Chapter 5

DISCUSSIONS

In this work we proved that the *Aspergillus giganteus* MTCC 8408 is able to produce an intracellular anti-yeast protein (Acp-N84) in addition to the previously well-characterized, α-sarcin, antifungal protein (AFP) and AFPNN5353 **[78, 83, 201].**

Two readily metabolized carbon sources including glucose and sucrose with three slowly utilized carbon sources such as maltose, lactose and soluble starch were investigated to keep their influence on cell growth and antifungal protein production, at the same level. Glucose and maltose carbon sources resulted lower level in μ value due to lower level in $Y_{p/x}$ value (**Table 4.6**). Sucrose appeared to be more beneficial to cell growth and antifungal protein production due to its slower catabolism rate than glucose or maltose. Although it was reported starch has been proved to be a better carbon source for antifungal protein production **[231]**, it produced the maximal biomass and antifungal protein titer in our study. Its linear, soluble fraction and amylopectin fractions served as carbon and reported as excellent growth substrates for mycelial biomass production. The value of μ was approximately twice higher than those obtained from glucose and four times higher than those obtained from maltose.

Our study showed that soluble starch was the best carbon source for intracellular antifungal protein production. This result was consistent with that reported by others **[1, 2, 78, 80-91].** Simultaneously, the metabolism of soluble starch is more complex than other investigated carbon sources prior to incorporate in glycolysis pathway.

Due to very higher residual lactose in the fermentation broths during harvest indicated that *Aspergillus giganteus MTCC 8408* inhibited the hydrolysis and utilization of lactose thus repressed.

During the period of metobolization of soluble starch, a small quantity of free hydrolyzed glucose occurred in the medium that makes a combination of starch and glucose as the carbon source **[226, 290]** and the starch metobolization starts only after complete glucose exhaustion. This not only avoided catabolite repression but also provided enough nutrients for cell growth and antifungal protein production. The antifungal protein was probably partially inhibited by carbon catabolite repression in the presence of readily metabolized carbon sources (glucose and sucrose) that causes the lowest μ value among the investigated carbon sources.

The influence of carbon source on antifungal protein production not only was controlled by regulating the metabolite biosynthesis but also was related to the fungal morphological differentiation. Like many other filamentous fungi, *Aspergillus giganteus* undergoes morphological and physiological changes when the abundance and complexity of nutrient sources are altered. In case of readily metabolized carbon sources (glucose and sucrose), the mycelium was developed thriftily to bigger and looser pellets covered with thicker filaceous and flourishing outer hairy region (image not shown). This led to hindrance of nutrients transfer into the pellets. In addition these carbon sources resulted in carbon catabolite repression and therefore lowered the μ value. The coordination of these morphological changes with the occurred carbon catabolite repression resulted in the inhibition to antifungal protein production.

While in presence of soluble starch, the medium were tardily assimilated for growth, self maintenance and survival of cell with fungal morphology developed to spongy filamentous with too much sporangium on the tips of hyphae.

This was one of the crucial factors that slowly utilized soluble starch results in higher specific antifungal protein titer and improved the excretion of this bioactive protein thereby stimulated the cell differentiation and growth associated metabolism during the fermentation (**Figure 4.4**).

Antifungal protein production in basal Olson media **[78]** supplemented with organic nitrogen as shown in **Table 4.2** was higher than those supplemented with inorganic nitrogen sources. Culture media supplemented with corn steep liquor produced higher antifungal protein yields than individual supplemented with beef extract, peptone and proteose peptone. Among these sources, combination of corn steep liquor (2%) and proteose peptone (1%) was appeared as best source of nitrogen for antifungal protein production (9.98 mg/l) with near complete soluble starch consumption. The lower final culture pH (6.2) indicated the stability of cationic bioactive protein suggested that the substrate utilization was better than other nitrogen sources.

The results of present study also showed the combination of beef extract (2%) and peptone (1%) was the second best nitrogen source for antifungal protein production but with higher final culture pH (6.4).

