the vacuole (Ye *et al*., 2001). The expression of *HXT7* is similar in aerobic and hypoxic conditions. Moreover, its expression is low when there are low levels of oxygen or no oxygen is present (Rintala *et al*., 2008).

HXT6 expression is very low compared to the one of *HXT7*. However, it is highly expressed in media with raffinose, low glucose concentrations, or non-fermentable carbon sources. Moreover, it is repressed at high glucose levels (Liang & Gaber, 1996). This transporter is usually more expressed in hypoxic conditions than in aerobic conditions. This happens in both steady state and non-steady state cultures as a response to induce an increase in sugar uptake when the oxygen levels are limiting (Rintala *et al*., 2008).

In spite of their expression differences, *HXT6* and *HXT7* genes are joined in tandem in the right arm of chromosome IV (Maier *et al.,* 2002). Hxt6p and Hxt7p are very related, as they are proteins of 570 amino acids and differ only in two residues, 293 (Valine/Isoleucine) y 556 (Threonine/Alanine). However, upstream differences in these genes indicate that their regulation is different (Boles & Hollenberg, 1997).

HXT6 allows the growth of V5 in glucose, while *HXT7* gives the greatest growth in this sugar. Hxt6p y Hxt7p compensate each other and act to obtain the correct fermentation termination. These high affinity hexose transporters are involved in one critical step of wine fermentation, as the consumption of the small sugar quantities that remain at the end of the fermentation is usually difficult (Luyten, 2002). In addition, as they have a

higher affinity than Hxt3p for fructose, their efficacy at the end of the fermentation can be better, as in this stage there is only fructose. The expression of these genes is repressed due to high hexose concentrations in the must, so it is likely that the expression of these genes is derepressed during the fermentation due to the sugar consumption (Luyten, 2002).

As these transporters act in the last stage of the fermentation, they need to be efficient in a medium with high ethanol quantities (Luyten, 2002). Therefore, both *HXT6* and *HXT7* have important activities with non-fermentable carbon sources by yeast, such as glycerol, ethanol, maltose, and galactose (Boles & Hollenberg, 1997).

1.4 Comparison between strains V5 and S288c

V5 *S. cerevisiae* strain was obtained by sporulation of a yeast strain specialized in wine production, concretely Champagne. Most lab strains cannot consume all the sugars present in the grape must, so a strain derived oenologically was needed (Luyten, 2002). Hexose transporters from *HXT1* to *HXT7* were eliminated in this strain to study the yeast's adaptation to the conditions of wine production (Luyten, 2002).

The strain with the deletion cannot live in a medium with a wide variety of glucose or fructose concentrations. This strain is unable to grow on glucose, so glucose flux and uptake are under the limits of detection. Therefore, any other hexose transporter that hadn't been eliminated could take up enough hexose to support the growth in this sugar (Luyten, 2002).

The *HXT6* and *HXT7* gene expression in V5 is used to compare the role of these molecules in glucose consumption in oenological conditions (Luyten, 2002). As V5 has no hexose transporters, maltose is needed for the growth of this strain. The consumption of this molecule doesn't start until the glucose concentration is low. Maltose is slowly absorbed, and ATP is needed to facilitate its absorption. Maltose is transported by specific maltose carriers, and then it is hydrolysed into glucose molecules (Serrano, 1977).

The use of maltose requires three genetic steps in which MAL genes take place. The maltose regulatory gene (*MAL-R*) is an activator gene that detects the presence of sugars and activates the other genes. Moreover, the maltose transport gene (*MAL-T*) codes for an active transport permease that allows specific sugar absorption. In addition, the maltase gene (*MAL-S*) produces maltase, which hydrolyses the links 1,4-1,6 to produce individual glucose molecules that are then decomposed in the glycolytic pathway. (Kodama et al., 1995).

S288c is a laboratory strain, which has been used for biochemical studies. It isn't flocculent in a minimal medium with specific nutrients (Mortimer & Johnston, 1986). It is a universal strain of *S. cerevisiae*. In comparison with the average *S. cerevisiae* strains, S288c has a superior proliferative rate on a rich medium, as it has high maltose utilization. However, its capacity to tolerate high levels of ethanol is lower than that one of the average strains. This strain is widely spread, so its unique phenotype is a matter of interest (Blomberg *et al*., 2011).

The V5 coding sequences of the genes *HXT3* and *HXT5* are one hundred percent identical to the ones of S288c. However, *HXT1*, *HXT2*, *HXT6* and *HXT7* have substitutions, deletions, or insertions. Some of these result in amino acid changes (Luyten, 2002). Nevertheless, the amino acid sequences of the corresponding proteins are identical in more than a 98%, The amino acid sequence of Hxt6p has the greatest number of changes, concretely 10 amino acids. Several of them are highly conserved in the *HXT* transporters family (Luyten, 2002).

Hxt6p and Hxt7p proteins are very similar, but they are more divergent in V5 than in S288c. In V5 they have an identity of 97.9%, whereas in S288c their identity is of 99.6% (Luyten, 2002). Moreover, the *HXT4* gene of the strain V5 has a nonsense mutation in the 123 codon, what changes the reading frame. This has happened due to the nucleotide 366 deletion (Luyten, 2002).

Regarding the promoters' sequences, there are no differences between the two strains for *HXT3,* and in the rest of promoters of *HXT* only small differences. Nevertheless, for the *HXT4,* in the open reading frame a big difference between the two strains was identified. The promoter sequence has various changes of only one base and one deletion. This elimination ends in the deletion of one recognition place of Mig1p, that has a function in the expression or repression of *HXT4* due to glucose (Luyten, 2002).

1.5 Strain BC187 of *S. cerevisiae*

It is a strain from America, concretely a California isolate, used in wine fermentation (Martínez-Garay *et al*., 2016). It is one of the vineyard strains with better capability at high temperatures (Deed & Pilkington, 2020). One characteristic of this strain is that it induces genes that respond to galactose before glucose is depleted, leading to galactose consumption. So, this strain can consume both glucose and galactose at the same time and has a short diauxic lag (Wang *et al*., 2015).

BC187 depletes glucose slowly, and before glucose consumption, it grows slowly. However, as BC187 is able to grow when glucose is exhausted, in approximately three hours, it can double its population size (Wang *et al*., 2015).

1.6 Strain DBVPG 6044 (402) of *S. cerevisiae*

DBVPG 6044, 402, CBS 405 or NCYC 3290 is a geographically isolated lineage of *S. cerevisiae* . Its natural habitat is West Africa, and its main source of isolation is Bili wine (Field *et al.,* 2015). DBVPG 6044 is characterized for being resistant to furfural, which is an industrial aldehyde obtained from agricultural subproducts (Field *et al*., 2015) . It is also important to emphasize that this strain has structured genome type (West *et al*., 2014)

1.7 Importance of this work

By the insertion of *HXT6* and *HXT7* carriers from different donor strains in *HXT1-7Δ* V5, this research provided evidence on the expression and important function of these two transporters. Concretely, this research studied the growth of V5 based on kinetic parameters such as the lag time, the maximum speed, and the area under the curve. In this way, it was compared the sugar transport of Hxt6p and Hxt7p from different *S. cerevisiae* strains that are less susceptible to genetic modifications.

This project demonstrated that, although Hxt6p and Hxt7p are more expressed at low hexose levels due to their high affinity for these sugars, they might not be repressed at high glucose and fructose concentrations. If there is a stress present in the cell, such as nitrogen starvation, these carriers could avoid the inhibition process. This would mean that these transporters take part at the end of fermentation, when sugar levels have been reduced. However, they can also play a role at the beginning of this process when they scape repression.

Furthermore, this study proved, that, although *HXT6* and *HXT7* are very related, they are regulated in a distinct manner. This confirms the hypothesis of the presence of duplications, recombinations and rearrangements between the loci of these two genes. This has led to the fact that in this research *HXT6* had a greater expression than *HXT7.* Nevertheless, in previous studies, *HXT7* was the *HXT* gene with the strongest function.

In addition, the carried-out analysis revealed the significance of Hxt6p and Hxt7p in wine fermentation. Strains containing these transporters contribute in a different way to the rate of this process. This allows the selection of the carrier that provides the most efficient sugar uptake for each fermentation.

