Chapter 13

The Emerging Role of the Yeast *Torulaspora delbrueckii* in Bread and Wine Production: Using Genetic Manipulation to Study Molecular Basis of Physiological Responses

Andreia Pacheco, Júlia Santos, Susana Chaves, Judite Almeida, Cecília Leão and Maria João Sousa

Additional information is available at the end of the chapter

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1. Introduction

Saccharomyces cerevisiae is the yeast primarily responsible for both grape must and bread fermentation and has been used for centuries in wine and bread making. Commercial yeasts utilized by these industries are essentially strains of *S. cerevisiae* that have been selected and optimized for these applications. Nowadays, the desire to produce consumer-directed wines and bread of differentiated styles has led to the emergence of new standard selection criteria for desirable yeast strains and has expanded the selection of yeasts to other environments and to non-*Saccharomyces* species (1, 2). In this context, strains of *Torulaspora delbrueckii* display particular traits that have caught the attention of the bread and wine industries. However, commercial exploitation of *T. delbrueckii* in wine production has only recently begun, and this yeast is not yet used consistently in the bread industry, apart from Japan (3). This is mainly due to the lack of knowledge of its genetic and physiological background, particularly when compared to *S. cerevisiae*, the yeast traditionally used and one of the best-studied microorganisms. In latest years, there has been a substantial effort to characterize *T. delbrueckii* strains potentially interesting for industrial use, especially at the physiological and biochemical levels. However, there have been few studies regarding their molecular characterization, due to the lack of efficient molecular biology tools and of a sequenced genome. Two strains of interest for the bread industry have been isolated from traditional corn and rye bread dough from the North of Portugal. Both strains are potentially interesting to use in frozen dough technology due to their particularly high resistance to osmotic and freeze stress (4-6), and have been the subject of detailed studies. These studies

focused on the characterization of traits that are relevant for a potential industrial application, such as growth rate and biomass yield on sucrose, maltose fermentative capacity, and patterns of sugar utilization and regulation when mixtures of different sugars are present (7).

Traceability is crucial in modern food technology, and it is thus essential to develop a method to discriminate between different *T. delbrueckii* strains. This would enable correct identification of the inoculated strain among the yeast flora present in bread dough or in wines. Recently, several methodologies of typing based on DNA polymorphisms that allow discriminating closely related yeast strains have been developed. Two different genetic fingerprinting techniques (karyotype analysis and mtDNA restriction analysis) have been used for detailed genotyping of *T. delbrueckii* strains. Mitochondrial DNA restriction analysis was not a good technique to differentiate among *T. delbrueckii* strains isolated from the same ecosystem that are genetically very closely related. In contrast, chromosome separation by pulsed-field gel electrophoresis revealed considerable variability in the chromosomal constitution of the strains studied, and turned out to be a useful method to discriminate among *T. delbrueckii* strains.

In the present chapter, we first review the current knowledge regarding the application of *T. delbrueckii* strains in the wine and bread industries, and discuss the physiological traits that make them valuable over *S. cerevisiae*. In the second part, we detail the biochemical and genetic characterization and disruption techniques used in *T. delbrueckii*, and conclude with future perspectives.

2. *Torulaspora delbrueckii* in wine production

2.1. *Saccharomyces cerevisiae* and non-*Saccharomyces* species in wine making: A brief overview

Saccharomyces cerevisiae is the yeast primarily responsible for grape must fermentation and has been used for centuries in wine making. Strains of this species are commonly found in nature on the surface of grapes and within the bioflora of wine cellars. Previous studies suggest that strains present in vats or in other winery equipment, rather than the strains found in the vineyards, are responsible for the fermentation of musts, although there is still some controversy on this subject (8, 9). Pure cultures of *S. cerevisiae* have been isolated from these environments and developed as starter cultures for conducting wine fermentations all over the world. Usually, start-up cultures of *S. cerevisiae* are employed to better control the fermentation process. In fact, in large scale fermentations and in the newer wine-producing countries, where a desirable natural flora may not be established in the vineyard and in the winery, there is a reluctance to rely on natural fermentation, and selected yeast cultures are inoculated into the grape must to induce fermentation (10, 11). Selection of strains for wine production is based on the identification of strains that can ferment grape must efficiently and produce wines of good quality. This selection is usually conducted within the genus *Saccharomyces* and using yeasts isolated in wine environments, particularly from cellars,

which allow for growth of the better adapted strains (1, 12). Isolation of strains is followed by characterization of their traditional oenological properties (13), which are divided into technological and qualitative properties. Technological characteristics influence the efficiency of the fermentative process, while qualitative characteristics relate to the chemical composition and influence on the sensory properties of wines (1). Some of these characteristics can be evaluated by monitoring fermentation progress and by chemical analysis of the levels of compounds present at the end of fermentation (2). As these strains are marketed as dried yeast, they must also be capable of maintaining viability during the dehydration/ rehydration process (14). The desire to produce consumer-directed wines of differentiated styles has led to the emergence of new criteria when selecting yeast, such as: ability to enhance wine colour via metabolic formation of stable pigments; absence of β-glucosidase activity, ability to prevent colour degradation; facilitation of colloidal stabilization in red wines by allowing over-lees aging (to help stabilize colour); appropriate enhancement of aroma via the production of volatile compounds such as esters and higher alcohols, along with limited production of off-flavours; and the bestowing structure and body via the production of polyalcohols such as glycerol and 2,3-butanodiols, and the release of mannoproteins and yeast polysaccharides. Because it is unlikely to find *S. cerevisiae* strains with an ideal combination of oenological characteristics, it became necessary to expand the selection of strains to other environments and to non-*Saccharomyces* strains (1, 2). The latter strains cannot compete with *S. cerevisiae* under oenological conditions, but have distinct characteristics that positively influence the sensory profile of the wine. *S. cerevisiae* strains isolated from Parmesan cheese serum degrade malic acid present in wine musts, until 50% of its initial concentration (15). Likewise, *Schizosaccharomyces pombe* proved to be effective in the deacidification of must through consumption of malic acid (16-18). *Candida stellata* and *Kloeckera apiculata* produce large amounts of glycerol (19), whereas *Candida colliculosa* produces acetaldehyde and n-propanol (1). These positive characteristics can be used in wine production and therefore contribute to wine sensory composition. The future of this industry points to individualization of consumers based on their genetic differences and on their olfactory profiles, which will be determinant in decisions of production and marketing of wine (20).

Flavours and aromas of wines are due to the grape itself and to biological activities carried out by the microorganisms. Several species of yeasts and bacteria and sometimes filamentous fungi may be present during fermentation of the must, and are responsible for the final characteristics of the wine (1). Besides *Saccharomyces*, other genus such as *Hanseniaspora*, *Kluveromyces*, *Candida*, *Metschnikowia*, and *Pichia* are usually found. Presence and persistence of these non-*Saccharomyces* genera are conditioned by several factors such as temperature of fermentation, addition of nutrients, aeration, contact with the peel of the grape, nature of the *Saccharomyces* strain used, and inoculation practices. Other important yeasts in wine production are those responsible for wine deterioration. This category includes the genera *Brettanomyces* and some species of *Zygosaccharomyces*, *Candida*, and *Pichia* (14). The non-*Saccharomyces* yeasts, which also include species of *Hansenula*, *Kloeckera*, *Schizosaccharomyces*, and *Torulaspora*, are present in the early stages of fermentation, growing

for several days until the fermentation is dominated by one or more of the *S. cerevisiae* strains (13, 21). During the initial stage of fermentation these yeasts, with low fermentation yield, produce high concentrations of long-chain alcohols, esters, aldehydes, and glycerol, which are important for the organoleptic characteristics of the wine (13). However, in the modern wine industry and particularly in large-scale production, as referred above, spontaneous fermentation is unlikely to be used due to lack of reproducibility of the wine quality. The main advantages of inoculated wine fermentations are a more rapid and even rate of fermentation and wine of more consistent quality (11, 22, 23). On the other hand, this practice has resulted in more uniform wines without the typical contribution of aromas and flavours from indigenous yeast flora. In an attempt to change this scenario, non-*Saccharomyces* yeasts have recently been used in commercial wine production, particularly in countries such as the United States and Australia. This practice represents a good alternative to problems that can result from spontaneous fermentation, without compromising the sensory profile of the wine. Due to the low ethanol tolerance demonstrated by some non-*Saccharomyces* yeasts, inoculation of these yeasts must be performed together with a more tolerant strain that assures fermentation is completed, usually mixed starter cultures or sequential inoculation (1). Mixed starter cultures of *Torulaspora delbrueckii* or *Kluyveromyces thermotolerans* together with *S. cerevisiae* have already been tested, and revealed promising results regarding the aroma and flavour obtained (24).

