CHAPTER V

General Discussion

The adhesive interactions that attract different types of cells to each other play a critical role in a wide variety of developmental and pathological events including intercellular communication, regulation of cell growth and differentiation, immune response and perhaps malignancy. Lectin-carbohydrate interactions have been shown to participate in many of such events [1]. When lectins, which have multiple combining sites, bind to the cell surface saccharides, they interconnect the cells causing them to agglutinate or clump together. Lectins are widely distributed in nature, being found in animals, insects, plants and microorganisms. Microbial lectins however, remain the least investigated [2].

Very few yeast lectins have been studied in detail. The lectin from pathogenic yeast sps. *Candida albicans*, which causes candidiosis in humans is best studied. Extracellular adhesins of different sugar specificities such as that for fucose, N-acetyl galactosamine, mannose; from *C. albicans* have been reported [3]. Spectrum of biological activities of yeast lectins includes killer activities with respect to yeasts of different sps. The intracellular lectin of *Pichia anomala* exhibited anti-*Candida* activity on account of selective interaction with β 1,6-glucan of *C. albicans* [4]. Another important biorecognition between different sex yeast gametes is controlled in part by a yeast lectin sensitive to α-mannan [2].

Yeast flocculation, a nonsexual adhesion of cells is also a type of cell aggregation in which involvement of lectin-carbohydrate interactions has been suggested along with other nonspecific interactions. Flocculation specific lectin (Kb-CWL1) from *Kluyveromyces bulgaricus* has been purified. Recently its use in the studies of cell targeting by glycosidic telomers has been reported, wherein Kb-CWL1 was used as the receptor and *K. bulgaricus* cells as the target cells [5]. Flocculation in *S. cerevisiae* has been studied in detail but role of the lectins in flocculation is not clear. Different phenotypes of the flocculent *S. cerevisiae* strains have been described depending on their flocculation characteristics. Highly flocculent *S. cerevisiae* NCIM 3528 used in the present studies was characterised with respect to its flocculation phenotype. Its constitutive expression of flocculation, mannose specificity and ability to flocculate in broad

pH range indicates that it belongs to the Flo1 group described by Stratford and Assinder [6]. Flo1 group contains the strains having dominant flocculation genes *FLO1*, *FLO5* and *FLO8*. Strains containing *FLO1* and *FLO5* genes also exhibit different flocculation characteristics with respect to the strength of flocculation as well as temperature and protease sensitivity of flocculation [7,8]. *S. cerevisiae* NCIM 3528 is a strain isolated from brewery, the genetic constitution of which is not known and it exhibits typical *FLO5* characteristics such as relative resistance to chymotrypsin and heat sensitivity indicating that the strain most probably contains *FLO5* gene.

Effect of tunicamycin on flocculation has produced contradictory results, previously [9,10]. Tunicamycin is highly specific in its action, inhibiting the first step in the *N*-glycosylation of proteins viz. synthesis of dolichol- pp- *N*-acetyl glucosamine [11]. In case of *S. cerevisiae* NCIM 3528 the growth gets inhibited completely in presence of tunicamycin at 6 μg/ml. Even at concentrations as low as 0.1 μg/ml only 50% growth as compared to the control was observed. However, flocculation of the cells was found to remain unaffected at any of the subinhibitory concentrations tested apparently indicating that *N*-glycosylation of the proteins is not required for flocculation of *S. cerevisiae* NCIM 3528. However, in case of *S. cerevisiae* NCIM 3528 mannose specificity of flocculation suggested involvement of carbohydrate in flocculation. When further studies on purified cell wall lectin were carried out, the lectin showed specificity towards branched trimannoside, a core structure of *N*-glycosylated mannoproteins of yeast cell wall. This implies that the lectin most probably uses carbohydrate moiety of *N*-glycosylated proteins as carbohydrate receptors in flocculation, which indirectly asserts the essentiality of the *N*- glycosylation of proteins in flocculation. This apparent contradiction can be explained as follows : In presence of tunicamycin yeast cells have been reported to be arrested in G1 phase and *N*-glycosylation of the protein has been shown to be absolutely essential for growth of the cells [12]. Increase in the size of cells can occur but not cell division. Thus at subinhibitory concentrations of tunicamycin, the observed