To sum up, contrasting the behaviour of Hxt6p and Hxt7p from different *S. cerevisiae* strains helped to understand the glucose transport in the yeast cell, as well as the molecular system behind this process.

2. Objectives

2.1 General objective

The general objective is to characterize *HXT6* and *HXT7* from different *S. cerevisiae* strains, to demonstrate the recombination between these transporters that occurred due to the similarity between their sequences.

2.2 Specific objectives

One of the specific objectives is to characterize the kinetic growth of V5 strain of Saccharomyces *cerevisiae* transformed with *HXT6* and *HXT7* from different selected strains. For this, lag time, maximum speed and area under the curve are compared.

Moreover, this project also specifically aims to characterize the fermentative capability of these transformed V5 yeasts, by doing glucose and fructose consumption trials in media with different sugar concentrations.

In addition, this research also has a specific objective, to prove if yeasts with recombinant *HXTs* obtain and consume glucose and fructose in the same way as yeast with not-recombinant *HXT*s or it changes. This is useful to improve the fermentation performed by yeast for wine obtention.

Finally, to evaluate the molecular expression of hexose transporters during the transformed V5 growth to select strains that allow a better use of the sugars present in the media is another specific objective.

3. Materials and methods

3.1 V5 MTF 255 verification and sequencing

To prove that the DNA from V5 MTF 255 was not degraded, this strain was incubated for 24h at 28ºC in an 20g/L agar plate with 200g/L maltose, 2.5 g/L bacteriological peptone and 2.5 g/L yeast extract. Then, a DNA extraction from this V5 strain was done. The lysis of yeast colonies from the culture was performed in 200 mM Lithium Acetate (LiAc) and 1% Sodium Dodecyl Sulphate (SDS), at 95ºC for 15 minutes. Then, the DNA was precipitated with ethanol (Lõoke *et al.,* 2011*)*. The extracted DNA was used in a colony-Polymerase Chain Reaction (PCR).

The PCR was done according to the standard protocol of Takara Ex (Takara Bio Inc., Shiga, Japan). For one reaction of 50 µL, 0.25 µL of enzyme, 5 µL of buffer 5X, 4 µL of dNTPs 2.5 mM, 2 µL of template DNA and 1 µL of each primer (Table 2) were used. The reaction general conditions were: 1 cycle of 5 min at 95ºC, 30 cycles of 30s at 95 ºC, 1 min at the optimal hybridization temperature for each nucleotide pair, a variable time (1 min for each Kb) at 72ºC for the extension, and the final extension cycle of 10 min at 72ºC. The last phase was the maintenance of the samples at 4ºC.

Table 2 Primers used for V5 verification. HXT3: hexose transporter three gene. HXT7: hexose transporter seven gene

***HXT3* S1 (forward)**	5'-GGTTGCATATAAATACAGGCGCTGT-3'
C2-*HXT7* (reverse)	5'-ATTATAGGAGAGGTATCTACGTA-3'

Takara Ex was used because it has a greater fidelity than most polymerases. With this PCR it was proved that the primers for the strain V5 MTF 255 worked, and they could be used for the next steps. The PCR products were separated by an electrophoretic run in an agarose gel 1% (P/V). The bands were observed with RedSafe™ staining. The approximate size of the bands was compared with a molecular marker (Genbiotech 100 bp DNA ladder). The obtained gels were examined, and a photo was taken using a Gelprinter Plus transilluminator with a dark camera for UV isolation.

For purifying the V5 strain, the High Pure PCR Cleanup Micro Kit from Roche for samples from 100 bp to 50 kb was used, as the sample had approximately 500 bp. Binding buffer was added to the PCR product. This was added to a column and centrifuged at maximum speed for one minute. The flowthrough was discarded, and the wash buffer was added. Centrifugation was done at 13 000 x g for one minute, and the flowthrough was discarded again. This step was repeated but adding less amount of wash buffer.

Then, the elution buffer was added, and after two minutes in which the membrane was absorbing the buffer, centrifugation was done at the same speed and time. An agarose gel 1% (P/V) was prepared and on it, the purified DNA sample from V5 strain and the 100 bp molecular marker were loaded.

In the Gelprinter Plus transilluminator the gel was seen (Figure AII.1). The DNA concentration was measured in the NanoDrop™ 1000 (NanoDrop Technologies, Wilmington, Delaware). by using the programme ND-1000 V3.8.1, concretely the nucleic acid section. The elution buffer was the blank.

The V5 strain was sequenced by Eurofins. At the beginning and at the end of the sequence there is no specificity in the sequencing, so both primers and the purified PCR product fragment were sent in order to obtain better results in the sequencing. In this way, the sequencing was more complete than if we only had sent one primer. The primers' and PCR product's concentrations were indicated by Eurofins. This sequencing was done to prove that the V5 strain DNA fragment where the *HXT* were going to be introduced was correct, as this strain had been conserved with glycerol 15% at -80ºC.

3.2 Donor strains DNA sequencing and culture

DNA extraction was performed with the zymolyase method (UNIVERSITY OF NOTRE DAME, 2021, 2021). Then, the extracted DNA was amplified by in vitro cloning (PCR) using the Phusion enzyme and its standard protocol (Phusion™ High–Fidelity DNA Polymerase, from Thermoscientific). Later, the purification of the obtained PCR product was performed with the High Pure PCR Cleanup Micro Kit from Roche, following the steps mentioned before.

Dimethyl Sulfoxide (DMSO) is used to lower the annealing temperature, as all the high-fidelity polymerases have a high annealing temperature, approximately 60 ºC. If this temperature is too high, the effectivity of the primers is lower than it should be, so DMSO is added.

The annealing time needed for this polymerase was 30 seconds every kb, and the length of the genes *HXT6* and *HXT7* is more than 2 kb. Therefore, the annealing time should have been more than one minute, so one minute and half was set in the thermocycler. The next step was to quantify the samples by using the ND-1000 V.3.8.1 nanodrop programme. Then, they were sequenced by Eurofins with the Sanger method. For the sequencing, the samples were prepared as stated by the company and as previously mentioned.

The sequencing was performed to find out the sequences of these strains, as they were not known, due to mistakes in the assembly and annotation, which vary depending on the technique. Afterwards, these strains were conserved in glycerol 15% at -25ºC. The strains were plated in Glucose-Yeast-Peptone Medium (GPY) (5 g/L yeast extract, 5 g/L bacteriological peptone 20 g/L glucose and 20 g/L agar) using the technique of triple streak. In this way, single separated colonies were obtained on the medium. The plate was incubated at 28ºC for three days to let the colonies grow.

3.3 Transformation primers' design and dilution

Sequences shown in Table 3 were designed taking into consideration the length, of approximately 25 nucleotides, as well as the percentage of CGs that the sequence should have. Moreover, the melting temperature (Tm) was

important, as it determined de annealing temperature. Furthermore, the primer's sequences were introduced in BLAST, to ensure that they were not homologous to other regions in the genome. The first part of the sequences was homologous to the part of the V5 DNA sequence where the *HXT6* and *HXT7* were going to be integrated, whereas the last part of the sequence was homologous to the *HXT6* and *HXT7* genes of the donor strains (Table 3).

The forward primer was designed by joining the V5 part of the sequence where the gene was going to be inserted and the beginning of the *HXT6* and *HXT7* sequences in the donor strain, that had to begin with ATG nucleotides, as this is the start codon for the protein synthesis (Table 3). The reverse primer was designed by doing the reverse complementary of the V5 DNA sequence where the gene was going to be inserted. This was joined to the reverse complementary of the nucleotides located 500 bp after the last part of the *HXT6* or *HXT7* sequence of the donor strains.

By designing primers in this way, translation mistakes were avoided, as ribosomes need these 500 bp to end the translation in a correct manner (Table 3). In the donor strains, as all the protein genes were of interest, the primers had to be placed at the beginning and at the end of the sequence (Table 3). If they had been in the middle of the sequence, a part of the protein would have been lost.

