Chapter 4
Debaryomyces hansenii: An Osmotolerant and Halotolerant Yeast

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Contents

Abstract The yeast *Debaryomyces hansenii* which was isolated from saline environments such as sea water, concentrated brines, salty food, is one of the most halotolerant species. It can grow in media containing as high as 4 M NaCl, while the growth of *Saccharomyces cerevisiae* is limited in media with more than 1.7 M NaCl. This species is very important for food industry as it is used for surface ripening of cheese and meat products. In the recent past, there is growing interest in understanding the molecular mechanisms of high halotolerance exhibited by *D. hansenii*. Availability of genome sequence of *D. hansenii* has opened up new vistas in this direction.

Keywords *Debaryomyces hansenii*, halotolerance, saline environment, halophily, osmotolerance

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4.1 Introduction

Debaryomyces hansenii is an osmotolerant, halotolerant, xerotolerant and a cryotolerant marine yeast. It was originally isolated from hyper-saline environ ments such as seawater (Norkrans, 1966) and concentrated brines (Onishi, 1963). It is the yeast most frequently found in traditional cheese and sausages, with a recognized contribution to special flavors in these products (Seiler and Busse, 1990; Saldanha-da-Gama et al., 1997; Mortensen et al., 2005; Mounier et al., 2005). In this respect, *D. hansenii* is different from other yeast species, the prevalence of which is cheese dependent (Fleet, 1990). It is also common in dairies as it is able to grow in the presence of salt at low temperature and to metabolize lactic and citric acids. Besides this it can be isolated from other sources such as meat, wine, beer, sakemoto, rennet, tobacco, salmon, fruit and soil (Barnett et al., 2000; Davenport, 1980) as well as from high-sugar products (a_w as low as 0.62) (Tilbury, 1980; Butinar et al., 2005). In the salterns on the Atlantic coast in Namibia and in the Great Salt Lake brine *D. hansenii* has been isolated (Butinar et al., 2005). This shows that *D. hansenii* can be found in many habitats with low water activity. The presence of this species of yeast in such environments is due to its high osmotolerance. It can tolerate salinity levels up to 4 M NaCl, whereas *Saccharomyces cerevisiae* growth is inhibited when salinity reaches above 1.7 M NaCl. It is capable of accumulating sodium ion at high concentrations without being intoxicated, even in the presence of low concentrations of potassium (Prista et al., 1997). Moreover, in environments where the concentration of potassium is low, as is frequently found in nature, sodium significantly improves its growth and protects it from additional stress factors such as high temperature and extreme pH (Gonzalez-Hernandez et al., 2004; Prista et al., 2005). For this characteristic, *D. hansenii* is being designated as the halophilic yeast. In the recent past, *Debaryomyces hansenii* has become a model of osmotolerance of eukaryotic microorganisms. Its high cryo- and osmotolerance is highly advantageous from biotechnological point of view as it allows quasi-non-sterile production under high osmolarity conditions that should reduce production cost dramatically. This species of yeast has enormous biotechnological potential in agro-food sector. Although *D. hansenii* is considered as non-pathogenic, one case of bone infection (Wong et al., 1982) associated with this yeast was reported and several clinical samples have been identified as *D. hansenii*. Wagner et al (2005) have reported a case of invasive infection due to *D. hansenii* and *Scopulariopsis brevicaulis* in a stem cell transplant patient receiving liposomal Amphotericin B and Caspofungin for suspected Aspergillosis. Further, association of this species with psoriasis, patient with angina, infected nails, infected hands and wounds has been shown (van Uden and Fell, 1968). Mattsson et al. (1999) identified feral pigeons as carriers of *D. hansenii.*

The peculiar behavior of this yeast together with its ubiquity in salty environments highlights that *D. hansenii* is a genetically and biochemically interesting

yeast with considerable biotechnological promise. This chapter introduces the phylogeny, general physiology, metabolism, genome organization and molecular biology of *D. hansenii* before discussing the current biotechnological applications.

4.2 Phylogeny

Debaryomyces hansenii is an ascomycetous yeast. Sequence of the 18S ribosomal DNA unambiguously placed *D. hansenii* within the hemiascomycete subdivision (Wilmotte et al., 1993). The hierarchical details of this species of yeast include: Kingdom, Fungi; Phylum, Ascomycota; Class, Saccharomycetes; Order, Saccharomycetales; Family, Saccharomycetaceae and Genus, Debaryomyces. Cai et al. (1996) examined the complete 18S rRNA gene (18S rDNA) sequences of three *Debaryomyces* species (*D. hansenii*, *D. udenii* and *D. castellii*) and *Candida guilliermondii* (anamorph of *Pichia* (*Yamadazyma*) *guilliermondii*). They suggested that the *Debaryomyces* species and *C. guillermondii* were closely related. However, due to the lack of additional 18S rDNA sequence data from other *Debaryomyces* species, their phylogenetic relationships remained unclear. Based on partial sequence analysis of D1/D2 regions of 26S rRNA gene, Kurtzman and Robnett (1998) showed that *Debaryomyces* species is not monophyletic. They also suggested that *Debaryomyces* species separate into four clades that are represented by *D. hansenii*, *D. polymorphous*, *D. melissophilus* and *D. etchellsii*, and stated that basal branches of these four clades were weakly supported and that additional data were needed before generic boundaries could be confidently drawn. ITS sequence analysis by Martorell et al. (2005) also suggested that genus *Debaryomyces* is polyphyletic confirming the earlier observations.

The species *D. hansenii* comprises of two varieties, *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*, these two show 56% DNA re-association. Kurtzman and Robnett (1998) found one base substitution and one base deletion between two varieties of *D. hansenii* in the D1/d2 domain. These two varieties differ by one base substitution in ITS2 region, however no substitution was found in ITS1 sequence (Martorell et al., 2005). Some yeast strains that were earlier considered as *D. hansenii,* based on phenotypic characters, were shown to belong to a new species *Debaryomyces prosopidis* (Phaff et al., 1998).

