Chapter 4
Molecular Mechanism of D-Xylitol Production in Yeasts: Focus on Molecular Transportation, Catabolic Sensing and Stress Response

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Abstract Xylitol is a naturally occurring non fermentable sugar alcohol. It can be produced by the microbial fermentation of xylose extracted from hemicellulose of lignocellulosic substrates like corn fiber, corn husk, sugarcane bagasse and birch wood. In last few decades, xylitol gained significant importance due to its applications in food and pharmaceutical industries. Sustainable production of xylitol from renewable sources is possible by fermentation process using xylose assimilating microbes. As chemical production of xylitol involves high temperature, pressure and expensive purification steps, highly efficient biotechnological production of xylitol using microorganisms is gaining more interest over chemical processes. For the economic production of xylitol, microorganisms with high osmotolerance, inhibitor resistance, fast conversion rates, and stress tolerance are required in the fermentation process. As xylose uptake might be a limiting factor for xylose fermentation, the study of xylose uptake with respect to xylose transporting proteins and improvement of utilization of sugar mixtures is necessary. This review is to provide an overall view of xylitol production by yeast strains under sugar, saline and different nutritive stress conditions. In addition this review emphasizes the role of molecular changes (genes) and pathways involved in the utilization and transport of sugars for increased xylitol production.

Keywords Xylitol · Stress response · Yeasts · Catabolic sensing · Molecular transport

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4.1 Introduction

Xylitol is a naturally occurring sweetener with one-third fewer calories than sucrose (Granström et al. 2007a). It is a high value polyalcohol produced by the reduction of D-xylose derived from the hemicellulose fraction of lignocelluloses. Xylitol can serve as a building block for organic synthesis and was listed as one of the top 12 value added materials produced from biomass (Werpy et al. 2004; Prakash et al. 2011). Xylitol is naturally found in fruits and vegetables like berries, oats, lettuce and cauliflower. It can be produced fermentatively from xylose extracted from the hemicellulosic fraction of lignocellulosic substrates like corn fiber, corn husk, sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switch grass, straws, stems, stalks, leaves, husks, shells and peels from fruits and cereals like rice, wheat, corn, sorghum and barley (Prakasham et al. 2009; Weber et al. 2010; Xu et al. 2011). The hemicellulosic fraction of lignocellulosic substrates is hydrolyzed into simple monomeric sugars (hexoses and pentoses) by chemical or enzymatic methods. During the hemicellulose hydrolysis by chemical methods, a number of sugars, mainly xylose, is derived in addition to various inhibitors (Chandel et al. 2010, 2011) which need to be eliminated prior to the fermentation process of xylitol production.

Industrial production of most sugar alcohols is performed by chemical hydrogenation of xylose in the presence of a metal catalyst, Raney nickel, with hydrogen gas at high temperature and pressure (Granström et al. 2007a) for which highly pure sugar substrates and costly chromatographic purification steps are required. In contrast, fermentative production of xylitol by microorganisms is becoming a more attractive sustainable alternative (Saha 2003; Lin et al. 2010) as it does not require pure xylose syrup at the starting point of the fermentation process, as the chemical synthesis does. This could be of high economical interest since low-cost hemicellulosic hydrolysates may become potential substrates (Milessi et al. 2011). Biotechnological production of xylitol is potentially attractive as it occurs under much milder process conditions with low purification costs, and it is relatively easy and environmentally safe (Rodrigues et al. 2011).

In the last two decades, bacteria, fungi and yeasts capable of producing xylitol have been identified (Table 4.1) (Cheng et al. 2010). Among the microorganisms, yeasts are considered to be the better xylitol producers (Singh and Mishra 1995; Guo et al. 2006). Some well-known xylitol-producing yeasts are *Candida guilliermondii*, *Debaromyces hansenii*, *Candida tropicalis*, *Candida boidinii*, *Pachysolen tannophilus* and *Pichia stipitis* (Prakasham et al. 2009; Chandel et al. 2011). Recent studies have indicated that insect guts harbor a vast diversity of yeasts belonging to genera *Arxula, Candida, Pichia, Cryptococcus, Debaryomyces*, *Dipodascus,* and *Endomyces*. Some of these insect gut yeast strains, such as *Spathospora passalidarum, C. jeffrisii,* and *P. stipitis* are known for xylitol production. Yeasts such as *C. tropicalis, Candida mogii, P. stipitis, Candida guillermondii* and *D. hansenii* are known to be good xylitol producers under oxygen-limited conditions (Granström et al. 2007b; Ko et al. 2008). Xylose can be converted to xylitol by recombinant

Table 4.1 Microorganisms screened for xylitol production

Yeast	Fungi	Bacteria
Petromyces albertensis	*Aspergillus niger*	*Corynebacterium sp.*
Candida boidinii	*Penicillium brevicompactum*	*Enterobacter liquifaciens*
C. maltosa	*P. citrinum*	*Mycobacterium smegmatis*
C. mogii	*P. expansum*	
C. tropicalis HXP2	*P. griseoroseum*	
Debaromyces hansenii UFV-170	*P. roqueforti*	
Hansenula polymorpha	*P. purpurogenum*	
Pachysolen tannophilus	*P. janthinellum*	
Pichia caribica	*P. chrysogenum*	
C. guilliermondii FTI-20037118	*P. italicum*	
C. intermedia	*P. crustosum*	

Sources Chen et al. (2010), Sampio et al. (2003), Ghindea et al. (2010)

microbes like *Escherichia coli*, *Bacillus*, *Saccharomyces cerevisiae* and also *Candida* (Jeon et al. 2011).

Xylitol is known to be metabolized through insulin-independent pathways in the human body; hence, it can be used as a sugar substitute for diabetics (Arrizon et al. 2011). It is used in foods like chewing gum, bakery products and chocolate as a sweetener with anticariogenic properties. It is also used in medicines as a sugar substitute for people with erythrocytic glucose-6-phosphate dehydrogenase deficiency (Kinterinwa et al. 2008). In rats, xylitol has been found to increase the activity of neutrophils, the white blood cells involved in fighting many bacteria (Renko et al. 2008). Xylitol has also shown potential to be used for the treatment of osteoporosis. As xylitol is highly beneficial to health, it has gained significant market demands corresponding to the current annual global value of $340 million per year and priced at $4–5 kg^{-1} (Kadam et al. 2008; Prakasham et al. 2009) with an annual commercial production of 40,000 tons (Granström et al. 2007a).

