Discussion
The incidence of life threatening mycoses caused by opportunistic fungal pathogens has increased dramatically in the recent years owing to the increase in the population of immuno-compromised patients with variety of underlying diseases such as AIDS, cancer, diabetes, etc and also due to advanced medical awareness leading to the use of exogenous immunosuppressive factors such as wide spectrum antibiotics, drugs in case of organ transplant, invasive surgery and other conditions. Among the opportunistic pathogens, *Candida albicans* remains the fourth leading cause of nosocomial infections worldwide causing considerable morbidity and mortality (5, 192). Basically a commensal, *C. albicans* can cause a variety of infections ranging from superficial muco-cutaneous infections to life threatening systemic infections (6, 7). The therapy of *C. albicans* infections is mainly hampered by the availability of relatively few antifungal agents, reduced efficacy of the available frontline antifungal agents due to the development of resistance by the fungus against them and also due to lack of reliable, specific and rapid diagnostic methods against this pathogen. Thus candidiasis remains a major social and clinical problem pressing the need for searching new fungal target molecules and novel diagnostic strategies for the prognosis. In the present work efforts were made to identify potential target molecule(s) of *C. albicans* with therapeutic and/or diagnostic value against candidiasis.

*C. albicans* is a very versatile pathogen and has a tremendous ability to survive in anatomically distinct sites each with its own specific set of environmental pressures. The fungus produces several virulence factors to overcome these pressures and establish in the host. These virulence factors include production of adhesins (to adhere to the host tissue), secretion of hydrolytic enzymes and host immuno-modulatory molecules, phenotypic and dimorphism dependent virulence traits, production of drug efflux pumps which confer drug resistance etc (13). Thus analysis of the proteins secreted by this pathogen during the course of infection may enable to identify potential molecules which could be attractive targets for the development of new therapeutic and diagnostic strategies.
Technically, proteins that are secreted in the *in vitro* medium by the pathogen are considered as secretory proteins (30). But, many proteins secreted by *C. albicans* reach cell wall where they co-exist with cell wall bound moieties contributing to the total cell wall proteins. There are many proteins that are cell wall associated in one condition but are secreted in another. N-acetyl-D-glucosaminidase is one such example which is cell wall associated in yeast form but is secreted during the germ tube formation (177). Phospholipase is cell wall associated when *C. albicans* is grown on yeast extract medium but has been found to be cell wall associated as well as secreted into the medium when cells were grown in contact with chorioallantoic membrane (31, 102). There are certain proteins which are thought to be cell wall associated but are recovered from the culture supernatant for example exo-β-(1,3)-glucanase, a β-glucan-branching enzyme and some mannoproteins (178, 193, 194). Thus there is an ambiguity in considering a protein to be called a cell wall or secretory protein. Cell wall of *C. albicans* is considered to be a very dynamic structure and important for virulence is the most explored structure for the development and identification of new target molecules. But secretory proteome still remains the underexplored area and due to the above mentioned reasons it is very difficult to recover all the secreted proteins. Also *C. albicans* secretes many hydrolases/proteases which result in the degradation of the other proteins that are secreted by it. Hence an alternative approach is required to recover the secreted proteins of *C. albicans*.

5.1. Utilization of proteins secreted by regenerating protoplast/spheroplast for the identification of potential target molecules

A protoplast is a cell devoid of cell wall with an intact cell membrane and a protoplast with traces of cell wall on its plasma membrane is called as a spheroplast. Both protoplast and spheroplast lack the rigidity provided by the cell wall and are vulnerable to the external environmental factors. These protoplast/spheroplast are capable of *de novo* synthesis of the cell wall when placed in an osmotically stabilized liquid nutrient medium. During the early stages of the regeneration process, proteins are secreted into the medium which include both secretory and cell wall proteins (32, 195). Thus
regenerating protoplasts/spheroplasts provides an excellent model not just to recover cell wall and secretory proteins but also these proteins are in their native conformation which enables their functional analysis. Also potential protein modifications of these proteins are prevented as they are collected from the growth medium and directly analysed for proteomic studies without the use of chemical agents or enzymes that may modify them. Some methods of extracting proteins from cell wall result in denaturing of the proteins rendering most of them inactive. This problem is also solved by the use of proteins produced by the regenerating protoplasts. Thus in the present thesis efforts were made to prepare protoplasts of both yeast and mycelial forms of *C. albicans* and recover proteins secreted by the regenerating protoplasts.

