Results
4.1. Conversion of yeast cells into mycelia

*C. albicans* yeast cells from stationary phase readily germinated into mycelia. Germ tube formation was seen in nearly 50% of the yeast cell population within 30 min in minimal media containing N-acetyl-D-glucosamine. Approximately 90%-95% of the yeast cells were converted into mycelia 16h post incubation at 37°C. The conditions such as inoculum size, pH of the media, incubation temperature, serum or serum replacement additives etc affected the conversion of the yeasts into mycelia. Mycelial cells exhibited greater adherence to glass or tissue culture flask surface compared to the yeast cells.

4.2. Protoplast preparation and their regeneration

Protoplast of yeast and mycelial forms were prepared using CelLytic™ Y Plus kit. A pre-treatment step for the reduction of disulfide bonds of the proteins present on the surface of the cell wall was performed which increased the efficiency of protoplast formation. For every 1 g of yeast/mycelial cells, 25 µl of lyticase enzyme was required in case of *C. albicans*. When observed under a phase contrast microscope the yeast cells/mycelia became translucent as the protoplasting proceeded, the cells gradually lost their shine and organelles became clearly visible. The cells also started flocculating to each other as the plasma membrane was exposed (Fig. 1). The protoplast formation was recorded spectrophotometrically where the OD values dropped by approximately 10% (from 0.169 to 0.03 approx.).

During the regeneration process proteins were secreted into the regenerating media for initial few hours. Further incubation of the yeast/mycelial protoplast in the regeneration media resulted in the formation of the cell wall as was evident by microscopic observation where the cells regained their shine and cells started becoming opaque. Protein concentration in the media decreased with the increase in regeneration time. A marked difference in the profile of the yeasts as well as mycelial secreted proteins was observed in SDS-PAGE (Fig. 2).
Fig. 1. Protoplast formation in *C. albicans* yeast cells and mycelia A. Normal yeast cells B. Yeast protoplast C. Mycelia, D. Mycelial protoplast. Clear difference in morphology could be seen in the protoplasts (yeast cells/mycelia) when compared with the normal cells. Protoplasts are less shining, more translucent, organelles are clearly visible.

Fig. 2. Proteins secreted by regenerating protoplasts on SDS-PAGE 1. Regenerating yeast protoplast secreted proteins, 2. Regenerating mycelial protoplast secreted proteins
4.3. Cell wall preparation and protein extraction

For cell wall preparation, various methods were followed such as multiple cycles of freeze and thaw, sonication, grinding in presence of liquid nitrogen, vortexing with glass beads on a common vortex mixture, and vortexing in bead beater. Cell breakage was more efficiently achieved with bead beater. The lysate was observed under phase contrast microscope and no intact cells were found after 10-12 cycles of beating in bead beater. A part of this lysate was also inoculated on SDA plate where no colonies were observed after 48 h of incubation at 28°C. No degradation of protein was noticed when the cell walls were stored at -80°C.

4.4. Protein estimation using 2D Quant kit

Protein quantification for all the experiments was done using 2D Quant kit. The procedure works by quantitatively precipitating proteins while leaving interfering substances in the solution. The assay is based on the specific binding of copper ions to protein. The unbound copper was measured with a colorimetric agent. The quantity of protein in the sample solution was calculated using the standard graph generated (Fig. 3). The colour density is inversely proportional to the protein amount in the sample solution.

**Fig. 3. Standard curve for protein estimation using 2D Quant kit**
The protein amount estimated in the samples is mentioned later in their respective sections.

4.5. 2D gel electrophoresis

Proteins secreted by regenerating protoplasts of yeast and mycelial form and their cell wall proteins were separated by 2D gel electrophoresis and silver stained to reveal the protein spots. Sample preparation included a protein precipitation step using TCA to remove sorbitol and other substances which otherwise would interfere with isoelectric focusing of the proteins. There was a clear difference in the profile of proteins secreted by the regenerating protoplast when compared with the cell wall protein profile in both yeast and mycelia. Also the proteins secreted by the regenerating protoplasts of yeast and mycelia varied significantly. The gels after silver staining were analyzed using Melani 2D gel analysis software. A total of 236 spots were detected in 2D gel of proteins secreted by regenerating yeast protoplasts (Fig. 4). The number of spots was significantly higher compared to that of yeast cell wall proteins the 2D gel of which showed only 137 spots (Fig. 5). Similarly, 2D gel of proteins secreted by regenerating mycelial protoplasts showed 292 protein spots (Fig. 6) which were also significantly higher than the number of spots in yeast cell wall protein 2D gel which showed 188 spots (Fig. 7). In all the 2D gels maximum spots were concentrated in a pI range of 4.0 and 7.0.