4.2 Metabolism of D-Xylose in Yeast

In the majority of yeasts and fungi, the conversion of D-xylose to D-xylulose needs two steps, a reduction step followed by an oxidation step. In yeasts and fungi, D-xylose is first reduced to D-xylitol by either NADH or NADPH-dependent xylose reductase (aldose reductase EC 1.1.1.21) (XR); the resulting D-xylitol was either secreted or further oxidized to D-xylulose by NAD or NADP-dependent xylitol dehydrogenase (EC 1.1.1.9) (XDH) (Chen et al. 2010). These two reactions were considered to be the rate-limiting steps in D-xylose fermentation and D-xylitol production. Some strains of yeast could metabolize D-xylulose to xylulose-5-phosphate by xylulokinase (EC 2.7.1.17) (XK). Xylulose-5-phosphate can subsequently enter the pentose phosphate pathway (Lachke and Jeffries 1986; Chen et al. 2010). This pathway consists of an oxidative phase that converts hexose

phosphates to pentose phosphates providing NADPH needed in biosynthetic pathways and a non-oxidative phase in which the pentose phosphates are converted into hexose and triose phosphates (Jeffries 1983). The pentose phosphate pathway also yields ribose-5-phosphate used for the synthesis of nucleic acids and histidine and of erythrose-4-phosphate which are necessary for the synthesis of aromatic amino acids. The phosphoketolase pathway presents an alternative route for the utilization of xylulose-5-phosphate (Prior et al. 1989) by conversion of xylulose-5-phosphate into glyceraldehyde-3-phosphate and acetyl phosphate. The process of xylitol formation cannot be stopped after the first step, when D-xylose is converted to xylitol. Cell growth depends on some of the above metabolic products and it is also necessary that the cofactors have to be regenerated through different steps in the metabolic pathway. Therefore, to obtain good yields of xylitol, the amount of xylose being converted to xylitol and the amount of xylitol which is available for further metabolism have to be well balanced.

4.3 Co-Enzyme Specificity

The first two enzymes, D-xylose reductase and xylitol dehydrogenase are the key enzymes in xylitol production by yeasts. They both require pyridine nucleotide cofactors exhibiting different cofactor specificity in different yeasts. Under anaerobic or oxygen-limited conditions, the difference in the cofactor requirements of these enzymes causes a redox imbalance which influences xylitol accumulation in yeasts (Bruinenberg et al. 1984). Xylitol formation is favored under oxygen-limited conditions, because of the NADH accumulation and subsequent inhibition of NAD-linked xylitol dehydrogenase. This phenomenon, known as the Custer effect, results from the incapability of the yeasts to compensate for excess NADH as they have no transhydrogenase activity (Van dijken and Scheffers 1986).

The variation in the ratio of NADH- to NADPH-linked D-xylose reductase activity with aeration conditions was first found in *P. tannophilus*; this infers that there was more than one form of n-xylose reductase in the yeast, which was further confirmed by Verdiyn et al. (1985). The same variations were also seen in the yeasts *Candida parapsilosis* ATCC 28474 and *C. boidinii* NRRL Y-17213. Under oxygen limitation, in contrast to all other D-xylose-fermenting and xylitol-producing yeasts, *C. boidinii* exhibits a NADH/NADPH ratio higher than 1 (Table 4.2). Referring to this, Vongsuvanlert and Tani (1988) found that in *C. boidinii* xylitol could be formed by two metabolic pathways: (1) D-xylose is directly reduced to xylitol and (2) D-xylose is initially isomerized by D-xylose isomerase to D-xylulose that is subsequently reduced to xylitol. In both reductions, NADPH was also active as a reductant but with less efficiency.

In most yeast cell-free extracts, D-xylose reductase has a higher preference for NADPH (Table 4.2). Though xylose reductase of *C. parapsilosis* ATCC 28474, *Candida shehatae* ATCC 22984, C. *shehatae* CBS 5813 and *P. stipitis* CBS 5773 is capable of using both NADPH and NADH, its affinity towards NADPH is very

Table 4.2 Enzyme activity of D-xylose reductase and xylitol dehydrogenase in various yeast with D-xylose as a substrate

Specific activity (U/g protein)				
Microorganism	D-Xylose reductase		Xylitol dehydrogenase	
	NADPH	NADH	NAD	NADP
C. boidinii (*Kloeckera* sp.) no. 2201	0.055	0.288	0.272	0.096
C. boidinii NRRL Y-17213	0.019	0.112	0.060	0.003
C. guilliermondii NRC 5578	0.521	0.050	n.r	n.r
C. guilliermondii NRC 5578	1.191	n.d	n.r	n.r
C.mogii ATCC 18364	0.160	0.060	0.220	n.r
C. parapsilosis ATCC 28474	0.416	0.161	n.r	n.r
C.shehatae ATCC 22984	0.333	0.100	0.240	n.r
C. shehatae CBS 5813	0.480	0.210	n.r	n.r
C. tropicalis IFO 0618	10.640	1.720	20.160	0.120
C.utilis CBS 621	0.075	n.d	0.280	n.d
Debaryomyces hansenii DTIA-77	0.091	n.r	0.047	n.r
P. tannophilus CBS 4044	0.220	0.009	0.910	0.070
P. tannophilus NRRL Y-2460	0.033	0.008	0.049	n.d
P. tannophilus U-U-27 (mutant)	0.173	0.118	0.160	n.d
P. stipitis CBS 5773	0.600	0.310	0.720	0.075

Source Winkelhausen and Kuzmanova (1998)
n.d not detected, *n.r.* not relevant

high. The lower NADH/NADPH ratio in *C. guilliermondii* NRC 5578 (0.1) compared to that in *C. parapsilosis* ATCC 28474 (0.4) explains the higher D-xylose reductase activity in *C. guilliermondii.*