Table 3 HXT-ampl primers' sequences. HXT-ampl primers: Transformation Hexose Transporter Amplifying primers

	Primer's sequence
***HXT*-ampl-1 (Forward)**	ATAGAATCACAAACAAAATTTACATCTGAGTTAAACAATCATG TCACAAGACGCTGCTATTGCA
***HXT*-ampl-2 (Reverse)**	AAGTGACGGGCGATGAGTAAGAAAGAAATAACTGACTCATTA GAGACAAGCCTTCTCTACTCTGTCG
***HXT*-ampl-3 (Reverse)**	AAGTGACGGGCGATGAGTAAGAAAGAAATAACTGACTCATTA GACCATAGATGCATTGTGAAAATTGAA
***HXT*-ampl-4 (Forward)**	ATAGAATCACAAACAAAATTTACATCTGAGTTAAACAATC ATGTCACAAGACGCTGCTGTCGCAG
***HXT*-ampl-5 (Reverse)**	AAGTGACGGGCGATGAGTAAGAAAGAAATAACTGACTCATTA GATTCAGGTTTATTGGCAAGCCTTCTC
***HXT*-ampl-6 (Reverse)**	AAGTGACGGGCGATGAGTAAGAAAGAAATAACTGACTCATTA GAATATCCTCTCCATAGATGCATTGTG

The *HXT*-ampl primers were incubated in the thermoblock 5 minutes at 42ºC, so that the DNA was completely dissolved. Then a dilution 1:10 was performed to obtain a final primer concentration of 10 µM.

The primers for *HXT7* in all the strains were *HXT*-ampl-1, which was the forward one, and either *HXT*-ampl-3 or *HXT*-ampl-6 as the reverse (both could be used). However, *HXT*-ampl-1 and *HXT*-ampl-2 were the forward and reverse primers, respectively, for *HXT6* from S288c, BC187, L-1528 and RM11-1a strains, whereas for the strains DBVPG 6044 (402), YIIC17-E5, YPS128 and DBVPG 6040, were the primers *HXT*-ampl-4 (forward) and *HXT*-ampl-5 (reverse).

3.4 Donor strains' *HXT6* and *HXT7* for transformation

The genomic DNA (gDNA) was extracted by using a method usually used for analysing the mitochondrial DNA, to obtain purer DNA for sequencing. Then, the Phusion enzyme was used for the PCR because it is of high fidelity, according to its standard protocol previously mentioned. The results of the PCR were seen in an agarose gel 1% (P/V) with RedSafe™ Nucleic Acid Staining Solution (iNtRION Biotech.) (Figure AII.2).

HXT6 and *HXT7* DNA were purified with a High Pure PCR Cleanup Micro Kit from Roche. It was for samples from 100 bp to 50 kb , as the samples had approximately 1700 bp. Later, *HXT6* and *HXT7* DNA were quantified in the NanoDrop™ 1000. Then, the purified samples were run in an agarose gel 1% (P/V) together with a 100 bp molecular marker (Genbiotech 100 bp DNA ladder).

3.5 V5 preculture for transformation

A medium containing 5g/L yeast extract, 5g/L bacteriological peptone and 200g/L maltose was prepared. This medium was used to grow the V5 for the transformation. Maltose is needed because the V5 strain doesn't have hexose transporters and wouldn't be able to grow if the medium had glucose.

The next day, a dilution 1/20 was done to 1 ml of the medium, and the Optical Density (OD) was measured in the spectrophotometer, using the maltose medium as the blank. The appropriate quantities of culture and medium were added to obtain a final OD of 0.2. This mix was let to grow in an Erlenmeyer at 28ºC with agitation until the following day.

3.6 Transformation

The preculture OD was measured and diluted to obtain an OD value between 1 and 2. Then the V5 culture was spun down for four minutes at 4000 revolutions per minute (rpm). The supernatant was removed, and the cells were resuspended in autoclaved dH_2O. Then the culture was centrifuged again in the same conditions. The supernatant was removed, and cells were resuspended in 100 mM LiAc. The following step was to do two aliquots of cell suspensions, each of them with an OD of 10.

The tubes were spun in the microcentrifuge at 13000 rpm for 1 minute, and the supernatant was removed. Moreover, single-strand salmon sperm DNA (ssssDNA) was denatured in the thermoblock at 95ºC for 10 minutes. The function of this DNA is to avoid the DNA of *HXT6* and *HXT7* stays in the cell

wall instead of going through the membrane pore. After 10 minutes, the ssssDNA was placed in ice.

Then a mix that contained 50% (w/v) Polyethylene Glycol-4000 (PEG-4000), 1M LiAc and 70 µl of a DNA solution was added, considering the concentration of the purified PCR products of *HXT6* and *HXT7*. For the transformation, 1 µg of DNA is needed. After these steps, the tubes were "wash boarded" to resuspend the pellet in the transformation mixture, so that it was homogeneous. The cells were incubated with agitation at 28ºC for 30 minutes.

Then they were incubated at 42ºC in the thermoblock for 25 minutes to do the thermal shock. Later, the tubes were centrifuged at 13 000 rpm for one minute. Then, the transformation solution was removed, and the tubes were incubated in the medium used for the V5 preculture (as the colonies were not totally transformed yet) for two hours at 28ºC in agitation.

After this time, the transformation product was plated in a minimum medium containing 0.05 g/L uracil, 6.70 g/L yeast nitrogen base (YNB) and 20 g/L glucose. Then the plates were incubated five days at 28ºC. The colonies that grew in the transformation plates were replated in the minimum medium, spreading the colonies to obtain more quantity of them. The plates were incubated at 28ºC, so that the yeasts grew for two days.

3.7 Transformation verification

The DNA from the colonies that grew in the transformation plate was extracted by the fast method (Lõoke *et al*., 2011), or, in case many colonies grew in the plates, a quicker method was used. It consisted of denaturing colonies in Sodium Hydroxide (NaOH) 0.02 N at 95ºC for five minutes. With this extracted DNA, a PCR was performed according to the standard protocol used for NZYTaq II DNA polymerase (NzyTech). The amplified samples were then loaded in an agarose gel 1% (P/V).

3.8 Transformants' PCR, purification and quantification

The colonies that in the gel showed a band of approximately 2.5 kb, higher than the one of V5, were probably transformants. This is because *HXT6* and *HXT7* gene fragments have a size of approximately 1700 bp, so considering the primers size, the total length is near 2000 bp. These possible transformants were amplified with the Phusion polymerase, according to its protocol. Then, they were purified following the protocol from Roche mentioned before. These purified samples were loaded in an agarose gel 1% (P/V) to ensure that the PCR product was present. Moreover, they were quantified in the NanoDrop™ 1000, with the program ND-1000 V.3.8.1.

3.9 Transformed colonies sequencing and preservation

Sequencing was done to verify that either the *HXT6* or *HXT7* genes were correctly inserted in the V5 strain. Eurofins sequenced the samples with the Sanger method. For the sequencing, the samples were prepared as stated by the company. In each Eppendorf, one of the primers (*HXT3* S1, C2 *HXT7* or

HXT-seq) (Table 4) and the purified PCR product, which had a concentration between 20 and 80 ng/µl, were introduced.

Apart from *HXT3* S1 and C2 *HXT7 primers*, *HXT*-Seq (hexose transporter sequencing primers) (Table 4) were used because they bind to a place closer to *HXT6* and *HXT7*, allowing the sequencing to be more specific. Moreover, they are shorter than the ones of *HXT*-ampl.

Table 4 HXT-Seq primers used for the verification of V5 transformants. HXT-Seq primers: Hexose transporters sequencing primers

***HXT*-Seq1-Forward**	5'-ATGTCACAAGACGCTGCTATTGCA-3'
***HXT*-Seq2-Reverse**	5'-GACAAGCCTTCTCTACTCTGTCG-3'
***HXT*-Seq3-Reverse**	5'-CCATAGATGCATTGTGAAAATTGAA-3'
***HXT*-Seq4-Forward**	5'-ATGTCACAAGACGCTGCTGTCGCAG-3'
***HXT*-Seq5-Reverse**	5'-TTCAGGTTTATTGGCAAGCCTTCTC-3'

The V5 colonies that had either the *HXT6* or *HXT7* gene correctly inserted, were conserved in 15% glycerol at -25ºC, as this compound maintains their protein structure.

3.10 SPECTROstar measurement and data analysis

For this measurement, V5-*HXT7*-S288c, V5-*HXT7*-BC187 and V5-*HXT6*-402 precultures in GPY were prepared. These were incubated for 24h at 28ºC with agitation. Then, they were centrifuged at 12000 x g one minute, and the supernatant was discarded. The colonies were washed twice with sterile distilled water.