The cell wall of *C. albicans* is a complex structure composed of chitin (cellulose-like biopolymer consisting predominantly of N-acetyl-D-glucosamine), glucan (a β-linked branched chain polysaccharide of glucose), mannans (α-linked polymer of mannose), mannanproteins and glycolipids (30). For the efficient preparation of the protoplasts pre-treatment with a reducing agent (β-mercaptoethanol) was done to reduce the disulfide bonds of proteins and to release the loosely adhered proteins which increased the efficiency of protoplast formation. This increase in efficiency may be due to the exposure of β-(1-3) glycosidic bonds hydrolysed by the lysing enzyme which otherwise were present hidden in the complex structure of the cell wall. Microscopic observation during the protoplast formation showed that cells became translucent and organelles were clearly visible in both yeast and mycelial form as the protoplasting proceeded (Fig.1). Also the OD dropped rapidly during protoplast formation. Regeneration of the yeast protoplast was carried out in defined minimal media (YNB) containing sorbitol to maintain the osmolarity. In general, serum is, required for conversion and maintenance of *C. albicans* yeasts in mycelial form. Use of serum would jeopardise the objective of recovery of proteins secreted by the regenerating mycelial protoplast from the medium as serum itself contains innumerable proteins. Thus regeneration of the mycelial protoplast was carried out in regeneration medium containing N-acetyl-D-glucosamine which was used as a serum replacement. The
conversion efficiency of yeast to germ tubes in presence of N-acetyl-D-glucosamine was high (90%-95%) and was comparable to that in the presence of serum. During regeneration process of both yeast and mycelial protoplast proteins were secreted into the medium in the initial few hours and the protein quantity recovered from the regeneration medium dropped rapidly when they were incubated for longer period in the medium. This drop in the amount of protein may be attributed to the incorporation of the released proteins into the newly formed cell wall by the protoplasts. Optimum amount of proteins was recovered in 2h of incubation of the protoplasts in the regeneration medium.

SDS PAGE analysis of the proteins secreted by yeast cells and mycelial form showed significant difference in their profile (Fig. 2). Further analysis of these secreted protein by 2D gel electrophoresis exhibited significant difference in the protein profile (Fig. 4 and 6). Most proteins secreted by both regenerating yeast and mycelial protoplast resolved in a pl range of 4 and 7. Further analysis of the 2D gels by Melani software detected about 292 protein spots in mycelial secreted protein gel and 236 protein spots in yeast secreted protein gel (Fig. 4 and 6). The profile of the secreted proteins from regenerating protoplast not only differed considerably from their counterpart cell wall proteins but also in the number of protein spots of both yeast and mycelia which were significantly higher as compared to the protein spots detected in the cell wall protein profile of both yeast and mycelia (Fig. 6). About 188 protein spots could be detected in mycelial cell wall protein 2D gel and around 137 protein spots in yeast cell wall protein 2D gel (Fig. 5 and 7). This increase in the number of protein spots detected in the proteins secreted by regenerating protoplasts of both yeast and mycelia suggest that there may be many proteins which are not cell wall associated but are secreted into the medium. These differentially expressed protein molecules may include important virulent factors required for the establishment and survival of the pathogen within the host. Thus regenerating protoplast of both yeast and mycelial forms offers an excellent model for the identification of potential new target molecules for therapeutic as well as diagnostic purpose.
Superficial and subcutaneous infections caused by *C. albicans* are easily diagnosed but diagnosis of systemic infections caused by this pathogen is difficult. The clinical symptoms of systemic candidal infections are nonspecific and similar to those of bacterial and viral infections. Diagnostic strategies based on the isolation of fungi from clinical samples often require an invasive procedure that is not always feasible and are also unreliable often leading to false positive results due to the presence of other colonising commensal organisms, or ubiquitous fungi. Precise diagnosis of the causative agent is required in order to start an appropriate therapy that will not only help in controlling the causative organism but also avoid the side effects associated with wrong therapy and the development of drug resistance by the organism against the available limited antifungal agents. A diagnostic method must be rapid, easy to perform and easy to assess the results without any ambiguity and over and above it should be specific for a particular species or a genus or a group of similar pathogenic organisms. Since the development of hybridoma technology by Kohler and Milstein (171), monoclonal antibodies are being used in the diagnosis and therapy of many diseases and disorders. Diagnosis and therapy based on monoclonal antibody technology not only combines the specificity of the antibody but also allows integrating it with advanced reporting technologies like fluorescence, chemiluminiscence, radiography etc that increases the sensitivity.