The two varieties exhibit marked differences in the growth temperatures. The maximum growth temperature of *D. hansenii* var. *fabryi* is 36–39°C, whereas *D. hansenii* var. *hansenii* can grow in temperatures up to 35°C. However, the var. *fabryi* is not very often found and is poorly characterized. Optimum growth temperature of *D. hansenii* is between 20 and 25°C, and the growth, however, has been reported between 5°C and 10°C and even below 0°C (Davenport, 1980). At 10°C, this yeast is capable of growth at pH 4.0–6.0 in water activities (a_w) up to 0.99 (van den Tempel and Jacobsen, 2000).

The techniques that have been used to discriminate between the two varieties includes hybridization to species specific sequences, pulsed-field gel electrophoresis

(PFGE), restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD) and Fourier-Transform Infrared Micro-spectroscopy. Corredor et al. (2000) have shown that two RAPD products F01pro and M18pro are useful for the identification and typing of *D. hansenii* strains. Both the probes hybridized specifically to the *D. hansenii* var. *hansenii* strains only. Unique probes corresponding to actin *ACT1*, glycerol-3-phosphate dehydrogenase *GPD1* and beta-glucosidase *LAC4* encoding genes recognized chromosomal bands from var. *hansenii* specifically and not from var. *fabryi* thus, strongly suggesting that strains of this variety actually represent a different taxon, as suggested by a number of previous authors (Nakase and Suzuki, 1985; Prillinger et al., 1999). Further, by using the technique of PFGE they have shown that the two strains differ markedly at the level of genomic organization (Corredor et al., 2003). Similar discrimination between the two varieties of *D. hansenii*, by using a combination of mitochondrial (mt) DNA RFLP and RAPD analysis, by using appropriate primers have been shown by Romano et al. (1996). *Debaryomyces* species can be identified both quickly and correctly by direct sequence comparison of the ribosomal 5.8S-ITS region. The two varieties of *D. hansenii* can be distinguished by following the techniques of ITS-PCR and mt-DNA RFLP (Petersen et al., 2001). Both the methods have been shown to be useful for subspecies typing and investigation of the microbial succession between strains of *D. hansenii* during the ripening process of surface ripened cheeses. In contrast to Corredor et al. (2003), results from the studies of Petersen and Jespersen (2004) showed that two varieties of *D. hansenii* couldn't be divided into separate groups. Wenning et al. (2002) have shown Fourier-transform infrared (FT-IR) micro-spectroscopy as a useful method for the identification of *D. hansenii* strains. The mean strain identification rate was 91% for *D. hansenii* and 92% for *S. cerevisiae*. Strains of *D. hansenii* can also be distinguished from other yeast species by using a chromogenic substrate salmon-Gluc and X-Gal (de-Siloniz et al., 2000). The taxonomic classification of the two varieties of *D. hansenii* seems likely to change in the near future as further analytical methods are developed and applied.

4.3 Sexual Reproduction

D. hansenii is haploid yeast that reproduces vegetatively by multilateral budding. It is a homothallic yeast (Dujon et al., 2004; Fabre et al., 2004). Homothallism is defined as a state that is caused by the gene conversion between the *MAT* locus and two *MAT*-like loci during cellular division of haploid cells (Herskowitz et al., 1992; Haber, 1998). *D. hansenii* has a single *MAT*-like locus that seems to be a mosaic of *MAT***a** and *MAT*α genes, which is in accordance with homothallic life style of this species. This is in contrast to what exists in other species, where *MAT* loci have either 'a' or 'alpha' type information. Sequence analysis revealed that although *D. hansenii* possesses all maturation factors and receptors of both **a** and α pheromones, but no gene encoding **a** factor precursor have been found. Further, no gene encoding

HO endo-nuclease homologue has been found, thus, indicating the absence of mating-type switching. Establishment of mechanism responsible for the homothallism in *D. hansenii* should, thus, provide precious information. The species has an essentially haplo-diplontic life cycle (Fabre et al., 2004). However, according to Forrest et al. (1987) the species has a haplontic life cycle.

Sexual reproduction occurs very rarely. According to van der Walt et al. (1977) somatogamous autogamy is the main agency of diploidization, and that the species is largely inbreeding. However, sexual reproduction via heterogamous conjugation had also been suggested (Forrest et al., 1987). Conjugation leads to a short diplophase that is followed by meiosis and ascospore formation. Ascus formation involves fusion between a mother cell and a bud while still attached to each other, via short protuberances developed between the cross wall between them. Nuclear fusion takes place in the channel that connects the two cells. Meiosis occurs in the mother cell. Generally, only one lobe of the meiotic nucleus is surrounded by a prospore wall and it becomes the nucleus of a spore (Kreger van Rij and Veenhuis, 1975). The rest of the nucleus disappears. The spores are spheroidal with a warty wall. The wartiness is not always distinct under that light microspore. With the scanning electron microscope, warts appear as small blunt protuberances or as small ridges (Kurtzman et al., 1975). Usually one, seldom two spores are formed per ascus. A unique wale type of surface ornamentation of ascospores in the two strains of *D. hansenii* was observed by Banno and Mikata (1985). The spores germinate by swelling in the ascus and forming one or more buds. The view of sexual reproduction by heterogamous conjugation was further supported by Forrest et al. (1987). Isogamous conjugation also occurs (Nakase et al., 1998).