4.4 Osmotic Stress in Yeast

Osmo-regulation is a basic biological process which controls cellular water content and turgor pressure. Accumulation of compatible solutes is a well conserved strategy in osmoregulation, though the solute accumulated differs between organisms (Yancey et al. 1982). The yeast *S. cerevisiae* employs glycerol, whose production and transmembrane flux are tightly controlled by osmotic changes (Hohmann 2002). Although the molecular changes and pathways involved in the response to saline or sorbitol stress are widely understood, less is known about how cells respond to high sugar concentrations (Hohmann and Mager 2003). Glucose (or catabolite) repression has been identified in many microorganisms, including budding yeast (Gancedo 2008; Santangelo 2006). According to this phenomenon, the transcription of genes whose products are essential for the catabolism of slowly fermentable or completely non fermentable carbon sources is repressed in the presence of a rapidly fermentable carbon source, such as glucose. This occurs with the genes involved in respiration (Krebs cycle and electron

transport chain), gluconeogenesis, the glyoxylate cycle, and utilization of alternative carbon sources such as galactose. The main glucose repression pathway involves the Snf1p kinase complex (Carlson et al. 1981) which, under glucose limitation conditions, inactivates Mig1p, thus preventing its interaction with co-repressor Cyc8p-Tup1p. When an excess of glucose is present, the Snf1p kinase is inactive (McCartney and Schmidt 2001) thus non phosphorylated Mig1p located in the nucleus exerts its repressing effects (Kaniak et al. 2004; Papamichos-Chronakis et al. 2004). The MAPK HOG (high osmolarity glycerol) pathway is the most prominent signaling pathway that allows *S. cerevisiae* cells to quickly respond to altered osmolarity (Capaldi et al. 2008; Jiménez-Martí et al. 2011). The activity of the HOG pathway can be known by immunological analysis of the level of phosphorylated Hog1 mitogen- activated protein kinase or by the mRNA level of target genes such as GRE2. Using such markers of HOG pathway activity, it has been found that a hyperosmotic shock leads to transient activation of the pathway. The HOG pathway controls glycerol production at a minimum of two levels. First, Hog1 activates the enzyme phosphofructo-2-kinase, leading to stimulation of glycolytic flux and enhanced glycerol production (Dihazi et al. 2004). In addition, Hog1 mediates enhanced expression of the genes GPD1 and GPP2 (Albertyn et al. 1994; Norbeck et al. 1996; Hirayama et al. 1995) and hence increased capacity to produce glycerol.

Under hyperosmotic stress conditions, Pbs2p phosphorylates Hog1p in the cytosol promotes its rapid nuclear accumulation (Ferrigno et al. 1998; Reiser et al. 1999), which is followed by an increase in the mRNA levels of osmo-induced genes (Posas et al. 2000; Rep et al. 1999a, b, 2000).

When 2 % (w/v) glucose was added to cells growing in raffinose, Hog1 phosphorylation was induced to the same extent as when treated with 110 mM NaCl. Further increase in the concentration of glucose led to the higher levels of Hog1p phosphorylation (Tomás-Cobos et al. 2004). Even though there is enough glucose available for growth, the cell has to counteract with the osmotic stress when exposed to high glucose concentration. Several reports have focused on the sugar stress condition by conducting global or particular transcriptomic analyses. In a microarray analysis of cells from the PSY316 strain exposed to 20 % w/v glucose (Kaeberlein et al. 2002), an up-regulation of glycerol and trehalose biosynthetic genes was found. Erasmus et al. (2003) reported the response of wine yeast Vin13 to 40 % w/v sugars compared to 22 % w/v sugars in Riesling grape juice. According to their studies, high sugar stress up-regulated the genes of the glycolytic and the pentose phosphate pathway and those involved in the formation of acetic acid from acetaldehyde, while genes involved in de novo biosynthesis of purines, pyrimidines, histidine and lysine were down-regulated. Proteomic characterization of *S. cerevisiae* response to high glucose concentrations has revealed that most of the proteins involved in glycolysis and pentose phosphate pathways are up-regulated under these conditions (Pham and Wright 2008). In a recent study by Guidi et al. (2010) when *S. cerevisiae* was exposed to 20 % w/v glucose, peroxiredoxin, a protein involved in protection against oxidative stress was found to be down regulated. Proteomic analysis of the mutant deletion strain

(Δyhr087w), YHR087W (RTC3) gene and its encoded protein reveals lower levels of several yeast Hsp proteins, which establishes a link between this protein and the response to several forms of stress. The relevance of YHR087W for the response to high sugar and other stress conditions and the relationship of the encoded protein with several Hsp proteins suggest applications of this gene in biotechnological processes in which response to stress is important (Jiménez-Martí et al. 2011).

Osmotic stress triggers a set of cellular responses that enables the yeast to adapt to the changes in the environment. For example, when ascomycetous yeasts are exposed to osmotic stress, polyols such as glycerol accumulate in the cytoplasm to counteract cell dehydration (Van Eck et al. 1993; Albertyn et al. 1994). The compounds that yeast cells produce and accumulate are also known as compatible solutes or osmolytes and their function is to increase the internal osmolarity after hyperosmotic shock. The cell regulates the concentration of intracellular osmolytes by either metabolic processes or by activating membrane transporters by controlling the inflow or the efflux of solutes and ions together with changes in the cell volume (Kayingo et al. 2001; Tamas and Hohmann 2003).