Virulence based on morphological transition is one of the strategies employed by *C. albicans* to counteract host immune system and to establish it within the host. Hyphal form is considered to be more virulent compared to yeast form or pseudohyphal form (26, 111). The profile of the proteins secreted by the regenerating mycelial protoplast not only differed significantly from that of yeast secreted proteins but also the number of protein spots was higher. The mycelial secreted proteins may include many virulent factors required for its survival and establishment which could be very good targets for the development of therapeutic and/or diagnostic monoclonal antibodies. Hence in the present study an attempt was made to generate and screen hybridomas.
producing monoclonal antibodies against proteins secreted by the regenerating mycelial protoplasts of *C. albicans* with therapeutic and/or diagnostic potential.

Antibody isotype is of immense importance in defence against fungal pathogens and its efficacy to clear fungal infection *in vivo*. In general antibodies of the isotype IgM, IgG₁ and IgG₃ are known to be protective against *C. albicans* (28). Although monoclonal antibody of IgM isotype is more efficient in clearing antigens and activation of complement system it has some disadvantages. Because of the robust nature of the antibodies of this isotype (IgM is a pentamer) it does not diffuse easily. Also IgM is the first immunoglobulin class produced in a primary response to the antigen by the B cells. The chances of the clones producing IgM isotype undergoing class switching are very high. This is the reason why IgM accounts for only 5-10% of total serum antibodies *in vivo*. Alternatively MAbs of the isotype IgG account for about 80% of the total serum antibodies and are very stable. Therefore most diagnostic techniques are based on IgG isotype. The immunization schedule has tremendous influence on the isotype of the resulting monoclonal antibodies. Longer immunization schedule results in the IgG isotype, while a shorter schedule results in IgM isotype. In this study longer immunization schedule of 60 days was followed seeking clones producing the MAbs with stable isotype.

In the present study primary screening after fusion resulted in the identification of 98 clones which were ELISA positive against proteins secreted by regenerating mycelial protoplasts. The number gradually decreased in subsequent screenings and only two stable clones (1A1 and 1C1) could be obtained which were consistently producing antibodies. Many factors are responsible for this low percentage of positive hybridoma production. The resulting hybrid cells are supposed to be in a state of shock and fragile and the first 10 days are most critical for the survival of the hybridomas. Further, the ploidy of the fused cells increases (either tetraploid or hexaploid) after fusion and therefore there will be chromosomal rearrangement within first few days and many of these chromosomes are lost
due to which cell death, cessation of cell growth and loss of ability to synthesize immunoglobulin takes place resulting in "non-producing" hybrids. In the present study single cell cloning was performed to get rid of these non-producing hybridoma clones. Experiments to identify the isotype of the MAbs produced by these clones revealed that MAb 1A1 was of the isotype IgG\(_1\) and MAb 1C1 was of the isotype IgA. Both the MAbs possessed light chain of the isotype "k" (Fig. 9). Preliminary characterization by Western blotting revealed MAb 1A1 cross reacted with two proteins of molecular weight \(~46\text{kDa}\) and \(~36\text{kDa}\). MAb 1C1 also showed similar banding pattern as that of MAb 1A1 and they were the same proteins (Fig. 8). Hence further characterization of the monoclonal and studies were carried out using MAb of the isotype IgG\(_1\) (MAb 1A1).

5.3. Characterization of MAb 1A1 for therapeutic and diagnostic potential

Further work was carried out for the characterization of MAb 1A1 to establish its potential to be used for either therapeutic or diagnostic purpose. Cross reactivity studies against cell wall and proteins secreted by the regenerating yeast protoplasts showed that the antigens recognized by the MAb 1A1 were also present in yeast form of \textit{C. albicans}. In addition, the study also showed that both the antigens were present in the cell wall proteins of mycelial form of \textit{C. albicans} (Fig. 10). This indicated that the antigens were constitutively expressed in the fungus and could be utilized as a marker molecule for the diagnosis of the pathogen. In order to check the diagnostic potential of MAb 1A1 cross reactivity studies were carried out with different strains of \textit{C. albicans}. Western blot analysis showed that both the antigens were present in all the strains tested. However the lower molecular weight protein (~36kDa) could be detected in very low amounts in two strains of \textit{C. albicans} (Fig. 12). It is a well know fact that most yeast proteins are very vulnerable and get degraded easily. The multiple bands seen in the Figure 12 may be the degradation products of one or both the proteins recognized by the MAb 1A1.
As mentioned before a diagnostic method should be specific for a particular species or a genus or a group of similar pathogenic organisms. In order to check the inter-species and inter-genus specificity of the MAb 1A1 Western blot study was carried out against different fungal species. Both the antigens recognized by MAb 1A1 were present in both the pathogenic yeasts tested (Candida parapsilosis and Cryptococcus neoformans). The MAb 1A1 did not cross react with any of the mycelial fungi (Aspergillus fumigatus, Trichophyton mentagrophytes, and Sporothrix schenki) (Fig. 13). Negative Western blotting results for the mycelial fungi may either be due to the absence of the antigens recognized by the MAb 1A1 in these fungi or due to the absence of epitope recognized by the MAb 1A1 on these proteins even if they are present. The studies clearly indicated that the MAb 1A1 has a broad spectrum capacity of recognising fungal species and may be utilized in the diagnosis of yeast like pathogenic fungi. C. albicans is a common commensal found coexisting with other organisms especially bacteria in normal healthy human beings. In order to check the specificity, cross reactivity studies were carried out against both Gram positive (Staphylococcus aureus, Bacillus cereus) and Gram negative (Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa) bacteria. MAb 1A1 did not exhibit any cross reactivity with any of the bacteria tested indicating its specificity towards fungi.

