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The role of mannitol in the central carbon metabolism of the yeast *Starmerella bombicola*

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Resumo

A frutofília é uma característica pouco comum entre os microrganismos, que consiste na preferência de frutose como fonte de carbono e energia. Em leveduras, até agora, esta característica foi reportada no género *Zygossacharomyces* e no clado *Wickerhamiella/Starmerella* (W/S). O comportamento frutofílico está relacionado com a presença do transportador de frutose, Ffz1, porém, o papel da frutose no metabolismo destas leveduras ainda não está completamente elucidado.

Uma das leveduras que se destaca no clado W/S é *St. bombicola* não só pela facilidade em manipular esta espécie geneticamente, mas também por ser produtora de elevadas quantidades de soforolípidos. Estes são biosurfactantes que têm características favoráveis do ponto de vista ambiental e, são aplicados em áreas como a cosmética e produtos de limpeza. Além disso, *St. bombicola* assim como outras espécies pertencentes ao clado W/S foram reportadas como produtoras de elevadas quantidades de manitol a partir de frutose através da enzima manitol desidrogenase (Mtdh) utilizando NADPH como cofator, regenerando assim $NADP^+$.

Tendo em conta que a produção de manitol é uma via metabólica dependente de frutose, o objetivo desta tese foi tentar perceber qual o papel do manitol na levedura *St. bombicola.* Como *St. bombicola* possui dois genes que codificam para duas desidrogenases do manitol foram utilizados três mutantes de deleção previamente construídos: um mutante onde o gene que codificava para a enzima Mtdh1 (*mtdh1Δ*) foi interrompido, outro para a enzima Mtdh2 (*mtdh2Δ*) e, finalmente um mutante duplo (*mtdh1Δmtdh2Δ*). A estirpe selvagem e mutantes de deleção foram cultivados, num meio onde o comportamento frutofílico é visível designado por 20FG (contendo 10 % (w/v) de frutose e 10 % (w/v) de glucose) onde a produção de manitol foi estudada. Não foi detetada produção de manitol pelos mutantes *mtdh1Δ* e *mtdh1Δmtdh2Δ,* porém foi detetada produção de manitol semelhante à estirpe selvagem pelo mutante *mtdh2Δ*, o que permitiu inferir que a principal enzima responsável pela conversão de frutose em manitol é a Mtdh1. Curiosamente, quando crescidos nesse mesmo meio a várias temperaturas (25 ºC, 30 ºC e 32.5 ºC), os mutantes de deleção (*mtdh1Δ* e *mtdh1Δmtdh2Δ*) não atingiam as mesmas densidades celulares que a estirpe selvagem às temperaturas mais elevadas. Além disso, excluímos também a possibilidade de os mutantes de deleção terem algum tipo de dificuldade em consumir especificamente um dos açúcares usados, glucose e frutose, cultivando-os em 20 % (w/v) glucose e em 20 % (w/v) de frutose uma vez que nestes meios os mutantes continuavam sem atingir as mesmas densidades celulares que a estirpe selvagem. Tendo em conta a elevada osmolaridade do meio utilizado assim como as diversas descrições do papel do manitol como osmoprotetor, foi avaliado o crescimento dos mutantes de deleção num meio onde a pressão osmótica é baixa, 2FG contendo 1 % (w/v) de frutose e 1 % (w/v) de glucose, a 30 ºC e 32.5 ºC. Curiosamente, a 32.5 ºC os mutantes de deleção não conseguiam sequer crescer. O facto de o defeito no crescimento ser ainda mais pronunciado neste meio de cultura permitiu-nos excluir a possibilidade de o manitol funcionar como osmoprotetor em *St. bombicola*. Além disso, a taxa de crescimento da estirpe selvagem e dos mutantes de deleção foi também analisada a 25 ºC, 30 ºC e 32.5 ºC. Porém, não foi detetada qualquer diferença na taxa de crescimento entre a estirpe selvagem e os mutantes de deleção a 25 ºC e a 30 ºC o que sugere que as diferenças que observamos são de facto no rendimento em biomassa e não na velocidade de crescimento.

A adição de sorbitol (18% (w/v) ao meio 2FG a 32.5 ºC repõe a capacidade de crescimento dos mutantes. Contudo, o sorbitol pode ser metabolizado quando presente em elevadas concentrações. No entanto, quando cultivámos um mutante duplo – onde o gene que codifica para uma enzima necessária para a metabolização do sorbitol (*SOR1*) foi interrompido assim como o gene *MtDH1*, *mtdh1Δsor1Δ* -

verificamos que esse mutante ainda consegue crescer o que indica que o sorbitol poderá substituir o papel do manitol na célula e que o poliol em si tem de facto um papel no crescimento a elevadas temperaturas. Esta conclusão é corroborada quando a estirpe selvagem e os mutantes de deleção foram cultivados no meio contendo 1 % (w/v) de glucose e 1 % (w/v) de frutose ao qual foi adicionado 18% (w/v) manitol e os mutantes de deleção atingiam densidades celulares semelhantes à estirpe selvagem a 32.5 ºC.

Tendo em conta que os mutantes de deleção não atingem as mesmas densidades celulares que a estirpe selvagem e, que é algo que parece estar intimamente relacionado com a temperatura a produção intracelular e extracelular de manitol foi analisada no meio 20FG a 20 ºC, 25 ºC e 30 ºC. Curiosamente, um aumento do rendimento intracelular de manitol é observado assim como extracelular o que sugere que o manitol desempenha um efeito protetor na célula contra o *stress* térmico, possivelmente tendo um papel estabilizador da membrana como a trealose em *S. cerevisiae*, em que os grupos hidroxilo formam pontes de hidrogénio com a bicamada lipídica da membrana. Esta explicação coaduna-se com o facto de, em *S. cerevisiae* a trealose ser necessária dos dois lados da membrana para garantir proteção uma vez que, em *St. bombicola*, não só a produção de manitol intracelular, mas também a quantidade extracelular de manitol aumenta com a temperatura.

Em suma, durante esta dissertação foi possível fazer uma contribuição significativa relativamente ao papel da produção de manitol em *St. bombicola*. Esse papel poderá residir no efeito termoprotector do próprio manitol, mas também na manutenção do equilíbrio redox das vias do metabolismo central.

Palavras-chave: *St. bombicola*; frutofilia; manitol; termoproteção; equilíbrio redox; soforolípidos.

Abstract

Fructophily is a rare trait that consists in the preference of fructose over glucose as carbon and energy source. In spite of the fact that it is already known that fructophily in yeasts is intimately related with the presence of a high capacity fructose transporter called Ffz1, the importance of fructose in the metabolism of fructophilic yeasts is not well established yet.

St. bombicola converts fructose directly into mannitol through a mannitol dehydrogenase (Mtdh) which uses NADPH as a cofactor. In order to understand the role of mannitol in this yeast, three deletion mutants previously constructed, where the genes encoding for Mtdh were disrupted (*mtdh1Δ*, *mtdh2Δ*, *mtdh1Δmtdh2Δ*) were used. Growth assays were performed at three temperatures 25 ºC, 30 ºC and 32.5 ºC and it was possible to observe that the *MtDH* deletion mutants did not reach the same cell densities as the wild-type. Also, at the highest temperature tested (32.5 ºC) and at low sugar concentrations, the *MtDH* deletion mutants were not even able to grow. The ability to grow was restored through the addition of exogenous polyols like mannitol and sorbitol, suggesting that these polyols could be replacing the function of mannitol produced by the cell. Also, the intracellular mannitol content produced by the wild-type increased at higher temperatures, suggesting a role as thermoprotector. It seems possible that mannitol, like trehalose in *S. cerevisiae*, can stabilize the lipid bilayer of the plasma membrane through hydrogen bonding.

In this thesis we were able to make a significant contribution to elucidate the role of mannitol production in *St. bombicola*. The first role may be as thermoprotector in the cell and the second role may be related with redox homeostasis.

Keywords: *St. bombicola*; fructophily; mannitol; thermoprotector; redox homeostasis; sophorolipids.

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List of Abbreviations

Acl	ATP-citrate lyase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Bp	Base pair
CDS	Coding sequence
DTT	Dithiothreitol
HPLC	High-performance liquid chromatography
kb	Kilobase pair
Mtdh	Mannitol dehydrogenase
NAD(H)	Nicotinamide adenine dinucleotide (hydride)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (hydride)
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PPP	Pentose phosphate pathway
OD_{640nm}	Cell density at 640nm
W/S clade	*Wickerhamiella/Starmerella* clade
w/v	Weight per volume

Frequently used species

G. oxydans	*Gluconobacter oxydans*
K. lactis	*Kluyveromyces lactis*
S. cerevisiae	*Saccharomyces cerevisiae*
Y. lipolytica	*Yarrowia lipolytica*
St. bombicola	*Starmerella bombicola*

Media used

YP	1 % (w/v) yeast extract and 2 % (w/v) peptone
20FG	YP supplemented with 100 g/L glucose and 100g/L fructose
20Glu	YP supplemented with 200 g/L glucose
20Fru	YP supplemented with 200 g/L fructose
2FG	YP supplemented with 10 g/L glucose and 10g/L fructose
2FG18S	YP supplemented with 100 g/L glucose and 100g/L fructose and 180 g/L sorbitol

1. Introduction

1.1. Carbon metabolism in yeasts: general aspects

Yeasts are unicellular fungi present in terrestrial, aerial and aquatic environments, which have been applied to different industrial fields. For example, *Saccharomyces* species are very well-known microorganisms in the wine and beer industry, while *Yarrowia lipolytica* is a model organism for lipid production (Dequin, 2001; Rakicka *et al*., 2015). The demands for yeast's metabolic products, not only by *S. cerevisiae*, require the understanding about their central carbon metabolism in order to provide possibilities to increase yields of desired products and decrease unnecessary products. This knowledge can also facilitate optimization of growth media, temperature and oxygen availability (Flores *et al.*, 2000; Mattanovich *et al*., 2014).
Yeasts can use different carbon sources like polyols and alcohols but, hexoses are their preferred carbon sources, namely glucose (Flores *et al.*, 2000). As outlined in Figure 1.1, in the cytoplasm, glucose is converted into glucose-6-phosphate by a hexokinase. After this step, Glucose-6-phosphate can be further metabolized through glycolysis being converted in the final step into pyruvate which in turn can enter the Krebs cycle or go to the fermentative pathway, which usually happens in low oxygen conditions (Rodrigues *et al.*, 2006). Alternatively, glucose-6-phosphate can be channelled to the pentose phosphate pathway.

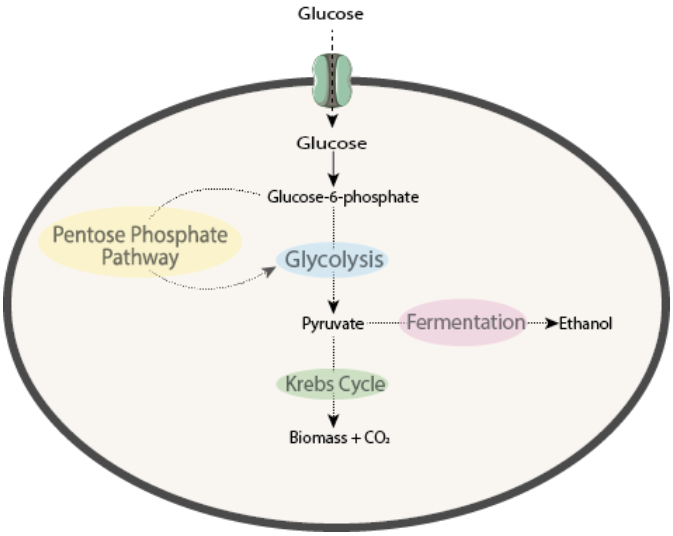


Figure 1.1 – The different metabolic pathways for glucose metabolism. Based on Rodrigues *et al.*, 2009.

1.1.1. Glycolysis

In glycolysis, the glucose uptake into the cell is converted into pyruvate through several enzymatic reactions. Until this step two ATP molecules are invested and two molecules of ATP as well as one molecule of NADH are produced per glucose molecule.

Pyruvate can have three different fates: fermentation to lactate, this usually happens in erythrocytes and in lactic acid bacteria and will not be approached in this thesis. Fermentation to ethanol that usually happen in yeasts and that is known as alcoholic fermentation, and finally respiration where pyruvate can be converted into acetyl-CoA and enter the Krebs Cycle. This happens in animals, plants and microorganisms.

The most used yeast in fermentation is *S. cerevisiae*, which has an uncommon behavior denominated as Crabtree effect. Crabtree positive yeasts conduct fermentation whenever the sugar levels are high regardless of oxygen levels. Therefore, under sugar excess *S. cerevisiae* can convert pyruvate derived from glycolysis into ethanol and carbon dioxide, which are the final products of alcoholic fermentation (Figure 1.2) (Deken, 1966). The conversion of acetaldehyde into ethanol is conducted by the enzyme alcohol dehydrogenase and regenerates NAD^+. As glycolysis needs NAD^+ to continue, the regeneration of this cofactor is very important for the normal carbon flux in this yeast and for redox homeostasis.