Replacing organic nitrogen sources with inorganic one resulted in poor antifungal protein production as well as cell biomass growth. The reduced final culture pH (3.42) using ammonium citrate indicated the instability of the intracellular bioactive protein with much reduced cell biomass (5.23 gdcw L^{-1}) and therefore found inconsistent with that reported by others. When ammonium citrate replaced the combination of corn steep liquor (2%) and proteose peptone (1%) as a nitrogen source, antifungal protein yields are halved (4.67 mg L^{-1}). These observations were attributed their lower yield to the composition of the nitrogen source in fermentation medium they used. Ammonium nitrates will normally cause an acid drift as they are metabolized like all the ammonium ion is utilized, and nitrate assimilation is repressed.

When the ammonium ion has been exhausted, there is an alkaline drift resulted much reduced antifungal protein yield (1.98 mg L^{-1}). In comparison with organic nitrogen sources, inorganic nitrogen sources resulted relatively lower mycelial growth and afp production. The observed variation certainly indicates the imperative role of optimization of all fermentation related factors in achieving the best possible productions. CSL is a complex substrate produced in the corn processing industry and composed of peptides, sugars, lactic acids, vitamins and metallic ions. CSL (1.5-3.0% nitrogen) supplies both nitrogen and carbon sources, and improves fungal growth during initial stages of colonization. CSL favors increased growth rates and including excess phosphate concentrations, have been shown to reduce pellet formation **[291, 292].**

Therefore, carbon and nitrogen sources such as soluble starch and corn steep liquor and proteose peptone were employed to study C/N ratio using Taguchi DOE L_8 OA statistical approach for further experiment.

Taguchi DOE has been successfully applied to foods, chemicals, and biological processes **[293**] and its theoretical and practical applications have been widely reviewed and shown to be very effective for analyzing the production of both antibiotics and industrial bioactive protein/enzymes **[294]**. Our screening experiments using OFAT approach to different carbon and nitrogen sources in the fermentation medium revealed that they significantly influenced antifungal protein production, with this being maximal when using soluble starch and corn steep liquor and proteose peptone, respectively. The experimental data of the C/N ratio for the production of antifungal protein is presented in **Table 4.6** and the ANOVA table is given in **Table 4.7**.

In this Taguchi DOE L_8 OA study, the average maximum antifungal protein yield reached $Y_{p/x}$ of 0.882 mg $gdcw^{-1}$ L^{-1} (Run 3) with C/N ratio of 27.4 obtained together with essential microelement ratio K^+/Ca^{2+} and Mg^{2+}/Na^+ at their assigned level 2 and 1, respectively, was even higher than obtained in OFAT approach in basal Olson medium.

This study also indicated that soluble starch with such C/N ratio (27.4:1) was propitious to the release of catabolite repression (Run 4) resulted much lower antifungal protein yield $Y_{p/x}$ value. This study also attributed the presence of mono- and divalent cation had significant effect on cell morphology. The effect of K^+/Ca^{2+} and Mg^{2+}/Na^+ ratio on Yp/x was inversely proportionate (Run 3, Run 7).

Na^+ stimulates dry weight production at low concentrations (Run 5) while K^+ inhibits it at high concentrations (Run 3) resulted filamentous form of growth was maintained with the hyphae being scarcely branched without bulbous cells **(Figure 4.4c).** Magnesium, calcium inhibits dry weight production at all concentrations (Run 2, Run 7).

The inhibition of dry weight production by sodium was encountered by magnesium and calcium at higher Mg^{2+}/Na^+ ratio and lower Mg^{2+}/Na^+ ratio (Run 7) resulted boisterous distribution, broken hyphal tips and concomitant cell wall immaturation **(Figure 4.4b).** Minimum concentration of calcium ions reduced the extent of the inhibition of growth brought about by sodium ions was 1.08 m-equiv, while the increased growth in the presence of added calcium ions of 3.2432 m-equiv resulted pelleted type morphology with significant reduction of $Y_{p/x}$ value **(Figure 4.4a).**

Magnesium was also as effective as calcium in reducing the extent of the inhibition brought about by sodium ions and the minimum concentration was 3.252 m-equiv.