The dimorphism from yeast-to-mycelial forms has been reported in *D. hansenii* (Cruz et al., 2000). Hyphal growth is induced when the organism is cultured continuously in a xylose containing media made from hemi-cellulosic acid hydrolysates of barley bran. This dimorphism has been ascribed to be dependent on both the dilution rates of the culture medium as well as the dissolved O_2 concentration. Further, the yeast-to-mycelia transition is induced by adding selected amounts of acid-soluble lignin (BBH) in media with xylose or glucose.

4.4 General Physiology

D. hansenii is a highly halotolerant yeast. Unlike *S. cerevisiae*, which is a 'sodium excluder', *D. hansenii* is the 'sodium includer' yeast (Prista et al., 2005). It can grow in a media containing as high as 4 M NaCl, while the growth of *S. cerevisiae* is limited in a media containing 1.7 M NaCl. In this species of yeast, NaCl and KCl has similar effect thus, indicating that NaCl created only osmotic effect and specific toxicity of sodium is not involved as has been shown in *S. cerevisiae* (Prista et al., 1997). Further, growth was even stimulated by 0.5 M NaCl. However, LiCl has a specific inhibitory effect, although relatively weaker than in *S. cerevisiae*. It can grow in a media containing as high as 0.6 M LiCl (our unpublished observation).

In the absence of Na^+ and presence of K^+ ions at a low concentration (as low as 50 mM), growth has been observed for both the species. However, under these conditions, growth of *S. cerevisiae* was completely inhibited by 0.6 M NaCl (Camacho et al., 1981). On the other hand, growth of *D. hansenii* was stimulated by NaCl at concentrations up to 1 M NaCl. Moreover, under these conditions, the value for the specific growth rate and the final biomass was close to the values obtained in normal growth medium. This difference in tolerance to Na^+ ions, however, is not due to the difference in the efficiency of transporters involved in sodium efflux (Prista et al., 1997, 1998). These results highlights that in *D. hansenii* LiCl is far more toxic than NaCl and, hence, different resistance mechanisms are involved.

Presence of salt appeared to stimulate the growth of *D. hansenii* at higher temperature that normally did not support its growth. Moreover, the stress effect of extreme pH had been shown to be relieved by presence of 0.25 M NaCl (Almagro et al., 2000). These studies further highlight the salt loving or halophilic nature of this species of yeast. Since *D. hansenii* is a highly halo- and osmotolerant yeast, it is quite plausible to think that genetic material from this species may confer tolerance to osmotic effect and salt stress to heterologous host. *S. cerevisiae* harboring genetic material from *D. hansenii* and thus, conferring a different phenotype like growth at high salt concentration or under alkaline conditions has been demonstrated (Almagro et al., 2001; Prista et al., 2002).

According to Norkans (1968), *D. hansenii* is capable of both respiration and fermentation. However, Gancedo and Serrano (1989) described it as exclusively respiratory. Further studies carried out by Neves et al. (1997) supported the observation made by Norkans. Recently, it has been shown that this species is 'almost' Crabtree negative yeast but clearly Pasteur negative yeast, probably due to its low fermentative capacity which is mainly because of its lower phosphofructokinase activity (Sanchez et al., 2006). Crabtree effect relates to the decrease in the production of ethanol in the presence of oxygen and Pasteur effect relates to the decrease of glucose consumption in the presence of oxygen.

Cyanide-resistant respiration (CRR), a widespread metabolic pathway among yeasts, is very common in Crabtree-negative yeasts (incapable of aerobic fermentation) and in non-fermentative yeasts. It is conferred by a salicylhydroxamic acid (SHAM)-sensitive alternative oxidase that transfers electrons from ubiquinol to oxygen, bypassing the cytochrome chain. Although the involvement of this pathway in the fine adjustment of energy provision to the cell has been proposed, its physiological role remains obscure (Veiga et al., 2003a, 2003b, 2003c). Under aerobic conditions, a respiratory pathway alternative to the cytochrome chain (i.e. CRR) is triggered by stress conditions (such as starvation under aerobic conditions, decreasing pH or incubation of the culture in a narrow temperature range below the maximum temperature for growth) in *D. hansenii*. Besides this, in *D. hansenii* the activity of mitochondrial alternative oxidase is also triggered by the presence of 1.5–2.0 M NaCl. Therefore, such a relationship between stress situations and CRR must be taken into account in studies on the performance of spoilage yeasts in the food processing environments where several forms of stress are common.

4.5 Genomic Exploration

Whether the gene content of the genome can be correlated to the adaptive properties of species with different lifestyles? Could the mechanism of eukaryotic genome evolution be answered by comparative genomics? Although complicated by the multiplicity of events that have taken place throughout the history of individual lineages, the comparison of sequences of several unexplored yeast that covers an evolutionary range comparable to the entire phylum of chordates provides with a unique opportunity to investigate how genes involved in adaptation have been shaped by evolution.

The whole genome of *D. hansenii* has recently been sequenced and annotated by the consortium Genolevures and is now available at http://cbi.labri.fr/Genolevures/. It is compared with the complete genome sequence of three species widely spread over the hemiascomycete phylum: *Candida glabrata*, the second most prominent causative agent of human fungal infection (candidiasis) and phylogenetically related to *S. cerevisiae*; *Kluyveromyces lactis*, a milk loving yeast and *Yarrowia lipolytica*, an alkane using yeast that shares a number of common properties with the filamentous yeast in the Genolevures project. Analysis of the genome sequences revealed the role of processes such as tandem gene repeat formation, segmental duplication, massive genome duplication and extensive gene loss in the evolution of these species (Dujon et al., 2004).