4.6. Generation of hybridomas and production of monoclonal antibodies

Fusion experiment for hybridoma production was carried out using spleen cells obtained from immunized mice (with proteins secreted by regenerating mycelial protoplast) and Sp2/O myeloma cell line. Multiplication of the resulting hybridomas in the selective medium was observed from day three. On day seven, screening for Candida-specific monoclonal antibody producing hybridoma cells was started using ELISA method, and continued till day eleven. At this stage clones appeared in rosette form, looking like a bunch of grapes. From this first round of screening 98 clones were identified that produced monoclonal antibodies against cell wall proteins of C. albicans.
Fig. 4. Silver stained 2D gel of proteins secreted by regenerating yeast protoplasts. A total of 236 spot were detected in the gel.

Fig. 5. Silver stained 2D gel of cell wall proteins of *C. albicans* yeast form. A total of 137 spots were detected in the gel.
Fig. 6. Silver stained 2D gel of proteins secreted by regenerating mycelial protoplasts of *C. albicans*. A total of 292 protein spots were detected.

Fig. 7. Silver stained 2D gel of cell wall proteins of *C. albicans* yeast form. A total of 188 protein spots were detected.
These clones were further sub cloned and re-examined by ELISA. Out of these, many clones lost their antibody producing capacity. After 3-4 rounds of sub-culturing and confirming by ELISA, eight clones were identified which were consistently producing monoclonal antibodies. All the other non-antibody producing clones were discarded. The selected clones were subjected to single cell cloning in order to separate the antibody producing and non-producing clone. Finally two positive clones which were producing the MAb’s consistently were identified and designated as 1A1 and 1C1. These two hybridoma clones were propagated in 24 well culture plates and finally expanded in 50 ml tissue culture flasks. Supernatant was collected and Western blot was performed against proteins secreted by the regenerating mycelial protoplasts after SDS-PAGE and transblot.

4.7. Western blot of MAb 1A1

The monoclonal antibody producing ability of the two positive hybridoma clones, 1A1 and 1C1, was also checked by Western blotting. Both MAb 1A1 and MAb 1C1 exhibited similar banding pattern. The antibodies produced by these two hybridoma clones recognized two protein bands of molecular weights ~46 kDa and ~36 kDa. The results were confirmed again by Western blotting (Fig. 8 A, B).

4.8. Iso-typing of the monoclonal antibody

Iso-typing of MAb’s was performed in order to check the isotypes of MAb 1A1 and MAb 1C1. The isotype of MAb 1A1 was found to be IgG1 and that of MAb 1C1 was of IgA. It was not possible to identify the isotype of the light chain by the capture ELISA method therefore Isostrip™ mouse monoclonal antibody isotyping kit was used which not only confirmed the isotypes identified by the capture ELISA method but also revealed the isotype of light chain (Fig. 9). The isotypes of both heavy chain and light chain of MAb 1A1 (Fig. 9 A, B) and that of MAb 1C1 (Fig. 9 C) were identified as follows.
Fig. 8. Western blot of positive hybridoma clones A. MAb 1A1 B. MAb 1C1. Both the MAbs exhibited similar banding pattern recognizing two proteins (~36kDa and ~46kDa).

Fig. 9. Isotype of monoclonal antibodies A. isotype of heavy chain of MAb 1A1 B. isotype of light chain of MAb 1A1 and C. isotype of heavy and light chain of MAb 1C1.
Since both the MAb's (MAb 1A1 and MAb 1C1) recognized the same proteins, MAb 1A1 was considered for further studies as this MAb is of the isotype IgG1. Under *in vivo* conditions IgG1 is the most abundant isotype (accounting for nearly 80% of the antibody population) and exhibit many important physiological functions. Antibody of the isotype IgA is mainly secretory in nature.