Addition of 1.08 m-equiv L^{-1} Ca^{2+} ions to the fermentation medium lowered the final dry cell weight by 22% as compared to 3.2432 m-equiv L^{-1} and increased the uptake of phosphate and sucrose, and afp production. At high K^{+}/Ca^{2+} Ratio (4.78:1) highly branched hyphae and numerous bulbous cells was observed **(Figure 4.4d)** while laminated layers, featured vesicles associated with the numerous inclusions was observed at high Mg^{2+}/Na^{+} ratio (0.94:1). Ca^{2+} Competed with magnesium and prevented growth almost entirely when present at 4.51 times in excess.

Optimization of operating parameters (e.g. effects of agitation, pH, temperature, slant age, inoculum volume and KH_2PO_4) using soluble starch with corn steep liquor and proteose peptone in submerged fermentation was further investigated using Taguchi DOE L_{27} OA. Inorganic phosphate concentrations was varied between 0.1 to 0.3 gm L^{-1} and showed a dramatic effect on antifungal protein including biomass/morphology production. Among the 8 most decisive factors studied, the effect of microelement (KH_2PO_4) and pH on higher afp production was inversely proportionate.

Decrease in KH_2PO_4 concentration caused higher afp production up to level 1 **(Figure 4.6;** Run 1**)** while increase in pH had higher effect on afp production at level 2 **(Figure 4.6;** Run 6**)**. Higher afp production was encouraged with decrease in slant age on afp production up to level 1 (17.947 mg L^{-1}) but decreased significantly with increase in slant age up to level 3 which was clearly reflected from cell morphology **(Figure 4.6;** Run 18**)** (16.872 mg L^{-1}). The inoculum level is generally recognized as one of the most important factors. Inoculum level had significant effect on higher afp production up to level 2 **(Figure 4.6;** Run 2**)** (17.766 mg L^{-1}) but decreased up to level 1 (17.114 mg L^{-1}) or 3 (17.529 mg L^{-1}).

This result was quiet consistent with that reported by others **[250-252].** The initial medium pH also plays an important role in fungal morphology **[253].** Increase in pH up to level 3 results procellous mycelial form of growth with swollen characteristics **(Figure 4.6;** Run 15**)** while decreasing facilitate convoluted pelleted type morphology **(Figure 4.6; Run 16)** with greater reduction of afp level.

Fungal morphology to a large extent is affected by agitation in a rotary shaker/bioreactor. Strong agitation will form free filaments. When pellets are formed, the pellet size, structure and survival are also affected by agitation. In general, less agitation yields smaller and more compact pellets **[255, 256].** Higher agitation (180 rpm) resulted free filaments with higher afp yield (12.3 mg L^{-1} at Run 3) while lower agitation (120 rpm) lead to pellets **(Table 4.8)** formed with much lower afp yield (4.5 mg L^{-1} at Run 17).

The regression analysis of the data, generated by Taguchi design experiments, was done to fit he response function (S/N ratio). The analysis of variance (ANOVA) was employed for the determination of significant parameters **(Table 4.10).** It was estimated by Fisher's statistical test. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the significance of each controlled factor on the tested model.

On analysis of variance by F-test, the F-value of 114.928 implied that the factor soluble starch was significant. Values of ‘$P<0.05$ indicated that analysis terms were significant. In this case the temperature term was found to be significant (29.302) followed by nitrogen source and pH. This implies a satisfactory representation of the process by this Taguchi DOE statistical model. The response was optimized by varying one factor over the range keeping the other factors in orthogonal array as shown in performance distribution of current condition versus improved condition (**Figure 4.8)**.