At the niche level, the fast conversion into ethanol can represent an advantage since ethanol can discourage the growth of other microorganisms (Rozpędowska *et al*., 2011). However, the biomass yield is lower, so more sugar must be consumed to achieve a high cell number. Another interesting feature is the possible consumption of ethanol through gluconeogenesis when no sugar is available, however in this case oxygen is necessary.

In respiration the energy yield is higher than in fermentation. In *S. cerevisiae*, 16 ATP are produced in respiration while in fermentation 2 ATP are produced per glucose molecule. However, oxygen is required since it is the final electron acceptor. In this case, the final products are biomass and carbon dioxide (Verduyn *et al.*, 1991; Barnett, 2003; Kayikci & Nielsen, 2015). Yeasts performing preferably this metabolic pathway whenever enough oxygen is present are said to be respiratory or Crabtree negative. *Kluyveromyces lactis* and *Y. lipolytica* are two examples of yeasts with this behavior (Veiga *et al*., 2000).

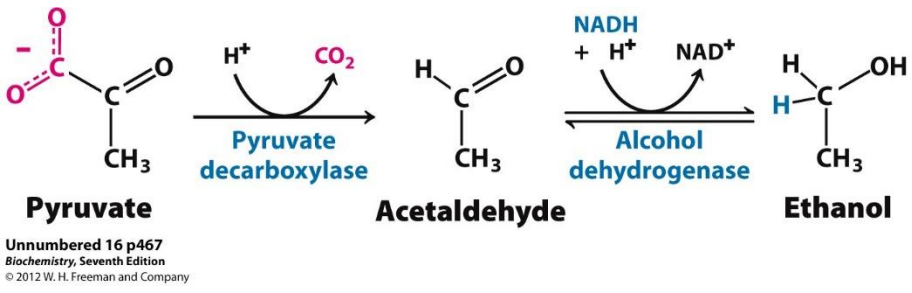


Figure 1.2 - Alcoholic Fermentation. Conversion of pyruvate into acetaldehyde by pyruvate decarboxylase. Alcohol dehydrogenase will convert acetaldehyde into ethanol using NADH as cofactor. Image from (Berg *et al*., 2012)

1.1.2. Pentose Phosphate Pathway

The Pentose Phosphate Pathway (PPP) is an alternative metabolic pathway branching from glycolysis - in glucose-6-phosphate - responsible for oxidation of glucose. The PPP is divided in two phases: the oxidative phase, which is represented in Figure 1.3, that comprises oxidative reactions, where glucose-6-phosphate is converted into ribose-5-phosphate with generation of two molecules of NADPH per one molecule of glucose. This cofactor has an important role in redox homeostasis and is essential for many intermediary metabolic reactions, namely in biosynthetic pathways like synthesis of fatty acids and sterols. Also, it can be used in the reduction of oxidized nitrogen sources to ammonia. In the non-oxidative phase, a series of non-oxidative reversible reactions produce five-carbon sugars for nucleotide biosynthesis and convert excess five-carbon sugar into intermediates of the glycolytic pathway (Mattanovich *et al*., 2014; Stincone *et al.*, 2016)

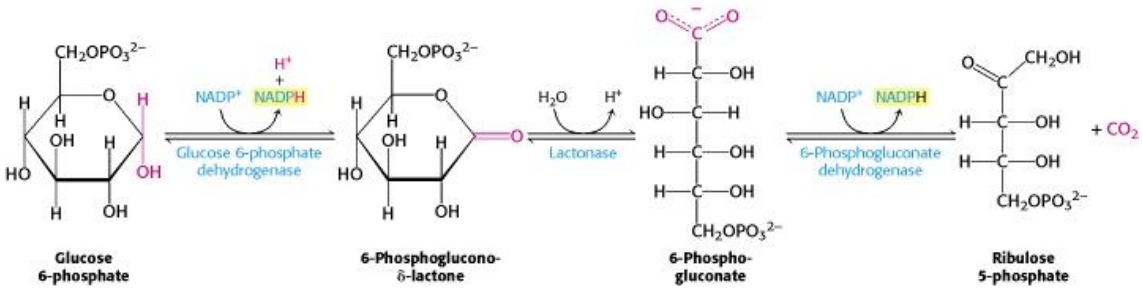


Figure 1.3 - The oxidative phase of the Pentose Phosphate Pathway. Glucose-6-phosphate is converted into 6-Phosphogluconate-δ-lactone by a glucose-6-phosphate dehydrogenase, followed by conversion into 6-Phospho-gluconate by a lactonase. Finally, ribulose-5-phosphate will be produced by a 6-phosphogluconate dehydrogenase. The first and last reaction in the oxidative phase of PPP can produce 2 NADPH molecules per 1 glucose molecule. Image from (Berg *et al*., 2012)

As referred in section 1.1, yeasts show differences in their central carbon metabolism. For example, while *S. cerevisiae* has been described as an organism with a glycolytic flux higher than the PPP flux, *K. lactis*, a Crabtree negative yeast, has, on the contrary, a higher flux through the PPP than through the glycolytic pathway (Jacoby *et al*., 1993; Saliola *et al.*, 2007). In *Y. lipolytica*, an oleaginous yeast, it has been reported that PPP is the principal provider of NADPH in the cell, namely for fatty acid synthesis (Wasylenko *et al*., 2015). While in *Y. lipolytica*, the PPP is the main source of NADPH for lipid biosynthesis, in *K. lactis* that is not the main function in the cell. Instead, the PPP seems to be the main metabolic pathway used by *K. lactis* to oxidize glucose and the NADPH produced will be coupled to mitochondrial respiratory chain (Overkamp *et al*., 2002). This does not happen in *Y. lipolytica* or in *S. cerevisiae* since their mitochondrial enzymes are not able to oxidize NADPH because they are only specific for NADH (Kerscher *et al*., 1999; Overkamp *et al.*, 2000).

In spite all the different roles that PPP or even the roles of NADPH in the cell, its main function is in the oxidative stress response and the maintenance of $NADP^+$/NADPH ratio. Also, we must keep in mind, that a higher flux in PPP does not mean an inexistent flux through the glycolysis or other metabolic pathway and vice-versa. Instead, both the PPP and the glycolytic pathway are dynamic and regulated processes that are interconnected, in the metabolic network that supplies energy and biomolecules needed for the biochemical processes. As environmental characteristics change, namely the availability of substrates, the metabolic adaptation must be fast. For that, metabolic reactions are regulated

increasing the production of metabolites needed as well as decreasing the synthesis of metabolites that are not needed, allowing the surviving and proliferation of the species (Stincone *et al*., 2016).

1.2. Fructophily: a peculiar characteristic of some microorganisms

Yeast and other organisms use sugar transporters for supplying cells with energy and carbon source. In *S. cerevisiae*, there are some membrane transporters, like hexose transporters, that operate through a facilitated diffusion mechanism that supports the use of glucose as preferred carbon source, which is called glucophilic behavior (Reifenberger *et al*., 1995). The glucophilic behavior is related with a problem that occurs in wine fermentations. In grape juice, the amounts of glucose and fructose are approximately equal (Berthels, Otero, Bauer, Thevelein, & Pretorius, 2004). When a glucophilic yeast is used it will consume glucose preferentially over fructose resulting in big differences between fructose and glucose levels can result in stuck fermentations with a high residual concentration of fructose (Berthels *et al*., 2004). Since fructose is sweeter than glucose, the high amount of this sugar at the end of the fermentation can modify the palate of wine (Lee, 1987).

The preference for glucose is possibly related with the existence of the transporters encoded by the *HXT* (HeXose Transporter) and *GAL2* (GALactose metabolism*)* genes that besides operating through a facilitated diffusion mechanism accept glucose as main substrate but can accept fructose and mannose too. Also, glucose sensors encoded by the *SNF3* (Sucrose NonFermenting) and *RGT2* (Restores Glucose Transport) genes are responsible for sensing and signalling the availability of glucose, controlling the uptake of glucose that could be a rate limiting step of glycolysis (Diderich *et al*., 1999; Kayikci & Nielsen, 2015). So far, there is no knowledge about the existence of an *HXT* gene essential for growth on glucose, instead as some of them have a high K_m (low affinity) and others a low K_m (high affinity) their expression is regulated according to the amount of glucose available, but also by osmotic pressure (Özcan & Johnston, 1999), starvation (Diderich *et al*., 1999) and physiological state of the cells (Özcan and Johnston, 1999; Luyten *et al*., 2002).

All these transporters were characterized individually showing a higher affinity for glucose than for fructose, forming a likely basis for glucophily. However, a small group of yeasts has a completely different behavior, consuming fructose faster than glucose what is defined as fructophilic behavior (Sousa-Dias *et al.*, 1996).

Fructophily was first associated to *Zygosaccharomyces* genus (Emmerich & Radler, 1983) but, recently was described the *Wickerhamiella/Starmerella* (W/S) clade within the *Saccharomycotina* sub phylum where are a majority of fructophilic yeasts. W/S clade encompasses species from the *Wickerhamiella* and *Starmerella* genera and closely related *Candida* species (Gonçalves *et al*., 2016). Interestingly, all the species in this clade are found in the floral niche – fructose rich environments - like fructophilic bacteria (Filannino *et al*., 2016). As fructophilic yeasts are able to quickly transport fructose, this could had represented an advantage to floral niche since probably it could confer a positive impact on fitness (Gonçalves *et al*., 2016).

Until now, fructophilic behavior was related with the presence of a membrane protein first described in the yeast *Zygosaccharomyces bailii*, Ffz1 (Fructose Facilitator of *Zygossacharomyces*). Ffz1 is a high capacity and low affinity uniporter specific for fructose (Pina *et al*., 2004). Interestingly, a second fructose transporter, Ffz2, was characterized in *Z. rouxii*, but with a difference at the substrate specificity level, since this transporter can accept both glucose and fructose as substrates (Leandro *et al.*, 2011). However, contrary to Ffz1, Ffz2 is not essential for fructophilic behaviour (Leandro *et al*., 2014). So

far, Ffz1 seems to be the only requirement for the preference of these organisms for fructose over glucose, since the deletion of the *FFZ1* gene abolished the fructophilic behavior in *Zygossacharomyces rouxii* (Leandro *et al*., 2014) and in *Starmerella bombicola* (Gonçalves *et al*., 2018).

The preference for fructose over glucose can be explained by the presence of *FFZ1*, but the role of fructose in the metabolism of these yeasts is still unknow. However, the knowledge of this theme could be of major importance for the wine industry as a possible solution for stuck fermentations.

1.3. *St. bombicola*: a frutophilic yeast and a sophorolipid producer

St. bombicola was first described in 1998 as a teleomorph of the yeast *Candida bombicola* (Rosa & Lachance, 1998). This yeast has an association with bees, flowers visited by bees and can be associated with some other floriculous insects (Kurtzman *et al*., 2011). Phylogenetically, *St. bombicola* belongs to the *Wickerhamiella/Starmerella* clade previously referred in section 1.2. *St. bombicola* is a fructophilic yeast which has gained much attention due to its biotechnological potential as a producer of sophorolipids, being capable of producing up to 400g/L of this type of lipids (*St. bombicola* ATCC 22214) (Elshafie *et al*., 2015) and is amenable to genetic manipulation (Saerens *et al*., 2011).

Sophorolipids are biosurfactants with diverse applications such as household cleaning, personal care and cosmetics and oil industry (Bogaert *et al*., 2011). Also, the applications of sophorolipids in nanotechnology is starting to be explored as well as their antimicrobial activity (Kapjung *et al*., 2002; Singh *et al*., 2009) So far, sophorolipids showed antimicrobial activity against gram-positive bacteria but not against gram-negative bacteria (Kapjung *et al*., 2002). However, they were not used with this purpose yet (Kapjung *et al*., 2002). Besides being produced by non-pathogenic species, which is an important characteristic, they have several advantages compared with regular surfactants, namely their low ecotoxicity, higher biodegradability, better environmental compatibility and higher selectively and specific activity at extreme pH, temperature and salinity. In contrast, the regular surfactants have a low biodegradability, high ecotoxicity and bio-accumulation (Elshafie *et al*., 2015; Kapjung *et al*., 2002; Van Bogaert *et al*., 2007, 2013). For these reasons, they have been intensively studied and a pathway for sophorolipids production has been established as well as several growth medias to optimize its synthesis.

Sophorolipids are considered secondary metabolites since they are produced in the stationary phase and the absence of their production does not influence cell viability. Also, it has been suggested that sophorolipids could work as a carbon storage since they are produced upon high carbon to nitrogen ratio which is linked with their niche. Also as they have antimicrobial activity this could represent advantages against other microorganisms (Van Bogaert *et al*., 2013). The pathway to produce sophorolipids can be longer or shorter depending on the availability of fatty acids in the medium. When no hydrophobic substrate is present, fatty acids are synthesized from acetyl-CoA derived from glycolysis, through *de novo* fatty acid synthesis. In this case, as outlined and explained in more detail in Figure 1.4, when a nitrogen depletion exists there are several metabolite alterations that result in an increasing of citrate in the cytoplasm. ATP-citrate lyase (Acl) will convert it into acetyl-CoA. The fatty acid synthetase (Fas) generates fatty acids and triacylglycerols (Papanikolaou, 2012; Ratledge, 2002). However, a hydrophobic substrate can be added into the medium to optimize sophorolipid synthesis like a fatty acid, a triglyceride, or a fatty acid methyl- or ethyl ester.