increase in the cell number indicates that some of the cells might have escaped the inhibition of *N*-glycosylation of at least some proteins essential for growth, as the concentration of tunicamycin is not enough to affect all the cells. These cells with probable limited *N*-glycosylation show unaffected flocculation, which implies that *N*-glycosylation of only some of the proteins generates enough carbohydrate receptors for the lectin. Flocculation uses only a small proportion of the available receptors which has been already shown by Stratford [13]. In the study using *mnn* mutants (defective in cell wall mannan), it was reported that *mnn9* mutants inspite of having small number of mannan side branches were able to flocculate [13]. The limited *N*-glycosylation of the proteins in presence of tunicamycin thus would not be able to affect the flocculation of *S. cerevisiae* NCIM 3528.

S. cerevisiae NCIM 3528 is a constitutively flocculent strain and flocculation level of the cells reaches its maximum at the late exponential phase. The floc size increases and the flocs become more tightly packed. When the time course of the presence of active lectin on the cell surface was studied and compared with the time course of flocculation a correlation between amount of lectin present in the cell wall and flocculation level of the cells was found. The active lectin was found to be present in the cell wall during all growth phases, and its amount increased at the end of the exponential phase when flocculation reaches the maximum level. Similar correlation of presence of *FLO1* mRNA with flocculation level in Flo1 strain has been reported [14]. Recently Bony *et al.* [15] have shown the appearance of Flop in the cell wall of Flo1 as well as Flo5 strains to coincide with the flocculation level.

Involvement of lectin molecules in asexual adhesion of the yeasts has been suggested two decades ago. During the search of active lectin molecules involved in flocculation; detection, isolation and partial purification of some cell wall proteins specific to the flocculent strains has been reported. All these proteins fall in a molecular weight range of 13-67 kDa and have been isolated from Flo1 as well as NewFlo strains [16-23]. However, regarding the

agglutinating activity of some of these proteins no information is available and many of them have been isolated in an inactive form. The mannose specific lectin from the cell wall of *S. cerevisiae* NCIM 3528 has molecular weight of 40 kDa which falls in the above mentioned molecular weight range of flocculation specific proteins (13-67 kDa). As Stewart *et al.* [21] have suggested, the variations of molecular weights obtained for these apparently related flocculation specific proteins could be due to the variations in strains, as well as due to different experimental conditions. The lectin isolated in the present studies has a tendency to aggregate. Aggregation of the other lectin molecules isolated in the previous studies also has been reported [22,23]. The different molecular sizes of the flocculation specific proteins reported in the literature may be due to the aggregates of different sizes induced due to different extraction conditions [21]. However, the possibility of proteolytic cleavage of the proteins during extraction also cannot be ruled out.

One of the most interesting aspects of microbial agglutinins is their location in the cell. Frequently these appear on the surface of the cell, on specific organelles such as bacterial fimbriae or pili. The lectin isolated and purified in present studies is shown to be located on the cell surface. Yeast cell wall has been reported to contain low molecular mass SDS extractable proteins and high molecular weight glucanase extractable proteins (30-410 kDa) [24]. Low molecular mass detergent extractable proteins usually are retained in the cell wall most probably by strong association with chitin, chitosan and glucan, while glucanase extractable proteins are anchored to the cell wall by GPI anchor [24,25]. The extractability of the cell wall lectin with detergents alone indicates its noncovalent linkage with the cell wall, most probably through hydrophobic and van der Waal's interactions. The predicted amino acid sequence of Flop, the putative flocculin, assumes the presence of GPI anchor [26]. Recently Bony *et al.* [27] have suggested the possibility of Flo1p being a true cell wall protein, noncovalently attached to the cell wall with no GPI anchor.

In some cases, microbial agglutinins when extracted from the organism lose their multivalent property and cease to cause agglutination. This property can sometimes be restored by either crosslinking the extracted adhesins or by their immobilization to an inert surface or particle. The monovalent nature of the protein involved in flocculation has been suggested previously [21]. Many of the mannose specific bacterial lectins such as type 1 pili lectin of *E. coli* have been shown to be monovalent in nature. Isolated *S. cerevisiae* NCIM 3528 lectin however, has ability to agglutinate the rabbit RBCs indicating its multivalency. Since the molecules have a tendency to aggregate the possibility of generation of apparent multivalency cannot be denied which in turn results in the agglutination of rabbit RBCs.