There are a number of yeast genera that survive in high sugar environments (Tokuoka 1993). These yeasts are termed osmophilic or osmotolerant and almost all species studied belong to the ascomycetous group (Tokuoka 1993; Van Eck et al. 1993). Basidiomycetous yeasts are mainly salt tolerant. In contrast, ascomycetous yeasts were more glucose tolerant than salt tolerant (Van Eck et al. 1993; Tekolo et al. 2010). Basidiomycetous yeasts accumulated glycerol, mannitol and arabitol intracellularly when exposed to $0.96a_w$. These polyols are the main solutes accumulated in other yeasts when subjected to osmotic stress (Spencer and Spencer 1978). Hounsa et al. (1998) found that trehalose is important for the survival of *S. cerevisiae*. When genes involved in trehalose synthesis were deleted, it was found that the survival of this mutant strain was reduced when grown in the presence of 3M NaCl as compared with wild-type strain. However, hyper accumulation of trehalose did not improve the survival rates. Furthermore, the work of Hounsa et al. (1998) indicated that trehalose does not act at any stage as a reserve for glycerol synthesis in *S. cerevisiae* when exposed to osmotic stress. The increase in intracellular sodium concentrations and decrease in potassium concentrations when grown in the presence of NaCl suggest that sodium might replace potassium as an intracellular cation under these conditions and may even be accumulated as was reported previously for the salt-tolerant ascomycetes *D. hansenii* (Prista et al. 1997; Gonzalez-Hernandez et al. 2004).

Due to the versatility of *S. cerevisiae,* it is used to study different stress responses, such as heat shock, osmotic and oxidative stresses, nutrient starvation, etc. (Mager and Ferreira 1993; Serrano 1996). In *S. cerevisiae* the existence of at least four different DNA elements that respond to stress signals and participate in the transcriptional regulation of different genes has been found. Among the best characterized is the heat shock element, which interacts with heat shock transcription factor (Parsell and Lindquist 1994). Other elements participate in the response to multiple stress conditions, such as the post-diauxic shift (PDS) element

that mediates gene activation during diauxic growth and in the stationary phase, and under heat shock stress (Boorstein and Craig 1990a, b; Werner-Washburne et al. 1993). The stress response element (STRE) contains a sequence that is activated by various stress conditions, such as nitrogen starvation, osmotic and oxidative stress, low external pH, weak organic acids, etc. (Belazzi et al. 1991; Marchler et al. 1993; Ruis and Schuller 1995). Although the transcription factor binding to STRE has not yet been identified, recent findings show that the transcription activators Msn2p and Msn4p are not only required for a high level of expression of genes activated via STRE, but also bind specifically to these elements in vitro (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). Finally, the AP-1 responsive elements have also been identified in the promoter region of genes typically involved in the response to oxidative stress (Gounalaki and Thireos 1994; Ruis and Schuller 1995).

One of the first responses, in organisms living under hyperosmotic conditions, was the synthesis of osmolytes or compatible solutes (Yancey et al. 1982). In *S. cerevisiae,* glycerol is the major osmolyte synthesized under hyperosmotic stress (Blomberg and Adler 1989). The enzyme responsible for the production of this osmolyte is glycerol-3-phosphate dehydrogenase, NAD+ (GPD). The GPD1 gene is induced under osmotic stress and the lack of its product renders the yeast osmosensitive (Albertyn et al. 1994). A second gene encoding a GPD isozyme, GPD2, has been described as being important for glycerol production, although it is not induced by osmotic stress conditions (Ansell et al. 1997).

In *S. cerevisiae*, glycerol is produced by the signal transduction pathway (Maeda et al. 1994). This transduction pathway consists of a MAP kinase cascade (MAPKKK, Ssk2/22p; MAPKK, Pbs2p; MAPK, Hog1p) (Brewster et al. 1993; Maeda et al. 1994) that results in the induction of several genes, including GPD1 (Albertyn et al. 1994; Schuffler et al. 1994). The kinase cascade is activated by the product of two different genes that encode osmosensors on the membrane of yeast cells: SLN1, which forms the two-component system with YPD1–SSK1 and SHO1, which acts on PBS2 through STE11 (Maeda et al. 1994, 1995; Posas et al. 1996; Posas and Saito 1997). This regulatory system is known as the high osmolarity glycerol (HOG) pathway as it was initially characterized as the regulatory mechanism for glycerol production during yeast growth in a high osmolarity medium (Brewster et al. 1993).

As in other yeasts, active glycerol uptake from the environment is observed (Lages et al. 1999), but does not contribute to osmoadaptation in *S. cerevisiae* (Holst et al. 2000). Rather, intracellular glycerol levels are controlled by passive glycerol export, which is mediated by Fps1 (Hohmann 2002; Luyten et al. 1995; Oliveir et al. 2003). Upon a hyperosmotic shock the transport capacity of Fps1 is rapidly diminished to ensure that glycerol is maintained inside the cell (Hohmann 2002; Luyten et al. 1995). A specific domain within the N-terminal extension of Fps1 is needed to control glycerol transport and deletion of this domain which makes Fps1 hyperactive (Tamas et al. 1999, 2003). Yeast cells that express this hyperactive Fps1, fail to retain glycerol and hence are sensitive to high external osmolarity. On hypo-osmotic shock Fps1 rapidly releases glycerol to prevent

excessive cell swelling. Therefore, mutants lacking Fps1 are sensitive to hypo-osmotic shock (Tamas et al. 1999). Fps1 is a member of the aquaglyceroporin subgroup of MIP channel proteins (Hohmann et al. 2001) and hence can mediate passive glycerol flux in both directions.

4.5 Salt Stress

Micro-organisms, like many types of yeast (Tokuoka 1993) that can tolerate high saline environments, develop systems to counteract the deleterious effects of the two components of salt stress: (1) intracellular ion toxicity and (2) osmotic stress (turgor and water loss). In yeast, defense responses to salt stress are based on osmotic adjustment by osmolyte synthesis and cation transport systems for sodium exclusion. Polyols, and especially glycerol, are the major osmolytes produced by yeast (Blomberg and Adler 1992, 1993). Both a Na+-ATPase and an H+/Na+ anti-porter have been used for sodium extrusion.

To determine maximum tolerance to salt stress, a study was performed on 42 different yeast species and found that *C. halophila* CBS 4019 proved to be the only yeast able to grow on glucose in the presence of a maximum of 4.5 and 5 M NaCl, respectively, without and with inoculum pre-adaptation to salt. Further, in comparison to other very salt-tolerant yeasts, it has shown considerably less reduction in cell volume as a response to sudden salt shock (Lages et al. 1999). Like other halophilic micro-organisms, *C. halophila* and *D. hansenii* grow better in the presence of a certain amount of salt than in its absence (Prista et al. 1997). Unlike halophillic bacteria, neither *C. halophila* nor *D. hansenii* actually depend on the presence of salt to survive.