Many of the serological diagnostic procedures available today are based on indirect method of detection of antibodies produced by the host against various metabolic proteins of the pathogen (22). These proteins are highly immunogenic and elicit a strong humoral response resulting in the production of antibodies by the patient’s immune system which can be detected in vitro by various techniques. However the sensitivity of diagnostic methods based on indirect serological detection of antibodies is very low owing to the fact that many patients with systemic fungal infections are immuno-compromised and have an impaired antibody response. Even in immuno-competent individuals, early diagnosis of systemic infection is not possible because of the time gap required to mount a formidable immune response since the time of infection. A diagnostic method based on the direct detection of the antigens secreted by
the fungal pathogen in patients during the course of infection is considered more efficient than the indirect method. The specificity of monoclonal antibodies is often employed in the direct detection of the antigens secreted by the pathogen. In this study in vivo diagnostic potential of the monoclonal antibody 1A1 was assessed in a mouse model of C. albicans. MAb 1A1 was used to detect the antigens secreted during the experimental course of infection by ELISA method. This method involves the use of enzyme conjugated secondary antibody specific for mice as the reporter system. Since the model of study was a mouse, the secondary antibodies would react with the antibodies present in the serum of even healthy animals and give false positive results. To overcome this problem MAb 1A1 was biotinylated and the antigens were detected using streptavidin conjugated with the reporter enzyme. Though the detection efficiency of MAb 1A1 was only 67% which is an average figure, MAb 1A1 did not give false positive results with control mice without any infection. The detection percentage can be improved by increasing the size of the sample. In another study an effort was made to detect the antigens recognized by the MAb 1A1 in the serum of patients positive for candidiasis. The results were encouraging and showed 77.78% efficiency. This percent efficacy could have been much higher if newer cases of candidiasis could have been included in the study. In the present study, many of the patient sera were kept under frozen condition for a long period and there are possibilities that the antigens got degraded during storage. MAb 1A1 did not give any false positive reactions with the sera of the healthy individuals. This was particularly important because C. albicans is a common commensal present even in healthy individuals.

Many monoclonal antibodies are reported to exhibit protection against C. albicans in vitro as well as in vivo (172, 173). Mycograb (NeuTec Pharma plc.) is a human genetically recombinant antibody against fungal Hsp90 which has entered clinical trial phase. This MAb shows activity against a wide range of yeast species and acts synergistically with amphotericin B both in vitro and in vivo (175). MAb 1A1 was also studied for its ability to confer protection in vitro. Adhesion of C. albicans to the host tissue is considered to be the first
step in the infection process. *C. albicans* produces many molecules of biological importance which help in adhesion of the pathogen to the host and thus contribute to the virulence exhibited by the pathogen (13). *C. albicans* exhibits intrinsic property of adhesion to composite material like polyvinyl chloride and poly carbonate plates. MAb 1A1 did not inhibit adhesion of *C. albicans* to the surface of the poly carbonate plate. Percent inhibition exhibited by cells treated with MAb 1A1 was only 19.35% (Fig. 14). Though the inhibition percentage was little higher compared to that seen in control (13.35%) it was lower compared to that of irrelevant antibody (28.5%). Therefore it may be concluded that the proteins recognized by MAb 1A1 are either loosely present on the cell wall or they are not directly involved in adhesion or both. MAb 1A1 was also tested for its ability to inhibit germination of yeast cells into mycelia which is one of the important virulence factors. But MAb 1A1 did not inhibit germination of yeast cells into mycelia. The results of this study were also in consensus with the fact that the MAb did not have any killing activity on *C. albicans* as indicated by MTT assay and killing assay tested for the ability of MAb 1A1 kill *C. albicans* as indicated by the reduction in the number of colonies. Since no direct evidence of protective role of MAb 1A1 was observed in the above *in vitro* experiments further evaluation of protective/therapeutic role of this MAb *in vivo* was not considered of worth in the present study.