When this type of molecule is supplied in the medium a lipase will hydrolyze it and the fatty acid will be taken up by the cell. Also, alkanes are widely used as hydrophobic carbon source to produce

sophorolipids, which will be oxidized into a fatty acid too. After this, several enzymatic steps occur as represented and explained in Figure 1.5, obtaining a sophorolipid in acidic form. The majority of sophorolipids are modified by lactonization and then acetylated through an acetyl transferase acetyl-Coenzyme A dependent. In this case, an acetylated lactonized sophorolipid is obtained. A minority of the sophorolipids in acid form are acetylated through the same enzyme originating an acetylated acidic sophorolipids (Bogaert & Saerens, 2007; Bogaert *et al*., 2011).

St. bombicola seems to excrete the sophorolipids produced to the culture media through a multidrug transporter (MDR) (Van Bogaert *et al*., 2013). However, this transporter does not seem to be essential for sophorolipid synthesis (Van Bogaert *et al*., 2013). So far, only two enzymes were experimentally tested and proven to be essential for sophorolipids synthesis. The deletion of *CYP52M1* encoding cytochrome P450 monooxygenase totally abolished sophorolipid production (Van Bogaert *et al*., 2013) and deletion of the gene encoding UDP-glucosyltransferase (*UGTB1*) also abolished production of sophorolipids but showed accumulation of glucolipids (Saerens *et al.*, 2011). These results suggest that both these genes encode essential enzymes for sophorolipid production (Saerens *et al.*, 2011; Van Bogaert *et al.*, 2013; Huang *et al*., 2014; Saerens *et al.*, 2015).

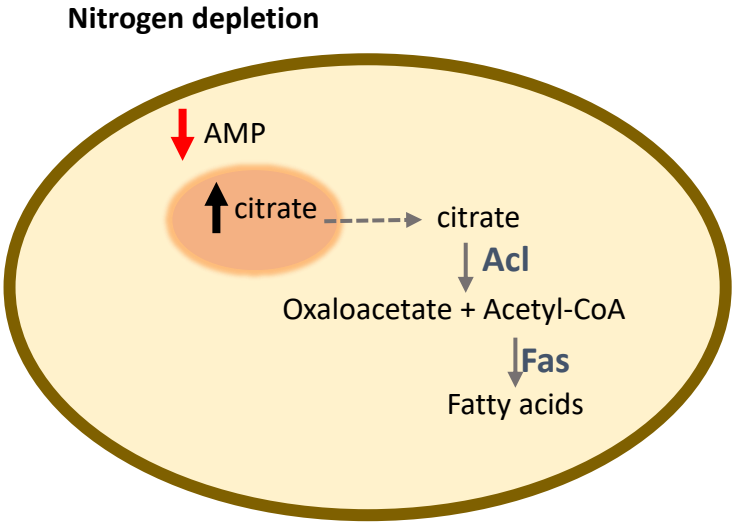


Figure 1.4 – Cellular alterations in oleaginous fungi in nitrogen depletion conditions. When a nitrogen depletion exists in the medium, the cellular AMP decreases. The lower levels of AMP lead to an alteration of Krebs cycle function. In this case, the enzymes activated by AMP decrease their activity altering the carbon flow towards accumulation of intra-mitochondrial citric acid. When citric acid levels are too high the cell secretes it to the cytoplasm. It is in the cytoplasm that ATP-citrate lyase (Acl) will convert citrate into acetyl-CoA. The fatty acid synthetase (Fas) generates fatty acids and triacylglycerols. Based on (Papanikolaou, 2012).

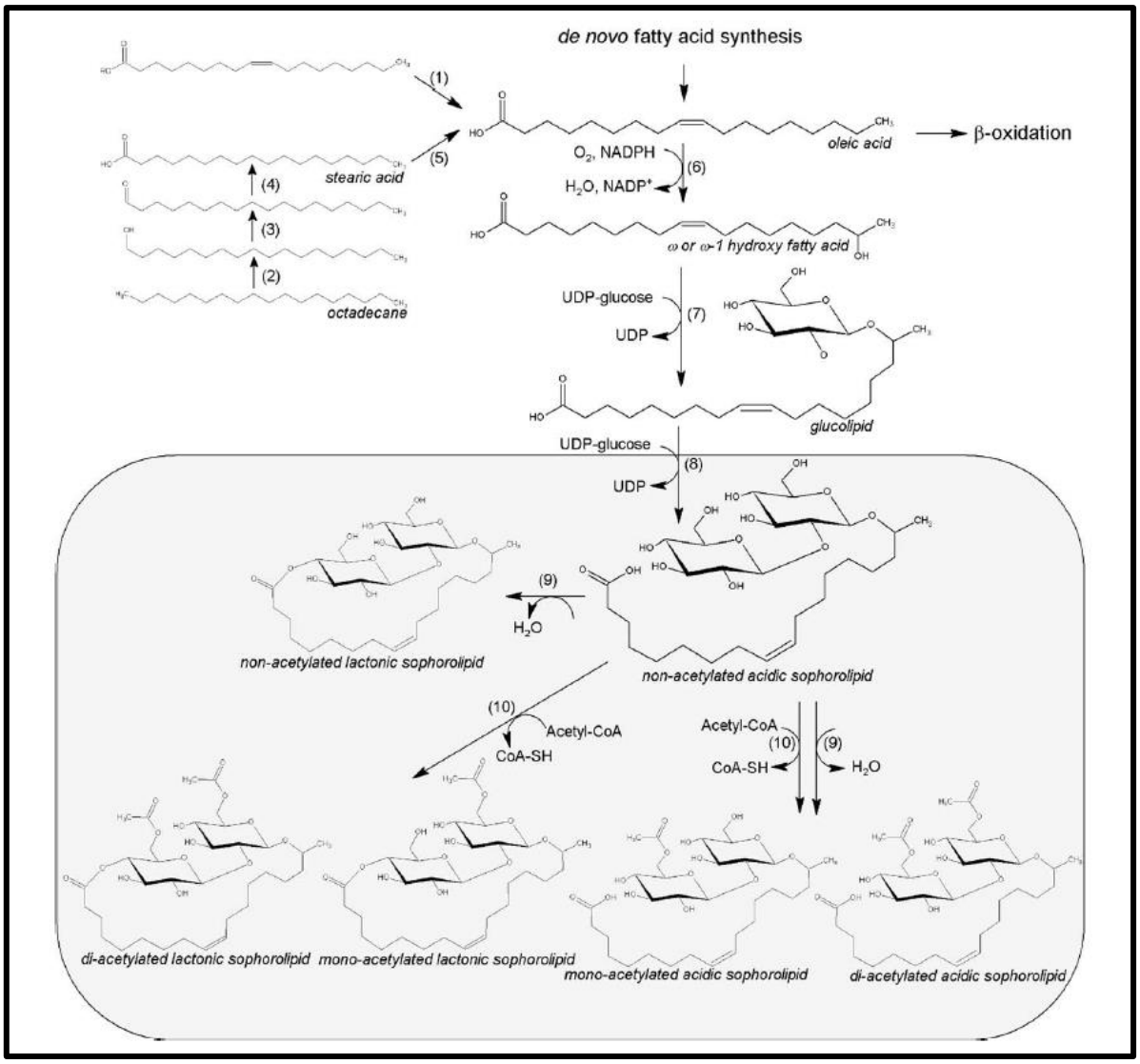


Figure 1.5 - **Proposed sophorolipid biosynthetic pathway.** The first step of sophorolipid production is the conversion of fatty acid into hydroxy fatty acid by a NADPH dependent cytochrome P450 monooxygenase. Then, through a glycosyltransferase I, glucose is glycosidically coupled to the hydroxyl group of the fatty acid. Then, a second glucose molecule is added by a glycosyltransferase II. Enzymes: 1) lipase, 2) cytochrome P450monooxygenase, 3) alcohol dehydrogenase, 4) aldehyde dehydrogenase, 5) desaturase, 6) cytochrome 0450 monooxygenase, 7) glucosyltransferase I, 8) glucosyltransferase II 9) lactonesterase, 10) acetyltransferase. Image from Van Bogaert *et al.*, 2011

As previously referred, sophorolipids are a particular type of lipid that only a few yeasts can produce. However, there are oleaginous yeasts (lipid content higher than 20 % (Boulton & Ratledge, 1981)) that produce other type of lipids biotechnologically important too due to the possibility of using them for biodiesel production. That is the case of *Rhodosporidium toruloides* and *Rhodotorula graminis*, that can produce lipids from sugars and from glycerol, however, glucose still is the preferential carbon source (Galafassi *et al*., 2012; Wiebe *et al*., 2012).

Fatty acid synthesis need large quantities of NADPH since two NADPH molecules are oxidized to $NADP^+$ for each elongation step and one NADPH is consumed for each fatty acid desaturation reaction (Dulermo *et al.*, 2015). *Y. lipolytica*, one of the most studied oleaginous yeasts so far, is a model organism for biofuel production. Because of this, fatty acids synthesis has been intensively studied in this organism. As oleaginous organisms need large quantities of NADPH, one of the most studied aspects in lipid production in *Y. lipolytica* is the source of NADPH. However, the origin of NADPH for

this metabolic pathway is not completely known. Malic enzyme, which is a cytosolic enzyme that converts malate into pyruvate with production of NADPH was considered for several years the main producer of cytosolic NADPH. However, it was proven that the malic enzyme does not have a significant impact on lipid synthesis since when the gene encoding for the malic enzyme was disrupted no significant decrease in fatty acid synthesis was observed (Dulermo *et al*., 2015). Instead, it has been suggested that the PPP could be the main producer of cytosolic NADPH that will be used in fatty acid synthesis (Wasylenko *et al*., 2015). On the other hand, in *K. lactis* mitochondrial respiratory chain seems to have a role in NADPH regeneration what does not happen in *Y. lipoytica* since the external mitochondrial enzymes use NADH instead of NADPH (Harder *et al.*, 2013; Kavscek *et al.*, 2015; Wasylenko *et al*., 2015). In addition, Dulermo *et al.* suggest a correlation between lipid synthesis and the synthesis of mannitol. In this study, mutants unable to produce mannitol have a higher yield in lipids. Also, they observe the opposite, since when lipid synthesis decreases, through the deletion of *ACL1*, mannitol yield increases instead. Due to the ability of *Y. lipolytica* to grow on mannitol and the typical role of lipids in the cell, the authors suggested a possible role of mannitol as carbohydrate reserve, since when one of these pathways were abolished a readjustment on carbon flow occurs (Dulermo *et al.*, 2015).

1.4 Polyols as a smart strategy to deal with high osmotic environments

In nature, the osmolarity usually changes with consequences to the integrity and hydration of the cell. When the external osmotic pressure is lower, it causes a water influx which can lead to swelling or lysis of the cell. But, when the external osmotic pressure is high, water efflux is the result leading to dehydration (Bubnová *et al*., 2014; Wood, 2015).

There are no active transport mechanisms for water, so microorganisms developed strategies to maintain the turgor and volume of the cell within boundaries acceptable for its normal physiology. *Aspergillus nige*r and *Penicillium chrysogenum* are capable of tolerating high external osmotic pressures using a common strategy: synthesis or uptake of polyols, namely glycerol, erythritol and mannitol (Hult *et al*., 1980).

Polyols or sugar alcohols are compatible solutes widely distributed in fungi, that can have a dual function: stress response and protection against dehydration. Structurally, they are polyhydric alcohols formed by the reduction of the carbonyl group of an aldose or a ketose monosaccharide to a hydroxyl group (Solomon *et al*., 2007). Polyols are synthesized or taken up by yeasts, especially the ones capable of growing in high sugar or salt environments – osmotolerant yeasts *(*Harder *et al., 2013;* Bubnová *et al*., 2014). Glycerol is the most common and the most studied polyol. However, there are more examples as mannitol, sorbitol and ribitol. Different organisms can use the same sugar alcohol for different purposes. Mannitol is a good example of that, since it has been assigned different functions in fungi like carbohydrate reserve, osmoregulation, coenzyme regulation and storage of reducing power (Jennings, 1984).

1.4.1. The importance of mannitol in fungi

1.4.1.1. Mannitol synthesis

Mannitol is one of the most abundant polyols in nature. It can be found in bacteria, plants, algae, lichens but specially in filamentous fungi. However, the pathway to synthesize mannitol is quite different between organisms (Figure 1.6). In the fungus *Alternaria alternata* a cycle with four enzymes was described: NADH-mannitol 1-phosphate dehydrogenase (M1pdh), $NADP^+$-mannitol 2-dehydrogenase (Mtdh), mannitol 1-phophate phosphatase (Mpp) and a hexokinase (HK). The mannitol cycle is a branching from glycolysis in fructose-6-phosphate, which is converted in mannitol 1-phosphate by M1pdh and Mpp will dephosphorylated it into mannitol. Then, mannitol is converted into fructose via a reversible NADPH-mannitol dehydrogenase (Mtdh). The hexokinase is responsible for the conversion of fructose into fructose-6-phosphate. The proposed role for mannitol cycle was regeneration or production of NADPH due to the balance of the cycle: NADH + $NADP^+$ + ATP in NAD^+ + NADPH + ADP + Pi (Hult & Gatenbeck, 1978). However, in some organisms the purpose of the production of mannitol does not seem to be the regeneration of $NADP^+$. Instead, in the last years several functions have been attributed to this polyol suggesting that mannitol can have different functions among organisms (Jennings, 1984; Patel & Williamson, 2016; Solomon *et al*., 2007).