2.2. The use of *Torulaspora delbrueckii* in the wine industry

The use of non-*Saccharomyces* wine yeasts in pure cultures as fermentation starters has shown that these have both advantages and undesirable fermentation characteristics. Among the latter, there is the production of acetic acid, ethyl acetate, acetaldehyde and acetoin at high concentrations that usually impairs the use of such strains as starter cultures. A number of authors have reported on the impact of non-*Saccharomyces* yeast species on wine quality under usual winemaking conditions (25-29). *Torulaspora delbrueckii* is reported to have a positive influence on the taste and aroma of alcoholic beverages (19, 25, 26), at the same time exhibiting low production of acetaldehyde, acetoin, acetate, and ethyl acetate (25, 27, 30), even in high-sugar must (31). Due to its high fermentation purity, its usage under standard conditions, in mixed or sequential culture with *Saccharomyces cerevisiae*, has been suggested as a strategy to reduce the acetic acid content of wine (25, 26, 32). Even though it is a low ethanol producer, *T. delbrueckii* still has useful potential in sweet wine fermentation, as it does not seem to respond to osmotic stress in the same way as *S. cerevisiae*. For instance, mixed cultures of *T. delbrueckii* and *S. cerevisiae* have been used in pineapple wine production as a strategy for the production of a distinct flavour complexity (33). In these fermentations, volatile acidity production remained constant throughout the entire process, in contrast with fermentation carried out by *S. cerevisiae*, where over 35% of the total production occurs in the initial stage of fermentation. The mechanisms of osmotic resistance in *T. delbrueckii* have been investigated, but are still not completely understood and are significantly different from those of *S. cerevisiae* (34). Survival of wine strains during the

fermentation process is also imperative for their application. Recent studies on wine ecology showed that non-*Saccharomyces* species survive during fermentation at significant levels for longer periods than previously thought (29). In high ethanol and moderate acetic acid concentrations, typical of stuck must fermentations, *T. delbrueckii* preserved its cell viability longer than *S. cerevisiae* (35), but on the other hand was unable to consume fructose under the same condition (35). On the contrary, *S. cerevisiae* presents a high fructose-consumption capacity but is much less resistant to ethanol and acetic acid. Attempting to combine the advantageous traits of these two yeasts, *T. delbrueckii*'s high ethanol and acetic acid tolerance and the high fructose consumption capacity of *S. cerevisiae*, Santos et al (35) created a hybrid strain of the two species by protoplast fusion. This hybrid (F1-11) displayed a fructose consumption capacity comparable to that of the *S. cerevisiae* parent strain and exhibited increased resistance to ethanol and acetic acid, displaying a lower cell death rate under the harsh conditions present in stuck fermentations. In addition to its potential to restart stuck fermentations, this hybrid could also be useful to conduct an entire fermentation that would benefit from its flavour properties.

3. *Torulaspora delbrueckii* in bread production

3.1. Baker´s yeast and its important traits for baking applications

In the history of human nutrition, a diversity of bakery products has been created which continues today. Bread is mostly made from flour dough that is allowed to rise (leaven) before baking in the oven. Making bread requires three main ingredients: flour, water, and yeast. The yeast's main role in the bread making process is to promptly ferment the sugars available in the flour of the dough or that have been added to it. As a result of an efficient fermentation, the yeast produces carbon dioxide (CO_2) and ethanol; the CO_2 is trapped within the gluten matrix of the dough, causing the leavening or rising, while the ethanol contributes to flavour development, along with other volatile compounds and flavour precursors that are formed during the fermentation process. Technically, the most important properties of baker's yeast comprise (1) the leavening ability in the dough, (2) the ability to adapt to different carbon sources, by expressing invertase and maltase activities, and (3) stress resistance, particularly osmo- and cryo-tolerance. Obviously yeast should also contribute to the flavour of the baked products, as well as grow rapidly in molasses, which are used in the culture media in their industrial production. Commercial baker's yeasts are domesticated strains, essentially of *Saccharomyces cerevisiae*, that have been selected and optimized for baking applications. These particular features are the result of natural adaptation from the continuous selective pressure generated by yeast manufacturers for many years. However, some parameters are still far from optimal. Fermentative capacity is one of the most important biotechnological challenges in the baking industry. Yeast's gassing rate is crucial in baking technology and mostly depends on characteristics of baker's yeast. Tolerance to different stresses, like osmotic or freezing, is also clearly insufficient. Baker's yeast cells subjected to osmotic stress dehydrate rapidly, which limits growth and gas-production capacity (36, 37). Consequently, proofing time (the period where the yeast is

allowed to leaven or raise the dough) increases and the bread volume is reduced. In frozen-sweet dough, freezing and thawing further reduce the water activity, aggravating this situation. Furthermore, freezing and frozen storage of dough has a negative impact on the baking performance due to cell damage (36, 37). Therefore, developing yeast strains with better gassing power in frozen and frozen-sweet dough is of great economic interest.

The physiological requirements of baker's yeast for optimal production and application represent an apparent contradiction (Figure 1). In fact, sugar-limited respiro-fermentative fed batch cultivation (yeast production phase) must render a yeast product that has developed a high fermentative capacity, although this requirement is not important during this phase. Subsequently, the gassing capacity (fermentation) is used in the application phase in the dough, under anaerobic, excess sugar conditions. Therefore the physiological flexibility of baker's yeast must be exceptional.

In addition to good fermentative capacities and high stress resistance, another major trait must be considered when selecting a yeast strain for the baking industry (38): effective biomass production in molasses. Because molasses are cheap and easily available and contain some nitrogen and several vitamins and minerals necessary for yeast growth, they are the main substrate used for large-scale baker's yeast production. However, molasses are considered a major factor of variation in the quality of baker's yeast (39). These substrates are highly variable and contain different proportions of sugars. Though sucrose is the major sugar present, there is also a quite high amount of glucose and fructose. Sucrose is cleaved outside the cell by invertase into glucose and fructose. Invertase is also capable of cleaving raffinose, a trisaccharide also present in molasses, into fructose and melibiose (glucose-galactose), but melibiose is generally not assimilated (40). The main fermentable sugar in plain bread dough is maltose, liberated from starch by amylase activity (α-glucosidase) in flour. This disaccharide is transported through a maltose permease and is subsequently hydrolyzed into glucose by maltase (figure 2). The order by which these different carbohydrates are fermented by *S. cerevisiae* is not random; rather, it is based on a specific hierarchy, with glucose being the preferred sugar. Consequently, in dough containing glucose, sucrose, and maltose, the disaccharides are fermented only when all the glucose is consumed. The monosaccharides glucose, fructose, and mannose are transported into cells across the plasma membrane by hexose transporter (Hxt) proteins, and are subsequently further metabolized in glycolysis. The endpoint of glycolysis is pyruvate, and whether it is used for respiration or fermentation depends on the growth conditions. When cells are grow in an anaerobic environment, sugars are fermented into CO_2 and ethanol. However, cells are also able to use glucose for fermentation when grown aerobically if glucose is present at high concentrations. This phenomenon is generally referred to as the Crabtree effect (42, 43). In an aerobic environment, glucose is catabolized exclusively through respiration only when cells are grown in low sugar concentrations. In addition to being the favourite carbon source for yeast, glucose controls different mechanisms that ensure its primary utilization, as well as general regulation of metabolism, cell growth, and development.

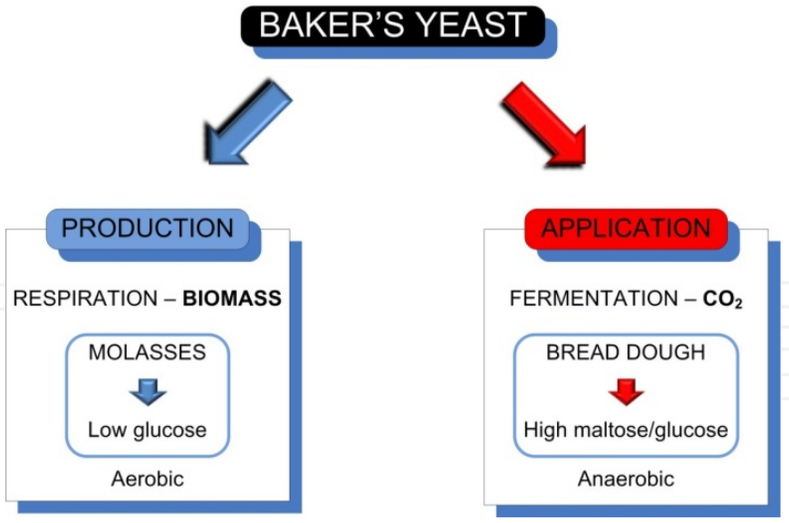

Figure 1. Baker's yeast production and application paradox. Baker's yeast must be able to readily ferment sugar to CO_2 (and ethanol) in doughs. At the same time it must grow on molasses in sugar-limited respiro-fermentative fed batch cultivations for yeast production. Therefore, when the metabolic flux is directed toward cell growth and biomass production, yeast is expected to display a good fermentative capacity. Adapted from (41).