The colonies were placed for two hours in 1 ml of PBS 1x. The following step was to measure the OD of each inoculum (Countess Automated Cell Counter 3 FL, Thermo Fisher Scientific, Waltham, MA). It was adjusted to an OD of 4 in one millilitre for each of the samples. 10 microplates of 96 wells (Figure 1) were read in a SPECTROstar Omega of BMG Labtech at 28ºC. This temperature was selected because it is the optimum one for yeast growth, so a possible inhibitory effect of this factor was avoided. The yeast growth was monitored at 600 nm.

To each well of the plates (Figure 1), 11µl of sample and 220µl of culture medium were added. The mediums contained 10x YNB and 0.05 g/L uracil with different concentrations of either glucose or fructose. The concentrations were from 0 mM to 325 mM. Due to experimental errors, the concentrations of the prepared media are usually different to the ideal ones. Therefore, the real sugar (fructose and glucose) concentrations were measured by using High-Performance Liquid Chromatography (HPLC) (Thermo Fisher Scientific, Waltham, MA).

The HPLC had a refraction index detector and an UV–Visible Spectrophotometer (UV/VIS) (210 nm) detector equipped with a HyperREZ™ XP Carbohydrate H+ 8 mm column (Thermo Fisher Scientific, Waltham, MA) and HyperREZ™ XP Carbohydrate Guard (Thermo Fisher Scientific, Waltham, MA) . Moreover, the conditions used for the analysis were: eluent, 1.5 mM of H_2SO_4; 0.6 ml min−1 flux and oven temperature of 50ºC.

The plates were placed in the SPECTROstar for 50h. Previously to each cycle, 10 seconds of shaking took place. Moreover, measures were taken every 30 minutes. The inoculates were always over the SPECTROstar detection level. This was determined by doing the comparison with a calibration curve previously stablished. Furthermore, the wells of the edges only had either distilled water or medium, without colonies. This was done to obtain the noise signal and, therefore, subtract it.

In total, 240 growth curves were analysed (20 different mediums x 3 strains x 4 replicates). In the following figure a scheme of how the inoculates were distributed in the plates can be seen.

	1	2	3	4	5	6	7	8	9	10	11	12
A			0 mM		0.125 mM		0.25 mM		0.5 mM			
B			---------- without inoculum ----------									
C			S288c	BC	S288c	BC	S288c	BC	S288c	BC		
D			S288c	BC	S288c	BC	S288c	BC	S288c	BC		
E			S288c	BC	S288c	BC	S288c	BC	S288c	BC		
F			S288c	BC	S288c	BC	S288c	BC	S288c	BC		
G			---------- without inoculum ----------									
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A			0 mM	0.125 mM	0.25 mM	0.5 mM	1 mM	2 mM	4 mM	8 mM		
B			---------- without inoculum ----------									
C			402	402	402	402	402	402	402	402		
D			402	402	402	402	402	402	402	402		
E			402	402	402	402	402	402	402	402		
F			402	402	402	402	402	402	402	402		
G			---------- without inoculum ----------									
H												

Figure 1 Distribution of strains and glucose medium concentrations in a SPECTROstar plate. This distribution was similar for the rest of the plates. The blue wells represent autoclaved distilled water, used as a contamination control. The wells without inoculum only had medium. The wells with BC187, S288c and 402 have the corresponding strain and medium. BC:BC187.

Growth curve analysis tool (GCAT) (MORGRIDGE INSTITUTE FOR RESEARCH, n.d.) was used to adjust the data and create growth curves. Three main parameters were considered: the lag time, the maximum speed growth rate, and the area under the curve (AUC).

4. Results and discussion

In this project it has been studied the different function of *HXT6* and *HXT7 S. cerevisiae* hexose transporters in different sugar concentrations. To perform this functional analysis, V5 strain, that does not have any hexose transporter, was used.

4.1 *HXT1-7*Δ V5 sequencing

The sequencing of V5 strain from *S. cerevisiae* was done to prove that the genome from this strain was the correct one. In Figure 2 it was seen that V5 sequencing results coincided with the reference sequence of this yeast. Concretely, it coincided with *HXT3,* which is the hexose transporter gene located before the section of sequence where *HXT6* and *HXT7* from donor strains were going to be introduced. Therefore, it could be said that the V5 strain was right. The comparison of sequences was performed with MEGA (Software package for phylogenetic analysis with a graphical user interface).

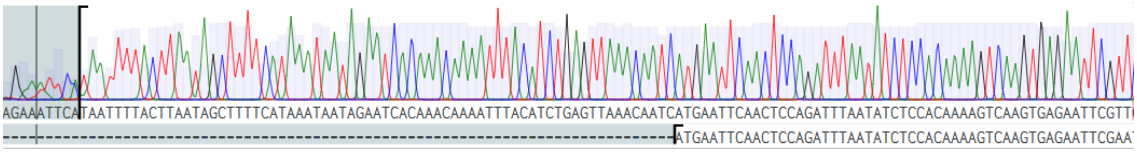

Figure 2 V5 sequencing results obtained with Mega programme. The reference sequence is located on top of the V5 real sequence. The hyphens show the part of V5 sequence where HXT6/HXT7 were going to be introduced.

4.2 Transformed V5 colonies

Figure 3 showed three agarose gels with bands corresponding to transformed and not transformed V5 colonies. Moreover, there were also negative controls of Milli-Q (MQ) water to prove that there was not contamination present. Transformed V5 colonies showed a band of approximately 2500 pb, as not only the size of the *HXT6* or *HXT7* carriers should be considered, but also the V5 region that was amplified by primers *HXT3* S1 and C2 *HXT7* (Figure 3 A and B) or *HXT*-ampl (Figure 3 C).

HXT3 S1 and C2-*HXT7* bound to a segment of V5 sequence that was located before and after the place where *HXT6* and *HXT7* should be inserted. Therefore, even if the hexose carriers were not inside the V5 genome, the region of the V5 sequence before and after the place where these transporters should be introduced was amplified, having a size of approximately 500 bp. In consequence, the bands that appeared at 500 bp corresponded to not transformed colonies. Nevertheless, the bands at 2.5 kb, were possible transformed colonies (they were later verified by sequencing).

With all this, it could be deduced that in Figure 3 A the V5-*HXT7*-402 was the one in well 1. The not transformed V5 colonies were the ones in wells 2,3,4 and 5. In wells 6 and 7 there were no bands due to errors in the PCR or in the DNA extraction of those colonies. In figure 3 B, wells 1 and 6 corresponded to V5-*HXT7*-BC187 colonies, while the rest were not transformed V5. However, as there were two bands, what was amplified in

the PCR was the DNA of more than one colony. To isolate colonies and obtain one band, serial dilutions were performed to the original group of colonies. The dilution 10^{-5} was replated in another plate containing the same medium. The separated colonies were verified again, and only one band of 2500 bp was obtained. In Figure 3 C, bands in wells 1,3,4,5,6 and 8 were V5-*HXT7*-S288c, while the rest were not transformed V5 colonies.

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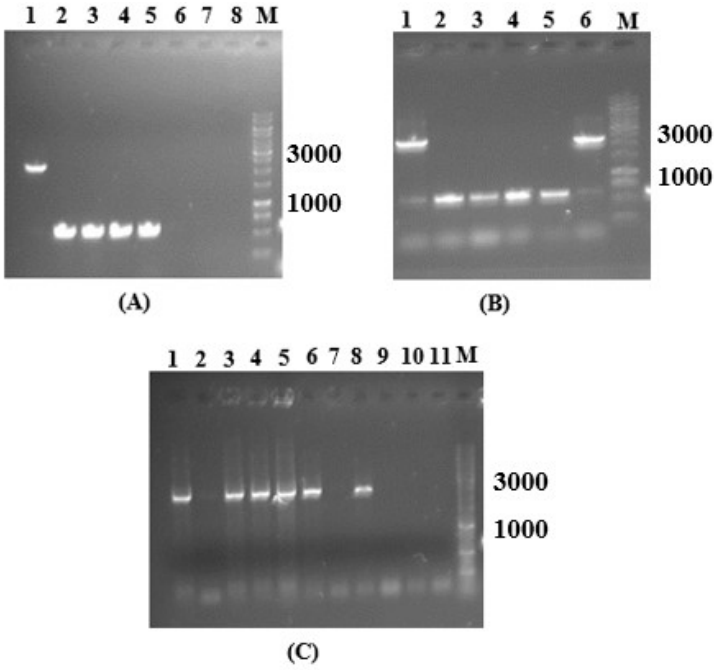


Figure 3 Agarose gels' electrophoresis of NzyTech PCR products from possible V5 HXT6/HXT7 transformants. (A) V5-HXT6-402 (1), not transformed V5 (2-5), not amplified colonies (6 and 7), MQ water negative control (8). (B) V5-HXT7-BC187 (1,6), not transformed V5 (2-5). (C) V5-HXT7-S288c (1,3,4,5,6,8), not transformed V5 (2,7,9) and V5 and mQ water negative controls (10,11). In all gels M: base pair marker (bp) of 1 kb.