The optimized pH parameters suggested by the Taguchi DOE L_{27} OA were pH of 5.8, inoculum size of 5.0% (v/v) and agitation at 180 rpm. The expected afp production at optimum conditions was 19.42 mg/l (based on SN = 25.765 mg L^{-1}) with total contribution from all the factors was 2.64 mg L^{-1} (based on SN = 8.294 mg L^{-1}) with grand average performance of 7.53 mg L^{-1} (based on SN = 17.47 mg L^{-1}). The experimental data showed an enhancement of afp yield by 2.46 fold i.e., from 7.53 mg L^{-1} (based on SN = 17.47 mg L^{-1}) to 18.54 mg L^{-1} (SN = 25.362 mg L^{-1}) with mycelial biomass 28.2 ± 0.9 gdcw L^{-1}, therefore; proving the validity of the method with the new modified culture conditions.

While OFAT approach resulted afp production enhanced from 5.47 ± 0.21 mg L^{-1} to 12.10 ± 0.11 mg L^{-1} with mycelial biomass enhanced from 21.77 ± 0.31 g L^{-1} to 26.6 ± 0.55 g L^{-1}as compare to basal Olson medium.

Kinetic study of biomass and anti-yeast protein production by *Aspergillus giganteus* MTCC 8408 in new modified (optimized by Taguchi DOE L_{27} OA) culture broth using soluble starch, was analyzed at the incubation temperature of 25°C **(Figure 4.9a).** This strain reached the stationary phase after 75 h. Prolonged incubation up to 120 h promoted degradation of the Acp-N84 but no lysis of biomass. No Acp-N84 was produced within first 9 h at 25°C, but it was produced during the active growth phase, and its concentration reached a maximum at 105 h, at the middle of the maximum stationary phase. The highest activity of protein against *C. albicans* was recorded between 90–105 h of incubation and decreased thereafter.

The pH dropped rapidly during the exponential phase, probably because of the strong production of cationic Acp-N84 associated with growth.

Cell-free intracellular crude afp production was drastically reduced as pH values value approaches 8.1 **(Figure 4.2a)** while it was stable upon treatment at different temperatures in submerged fermentation in the range 20-30^{0}C **(Figure 4.2b)** while.

Dynamics of anti-yeast protein production was described reasonably well by considering the inhibition effect of soluble starch above 30 g L^{-1} **(Figure 4.1a).** It was mentioned earlier that anti-yeast protein production was affected by the carbon and nitrogen sources, and the conditions of fermentation, e.g., pH value of the broth. Because of the high initial anti-yeast protein concentration and keeping the pH value of the broth at < 6.0, the protease activity was kept low most of the time during the fermentation. The increase of protease activity near the end of the culture might be caused by cell lysis. This may explain the drop in anti-yeast protein activity after 120 h submerged fermentation **(Figure 4.9a).**

The usefulness of the developed unstructured mathematical model (Logistic) was evaluated for growth kinetics with soluble starch consumption by *Aspergillus giganteus* MTCC 8408 under the fermentation conditions optimized by Taguchi DOE L_{27} OA. The experimental results for biomass growth dynamics related (μ_m , *Ks*, E and S) parameters were evaluated using simulation **(Figure 4.12)** of the proposed mathematical model equations. The experimental results and corresponding fits to the model equations are shown in **(Figure 4.11).** Biomass growth related (ν_{max} and λ_x) parameters were evaluated using the proposed mathematical model.

Following the lag phase, the cells entered the exponential phase, eventually reaching the asymptotic level of maximum growth. This phase of the growth curve is characterized by the production of two metabolically associated products, i.e. biomass and antifungal protein. The specific growth rate (μ_{max}) decreased from 1.56 to 0.587 h^{-1} with an increase in substrate concentration (*S*) from13.75 to 21.25 g L^{-1}. However, maximum biomass production (X_m = 24.0 g L^{-1}) was observed in the 21.25 g L^{-1} soluble starch supplemented nutrient medium after 90 h.