Under *in vivo* conditions antibodies exhibit innumerable functions such as neutralization of the toxins secreted by the pathogens, activation of the complement, participation in antibody dependent cell cytotoxicity, opsonisation of the pathogens along with the activated complement. Among the host factors to counteract the invading pathogen, phagocytosis by mononuclear phagocytic cells such as macrophages, monocytes, etc. is considered to be an important step. Antibodies especially of the isotype IgG1 and IgG3 exhibit high affinity towards the Fc receptors on the phagocytes and thus play an important role in opsonisation mediated phagocytosis (196). Since MAb 1A1 turned out to be of the isotype IgG1 its ability to opsonise *C. albicans* yeast cells and germ tubes and enhanced phagocytosis by mouse
macrophages was studied using J774 cell line. The process of phagocytosis could be seen within an hour of co-culturing of the MAb treated and PBS treated cells with the macrophages. Marked enhancement of phagocytosis could be noticed in case of MAb 1A1 treated cells compared to that of PBS treated cells (Fig. 16). About 89% phagocytosis was observed in case of yeast cells treated with MAb 1A1 when compared to control where only 27% was seen. In case of germ tubes treated with MAb 1A1, 75% phagocytosis was noticed when compared to that of the PBS treated cells (36%). Thus MAb 1A1 contributes to the protection against *C. albicans* in an indirect way by enhancing phagocytosis (Fig. 16). The study also indicated that the antigens recognized by the MAb 1A1 were present on the surface of the pathogen and were not hidden in the complex structure of the cell wall.

Proteins with distinct functions exhibit a distinct localization pattern. Some proteins are only found at the tip of actively growing germ tubes and mycelia, while others are differentially expressed in either of the morphologies. Western blot studies indicated that the proteins recognized by MAb 1A1 were present in all the morphological forms. In order to localize these proteins on the surface of different morphological forms of *C. albicans* epitope localization studies were carried out. Localization studies indicated the presence of the antigens in yeast form, germ tubes and also on the surface of the mycelia (Fig. 17) which is in consensus with the Western blotting results. The antigens were found to be present uniformly on the surface of all the morphologies and in abundance. These localization studies also indicates that the antigens are expressed during morphological transition as was evident by their presence in abundance and uniform distribution on the surface of the newly emerging germ tubes (Fig. 17B). This implies that the antigens are constitutively expressed and might have an important role in the cell integrity or survival of the pathogen. This observation also complements the phagocytic studies as above. The enhanced phagocytosis after opsonisation of the yeast cells and germ tubes by MAb 1A1 may be because of the abundant availability of the antigen on the surface of all the morphological forms of *C. albicans*.
5.4. Purification, identification and characterization of the antigens recognized by MAb 1A1

Many protein purification methods are available based on different principles such as physical and chemical properties of proteins like size, charge, hydrophobicity, polarity or affinity for other molecules. There are various modes for column chromatography of proteins, including ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), size exclusion (SEC), and affinity chromatography (AC). Among these methods, purification of proteins using affinity chromatography allows the possibility of obtaining several fold purification with high recovery in fewer steps. Antibody based affinity chromatography has long been employed for the purification of bio-molecules due to the unique specificity that they display (197). Monoclonal antibody 1A1 is unique in the sense that it recognizes two different proteins having an epitope which is similar at least in part. In the final part of the study an attempt was made to purify both the antigens recognized by the MAb 1A1, identify them and characterize them partially. Purification of the antigens recognized by MAb 1A1 would enable to understand better the importance of the monoclonal antibody (whether it can be used for diagnostic or therapeutic purpose) and also to characterize the purified proteins at least partially.

