Chapter 3

MATERIALS AND METHODS

3.1 Strain and culture maintenance

Aspergillus giganteus (CBS-KNAW Fungal Biodiversity Center, Netherlands; Type: F; Strain Designation No: 515.65), MTCC (Microbial Type Culture Collection): 8408 was maintained in Czapek's yeast extract agar slant containing (g L^{-1}): K_2HPO_4 1.0; yeast extract: 5.0; sucrose: 30.0; agar: 15.0; $NaNO_3$: 0.3; KCl: 0.05; $MgSO_4$, $7H_2O$: 0.05; $FeSO_4$, $7H_2O$: 0.001; $ZnSO_4$, $7H_2O$: 0.001; $CuSO_4$, $5H_2O$: 0.0005 for 7 day at 25^0C at initial pH 6.0 and stored at 4^0C for maximum period of 14 d. Unless otherwise stated, all chemicals used in this work were purchased from Merck (India) and Sigma (USA). Deionized water was used throughout the experiment to reduce undue effect of metal ion contamination in growth or production medium.

3.2 Inoculum preparation and submerged fermentation in rotary shaker

Aspergillus giganteus MTCC 8408 culture was grown in 250 ml Erlenmeyer flasks containing 60 ml of Czapek's yeast extract broth (except agar); at temperature 25^0C, orbital rotator speed 150 rpm for 24 h at initial pH 6.0, to use as inoculum (A_{600nm} ≥0.9) in next phase submerged fermentation.

3.3 Production media design

3.3.1 OFAT (one factor at a time) approach

Submerged batch fermentation was carried out separately in modified Olson **[78]** medium containing 0.28% K_2HPO_4, 0.06% $MgSO_4$, 0.15% $CaCl_2$ and 0.001% $FeSO_4$, in presence of

different carbon sources (glucose, maltose, lactose, sucrose and soluble starch) with various organic nitrogen sources: peptone (PEP), beef extract (BE), corn steep liquor (CSL), proteose peptone (PP) and inorganic nitrogen sources: Ammonium nitrate (An), Ammonium citrate (Ac), Ammonium chloride (Acl), Sodium nitrate (Sn) at initial orbital rotator speed 120 rpm for 144 hours at initial temperature 25^0 C with 5% (v/v) of the seed culture. Initial pH and temperature were also varied to approximate the control factors and to make the results become reliable and reproducible.

3.3.2 Taguchi statistical DOE methodology

Taguchi method uses various types of signal-to-noise (S/N) ratios to measure the variability around the target performance. Statistical optimization methodology adopted in the present study was divided into 4 phases' viz., design of experiments (planning); Submerged fermentation (experimentation); Analysis of experimental data and prediction of performance (software analysis); and validation of results. Objectives of each phase are interconnected in sequence to achieve the overall bioprocess strategy development process **[264-273].**

$$SN_i = -10log\left[\frac{1}{N_i}\sum_{u=1}^{N_i}\frac{1}{y_u^2}\right]. \qquad \text{..... (3.1)}$$

The performance quality "larger the better" have been used to define the optimum conditions and evaluated using Equation (1): Where N_i, the number of trials for experiment i designed at their assigned level, i, the experiment number, u, the trial number and y, experimental value of each trial. The effect of each of these factors was identified using main effect, severity index (SI) and analysis of variance (ANOVA) study. Data obtained were analyzed using Qualitek-4 (Nutek Inc., MI, USA).

3.3.3 C/N ratio and essential microelement sources (Taguchi DOE L_8)

Based on the results of OFAT approach, the suitable carbon and nitrogen sources were pooled and effect of individual Na^+ (5.1724 and 10.3448 m-equiv.), K^+ (1.7214 and 5.1724 m-equiv.), Mg^{2+} (3.252 and 4.878 m-equiv.), and Ca^{2+} (1.08 and 3.2432 m-equiv.) ions on biochemical yield coefficient, $Y_{p/x}$ and cell biomass during submerged fermentation of *Aspergillus giganteus* MTCC 8408 was elucidated at various assigned ratio of C/N, K^+/Ca^{2+} and Mg^{2+}/Na^+. Taguchi design of experiments (DOE) was employed with inner array to carry out three interaction columns (1 × 2, 1 × 4 and 2 × 4) in 8 well defined experiments, L_8 **(Table 3.1).**

Table 3.1 Selection of factors at their assigned level (Taguchi DOE L_8 OA)

Columns	Factors	Level 1	Level 2
1	C/N ratio	27.4:1	54.9:1
2	K^+/Ca^{2+}	1.59:1	4.78:1
3	INTER COLS 1 × 2	–	–
4	Mg^{2+}/Na^+	0.62:1	0.94:1
5	INTER COLS 1 × 4	–	–
6	INTER COLS 2 × 4	–	–

INTER COLS represents the interaction column data among the investigated 6 columns.