4.5.1 Overview of the Genome

The genome of *D. hansenii* var *hansenii* type strain CBS767 is made of seven chromosomes ranging in size from 1.25 Mb to 2.33 Mb (Dujon et al., 2004). The total size of the genome is 12.2 Mb excluding ribosomal DNA (rDNA). It has the highest coding capacity among yeasts explored in the Genolevures project, amounting to 79.2% of the genome. The putative number of coding sequences (CDs) is 6906. Three distinct intra-chromosomal rDNA repeat loci are present in *D. hansenii*, whereas, single, seven and two loci are present in *S. cerevisiae*, *Y. lipolytica* and *C. glabrata* respectively. Variability also exists for the 5S rRNA gene copies. With a complement of 205 tRNA gene types corresponding to 43 types of tRNA, it has the largest number of potentially co-transcribed tRNA gene pairs. As compared to *S. cerevisiae*, *C. glabrata* and *K. lactis* which possess 42-tDNA set, *D. hansenii* has a 43-tDNA set. tDNA pairs are made of two distinct tRNA genes in the same orientation and separated from each other by a short distance (distance being shorter than the minimal 5′′ sequence required for transcription, thus, suggesting a common transcription of the two genes). Unlike the other three species of yeast, *D. hansenii* possesses eight identical copies of tDNA-lys that are present in tandem and are separated by intergenic distances sufficient for independent transcription. Like *C. albicans*, it uses an alternative genetic yeast code in which the CUG codon (leucine) is used as a serine codon (Tekaia et al., 2000; Sugita and Nakase, 1999).

D. hansenii is also the yeast with the most redundant genome, with an overall redundancy of 49.2%. Gene redundancy here is defined as the presence of genes representing conserved gene families that were present in organisms' last ancestor as well as gene families that have emerged or disappeared since specification. The global degree of genome redundancy, estimated from the number of paralogous gene copies per protein family indicates that this species of yeast has the most duplicated genome (901 sets) than *S. cerevisiae*, *C. glabrata, Y. lipolytica* and *K. lactis*. Among 97 newly identified members of paralogous gene families, 23 genes have putative role in transport, thus, indicating an important over-representation of this type of activities in *D. hansenii* as compared to *S. cerevisiae*. Similar over-representation exists for a large number of orthologues within a functional class however most striking is with the orthologues involved in 'transport facilitation'. The largest excess is for the 'allantoin and allantoate transporters' class. Besides this, multi-genic families encoding multi-drug resistance proteins and hexose transporters are more expanded in *D. hansenii* than in the other four yeasts. Tandem gene duplications are 5 to 10 times more frequent in this yeast. Tandem paralogues identified in *D. hansenii* are not strictly identical in sequence, indicating possible functional specialization and hence, limiting their destruction by popout. Few duplicated blocks (5 blocks, 3 in tandem) are present (Lepingle et al., 2000). Acquisition of new genes by horizontal gene transfer, a phenomenon rare in hemiascomycetes, has also been detected in *D. hansenii* (Dujon et al., 2004). Transposable elements are found in all eukaryotic organisms. In fungi, they participate to a large extent to genome plasticity through transposition and homologous recombination. Transposable elements are also present in *D. hansenii* and are more related to those found in higher eukaryotes (Lepingle et al., 2000).

4.5.2 Transporters

Trans-membrane transporter proteins make up to 10% of the coding genes in the hemiascomycete phylum (Hertogh et al., 2006). These may be considered as metabolic checkpoints for complex anabolic or catabolic pathways. These proteins have been classified according to the transport classification system (TC) (Saier, 2000). This classification system allocates five digits to each phylogenetic cluster of transporters in the order: class, subclass, families or super families, subfamilies and finally the fifth digit (clusters) that identifies the substrate or the range of substrates transported. Comparative analysis of the sequences encoding these proteins has allowed the identification of 'species-specific' transporter subfamilies that have either emerged or lost in the hemiascomycete phylum and to distinguish them from 'ubiquitous' subfamilies of transporters. It allows identifying the 'homoplasic' subfamilies of transporters that are conserved transiently in the different hemiascomycete species. A few examples of subfamilies in *D. hansenii* are: anion: cation symporter subfamily that takes up anionic vitamins in symport with protons or other cations (27 transporters including 9 members of the 'allantoate clusters'),

sugar porters (48 transporters, 21 members are gained and their substrates are undetermined), drug: proton antiporter-1 subfamily that pumps out a variety of hydrophobic drugs (24 transporters in *D. hansenii* as compared to 12 and 8 in *S. cerevisiae* and *K. lactis*) and large amino acid-polyamine-organocation family that transports a variety of amino acid in yeasts (24 members compared to 14–18 in the other species). Further, the species that exhibit the highest number of unique Hemiascomycete transporters is *D. hansenii* (9 specific transporters) compared to 5 in *Y. lipolytica* and one each in *S. cerevisiae*, *K. lactis* and *C. glabrata*. Mitochondrial carriers although are present in *Y. lipolytica* but are absent in *D. hansenii* and afterwards. This shows that the emergence of *Y. lipolytica* and *D. hansenii* is accompanied by a drastic gain of transporters compared to those during emergence of the other three species and thus, establishes the involvement of transporters in the evolution mechanism of speciation. The molecular mechanism of emergences or losses of these transporters genes during evolution are not understood.

Besides this, cell employs coordinated functions of different transporters for cation influx and efflux. In natural environments, sodium belongs to the abundant and potassium to the scarce ions. High internal concentrations of Na^+ (or its analogue Li^+) are generally toxic for cells. On the other hand, K^+ is required for many physiological functions such as regulation of cell volume and intracellular pH, protein synthesis, enzyme activation and this cation is accumulated in cells at a fairly high concentration. To maintain an optimum cytoplasmic concentration of potassium and a stable high intracellular K^+/Na^+ ratio, cells employ three distinct strategies: strict discrimination among alkali metal cations at the level of influx (higher affinity of transporters for potassium than for sodium), efficient efflux of toxic cations from cells, and selective sequestration (compartmentation) of cations in organelles (Sychrova, 2004).