Preparation of affinity column of MAb 1A1 required purification of the MAb as both the sources of the MAb viz., culture supernatant and ascites contained large amounts of other protein molecules which may exhibit affinity towards non specific proteins of *C. albicans* hampering the purification of the intended antigens. Purification of the MAb 1A1 was carried out using Protein G column. Protein G is a bacterial protein expressed on the surface of the bacteria *Streptococci* spp. which binds tightly to Fc region of an antibody without interfering with the antigen binding sites (198). The affinity of the protein varies widely depending upon the species of origin of the antibody. Protein G exhibits an affinity for IgG class in the range of $10^8$ – $10^{10}$ M$^{-1}$ (199, 200), depending on species and subclasses. The protein exhibits high affinity towards the mouse antibody isotype IgG$_1$ at neutral pH. The interaction of protein G with the antibodies of isotype IgG is very strong and is very difficult
to break under normal conditions. A strong denaturation step of either low pH or very high pH conditions are required to break the interaction of the antibody with protein G. The eluents were brought to neutral conditions immediately as such extreme conditions may denature the MAb permanently resulting in loss of activity.

MAb 1A1 affinity column was prepared by coupling purified MAb to cyanogen bromide activated sepharose. Cyanogen bromide reacts with hydroxyl groups on sepharose to form reactive cyanate ester groups. Proteins, peptides, amino acids or nucleic acids can be coupled to CNBr-activated sepharose, under mild conditions, via primary amino groups or similar nucleophilic groups. The activated groups react with primary amino groups on the ligand to form isourea linkages. A multipoint coupling reaction occurs between the MAb and CNBr activated sepharose spontaneously. Though the multi-point attachment ensures that the ligand does not hydrolyze from the matrix, it compromises with the number of sites available for the binding of the antigen molecules as coupling of antibody to the activated sepharose may also occur at the hypervariable region of the antibody responsible for antigen binding. Purification of the antigens recognized by MAb 1A1 was carried out using proteins secreted by regenerating mycelial protoplast. Only one protein of low molecular weight (~36kDa) could be purified using CNBr coupled MAb 1A1 (Fig. 19A). The purified low molecular weight protein was the same protein recognized by MAb 1A1 as confirmed by Western blot study.

Purified low molecular weight protein resolved into four separate spots on further analysis by 2D gel electrophoresis. These protein spots were of same molecular weight but differed in their pl (ranging between 6 and 7) (Fig. 21). Efforts were made to identify the protein spots by N-terminal protein sequencing. N-terminal sequencing of intact proteins can be employed to identify the amino acid sequence at the N-terminal of the protein. A short sequence of 5-15 amino acids, named protein N-terminal sequence tag, can be obtained from the N-terminus of the intact protein by mechanized Edman degradation method. Owing to the specificity of these tags, it is possible to identify the proteins of the organism by using bioinformatic tools such as
BLAST (Basic Local Alignment Search tool). For N-terminal protein sequencing the purified protein was blotted onto PVDF membrane (PVDF membrane is compatible for N-terminal sequencing) after SDS PAGE and band was excised after amido black staining. Since the purified protein was a mixture of four proteins, the spots were also blotted onto PVDF membrane after resolving by 2D gel electrophoresis and three distinct spots were processed to obtain N-terminal protein sequence. The individual sequences obtained by N-terminal sequencing were subjected to BLASTp search in order to identify the proteins. All three protein spots from 2D gel blot and the single protein band were identified to be the isoforms of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *C. albicans* (Accession no Q92211). Clustal W analysis showed that the N-terminal sequences of these spots matched with *C. albicans* GAPDH sequence at N-terminal with high scores ranging between 80 and 100.

Glyceraldehyde-3-phosphate dehydrogenase is a glycolytic protein (EC 1.2.1.12) of 331 amino acids expressed by *TDH1* gene in *C. albicans*. Orf19.6814 is a putative gene sequence present in the genome of *C. albicans* encoding GAPDH. At least three isoforms in *S. cerevisiae* (*TDH1*, *TDH2* and *TDH3*) and one in *S. pombe* (*GPD3*) of this gene are known to be present. Although only one active gene of GAPDH is present in *C. albicans*, several isoforms of this protein are known to exist which have the same molecular weight but differ in their pI. The variation in pI of these isoforms is because of the post translational processing of the protein molecule. The pI of isoforms of GAPDH in *C. albicans* varies between 6.31 and 7.4 (Compluyeast 2D-PAGE database) and their known molecular weight is 35.5 kDa. The observed molecular weight of the purified protein (~36kDa) and the pI (6-7) of these spot on 2D gel electrophoresis were comparable to that of the actual values mentioned in the database available.