To exhibit salt stress response, *C. halophila* either (1) produces a higher amount of osmolyte than other less tolerant yeasts (Blomberg and Adler 1989), (2) possesses efficient ion extrusion transporters or pumps (Ramos 1999) and has active transport systems able to cope efficiently with osmolyte retention needs under stress, i.e., when cells should be demonstrating increasing difficulty in maintaining a membrane potential favorable for active nutrient transporters, (3) possesses channels able to regulate osmolyte retention in response to sudden changes in the environment (Tamas et al. 1999) or (4) has a membrane lipid composition that does not allow leakage of osmolytes or any other lipid-soluble compounds, thus maintaining integrity of the intracellular environment (Yoshikawa et al. 1995). Finally, the control of metabolism through an efficient redox regulation could also contribute to such a high tolerance.

When *C. tropicalis* and *S. cerevisiae* were grown in the absence and presence of 1 M NaCl, *C. tropicalis* was able to grow much faster than *S. cerevisiae* due to its respiratory metabolism (Gancedo and Serrano 1989). In the absence of NaCl the growth rate of *C. tropicalis* was 0.43 h^{-1}, whereas that of *S. cerevisiae* was 0–26 h^{-1}. In the presence of 1 M NaCl, *C. tropicalis* showed a 30 % reduction in growth rate while in the case of *S. cerevisiae* the reduction of growth rate was

much greater, i.e., a 70 % decrease and also the lag phase was increased (to 40 h) in the presence of 1 M NaCl. These results suggest that *C. tropicalis* grows better than *S. cerevisiae* at high NaCl concentrations, not only because of its more vigorous respiratory metabolism but also because of its higher salt tolerance.

When sensitivities of both yeast species to different chloride salts (NaCl, KCl, LiCl) and to sorbitol were studied, all these solutes produced an increase in both the generation time and the lag phase of yeast cultures. It was found that lithium is much more toxic than sodium while KCl is not toxic and only poses osmotic stress similar to sorbitol. KCl was the least inhibitory solute for both *S. cerevisiae* and *C. tropicalis.* Taking KCl as a reference, sorbitol was strongly inhibitory for *C. tropicalis* but much better tolerated by S. *cerevisiae*. NaCl and LiCl were much better tolerated by *C. tropicalis* than by *S. cerevisiae.* The above results indicate that these yeast species differ in their relative sensitivities to osmotic stress (sorbitol) and cation toxicity (sodium and lithium). The low relative toxicity of KCl indicates that chloride posses no toxicity problem for either yeast species. The fact that KCl is much less inhibitory for *C. tropicalis* than sorbitol could be explained if K+ uptake could be utilized for osmotic adjustment in this species. The difference between KCl and sorbitol toxicities is much less apparent in *S. cerevisiae*.

4.6 Dehydration Stress

The osmotolerant yeast *D. hansenii* is highly resistant to dehydration stress and this tolerance was higher for cells taken from the exponential growth phase than from the stationary phase. Growth of *D. hansenii* in medium containing 10 % (w/v) NaCl resulted in an additional increase in cellular resistance to dehydration, which was most prominent for stationary phase cells. Mechanisms of yeast cell responses to changes in external osmolarity were found to have similarities with exposure to dehydration–rehydration. Such similarities are particularly evident during the early stages of the response to these stress treatments. There are important species and even strain-dependent differences in stress response and stress tolerance. Since such differences might prove useful in revealing new mechanisms of tolerance, comparison was made between the sensitivity to dehydration–rehydration of cells of the extremely osmotolerant yeast *D. hansenii* with that of *S. cerevisiae*.

When the viability of dehydrated cells of *D. hansenii* were grown in basal medium and the same medium containing 10 % NaCl, it is found that growth at high salinity improves dehydration tolerance. This fact is applicable to both log phase cells and stationary phase cells, although the effect of NaCl addition was only marginal for cells taken from the exponential phase. The above results indicate that intracellular glycerol accumulation does not play any role in the tolerance to dehydration stress by *D. hansenii*. Exponential cells that accumulate intracellular glycerol show little improvement in stress tolerance to high levels of salinity (Adler and Gustafsson 1980; Larsson et al. 1990), whereas stationary

phase cells lack glycerol but instead accumulate high concentrations of arabinitol and showed improvement in dehydration resistance upon exposure to saline growth conditions. The arabinitol content of *D. hansenii* cultured in basal medium is higher in mid-exponential cells than in stationary phase cells (Adler and Gustafsson 1980) which seems to indicate that arabinitol, but not glycerol, might have a protective function under dehydration conditions. Further investigation of anhydrobiosis of *D. hansenii* might reveal new effective mechanisms by which eukaryotic cells can maintain viability under strongly unfavorable dehydration conditions, specifically mechanisms for dehydration resistance of exponentially growing cells as well as of their capability of tolerating extreme dehydration. It is well known that the characteristic features of yeasts growing in exponential growth phase have enhanced content of nucleic acids and proteins; this could be one of the main reasons which would determine the high sensitivity of the yeast cell to dehydration (Beker and Rapoport 1987). This hypothesis was substantiated by the idea that for the maintenance of the conformation and integrity of these molecules it would be necessary to retain high quantities of the bound water. This means that on dehydration down to relative humidity of about 8–10 %, cells loose much of their bound water, which finally results in irreversible changes in the structure and conformation of membranes and macromolecules.