4.9. Production of monoclonal antibodies (MAb 1A1) under serum-free conditions

For performing some sensitive experiments where serum proteins interference affects the results, the hybridoma clone MAb 1A1 was grown in serum free conditions with gradual change of RPMI 1640 medium containing FBS to serum free low protein medium. The monoclonal antibody producing ability of the clone decreased considerably under serum free conditions but was not lost. Approximately 0.2-0.4 mg/ml MAb was obtained under serum free conditions as estimated after ammonium sulphate precipitation and dialysis.

4.10. Cross reactivity of MAb 1A1 with *C. albicans* yeast form proteins

Cross reactivity of MAb 1A1 was assessed with the cell wall proteins of both mycelial and yeast forms of *C. albicans* and also with the proteins secreted by the regenerating yeast protoplasts. Both the protein bands were present in the cell wall proteins of yeast and mycelial forms as well as the proteins secreted by the regenerating yeast protoplast (Fig. 10).

4.11. Cross reactivity of MAb 1A1 under native condition of secretory proteins

Native gel electrophoresis followed by transblot of the proteins secreted by regenerating protoplasts of both yeast and mycelial forms was performed in order to check the cross reactivity of MAb 1A1 with these proteins under native conditions. MAb 1A1 cross reacted only with low molecular weight
Fig. 10. Cross reactivity of MAb 1A1 with cell wall proteins and secreted proteins 1. Proteins secreted by regenerating mycelial protoplast 2. Mycelial cell wall proteins 3. Proteins secreted by regenerating yeast protoplast and 4. Yeast cell wall proteins.

Fig. 11. Cross reactivity of MAb 1A1 under native conditions of secretory protein 1. Proteins secreted by regenerating mycelial protoplast 2. Proteins secreted by regenerating yeast protoplast.
protein (~36kDa) under native conditions as revealed by the Western blotting results (Fig. 11).

4.12. Cross reactivity of MAb 1A1 with other fungi and different strains of C. albicans

MAb 1A1 was tested for its ability to cross react with cell wall antigens of other C. albicans strains, other yeasts, and mycelial fungi. Seven strains of C. albicans (4 patient isolates, ATCC10231, ATCC14053, MTCC183) and five species of other fungi viz. Aspergillus fumigatus, (patient isolate), Trichophyton mentagrophytes, Sporothrix schenkii, Cryptococcus neoformans (ATCC 66031), and C. parapsilosis (ATCC 22019) were tested. MAb 1A1 found to cross react with all the C. albicans strains tested. The high molecular weight protein band was distinct in Western blot in all the C. albicans strains tested where as the low molecular weight protein was distinct in all the strains except for one patient isolate and MTCC183 where it was very faint (Fig. 12). Multiple bands were seen in few C. albicans strains which may be due to the degradation products of the proteins recognized by the MAb 1A1. MAb 1A1 also cross reacted with yeast like fungi Cryptococcus neoformans and C. parapsilosis recognizing three bands in case of first one and two in the later. MAb 1A1 did not cross react with any of the mycelial fungi (Aspergillus fumigatus, Trichophyton mentagrophytes, and Sporothrix schenkii) (Fig. 13).

4.13. Cross reactivity of MAb 1A1 with bacteria

Cross reactivity studies of MAb 1A1 was also performed against both Gram positive (Staphylococcus aureus, Bacillus cereus) and Gram negative (Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa) bacteria. Total bacterial cell protein was isolated by boiling the cell pellets with SDS PAGE sample loading buffer. Western blot analysis was negative for Gram positive as well as Gram negative bacteria as no band could be recognized. This showed that MAb 1A1 did not have any cross reactivity with bacterial proteins.
Fig. 12. Cross reactivity of MAb 1A1 with different strains of *C. albicans* 1, 2, 3, 6-patient isolates, 4- ATCC14053, 5-ATCC10231, and 7-MTCC183

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Fig. 13. Cross reactivity of MAb 1A1 with different fungal species

\textit{In vivo} diagnostic potential of the monoclonal antibody 1A1 was assessed in a mouse model of \textit{C. albicans} using BALB/c mice. The mice were injected with a sub lethal dose of \textit{C. albicans} yeast cells and mice without any infection served as control. After the establishment of infection, serum from each mouse was collected at an interval of three days and presence of the fungal antigens was assessed by using MAb 1A1. Since the model is a mouse model and the hybridoma was developed by the fusion of splenocytes from the same species, the secondary antibody used against MAb 1A1 may also bind with antibodies present in the mice serum thus giving false positive results. This problem was overcome by coupling biotin to MAb 1A1 and detection was done using HRP conjugated to streptavidin. Streptavidin-biotin interactions are one of the strongest and stable interactions thus increasing the reliability of the test. Using ELISA, the \textit{C. albicans} antigens could be detected significantly in four of the six mice. No antigen could be detected in control mice. Thus the detection efficiency of the MAb 1A1 was found to be 67\% in the present study.

