High intracellular concentrations of salt adversely affect protein structure and thus would inhibit many metabolic reactions and membrane functions nonspecifically (Yancey *et al.*, 1982). Recent studies, in model organism such as *S. cerevisiae*, showed that in addition to these relatively nonspecific effects, some enzymes may be specially, sensitive to inhibition by salt at much lower concentrations. These metabolic reactions are critical factors in determining the fitness of the organism to survive under high salt stress. It is quite plausible that in halotolerant organisms, "these specific targets of salt toxicity" have been evolved to be more robust and less amenable to salt inhibition. Therefore, the identification and characterization of these molecules from halotolerant organisms, is of crucial importance for understanding the mechanism of salt toxicity and for the manipulation of salt tolerance. Molecular genetic analysis revealed that *HAL2* is an important target of salt toxicity in *S. cerevisiae*. This gene encodes a 3'(2'),5'-bisphosphate nucleotidase. It is highly sensitive to monovalent cations like lithium and sodium ions. The activity of this enzyme is rate limiting for the growth of yeast under salt stress. Homologues of *HAL2* seem to be ubiquitous in eukaryotes. It has been identified and characterized from plant and mammalian sources. They exhibited a varying degree of cation sensitivity. Interestingly, so far no study has been carried out with such an important determinant of salt tolerance from halotolerant species. Highly halotolerant yeast *Debaryomyces hansenii* could be one such source for this type of genes. It was isolated from saline environments such as sea water and concentrated brines. This species can grow in the media containing 4 M NaCl, whereas growth of *S. cerevisiae* is limited in the media having more than 1.7 M NaCl. In this context, the work in this thesis was focused on the detailed molecular, biochemical and functional characterization of a *HAL2* homologue (*DHAL2*) from *Debaryomyces hansenii*.

In *S. cerevisiae*, *HAL2* is allelic to *MET22* and mutation in this gene results in methionine auxotrophy. Therefore, homologous gene from *D. hansenii* was isolated by complementation of this phenotype. From the nucleotide sequence of the clone, an open reading frame of 1260bp encoding a protein of 420 amino acids was identified. Near the 5' end of the ORF sequence, three in frame ATG codons at positions +1

(M1), +13 (M2) and +163 (M3) were identified that could potentially serve as start codon. Comparison of the deduced amino acid sequence revealed that it showed substantial homology with the *HAL2* homologue identified earlier from other yeast species thus, indicating that our clone could be a *HAL2* homologue. Hal2p belongs to inositol monophosphatase protein family (Albert *et al.*, 2000). Analysis of the nucleotide derived amino acid sequence revealed that Dhal2p also possessed the signature sequence motifs, DPIDGT and WDXAAG of the phosphatase superfamily (Neuwald *et al.*, 1991; York *et al.*, 1995). However, the most distinguishing feature of the Dhal2p was a unique 'hydrophobic' stretch of 54 amino acid residues at its N-terminus. Upon over-expression, Hal2p improves halotolerance in *S. cerevisiae* (Glaser *et al.*, 1993). Phenotypic analysis displayed that Dhal2p also conferred a similar phenotype. Unlike *S. cerevisiae* homologue, Dhal2p could improve the salt tolerance to a similar level irrespective of its copy number.

Expression of the full-length Dhal2p was toxic to *E. coli*. However, a truncated form of Dhal2p, without the N-terminal 54 amino acid residues could be expressed and purified. This form of Dhal2p also retained the enzyme activity. Therefore, biochemical characterization was carried out with this. The purified recombinant Dhal2p could hydrolyze the substrate over a broad range of temperature and pH. Both Mg^{2+} and Mn^{2+} were used as cofactors and optimum concentrations for both were 0.5mM. Unlike the other homologues, excess magnesium did not inhibit the enzyme even at very high concentrations. The enzyme could efficiently utilize PAP, PAPS or 2'PAP as substrate. However, it displayed very little activity against other phosphoesters like ATP, ADP etc. Some of the *HAL2* homologues such as *SAL1* (Quintero *et al.*, 1996) and *RnPIP* (Lopez-Coronado *et al.*, 1999), showed very high inositol polyphosphate-1-phosphatase activity and therefore considered as enzymes having dual specificity. These enzymes have been speculated to function in both PAP metabolism as well as phosphoinositide signaling. Activity of Dhal2p against inositol-1,4-bisphosphate was quite low (~15.58%). Furthermore, the comparison of the kinetic parameter suggested that PAP was preferred substrate. Therefore, Dhal2p could be a typical PAP nucleotidase.