For the most part, regulation is mediated by catabolite repression, acting at early steps in various catabolic pathways. The aim of the regulation is to induce utilization of most favoured carbon source (glucose), and to exclude utilization of other carbon sources if a sufficient amount of glucose is available. Therefore, in most strains of *S. cerevisiae*, glucose represses genes responsible for maltose transport and hydrolysis, as well as invertase that hydrolyzes sucrose to glucose and fructose (42). Although high levels of invertase activity are required for growth in molasses, there is evidence that the capacity of *S. cerevisiae* to ferment high sucrose concentrations, like those present in sweet bread doughs, is inversely related to the activity of this enzyme (43).

3.2. *Torulaspora delbrueckii* as an emergent yeast in the baking industry

Nowadays, the baker's yeast strains used have been developed as a result of centuries of experience and selection, resulting in a high degree of domestication best suited for bread making. Nevertheless, research to improve yeast strains continues. Although methods of classical genetics (selection, mutation, and hybridization) are still very useful, novel methods such as protoplast fusion and genetic engineering have resulted in baker's yeast strains with even better technological properties (35, 37, 44). *Saccharomyces cerevisiae* strains are generally used as baker's yeast, nevertheless the use of alternative species in bread making may allow coping with the new and more demanding challenges in the baking industry. Dough leavening ability has been reported for yeast other than *S. cerevisiae*. *Issatchenkia orientalis*, *Pichia membranaefaciens*, and *Torulaspora delbrueckii* were the most abundant non-*Saccharomyces* species present in homemade corn and rye bread dough (6, 45). Among them, the biotechnological interest in *T. delbrueckii* has increased in recent years due to its particularly high freezing and osmotic tolerance

(4, 5, 46). Thus, some *T. delbrueckii* strains are commercialized in Japan for regular (5% sucrose) frozen dough applications (47). In rural areas of Portugal, bread is sometimes prepared with dough carried over from a previous making. Following an isolation program of yeasts from homemade corn and rye bread doughs, two strains of *T. delbrueckii*, PYCC 5321 and PYCC 5323, were selected on the basis of simultaneously combining high growth rates and leavening ability, characteristics desirable in baker's yeast, as well as an exceptional freeze tolerance (6). The leavening activity of *T. delbrueckii* PYCC 5321 and PYCC 5323 upon freeze-thaw appeared to be unaltered, in contrast with the decrease of fermentative capacity of *S. cerevisiae* baker's yeast (4). In addition, these report from Almeida and Pais (1996b) (4) showed that *T. delbrueckii* strains are not affected by a period of fermentation before freezing. This yeast also displayed higher leavening ability than *S. cerevisiae*, under conditions of hyperosmotic stress in bread dough containing 20% sucrose and 2% salt (46). This feature is in agreement with a low invertase activity, a slow rate of trehalose mobilisation and the ability to respond rapidly to osmotic stress. In summary, *T. delbruecckii* strains studied gather the most important traits needed in a baker's yeast as rapid growth, high biomass, a high leavening activity in lean and frozen dough and extend these properties to sweet and frozen sweet dough. Thus, application of these strains in bread making would have additional advantages, since a single strain can be used for most or all baking applications with the highest performance.

Although this yeast is widely commercialized in Japan, the regular utilization of this species in the bread-making industry has not been established due to several drawbacks. *T. delbrueckii* strains are usually resistant to osmotic stress, but they show considerable variation in their abilities to ferment and to assimilate carbon compounds, as galactose or maltose (48), as well as variable maltase activity and fermentative capacity (46). Moreover, its small cell size is a major disadvantage in the industrial dehydration process, as filtration of cells for dehydration requires a long time and, even worse, cannot be performed continuously because the filter used for dehydration becomes clogged and must be changed frequently (49). Additionally, there is a lack of knowledge on the physiology and molecular biology of this yeast. Despite its phylogenetic closeness to *S. cerevisiae*, the differences observed between the two species demonstrate that the behaviour of *T. delbrueckii* cannot be directly inferred from that of *S. cerevisiae*.

4. Sugar metabolism in *Torulaspora delbrueckii*

As referred above, *Torulaspora delbrueckii* is an important case study among the non-*Saccharomyces* yeast species, of particular relevance to the baking and wine industries. In order to better evaluate the potential offered by *T. delbrueckii*, several physiological and biochemical studies have been carried out with this yeast. In particular, our group studied sugar utilization patterns, maltase and invertase activities, respiration/fermentation rates, and sugar uptake, thus contributing to a better understanding of the mechanisms underlying regulation of sugar metabolism in this yeast.

4.1. Sugar utilization patterns and respiro-fermentative metabolism

One of the leading characteristics of a valuable baker's yeast is its dough-leavening ability, which implies there is efficient fermentation of both the maltose and glucose that are present in the dough. Another important feature is its ability to generate high biomass yields on sucrose, the major sugar present in molasses. On the other hand, glucose and fructose are the main sugars present in grape musts, and thus efficient fermentation of these sugars is also of great importance for utilization of a yeast strain in wine fermentation.

The behavior of *T. delbrueckii* PYCC 5321 in Yeast Peptone (YP) medium with glucose, sucrose, and maltose, either as single carbon and energy source or in mixtures, has been compared with that of a commercial baker's yeast, used as reference (7). When single-sugar media were used, the growth rate of both yeasts was similar in both glucose and sucrose-containing media and slightly lower in maltose-containing medium. For *Saccharomyces cerevisiae*, these values were similar to those obtained in mixed-sugar media, but for *T. delbrueckii* the values obtained in mixed-sugar media with maltose (Glucose-Maltose (G-M) and Sucrose-Maltose (S-M) mixtures) were again slightly lower. Since analysis of the specific sugar consumption rates and transport capacities suggested that sugar transfer rates into the cell limit fermentation efficiency (50), the lower growth rates obtained in the presence of maltose were probably a result of inhibition of glucose uptake by maltose, which is known to occur in both *S. cerevisiae* (51) and *T. delbrueckii* (7). In G-M mixtures, maltose was consumed only after glucose was exhausted from the medium, and the increase in maltose transport and maltase activities clearly correlated with the beginning of maltose consumption. Therefore, it seems that the maltose transporter and maltase are co-regulated, and are subject both to induction by maltose and to repression by glucose. On the contrary, commercial baker's yeast began to consume maltose when glucose was still available, suggesting glucose control over maltose metabolism is higher in *T. delbrueckii* than in commercial baker's yeast. Under laboratory conditions, this distinct performance of the two species could imply an unwanted delay in CO_2 production in maltose-containing bread dough by *T. delbrueckii*. However, this apparent advantage of *S. cerevisiae* is counteracted by the higher osmotolerance of *T. delbrueckii* under the conditions prevailing in bread dough. In S-M mixtures, maltose consumption is also inhibited until sucrose is exhausted, although this inhibition is not particularly efficient and maltose levels slightly decrease concomitantly with sucrose consumption. These results suggest that glucose released from sucrose through invertase activity inhibits maltose metabolism. When cells were grown in media with either only one sugar or with mixtures of sugars as carbon sources, the invertase activities obtained were also in accordance with a regulation pattern similar to that observed in *S. cerevisiae*. In fact, while invertase activity was subject to glucose control, it was not dependent on induction by sucrose.

T. delbrueckii displayed biomass and ethanol yields typical of fermentative metabolism in all media. Despite this clear fermentative metabolism exhibited by *T. delbrueckii* in batch cultures with each of the sugars tested (glucose, maltose and sucrose), the specific rates of CO_2 production and O_2 consumption, estimated with the Warburg manometric technique,

showed there was a higher contribution of respiration to the overall sugar metabolism than that observed in *S. cerevisiae*. It is worth noting that during batch cultivation the available oxygen rapidly reaches limiting concentrations, thereby favoring fermentative metabolism. In fact, when biomass yields were determined in YP medium containing glucose, sucrose, or maltose, using higher aeration rates resulted in a very significant increase in biomass yields (from 20 % in glucose or sucrose medium to 80 % in maltose medium). The more efficient modulation of the respiratory metabolism in *T. delbrueckii* under aerobic conditions thus represents an asset for the large-scale production of this yeast.