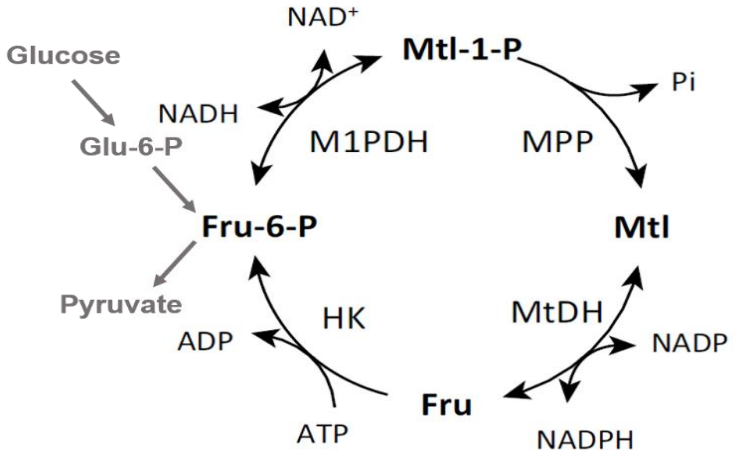


Figure 1.6 - Mannitol cycle. Fructose-6-phosphate is converted to mannitol-1-phosphate by mannitol-1-phosphate dehydrogenase (M1pdh). Mannitol-1-phospate is converted by mannitol-1-phosphate phosphatase into mannitol, which will be converted in fructose by mannitol dehydrogenase (Mtdh). Fructose will be phosphorylated by a hexokinase (Hk).Abbreviations: Fru: fructose; Fru-6-P_ fructose-6-phosphate; Mtl: mannitol; Mtl-1-P: mannitol-1-phosphate. Adapted from Patel and Williamson, 2016.

However, in some fungi like *Aspergillus niger* Mtdh is expressed only in spores and M1pdh, which seems to be the major enzyme in mannitol metabolism, is expressed only in mycelia (Ruijter *et al*., 2003). Also, in *Basidiomycetes* the absence of mannitol 1-phosphate dehydrogenase or mannitol 1-phosphatase activities is described, suggesting that mannitol metabolism is not always a cycle *(Hult et al*., 1980).

In *Stagonospora nodorum* the absence of M1pdh causes alterations on central metabolism, since when grown on glucose, the levels of arabitol and mannitol were reduced but more trehalose was detected. When grown on fructose, mannitol production is not affected suggesting that fructose can be reduced to mannitol via Mtdh. The fact that mannitol metabolism is not always part of a cycle as well as the fact

that in *S. nodurum* the absence of M1pdh or Mtdh had effects on central metabolism and in asexual sporulation suggests a versatile role of this polyol in the cell. So, the function of mannitol is not necessarily storage compound or redox balance, instead mannitol can have different functions and its absence can have strong effects in cell physiology (Solomon *et al*., 2006).

1.4.1.2. The different functions of mannitol in microorganisms

One of the reported functions of mannitol is that of compatible solute (Zahid *et al*., 2015). To deal with low water activity – high osmotic pressure – many bacteria synthesize and accumulate polyols. *Gluconobacter oxydans* lives in sugar rich environments like fruits, nectars, wine and honey syrups being subjected to a high osmotic pressure. The osmotolerance of these bacteria is improved by production and accumulation of intracellular mannitol. Also, in different species of bacteria like *Pseudomonas fluorescence, Acinetobacter baylyi* and in hetero-fermentative lactic acid bacteria it has been reported that mannitol works as osmoprotector as in *G. oxydans* (Kavanagh *et al*., 2002; Sand *et al*., 2013; Wisselink *et al*., 2002; Zahid & Deppenmeier, 2016).

Also, the conversion of mannitol into fructose is a unique enzymatic step that can provide a faster start of glycolysis. In *Alternaria alternata*, mannitol seems to have a role as storage compound too. However, it does not seem essential since spores of mannitol deletion mutants (*mtdhΔ, mpdhΔ* and *mtdhΔmpdhΔ*) could germinate. Otherwise, when mannitol levels decrease the disaccharide content increases, suggesting that mannitol is not essential as storage compound since it can be substituted by other carbohydrates, but when it is present it performs that role in the cells (Vélëz *et al*., 2007).

Mannitol is also used as carbohydrate reserve in pathogenic fungi (Solomon *et al*., 2007). Many plants are unable to metabolize mannitol, so fungal pathogens or mutualists can convert hexoses to mannitol. This limits the use of the carbon source by the plant, which is an advantage for the fungus *(*Solomon *et al*., 2007). But, mannitol can also have another function in plant-pathogen interaction. When invaded, plants produce Reactive Oxygen Species (ROS) in the extracellular space or apoplast which are signals to start the defense responses. As mannitol can quench the ROS produced by the plant it will block the defense signal itself. In *Cladosporium fulvum*, a tomato fungal pathogen, the mutants unable to produce mannitol were non-pathogenic showing that, in this case, mannitol is necessary for pathogenicity (Patel & Williamson, 2016). This can be due to the role of mannitol as quencher of ROS, but it can also be related with an intrinsic mechanism to defend the pathogen against the stresses associated with plant defense responses.

In 2006, mannitol was described for the first time as fundamental for asexual sporulation in *S. nodorum*. In this study, mutants unable to synthesize mannitol did not sporulate and when mannitol was added in the medium the ability to sporulate was restored. Although it has not been proven so far, mannitol could work as a signalling molecule for the initiation of asexual conidiation in *S. nodorum* (Solomon *et al.*, 2006).

However, mannitol can have yet another role in sexual structures in fungi. In *Aspergillus fischeri*, the deletion mutant unable to convert fructose-6-phosphate into mannitol-1-phosphate exhibit delayed formation of ascospores and some of ascospores were incompletely formed. Also, conidia germination after exposure to stress conditions was lower than wild-type showing that conidia were more sensitive to heat and oxidative stress. However, there were no differences in vegetative growth indicating that mannitol seems to be fundamental for conidia germination (Wyatt *et al.*, 2014).

Some authors described a dual function for mannitol in the rust fungus *Uromyces fabae*. In this case, mannitol was found in spores but disappeared rapidly upon infection structure formation leading the authors to suppose that mannitol could have a role as carbohydrate storage. But mannitol also seems to work as a scavenger of Reactive Oxygen Species (ROS) in this species. This shows that mannitol has different functions not only depending on the organism, but it can also have different roles in the same organism emphasizing its versatility, which by itself is very interesting (Voegele *et al*., 2005).

In summary, so far mannitol has been described to have roles in stress tolerance in high osmotic environments, can work as a quencher of ROS, as carbohydrate reserve or it can have a role in sexual and asexual development in fungi. Several microorganisms also produce high amounts of mannitol including yeasts belonging to the W/S clade (Gonçalves, 2018). But, so far, the role of mannitol has not been established. *Candida magnoliae*, a yeast belonging to the W/S clade, has been described as a producer of high amounts of mannitol. Still its function was not elucidated (Lee *et al*., 2003; Song *et al*., 2002). Probably due to the presence of this species in nectar where the osmotic pressure is high, mannitol could be involved in osmoprotection. As well as other yeasts belonging to W/S clade (Gonçalves, 2018), *St. bombicola* produces high amounts of mannitol and lipids. In turn, *Y. lipolytica* produces high amounts of lipids, a pathway that has already be showed (see section 1.3) to be interconnected with mannitol synthesis in this yeast. In this way, the mannitol and lipid synthesis could also be interconnected in *St. bombicola*.

1.5. Objectives

Ffz1 is essential for fructophilic behavior, however it does not seem to be the only requirement for the preference for fructose over glucose, so it is possible that fructose dependent metabolic pathways may play a role in fructophilic yeasts. W/S clade yeasts, namely *St. bombicola*, produces high amounts of mannitol from fructose through Mtdh. Since this is a fructose dependent pathway, the first objective of this study was to understand if the absence of this pathway could affect the fructophilic behavior of *St. bombicola*.

1. Introduction

2. Materials and Methods

2.1. Yeast strains

St. bombicola PYCC 5882 (will be referred in this work as wild-type strain) was isolated from a flower of *Ornithogalum narbonense* in Cascais and is deposited in the Portuguese Yeast Culture Collection (PYCC) in Caparica, Portugal. All the deletion mutants used in this work were constructed in this strain background. The culture was maintained in YPD medium (1% (w/v) yeast extract (Difco), 2% (w/v) peptone (Difco), 2% (w/v) glucose and 2% (w/v) agar (Labchem).

2.2. Growth experiments with *St. bombicola* and deletion mutants

For growth assays, the strains (wild-type, *mtdh1Δ*, *mtdh1Δ mtdh2Δ* and for some assays *mtdh2Δ* and *mtdh1Δ sor1Δ*) were pre-grown overnight (o/n) in 100 mL flasks containing 20 mL of culture medium at the temperature used for the growth assay. The inoculum was set at OD_{640nm}=0.2 in 30 mL of medium YP (1% (w/v) yeast extract and 2% (w/v) peptone) supplemented with a carbon source in 250 mL flasks. The cell density was measured at 640nm and was monitored for 100-150h and supernatant samples were collected for further metabolites analyses.

2.2.1 Assimilation tests

To test if sorbitol could be assimilated as a carbon source, wild-type and the deletion mutant available at the lab *sor1Δ* (sorbitol dehydrogenase) were grown on 100 mL flasks containing 20 mL of Yeast Nitrogen Base medium (YNB, Difco) supplemented with 2% (w/v) or 18% (w/v) sorbitol. Growth was monitored for 7 days.

To test if mannitol could be assimilated as a carbon source wild-type and *MtDH* deletion mutants were grown in the YP supplemented with 18% (w/v) mannitol. Growth was monitored for 7days.

2.3. Metabolite Analyses

2.3.1. Analysis of extracellular metabolites: quantification by HPLC

Extracellular metabolites were analysed by HPLC. 1 mL of culture was centrifugated for 1min at 16000g and the supernatant was collected. If necessary, the sample was diluted in a sodium azide solution in distilled water (0.025% w/v) prior to filtration through Q-Max RR Nylon Filters with 0.22µm pores (Frisenette). 10µL was injected into a Dionex P680 instrument equipped with an ASI-100 Automatic

Sample Injector and a 2142 Differential Refractometer. For these analyses, the eluent was a sodium azide solution in bi-distilled water (0.025%) at a flux of 0.6 mL.min^{-1}. The Aminex HPX-87P (300mm x 7.8 mm, BioRad) column was operated at 80 ºC. Solutions containing glucose, fructose, ethanol, glycerol, erythritol and mannitol with concentrations between 10 and 15 g/L were sequentially diluted for construction of a calibration curve.

To detect citric acid, the column used was Aminex HPX-87H (300 mm x 7.8 mm, Biorad) at 65 ºC with an Ultimate 3000 Diode Array Detector at 210 nm. The mobile phase a solution of 5 mM of Sulfuric Acid at 0.6 mL.min^{-1} in bi-distilled water. The pure standard of citric acid was 5 g/L which was sequentially diluted for construction of a calibration curve.

The results were treated and integrated using the software Chromeleon v6.8 (Dionex). Two replicates were analysed.

2.3.2. Determination of metabolite yields

After the concentration in the growth medium was determined by HPLC, the yield of each metabolite was calculated per gram of sugar consumed. For that, the sugar consumed was considered and equation used is shown:

Equation 2.1 $$Metabolite\ Yield\ (g/g\ sugar\ consumed) = \frac{Metabolite\ (g/L)}{Sugar\ consumed\ (g/L)}$$

2.3.3. Analysis of Intracellular Metabolites

2.3.3.1. Preparation of crude extracts for intracellular metabolite analysis

Wild-type and *MtDH* deletion mutants were grown in duplicate for 72 h in YP supplemented with 20% (w/v) of fructose and glucose (1:1) at the temperatures 20 ºC, 25 ºC and 30 ºC at 180 r.p.m (rotations per minute). After this time, cells were harvest by centrifugation (4 ºC, 10 min, 8000g) and 1mL of supernatant was collected for HPLC analysis. Then, the cells were washed twice with 100 mM phosphate buffer pH = 7 and stored at -20 ºC. After thawing, the cells were resuspended in 400 µL of TRIS Lysis buffer (0.1 M triethanolamine hydrochloride, 2 mM $MgCl_2$, 1 mM DTT and 1 µM PMSF) and 200 µL glass beads (212-300µm). The cells were lysed by six alternate cycles of 1 min vortexing followed by 1 min cooling on ice. The cell debris were removed by centrifugation (4ºC, 20min, 9000 g) and the supernatant collected for intracellular analysis by HPLC. For protein quantification 10 µL of the supernatant was collected.

2.3.3.2. Protein Quantification

For protein quantification Qubit fluorimeter using the manufacturer's protocol.