It was observed that substrate saturation constant (K_s) increased from 0.1 to 3.81 g L^{-1} with an increase in substrate concentration (*S*) from13.75 to 21.25 g L^{-1} after 25 h. This substrate concentration dependent biomass production suggested that soluble starch concentration regulates the growth pattern of *Aspergillus giganteus.*

Antifungal protein production was only associated with the exponential growth phase and not detected in the lag phase, indicating that production in this strain is growth associated, similar to other bio-active metabolites produced by microbial strains **[225]** Antifungal protein production increased progressively with an increase in soluble starch concentration up to 20 g L^{-1}, reaching 18.54 mg L^{-1}, but further increase in substrate concentration led to a decrease in antifungal protein production.

Critical analysis of the antifungal protein production pattern further indicated that $Y_{p/s}$ values during the exponential phase of microbial fermentation showed about 18% variation, suggesting that economic production of this antifungal protein could be achieved with the use of a low substrate concentration.

Analysis of the yield factor for biomass production ($Y_{x/s}$) suggested less substrate was consumed at the lowest (at 10 g L^{-1}) and highest (at 40 g L^{-1}) substrate concentrations than at the intermediate (at 20 and 30 g L^{-1}) substrate concentrations studied. The calculated yield factors for antifungal protein production based on were 1.7 gdcw g^{-1} L^{-1} (lactose) followed by 1.49 gdcw g^{-1} L^{-1} (soluble starch), 1.19 gdcw g^{-1} L^{-1} (maltose and sucrose) and 1.12 gdcw g^{-1} L^{-1} (glucose), indicating that effective antifungal protein production could be achieved in soluble starch with highest yield factor for product concentration ($Y_{p/s}$=0.98 mg g^{-1} L^{-1}) studied **(Table 4.12**).

Approximately 80–95% of available soluble starch was consumed by the growing *Aspergillus giganteus* strain in the substrate concentration range 10–50 g L^{-1}, with effective utilisation (>94.5%) being observed at 20 g L^{-1} substrate concentration.

This may be explained based on the fact that the organism uses consumed soluble starch more for antifungal protein production than for biomass growth. This could be further confirmed based on the fact that the organism μ value was highest (0.9 h^{-1}) at 20 g L−1 substrate concentration. All values predicted by the nonlinear adjustments showed high coefficients of linear correlation with the observed values ($R \geq 0.9$). The data fitted the proposed mathematical model very well. Fitness of the experimental results with respect to probability (P) and Fisher (F) values in the proposed unstructured mathematical model was satisfactory graphically and statistically.

The highest antifungal activity against *C. albicans* strain was present mainly in the fraction precipitated with 50-70% ammonium sulfate **(Figure 4.13)**. Fractions precipitated with 30-50% ammonium sulfate exhibited very weak inhibition. The supernatant obtained after 50-70% ammonium sulfate precipitation clearly did not exhibit any antifungal activity. The antifungal substance present in the 50-70% cut-off also inhibited germ tube formation in *Aspergillus niger* (model organism). As is clear from purification profile **Table 4.15**, ammonium sulfate precipitation resulted in an approximate 6-fold increase in specific activity.

After ion-exchange chromatography using CMC, the adjacent fractions eluted in first 12-16 minutes in the chromatogram showed biological activity as F-4 **(Figure 4.13a),** and the specific activity increased 8-fold. After gel filtration, the recovery was approximately 9-fold. Based on the purification steps summarized in **Table 4.15**, it was concluded that the total active anti-yeast protein recovered was 17.18% only.

After gel filtration, partially purified active pooled fractions (30 μL), were loaded onto silver stained gel showed a corresponding band that was responsible for the biological activity. Based on the polypeptide molecular weight marker, the molecular mass of the active peptide was estimated to be approximately 24.3 kDa **(Figure 4.22).**

The first 12 amino acid residues of the N-terminal determined were LRHDPKTIEELT, and the same partial sequence was matched for homology. Complete homology was found in the NCBI BLAST, Uni-Prot and AspGD search result. Analysis of the major N-terminal sequence revealed the presence a feature of class putative GTP-binding protein **(Table 4.16).**

Based on analysis the combined peptides having 64 amino acid residues were assembled. Individual peptides having m/z 918, 2149 and 879 **(Table 4.17)** were found. The combined peptide possessed R/K/D/E = 5/11/3/4 amino acid ratio **(Table 4.18).**

The peptides did not significantly match any known proteins present in the MASCOT and BLAST databases. The amino acid sequence of Acp-N84 (64 residues) obtained from peptide fragments after digestion of the anti-yeast protein with trypsin was analyzed by MALDI-TOF MS spectra.