Aiming to provide new insights into the molecular mechanisms underlying energy source signaling in *T. delbrueckii*, a gene coding for a putative protein with high similarity to the *S. cerevisiae* carbon catabolic-derepressing Ser/Thr protein kinase Snf1 was recently identified, and named Tdsnf1p (52). It seems that, like Snf1p, Tdsnf1p is required for growth in ethanol, low glucose (0.05%) and raffinose-containing medium, although in the last case the phenotype was not as pronounced as that of the *S. cerevisiae snf1*Δ mutant. In contrast, the *tdsnf1*Δ mutant displayed increased Li^+ tolerance, a phenotype not observed in the *S. cerevisiae snf1*Δ mutant, further highlighting the differences between these two yeasts.

In summary, the overall patterns of sugar utilization and regulation in mixed sugar media by *T. delbrueckii* are equivalent to those described for *S. cerevisiae* (42, 53, 54), though some critical differences were identified. In addition, given that specific growth and fermentation rates in maltose-containing media were lower than those obtained in glucose- and sucrose-containing media, and that growth rate seems to be limited by transport capacity, these studies also indicated that maltose uptake is a good target for metabolic engineering and improvement of *T. delbrueckii*'s performance in bread doughs.

4.2. Hexose transport in *Torulaspora delbrueckii*

Yeast sugar transporters: a brief introduction

The sugar porter family is the largest within the major facilitator superfamily (MFS), which includes proteins from Bacteria, Achaea, and Eukarya, with very diverse sequences and function (55-57). Proteins belonging to the MFS exhibit highly structural conservation, though they share little sequence similarity (58). Generally, these permeases have 12 putative transmembrane segments, and consist of a single integral membrane protein with two sets of six hydrophobic transmembrane-spanning (TMS) α-helices connected by a hydrophilic loop, whose amino- and carboxy-terminal regions localize to the cytoplasm (59-61). The strong similarity between the two sets of hydrophobic TMS of MFS proteins and their overall structure supports the theory that they result from a gene duplication event that probably took place before de divergence of MFS families (57, 59). Sugar transport across the plasma membrane is the first and obligatory step of its utilization. Yeasts can use different carbon sources for growth, but evolution has selected mechanisms for the preferential utilization of glucose. As permeability of biological membranes is quite restricted, most of the cellular nutrients must enter the cell via specific transport systems and both facilitated diffusion and proton-symport transport systems for sugars have been

described in yeasts. In facilitated diffusion, solutes are transported down a concentration gradient by a uniport mechanism. Secondary active transport uses accumulated energy from an electrochemical gradient to transport molecules against their concentration gradient, coupled with the simultaneous movement of another molecule (normally H^+ or Na^+) in the same (symport) or opposite (antiport) direction (62). Such a mechanism becomes fundamental during growth in very low extracellular sugar concentrations, when an intracellular accumulation of hexoses may be necessary to allow hexose kinases to function optimally. Evidently, yeast species possessing proton-hexose symport systems are better adapted to grow in low hexose concentrations (63, 64). Since a facilitated diffusion transport system is most efficient only under reasonably constant levels of the carrier substrate, this system might not be appropriate for yeasts like *Saccharomyces cerevisiae*. However, this yeast uptakes hexoses only by facilitated diffusion, and has clearly overcome this setback by developing an unusual diversity of hexose transporter proteins (Hxtp) with specific individual properties and kinetics (65).

Multiple hexose carriers have been characterized genetically in *S. cerevisiae*. Among about 5600 protein-coding genes in *S. cerevisiae*, at least 271 encode predicted or established permeases (66). Twenty encode hexose transporters and related proteins, the so-called HXT gene family (60, 65). Of these, only *HXT1–HXT7* encode transporters that are important for growth and metabolism of glucose (51, 67). The galactose transporter, encoded by *GAL2*, is also a member of the *HXT* gene family (68, 69). Two members of the family, encoded by *SNF3* and *RGT2*, have lost the ability to transport hexoses; instead they function as sensors of the extracellular glucose concentration. This glucose signal is involved in the transcriptional regulation of various *HXT* genes (70). The remaining members of the family (*HXT8–HXT17*) are phenotypically silent, and may not be expressed under normal physiological conditions (51). As discussed by Wieczorke et al. (71), the large number of hexose transporter proteins in *S. cerevisiae* seems to reflect its adaptation to the variety of environmental conditions to which yeast cells are exposed. Two kinetically distinct glucose uptake systems were initially described in *S. cerevisiae*: a constitutive low-affinity system (K_m=15–20 mM) and a glucose-repressible high-affinity system (K_m=1–2 mM) (reviewed in 70). It is now considered these two components consist of several different transporters contributing to the overall kinetic properties of the systems. The affinity of the major Hxt proteins for glucose was determined by individual expression of these transporters in a *hxt* null strain (72), and differs significantly; for example, Hxt1p and Hxt3p have a low-affinity for glucose, whereas Hxt2p, Hxt6p, and Hxt7p have a high-affinity (72). The low-affinity transporters are expressed when cells are grown in media containing high concentrations of glucose, whereas the high-affinity transporters are expressed when cells are grown in media containing low concentrations of glucose (70). It should however be noted that most of the data pertaining to the kinetics of glucose transport was obtained from individual expression of *HXT* genes in a *hxt* null mutant, and thus the results may not reflect the *in vivo* functions of Hxt proteins. When expressed in the absence of other Hxt proteins, an individual Hxt protein might display a different affinity for glucose, as it may be modulated by means of interactions between different Hxt proteins. Furthermore, any one *HXT* gene may be important for regulation of the expression of the other *HXT* genes (70, 73).

The number of hexose transporters is very variable among yeasts, ranging from 20 hexose transporters in *S. cerevisiae* and *Candida albicans* to six in *Schizosaccharomyces pombe*. *In silico* analysis of the *Kluyveromyces lactis* genome (74) showed that this yeast has 20 sugar transporter genes, but only seven of them have been characterized. Based on protein sequence homology (TBLAST search) with CaHgt1, the first gene encoding a glucose transporter in *C. albicans* (75), 19 other putative glucose transporters were uncovered and designated Hgt2-Hgt20 (76, 77). In the fission yeast *S. pombe*, six hexose transporter genes (*GHT1-GHT6*) have been identified (78), which are highly similar at both the nucleotide and amino acid level. In *Pichia stipitis*, three genes encoding glucose transporters *SUT1*, *SUT2* and *SUT3* (sugar transporters) have been identified, which probably constitute only a subfamily of glucose transporters (79). With the complete sequencing of the *P. stipitis* genome, several additional putative sugar transporter genes were also uncovered (80). Stasyk et al. (81) described Hxt1p, the first functional hexose transporter identified in the methylotrophic yeast *Hansenula polymorpha*. Wei et al. (82) found at least 17 putative hexose transporters in the genome of *Aspergillus nidulans*. So far, only one hexose (particularly fructose) transporter has been described in *S. pastorianus* (83) and in *Zygosaccharomyces bailii (84)*. Fsy1p (fructose symport) is a specific fructose/H+ symporter which mediates high-affinity fructose uptake (it does not transport glucose) in *S. pastorianus* (83). In *Z. bailii*, Ffz1p (fructose facilitator of *Zygosaccharomyces*) does not accept glucose as a substrate and displays low affinity for fructose (84).

Hexose transport in *Torulaspora delbrueckii* and cloning of *LGT1*

Two natural habitats of *Torulaspora delbrueckii* are bread doughs and fruit juices, such as grape juice, environments that are rich in sugars. As a consequence of growth and fermentation of these sugars, this yeast experiences dramatic changes in its physicochemical environment, and thus must adapt to these varying conditions in order to sustain its growth. The sugar concentration may decline from 1 M to 10^{-5} M during fermentation, and the overall composition of the medium will be altered by yeast metabolism. The sugar transport activity of the cell and the proteins that mediate sugar transport must be responsive to these changing conditions, and thus the capacity and kinetic complexity of hexose transport in the yeast may be a reflection of the existence of a large number of sugar transporter genes in its genome. Based on this assumption, multiple hexose transporters with different affinities for glucose probably exist in *T. delbrueckii*. This yeast displays a mediated glucose transport activity best fitted assuming a biphasic Michaelis–Menten kinetics with a low- (apparent K_m = 8.32 ± 0.55 mM) and a high-affinity component (apparent K_m = 1.30 ± 0.34 mM) (50). A kinetic compatible with the presence of these two components was observed in either glucose-, frutose- or maltose-grown cells. Aiming to identify glucose transporters in *T. delbrueckii*, a complementation screen of a *S. cerevisiae* hexose transport-null mutant strain (71) yielded a genomic DNA fragment containing a gene encoding Lgt1p, a low-affinity glucose transporter (50). When expressed in the *hxt* null strain, Lgt1p exhibited an apparent Km value for glucose of 36.5 ± 3.1 mM, in the range of the low-affinity component, and a V_{max} of 1.1 ± 0.04 nmol/s/mg dry weight. This transporter is also able to mediate significant fructose uptake in the *hxt* mutant,