In *S. cerevisiae*, sodium ions have been shown to enter the cell through the potassium transport system(s) encoded by the *TRK* genes (*TRK1* and *TRK2*) (Borst-Pauwels, 1981; Gaber, 1992; Rodriguez-Navarro and Ramos, 1984). However, specific sodium uptake system has not been identified as yet. Similarly in *D. hansenii*, nothing has been published in relation to the genes encoding the proteins mediating influx of sodium and lithium ions. The existence of genes orthologous to the *TRK* in *D. hansenii* has been reported (Prista et al., 2005).

Norkrans and Kylin (1969) explored the capacity of this species of yeast to tolerate high concentrations of NaCl in the growth medium. They have shown that *D. hansenii* when grown in presence of NaCl, accumulates high concentration of Na^+ inside the cell. But interestingly, when placed in presence of K^+ or Rb^+, it rapidly extrudes Na^+ to maintain high concentrations of K^+ inside the cell. The uptake of Rb^+ ions was found to be stimulated by the presence of NaCl in the medium (Prista et al., 1997). These results were further supported by the studies done by Thome-Oritz et al. (1998). The authors have postulated the existence of (i) an ATPase functioning as a proton pump and thus, generating a membrane potential difference that would drive K^+ ions through K^+ ion uniporter (ii) a K^+/H^+ exchange system (iii) a cation/cation exchange system. Analysis of the genome data also indicated the existence of homologues of Pma1p and Vma2p in *D. hansenii* (Prista et al., 2005). Similar

proteins in *S. cerevisiae* have been shown to generate a transmembrane potential and a pH gradient by massive proton efflux (Thome-Oritz et al., 1998; Gonzalez-Hernandez et al., 2004).

Ion transport systems mediating the efflux of sodium ions either out of the cell (Na^+ -ATPases and Na^+/H^+ antiporters) or their sequestration into the vacuoles have been identified. In *S. cerevisiae,* Na^+ -ATPases are encoded by *ENA1*/*PMR2* (*ENA* for efflux of natrium), the first unit of tandem array of four to five genes, depending on the strain, that encode the homologous P-type ATPases (Garciadeblas et al., 1993; Wieland et al., 1995). The expression of *ENA1* is modulated by osmotic stress and high pH values at the level of transcription. Under low salt concentrations (0.3 M), induction is mediated by HOG-MAPK kinase pathway, whereas under high salt conditions (0.8 M) by calcineurin which antagonizes the negative regulator, cAMP-dependent protein kinase (Marquez and Serrano, 1996; Hirata et al., 1995). In addition, it is also regulated post translationally by calcium-calmodulin via a calcineurin-independent mechanism (Wieland et al., 1995). *ENA1* encoded Na^+ -ATPases functions at alkaline pH values. In contrast, *NHA1* encoded Na^+/H^+ antiporters mediates Na^+ and K^+ efflux through the plasma membrane, required for alkali cation tolerance at acidic pH (Banuleos et al., 1998). Bedsides this, Na^+/H^+ antiporters are also involved in the regulation of intracellular pH. Thus, both systems have a complementary action to maintain the intracellular steady-state concentration of K^+ and Na^+. The two genes (*DhENA1* and *DhENA2*) encoding *ENA* homologues, which are involved in Na^+ extrusion, have been isolated and characterized (Almagro et al., 2001). *DhENA1* is expressed under conditions of high Na^+ concentrations. However, a high pH value along with the high Na^+ concentration is required for the *DhENA2* expression. It is suggested that these Ena proteins do not determine the Na^+ extrusion, as in *S. cerevisiae*, but plays an important role in maintaining the balanced levels of intracellular cations. These proteins are, thus, involved in ionic homeostasis of the cell.

The gene (*DhNHA1*) encoding *NHA1* homologue has been isolated and characterized from *D. hansenii* (Velkova and Sychrova, 2006). It shares the highest degree of identity with the antiporters of two other osmotolerant yeast species, *C. albicans* (*CNH1*) (Kinclova et al., 2001a) and *P. sorbitophila* (Banuelos et al., 2002). Thus, like the Na^+/H^+ antiporters from these yeast species, *DhNHA1* also exhibits broad substrate specificity. These antiporters might also play role in cell volume, intracellular pH and maintenance of cell cation homeostasis. On the other hand antiporters from *Zygosaccharomyces rouxii* (Kinclova et al., 2001b) and *Schizosaccharomyces pombe* (Jia et al., 1992; Kinclova et al., 2002) are able to transport only Na^+ and Li^+. Thus, depending upon the substrate specificity, yeast Na^+/H^+ antiporters can be divided into two subfamilies with different functions in yeast physiology. The gene encoding *DhNHX*, i.e. the homologue of *NHX1* gene of *S. cerevisiae* has been annotated in *D. hansenii* (Prista et al., 2005). *NHX1* functions to sequester the sodium ions into the cell vacuole. These results suggests that the ability of *D. hansenii* to adapt to high salt concentrations is not due a particular Na^+ ion extrusion system, but is due to the mechanism leading to the intrinsic resistance to the toxic effect of cations (Gonzalez-Hernandez et al., 2004).

4.6 Salt Toxicity and Defense Responses

The genetic analysis of salt tolerance in yeast has disclosed several adoptive mechanisms that cell has developed to counteract the stress due to changes in the osmolarity of the surrounding medium. The most important ones among these are the reactions crucial for maintaining ion homeostasis and cellular targets of salt toxicity. In the following sections these aspects are dealt with in detail.