There are several antifungal proteins from several *Aspergillus* species and other species origin, but anti-yeast peptides or proteins are very rare. *Pseudomonas syringie*, Penicillium species, *E. coli* and some Bacillus species also produce antifungal peptides, but no such reports about *Aspergillus giganteus* producing anti-yeast protein were found.

The identified *Aspergillus giganteus* strain produces the anti-yeast protein, intracellularly. The activity of the Acp-N84 was stable up to temperature 72°C for 24 minutes (data not shown) but the activity was lost after boiling and autoclaving.

While quiet similar results have been reported for bacillomycin D from B. subtilis **[295]** and durancin L28-1A from E. durans **[296]**, was inactivated when subjected to 121°C for 20 min.

The anti-yeast property of the Acp-N84 also remained unaffected in the pH range of 5.7–6.9. At pH values of 4.5 and 8.1, however, the activity was drastically reduced and activity was lost completely. These results are similar to those reported for the bacteriocin produced by *E. mundtii* **[297]**. Several bacteriocins produced by enterococci are known to exhibit a wide range of pH stability.

When the dialyzed 50–70% fraction was treated with the reducing agent DTT, no decrease in inhibitory activity was observed, indicating that disulphide bonds are not responsible for biological activity. It was also observed that storage of Acp-N84 at −48°C for 3 months did not significantly affect biological activity. Ammonium sulfate salt as well as sodium acetate buffer did not inhibit its activity at the concentration used and did not modify the result of the assay. The dialyzed concentrate of 50–70% fraction, dissolved in 0.05 M sodium acetate buffer, weakly bound with the CMC matrix, indicating that the Acp-N84 bears positive charges. Being weakly positive, it was separated easily in native polyacrylamide gel electrophoresis. After purification by ammonium sulfate fractionation, dialysis, cation exchange chromatography and gel filtration, the final amount of recovered protein was found 17.18%. This could be an indication of effective purification strategy and optimization methods.

Comparing the partial amino acid sequence of the purified anti-yeast protein to other antimicrobial peptides by using NCBI BLAST, UniProt revealed no complete homology with other known AMPs. The combined N-terminal sequence had high amounts of lysine (17.2%), leucine (10.9%), glycine (7.8%) **(Table 4.18)**. This has been observed in many antimicrobial peptides published earlier by researcher **[298, 299].**

It was reported earlier that the lysine or glycine-rich antifungal peptide tenacin-3 enters the *C. albicans* cytoplasm [**300**]. Penaedins characterised from shrimps and prawns had a high content of Arg/Gly residues in the extended N-terminal domain [**301**].

ACLA from *Aspergillus clavatus* has high lysine and glycine residues, resulting in calculated grand average of hydropathicity (GRAVY) of −0.017 while ANAFP1 from *Aspergillus niger* strain KCTC 2025 has high alanine and glutamine residues resulting in calculated grand average of hydropathicity (GRAVY) of−0.218 and ANAFP2 from *Aspergillus niger* strain CBS 513.88 has high alanine and leucine residues, resulting in calculated grand average of hydropathicity (GRAVY) of −0.241. The presence of Ala-Gly-Leu hinges in antimicrobial peptides like ACLA and ANAFP supports the antimicrobial potential of Acp-N84, wherein a quiet similar sequence was observed. The regional flexibility provided by proline was sometimes enhanced by the presence of glycine residues [**302**]. This information harbors the idea that the anti-yeast protein in the present study could interact with the cell wall of Candida as a primer for antimicrobial action.