although with a lower affinity than that for glucose, with an apparent K_m value of 51.4 ± 3.0 mM. Glucose transport in this mutant (expressing Lgt1p) was inhibited by the presence of frutose, manose and maltose. Expression studies of the *T. delbrueckii LGT1* gene in *S. cerevisiae* strains, including wild-type, using a fusion of the *LGT1* promoter to the reporter gene l*ac*Z, revealed that it was induced by high glucose concentrations, and its expression was elevated in media containing 4% glucose and almost undetectable in medium containing galactose as the sole carbon source. The transcription factor Rgt1p was necessary for repression of *LGT1* in the absence of glucose; however, and in contrast with the activity of Rgt1p as a bifunctional regulator in *S. cerevisiae* strains, full induction of *LGT1* by high glucose concentrations does not require functional Rgt1p. Even though Mig1p-binding sequences were identified in the promoter region of the *LGT1* gene, the general repressor of *S. cerevisiae* had no effect in the regulation of *LGT1* gene expression. However, disruption of *MIG2* in a *mig1* background led to high levels of *LGT1* expression in high glucose concentrations, indicating that either Mig2p or both Mig1p and Mig2p acting redundantly, function as repressors of *LGT1* expression under these conditions, consistently with their function in *S. cerevisiae*.

Even though just one glucose transporter has been identified in *T. delbrueckii* until now (the low-affinity glucose transporter *LGT1* (50)), it is likely that *T. delbrueckii* possesses high-affinity transporters, which is supported by the biphasic Michaelis–Menten kinetics of glucose transport. These results suggest there are additional physiological relevant glucose transporters. Identification of novel transporters will provide new clues regarding the mechanisms underlying regulation of sugar transport, and as a consequence the fermentative capacity of this biotechnologically relevant yeast.

4.3. Cloning and characterization of the *Torulaspora delbrueckii MAL11*, encoding a high-affinity maltose transporter

The genes involved in the utilization of maltose have been characterized in detail in laboratory strains of *Saccharomyces cerevisiae* (85). Genetic analysis revealed there are five MAL loci, *MAL1-MAL4* and *MAL6*, located on different chromosomes (86), but with a high degree of similarity (87). Each locus contains a set of three different genes that encode a maltose transporter (MALT - *MALx1*, where x represents the number of each locus), α-glucosidase (MALS - *MALx2*), and a regulatory protein (MALR - *MALx3*) (53). *MALx1* genes code for high affinity maltose-H^+ simporters with a K_m of approximately 5 mM (88), but with different specificities for various substrates. While Mal11p/Agt1p transports a wide range of substrates, including several α-glucosides (89), Mal31p and Mal61p seem to primarily use maltose, maltotriose, and turanose (89, 90). Nevertheless, only one fully functional locus seems to be found in standard laboratory strains, MAL1, which is heavily regulated through repression by glucose and induction by maltose (91). Comparatively, industrial yeasts contain multiple fully or partially functional MAL loci (92, 93). Additional analysis showed there are considerable variations in the *MALR* gene (94), leading to non-sensitivity to glucose and lack of control by maltose. These special features were the result

of applying successive programs directed to a rapid adaptation to the fermentation of maltose.

Characterization of maltose transport rates in *Torulaspora delbrueckii* indicated it contains an inducible active transport system that co-transports protons with maltose, with the following kinetic parameters: V_{max} 1.03 ± of 0.05 nmol s^{-1} (mg dry weight) $^{-1}$ and K_m 2.26 ± 0.27 mM maltose (95). This transport system was subject to glucose repression and was competitively inhibited by the presence of sucrose, melizitose, and melibiose, suggesting these sugars likely share the same transporter(s) with maltose.

A DNA fragment containing the *MAL11* gene from *T. delbrueckii* (*TdMAL11*) was isolated by complementation cloning in *S. cerevisiae* with a *T. delbrueckii* genomic library (95, 96). DNA sequence analysis revealed the presence of an ORF of 1884 bp encoding a putative 627-amino acid membrane protein with 10 transmembrane domains, highly similar to other yeast maltose transporters. Upstream of *TdMAL11*, the DNA insert included a partial ORF (*TdMAL12*) on the opposite strand and direction, highly similar to the *S. cerevisiae MAL12* gene. Two consensus binding sites of the repressor Mig1p (positions -362 to -378 and -459 to -475) are evident in the divergent promoter. Interestingly, the first overlaps with a binding site of a MAL activator (-451 to -461). A similar situation was described in the intergenic region *MAL61-MAL62* in *S. cerevisiae*, where one of the two Mig1p binding sites is very close to a UAS_{MAL} site (97). This overlap seems to have a functional role in the transcriptional regulation of *MAL61* and *MAL62* genes, as occupation of the UAS_{MAL} site will result from direct competition between the two regulators for the binding region (98). Sequence analysis, Northern blot, and transport measurements, indicated that *TdMAL11* expression is regulated by carbon source, and is subjected to repression by glucose and induction by maltose. Attempts to disrupt *TdMAL11* and Southern blot analyses revealed the presence of two functional *MAL loci*. Disruption of a single copy decreased the V_{max} of maltose transport, but not the K_m, whereas the double disruption abolished the uptake of this sugar in *T. delbrueckii* (95).

As referred above, the activity and regulation of the maltose uptake system in *T. delbrueckii* cells could be a good target for improvement of its leavening ability in bread dough, and the identification of maltose transporter genes in this yeast can now open the door to these studies.

5. Freezing tolerance of *Torulaspora delbrueckii*: Cellular and biochemical basis

Baker's yeast and stress resistance

In the baking industry, yeasts encounter numerous stresses. During production, they must adapt to low sugar and high aeration, repressing fermentation to produce large amounts of biomass. Cells are then preserved in a cold, frozen or dry state until use, when rehydration or thawing and inoculation cause osmotic shock in a new environment that

requires the induction of enzymes for maltose utilization under semianaerobic conditions. The low stress resistance of yeast during active fermentation is disadvantageous for its use in industrial applications, and it would be highly advantageous to have yeast strains available that do not lose their stress resistance during fermentation (36). Furthermore, human food habits have changed in the past few years and there has been an increasing usage of frozen dough for bread production. Yeasts under such stress conditions reduce fermentation performance, compromising product quality (36, 99). Namely, reduced yeast vitality after freezing and thawing the dough causes loss of fermentation capacity and makes it necessary to use higher yeast amounts and longer proofing times (i.e., the resting period after mixing during which fermentation takes place), consequently decreasing product volume (100). As a result, these effects have a great technological and economic impact in the baking industry. Undoubtedly, the ability of baker's yeast to cope with stress conditions is an essential physiological requirement in this industry, which evidently would greatly benefit from the availability of yeast strains with improved freeze resistance.

One of the first stresses encountered by baker's yeast cells during the preparation of frozen dough is the cold-shock, i.e. the decrease in the environment temperature after mixing. This change impairs the correct functioning of both the membrane and the translational apparatus as a result of reduced membrane fluidity and stabilization of the secondary structures of DNA and RNA (101, 102). While positive cold temperatures lead to the synthesis of specific proteins associated with the development of transient phenotypic adaptation (103, 104), freezing is frequently a lethal stress to cells. At sub-zero temperatures, the damaging effects on yeast cells depend on the freezing rate. In the case of rapid freezing, cells are injured by the formation of intracellular ice crystals, which leads to membrane disruption (105). Structural examination of these cells shows discontinuous nuclear membranes, disappearance of vacuoles, and spreading of DNA all over the cells (106). On the other hand, in cells exposed to low freezing rates, osmotic shrinkage of the cells and frozen extracellular water is observed. Therein, cells become exposed to hyperosmotic solutions and try to compensate by moving water across the membranes (107). In this case, cells suffer cellular damage similar to that caused by dehydration. During frozen storage, the growth of ice crystals can further deteriorate the plasma membrane and damage the activity of different cellular systems. Taken together, these findings indicate that freezing is a very complex stress, in which different stress responses appear to play important roles. Therefore, freezing tolerance likely involves different mechanisms working in concert.