The cell wall of *S. cerevisiae* consists of mannoproteins, glucan and chitin. Mannan in the cell wall is mostly in the form of mannoproteins and the linkages are α-1,2, α-1,3 and α-1,6. Heavily mannosylated proteins are composed of *N*-linked and *O*-linked mannose side chains. Purified *S. cerevisiae* NCIM 3528 lectin showed affinity to the core structure of the carbohydrate moiety of *N*-linked glycoproteins similar to the legume lectin Con A. The N-terminal domain of the putative *FLO1* gene product shares common features with legume lectins [14,28]. Flocculation of *S. cerevisiae* NCIM 3528 is inhibited by concanavalin A which can be relieved by the addition of methyl-α mannoside and this is consistent with the previous reports [29,30]. These observations indicate that the lectin involved in flocculation shares a common carbohydrate receptor with Con A. The cell wall lectin from *S. cerevisiae* NCIM 3528 showed high specificity and affinity towards the branched mannose trisaccharide. The inhibition of flocculation by the branched trimannoside provides direct evidence of involvement of the lectin in flocculation and strongly suggests that trimannoside could be the putative carbohydrate receptor of the lectin during flocculation. The fact that trisaccharide is a much better inhibitor than disaccharide or pentasaccharide of mannose indicates the possibility that the combining site corresponds to the size of trisaccharide. Con A has high affinity for the branched

trisaccharide of mannose but also is inhibited by the disaccharide of mannose [31]. The lectin however, is apparently insensitive to the inhibition by mannose disaccharide. Stratford *et al.* [13], who in an attempt to elucidate the receptor structure using *mnn* mutants has suggested outer linear chains of yeast mannan, two or three residues in length to be putative receptors of the lectin. Inhibition of the lectin by linear trisaccharides has not been checked in the present study, but lack of inhibition of the lectin by mannobioses is contradictory to the above results. However, it is also possible that the lectin although does not show high binding affinity towards soluble disaccharide it may bind to linear di and trisaccharides of mannose bound to the cell wall. The arithmetic increase in the sugar density on the cell surface leads to logarithmic increase of the binding affinity, which is more than can be accounted by the statistical increase in the sugar concentration and is termed as glycoside clustering effect [32]. In case of flocculation it is quite possible that although the isolated lectin does not show affinity towards soluble disaccharides of mannose at millimolar concentrations tested, in vivo, the disaccharides with which the yeast cell surface is almost bathed may exert glycoside clustering effect on the cell bound lectin.

Another class of mannose specific lectins, besides legume lectins, is bacterial surface fimbrial and nonfimbrial lectins. Fimbrial lectins from *E. coli* and other enteric bacteria are highly hydrophobic agglutinins in a molecular weight range of 18-22 kDa. Although all of them are generally termed as mannose specific their specificity towards mannose derivatives differs considerably from each other [33]. Teunissen and Steensma [34], based on the sugar specificities, have compared the lectins from Flo1 strains with *E. coli* pili lectins. Similar to pili lectins, the lectin from flocculent strain NCIM 3528 also is a hydrophobic lectin with about 44% hydrophobic amino acids and has a tendency to aggregate. Although both are mannose specific they differ in their oligosaccharide specificity. *S. cerevisiae* lectin shows affinity towards branched trimannoside unlike *E. coli* fimbrial lectin which is highly specific towards linear trisaccharide Man α-1,3-Man β-1,4-GlcNAc. *S. cerevisiae* lectin is not inhibited

by *p*-nitrophenyl mannoside at the concentration of 10 mM in contrast to *E. coli* pili lectin [33] but the possibility of inhibition at the higher concentration cannot be ruled out.