In fact, the increase of hydrophobicity of the peptides is directly correlated with fungicidal activity [**304**]. In accordance with many other bacteriocins of LAB e.g., lactococcin A [**305**], lactacin F [**306**], and curvaticin FS47 [**303**], a high proportion of glycine was likely to provide a significant amount of flexibility to the molecule. In our study conducted on Acp-N84, the neutral (Gly [7.8%]) and hydrophobic (Ala, Ile, Leu, Val, Pro, and Phe [35.9%]) residues constitute a significant proportion to cell wall interactions with Candida therefore may consider the Acp-N84 enter cells through the cytoplasm (without membrane lysis) and inhibit the activity of specific molecular targets followed by cell death.

Other studies with model amphipathic all L-amino acid peptides with the sequence KX3KWX2KX2K, where X= Gly, Ala, Val, or Leu showed that the leucine-rich peptide, rather than the Ile- or Val-containing peptide, was particularly antimicrobial [**307**]. Our result is in agreement with this observation (total=20.3%): leucine: 10.9 %,; proline: 1.6% and arginine: 7.8%.

We have used the PSIPRED (Protein Structure Prediction) server to predict the structure. The sequence showed alpha helical structure, which is characteristic of many antimicrobial peptides [**307]**. This has been observed in FTIR study and harbors the justification of predominantly α-helical secondary structures exhibited by amide I band at1650–1658 cm^{-1} and 1635.6360 cm^{-1}.

Syringostatin A and syringotoxin B were the two antifungal proteins obtained from Pseudomonas, which were reported to be lethal to *C. albicans* and A. fumigatus at MICs of 3.2 and 5.0 µg mL^{-1}, respectively **[308]**. In vitro, the MIC of cepacidine A ranged from 0.049 to 0.391 mg mL^{-1} for *Candida* species. The MICs of trichopolyns A and B for *C. albicans* 0.78 to 6.25 mg mL^{-1} **[309]**. Acp-N84 was able to only inhibit the growth of yeast at its relative low concentrations (3.6µg mL^{-1}) and it shows our result is in quiet good agreement with this observation **(Table 5.1).** In susceptibility tests strain of *C. albicans* and other emerged fungal pathogen which can cause serious cutaneous, mucosal and/or systemic infections were involved.

Table 5.1 In vitro MICs values of Acp-N84 and other conventional antifungal agents (peptides and synthetic) for the investigated fungal isolate, *Candida albicans*

Peptide	Source	MIC (μg mL^{-1})	Mode of action
	Bacterial/fungal		
Acp-N84	***Aspergillus giganteus MTCC8408***	**3.6**	Unknown
ACP	*Enterococcus faecalis*	133	Unknown
NFAP2	*Neosartorya fischeri* NRRL 181	0.781	Unknown
NFAP	*Neosartorya fischeri* NRRL 181	>200	Unknown
PPEBL21	*Escherichia coli* BL21	15.62	Unknown
Pentocin TV35b	*Lactobacillus pentosus*	------	Unknown
Aculeacins	*Aspergillus aculeatus*	0.2–6.3	Glucan synthesis
Echinocandin B	*A. nidulans*	0.625	Glucan synthesis
FR900403	*Kernia* sp.	0.4	Chitin synthesis
Helioferin A, Helioferin B	*M. rosea*	5.0	Unknown
Leucinostatin A	*P. lilacinum*	10.0	Unknown
Leucinostatin H	*P. marquandii*	25.0	Unknown
Leucinostatin K	*P. marquandii*	0.97	Glucan synthesis
Pneumocandin A0	*Z. arboricola*	0.12-2.0	Glucan synthesis
Schizotrin A	*Schizotrix* sp.	0.02	Unknown
Syringotoxin B	*P. syringae*	3.2-50.0	Lysis
WF11899 A, WF11899B	*C. empetri*	0.008-0.16	Glucan synthesis