GAPDH is a constitutively expressed protein present in cytoplasm as well as on the cell surface in the cell wall of all the morphologies of *C. albicans* viz., yeast, pseudomycelia, and mycelia (201). This enzyme is known to be present even in mycelial fungi and also in some bacteria (202). This is in
contrast to the observations in cross reactivity studies which suggest that the epitope recognized by MAb 1A1 is not present in the mycelial fungal GAPDH as well as the bacterial GAPDH. *C. albicans* GAPDH is also secreted during the course of infection and is a major protein found circulating in the serum of the infected individuals. Thus, it acts as a perfect marker molecule to be targeted for diagnostic purpose. GAPDH is also known to promote adhesion of *C. albicans* to host tissues by binding to laminin and fibronectin (203). MAb 1A1 did not inhibit binding of *C. albicans* to the surface of the polycarbonate plates although one of its target antigens is GAPDH. This is because GAPDH binding is specific for laminin and fibronectin proteins. Another reason may be that the epitope recognized by the MAb 1A1 may not be involved in binding to surfaces in *in vitro* conditions. It is well known that GAPDH is abundantly present on the surface of *C. albicans* yeast and mycelia and this fact could be proved in the present phagocytosis studies as well as epitope localization studies (Fig. 16 and 17).

The protein purification process using MAb 1A1 using protein G column yielded only the low molecular weight protein although MAb 1A1 recognized two protein bands. The high molecular weight protein recognized by this MAb could not be purified by this method. One reason may be that purification of MAb 1A1 using protein G column was done under low pH conditions this may have resulted in the loss of affinity of this MAb towards the antigen of high molecular weight (~46kDa). Antibody purification under extreme pH conditions induce structural changes in the antibody which may increase or decrease the affinity of the antibody towards its antigen. Another reason may be that the epitope recognized by MAb 1A1 on the protein may not be available for binding under native conditions. This assumption is based on the fact that only one protein band of low molecular weight protein was detected (Fig. 11) in Western blot studies on native proteins. Further, the epitope may be present hidden inside the protein folds which may be exposed for MAb 1A1 binding only under denatured conditions. Direct covalent coupling of MAb 1A1 to protein A was done in order to rule out the possibility of structural changes and loss of affinity of the MAb 1A1 towards the high molecular weight protein.
by purification under extreme pH conditions. Protein A is a bacterial protein isolated from *Staphylococcus aureus* which in general has very low affinity for mouse monoclonal antibody of the isotype IgG that increases with the increase in salt concentration. At very high salt concentration (3 M) protein A has the affinity as exhibited by protein G (197). Protein A also binds to the Fc region of the antibody. After binding of the MAb 1A1 with protein A, covalent coupling was performed using dimethylpimelimidate. The antigen of high molecular weight could be purified from denatured proteins (Cell wall protein extracted by SDS) in the presence of high salt concentration (Fig. 22). Thus it is clear that the epitope recognized by MAb 1A1 is present hidden in the native form of high molecular weight protein. Western blotting studies confirmed that this purified protein was the same protein recognized by MAb 1A1 (Fig. 23).

One of the virulence trait exhibited by *C. albicans* is immunomodulation of the host during the process of infection which enables it to escape killing by the cells of host immune system and establish itself within the host. A common mechanism employed by *C. albicans* to overcome host immune responses is suppression of pro-inflammatory cytokine production (Th1 response) and promoting the production of anti-inflammatory cytokines (Th2 response). *C. albicans* produces many biomolecules in to the blood stream of the patient during the course of infection. Many of these molecules are known to participate in the immunomodulatory functions. Mannan and mannoproteins, β-glucan, mannoproteins, phospholipomannan and recently concluded glycoprotein, CA-SIIF have been shown to be immunomodulatory and result in Th2 response leading to immunosuppression (131-133, 204). The cytokine responses of many molecules produced by *C. albicans* are not known. Since the antigens recognized by MAb 1A1 are immunodominant proteins and are released into the serum during the process of infection, the immunological responses of the host against these antigens was studied by measuring the cytokine responses against these proteins. Three Th1 cytokines (TNF-α, IFN-γ and IL-2) and two Th2 cytokines (IL-4 and IL-5) responses of these antigens were measured using splenocytes (T-cells, B-cells and phagocytic cells).
Discussion

Spleen is a secondary lymphoid organ which traps antigens directly from the blood and it is the place where extensive immunological activity such as antigen recognition, antigen processing and the release of cytokines of the type Th1 or Th2 takes place.