Torulaspora delbrueckii and freezing resistance

The high freezing resistance of *T. delbrueckii* PYCC 5321 and 5323 strains was the main characteristic that set them apart as potential candidates for the baking industry. In fact, even after freezing these *T. delbrueckii* cells at –20 ºC for at least 120 days, they retained nearly 100% cell viability, estimated as colony forming units (CFU). In contrast, viability of a *Saccharomyces cerevisiae* commercial baker's yeast was less than 20% viability by the 15th

day (5). The resistance of *T. delbrueckii* was due to its ability to maintain the integrity of the plasma membrane, and it diminished in the presence of cycloheximide in the freezing medium. Membrane integrity, evaluated by flow cytometry with propidium iodide staining, correlated directly with the CFU counts for both yeasts, validating the utilization of flow cytometry to measure viability of yeast cells subject to freezing stress. The ability of *T. delbrueckii* to preserve plasma membrane integrity during freezing does not correlate with the concentration of intracellular trehalose (T_{in}) at the time of freezing, since the values of T_{in} were high and in the same order of magnitude in both *T. delbrueckii* and *S. cerevisiae* strains. In addition, *T. delbrueckii* was able to retain much higher cell viability when subject to a period of fermentation before freezing. Under those circumstances, there was also no correlation between membrane integrity and T_{in}. During the period of prefermentation, the concentration of T_{in} fell at a high rate and to similar values in both strains, consistent with a similar pattern of activation of trehalase (s) by glucose, determined in cellular extracts of *T. delbrueckii* and *S. cerevisiae*. In contrast, the higher capacity to preserve the plasma membrane observed in *T. delbrueckii* seems to be related to a smaller increase of lipid peroxidation during the freeze storage period. These results suggest that the ability of *T. delbrueckii* to avoid damage from oxidative stress in the plasma membrane during freezing can contribute to its freeze-resistant phenotype. In agreement with these results, which seem to imply oxidative damage is involved in the loss of plasma membrane integrity during freezing, pre-treatment of *S. cerevisiae* cells with the radical scavenger N-tert-Butyl-α-phenylnitrone (PBN) led to a reduction in the loss of membrane integrity.

6. Gene disruption in *Torulaspora delbrueckii*

The use of gene deletion mutants is an important tool to decipher the role and physiological relevance of the proteins encoded by different genes. Results from these studies can increase the knowledge of the physiology, biochemistry and molecular biology of an organism. To this purpose, construction and analyses of *Torulaspora delbrueckii* mutant strains are of highest importance; however, the genetic tools available for this yeast are very scarce. Disruption of genes followed by phenotypic analyses is a vital tool for understanding yeast gene function. However, the efficiency of gene disruption is highly variable among species, and is often quite low for non-conventional yeasts. In yeast, gene disruption is usually accomplished by transforming cells with a gene-targeting section (cassette) containing a selectable marker, conferring drug resistance or nutrient autotrophy, flanked by upstream and downstream sequences of the gene of interest (108). These cassettes are frequently generated by PCR, using primers containing both bordering regions of the target gene and part of the selectable marker gene, and subsequently used to transform yeast cells through various transformation protocols, but usually by the lithium acetate TRAFO method (109). There has been widespread use of gene disruption cassettes generated by PCR (110, 111), since very short sequences of yeast DNA flanking the marker gene are sufficient for efficient integration into the *Saccharomyces cerevisiae* genome by homologous recombination (112).

Therefore, it is possible to screen a relatively small number of the transformants growing on the selection media (usually by PCR) and confirm correct integration of the cassette. When recombination efficiency is very low but cells are under selection pressure, cassettes are often integrated in the wrong locus, giving rise to a large number of false positives (cells that are able to grow on the selection media, but not actual disruptants), and the number of true positives may be lower than 1 in a 100. It then becomes necessary to perform a secondary screen, which is only possible when deletion of the gene of interest results in a readily identifiable phenotype. Evidence of the low efficiency of *T. delbrueckii* homologous recombination emerged when disruption of the *TdMAL11* gene was attempted (95); however, in that case it was possible to screen the transformants through phenotypic analysis, since *TdMAL11* null mutants exhibit deficient growth on medium containing maltose as the sole carbon source. However, it was not possible to use the same strategy to disrupt *LGT1*, the first gene identified as coding for a hexose transporter in *T. delbrueckii*, since there was evidence that other hexose transporters exist in this yeast (50). Therefore, we could not screen for potential *T. delbrueckii LGT1* disruptants by searching for a clear-cut phenotype, because loss of *LGT1* might be compensated by the activities of other genes and was thus not expected to impair glucose growth capacity. Therefore, using the conventional method of transforming a PCR-amplified disruption cassette with a short flanking homology (SFH-PCR) (113), we were unable to generate Δ*lgt1* mutants. Our strategy was thus first to obtain a TdLGT1-targeting cassette harboring longer homology arms, and then to attempt further optimization of the yield of *LGT1* disruption, by testing how different parameters inherent to the lithium acetate method described by (109) contributed to the transformation efficiency (114). We constructed a cassette with longer flanking regions by inserting a marker-resistance module into the core of the *LGT1* gene, and then used this construction as a template for PCR amplification of a TdLGT1 long-flanking homology disruption cassette (figure 2). Then, to further improve the yield of *LGT1* disruption, we reformulated some parameters of the transformation method. Mainly, after heat shock, cells were pelleted and resuspended in rich medium supplemented with low concentrations of geneticin (100 mg mL^{-1}) and incubated overnight (instead of the usual 4-h recovery time). Finally, cells were plated onto selective YPD plates supplemented with higher concentrations of geneticin (300 mg mL^{-1}), to select against false positives, and incubated for up to 4 days.

Integration efficiency using this strategy was extremely high when compared with the conventional method (no disruptants from using the conventional method and 12/16 using this improved method). We thus concluded that two important modifications were the most relevant for our global strategy: the size of the disruption cassette and the new recovery period of cells during the transformation protocol. As a result, this method demonstrated to be a valuable alternative to the conventional PCR-based gene disruption for the yeast *T. delbrueckii*. This methodology could also be advantageously applied to other non-conventional yeasts, where correct gene disruption with the commonly used short flanking homology cassettes is frequently very low.

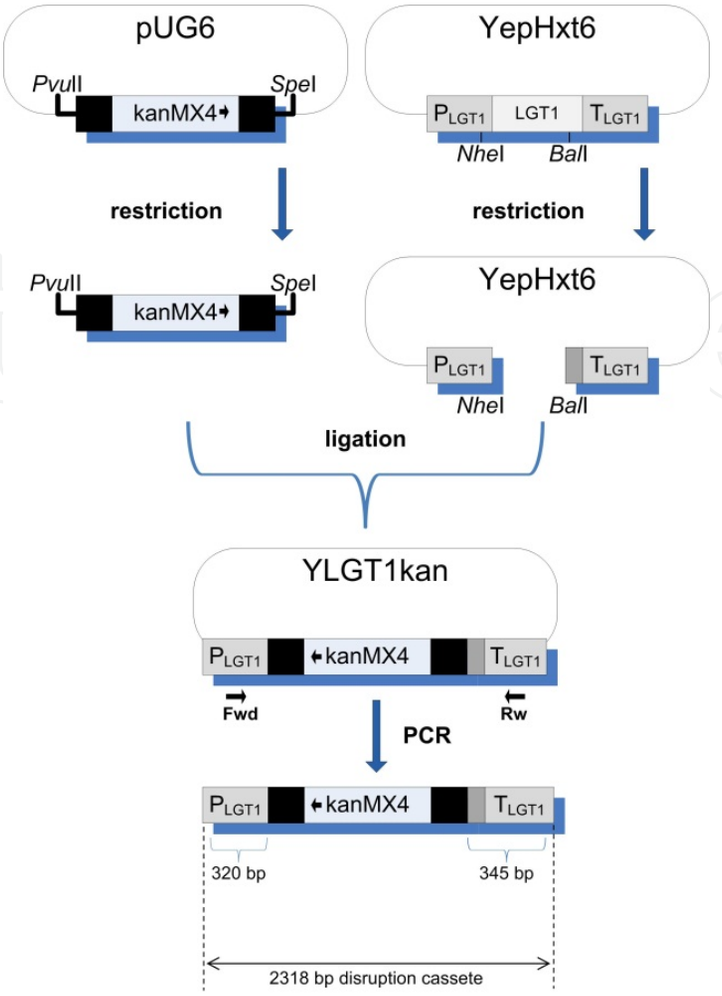

Figure 2. **Schematic illustration of the construction of *Torulaspora delbrueckii* *LGT*1** disruption cassete. pUG6 plasmid was digested with *Pvu*II and *Spe*I to release de KanMX4 module, which confers resistance to geneticin (left side of the scheme). In parallel YepHxt6 plasmid (which contains *LGT*1 ORF and part of the gene promoter and terminator regions) was restricted with *Nhe*I and *Bal*I, removing nearly the entire ORF (right side of the scheme). Afterward the PvuII-loxP-KanMX-loxP-SpeI released from pUG6 was cloned into *Bal*I/*Nhe*I restricted YepHxt6, creating YLGT1kan plasmid, the template to generate the *LGT*1 disruption cassette. Using specific primers to *LGT*1 promoter and terminator regions, the disruption cassette (2318 bp) containing the marker module flanked by 320 and 345 bp (5′ and 3′ sides, respectively) *LGT*1 homologous regions was generated by PCR. This cassette was used to transform *T. delbrueckii* with a modified LiAc transformation protocol (described in the text). P_{LGT1} and T_{LGT1} are the promoter and terminator regions, respectively, of *T. delbrueckii LGT1* gene. Restriction enzyme sites and the sizes of the DNA fragments are shown. Arrows at either end of the module represent the oligonucleotides used for PCR.