S. cerevisiae lectin is more closer to nonfimbrial mannose specific surface lectins from *S. marcescens* and *E. coli* with respect to molecular mass. Nonfimbrial lectins of bacteria are in the molecular mass range of 37- 39 kDa. Pili have not been detected in *S. cerevisiae* NCIM 3528 although it can be partly due to the drying procedure used during the preparation of the sample for electron microscopy. The difficult extractability of the lectin isolated in the present study as compared to that of bacterial and yeast pili however lowers the possibility of this lectin being a fimbrial protein. To date there are no reports of isolation of agglutinating activity associated with yeast pili. Purification of hydrophobic flocculin associated with pili of flocculent strain exhibiting NewFlo phenotype has however been reported [35]. This high molecular mass (300 kDa) heavily glycosylated flocculin has been proposed to be the putative Flo1p since it shows 69% N-terminal sequence homology with the *FLO1* gene. The protein was shown to be easily extractable from the cell wall unlike that suggested for Flo1p. This protein does not show any agglutinating activity and thus its role in the flocculation has been proposed as the flocculation ligand for the lectin. But this was contradicted since the receptors identified were *N*-linked mannan in contrast to the predicted heavy *O*-glycosylation of Flo1p [36]. In addition, the carbohydrate receptors were shown to be abundantly present in the cell wall at all times [6] but there is no genetic evidence for the constant existence of Flo1p in the cell wall [28].

Very recently however, the isolation of Lg-*FLO1* gene (a homologue of *FLO1* gene from NewFlo lager strain) has been reported and this gene has been shown to code for a glucose-mannose specific lectin [37]. This gene has been shown to have complete N-terminal sequence homology with the hydrophobic flocculin isolated by Straver *et al.* [35]. The replacement of the *FLO1* gene by Lg-

FLO1 gene changed the specificity of Flo1 strain making it glucose mannose specific indicating that the *FLO1* gene codes for mannose specific lectin [37].

S. cerevisiae NCIM 3528 is likely to be a *FLO5* strain as indicated by its strong, heat sensitive and chymotrypsin resistant flocculation. The sequence of *FLO1* and *FLO5* genes are 95% similar and the lectin purified from *S. cerevisiae* NCIM 3528 shows 70% N-terminal sequence similarity with both of these genes. This strongly indicates that the cell surface lectin involved in flocculation has been coded by one of the *FLO* genes. Moreover, the firm attachment of this lectin in the cell wall is indicated by the repeated extraction and is comparable to that described by Bony *et al.* [27] for cloned and expressed Flo1p. The lectin however, differs from the predicted *FLO1/FLO5* gene product with respect to size and amino acid composition. The possibility of post translational modification of the Flo1p has been indicated previously [27] and the reduced size of the lectin could be the result of post translational modification. However, chances of the proteolytic cleavage of the lectin during extraction procedure also cannot be denied.

Industrial importance of yeast flocculation, especially in ethanol fermentations, is well documented. The yeast strains usually used in industrial fermentations have a number of limitations such as inefficiency in fermenting substrate, variation in flocculation properties, limited control over the flavours produced, limited thermostability, ethanol and osmotolerance, contamination of fermentations etc [38]. Hence there is a constant need for the construction of the improved strains possessing better qualities for alcohol fermentations. However, industrial yeast strains are usually polyploid in nature and thus relatively resistant to physical/chemical mutagenesis. Sexual processes are not frequently available due to their homothallism and inability to sporulate [39] although these processes still prove to be beneficial for improvement of some of the strains [40-44]. Thus transformation or protoplast fusion are the methods of choice under such circumstances. Protoplast fusion is easy to perform, simple, cheap and rapid technique and allows the transfer of relatively large segments of DNA [45]. This

technique has been used to improve many industrial strains for different properties such as the ability to use wider range of carbohydrates, modification to the processes responsible for flavour production, introduction of killer property; introduction of flocculation or changes to the flocculation of yeasts, ethanol tolerance and thermotolerance [46-50].

The protoplast fusion essentially involves the removal of the cell walls so that protoplsts are formed in presence of osmotic stabilizer, aggregation and subsequent fusion of them under influence of fusogenic agent or strong electric field (electrofusion), regeneration of the fusion products on osmotically stabilized medium and selection of the fusants with desirable characters by applying suitable selection pressure [51]. Isolation and regeneration of protoplasts has been well established in case of *S. cerevisiae*. Use of novozyme and reducing agents for protoplasting has been suggested long back [52-54]. However some degree of standardisation with respect to each strain is required owing to the individual differences. In an attempt to standardize the procedure for protoplast isolation and regeneration we used *S. cerevisiae* NCIM 3458 since it is industrial strain, polyploid in nature. The pretreatment of the cells with DTT was found to be essential. The effect of different osmotic stabilizers on protoplast formation and regeneration was studied. Among various osmotic stabilizers used, 0.8 M NaCl was found to be the best osmotic stabilizer leading to completion of protoplasting of the cells within a very short time period (15 min) which in turn resulted into high regeneration frequency of around 80%.