4.3 Transformation efficiency

Transformation efficiency refers to the number of colony forming units (cfu) which are created by transforming 1 µg of purified PCR product into competent cells (Figure 4) (Carson *et al*., 2019).

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol. at recovery (mL)}}{\text{vol. plated (mL)}} = \text{Number of transformants per µg}$$

Figure 4 Formula of transformation efficiency. Vol: volume (EDVOTEK, 1987).

The number of transformants used for the calculations of Table 5 was the number of cells that grew in the transformation plates. However, there were many false positives, as when these cells were spread in another plate to obtain enough quantity for DNA extraction, only some of them grew. Moreover, in the verification of transformants by PCR amplification, most of them were negative. The exceptions for this were one V5-*HXT6*-402 colony

(Figure 3 A), two V5 colonies with *HXT7* from BC187 (Figure 3 B) and 6 V5 colonies with *HXT7* from S288c (Figure 3 C). All these colonies were checked by sequencing and were positive, except for the colonies whose bands are in wells 6 of Figure 3 B and in wells 3, 4, 5 and 8 in Figure 3 C.

From each transformation, two plates were obtained, as in one of them 100 µl of cells were plated and, in the other one, 150 µl. Therefore, the total number of plated cells was 0,25 mL. Cells were plated in two different plates to observe the differences between both plates and in case one of them was contaminated, to not lose all the transformants. In general, both plates had a similar number of transformants, independently of the cell volume plated.

The final volume recovery corresponded to the sum of the number of competent cells used for the transformation and the number of liquid media added to those cells for their recovery and posterior plating. In the transformations shown in Table 5, the volume of competent cells used was of 100 µl, as this amount was enough to obtain a final OD of 10 for each transformation reaction. The volume of medium added was 250 µl, which was the necessary quantity for cell recovery and growth after heat shock.

The number of transformants per µg of purified PCR product should be in a range between $1x10^4$ and $1x10^8$ (EDVOTEK, 1987). However, as it was observed in Table 5, the transformation efficiency was low due to possible errors such as a wrong heat shock temperature and time, a not efficient medium specific for the selection of only positive transformants, or not waiting enough time for the cells' recovery.

If the heat shock time and temperature were not correct, the *HXT6* or *HXT7* PCR products could not be inserted in yeast cells. This is since, at high temperatures, not only yeast synthesize heat shock proteins (Hsps), but also the lipidic composition of membranes changes, creating pores through which the PCR amplified genes could enter the cells (Mejía-Barajas *et al*., 2016).

Moreover, if the medium used had, apart from glucose, other nutrients that the yeasts could use as a source of energy, the V5 was not transformed with effectiveness. In addition, the cell recovery time should have been enough to allow the lipid membrane composition turn back to the original state. In this way, pores are closed and the introduced amplified *HXT* genes are not lost. If this time had been too short, transformants would have been lost.

To try improving the transformation efficiency, several conditions were changed. For example, the time and temperature of the thermal shock. Instead of 25 minutes at 42ºC, it was changed to 35ºC 25 minutes, 40ºC 15 minutes and 40ºC 25 minutes in several transformations. However, the combination of time and temperature that best results gave was the one of 42ºC for 25 minutes. Moreover, the medium where the colonies from the transformation were incubated was also changed, in total three different media were used. They were the minimum medium previously mentioned, GPY with 20 g/L glucose, and GPY with 1 g/L glucose.

The differences in yeast growth between these different media were that in GPY with 20 g/L glucose many colonies grew, but very few were positive after the verification by PCR and posterior sequencing. The same happened in the minimum medium, although fewer colonies grew than in GPY. This happened because GPY was a rich medium, as it had more amino acids and other nutrients than the minimum medium used. Moreover, in GPY with 1 g/L glucose very few colonies grew, but most of them were positive. This might have happened because *HXT6* and *HXT7* are high affinity transporters, so with low glucose concentrations they could provide cell survival.

The last factor that was changed was the recovery time. The cells were incubated at 28ºC during two, three and six hours, or even during the whole night, before plating them. After several experiments with these varying times, most transformed colonies were obtained with a recovery time of three hours. Less time was not enough, and mutated colonies were lost because the alterations induced by chemicals in the cell wall and membrane were not restored. However, more recovery time was also prejudicial because cells stopped growing due to the run out of medium.

Another factor that could have contributed to having a low transformation efficiency was the donor strains used. Maybe when *HXT* genes from these strains were introduced in V5 strain, transformants needed different sugar concentrations for their recovery.

Table 5 Transformation efficiency of V5 with HXT6 or HXT7 from different strains. HXT: hexose transporter gene

Strain	HXT6/ HXT7	Transformants	Positives	Negatives	Puridifed PCR product (µg)	Final recovery volume (ml)	Plated volume (ml)	Trasnformation efficiency
S288c	HXT6	4	0	4	1	0,35	0,25	6
	HXT7	9	2	7	1	0,35	0,25	13
402	HXT6	53	2	51	1	0,35	0,25	74
	HXT7	35	0	35	1	0,35	0,25	49
BC187	HXT6	64	0	61	1	0,35	0,25	90
	HXT7	33	1	32	1	0,35	0,25	46
L-1528	HXT6	43	1	42	1	0,35	0,25	60
	HXT7	14	0	14	1	0,35	0,25	20
RM11-1a	HXT6	19	0	19	1	0,35	0,25	27
	HXT7	5	1	4	1	0,35	0,25	7
DBVPG 6040	HXT6	30	0	30	1	0,35	0,25	42
	HXT7	23	0	16	1	0,35	0,25	32
YPS128	HXT6	14	0	14	1	0,35	0,25	20
	HXT7	15	0	15	1	0,35	0,25	21
YIIC17-E5	HXT6	68	0	68	1	0,35	0,25	95
	HXT7	24	0	24	1	0,35	0,25	34

4.4 Effect of *HXT6* and *HXT7* alleles on V5 strain on growth

Isolated *HXT6* and *HXT7* genes from donor strains S288c, BC187 and 402 (DBVPG 6044) were expressed in strain V5 by homologous recombination. The growth by the analysis of the lag time, maximum speed, and AUC of the three V5 *HXT* mutated strains was assessed at concentrations of glucose and fructose ranging from 0 to 325 mM. These concentrations were chosen to observe differences in the growth of *HXT* mutated V5 at low, medium, and high glucose and fructose levels.

Moreover, low sugar levels were very close to each other, as well as high sugar concentrations, to better see growth variances between V5 transformants. However, in the media containing the lowest sugar concentrations yeast didn't grow well, and precise data for the lag time and AUC parameters were not obtained. Models are useful for the characterization of yeast growth under different conditions. For the building of these models, growth needs to be measured. The logarithm of the number of organisms against time is the growth curve, which is sigmoidal (Zwietering *et al*., 1990).

In the studied conditions, fermentation starts after a short lag phase. This phase is followed by the exponential one, which is usually brief. In this time the fermentation rate, which is proportional to CO_2 production, increases parallelly to the number of yeast cells (Luyten, 2002). Just after the highest value of the fermentation rate is obtained, the stationary phase starts, due to nitrogen starvation and a decline in intracellular amino acids. In addition, there is a reduction in ethanol and glycerol, which are the main fermentation products. Moreover, CO_2 levels decrease until they reach 0 (Minebois *et al*., 2020). After this, yeast run out of sugars and the rate of fermentation is reduced, which leads to the end of the fermentation process (Luyten, 2002).