4.6.1 Metabolism

When exposed to high osmolar medium, yeast cells respond by synthesizing and accumulating compatible solutes, called as osmolytes, inside the cell to counterbalance the external osmotic pressure (Mager and Varela, 1993). Glycerol accumulates as a major osmolyte in *S. cerevisiae*. It is synthesized in the cytosol via reduction of dihydroxyacetone phosphate in two steps that are catalyzed, respectively, by cytosolic NADH-dependent glycerol-3-phosphate dehydrogenase and a glycerol-3-phosphatase (Albertyn et al., 1994). The first enzyme in this pathway is encoded by two genes, the osmoresponsive *GPD1* (Larsson et al., 1993) and *GPD2* (Eriksson et al., 1995). The second enzyme is again encoded by two genes, the constitutively expressed *GPP1* and the osmotically induced *GPP2* (Norbeck et al., 1996). Increased accumulation of glycerol during osmotic stress is due to the enhanced activity of *GPD1*, thereby, resulting in the enhanced production and also because of the enhanced retention inside the cell, achieved by regulating the activity of *FPS1* (Olz et al., 1993; Blomberg and Adler, 1989; van Aelst et al., 1993). Fps1 behaves as a glycerol channel, responsible for glycerol leakage, that would remain closed in hyper-osmotic environments keeping glycerol inside the cells (Oliviera et al., 2003). Both the genes, i.e. *GPD1* and *FPS1* are regulated by HOG pathway (Albertyn et al., 1994). Although in *D. hansenii* glycerol accumulates as a dominant polyol during log phase like *S. cerevisiae* and arabinitol as dominant solute in the stationary-phase cells (Gustafsson and Norkans, 1976; Adler and Gustafsson, 1980; Nobre and da Costa, 1985), the information regarding the glycerol response in this yeast is still scarce. Besides this, trehalose, glutamic acid, erythritol, mannitol and alanine could also be accumulated in the cell in response to the osmotic stress. Recently, the gene encoding glycerol-3-phosphate dehydrogenase (*DhGPD1*) has been isolated from *D. hansenii* (Thome, 2004). A key role for this enzyme in glycerol production in *D. hansenii* has been demonstrated (Adler et al., 1985). The enzyme has been purified and characterized in vitro (Nilsson and Adler, 1990). Increased transcriptional activation of *DhGPD*, in presence of high salt stress, has been demonstrated (Lucas et al., 1990; Thome and Trench, 1999). Further, the ability to synthesize glycerol under osmotic stress does not differ much between *D. hansenii* and *S. cerevisiae* but the former is capable of retaining more glycerol than the other (Edgley and Brown 1983;

Brown, 1978). This is because of the presence of both an osmoregulatory-active glycerol transport system (Adler et al., 1985; Lucas et al., 1990) and by its ability to regulate the permeability of the plasma membrane (Oliviera et al., 2003). The first osmoregulatory-active glycerol transport system in *D. hansenii*, that accumulates glycerol up to 150-fold in the presence of 1 M NaCl, was described by Lucas et al. (1990). On the basis of their studies, these authors suggested the involvement of sodium-glycerol symporter in *D. hansenii*.

It is quite plausible that in halophilic organisms, the expression of genes encoding the enzymes of the central metabolic pathways could be modulated in order to increase transcription and enzyme synthesis, to overcome the negative effects of high salt concentration on enzyme activity. In this regard, Guerrero et al. (2005) studied the expression level of NADP-glutamate dehydrogenase involved in glutamate biosynthesis and ammonium assimilation in *D. hansenii*. They have shown that the expression of *DhGDH1* increases several fold in the presence of NaCl, whereas reverse is true for *DhGLN1*. Furthermore, the salt regulated expression of these two genes is by the action of a particular mechanism specific to *D. hansenii*, as its heterologous expression is not modulated by NaCl.

4.6.2 Signal Transduction Pathways

In eukaryotes, mitogen activated protein kinase (MAPK) cascades constitute very important signal transduction pathways that are conduits of various extracellular stimuli arising out of fluctuations in the environment or cell-cell communications. These cascades control various aspects of cellular physiology such as cell proliferation, differentiation and adaptive response to changes in the extracellular environment. A typical MAPK cascade is composed of three conserved families of protein kinases; the MAPK (also known as ERK, i.e. extracellular signal regulated kinase); a MAPK activator: MAPK kinase (MAPKK, MEK, MKK, extracellular signal regulated kinase kinase); and a MEK activator: MAPK kinase kinase (MAPKKK, MEK kinase [MEKK], extracellular signal regulated kinase kinase kinase) (Robinson and Cobb, 1997; Banuett, 1998). These kinases act in succession to form a cascade that ultimately modulates the activity of both cytoplasmic and nuclear targets by phosphorylation (Martinez-Pastor et al., 1996; Raitt et al., 2000). MAPK pathways seem to be essentially conserved from lower eukaryotes such as yeast to higher organisms, though their numbers and functional complexities vary. In *S. cerevisiae,* five MAPK modules have been identified so far, they regulate: mating, filamentation, sporulation, high-osmolarity responses and cell wall remodeling (Gustin et al., 1998; reviewed by Herskowitz et al., 1995; Madhani and Fink, 1998). Among these pathways, one is required for spore wall assembly and normally not present in growing cells, whereas other four are present in growing cells.