	Insect/amphibian		
Antifungal peptide	*S. peregrine*	25.0	Lysis
Magainin 2	*X. laevis*	80.0	Lysis
	Plant		
Zeamatin	*Z. mays*	0.5	Lysis
	Synthetic		
Amphotericin B		8.0	
Itraconazol		>64.0	
Fluconazole		>64.0	
Caspofungin		16.0	

The result indicated that *Fusarium oxysporum* and *Aspergillus fumigatus* were not susceptible (<20% inhibition) to Acp-N84 in its investigated concentration range. In contrast to these results, the applied concentrations of Acp-N84 could inhibit the growth of *A. niger and Botrytis cinerea* (<40% inhibition), and considered as ineffective against these isolates in the investigated concentration range. The difference in sensitivity of different *Aspergillus* species to treatment with antifungal proteins isolated from *Moringa oleifera* and *Indigofera oblongifolia* was also observed.

Time killing assay the conventional antifungal agents showed higher MIC than Acp-N84. The observed extremely low MICs render commercial utility as potential antifungal for this protein. In the present study, the results of cytotoxicity assay appeared to be of great interest. Acp-N84 showed dose dependent cytotoxicity with inhibitory concentration (IC_{50}) value of 5µM up to a tested concentration of 20 µM against HeLa cell line.

Among the tested fungal peptides 1-4 **(Figure 4.13b)**, pep-2 found to be most active while pep-1 was the least effective. Fluorescence staining with Hoechst 33342 and propidium iodide dye for 24h indicated the presence of early and late apoptotic cells. In early apoptotic cells, chromatin condensation and fragmentation with bright blue fluorescence was observed, whereas, in case of late apoptotic cells condensed and fragmented nuclei with pink fluorescence was present.

Based on our observations it could be proposed that the main antifungal mechanism of Acp-N84 on yeast cell is the cell membrane shrinkage followed by disruption of the plasma membrane leading to vesicle formation and cell lysis. However further experiments are required to elucidate and to reveal the exact mechanism of action. Membrane disruption activity of several cationic antimicrobial peptides from plants and human on *C. albicans* has been described **[308, 309].**

It is described that the membrane disruption ability of antimicrobial proteins is in tight connection with the high abundance of arginine and lysine residues which render high positive charge to them **[310, 311]**. Here Acp-N84 is corroborated by its cationic character (pI of 9.90) due to the presence of five arginine and eleven lysine residues.

The N-terminal amino acid sequence of Acp-N84 (identified as putative GTP-binding protein) is LRHDPKTIEELT. A MASCOT search of Acp-N84 for homology with other reported antifungal protein sequences showed that the above sequence had no significant similarity with any antifungal proteins reported so far. Peptide mass fingerprinting also revealed the best matches with putative GTP-binding protein from six different strains of *Aspergillus*. These matched peptides were shown to be present in 33% sequence coverage and similarity with the sequence of putative GTP-binding protein of *Aspergillus calidoustus* available in the NCBI BLAST, Uni-Prot and AspGD protein database.

Acp-N84 appeared as a single band in silver stained PAGE and had a molecular mass of 24.3 kDa, corresponded well to the calculated mass of putative GTP-binding protein of *Aspergillus calidoustus* (21736 Da), *Aspergillus ruber* (21590 Da), *Aspergillus glaucus* (21590 Da), *Aspergillus clavatus* (21665 Da), *Aspergillus fumigatus* (22530 Da) and *Aspergillus novofumigatus* (22558 Da) **(Table 4.16).**

Similarly to NFAP and NFAP2 **[87]**, Acp-N84 also proved to be heat-stable owing to its folded and disulfide bridge-stabilized tertiary structure. This structure is common within the group of ascomycetes cysteine-rich antifungal proteins and experimentally proved at AFP **[9, 10]**; PAF (**[80, 81, 204]**); and BPI **[127-131]**.

Results from this study render Acp-N84 of antifungal highly interesting compounds for development of a new antifungal strategy as well as antitumor agent after further studies focusing on its bulk production, antifungal mechanism, toxicity and in vivo activity.