The CN ratio was evaluated using Equation (css.cornel.edu) (2):

$$CN\ ratio = \frac{\mathrm{Q1[C1(100-M1)]+Q2[C2(100-M2]+\cdots Qn[Cn(100-Mn)]}}{\mathrm{Q1[N1(100-M1)]+Q2[N2(100-M2]+\cdots Nn[Cn(100-Mn)]}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.2)$$

$\mathrm{Qn} = \text{sample weight taken};\ \mathrm{Cn} = \%\ \text{carbon}\ ;\ \mathrm{Nn} = \%\ \text{nitrogen};\ \mathrm{Mn} = \%\ \text{moisture}$

3.3.4 Final phase fermentation (Taguchi DOE L_{27} OA)

Based on the results of Taguchi DOE L_8 approach, final phase fermentation was performed in rotary shaker containing in various combination with six most decisive factors at their assigned individual level (pH: 5.2, 5.8, 6.4; temperature: 25, 29, 33^0C; slant age: 3, 7, 11 d; inoculums vol.: 2.5, 5.0, 7.5% (v/v); agitation: 120, 150, 180 rpm; KH_2PO_4: 0.1, 0.2, 0.3 g L^{-1}) and carbon, nitrogen sources at their assigned individual level as per Taguchi's DOE L_{27} OA. The upper and lower limits of carbon and nitrogen variable were chosen to encompass the range and to reflect results after a preliminary investigation of the limits in Taguchi DOE L_8 results. All experiments were performed in duplicate (mean ± standard deviation of double determination). Total volume of fermentation broth was 3 L.

3.4 Kinetic model development

The growth kinetic model was elucidated linking microscopic to macroscopic parameters of the kinetics of fungal mycelial growth. A microscopic description was based on the assumption that mycelial growth *of Aspergillus giganteus* MTCC 8408 can be represented approximately by branching level parameter (k) which is logarithmically related to symmetric binary tree where every branching level was associated with a number of tips (N_t) and segments (N_s) **[274-276].**

The elucidation of microscopic parameters was based on simple growth kinetics in terms of calculated as

$$\mathbf{N_t = 2^k} \qquad \text{..... (3.3)}$$

The no of segments (N_s) with average length (L_{av}) was elucidated, as

$$\mathbf{N_s = (2^{k+1} - 1)} \qquad \text{..... (3.4)}$$

And $$\mathbf{L_{av}} = \frac{\mathbf{L_t}}{\mathbf{N_s}} \quad \text{..... (3.5)}$$

Where, $\mathbf{L_t}$= total hyphal length. The hyphal growth unit (**G**) was measured, as

$$\mathbf{G} = \frac{\mathbf{L_t}}{\mathbf{N}_t} = (\mathbf{L_{av}} \times \boldsymbol{\alpha}) \quad \text{..... (3.6)}$$

Where, $\boldsymbol{\alpha} = \frac{\mathbf{N_s}}{\mathbf{N_t}} = \frac{(\mathbf{2^{k+1}} - 1)}{\mathbf{2^k}}$. If, X_c is the critical biomass concentration at maximized antifungal protein production, then

$$\mathbf{X_t} = (\mathbf{X_c} \times \mathbf{N_s}) \quad \text{..... (3.7)}$$

If E is the mean hyphal extension rate, an expression can be derived for the specific growth rate of the mycelium, μ_x

And, $$\mu_x = \left(\frac{(2 \ln 2) \times \mathbf{E}}{\mathbf{G}} \right) \quad \text{..... (3.8)}$$

Therefore; under optimal growth condition, combining morphological parameters effect on biomass growth with hyphal growth unit (G) of fermentation parameter, provided,

$$\frac{dX}{dt} = \left(\frac{\mathbf{1.386} \times \mathbf{E}}{\mathbf{G}} \right) (\mathbf{X}) \quad \text{..... (3.9)}$$

Equation (9) allows consideration of the extent of mycelial branching and its effect on the specific growth rate (μ_x). It also allows prediction of specific growth rate, determined in terms of biomass through incorporating 'macroscopic' model predicts the observed difference in specific growth rate between germ tubes and exponentially growing mycelia during batch culture. The model therefore may provide a new approach to descriptions of mycelial growth and is valuable in linking morphological properties to kinetics. Testing of the model will be facilitated with the image analysis techniques, available for quantification of mycelial morphology.