4.15. Detection of antigens recognized by MAb 1A1 in \textit{Candida} positive patient sera using ELISA

The ultimate goal of \textit{C. albicans} antigen detection by MAb 1A1 was to check its diagnostic ability during the course of infection in patients suffering with candidiasis. For this purpose eighteen patient (\textit{Candida} positive) sera and nine sera from healthy donors were screened for the presence of \textit{C. albicans} antigens. Here TMB was used in place of OPD due to its higher sensitivity in ELISA. ELISA titre in serum from healthy donors was considered as control to fix the base line for OD values. In fourteen out of eighteen patient sera samples, antigen could be detected in the present study (77.78\%).

4.16. Inhibition of attachment of \textit{C. albicans} with composite material

\textit{C. albicans} exhibits an intrinsic property of adhesion to the surface of different substances like dental enamel, surfaces of prosthetic devices, and other composite materials used in implants. In laboratory these conditions may be
mimicked using composite material like polyvinyl chloride and poly carbonate plates. MAb 1A1 was tested for its ability to reduce or prevent the adhesion of the pathogen to these materials. MAb 1A1 exhibited lower ability to prevent C. albicans yeast cells from adhering to the surface of polyvinyl plate. Percent inhibition of attachment to composite material exhibited by cells treated with MAb 1A1 was 19.35% while the cells treated with irrelevant antibody and DPBS (control) was found to be 28.5% and 13.35% respectively. Number of cells sticking to the surface of the polyvinyl plate is shown in graphical form (Fig. 14.)

![Graph showing inhibition of attachment of MAb 1A1 to composite material](image)

**Fig. 14. Inhibition of attachment of MAb 1A1 to composite material.**

4.17. Inhibition of germination of *Candida* cells

Germination of blastospore and formation of hyphae is considered as an important virulence trait of *C. albicans*. MAb 1A1 was tested for its ability to inhibit the germination of yeast cells in to mycelia. No significant reduction in the percentage of germination of yeast cells treated with MAb 1A1 could be observed. Approximately 80%-85% yeast cells showed germination as compared to those without any treatment (DPBS) where it was approximately 90-92%. This implies that the proteins recognized by the MAb 1A1 may not be involved in the process of germination of the yeast cells.
4.18. Effect of monoclonal antibodies on colony forming units (CFU)

Some monoclonal antibodies exhibit *in vitro* cidal activity against *C. albicans* when the pathogen is treated with these MAbs for a specific period of time. MAb 1A1 was assessed for its ability to kill *C. albicans* as indicated by the reduction in the colony forming units (CFU). In order to determine the effect of MAb 1A1 on CFU, the cells of *C. albicans* were incubated with MAb 1A1 and CFU was determined. No significant reduction in the colony forming ability was noticed in the cells treated with MAb 1A1 on comparison with control (DPBS). 94.75% cells treated with MAb 1A1 formed colonies comparable to that of control where cells treated with PBS showed 95.75%.

4.19. MTT assay

The cidal activity of MAb 1A1 was also assessed by MTT reduction assay where a maximum reduction of 11.11% in viability was observed which was insignificant (Fig. 15). This was in consensus with the results obtained in the previous experiment. The cells treated with an irrelevant antibody showed 12.5% reduction in viability. Figure 15 represents average data from one of the three experiments performed in triplicate.

