SUMMARY

The major goal of this study has been to examine if we can identify possible reasons that might have led to the evolutionary development of *Schizosaccharomyces pombe* as a natural inositol auxotroph. We have looked for the molecular basis for this auxotrophy, and the reasons that could have led to this phenotype.

We transformed *S. pombe* with two libraries that were made from inositol prototrophic organisms made in *S. pombe* vectors. The first library was a human cDNA, and the second was a *Pichia pastoris* genomic library. We selected for transformants that could grow on inositol lacking medium. No transformants were obtained with the human cDNA library. However, we obtained 11 transformants with the *P. pastoris* library.

From these 11 transformants, plasmids were isolated. Three independent plasmids were obtained that had the ability to confer inositol prototrophy to *S. pombe*. However, overlapping restriction patterns indicated that they contain a common fragment that was conferring the phenotype. One of the plasmids was selected for further study. A detailed restriction map was made. Subcloning and complementation data revealed a minimum complementing, 2.2 kb *Bam*H I-*Sal* I fragment within this 6.0 kb insert. This minimum complementing fragment was sequenced on both the strands. Sequence analysis revealed that we had isolated the *INO1* gene of *P. pastoris* as a gene complementing inositol auxotrophy in *S. pombe*. The smallest fragment contained only 60 bp of promoter. The sequence of the complete promoter was also determined. Complementation of inositol auxotrophy in *S. pombe* by the *INO1* gene of *P. pastoris* suggested the loss or nonfunctionality of *INO1* gene in *S. pombe*. We carried out Southern blotting with *INO1* gene of *P. pastoris* as a probe and could detect no band even at lower stringency conditions which suggested loss of *INO1* gene in *S. pombe* as the cause of inositol auxotrophy.

We next investigated the probable reasons for the loss of *INO1* gene in *S. pombe*. As inositol biosynthesis is regulated in *Saccharomyces cerevisiae* through a

complex network, we thought that perturbance in the inositol homeostasis in *S. pombe* might yield some clues in this directions. Inositol homeostasis was disturbed by overproducing inositol by overexpressing *INO1*. A 3.3 kb *Bam*H I fragment of *INO1* gene of *P. pastoris* was cloned downstream of the strong and tightly regulated *nmt1*$^+$ promoter of *S. pombe*. As *nmt1*$^+$ promoter is regulated by thiamine, under derepressed conditions (i.e. in the absence of thiamine), *INO1* gene was overexpressed, producing high levels of intracellular inositol. Inositol was excreted in the medium indicating overproduction of intracellular inositol. However, we did not see any other observable phenotype. The phospholipid composition and growth were normal. There were no significant differences or any adverse effects on cells due to the disturbance in inositol homeostasis. We also checked for the synthetic lethality by expressing multicopy plasmid genomic library into *INO1* overexpressing cells. This also did not show any lethal phenotype.

Results of the cloning and overexpression studies suggested to us that in looking for explanations for the loss of the *INO1* gene in *S. pombe*, we would also need to examine the external environmental factors. The natural environment of *S. pombe* contains significant amount of phytic acid (i.e. inositol hexaphosphate). We therefore checked the ability of *S. pombe* to utilize phytic acid as a source of inositol. Utilization of phytic acid would presumably require the secretion of phosphatases. *S. pombe* and *S. cerevisiae* both secrete acid phosphatases, the bulk of which is phosphate repressible. We, therefore, carried out the growth experiments with *S. pombe* and an *S. cerevisiae ino1Δ* strains under high and low phosphate conditions. We observed that both *S. pombe* and *S. cerevisiae ino1Δ* could utilize phytic acid as a source of inositol, but in different conditions. *S. pombe* preferred more acidic pH for utilization of phytic acid compared to *S. cerevisiae*. This might be one possible reason for *S. pombe* to have evolved as an inositol auxotroph.

The ability of the *INO1* gene of *P. pastoris* to complement natural inositol auxotrophy in *S. pombe* suggested to us that we might be able to exploit the

observation for the construction of novel vectors. We examined, whether the plasmid containing *INO1* gene could be directly selected on inositol deficient plates. We also constructed a plasmid which did not have any other selection marker apart from *INO1* gene of *P. pastoris*. Transformation efficiency of this plasmid was same as that of other plasmids when selected directly on MM-Ino medium although colony appearance was 1 day later. All these plasmids were also checked for their ability to transform other members of *Schizosaccharomyces* genus. We were able to transform *S. malidevorans* and *S. pombe* var *malidevorans* with these plasmid, selecting them for inositol prototrophy. However, we did not get any transformants for *S. japonicus* and *S. octosporus*. As these species differ significantly from *S.pombe*, so much that some researches beleive they should be placed in a separate genus, we are not sure whether these strains did not get transformed, or whether the *INO1* gene of *P. pastoris* did not express in the cells. The *INO1* gene of *P. pastoris* nevertheless thus be used as a selection marker in vectors for *S. pombe, S. malidevorans* and *S. pombe* var. *malidevorans*.