7. Molecular characterization of *Torulaspora delbrueckii* strains

Before we can exploit the potential of *Torulaspora delbruecckii* in industrial processess, we must be able to identify it and distinguish between strains, using reliable techniques. The accessibility of typing techniques that enable a rapid and accurate differentiation at the strain level is imperative for both wine and baker's yeast users and producers, to assure that

the commercialized yeast corresponds to the strain selected originally. Thus, developing practical typing techniques that enable discrimination between *T. delbrueckii* strains is an essential tool for its implementation in the baking and wine industries.

In order to determine the suitability of mitochondrial DNA restriction analysis for *T. delbrueckii* strain differentiation, we selected additional autochthonous yeast strains from the yeast flora present on the home-made corn and rye bread doughs in the northern area of Portugal (unpublished results from our laboratory). We first screened 134 isolates by restriction pattern analysis of both PCR-amplified 5.8S rRNA gene and internally transcribed spacers ITS1 and ITS2, as previously described (115), selecting only *T. delbrueckii* species. The total length of the ITS1-5.8S-ITS2 regions of the 5.8S rRNA gene is identical for all *T. delbrueckii* strains, and for that reason this method cannot discriminate at the strain level (45, 115-117). Three isolates (45A, 45D and 62C) were selected and placed in the CBMA yeast culture collection, Department of Biology, University of Minho, Braga-Portugal. To discriminate between *T. delbrueckii* strains, both mitochondrial DNA restriction fragment length polymorphism and pulsed-field gel electrophoresis (PFGE) were applied to the three selected isolates, to *T. delbrueckii* PYCC 5321 and PYCC 5323, and to type strain ISA1082 (Portuguese Yeast Culture Collection, Institute Gulbenkian de Ciência, Oeiras –Portugal) for a comparative pattern.

Mitochondrial DNA restriction fragment length polymorphism (mtRFLP) analysis has been widely applied to the characterization of reference and commercial *Saccharomyces cerevisiae* wine yeast strains (118-123), as well as strains belonging to other species (116, 124). Not all the enzymes used in this method detect the same degree of polymorphisms, which depend greatly on the species. mtRFLP using *Hinf*I is associated with the detection of a high polymorphism and is a widely used genetic marker to distinguish *S. cerevisiae* wine strains (118, 122, 125, 126). On the other hand, GC clusters of the mitochondrial genome are the main source of the polymorphisms, and a large portion of these contains restriction sites for *Hae*lll.

For mtRFLP, DNA was isolated from yeast cells grown in YPD and digested with *HinfI* or *Hae*III restriction enzymes. Restriction fragments were separated in horizontal agarose gels (figure 3). mtRFLP's of the six strains using *Hinf*I or *Hae*lll resulted in two distinct profiles, with slight variability. The major difference was found in the upper bands, where the resolution is better (figure 3 arrows). Apart from these bands, the pattern of the profiles provided by each enzyme is identical, indicating that these strains are genetically very closely related. Restriction with *Hinf*I resulted in one profile including *T. delbrueckii* ISA1082 (type strain), *T. delbrueckii* PYCC 5321, 45A and 45D, and in another profile including *T. delbrueckii* PYCC 5323 and 62C strains (Fig.3A). Restriction analysis with *Hae*III resulted in a profile including *T. delbrueckii* PYCC 5321 and 45A, and a second profile that includes *T. delbrueckii* ISA1082, *T. delbrueckii* PYCC 5323, 45D, and 62C strains (figure 3B). This method is therefore not suitable to discriminate between *T. delbrueckii* strains.

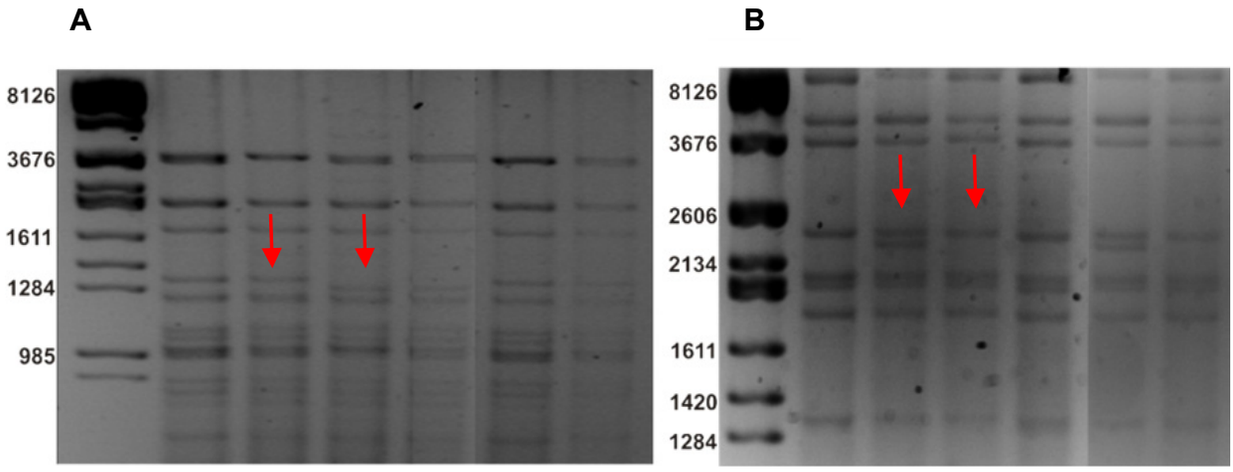

Figure 3. Mitochondrial DNA restriction profiles of *T. delbrueckii* strains obtained with the (A) *Hinf*I and (B) *Hae*III restriction endonucleases in 1.5% agarose gel (unpublished results from our laboratory). Lane 1 – Molecular Marker Lambda DNA/Eco47I (*Ava*II) from Fermentas; Lane 2 – *T. delbrueckii* ISA1082 (reference strain); Lane 3 – *T. delbrueckii* PYCC 5321; Lane 4 – *T. delbrueckii* PYCC 5323; Lane 5 – 62C; Lane 6 – 45A; Lane 7 – 45D. Arrows indicate main differences between profiles I and II. Yeast cells were cultivated in 1 ml YPD (1% yeast extract, 2% peptone, 2% glucose) for 24 h at 30 ºC and 160 r.p.m and DNA isolation was performed as previously described (126). Digestion was carried out with *Hinf*I or *Hae*III restriction enzymes overnight at 37 ºC, in a final volume of 20 µl as previously described (123). Restriction fragments were separated in horizontal 1.5% agarose gels run in 0.5X TBE buffer at 60 V for six hours and visualized in a UV transilluminator (Eagleeye II Image Acquisition System, Stratagene, La Jolla, CA) after ethidium bromide staining.

Karyotype analysis is a highly efficient technique to differentiate strains of *S. cerevisiae*, and was applied by numerous authors to characterize reference and commercial yeasts belonging to different species (118, 120, 122, 123, 127). The electrophoretic karyotypes of the strains under study were therefore also compared. Intact DNA for pulsed field gel electrophoresis (PFGE) was prepared in plugs as previously described (128) and PFGE was run in a CHEF-DRII Chiller System (Bio-Rad, Hercules, CA). Under the conditions used, six chromosome bands were detected in all the strains, which is in agreement with previous studies indicating *T. delbrueckii* has six chromosomes (129). PFGE gel electrophoresis revealed that the chromosomal DNA banding profiles of the strains differ substantially (figure 4), and six different karyotypes could be defined on the basis of the size of putative chromosomes, thereby allowing the discrimination of 4 strains that were not indistinguishable by mtRFLP.