In the experiments described by Khroustalyova et al. (2001), it was possible to reach not only the humidity level required for the anhydrobiotic state of yeast (that is, about 8–10 %) but to go significantly below it (down to about 5 %), without experiencing a decrease in the viability of the population of dehydrated cells. The above phenomenon can be explained in two ways: (1) the existence of protective molecules in the dehydrated cells which are able to substitute for the water released during drying (Beker and Rapoport 1987; Blomberg and Adler 1992) and (2) maintenance of the stability of the molecular organization of intracellular membranes which usually determines cell sensitivity to dehydration (Crowe et al. 1989; Hoekstra et al. 1992). The most well known protective substance is trehalose which could be synthesized in yeast cells in the stationary phase of growth (Panek 1995). It is known that trehalose could significantly increase the resistance of cells to dehydration–rehydration (Leslie et al. 1994) as well as to stabilize such biological molecules as proteins. Furthermore, investigations of cell resistance mechanisms to dehydration– rehydration stress should also include other protective compounds such as polyols (Krallish et al. 1997). Therefore, further investigations should be carried out by studying protective substances and high stability of the molecular organization of the yeast's intracellular membranes.

Results from the work of Khroustalyova et al. (2001) indicate that further significant progress in investigations of anhydrobiosis mechanisms have to be linked with a 'return' to a more ecological approach. It is now necessary to investigate in detail those yeasts which exist in nature in conditions like high salinity desert conditions leading at times to transfer to the state of anhydrobiosis. There are several alternative directions for this research; one of these involves construction of dehydration resistant strains by genetic approaches.

4.7 Phosphate Limitation Stress

Phosphate is known to play an important role in yeast metabolism, especially for enzyme activation or repression and for biosynthesis of membrane phospholipids (Teixeira de Mattos and Neijssel 1997; Jones and Gadd 1990) which could lead to alterations in membrane permeability thus allowing xylitol freely to cross the membrane. On the other hand, kinase activation/repression, such as the xylulokinase which catalyses the step of xylulose phosphorylation might play a role in xylitol accumulation under phosphate-limited growth. It was found that for xylose and potassium limitation, fully oxidative metabolism occurred leading to the production of biomass and CO_2 as the only metabolic products. However, potassium-limiting cultivation was the most severe nutritional stress of all tested, showing highest xylose and O_2 specific consumption rates along with the lowest biomass yield of 0.22 g g^{-1} xylose. Carbon was mainly assumed to meet the cellular energy requirements for potassium uptake. For the other limiting nutritional conditions, increasing amounts of extracellular xylitol were found for ammonium, phosphate and oxygen limitation. Although xylitol excretion is not significant for ammonium limitation, but where as for phosphate limitation the xylitol productivity reached 0.10 gl^{-1} h^{-1} about half of that found under oxygen-limiting conditions, 0.21 g gl^{-1} h^{-1}. It is the first evidence that xylitol production by *D. hansenii* might not only be a consequence of a redox imbalance attained under semi-aerobic conditions, but additional physiological mechanisms especially phosphate limitation also must have been involved. Cell yields changed drastically as a function of the limiting nutrient, being 0.22, 0.29, and 0.39 g $g^3$1 xylose for potassium, oxygen and phosphate limitation, respectively, and are a good indicator of the severity of nutritive stress (Tavares et al. 1999).

When effect of different nutritional stress on the growth and metabolic rates of *D. hansenii* grown at constant dilution rate (D) = 0.055 h^{-1}, for different growth-limiting essential nutrients were studied, Surprisingly it was found that carbon-, ammonia- and phosphate-limited cultures led to similar oxygen consumption, xylose consumption and carbon dioxide production rates, indicating that the dilution rate of 0.055 h^{-1}, did not provoke a severe growth stress. The cell yields of carbon- and ammonium-limited continuous cultivation was of the same magnitude, while the phosphate-limited culture was slightly lower. Whereas, oxygen- and potassium-limited chemostat cultivation was found to provoke drastic changes in metabolic rates leading to severe growth stress, with cell yields of 0.29 and 0.22 g g^{-1}, respectively. Moreover, potassium limited chemostat cultivation showed the highest oxygen consumption and CO_2 production rates, being more than three times higher than for carbon-limited cultivation (Tavares et al. 1999).

When *D. hansenii* was grown under both oxygen and phosphate limitation, xylitol was excreted into the culture medium. However, the xylitol yield was two times higher for oxygen-limited growth than for phosphate-limited growth, 0.43 and 0.18 g g^{-1}, respectively. Under oxygen-limited conditions, xylitol accumulation is assumed to occur due to the NADH surplus generated in the second

enzymatic step of xylose metabolism that cannot be reoxidised by the first enzymatic reaction.

The xylitol overproduction by *D. hansenii* under oxygen-sufficient cultivation (phosphate-limited chemostat) is the first evidence that xylitol production by yeasts might not be only a consequence of the intracellular redox unbalance due to NADH surplus under low respiratory capacity because this does not occur for the other oxygen-sufficient *D. hansenii* chemostats. This indicates that other regulatory mechanisms associated with xylitol overproduction are related to low-phosphate growth conditions.

4.8 Sugar Transporters in Efficient Utilization of Mixed Sugar Substrates

Among a variety of issues on utilization of sugar mixtures by the microorganisms, recent studies have started to reveal the importance of sugar transporters in microbial fermentation performance. Since most microorganisms cannot effectively utilize raw cellulose or hemicellulose because of their inability to biodegradation, biological production of fuels and chemicals from lignocellulosic biomass need depolymerization of lignocelluloses by enzymatic or chemical hydrolysis in a saccharification process. The resultant monomeric sugars can be converted to desired product by microbial action. The ability of a host organism to efficiently uptake the saccharified sugars greatly impacts microbial efficiency, whereas transporters for glucose derived from the cellulosic fraction are common among most microbial species. Hexose (e.g. mannose and galactose) and pentose (e.g. xylose and arabinose) that are derived mainly from the hemicellulosic fraction are much less common. Hexose and pentose transport systems in yeasts work by an energy-independent and facilitated diffusion mechanism. Hexose (Hxt) and galactose (Gal2) transporters naturally exhibit different affinities and specificities for their substrates. They are abundant in *S. cerevisiae* but less in other industrial important yeast strains including *Kluveromyces lactis* and *P. stipitis* (Boles and Hollenberg 1997). Besides D-glucose, many Hxt proteins transport D-fructose as well as D-mannose (Leandro et al. 2009), indicating that substrate specificities of Hxt proteins in yeast are generally broad. In *S. cerevisiae* Gal2, Hxt1, Hxt2, Hxt4, Hxt5, and Hxt7 catalyze the uptake of xylose (Hamacher et al. 2002; Saloheimo et al. 2007). However, their affinity for xylose is much lower than that for glucose and the xylose uptake by the transporters is strongly inhibited by glucose (Saloheimo et al. 2007). *P. stipitis* is the best studied xylose-fermenting yeast (Jeffries 2006). Three glucose transporters, encoded by the genes SUT1, SUT2, and SUT3, have been experimentally characterized in *P. stipitis* (Weierstall et al. 1999). Wild-type *P. stipitis* shows biphasic kinetics in the uptake of xylose whereas a SUT1 mutant loses the low-affinity component, suggesting that Sut1 protein achieves xylose uptake using the low affinity component. Few high-affinity