Very little is known about the signal transduction pathways in the marine yeast, *D. hansenii*, except the high osmolarity glycerol response pathway (HOG pathway) that mediates cellular adaptation process under high osmolar conditions. In *S. cerevisiae*

HOG pathway is specifically stimulated by osmotic shock (Brewester et al., 1993). This pathway receives signal from two upstream branches. One branch is the two component signaling system comprising Sln1, Ypd1 and Ssk1 (Maeda et al., 1994; Posas et al., 1996). Ssk1p activates the functionally redundant MAPKKKs Ssk2p/Ssk22p. A second branch of HOG1 is activated by the osmosensor Sho1p (Maeda et al., 1995), which signals to MAPKKK Ste11p (Posas and Saito, 1997). These MAPKKKs independently activate the MAPKK Pbs2p, which in turn dually phosphorylates and activates the MAPK Hog1p. Besides its role as a MAPKK, Pbs2p also functions as a scaffold protein in HOG pathway. Thus, Pbs2p plays a very complex role as it receives signals from both the upstream branches and allows the HOG pathway to operate over a wide range of sensitivity. Analysis of the genome sequence revealed that most of the components of HOG pathway are present in *D. hansenii*. Recently homologues of *HOG* and *PBS2*, has been isolated and characterized from *D. hansenii* (Bansal and Mondal, 2000; Bansal et al., 2001). Although *DHOG1* complemented the *hog1* mutation, the *PBS2* homologue (*DPBS2*) could partially complement the osmo-sensitivity of *pbs2* mutation in *S. cerevisiae* (Bansal et al., 2001). This partial complementation could be abrogated by replacing the C-terminal region of Dpbs2 with the homologous region of Pbs2p (Sharma and Mondal, 2005). This was due to an increase in nuclear translocation of Hog1p upon osmo-stress. Therefore, the C-terminal region of Pbs2p has an important role in nuclear translocation of Hog1p. Moreover, *DPBS2* was found to complement the polymyxin B sensitivity phenotype but had no perceptible effect on the calcofluor resistant phenotype of the *pbs2* mutation. Another interesting feature of Dpbs2p is the presence of a MAPK docking motif that appears to be essential for its function (Sharma and Mondal, 2006).

In *S. cerevisiae*, the activity of the HOG pathway is tightly controlled. Exposure to hyper osmolarity leads to rapid but transient activation of Hog1p by phosphorylation. Phosphorylated Hog1p translocated to the nucleus for a brief period to elicit the transcriptional responses necessary for osmoadaptation (Ferrigno et al., 1998; Reiser et al., 1999). Deactivation and subsequent re-entry of the Hog1p in the cytoplasm precedes the resumption of cellular growth. The prolonged activation of this pathway is detrimental for cell growth in *S. cerevisiae*. Under moderate stress conditions (0.7 M NaCl) the activation of HOG pathway in *D. hansenii* is quite similar to that in *S. cerevisiae*. However, the dynamics of the HOG pathway activation in *D. hansenii* differs considerably under severe osmotic stress. Sustained activation of Dhog1p has been observed in *D. hansenii* in the presence of 2.0 M NaCl. Interestingly, except for a brief period in the nucleus, the activated Dhog1p mostly remained in the cytoplasm (Sharma et al., 2005). These studies thus indicated important cytoplasmic role of phosphorylated Dhog1p in *D. hansenii*. Identification of the cytoplasmic targets of Dhog1p could provide us important clues for understanding the high osmotolerance exhibited by this yeast. HOG pathway is the only stress activated protein kinase pathway in yeast. The types of stress conditions that activate HOG pathway differ among yeast species. In *D. hansenii* this pathway is activated by osmostress, oxidative stress and UV stress. However, the heat stress did not activate this pathway. In this regard *D. hansenii* is quite similar to *Candida*

albicans (Sharma et al., 2005). UV induced activation of this pathway could be mainly as a reaction to oxidative stress due to formation of free radical.

4.6.3 Halotolerance Genes

Halotolerance genes are defined as the genes that have the capability to improve salt tolerance. Using *S. cerevisiae* as a model system, a few genes that are importance for halotolerance in yeast have been identified. Most prominent among them are *HAL1, HAL2, HAL3, HAL4* and *HAL5. HAL1* (Gaxiola et al., 1992; Rios et al., 1997) and *HAL3* (Ferrando et al., 1995) modulate intracellular sodium and potassium concentrations and encode components of the regulatory machinery for ion homeostasis. Their effects are mediated by the *ENA1*/*PMR2* gene. *HAL4* and *HAL5* encoded protein kinases are also required for ion homeostasis however their action is mediated through the coordinate regulation of potassium ion transporters of yeast cells, *TRK1* and *TRK2* (Mulet et al., 1999). On the other hand, *HAL2* encodes an enzyme, 3′(2′)5′-bisphosphate nucleotidase that catalyzes a side reaction essential for sulfate assimilation in yeast (Glaser et al., 1993). These enzymes are highly sensitive to sodium and lithium (Murguia et al., 1995) and thus, perform a crucial rate-limiting metabolic step during salt stress in yeast (Murguia et al., 1996). Genome sequence revealed that except *HAL1*, all these genes are present in *D. hansenii*.

Recently, a *HAL2* homologue, *DHAL2*, has been isolated and characterized from *D. hansenii* (Aggarwal et al., 2005). Biochemical as well as in vivo studies has revealed that Dhal2p has much higher intrinsic salt tolerance than that of the other homologues. It is quite plausible that in halotolerant organisms 'specific targets of salt toxicity' have been evolved to be more robust and less amenable to salt inhibition. It could tolerate much higher concentration of Na^+ and Li^+ (IC_{50} = 180 mM and 2.4 mM respectively) than most of the known homologues. With respect to sodium sensitivity, Dhal2p is comparable to *SAL2* of *A. thaliana*, which exhibited maximum salt tolerance (IC_{50} for Na^+ and Li^+ = 200 mM and 10 mM respectively). However, in case of lithium, Dhal2p seems to be more sensitive. Hal2p belongs to a larger super-family of phosphatases, Mg^{2+} -dependent Li^+ sensitive phosphomonoesterases. A number of *HAL2* homologues from plant, mammal and yeast have been characterized. Although they require Mg^{2+} for their activity, higher Mg^{2+} concentration has inhibitory effect on these enzymes. In this regard, *D. hansenii* homologue is quite different (Aggarwal et al., 2005).