The different karyotypes of the six strains are consistent with their different phenotypes. Indeed, *T. delbrueckii* PYCC 5321, PYCC 5323, and *T. delbrueckii* ISA1082 (type strain) have already been established as different strains (4) and several physiological and biochemical studies of the other three isolates indicated they also correspond to different strains (our unpublished results). Contrary to reports of molecular typing of other yeasts (123), where both methods allowed discriminating strains in a similar manner, our results show that karyotyping analysis displayed a much higher discriminative power than mtRFLP for *T. delbrueckii* strains. A reasonable explanation for this difference may be the need for higher

stability of an intact mitochondrial genome in this species than in *S. cerevisiae*. For instance, we have already shown that the relative contribution of respiration to sugar catabolism is higher in *T. delbrueckii* than in *S. cerevisiae* (7). Although mitochondrial genomes contain a very similar set of genes common to all organisms, mtDNA molecules among species are extremely variable in size and organization (130). Furthermore, the stability of the mitochondrial genome can be evaluated by the ability to form petite mutants. *S. cerevisiae* spontaneously produces these mutants, which are deficient in the capacity to respire aerobically. The petite phenotype is correlated with gross alterations and extensive deletions or loss of mtDNA (131, 132). On the contrary, *T. delbrueckii* is a petite-negative species, as it doesn't have the ability to form these respiratory mutants even after prolonged treatment with ethidium bromide (133-135). The high resolutive capability of the CHEF technique allowed us to differentiate between strains isolated from the same environment and that could not be distinguished by mtRFLP. These results underline this technique as a powerful tool for *T. delbrueckii* strain differentiation, although there are some factors that limit its applicability, since it is complex and time-consuming and not suitable as a routine technique for strain identification.

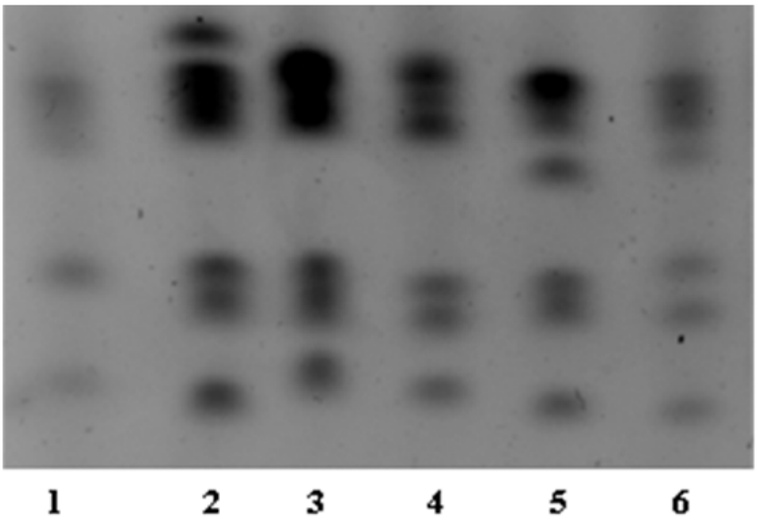

Figure 4. Electrophoretic karyotype comparison of *T. delbrueckii* strains (unpublished results from our laboratory). Lane 1- *T. delbrueckii* ISA1082 (reference strain): Lane 2 - *T. delbrueckii* PYCC 5321; Lane 3 - *T. delbrueckii* PYCC 5323; Lane 4 - 62C; Lane 5 - 45A. Lane 6 - 45D. DNA for pulsed field gel electrophoresis (PFGE) was prepared in plugs as previously described (128). PFGE was run in a CHEF-DRII Chiller System (Bio-Rad, Hercules, CA). PFGE gels were run in 0.5% Tris borate-EDTA buffer at 12 °C with an angle of 120° with the following voltage and switch times: 480s → 900s, 3 v/cm for 10 hours; 240s → 480s, 3 v/cm for 15 hours; 120s → 240 s, 3 v/cm for 15 hours; 90 s, 6 v/cm for 10 hours and 60 s, 6 v/cm for 5 hours. Thereafter, gels were stained with 0.8% ethidium bromide for 45 min and de-stained for 20 min. Gels were visualized under UV light and analyzed using the EagleEye II Image Acquisition System (Stratagene, La Jolla, CA).

In summary, the karyotyping profiles and RFLP's of mitochondrial DNA of the *T. delbrueckii* strains PYCC 5321 and PYCC 5323 were clearly different. These data corroborate and complement the results obtained in the past by the classical biochemical methodology (6),

representing an update to the understanding of *T. delbrueckii* populations present in bread doughs. Furthermore, the availability of functional typing tools that enable differentiation at the strain level is extremely important to the bread and wine industries, to assure traceability of the selected strains.

8. Conclusion

The biotechnological interest in *Torulaspora delbrueckii* has increased in recent years due to its particularly high freezing and osmotic tolerance (4, 5, 46). These features made this yeast species a candidate of potential value for the baking industry. However, the existing knowledge on this yeast is still far from the vast knowledge on the traditional baker's yeast *Saccharomyces cerevisiae*. Therefore, studies have been developed to gain insight into the physiology, biochemistry, and molecular genetics of *T. delbrueckii*.

While two of the most important traits for large-scale baker's yeast production are its growth rate and biomass yield on sucrose, its leavening ability depends mainly on its capacity to ferment maltose. The pattern of sugar utilization and regulation also determines the yeast capacity to rapidly adapt when changing from sucrose-rich growth medium to the dough. Physiological and biochemical studies of *T. delbrueckii* in batch cultures with the sugars present in molasses and in bread dough, both alone and in mixtures, showed that *T. delbrueckii* behaves very similarly to *S. cerevisiae* with respect to sugar utilization and regulation patterns. However, this yeast modulates respiratory metabolism under aerobic conditions more efficiently, an asset for large-scale production of the yeast. Furthermore, comparative analysis of specific sugar consumption rates and transport capacities suggested that it is the transport step that limits both glucose and maltose metabolism.

So far, only one glucose transporter has been identified in *T. delbrueckii*, the low-affinity glucose transporter *LGT1* (50). Southern blot analysis of the *T. delbrueckii* genome revealed the existence of several genes with high similarity to *LGT1*, suggesting there are several hexose transporters in this yeast, which hampered disruption of the *LGT1* gene. The existence of several hexose transporters had first been suggested by the isolation of several plasmids from a genomic library of this strain that could complement the glucose growth defect of the *S. cerevisiae* hexose transport-null mutant (50). Despite the phylogenetic closeness of *T. delbrueckii* and *S. cerevisiae*, the differences observed between the two species show that the behavior or even the methods that can be applied to the former yeast cannot always be inferred from those of *S. cerevisiae*. For instance, when we attempted to disrupt the *T. delbrueckii LGT1* gene, the current methods used for *S. cerevisiae* were not suitable, and an optimized disruption method had to be developed.

In modern food technology, traceability is a crucial requirement, and thus establishing a rapid method to discriminate between *T. delbrueckii* strains is of upmost relevance. This technique would enable correct identification of the inoculated strain from the remaining yeast flora present in the bread dough. In the last years, several methodologies of typing based on DNA patterns have been developed which allowed discriminating closely related

yeast strains. In this chapter, two different genetic fingerprinting techniques (karyotype analysis and mtDNA restriction analysis) were presented for detailed genotyping of *T. delbrueckii* strains. Mitochondrial DNA restriction analysis was not a good technique to differentiate among *T. delbrueckii* strains isolated from the same ecosystem and genetically very closely related. Chromosome separation by pulsed-field electrophoresis revealed considerable variability in the chromosomal constitution of the strains studied, and turned out to be a useful method to discriminate among *T. delbrueckii* strains. However, this method of chromosome karyotyping may be too complex, laborious, and time-consuming for the analysis of numerous yeast isolates, in contrast with mtDNA restriction analysis.

Nowadays, yeast strains used in bread industry are involved in large-scale processes and hence are exposed to more extreme stress conditions. On the other hand, development of new products and more versatile processes also require yeast strains with new traits. This chapter aimed to highlight some of these emergent problems/needs in the wine and bread-making industries, including selecting, characterizing, and constructing resistant yeast strains, and strains with important qualities for application in the baking and wine industries, as is the case of some studied strains of *T. delbruekii***.**

Despite the accomplishments reported in this chapter, many important questions remain to be answered regarding sugar transporters and freezing resistance in *T. delbrueckii*. How many hexose transporters are present in *T. delbrueckii*? What are their affinities and regulation? Is *T. delbrueckii* similar to *K. lactis*, as speculated by Alves-Araújo (50), based on comparison of sequencing data and regulatory studies of *LGT1* expression? Or is this yeast more comparable to *S. cerevisiae* as is suggested by their similar sugar utilizations patterns (7)? Evidently, it would be important to continue the characterization of *T. delbrueckii* strains, as their biotechnological potential has already been established (5, 7, 46). It is clear that answers to these questions may only arise from future studies. Characterization of *T. delbrueckii* at the different levels will narrow the gap towards its industrial exploitation and increase knowledge on the so-called non-conventional yeast species.

Author details

Andreia Pacheco, Susana Chaves and Judite Almeida
Molecular and Environmental Research Centre (CBMA)/Department of Biology, University of Minho, Braga, Portugal

Júlia Santos and Cecília Leão
Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

Maria João Sousa
Corresponding Author
Molecular and Environmental Research Centre (CBMA)/Department of Biology, University of Minho, Braga, Portugal

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