![Graph](image)

**Fig. 15. MTT assay performed by treating *C. albicans* cells with MAb 1A1.**

4.20. Phagocytic assay

Phagocytosis is an important step in counteracting the attacking pathogen by the host. Antibodies and complement factors are known to enhance the phagocytic process by opsonisation of the pathogen. Antibodies of the isotype
IgG are especially implicated in the process of opsonisation and phagocytosis. The ability of MAb 1A1 to opsonise yeast and germ tubes of *C. albicans* and the resultant enhanced phagocytosis by mouse macrophages was studied using J774 cell line. Yeast cells and germ tubes treated with PBS served as control. The process of phagocytosis could be seen within an hour of co-culturing of the MAb 1A1 treated as well as PBS treated cells with the macrophages. Marked enhancement of phagocytosis was observed in case of MAb 1A1 treated cells compared to those treated with PBS (Fig. 16). After three hours of co-culturing, phagocytosed yeasts and germ tubes were counted under a microscope after fixing and staining the phagocytes. An average of 7-8 yeast cells or germ tubes treated with MAb 1A1 were phagocytosed by the macrophages. Only 2-4 yeast cells or germ tubes per macrophage was seen in case of PBS treatment. Percent phagocytosis was calculated by counting the number of cells present within the phagocytes per 100 macrophages. About 89% phagocytosis was observed in case of yeast cells treated with MAb 1A1 as compared to control where only 27% phagocytosis was observed. In case of germ tubes treated with MAb 1A1, 75% phagocytosis was recorded as compared to those treated with PBS (36%).

### 4.21. Epitope localization on the surface of *C. albicans*

Epitope localization of the proteins recognized by MAb 1A1 was performed to study the distribution of these antigens on the surface of the pathogen in yeast form, germ tubes and mycelia. Immuno-fluorescence was used for this study using FITC labelled secondary antibody against the MAb. The study indicated the presence of antigens recognized by the MAb 1A1 in yeast form, germ tubes and also on the surface of the mycelia (Fig. 17). The study also indicated that the antigens are expressed during morphological transition as could be observed by their abundance and uniform distribution on the surface of the just 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.
Fig. 16. Phagocytosis of *C. albicans* yeast cells and germ tubes by mouse macrophage cells J774. A. Resting macrophages, B. Non-treated yeast cell phagocytosis, C. Non-treated yeast cell phagocytosis (100x), D. MAb 1A1 treated yeast cell phagocytosis, E. MAb 1A1 treated yeast cell phagocytosis (100x), F. Non-treated germ tube phagocytosis, G. Non-treated germ tube phagocytosis (100x), H. MAb 1A1 treated germ tube phagocytosis and I. MAb 1A1 treated germ tube phagocytosis (100x).
Fig. 17. Epitope localization of the antigens recognized by the MAb 1A1 using indirect immuno-fluorescence microscopy A. yeast cells, B. just germinating blastoconidia, C. germ tubes and D. mycelium. Abundant presence of the antigen localized evenly in all the morphological forms of *C. albicans* could be seen clearly. There is no change in the intensity of the antigen during morphological transition (B) which indicates that the antigens are constitutively expressed at all the times.

### 4.22. Monoclonal antibody 1A1 production by ascites growth

Almost seven days after the intraperitoneal injection of the Freund’s incomplete adjuvant, swelling of the mouse peritoneal cavity was observed indicating the formation of the ascites. MAb 1A1 producing hybridoma cells in
log phase were implanted in the peritoneum. The mice were observed daily for the enlargement of the peritoneum indicating the production of MAb. Tapping of the ascites was carried out from day 10 from the date of inoculation of the hybridoma. An average of about 4-5 ml ascites was obtained from each mouse in the first tapping. Two more tapings were followed subsequently on alternate days. Total of 35 ml ascites fluid was harvested from all the mice. The ascites fluid was centrifuged and filtered through 0.22 µm syringe filter to remove cells and the cell debris and stored at -20°C until further use.

4.23. Purification of monoclonal antibody

Purification of the MAb 1A1 was carried out using HiTrap Protein G HP column from either culture supernatant or ascites fluid to remove other unrelated protein molecules that may interfere in certain studies. Protein G exhibits high affinity for mouse antibody isotype IgG₁ at neutral pH. Ascites fluid or culture supernatant was diluted in binding buffer (1:6) to provide the required pH to increase the binding of the MAb to protein G. Binding of other proteins to the column material was prevented as much as possible by washing the column thoroughly with the binding buffer. 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, either at very low pH or at very high pH is required which results in the breakage of the interaction between protein G and the antibody. Such extreme pH may lead to the denaturation of the antibody resulting in the loss of activity. Hence it is important to bring the eluted antibodies to neutral pH as soon as possible. Protein G bound MAb 1A1 was eluted using glycine-HCl (pH 2.7) and the eluents were collected directly in microfuge tubes containing tris-HCl (pH 9.0). The purity and the presence of the monoclonal antibody was confirmed by SDS PAGE (Fig. 18) and the fractions containing pure MAb 1A1 were pooled and stored at -20°C after estimating the protein value. MAb 1A1 with an average of 1 mg/ml (protein value) from culture supernatant and 5 mg/ml (protein value) from ascites fluid was obtained after purification.
Fig. 18. Protein G purified monoclonal antibody MAb 1A1. Lane numbers indicate different fractions of the eluents. “H” indicates heavy chain of the antibody and “L” indicates light chain.