Another interesting and distinguishing feature of Dhal2p is that, two distinct isoforms of this enzyme existed in *D. hansenii* (Aggarwal and Mondal, 2006). One of them is a constitutively expressed cytosolic form. Like the other known homologues, this form of Dhal2p is a typical PAPase. Whereas the other isoform of Dhal2p is membrane bound. It is accumulated in the endoplasmic reticulum only in response to high salt stress conditions and is a PIPase, i.e. enzyme acting on both inositol-1,4-bisphosphate and PAP. In this respect, the membrane-bound form was like a dually active BPntase prevalent in multi-cellular organisms (Lopez-Coronado et al., 1999). These types of enzymes have been suggested to play an important role

in inositol signaling. Modulation of Na^+ efflux system by the expression of *SAL1* in *S. cerevisiae* (Quintero et al., 1996), increase in sensitivity to abscisic acid and to damage by drought, low temperature or salt stress by mutation in *SAL1*/*FRY1* in *Arabidopsis thaliana* (Xiong et al., 2001) are few examples to quote. Studies done by Aggarwal and Mondal (2006) have further shown that the *S. cerevisiae* cells expressing both the cytosolic and membrane bound isoforms exhibited a higher level of salt tolerance than those expressing only the cytosolic form of Dhal2p. These studies thus, clearly suggested that the membrane-bound form of Dhal2p, unique to *D. hansenii*, played an important role under high salt stress conditions. Moreover, such a modulation of sub-cellular localization and substrate specificity of Dhal2p could be an important adaptive mechanism for growth of *D. hansenii* under high salt stress conditions. These studies, furthermore, highlight the importance of *D. hansenii* as a model to study halotolerance.

4.7 Biotechnological Applications

Studies done in recent past have shown that *D. hansenii* has enormous biotechnological potential in agro-food industry. It is commonly found in cheeses and sausages (Seiler and Busse, 1990; Saldanha-da-Gama et al., 1997) and in fact, plays an important in cheese making. The characteristic properties of the genus *Debaryomyces* that endows it with such a potential has recently been reviewed by Breuer and Harms (2006). It includes its ability to adhere to a solid surface (a prerequisite for initiation of growth to occur) (Mortensen et al., 2005), its salt tolerance, capacity to grow at low temperature and low water activity (a_w), ability to produce proteolytic and lipolytic enzymes that can metabolize milk proteins and fat (Besancon et al., 1992; Davenport, 1980; Fleet and Mian, 1987; Roostita and Fleet, 1996) and ability to inhibit the germination of undesired microorganisms in cheese brines (Deiana et al., 1984; Fatichenti et al., 1983). Furthermore, in comparison to other cheese ripening yeast, *D. hansenii* produces volatile sulphur compounds (Arfi et al., 2002). Similarly the involvement of *Debaryomyces* in meat fermentation has been known for a long time. Besides producing volatile compounds, the yeasts also influence the sensory properties of the meat (Dura et al., 2004a, 2004b). Recently few enzymes like prolyl amino peptidase, arginyl amino peptidase (Bolumar et al., 2003a, 2003b) and glutaminase (Dura et al., 2004c) of *D. hansenii* has been isolated and characterized. However, much less is known about the possible involvement and their role in ripening of a fermented sausage and meat fermentation.

One of attractive biotechnological potential of *D. hansenii* is its use in the production of xylitol from D-xylose or wood hydrolysate (Girio et al., 1996, 2000; Parajo et al., 1997). Xylitol, as a sweetener, is an important raw material in food industry for the production of sugar-free confection and food (Cruz et al., 2000). Conversion of xylose to xylitol by *D. hansenii* has been studied quite extensively. *D. hansenii* could use both pentoses and hexoses individually as well as in mixture. The presence of hexoses particularly glucose did not inhibit the consumption of pentoses such as xylose (Nobre et al., 1999). This interesting property needs further

investigation, as it will be useful for developing strains of other yeast species to utilize sugars in a mixture simultaneously. The initial steps for the metabolism of xylose in *D. hansenii* involve a NADPH-dependent xylose reductase and a NAD^+ linked xylitol dehydrogenase (Converti and Dominguez, 2001; Girio et al., 1994). Since these two enzymes utilize different co-actor, the availability of oxygen during the fermentation has tremendous influence in the overall yield of xylitol from xylose. Although production of xylitol by *D. hansenii* awaits commercial exploitation at present, insights into the regulation of pentose metabolism will definitely help towards achieving this objective. Besides xylitol, *D. hansenii* can be used to synthesize a range of other useful product. It produces xylitol as well as arabinitol from pentose sugar. D-Arabinitol is produced in the stationary phase simultaneously with the excretion of riboflavin in the batch cultivation. The importance of this yeast species as cell factories for producing chemicals like pyruvic acid, D-arabinitol has recently been highlighted (Breuer and Harms, 2006). Some strains of *D. hansenii* exhibit anti-fungal activity. Effectiveness of these strains as a biocontrol agent of fruit spoilage fungi has also been demonstrated (Fleet, 1992; Droby et al., 1989; Ramirez-Orozco et al., 2001).

4.8 Future Perspectives

Although *D. hansenii* had been known in the literature for a long time, the surge of scientific interest in this organism is very recent. This is evident from the increase in the number of publications on this organism in the past ten years. A cursory glance through the published literature, certainly suggests that this is an interesting yeast, with enormous potential for both fundamental research and applied research. The availability of the whole genome sequence of *D. hansenii* offers new possibility for an integrated approach to unravel the extremophilic nature of this organism. Comparative genomics could be very useful. Development of molecular genetic tools is an urgent requirement. This will facilitate the development of better strains for commercial exploitation. It will also help to garner the detailed information about the signal transduction pathways, genetic network and regulation, an important step for understanding and exploitation of the halotolerance/halophily of this organism.

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