xylose transporters have been identified in yeast despite early evidence of the existence of proton-linked symporters in some strains (Hofer and Misra 1978; Lee et al. 2002). The rapid growth of *Candida intermedia* PYCC4715 on xylose was attributed to glucose/xylose proton-linked symporter (Gardonyi et al. 2003; Leandro et al. 2006). The symporter protein produced in *S. cerevisiae* (Leandro et al. 2008) showed much higher affinity to xylose than xylose transporting uniporters from *S. cerevisiae* and *P. stipitis*. However, affinity of the protein for glucose is still higher than that for xylose.

4.9 Xylose Transport Within the Yeast Cell

The first step in the metabolism of D-xylose is the transport of the sugar across the cell membrane. Under aerobic and oxygen limited conditions the rate of transport can limit the utilization of D-xylose in *P. stipitis* CBS 7126 and C. *shehatae* ATCC 22984 (Kilian and van Uden 1988). Under anaerobic conditions, D-xylose metabolism has not appeared to be transport-limited either in C. *shehatae* or in *P. stipitis.* Instead, the limitation was in the two initial steps of D-xylose metabolism, reduction of D-xylose and subsequent oxidation of xylitol. Studying the oxygen requirement for D-xylose uptake, Skoog and Hahn-Hagerdal (1990) found that oxygen induces or activates a transport system in *P. stipitis* CBS 6054. Starvation induced both proton symport and a facilitated xylose uptake diffusion system in *C. shehatae* CBS 2779. In non-starved cells, D-xylose was transported by a facilitated diffusion system. Kilian and van Uden (1988) reported on a low-affinity and a high-affinity xylose proton symport operating simultaneously in both starved and non-starved cells of *P. stipitis* IGC 4374. From the differences between the kinetic parameters of *C. shehatae* CBS 2779 (*K*m varies from 1 to 125 mM) and *P. stipitis IGC 4374 (K*m varies from 0.06 to 2.26 mM) it is found that transport systems in *P. stipitis* are more efficient. A transport system study in *C. mogii* ATCC 18364 (Sirisansaneeyakul et al. 1995) infers that D-xylose uptake rate followed Michaelis–Menten kinetics which suggested a carrier mediated facilitated diffusion transport system.

It was recently proved that *S. cerevisiae* can grow slowly in the presence of xylose as sole carbon source, in aerobic conditions. Until recently, it was considered that S. *cerevisiae* does not present specific transporters for xylose and that it grows poorly in its presence (Kinterinwa et al. 2008). The initial studies regarding the xylose transport alternatives within the yeast cell involved strains of *P. stipitis, P. heedii, C. shehatae* and *C. intermedia* and proved the existence of two transport systems:

1. A facilitated diffusion system, with low affinity—the genes involved here are, for example, SUT1 (sugar transporter 1) for *P. stipitis* or GXF1 (glucose/xylose facilitator 1) for *C. intermedia*, which are constitutively expressed genes that code the glucose/xylose transporter proteins (Jeffries and Jin 2004).

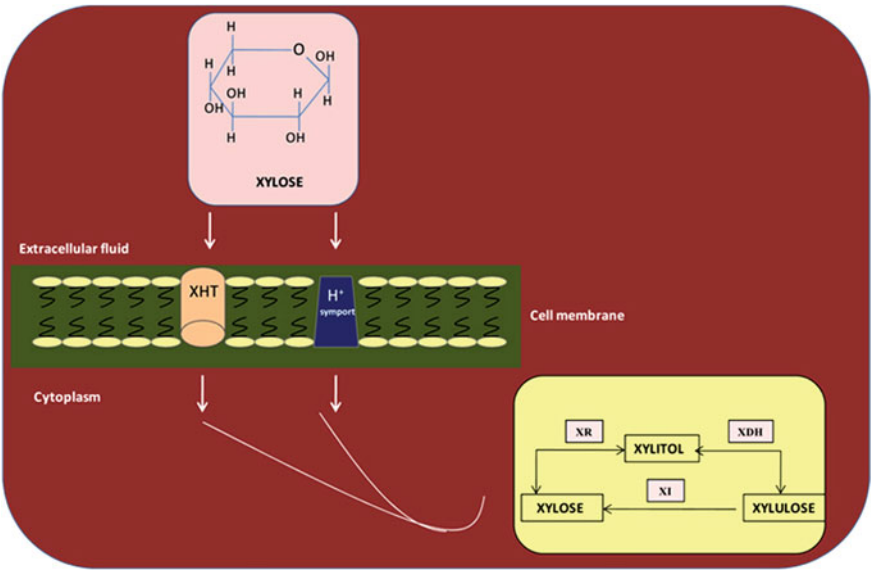

Fig. 4.1 Xylose transport within the yeast cell (*XHT* hexose transpoters, *XR* xylose reductase, *XDH* xylitol-dehydrogenase, *XL* xylose–isomerase (*Source* Ghindea et al. 2010)

2. A symport xylose-proton system, with high affinity—GXS1 (glucose/xylose symporter) that codes proteins involved in the symport monosaccharides-protons transport in various species of yeasts and fungi. In the case of *S. cerevisiae* species, the facilitated diffusion of xylose takes place with the aid of transporter proteins coded by HXT (hexose transporter) genes (Fig. 4.1). The xylose transport in the *S. cerevisiae* cell is, however, less efficient than glucose transport, the transport proteins (code by the genes XHT2, XHT6, XHT7) showing higher affinity for glucose.