2.3.3.3. Analysis of Intracellular Metabolites: Quantification by HPLC

The intracellular samples were analysed at the Biological and Chemical Analysis Facility at UCIBIO. The samples (10µL) were eluted with NaOH 0.6 M at a flux 0.4 mL/min at 25 ºC in a CarboPac MA1 column and the peaks were identified with a pulsed amperometric detector. The presence of glucose, fructose, ethanol, glycerol, erythritol and mannitol was analysed. Two replicates were analysed.

2.3.4. Determination of intracellular metabolites

After quantification of intracellular metabolites, the metabolites produced were analysed, however, the amounts of intracellular metabolites could be influenced for the efficiency of the lysis. So, to control the efficiency of cell lysis, the total protein was measured as explained in section 2.3.3.2. Thus, to be able to compare the intracellular results from the three temperatures, we decided to consider the total protein of the crude extracts (described in 2.3.3.1). Therefore the follow formula was used:

Equation 2.2 $$Metabolite\ (g/mg\ total\ protein) = \frac{Metabolite\ (g/L)}{Total\ protein\ (mg/L)}$$

2. Materials and Methods

3. Results

The results obtained during this thesis are partially confidential. Therefore, the results presented in this section are only a part of the work performed which correspond to the non-confidential results.

3.1. Understanding the function of mannitol and mannitol dehydrogenase in *St. bombicola*

The preference for fructose over glucose as carbon source was previously shown to be related with the presence of the Ffz1 fructose transporter (Pina *et al*., 2004). In *St. bombicola,* as well as in *Z. rouxii*, the deletion of the gene encoding for this transporter totally abolished the fructophilic behavior, however the relevance of fructose in their metabolism has not been elucidated yet (Gonçalves *et al*., 2018; Leandro *et al*., 2014). In addition to being a carbon source, fructose can also be a final electron acceptor to re-oxidation of NAD(P)H in fructophilic bacteria, resulting in the formation of mannitol (Zaunmüller *et al*., 2006).

Candida magnoliae, a species from the W/S clade, has already been described as a producer of high amounts of mannitol from fructose (Lee *et al*., 2003; Song *et al*., 2002). Moreover, the mannitol yield of several species from the W/S clade was determined and all of them, with the exception of *St. bacillaris,* seem to produce high amounts of mannitol when grown on fructose as carbon source (Gonçalves, 2018).

In *St. bombicola,* mannitol seems to be produced directly from fructose through the enzyme mannitol dehydrogenase which uses NADPH but not NADH as a cofactor (Gonçalves, 2018). Also, in the genome of *St. bombicola* two genes encoding for two mannitol dehydrogenase (Mtdh) were found. Single and double deletion mutants were previously constructed (*mtdh1Δ*, *mtdh2Δ*, *mtdh1Δmtdh2Δ)*. In order to understand if mannitol dehydrogenase could have an impact in fructophily, the different mutants were grown in YP medium supplemented with 100 g/L glucose and 100 g/L of fructose (henceforth this medium will be named 20FG) since it is a medium where fructophily is apparent. In figure 3.1 where the growth curves are represented it is possible to see that the deletion mutants *mtdh1Δ* and *mtdh1Δmtdh2Δ* are not able to reach the same cell densities as the wild-type. However, the *mtdh2Δ* deletion mutant grows as well as the wild-type suggesting that Mtdh1 is the major enzyme involved in mannitol synthesis. Measuring the mannitol production, we were able to observe that *mtdh1Δ* and *mtdh1mtdh2Δ* do not synthesized mannitol, while *mtdh2Δ* is able to produce mannitol what corroborates the previous results that Mtdh1 is the major enzyme involved in mannitol synthesis in *St. bombicola.* To try to explain why these deletion mutants reach lower cell densities, we first assessed whether this effect was also observed when a single sugar was present (either glucose or fructose). *MtDH* encoded an enzyme that converts fructose into mannitol, so the deletion of this gene could have more impact on the metabolism of fructose. To evaluate that, the osmotic pressure was maintained (20 % (w/v)), but we added either only glucose or only fructose to the growth medium (Figure 3.2). As it is possible to observe, in both media tested, we saw that the *MtDH* deletion mutants did not reach the same cell densities as the wild-type independently of the sugar, suggesting that the lower cell densities of the

MtDH deletion mutants are not related with one specific sugar, since their behavior is observable in presence of both glucose or fructose.

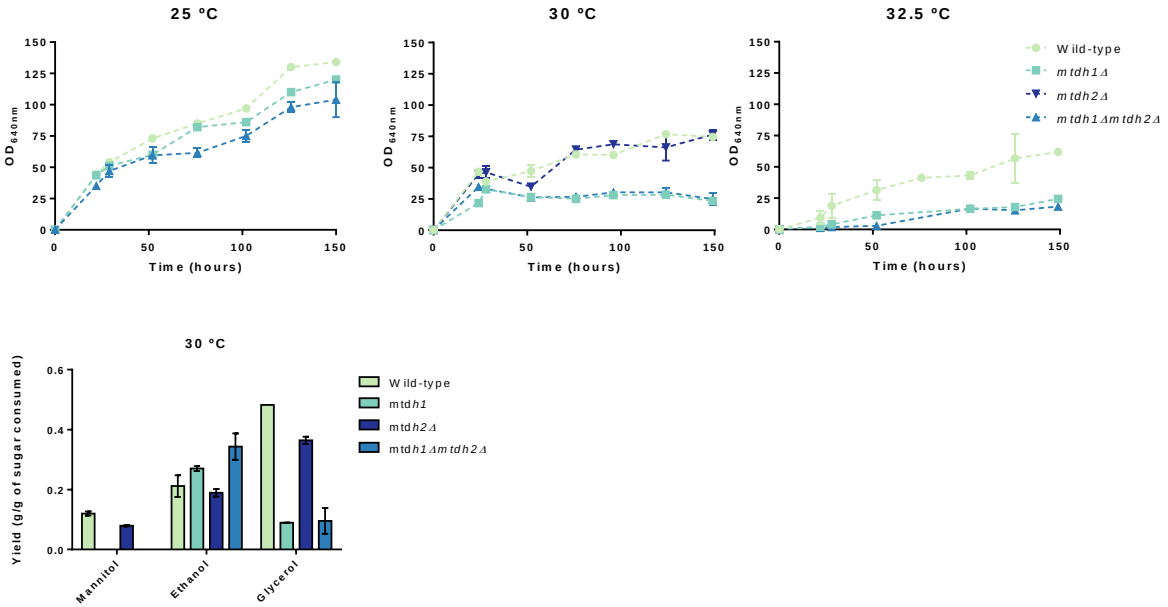


Figure 3.1 – Growth curves of wild-type and *MtDH* deletion mutants at 25 ºC, 30 ºC and 32.5 ºC and Mannitol, Ethanol and Glycerol yields at 30 ºC, 72 h. The strains were grown in YP supplemented with 100 g/L Glucose and 100 g/L Fructose at 25 ºC, 30 ºC and 32.5 ºC for 150 h**.** The yields represented were determined at 72 h at 30 ºC.The experiments were performed with two biologicals replicates in two independent experiments and the error bars represent the standard deviation n=4.

Among the different functions that mannitol can have in the cell, one of the most studied so far is its role as osmoprotector. *Gluconobacter oxydans* accumulates mannitol as a compatible solute under osmotic stress conditions. But, when a double deletion mutant lacking the two mannitol dehydrogenases is grown in a high osmotic medium, the deletion mutant presents a defective growth and has an intracellular mannitol amount lower than the wild-type. These results suggests that mannitol synthesis in *G. oxydans* is involved in osmoprotection. (Zahid *et al*., 2015; Zahid & Deppenmeier, 2016). In our experiments with *St. bombicola* the partial growth impairment of the mannitol dehydrogenase mutants could be related with the high osmolarity of the medium (20 % (w/v) sugar). To test this, the wild-type strain and the *MtDH* deletion mutants were cultivated at the temperatures where the growth impairment was more pronounced, in this case 30 ºC and 32.5 ºC and in lower sugar concentrations where osmotic pressure is reduced, namely YP medium supplemented with 10 g/L fructose and 10 g/L glucose, henceforth referred to as 2FG.

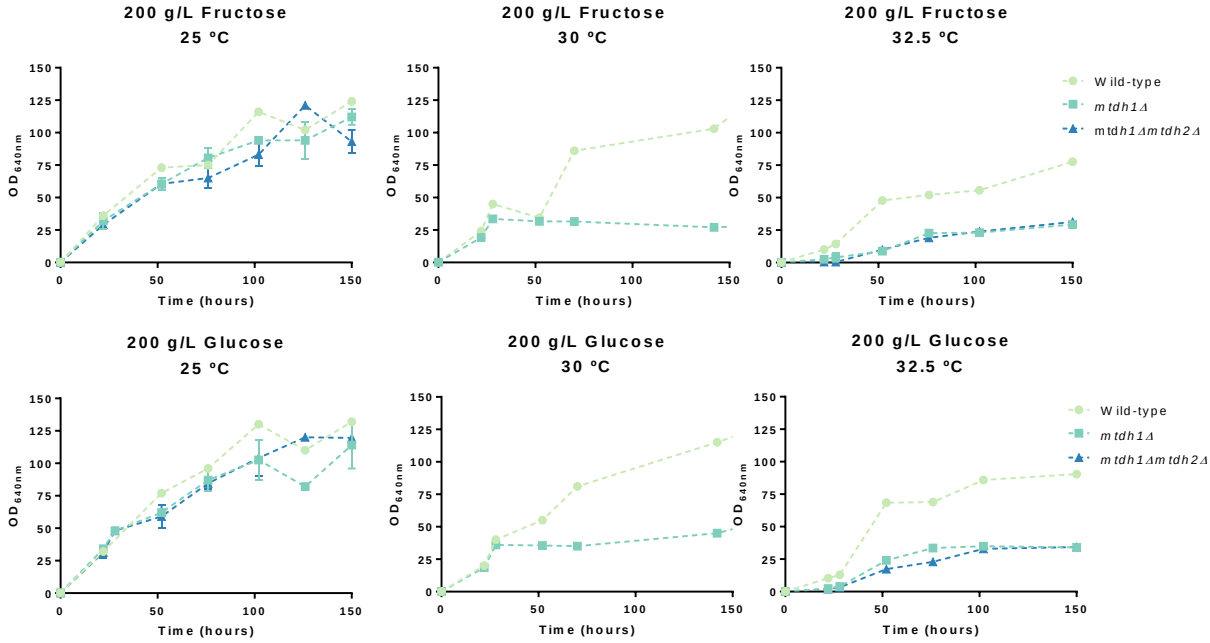


Figure 3.2 - Growth curves of wild-type, *mtdh1Δ* and *mtdh1Δmtdh2Δ* deletion mutants at 25 ºC, 30 ºC and 32.5 ºC in fructose or glucose. The strains were grown on YP supplemented with 200 g/L Fructose (first row) and 200 g/L glucose (second row) at 25 ºC, 30 ºC and 32.5 ºC for 150 h. The error bars represent the standard deviation of two biological replicates

In figure 3.3 the growth curves of wild-type and *MtDH* deletion mutants in 2FG are represented. It is observable that at 30 ºC there are no differences in cell densities comparing with the wild-type. However, when grown at 32.5 ºC in the same medium, *MtDH* deletion mutants are not even able to grow, suggesting that the absence of mannitol/mannitol dehydrogenase in 2FG medium at 32.5 ºC is indispensable for growth. On the other hand, these results suggest that mannitol itself or mannitol synthesis is not involved in protection against osmotic stress in *St. bombicola*.

So far, we know that *MtDH* deletion mutants do not reach the same cell densities as the wild-type, but the reason for a lower cell density is not understood yet. Also, it is not known whether only the biomass yield is affected or the growth rate is also affected by the absence of mannitol/mannitol dehydrogenase. To test this we determined the growth rates during the exponential phase. Table 3.1 lists the specific growth rate of *MtDH* deletion mutants in different media and temperatures.

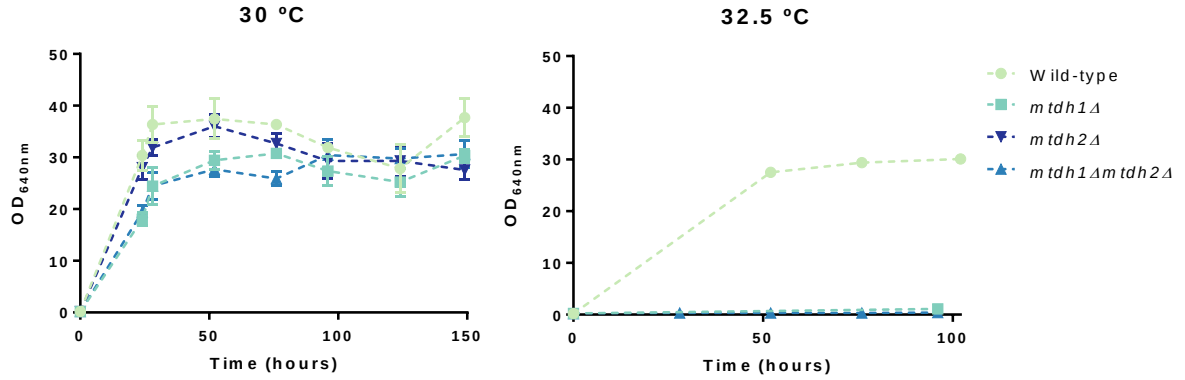


Figure 3.3 - Growth assays in YP supplemented with 10 g/L glucose and 10 g/L of fructose at 30 ºC and 32.5 ºC for 150 h and 100 h respectively. The error bars represent the standard deviation of 4 assays which were performed twice with two biological replicates (n=4).