PAM$_3$CSK$_4$ is a selective agonist of potent stimulant of TLR2 receptors inducing Th1 response (205). Both the antigens elicited TNF$_\alpha$ response in a dose dependent manner (Fig. 24). TNF$_\alpha$ promotes growth and differentiation of T cells and activation of host immune system against the invading pathogens. In the present study the response of TNF$_\alpha$ was found to be more against antigen 1 (~46kDa protein) compared to that of antigen 2 (GAPDH). Further, a very high response of TNF$_\alpha$ was noticed in case of PAM$_3$CSK$_4$ which is a potent stimulant of TLR2 receptors (Fig. 24). IFN$_\gamma$ activates macrophages and NK cells which are very effective against the invading pathogens. The response of IFN$_\gamma$ was also found to be in a dose dependent manner for PAM$_3$CSK$_4$ and GAPDH but in case of antigen 1 this response decreased initially and subsequently increased with concentration. However, IFN$_\gamma$ response of GAPDH was found to be higher than that of antigen 1 where as no IFN$_\gamma$ was noticed in case of untreated control cells (Fig. 25). IL-2 is considered to be a cytokine required for the proliferation and clonal expansion of the T cells. In the present study IL-2 response varied with the concentration of the antigens, the response was higher in case of antigen-1 and almost equal in case of GAPDH compared to PAM$_3$CSK$_4$ (Fig. 26). Interleukin-4 and interleukin-5 are considered to be cytokines of Th2 type. In this study the responses of IL-4 and IL-5 varied with the concentration of the antigens and the overall response was comparable to that observed against PAM3CSK4. The response of the ~46kDa antigen was almost similar for both the interleukins where the response increased with the concentration and decreased as the concentration increased further. This response was marked in case of IL-4 and also the interleukin production was higher compared to PAM$_3$CSK$_4$ at 10 $\mu$g concentration (Fig. 27). In case of IL-5 the decrease in the response was not significant as seen in case of IL-4 at 15 $\mu$g concentration but was comparable to the response of PAM$_3$CSK$_4$ (Fig. 28).
GAPDH exhibited a concentration dependent response in case of IL-4 and the response was higher compared to PAM₃CSK₄ (Fig. 27). However IL-5 concentration first decreased and then increased with concentration for this antigen. The response of IL-5 at 15 μg concentration of GAPDH was found to be very high in the present study (Fig. 28). It can be concluded that the overall response of interleukin production by the splenocytes against these antigens is of Th1 type which is similar to that against PAM₃CSK₄. Therefore it may be concluded that the antigens are activators of the host immune system eliciting a protective response. This is also supported by the fact that circulating antibodies are found in the serum of the patient infected with C. albicans against GAPDH (30).

Conclusion

In the present study protoplast of both yeast and mycelial forms of C. albicans were prepared and proteins secreted by these protoplasts during their regeneration process were compared with the cell wall proteins in order to find the differentially expressed proteins which could be secretory proteins. The analysis of these proteins was carried out using 2D gel electrophoresis which showed a tremendous difference in the protein profile of secreted proteins by regenerating protoplasts of both yeast and mycelia from their cell wall counterparts. Large number of protein spots could be detected in 2D gels of protein secreted by regenerating protoplasts compared to cell wall proteins and the profile of proteins secreted by yeasts and mycelia differed significantly. Thus it is clear that regenerating protoplasts offer an excellent model for the recovery of both cell wall and secreted proteins which are not only in their native form but are also free from modifications. Further the proteins secreted by the regenerating mycelial protoplast (virulent form) were used to generate hybridomas producing monoclonal antibodies against these proteins with therapeutic or diagnostic potential. Two stable hybridoma clones designated MAb 1A1 and MAb1C1 (IgG₁ and IgA isotype respectively) were obtained from the fusion experiment. Initial characterization of these MAbs showed that they both recognized the same proteins (two proteins of ~36kDa and ~46kDa) and hence further studies were carried out using MAb 1A1.
Discussion

(subclass IgG1). Western blot analysis showed that the proteins recognized by MAb 1A1 is a constitutively expressed proteins and are present in all yeast pathogens tested but not in the pathogenic mycelial fungi. Thus it has a broad pathogenic spectrum and could be effectively utilized for diagnostic purpose. MAb 1A1 did not show any in vitro protective efficacy therefore further evaluation for protection under in vivo conditions was not considered. The monoclonal MAb 1A1 exhibited diagnostic potential (66.7%) in in vivo experiments which could be enhanced by increasing the population size. The MAb 1A1 exhibited a good potential in in vitro screening (72.4%) of Candida positive patient sera for the presence of antigens recognized by the MAb. Purification of the proteins was carried out by affinity chromatography using MAb 1A1. N-terminal sequencing of lower molecular weight protein recognized by the MAb 1A1 was found to be GAPDH which is considered to be a virulent factor and secreted in to the blood stream of the patient during the course of infection. Cytokine studies showed that both GAPDH and the high molecular weight protein promote Th1 type response in mouse splenocytes.