4.10 Transport and Pentose Utilization in Yeast

Transport of sugar across the membrane does not limit the endogenous metabolism of sugars, but it may limit exogenous pentose metabolism. Without any genetic modifications, *S. cerevisiae* will transport pentoses across the cell membrane through native hexose transport proteins: Hxt7p, Hxt5p, Hxt4p, Hxt2p, Hxt1p and Gal2p (Becker and Boles 2003; Hamacher et al. 2002; Saloheimo et al. 2007). However, these proteins have a much higher affinity for their native hexose substrates, which may create unfavourable competitive inhibition and lead to diauxic growth in a hexose-pentose co-fermentation. Hence, dedicated pentose

transport may help to improve the simultaneous use of hexoses and pentoses. Gardonyi et al. (2003) reported a flux control coefficient of 0.2 for xylose transport in *S. cerevisiae* TMB 3001 (CEN.PK-2 XR/XDH/XKS) irrespective of xylose concentration, and rising to 0.5 in *S. cerevisiae* TMB 3206 (TMB 3001 with over expressed XR) at xylose concentrations below 0.6 g/l (Gardonyi et al. 2003). An evolutionary engineering experiment performed recently (aided by continuous culturing in xylose), using an optimized *S. cerevisiae* strain over-expressing six key xylose metabolic enzymes (including XI, XKS, TAL and TKL) (Kuyper et al. 2005), accumulated mutations over time resulted in greatly altered xylose transport kinetics, doubling V_{max} (15.8–32 mmol per dry weight per h) and reducing *K*m by 25 % (132–99 mmol/lM). This result infers that xylose transport is a limiting step, especially in metabolically optimized strains with higher downstream flux capacity. Experiment with recombinant *S. cerevisiae* found an increase in expression of the hexose transporter gene HXT5 (Wahlbom et al. 2003) showing that cells choose to increase transport activity when selected on xylose.

Heterologous transport proteins from plant, bacteria and other yeasts have been cloned and expressed in recombinant *S. cerevisiae*. However, only the class of transporters native to yeast, the major facilitator super family (MFS) (Pao et al. 1998) has been investigated. Other classes, such as the ATP binding cassette (ABC) transporters and the bacterial phosphoenolpyruvate (PEP) dependent transporters, were ineffective, because of expression difficulty and high relative energy requirements (Leandro et al. 2009). Xylose uptake in *S. cerevisiae* cells have shown two kinetically distinct uptake components with *K*m values of about 0.19 and 1.5 M, demonstrating that the monosaccharide transport in *S. cerevisiae* has nearly a 200-fold lower affinity for xylose than for glucose (Kotter and Ciriacy 1993). Isolation of transport protein with xylose as its highest affinity substrate would greatly enhance the prospects of a glucose-xylose co-fermentation. The most successful improvement of xylose transport to date is the expression of *C. intermedia* PYCC 4715 transport proteins (Leandro et al. 2006). Both *C. intermedia* GXF1 and GXS1 conferred significantly improved growth phenotypes in recombinant *S. cerevisiae* when glucose and xylose were used as sole carbon sources. Subsequent work has evaluated co-expression of the two proteins (Leandro et al. 2008) as well as more in-depth fermentation analysis (Runquist et al. 2009). Both of these transporters are efficient xylose transporters, yet also have a high affinity for glucose. Therefore, they remain primarily hexose transporters, despite the excellent xylose transport characteristics.

Heterologous xylose transport phenotypes were observed in yeast expressing *Arabidopsis thaliana* At5g59250 and At5g17010 (Hector et al. 2008). Where this was not supported was in a second study in which At5g59250 expression did not confer growth on xylose (Hamacher et al. 2002). In the first study with At5g59250, a standard strain of yeast was used whereas in the second, a strain lacking the HXT family of proteins was used. In this regard, the *A. thaliana* proteins may act as sensors or activators of HXT family transporters. These results show the importance of genotype on transporter characterization studies. Moreover, they emphasize the need for simultaneous optimization of both transport processes and

metabolic pathways. Further investigation is needed, as *P. stipitis* is known to have an excellent xylose transport system, yet to date no high-affinity xylose transporters have been isolated.

4.11 Conclusion and Future Recommendations

Xylitol producing yeast should have inherent characteristics of high osmotolerance, salt tolerance and inhibitor tolerance for the economization of xylitol production. All the above studies make up work concerning heterologous transport protein expression in *S. cerevisiae*, and motivate for further dedicated xylose transporters. However, future studies in this area must proceed with caution. Owing to the native transport characteristics of yeast, efficient transporters may need to be discovered through the use of HXT family knockout strains. It is also unknown how transporter proteins interact with each other to produce certain phenotypes. Naturally, as the internal metabolic pathway is improved, pentose transport will become a greater limitation. Therefore, novel tools and approaches must accomplish simultaneous optimization of transport and metabolism. Metabolic engineering approaches must be adapted to address the challenges for pathway and global cellular optimization that currently limit the construction of an integrated, efficient pentose pathway. Although much progress has been made towards lignocellulosic biomass conversion by yeasts, the approach of identifying limiting steps, and optimizing, must accelerate. More emphasis on host genome and regulatory structure must occur in future projects to understand the full effect of biological complexity on a pathway. In this regard, classic approaches combined with next-generation technologies may allow simultaneous optimization of all steps in a pathway. Although steady progress has been made in improvement of mixed-sugar utilization by microorganisms, more work is required for utilization of lignocellulosic biomass resources by microorganisms to make the process economically viable. Another hurdle to be cleared for efficient microbial utilization of mixed sugar substrates is the absence of a versatile method for over production of membrane proteins. In this respect, more efforts to explore and characterize sugar transporters should be expended. Hopefully, attempts to alter substrate specificities of sugar transporters by protein engineering will become more successful in order to improve utilization of pentoses. Finally, new methods that enable fast and simple assay of sugar uptake will help to facilitate finding and engineering of sugar transporters.

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