The members of this family have been shown to be highly sensitive to monovalent cations particularly Li^+ and Na^+ ions (York *et al.*, 1995), therefore, monovalent cation sensitivity of Dhal2p was determined *in vitro*. Dhal2p could tolerate much higher concentrations of salt (IC_{50} for Na^+ and Li^+ 180mM and 2.4mM respectively) than most of the homologues. According to the earlier reports, *SAL2* from *A. thaliana* (Gil-Mascarell *et al.*, 1999) exhibited maximum salt tolerance (IC_{50} for Na^+ and Li^+ 200mM and 10mM respectively). Therefore, with respect to sodium sensitivity these two enzymes were quite comparable but in case of lithium Dhal2p seemed to be more sensitive. Interestingly, activity of *SAL2* enzyme was inhibited at lower concentrations of K^+ (IC_{50} = 300mM) compared to Dhal2p (IC_{50} = 1.0M).

Recently the crystal structure of *S. cerevisiae* Hal2p has been solved (Albert *et al.*, 2000). Therefore, based on this structure, homology based model of truncated Dhal2p was generated to obtain structural insight of its function. Like other members of the family (Bone *et al.*, 1992, 1994a, b; York *et al.*, 1994; Xue *et al.*, 1994; Villert *et al.*, 1995), this enzyme seems to have a common core fold of two domains, one $\alpha+\beta$ and one α/β. A detailed comparison of the structures revealed that the residues as well as the ionic interactions involved in coordinating phosphate ions, metal ions and substrate at the active site, were absolutely conserved (Albert *et al.*, 2000). To confirm their vital role in the catalytic activity of the enzyme, mutations at these residues (D201N, T206A, E133Q and D363N) were introduced. *In vitro* assay showed that these mutants retained very little (5-9%) activity. These results corroborated with the previous studies with human IMPases (Pollack *et al.*, 1993; Gore *et al.*, 1993) where mutations at these residues resulted in an enzyme exhibiting less than 0.2% of the wild type activity. These mutants of Dhal2p failed to complement the *hal2* mutation in *S. cerevisiae*. These results, thus, established the contribution of these residues towards the functionality of the enzyme.

Previous studies have shown that mutation either at V70 or E238 in *S. cerevisiae* Hal2p dramatically improved its tolerance to monovalent cations (Albert *et al.*, 2000). The corresponding residues in Dhal2p are V131 and E301, which are

conserved. Therefore, the effect of these mutations on the catalytic activity as well as cation sensitivity of Dhal2p was examined. The mutants V131A and E301K, exhibited reduced phosphatase activity as evident by their K_m and K_{cat}/K_m values. It was evident from the IC_{50} values for lithium and sodium ion that the cation sensitivity of V131A mutant was improved marginally. In contrast, E301K mutation resulted in the generation of Dhal2p mutant that was virtually insensitive to the monovalent cations *in vitro*. The observed improvement in the salt tolerance of mutated Dhal2p was presumably due to the displacement of helix α5, resulted upon E310K mutation, with respect to its position in wild type Dhal2p. This displacement of helix α5 in turns weakened the salt bridge interactions between R211 and D332.

By site-directed mutagenesis, we have demonstrated for the first time the role of the 'flap region' (98-106) and a 'loop region' (135-138) present C-terminal to EE consensus motif, in the phosphatase activity and cation sensitivity of the enzyme. Mutations in the flap region caused a significant decrease in the salt tolerance of the enzyme. On the other hand, mutations in the loop region resulted in a generation of highly salt tolerant form of Dhal2p. Homology model revealed that the flap region made contacts with the residues (Y296 and K299) present on the loop from the other side of the active site thus, forming a protective shield. Furthermore, flap and loop regions interact via hydrophobic as well as ionic interactions. It is possible that these mutations affect the above mentioned frame work interactions of the flap region with the residues on neighboring loops, thus, resulting in an observed differences in the cation sensitivity of the mutant enzyme. The crystal structure of the enzyme has to be solved to fix with precision the changes in the conformation or the framework interactions responsible for the observed differences in the cation sensitivity of the enzyme.