In Table 3.1 are represented the specific growth rates in different conditions, 20FG at 30 ºC and 2FG at 25 ºC, 30 ºC and 32.5 ºC. Interestingly, looking into the specific growth rates of *MtDH* deletion mutants, they grow at the same rate as the wild-type in all conditions tested. This could indicate that mannitol or mannitol dehydrogenase could influence the biomass yield but not the growth rate. Also, comparing the specific growth rates at 30 ºC between 20FG and 2FG it is possible to observe that the specific growth rate is higher in wild-type and in *MtDH* deletion mutants in a high sugar concentration medium.

Table 3.1 – Specific growth rates of wild-type and *MtDH* deletion mutants. Standard deviation results from two different experiments performed with biological replicates. NG means that the strains did not grown in the condition tested.

Conditions		Specific Growth Rate (h^{-1})		
Temperature (ºC)	Culture medium	Wild-type	*mtdh1Δ*	*mtdh1Δ mtdh2Δ*
25	2FG	0.321 ± 0.006	0.314 ± 0.005	0.312 ± 0.001
30	20FG	0.347 ± 0.006	0.373±0.0002	0.338±0.0004
	2FG	0.234 ± 0.004	0.224 ± 0.011	0.224 ± 0.010
32.5	2FG	0.24	N.G.	N.G.

As mannitol is synthesized directly from fructose, the sugar consumption in 20FG at 25 ºC, 30 ºC and 32.5 ºC was analysed (Figure 3.4) in order to understand if the absence of this metabolic pathway could alter the consumption of fructose. The preference for fructose over glucose is observable at all temperatures and all strains tested. However, the *MtDH* deletion mutants does not consume fructose as the wild-type. For example, at 30 ºC at 124 h the wild-type consumed all the sugar in the medium, while the *MtDH* deletion mutants still have about 10 g/L of residual fructose available. This is more noticeable at 32.5 ºC where the *MtDH* deletion mutants could not consume all the fructose in the medium in 150 h when the wild-type consumes all fructose in 102 h. The consumption of glucose seems to be different too but only at 32.5 ºC. From 102 h *MtDH* deletion mutants barely consume the sugars, namely fructose. Besides that, at 150 h in the *MtDH* deletion mutants there is approximately 50 g/L of glucose in the medium, while the wild-type consumed all the glucose present in the medium. The absence of Mtdh seem to attenuate the fructophilic behavior. So, to understand if this only happen when both sugars are present or if it happens even when only one sugar is present, the sugar consumption profile in medium containing 200 g/L fructose or 200 g/L glucose was analysed (Figure 3.5).

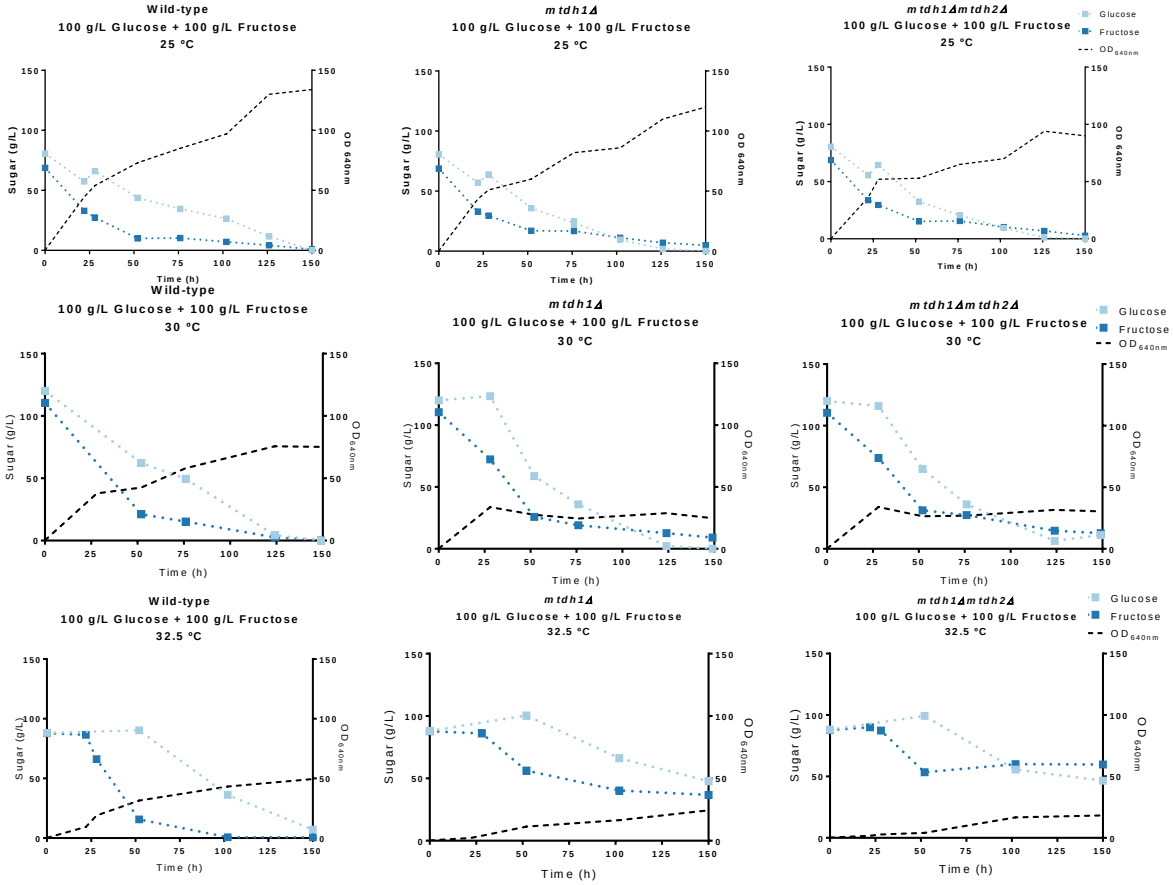
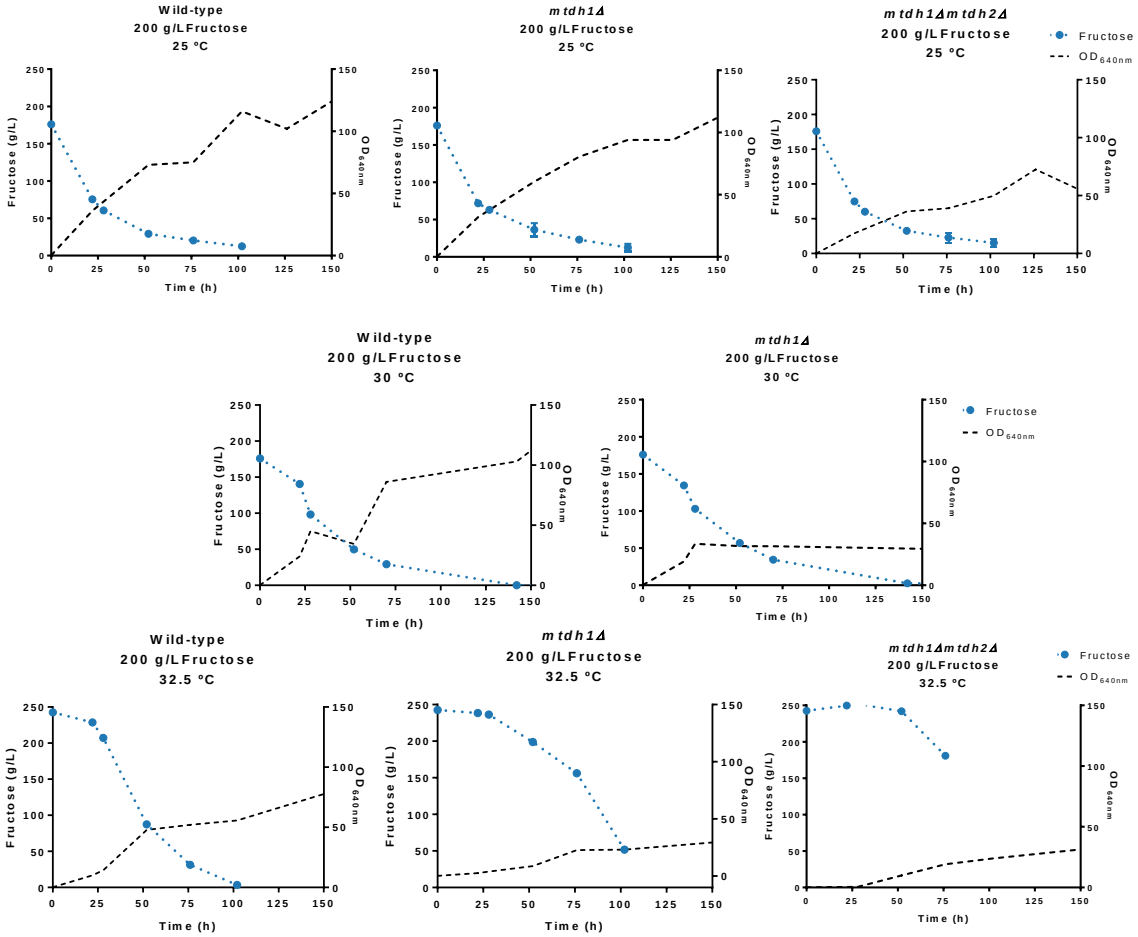


Figure 3.4 - Sugar consumption of wild-type and *MtDH* deletion mutants in YP supplemented with 100 g/L fructose and 100 g/L glucose at 25 ºC, 30 ºC and 32.5 ºC. The assays were performed in duplicate, however only one experiment is shown.

Figure 3.5A shows fructose consumption at 25 ºC, 30 ºC and 32.5 ºC. At 25 ºC there are no differences in fructose consumption profiles, since fructose is consumed in 102 h both in the wild-type and in the *MtDH* deletion mutants. However, when the temperature increases, the wild-type consumes the sugar slower than at 25 ºC. This effect is more remarkable at 32.5 ºC where until 24 h the wild-type barely consumes the sugar available in the medium. Looking into sugar consumed by the *MtDH* deletion mutants, is observable that they do not consume fructose as the wild-type, since at 32.5 ºC, the wild-type consumed all the fructose in the medium after 102 h while at that time point the *mtdh1Δ* mutant still had 50 g/L of sugar in the growth medium.

When glucose is the carbon source in the medium (Figure 3.5B), there does not seem to be a difference in sugar consumption between wild-type and *MtDH* deletion mutants. However, when sugar consumption in 200 g/L fructose is compared with 200 g/L glucose at 32.5 ºC by *mtdh1Δmtdh2Δ* it is noticeable that there is a quite difference since the glucose consumption starts at ~28 h while fructose consumption starts at 52 h. Interestingly this is not observable in the *mtdh1Δ* since both glucose and fructose are consumed from ~28 h as in the wild-type. This seems to indicate that fructose consumption is affected by the absence of Mtdh enzyme, while glucose consumption does not seem to be affected when only one sugar is present in the medium.