Models are used to describe the growth curve and to find interesting parameters among all the measured data. These models describe the number of organisms without the substrate consumption, as there is enough substrate to reach high organisms' numbers (Zwietering *et al*., 1990).The three main parameters used for the description of the growth curve are the lag time, the maximum specific growth rate, and the AUC.

The maximum specific growth rate (μ_m) is the slope of the line when there is an exponential growth of the organisms. Moreover, it is the tangent in the inflection point. It is decided which curve part is approximately linear, and after this, the slope of this curve part is calculated by linear regression (Zwietering *et al*., 1990). The lag time (λ) is the x-axis intercept of the maximum specific growth rate (Zwietering *et al*., 1990). The AUC is the area between the x axis and a curve. To calculate the AUC between two points, it is done the integral between these two points (VEDANTU, n.d.)

In this research, the general growth of the transformed V5 strains was measured at different glucose and fructose levels with the SPECTROstar Omega of BMG Labtech at 28ºC. Then, the mentioned parameters were calculated by using the GCAT (MORGRIDGE INSTITUTE FOR RESEARCH, n.d.). The models used in this project to calculate these parameters were the ones better adapted to the data obtained: Gompertz, Richards and the logistic. The growth of the different *HXT* mutated V5 strains was compared.

4.4.1 Effect of *HXT6* and *HXT7* alleles on V5 strain lag time

Lag time is the time yeast need to change from a dormant state to an active metabolism. In Figure 5 it was observed that the lag time of both V5-*HXT7*-S288c and V5-*HXT7*-BC187 was much higher in glucose (Figure 5 A) than in fructose (Figure 5 B). However, this time was very similar for V5-*HXT6*-402

in both media. This might mean that *HXT7* had more affinity for fructose than for glucose. However, *HXT6* had the same affinity for both sugars.

Moreover, in Figure 5 A, clear difference was seen in the lag time at different sugar concentrations for V5-*HXT7*-BC187, being the highest values at low glucose levels. The same happened in the fructose media, although the variation was lower. This could mean that glucose and fructose are essential components for the fermentation process. However, for V5-*HXT6*-402 the lag time was constant and brief at all glucose levels, meaning that there was an early and great glucose uptake. In the medium containing fructose, the variation was higher, being the highest lag time at 111.2 mM (Figure 5). V5-*HXT7*-BC187 had the highest lag time in both media, what meant that it was the transformant that needed more time to start fermentation. This was shown overall in concentrations from 121.6 to 191.3 mM in the glucose medium, where the differences in lag time with V5-*HXT7*-S288c and V5-*HXT6*-402 were great (Figure 5).

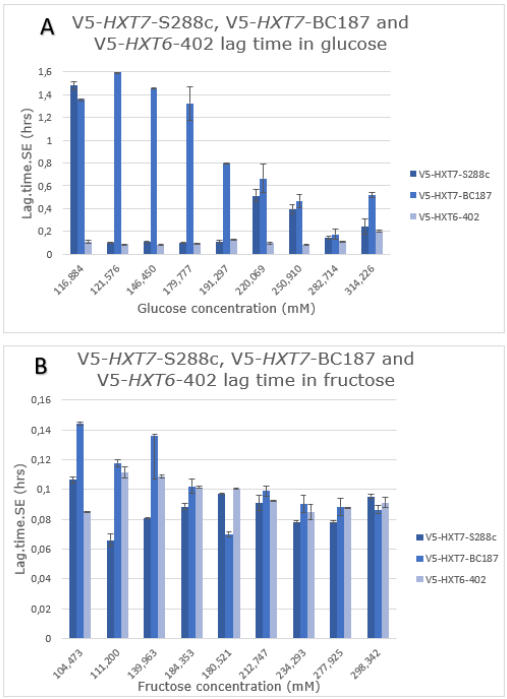


Figure 5 Lag time profile of wild type V5 strain carrying either HXT7 from S288c or BC187 and HXT6 from 402 at different glucose and fructose concentrations. The values are displayed as mean and standard deviations from quadruplicate experiments. SE: standard error

4.4.2 Effect of *HXT6* and *HXT7* alleles on V5 strain maximum speed

As shown in Figure 6, at the four smallest sugar concentrations none of the transformants grew enough in neither of the two media to see an increase in its maximum speed or slope. Despite this, when the sugar concentration started to increase, the V5 with *HXT7* from either S288c or BC187 showed a higher maximum speed than V5-*HXT6*-402 in both media.

Moreover, at these same concentrations in glucose, the V5-*HXT7*-BC187 was faster than V5-*HXT7*-S288c (Figure 6 A). However, in fructose, V5-*HXT7*-S288c showed a higher maximum speed than V5-*HXT7*-BC187 (Figure 6 B). The maximum speed of V5-*HXT6*-402 started to increase at a concentration of 8.7 mM in the case of the glucose medium (Figure 6 A), but at 4.1 mM in the medium containing fructose (Figure 6 B). The maximum growth rate of this V5-*HXT6*-402 got higher than the one of the other transformed V5 strains at middle sugar concentrations. This difference was greater with glucose as main sugar than with fructose (Figure 6).

When comparing V5-*HXT7*-S288c and V5-*HXT7*-BC187, at the lowest and highest glucose levels, V5-*HXT7*-S288c was the one growing more slowly. However, this mutant was the one that achieved the highest growth rate of the three transformants at 146.5 mM (Figure 6 A). In addition, in the medium containing fructose, V5-*HXT7*-S288c only had a smaller maximum speed than V5-*HXT7*-BC187 at the smallest sugar levels (Figure 6 B).

When the slope of the yeast growth was higher, yeast grew faster due to the high number of sugars they took. Therefore, results from Figure 6 suggested that *HXT6* allowed a better hexose transport than *HXT7* at middle sugar concentrations, allowing a higher maximum speed of the transformants containing this transporter. However, the graphs represented a higher affinity of *HXT7* for either glucose or fructose at low sugar levels. Concretely, *HXT7* from BC187 showed the highest slope values. On the contrary, *HXT7* from S288c acted better at high glucose and fructose levels. Despite this fact, at the highest glucose levels, there was a decrease in the maximum growth rate of V5 containing this transporter (Figure 6).

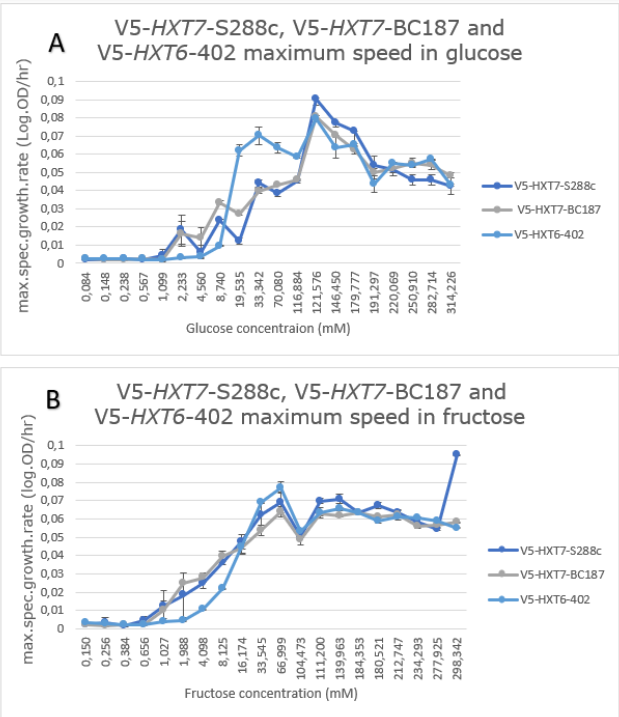


Figure 6 Maximum speed profile of wild type V5 strain carrying either HXT7 from S288c or BC187 and HXT6 from 402 at different glucose and fructose concentrations. The values are displayed as mean and standard deviations from quadruplicate experiments. OD: optical density

4.4.3 Effect of *HXT6* and *HXT7* alleles on V5 strain AUC

AUC.OD is the abbreviation for the area under the curve optical density. AUC takes into consideration the initial size of the yeast population, the carrying capacity, and the rate of growth. In consequence, it is used as a parameter that represents a growth curve. V5-*HXT6*-402 was the yeast that showed the highest values of AUC in all glucose and fructose levels, except for fructose 111.2 mM.