4.24. MAb 1A1 column preparation and antigen purification

Affinity column of the purified MAb 1A1 was prepared using cyanogen bromide activated sepharose beads. Antibodies can be coupled to CNBr-activated sepharose via primary amino groups. Activation of the sepharose beads was done in a pH range of 10.5 to 11.0 as this is the most favourable pH range for the activation. The pH was not allowed to fall below 10.5 and also rapid fluctuations in pH were avoided as this would lead to the breakage of the beads. Cyanogen bromide is very unstable and releases cyanogens gas at RT which is very toxic and hence the entire operation was carried out in a chemical fume hood. Purified MAb 1A1 was coupled to the activated sepharose beads in an overnight reaction. Coupling of the MAb 1A1 to the activated sepharose beads was confirmed by Bradford test. The initial OD of the MAb 1A1 solution in coupling buffer was 1.86 which dropped to 0.84 after coupling (flow through). The beads were washed and used for antigen purification. Purification of the antigens recognized by the MAb 1A1 was carried out using MAb 1A1 affinity column.
Fig. 19. Purified lower molecular weight protein recognized by MAb 1A1. Proteins secreted by regenerating mycelial protoplast were affinity purified using CNBr activated sepharose coupled purified MAb 1A1. A. Coomassie stained protein after SDS PAGE, B. Western blotting of the purified protein.

Proteins secreted by the regenerating protoplasts were used as the source of the antigens. Binding of antigen with MAb 1A1 column was carried out at neutral pH and elution was done at low pH (pH 2.7). The fractions were neutralized immediately to prevent permanent damage to the proteins. The fractions were checked by SDS PAGE for the presence of the purified antigens. Of the two antigens recognized by the MAb 1A1, only one protein (low molecular weight) could be purified (Fig. 19A) and the protein was confirmed by Western blotting (Fig. 19B).

4.25. 2-D gel electrophoresis and Western blotting of the purified protein for N-terminal sequencing

The low molecular weight protein purified above was further resolved using 2D gel electrophoresis and confirmed by Western blotting. The purified protein was resolved into four spots of same molecular weight but with different pI.
Fig. 20. Western blotting of the purified protein resolved after 2D gel electrophoresis. Four spots were identified by MAb 1A1 shown by the arrows.

Fig. 21. Purified protein was further resolved by 2D gel electrophoresis and trans-blotted onto a PVDF membrane. The spots (1, 2 and 3) were identified by N terminal protein sequencing.
(ranging between 6 and 7) (Fig. 20). These four protein spots were tranblotted onto a PVDF membrane after 2D gel electrophoresis and stained with amido black for N terminal protein sequencing (Fig. 21). Three spots (spot-1, spot-2 and spot-3) along with the band of the purified protein resolved on SDS PAGE were sent to IMTECH, Chandigarh for N terminal sequencing. The N terminal protein sequencing results are as follows

Spot-1: AIKWTNDFGRDAAL
Spot-2: GIKIGINGFGRIGRL
Spot-3: GIKIGINGFGRIKGL

Purified protein in single dimension: AIKIGIRGFGRIKGL

BLASTp was used on ExPASy for the identification of these proteins (see appendix). All the protein spots were found to be isoforms of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Glyceraldehyde-3-phosphate dehydrogenase is a constitutively expressed protein present inside the cell as well as on the cell surface. Clustal W analysis showed that the sequences matched with *C. albicans* (Ac. No. Q92211) GAPDH sequences with high scores. BLASTp E-values and clustal W scores are as follows.