Analysis of the Dhal2p sequence revealed that it possessed a unique hydrophobic stretch of 54 amino acid residues at its N-terminus. Further, the expression of the full-length Dhal2p was toxic to *E. coli*, therefore, it was presumed that it could be a membrane protein. To test this hypothesis, expression profile of the

Dhal2p in *S. cerevisiae* expressing either the full-length or the truncated *DHAL2* (without N-terminal 54 amino acid) was analyzed. With the truncated *DHAL2* a single band of 41 kDa was detected, whereas with the full-length *DHAL2* two bands of 41 and 44kDa were observed. Moreover, 41kDa was found in the cytosol and 44kDa band was localized exclusively in the membrane fractions. Thus, these results clearly indicated the existence of two isoforms of Dhal2p. One of the isoform was present in membrane and the N-terminal hydrophobic region was essential for its membrane localization. The expression of full-length *DHAL2* ORF resulted in the synthesis of two isoforms. Three potential initiation codons at positions +1 (M1), +13 (M2) and +163 (M3) were present at the 5' end of the ORF sequence. It was observed that M1/M2 ATG mutant expressed only the cytosolic 41kDa form. On the other hand, M3 ATG mutant expressed only the membrane bound 44kDa form. Thus, alternative translation initiation was the major mechanism regulating the synthesis of these two forms. This form of regulation has been previously documented in yeast. A number of genes in *S. cerevisiae* such as *LEU4*, *FUM1*, *VAS1*, *TRM1* and *HTS1* (Beltzer *et al.*, 1986; Chatton *et al.*, 1988; Chui *et al.*, 1992; Ellis *et al.*, 1989; Wu and Tzagoloff, 1987; Natsoulis *et al.*, 1986)) encode two protein species. Like *DHAL2*, these genes also have ORFs with more than one in frame ATG codon near the 5' ends. The smaller protein is produced from 3' ATG whereas the larger form utilizes the 5' ATG as initiation codon. Interestingly, in these examples the two forms of the protein are also associated with different intracellular locations, cytosol and mitochondria. To monitor the intracellular localization of Dhal2p isoforms, they were fused to GFP and fluorescence microscopy was done. It was observed that the truncated Dhal2p was cytosolic whereas the full-length form was localized in endoplasmic reticulum.

Subsequently, the two isoform was purified by utilizing PAP-agarose as affinity matrix and their activity was measured against either PAP or inositol-1, 4-bisphosphate as a substrate. These results indicated that the truncated Dhal2p was a typical PAPase. However, the full-length membrane bound isoform was a PIPase as it exhibited very high level of activity against inositol-1, 4-bisphosphate. PIPases are prevalent in multi-cellular organisms where they have been shown to play a role in

phosphoinositide signaling. In *A. thaliana*, IPPase activity of *SAL1/FRY1* has been demonstrated to play a role in phosphoinositol (IP_3) mediated absicsic acid and stress signal transduction (Xiong *et al.*, 2001). Interestingly, the expression of *SAL1* in *S. cerevisiae* has been shown to modulate the Na^+ efflux and the IPPase activity of *SAL1* is involved in this process (Quintero *et al.*, 1996).

To substantiate the existence of the Dhal2p isoforms in *D. hansenii*, western blot analysis was performed with the membrane and cytosolic fractions of *D. hansenii* cultures grown either in absence or presence of salt. Immunoblot analysis clearly demonstrated the existence of both the forms in *D. hansenii*. Interestingly, the full-length form accumulated in the cell, presumably in ER, specifically in response to salt stress. These results clearly suggested an important physiological role for the membrane bound form in high salt stress. The observation that *S. cerevisiae* cells expressing both the isoforms exhibited higher level of salt tolerance that those expressing only the cytosolic form of Dhal2p argued in this direction.

Finally, the most interesting and a novel facet of this investigation is the discovery of a membrane bound isoform of bisphosphate nucleotidase in *D. hansenii*. To date, a number of bisphosphate nucleotidases from different sources have been characterized but ours is the first example of membrane bound form. This form is accumulated in endoplasmic reticulum specifically in response to salt stress. Moreover, unlike the cytosolic form, this form is a PIPase that could utilize inositol-1,4- bisphosphate as a substrate. Together, these results illustrates that the modulation of sub-cellular localization and activity of Dhal2p could be an important adaptive mechanism for the growth of *D. hansenii* under high salt stress. What physiological advantage does this organism achieve by adopting such a mechanism? Inositol is a key metabolic sensor and inositol levels regulate important ER functions such as membrane biosynthesis (Nunnari and Walter, 1996). In *S. cerevisiae*, the role of inositol polyphosphates in maintaining cell wall integrity, salt stress tolerance, endocytosis and mRNA export has been elaborated (Cooke *et al.*, 1998; Odom *et al.*, 2000; Dubois *et al.*, 2002). Recent studies indicated that the salt stress markedly

influenced the level of inositol mono and polyphosphate in *S. cerevisiae* (Navarro-Avino *et al.*, 2003). A number of important regulators of phosphoinositide signaling in yeast are located in the ER. Therefore, it is possible that the full-length Dhal2p plays important role in maintaining the inositol homeostasis under salt stress. Elucidation of the role of the membrane bound Dhal2p in this process could unravel interesting aspects of salt tolerance in *D. hansenii*.