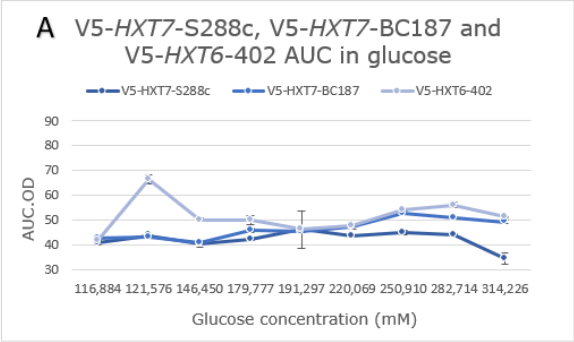
Moreover, in glucose it had its maximum at a low concentration, but in fructose it had it at the highest concentration. These data suggest that there had been a greater growth of this transformant than of V5-*HXT7*-BC187 or V5-*HXT7*-S288c at sugar levels shown in Figure 7, meaning that *HXT6* allowed a better hexose transport at high fructose and glucose concentrations than *HXT7*.

Furthermore, when doing the analysis of *HXT7* from strains S288c and BC187, there were differences in the growth in glucose medium (Figure 7 A) and in the fructose one (Figure 7 B). *HXT7* from BC187 was more efficient in transporting glucose than this hexose transporter from S288c, as the parameter of area under the curve was higher for V5-*HXT7*-BC187 than for V5-*HXT7*-S288c.

In contrast, the AUC values of transformants containing *HXT7* from either of the two strains were very similar when fructose was the sugar present in the medium (Figure 7 B). Therefore, the growth of these transformants was of the same magnitude. This could mean that these two yeasts had a more similar growth behaviour in a medium containing fructose than in one containing glucose.

It should also be considered that AUC in V5-*HXT7*-S288c, V5-*HXT7*-BC187 and V5-*HXT6*-402 increased with fructose concentration, while in glucose this linear growth only occurred in transformants containing *HXT7* from BC187 (Figure 7). By observing the data obtained, it could be deduced that the expression of hexose transporters *HXT6* and *HXT7* in V5 resulted in a higher glucose and fructose uptake.

Taking everything into consideration, *HXT6* and *HXT7* were able to restore V5 growth in media with different glucose and fructose levels. It should be highlighted that *HXT6* had a better affinity for fructose and glucose than *HXT7* in the conditions in which this study was conducted. Therefore, a lower lag time and a higher maximum speed and AUC were obtained for V5 containing *HXT6.*



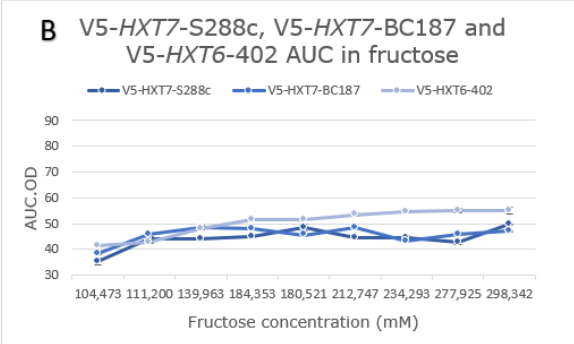


Figure 7 AUC profile of wild type V5 strain carrying either HXT7 from S288c or BC187 and HXT6 from 402 at different glucose and fructose concentrations. The values are displayed as mean and standard deviations from quadruplicate experiments. OD: optical density

4.4.4 V5 with *HXT6* and *HXT7* growth study

As *HXT6* and *HXT7* transporters could consume fructose at low concentrations, they showed high affinity for this sugar, which is the more present at the fermentation end (Luyten, 2002). This allows Hxt6p and Hxt7p to be efficient at a point that is critical for wine fermentation, as they can use as a source of energy the remaining sugars in the last fermentation steps.

As Hxt6p and Hxt7p are high-affinity transporters, results obtained in the research of this project proposed that they are expressed during fermentation, where sugar concentration is reduced due to its consumption. These results coincide with the fact that Hxt6p and Hxt7p have an important function at constant low glucose levels, which was stated by Boles and Hollenberg (1997).

Yeasts can grow on 0.1% glucose by only expressing either *HXT6* or *HXT7* genes (Maier *et al*., 2002). This glucose concentration is approximately 8 mM, so the results corroborate this, as at 8 mM the maximum speed of V5 *HXT6* and *HXT7* mutants was already high. Moreover, as stated by Diderich *et al*. (1999), the expression of *HXT6* and *HXT7* genes augmented at glucose levels of 100 mM. This is corroborated by data obtained in this project, as from approximately this concentration, the three studied parameters indicated an increase in transformant's growth.

Boles and Hollenberg (1997) showed that Hxt6p and Hxt7p are high-affinity glucose transporters which are repressed at high levels of glucose, due to their saturation by the accumulation of sugar molecules in the medium. Hxt6p repression occurs by the glucose repression pathway, and it is mediated by sucrose non-fermenting protein (Snf3p). However, the way in which Hxt7p is repressed is still unknown.

Data obtained for lag time, maximum speed, and AUC showed a decrease in the Hxt6p expression at high glucose concentrations, although it was not completely repressed. Maybe higher concentrations of glucose than the ones used in this study were needed for the complete repression of Hxt6p. Regarding Hxt7p, at high glucose levels this transporter started before its metabolic activity than when there was not much glucose in the medium. However, its maximum speed and AUC were reduced at high glucose concentrations. Nevertheless, as it happened with Hxt6p, its complete repression did not occur.

The fact that *HXT6* and *HXT7* were expressed even in the presence of high glucose and fructose levels also occurred in the experiments performed by Perez *et al*. (2005). Therefore, these carriers may not have a role only at the fermentation end, but also at the beginning. However, this *HXT6* and *HXT7* regulation does not coincide with the previously mentioned repression of these transporters by glucose. As even if there is growth arrest, their expression is still inducted, they might escape glucose repression by different mechanisms (Perez *et al*., 2005). One of them could be a stress response, as usually growth arrest occurs due to the running out of nitrogen. When this happens, yeast cells develop a stress response. Specifically, *HXT7* carrier is stress-inducible to some degree (Perez *et al*., 2005).

Ye *et al*. (2001) stated that Hxt7p was in the plasma membrane when, at high glucose levels, this transporter was expressed in a strain that did not have any other hexose carrier. To find out this, they created a Hxt7p:Green fluorescent protein (GFP) fusion protein and studied its localization in the cell. However, when glucose exhaustion occurred, Hxt7p was endocytosed and degraded in the vacuole. Therefore, it is needed a high glucose transport capacity for the *HXT7* repression and Hxt7p degradation at high glucose levels (Ye *et al*., 2001).

HXT6 and *HXT7* are similarly transcriptionally regulated, but in some laboratory strains *HXT7* is more expressed. In fact, *HXT7* is the most greatly expressed *HXT* gene under non repressing conditions (Boles & Hollenberg, 1997). However, the results of this project are not consistent with this statement, as they show a higher expression of *HXT6* than of *HXT7*.

According to Diderich *et al*. (1999), genetic modifications occurred in *HXT6* in chemostats with low glucose levels. Concretely, rearrangements and duplications happened in the tightly linked *HXT6* and *HXT7* loci, which led to higher levels of *HXT6* mRNA. Nonetheless, the behaviour of *HXT7* was not like the one of *HXT6*. For this reason, these two transporters are differentially regulated, in spite of their tight link and equal open reading frame sequences (Diderich *et al*., 1999).

Moreover, Hxt6p and Hxt7p are expressed independently of the carbon source (Boles & Hollenberg, 1997). The results obtained in this research coincide with the fact that, when using a different carbon source such as fructose, these hexose carriers are also expressed.

Hexose Transporter Proteins (Hxtp) can independently act as transporters of glucose. Moreover, together with Hxt1-4p, Hxt6-7p are the most relevant transporters in *S. cerevisiae* (Maier *et al*., 2002). As results show, only expressing either *HXT6* or *HXT7* on V5, this strain was able to grow at different glucose levels, so they are important in *S. cerevisiae.* The shape of *HXT6* and *HXT7* components of the hexose transporter family might have been modified by the growth and fermentation of *S. cerevisiae* in mediums containing glucose and fructose as main sugars, such as wine or grape must (Luyten, 2002).