<table>
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<tr>
<th>Protein</th>
<th>BLASTp E-value</th>
<th>Clustal W score</th>
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<tbody>
<tr>
<td>Spot-1</td>
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<td>80</td>
</tr>
<tr>
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4.26. Purification of high molecular weight protein by Protein A–MAb 1A1 coupled column

In another experiment MAb 1A1 was coupled covalently to protein A for purification of the high molecular weight protein recognized by it. Protein A is a bacterial protein isolated from *Staphylococcus aureus* and has very low affinity for mouse monoclonal antibody of the isotype IgG which increases with salt concentration. At very high salt concentration of 3M, protein A has an
Results

Fig. 22. SDS PAGE of the purified high molecular weight protein recognized by MAb 1A1. The protein was purified from mycelial cell wall proteins under high salt concentrations using MAb 1A1 coupled covalently to protein A sepharose beads.

Fig. 23. Western blot of purified high molecular weight protein
affinity equivalent to that of protein G. Dimethylpimelimidate was used to couple MAb 1A1 to protein A covalently, and the coupling was confirmed by SDS PAGE where no antibody bands were observed after coupling.

The second antigen (high molecular weight protein) recognized by MAb 1A1, proteins extracted from cell wall of mycelial form was used as this protein was also present in the cell wall. SDS present in the cell wall protein sample was removed using SDSOut precipitation kit which precipitated the SDS present in the sample. Different conditions such as pH and salt concentrations were used to standardize the binding of high molecular protein to protein A coupled 1A1 column avoiding non specific interactions with other proteins. The protein could be finally purified under high salt conditions (1M NaCl). Elution was done at low pH conditions (pH 2.7) and the eluents were neutralized immediately to avoid any permanent structural changes. The purification of the protein was confirmed by SDS PAGE (Fig. 22). Western blotting was also performed against this purified band to confirm further that it is the same protein that was recognized by MAb 1A1 (Fig. 23).

4.27. Cytokine response of mouse splenocytes against the purified antigens

Cytokine response of the mouse splenocytes against the two purified antigens (as above) was studied using CBA kit. Three concentrations of each antigen (5 μg, 10 μg and 15 μg) were used and five cytokines (TNFα, IFNγ, IL-2, IL-4 and IL-5) were estimated after 24h of stimulation. TLR2 agonist PAM3CSK4 was used as the positive control. Both the antigens elicited TNFα response in a dose dependent manner. TNFα in general is produced in the initial stages of stimulation and usually a heightened response is seen. The response of TNFα was more in case of antigen 1 (~46kDa protein) compared to that of antigen 2 (GAPDH). A very high response of TNFα was noticed in case of PAM3CSK4 which is a potent stimulant of TLR2 receptors (Fig. 24). A mild response of this cytokine was also noticed in case of non stimulated control macrophages which indicate that the cell may have been in stress.
The response of IFNγ was also found to be in a dose dependent manner for PAM3CSK4 and GAPDH but in case of antigen 1 the response decreased and then increased with concentration. Further, the IFNγ response of GAPDH was higher than that of antigen 1 and no IFNγ was noticed in case of control cells (Fig. 25). IL-2 is considered to be a cytokine required for the proliferation of the T cells. A suppressive response of T cell proliferation was noticed in case
of antigen 1 which showed decrease in IL-2 response with increase in concentration from 5 μg to 10 μg but later increased mildly. In contrast GAPDH showed a proliferative response where the response increased with concentration though it decreased later. IL-2 increased with increasing concentration of PAM$_3$CSK$_4$ while it first decreased and then mildly increased in case of antigen 1. No IL-2 production could be seen in non stimulated macrophages (Fig. 26).

![IL-2 response of splenocytes against the stimulants](image)

**Fig. 26. IL-2 response of splenocytes against the stimulants**

Interleukin-4 and interleukin-5 are considered to be cytokines of Th2 type. While the TLR2 ligand PAM$_3$CSK$_4$ exhibited a concentration dependent increase in response for the production of both IL-4 and IL-5 cytokines, the responses of the two antigens varied with their concentrations. The response of the high molecular weight antigen was almost similar for both the interleukins where the response increased with the concentration and then 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 μg concentration (Fig. 27). In case of IL-5 the decrease in the response was not significant and was comparable to the response of PAM$_3$CSK$_4$ (Fig. 28).
Results

**Fig. 27.** IL-4 response of splenocytes against the stimulants

**Fig. 28.** IL-2 response of splenocytes against the stimulants

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. It can be seen that the increase in the response of IL-5 at 15 µg concentration was very high (Fig. 28). Response was not significant and was comparable to the response of PAM3CSK4 (Fig. 28).