A


Wild-type
200 g/L Fructose
25 °C
mtdh1Δ
200 g/L Fructose
25 °C
mtdh1Δmtdh2Δ
200 g/L Fructose
25 °C
Fructose
OD640nm
Wild-type
200 g/L Fructose
30 °C
mtdh1Δ
200 g/L Fructose
30 °C
Wild-type
200 g/L Fructose
32.5 °C
mtdh1Δ
200 g/L Fructose
32.5 °C
mtdh1Δmtdh2Δ
200 g/L Fructose
32.5 °C
Fructose (g/L)
OD640nm
Time (h)

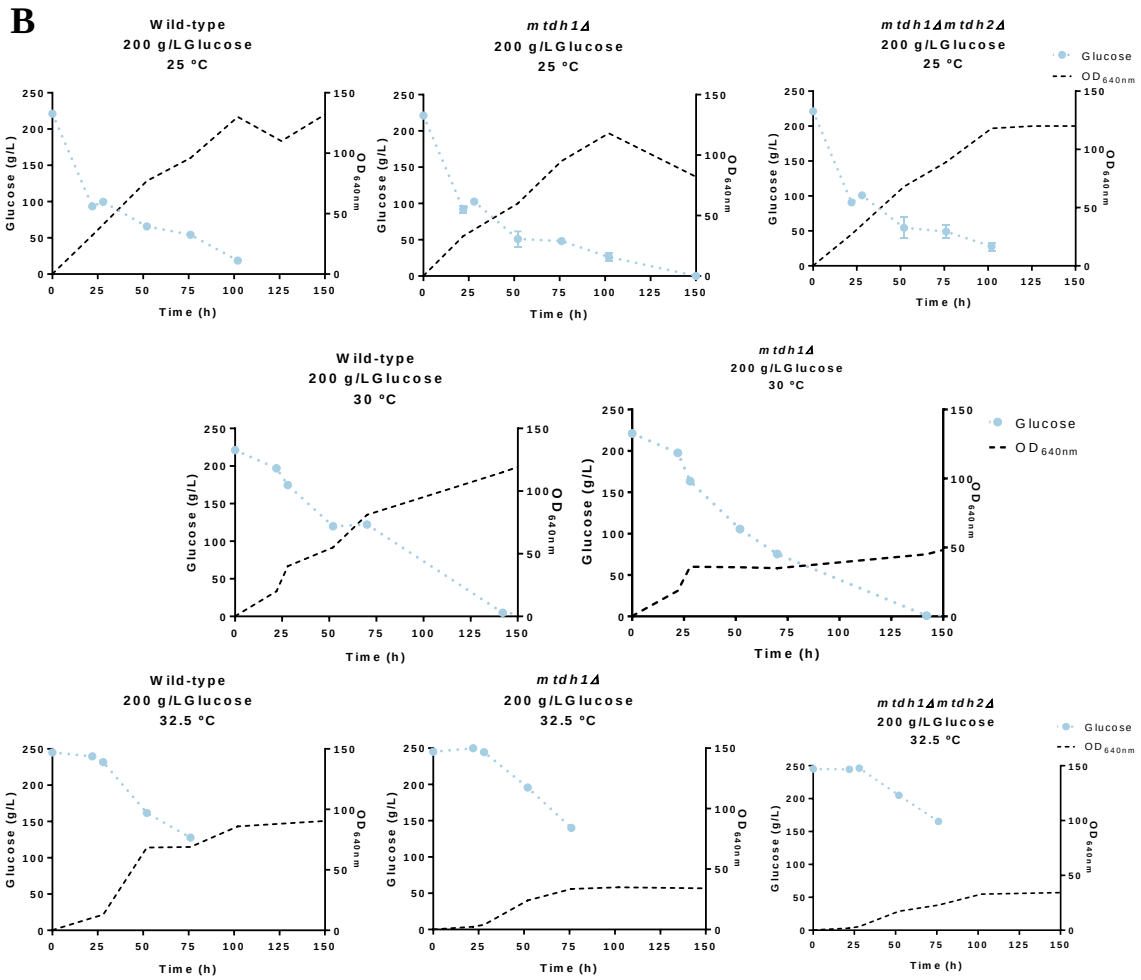


Figure 3.5 - A) Fructose consumption in YP supplemented with 200 g/L fructose at 25 ºC, 30 ºC and 32.5 ºC. B) Glucose consumption in YP supplemented with 200 g/L glucose. The experiments were performed in duplicate, however only one experiment is shown.

It has been often observed in yeasts that, when synthesis of an important metabolite for the cell is impaired, there is a compensation with the production of another one. For example, in *S. cerevisiae* or in *St. bombicola* when the ethanol synthesis is impeded, glycerol is produced instead (Gonçalves *et al*., 2018; Smidt *et al*., 2011). To understand if when mannitol synthesis was abolished another metabolite could be produced, the extracellular metabolites produced were analysed by HPLC. Interestingly, the *MtDH* deletion mutants produce low amounts of erythritol while wild-type does not (figure 3.6).

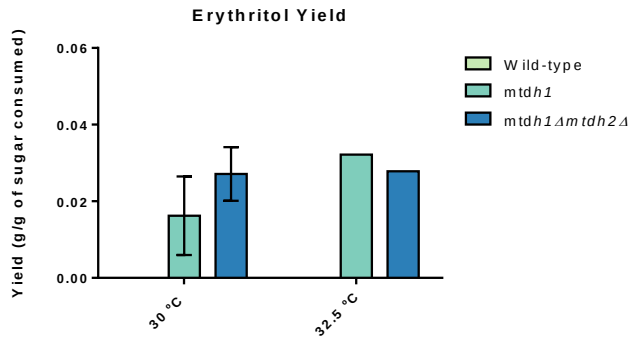


Figure 3.6 - Erythritol production in different conditions. Erythritol production by wild-type and *MtDH* deletion mutants at 72 h in YP supplemented with 10 % (w/v) glucose and 10 % (w/v) fructose at 30 ºC and 32.5 ºC. The error bars represent the standard deviation from duplicates in two biological replicates

3.2 Growth defects of *mtdhΔ* mutants are rescued by exogenous polyols

To distinguish if the growth defects of the *MtDH* deletion mutants were due to the need for mannitol itself or for the $NADP^+$ regenerating role of the production of mannitol from fructose, we attempted to rescue the growth defects of the mutants by adding of various polyols to the growth medium.

We started by using sorbitol since it is also a common polyol and is an isomer of mannitol. We also tested if sorbitol could be metabolized by *St. bombicola*, using 2 % and 18 % (w/v) of sorbitol, and found that growth was only observed at the higher concentration (wild-type grown on 18% (w/v) sorbitol is represented in figure 3.7). This could mean that this yeast lacks a high affinity sorbitol transporter.

Figure 3.7 - Growth test on YNB supplemented with 18 %(w/v) sorbitol. The assay was performed in duplicate, however only the first experiment is shown.

Growth of the wild-type and *MtDH* deletion mutants at 30 ºC and 32.5 ºC in YP supplemented with 1% fructose, 1% glucose and 18% (w/v) sorbitol (2FG18S) was monitored and the results are shown in Figure 3.8.

As it is possible to observe in Figure 3.8, in the presence of sorbitol a striking rescue of the growth defect of the mutants in 2FG medium at 32.5 ºC is observed (see Figure 3.3). Since the difference between the two sets of experiments at 32.5 ºC was only the presence of 18 % (w/v) sorbitol in the

experiment depicted in Figure 3.8 it seemed important to evaluate whether the observed growth rescue was due to metabolization of sorbitol.

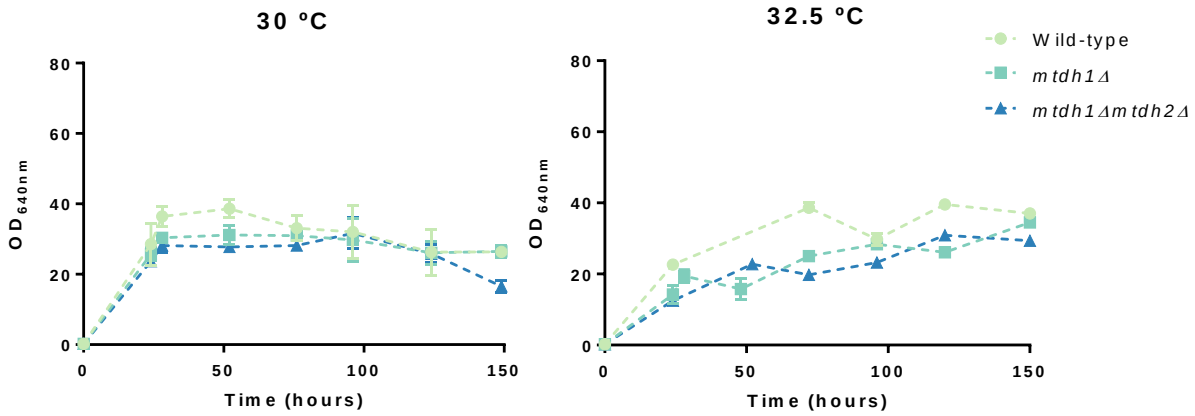


Figure 3.8 - Growth curves of wild-type and *MtDH* deletion mutants on YP supplemented with 1 %(w/v) glucose, 1 %(w/v) fructose and 18 % (w/v) sorbitol (2FG18S) at 30 ºC and 32.5 ºC. The experiments were performed in duplicate with two biological replicates.

St. bombicola has a gene encoding for a sorbitol dehydrogenase (*SOR1*). We observed that the *St. bombicola* deletion mutant *sor1Δ* does not grow with sorbitol as sole carbon source, suggesting that this is the enzyme involved in its metabolization (Figure 3.7).

Therefore, we subsequently used a double deletion mutant where *SOR1* and *MtDH1* were disrupted already available in the laboratory to test to which extent sorbitol metabolization contributed to the rescue of growth of the *MtDH* deletion mutants observed in Figure 3.8. To do that *mtdh1Δsor1Δ* was grown at 32.5 ºC in 2FG18S.

The results, shown in Figure 3.9 show that sorbitol metabolization is largely dispensable for growth rescue of the mutants. Therefore, exogenous sorbitol can compensate for the lack of the ability of these mutants to synthesize mannitol, which in turn can mean that the inability of the mutants to grow in the conditions shown in Figure 3.3 is not due mainly to the $NADP^+$ regenerating role of mannitol synthesis, but rather with the role of the compound itself in the cell.

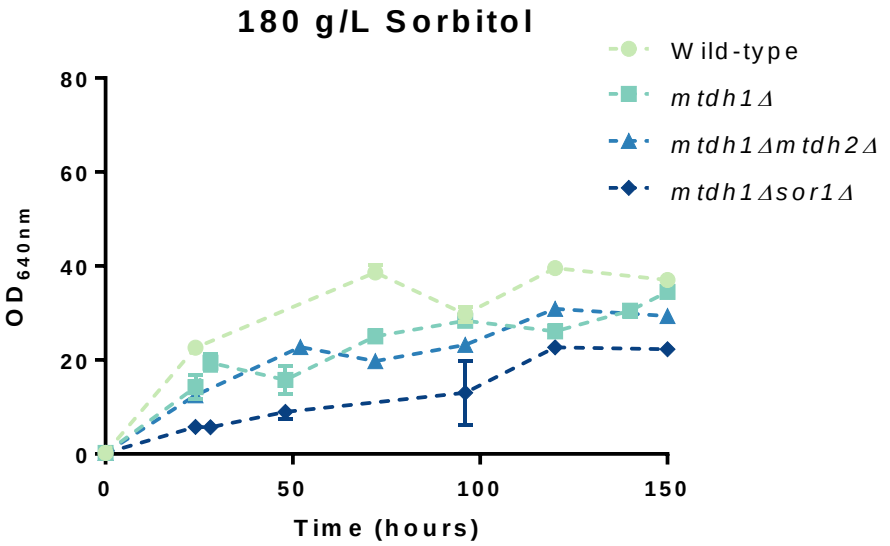


Figure 3.9 - Growth curves of wild-type and *MtDH* deletion mutants on YP supplemented with 1 % (w/v) glucose, 1% (w/v) fructose and 18 % (w/v) sorbitol at 32.5 ºC. The error bars represent the standard deviation of two biological replicates from two independent experiments.

Instead using of sorbitol in the medium, we decided to replace it for mannitol itself. For that, the ability to use mannitol as carbon source was tested. The wild-type and *MtDH* deletion mutants were grown on YP supplemented with 18 % (w/v) of mannitol. Until 150 h neither the wild-type nor the *MtDH* deletion mutants used it as carbon source. Also, during the growth assays mannitol consumption was never observed, suggesting that mannitol is not used as a carbon source.

We next included mannitol at identical concentrations (180 g/L) in the growth medium and found that it was equally largely capable of rescuing growth of the *MtDH* deletion mutants in 2FG/32.5 ºC (Figure 3.10). Interestingly, the *MtDH* deletion mutants reach higher cell densities in the presence of mannitol than sorbitol what suggests that sorbitol cannot replace totally the role of mannitol in the cell. Although, the addition of mannitol in the growth media is not enough to restore the ability of *MtDH* deletion mutants to reach up to the same cell densities as the wild-type, it is able to restore the ability of *MtDH* deletion mutants to grow. The same happens with sorbitol suggesting that addition of exogenous polyols can restore the ability of *MtDH* deletion mutants to grow in a low sugar medium at 32.5 ºC.

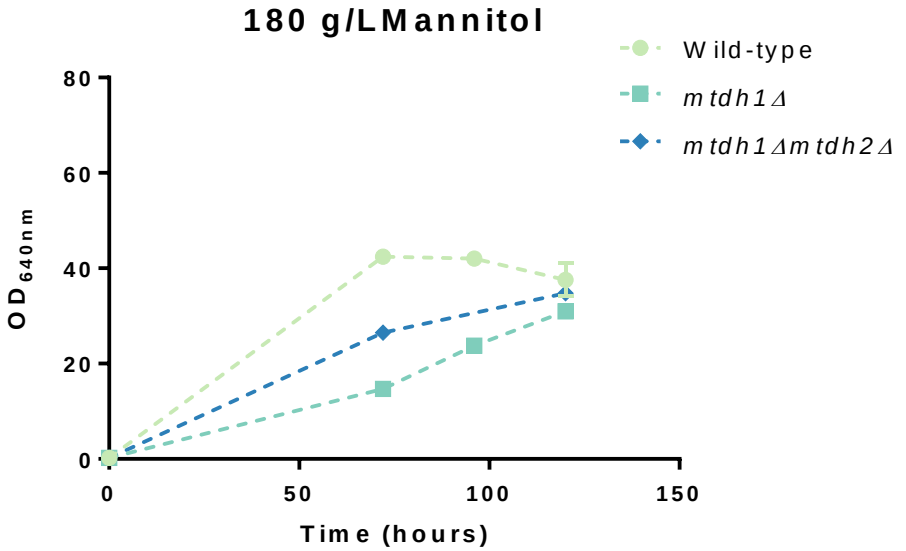


Figure 3.10 - Growth curves of wild-type and *MtDH* deletion mutants in YP supplemented with 1 % (w/v) fructose, 1 % (w/v) glucose and 18 % (w/v) mannitol at 32.5 ºC.