Data obtained in this project added evidence to the fact that the function of hexose carriers in sugar uptake is very relevant in the wine fermentation rate. A possible consequence of sugars such as glucose or fructose being exhausted is that their uptake is reduced, which might result in extremely slow or not completed fermentations.

Broader knowledge and a better comprehension of the members of the hexose carrier family and their function across the plasma membrane are useful to avoid prolonged fermentations. This can be achieved by the optimization of yeast strains such as *Saccharomyces cerevisiae* in wine production processes (Luyten, 2002).

5. Conclusion

1. Results from this research corroborate that *HXT6* and *HXT7* and the different alleles have a relevant function in sugar transport. The kinetic characteristics, using different glucose and fructose concentrations, of the *S. cerevisiae* strains that expressed the different alleles of *HXT6* and *HXT7* have been determined.

2. Hxt6p and Hxt7p high-affinity transporters are expressed at low hexose concentrations, according to the growth data, while they are repressed at high hexose levels. They can scape this repression at high amounts of glucose. These data suggested the role of these carriers in both the beginning and the end of fermentation.

3. *HXT6* and *HXT7* are differentially regulated, in spite of their tight genetic connection. This might be caused by recombinations and rearrangements between *HXT6* and *HXT7* loci.

4. In this study, it is emphasized the relevance of the different genetic variants Hxt6p and Hxt7p, and, therefore, the yeasts containing these transporters, in glucose and fructose uptake, and how it influences the wine fermentation rate.

5. Comparing the components of the huge sugar transporters' family from yeast in terms of function and structure can contribute to the sugar transport molecular characterization. Moreover, it should throw light on the molecular mechanisms that drive the transport of substances across cell membranes.

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7. Annexes

Annex I. Relationship of the project with the Sustainable Development Goals (SDG) of 2030 Agenda

The Sustainable Development Goals (Table AI.1) are seventeen interconnected global objectives that were designed in September of 2015 to improve life in the planet and create a better future for all living beings. Therefore, it is of great relevance to accomplish these aims not only at a professional level, but also in every field.

Table AI.1 Relationship of the project with the Sustainable Development Goals of 2030 Agenda

Sustainable Development Goals	High	Medium	Low	Not applicable
SDG 1. No poverty				x
SDG 2. Zero Hunger				x
SDG 3. Good Health and well-being				x
SDG 4. Quality education				x
SDG 5. Gender equality				x
SDG 6. Clean water and sanitation				x
SDG 7. Affordable and clean energy				x
SDG 8. Decent work and economic growth	x			
SDG 9. Industry, innovation and infrastructure				x
SDG 10. Reduced inequalities				x
SDG 11. Sustainable cities and communities				x
SDG 12. Responsible consumption and production	x			
SDG 13. Climate action				x
SDG 14. Life below water				x
SDG 15. Life on land				x
SDG 16. Peace, justice and strong institutions				x
SDG 17. Partnerships for the goals				x

This project is strongly related with the SDG 8. Decent work and economic growth. This SDG has as an objective to develop a sustained and inclusive growth in the economy, as well as to promote a respectable and productive employment for everyone. Concretely, it can be related to the point 1.2 "the production and economic benefits derived from yeasts are higher than the ones produced by any other industrial microorganism" (REPORTLINKER, 2018). Moreover, if wineries use high affinity transporters like *HXT6* and *HXT7*, they will need less sugar, what will lead to reduced expenses, and therefore, an economic development.

This research can also be connected to the SDG 12. Responsible consumption and production, that is focused on improving the efficiency and management of natural resources. In particular, it can be related with point 4.4.4. "Hxt6p and Hxt7p have an important function at constant low glucose levels" (Boles & Hollenberg, 1997). If by-products different from glucose originated in cell metabolic routes are used for wine fermentation, a low hexose concentration will be transported by Hxt6p and Hxt7p. In this way, a reduced sugar quantity would be used for wine production, resulting in a more ecological product.

In conclusion, the research of this project contributes to the sustainable production of quality foodstuff. Moreover, it contributes to its security and consumer acceptance, as well as to economic growth.

Annex II. Gels' results

Some results of intermediate steps of materials and methods are shown in this annex. In Figure AII.1 it is clearly seen that the PCR product from V5 was correctly purified, as clear bands are seen in wells 1 and 2. Moreover, there is no contamination, due to the lack of band in well 3.

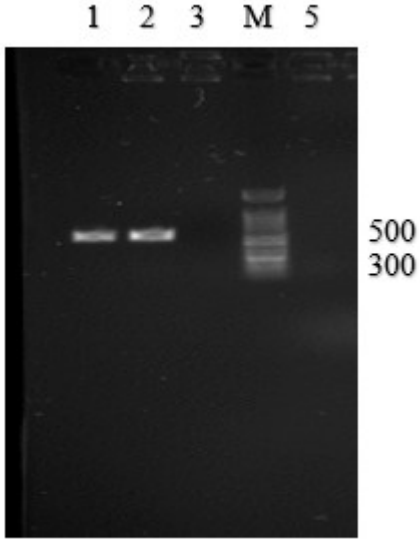

Figure AII.1 V5 purification verification. V5 purified PCR product (1 and 2), MQ water control (3). No added sample (5) M: base pair marker (bp) of 100 bp

In Figure AII.2 it is observed that in all wells there is a band corresponding to a size of approximately 2 Kb. This is the size of *HXT6* and *HXT7* transporters of different strains amplified by *HXT*-ampl primers, so the PCR amplification of these genes was correct. However, in wells 29, 30 and 33 no bands are seen.

They correspond to RM11-1a *HXT7* (29 and 30) and DBVPG 6040 *HXT7* (33). Maybe these genes were not amplified due to errors in DNA pipetting, or it is possible that for RM11-1a *HXT7* it was needed a different annealing temperature, as neither of the two bands were seen. There are two bands for these transporters because, as mentioned in the Materials and methods section, for their purification 100 µl of PCR product were needed, so two Phusion PCR products of 50 µl were used.

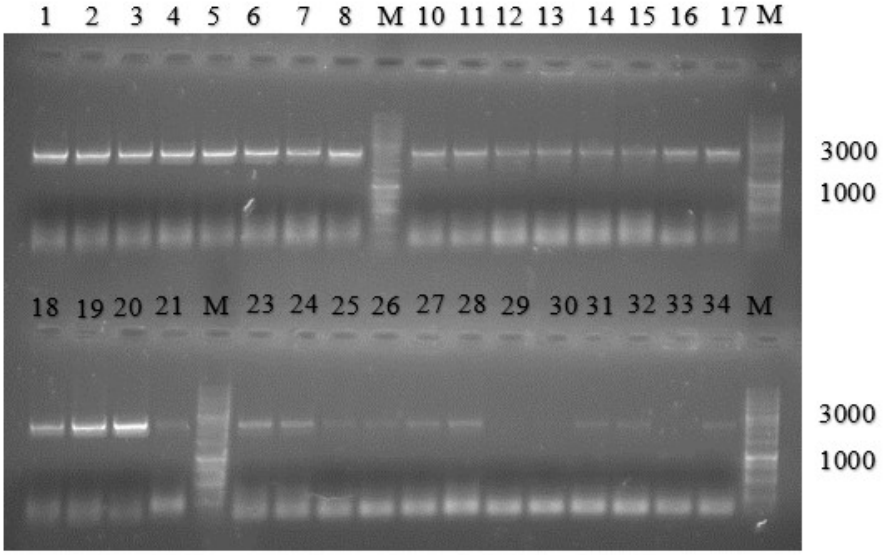

Figure AII.2 Agarose gels' electrophoresis of Phusion HXT6 and HXT7 PCR products from different donor strains. There are two wells for each strain transporter located together. S288c, BC187, L-1528, RM11-1a, 402, YIIC17-E5, YPS128, DBVPG 6040 HXT6 (1-17). S288c, BC187, 402, L-1528, Y11C17-E5, RM11-1a, YPS128, DBVPG 6040 HXT7 (18-34). M: base pair marker (bp) of 1 Kb.