Parental strains used in protoplast fusion are generally mutants with auxotrophic markers [55-63], antibiotic resistance markers [64,65], respiratory deficiency [66-68] and morphological markers [69,70]. Protoplast fusion involving inactivation or death of one of the parental strains also has been successfully applied. Protoplasts of one of the parental strains are rendered nonviable by heat treatment [71,72], antibiotic treatment [73] or UV radiations [74-76]. Chemical inactivation of yeast protoplasts prior to fusion by fungicide treatment also has been reported [77]. The ability of some strains to produce killer

toxins has been exploited for selection of fusion hybrids [78]. Recently, Palkova *et al.* [79] have described the killer plaque technique, a simple version of the selection procedure based on application of the killer toxins.

In the present study, the intention was to construct a yeast strain possessing both killer as well as flocculent phenotypes. Since no selectable markers were available on both strains, we thought of using dead donor technique [76] to construct the hybrid strains. Fusion was carried out between live protoplasts of flocculent strain and UV killed protoplasts of the killer strain using PEG as a fusogenic agent. In spite of obtaining high protoplast regeneration frequency the fusion frequency was low. Use of autoclaved PEG has been shown to reduce the fusion frequency [80]. However, the low fusion frequency obtained in this study also could be due to the formation of nuclear hybrids after the fusion of two polyploid strains. Nuclear recombination is rare when either of the parents is polyploid and the use of UV-killed protoplasts during fusion still lowers the possibility of nuclear fusion [76]. However, in the present study, the fusants were selected on medium containing benomyl, and thus the selection pressure was in favour of nuclear hybrids, benomyl being the nuclear marker.

Moderately flocculent fusants with a killer character were isolated. The tolerance of the parental flocculent strain to high molasses sugar concentration [81] remained unaltered in fusants as indicated by equivalent fermentation performances by all the four fusants even at 30 % molasses sugar concentration. Efficient fermentation at high molasses sugar concentration along with high initial ethanol productivities of the fusants indicated their good fermentation capabilities for ethanolic fermentation of molasses.

CONCLUSIONS

1. *S. cerevisiae NCIM 3528, a highly flocculent strain, was characterised with respect to its flocculation phenotype and it exhibits typical Flo1 phenotype such as constitutive expression of flocculation, sensitivity to mannose and flocculation in broad range of pH.*
2. *Flocculation of S. cerevisiae is sensitive to heat and broad specificity proteases but relatively resistant to specific proteases such as chymotrypsin, similar to the strains having FLO5 phenotype.*
3. *The cell wall lectin purified from S. cerevisiae NCIM 3528 is a 40 kDa polypeptide with a pI of 4.0.*
4. *It is mannose specific having high affinity and specificity towards branched trimannoside.*
5. *The purified lectin shows 70% N-terminal sequence homology with the FLO1 gene product.*
6. *Immunolocalization studies indicated the presence of the lectin on the cell surface.*
7. *Involvement of the lectin in flocculence of S. cerevisiae is supported by the following findings -*
   a. *Specific antibodies raised against the purified lectin could prevent the flocculatiom*
   b. *The strongest ligand of the lectin, a branched trimannoside, inhibited the flocculation*
   c. *There is a positive correlation between the lectin stability and flocculation of the cells under different environmental conditions.*
8. *The protoplast isolation and regeneration protocol, standardised using industrial yeast strain, leading to rapid release of protoplasts and improved regeneration frequency was achieved.*

9. ***Strain improvement of S. cerevisiae NCIM 3528 was carried out using protoplast fusion technique. The flocculent strain with a killer character was constructed.***
10. ***The fusants were capable of fermenting cane molasses with equal efficiency as that of the parental strains.***

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