3.3 Mannitol seems to be a thermoprotector in *St. bombicola*

The results described in the previous section show that mannitol itself seems to be important for normal cell growth, although it remains likely that mannitol production also plays a role in cellular redox balance through the regeneration of $NADP^+$. In addition, the most striking phenotype observed in *MtDH* deletion mutants is the pronounced growth defect at higher temperatures, which might suggest that mannitol has a role as thermoprotector. If this is the case, intracellular mannitol concentrations would be expected to increase at higher temperatures.

To assess this, the wild-type was cultivated in a medium with high sugar concentrations (20FG) for 72 h at three different temperatures, namely 20 ºC, 25 ºC and 30 ºC and extracellular and intracellular levels of mannitol were measured. As shown in Figure 3.11, intracellular levels of mannitol increased at higher temperatures. This is consistent with a role of mannitol during the thermal stress in *St. bombicola*. Extracellular mannitol yield also increases sharply with temperature.

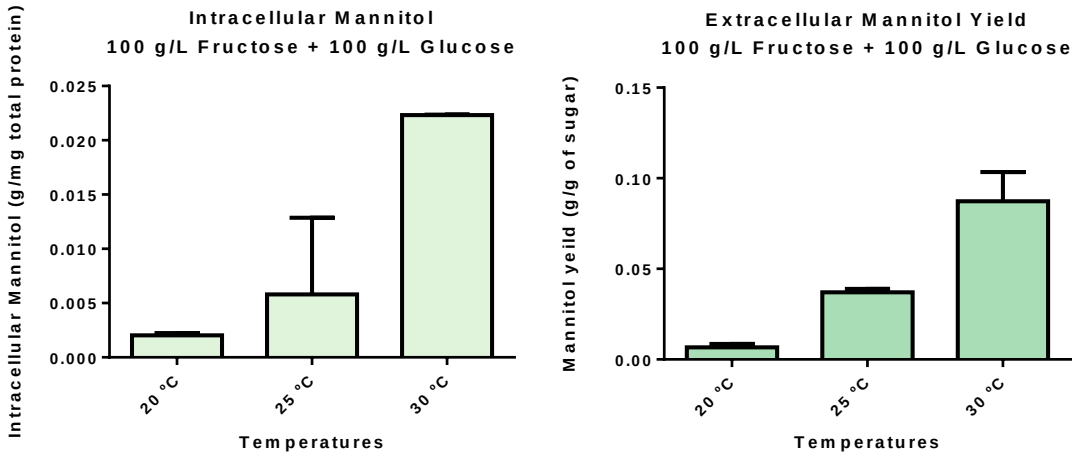


Figure 3.11 - Correlation between mannitol production in the wild-type and temperature. The cells were grown for 72 h at 20 ºC, 25 ºC and 30 ºC on YP supplemented with 100 g/L fructose and 100 g/L glucose (20FG). The error bars are the standard deviation resulted from two independent experiments (n=2).

To clarify if mannitol was the only metabolite produced by *St. bombicola* which increased at higher temperatures either intracellular or extracellular, the other metabolites produced were analysed (figure 3.12). At intracellular level, glycerol and erythritol were detected but not ethanol while, extracellularly glycerol and ethanol were detected but not erythritol. Intracellularly, at 30 ºC glycerol (0.0029 g/mg total protein) and erythritol (0.0007 g/mg total protein) have a much lower yield than mannitol (0.0223 g/mg total protein) in *St. bombicola*. However, it is possible to observe a slight increase at higher temperatures not so evident as in mannitol case. At extracellular level, glycerol levels are similar at all temperatures tested. Also, ethanol yield is very similar between the three temperatures. This is different from what was observed in extracellular mannitol yield.

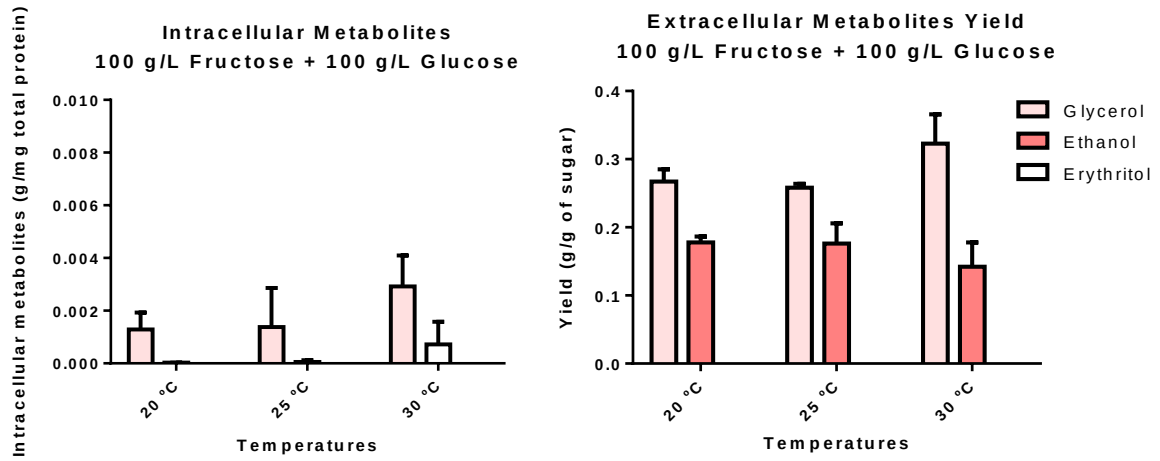


Figure 3.12 – Intracellular and extracellular levels of glycerol, ethanol and erythritol. The cells were grown for 72 h at 20 ºC, 25 ºC and 30 ºC on YP supplemented with 100 g/L fructose and 100 g/L glucose (20FG). The error bars represent the standard deviation resulting from two independent experiments (n=2).

4. Discussion

St. bombicola seems to be adapted to the flouriculous niche which is rich in sugars as fructose, glucose and sucrose. Also, in the last years, several studies gave us an insight of evolution of fructophily and the adaptation of this yeast to its natural environment, namely the loss and re-acquisition of ability to conduct alcoholic fermentation (Gonçalves *et al*., 2018). The most interesting aspect is its differences in the central carbon metabolism comparing to well-study species. Namely, the ability W/S clade species to produce mannitol directly from fructose could be an adaptative behavior to the floriculous environment.

In other organisms it was reported a role for mannitol as carbohydrate reserve since mannitol can be directly converted into fructose. As plants are not able to consume mannitol this could reveal an advantage for plant-fungal interactions (Patel & Williamson, 2016). However, it does not seem to be the case in *St. bombicola*. Although Mtdh showed activity in both directions (Gonçalves, 2018), when growth on mannitol was tested wild-type did not grow.

The *MtDH* deletion mutants do not reach the same cell densities as the wild-type. As previously described, one of the major functions of mannitol in cells is the protection against high osmolarity. However, in *St. bombicola* does not seem to be the case. The lower cell densities reached by *MtDH* deletion mutants do not seem to be due to a possible role as osmoprotector for several reasons. First, *MtDH* deletion mutants were grown in media with high osmotic pressure (20FG, 20Glu and 20Fru) where it was possible to observe lower cell densities than wild-type only at higher temperatures. Also, when we used polyols – sorbitol and mannitol – the medium had the same osmotic pressure of the previous media (20%) and interestingly, the *MtDH* deletion mutants reach similar cell densities as the wild-type. Moreover, when the *MtDH* deletion mutants were grown in low sugar concentrations – low osmotic pressure – the mutants could not even grow at the highest temperature tested suggesting that the lower cell densities are not due to the high osmotic pressure of the medium. This difficulty to grow in this condition could be related with some instability on the membrane that affects sugar transport. In *S. cerevisiae*, the hydroxyl groups of trehalose – a thermopotector - are able to interact and stabilize the membrane (Magalhães *et al*., 2018). Studies where simulations were performed showed that trehalose is able to minimize the disruptive effect of high temperatures in the membrane. During thermal stress, trehalose molecules interact with the membrane through hydrogen bonds stabilizing it (Pereira *et al*., 2004). In *MtDH* deletion mutants, growth is restored with addition of exogenous polyols – sorbitol and mannitol – in the growth media. As the metabolization of these polyols does not seem to be related with the growth rescue, it is reasonable to think that these polyols can possibly stabilize the membrane as trehalose and the cell can transport the sugar and grow. In 2006, it was suggested, with similar experiments done with trehalose, that maltose and glucose could also prevent the disruption of the bilayer due to thermal stress (Pereira & Hünenberger, 2006). This is very interesting, since *MtDH* deletion mutants are not able to grow at 32.5 ºC when the culture medium has low sugar content, while they can grow at the same temperature in high sugar amounts or with addition of polyols. This reinforces the possibility that at 32.5 ºC, that sugars and polyols could both stabilize the bilayer in *St. bombicola*,

namely it seems that absence of mannitol at 32.5 ºC in low sugar mediums is crucial to its inability to grow, suggesting that in these conditions, mannitol could be the main metabolite to stabilize the membrane, working as a protector against thermal stress. To clarify if in fact mannitol could be a thermoprotector in *St. bombicola*, the intracellular mannitol levels were analysed. The analysis suggests an increasing in mannitol content at higher temperatures. Moreover, the extracellular mannitol levels were also analysed at the same temperatures. Curiously, wild-type produces more mannitol per gram of sugar consumed at higher temperatures, this agrees with what was described about the role of trehalose in *S. cerevisiae* since it comprises trehalose inside and outside of the cell to guarantee full protection. This behavior is similar to what is observed in *St. bombicola*. Although intracellular levels increased at higher temperatures, the extracellular levels also increased. As previously suggested, probably mannitol could be stabilizing the membrane through hydrogen bonding. However, mannitol is not the only metabolite that increases intracellularly at higher temperatures, instead it was showed that glycerol and erythritol also increase with temperature however, their levels are lower comparing with mannitol levels and increase much less with temperature. In this way, all these three metabolites could, probably, interact with the bilayer, stabilizing it.

In addition to mannitol having a role in protection against thermal stress in *St. bombicola*, at higher temperatures the metabolism is faster and the production of fermentation products also increases. However, when other extracellular metabolites were quantified (glycerol and ethanol) their amounts are very similar at different temperatures, showing an opposite behavior when comparing with mannitol. The higher levels of extracellular mannitol indicate that is being excreted as a fermentation product, just like ethanol in *S. cerevisiae*. In the baker's yeast and in *St. bombicola*, when production of ethanol is deactivated, there is an increase in glycerol yield what could be related to the requirement for NAD^+ for glycolysis (Gonçalves *et al*., 2018; Smidt *et al.*, 2011). In this way, when metabolites produced by *MtDH* deletion mutants were analysed small increases of erythritol were detected. The first steps for erythritol production occur during the PPP with production of erythrose-4-phosphate that will be converted into erythrose. In turn, erythrose will be converted into erythritol trough erythrose reductase, which releases $NADP^+$ just like in mannitol synthesis. So far, this suggests that mannitol production could also have an important role in $NADP^+$ regeneration.

At higher temperatures, for example at 30 ºC, the metabolism is faster, the requirement for cofactors is higher. As referred before (section 1.1.2), PPP uses $NADP^+$, to regenerate reducing power to the cell (NADPH). Furthermore, the high activity of PPP has been reported in several yeasts, namely *Y. lipolytica* and *K. lactis*. Though this has not been investigated yet in *St. bombicola* it is possible that PPP is very active.

As previously referred (see section 3.1), *St. bombicola* produce mannitol through a NADPH-dependent reaction which releases $NADP^+$ that could be necessary for redox homeostasis, namely for the presumably very active PPP. Since at higher temperatures, in the absence of this metabolic pathway, *MtDH* deletion mutants could have a depletion of $NADP^+$ that could affect the PPP and consequently, reduce the biomass yield.

5. Conclusions and Future Perspectives

In the course of this work, we were able to give an insight of the role of mannitol. Initially, we were able to identify the role of mannitol as thermoprotector. The inabilities of *MtDH* deletion mutants to reach the same cell densities as the wild-type could not be due only to its role as thermoprotector but also due to a depletion of $NADP^+$ that could influence the function of PPP consequently, decreasing the biomass yield.

It would be exciting confirm if in fact the PPP is more active than the glycolysis in the wild-type. It would also be interesting quantify the ration NADPH/$NADP^+$ in the wild-type and *MtDH* deletion mutants to understand if in fact *MtDH* deletion mutants reach lower cell densities due to a depletion of $NADP^+$.

Also, it would be interesting to quantify the intracellular and extracellular levels in low sugar concentration at 20 ºC, 25 ºC and 30ºC in order to understand if accumulation of mannitol also responds to temperature under these conditions. Erythritol is also a polyol and is produced through a NADPH dependent pathway, and during this thesis it was observed that wild-type accumulates erythritol intracellularly and *MtDH* deletion mutants seem to release erythritol to the culture medium. Due to the common features of these polyols it would be interesting quantify the erythritol intracellular content in *MtDH* deletion and compare it with the wild-type.

6. References

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