A macroscopic description was based on the assumption that carbon source is the only limiting factor during fermentation and metabolite production is intracellular in nature and growth associated with cell biomass. The unstructured growth kinetic model was evaluated by comparing similarities and differences between the six models used in published available data on the basis of steps, included: Acquisition of data; Fitting the data; statistical analyses; model prediction and validation.

In order to process modeling, the experimental data with good enough precision were taken to elucidate the effect of different concentration of the substrate on the growth of *Aspergillus giganteus* MTCC 8408 measured over several hours. Ordinary differential equations were simultaneously solved by the Runge-Kutta-4 integration technique using Berkeley Madonna software (9.0.126, USA). All parameters optimized with the functions 'multiple curve fit' "batch run" and 'parameter slide'.

Table 3.2 Various mathematical models for growth kinetics and modeling used in this study

Name	Model	Parameter
Monod	$\mu_m \frac{S}{S + K_s}$	2
Moser	$\mu_m \frac{S^n}{S^n + K_s};\ n\rangle 0$	3
Tessier	$\mu_m \left(1 - e^{S/K_s}\right)$	2
Blackman	$\mu_m \frac{S}{2K_s};\ S\langle 2K_s$ $\mu_m \quad ;\ S\rangle 2K_s$	2
Haldane	$\mu_m \frac{S}{\frac{S^2}{K_i} + S + K_s}$	3
Logistic (Verhlust)	$\mu_m \left(1 - \frac{X}{X_m}\right)$	2

To decide whether there is a statistically substantial difference between models (**Table 3.2**) with different number of parameters, in terms of the quality of fit to the same experimental data was statistically assessed through various methods such as the root mean square error (RMSE), adjusted coefficient of determination (adjR^2) and corrected AICc (Akaike Information Criterion) **[277].** An adjusted R^2 was used to calculate the quality of nonlinear models as in nonlinear regression the adoption of the model does not readily provides comparable analysis.

The Akaike information criterion (AIC) measured the relative quality of a given statistical model for a given set of experimental data. The more the parameters, the less preferred the output or the higher the AICc value. Hence, AIC not merely rewards goodness of fit, but in addition does not encourage using more complicated model (over fitting) for fitting experimental data. Since the data in this work is small compared to the number of parameter used a corrected version of AIC, the Akaike information criterion (AICc) with correction was used instead **[278].**

3.5 Isolation and purification of protein

3.5.1 Crude protein extraction

The harvested mycelia was filtered, washed with deionized water (conductivity <1 mS) and kept in minimum volume buffer (pH 8.0 ± 0.2) comprised of 100 mM Tris-HCl, 10% ethylene glycol, 0.05 mM EDTA, 1 mM TCEP, 1 mM aprotinin (protease inhibitor from Sigma, USA). Sonication (UP200S, hielscher) was applied (60% amplitude and 0.5 cycles) under a constant temperature 4^0C in cooling water bath. The mycelial biomass was measured after repeated washing of the mycelia with 0.1 M PBS (phosphate buffered saline) at pH 7.4±0.2 followed by deionized water and kept in hot air oven for overnight (at 85^0C) to constant weight.

3.5.2 Protein fractionation and purification

The crude proteins fractionated and purified **[279]** using pure ammonium sulfate (0–30%, 30–50%, 50–70% and 70–90%). Each fraction was pooled in minimum volume of Tris-HCl buffer (pH 7.4 ± 0.2) and dialyzed overnight (< 2kDa, 9 mm benzoylated, Sigma) in the same buffer at 4^0C. Antimicrobial assay was performed with each crude fraction. Active and potent fraction, designated as antifungal protein (afp) was then purified using cation-exchange resin carboxymethyl cellulose (CMC) column (2.8 × 20 cm^2) followed by Sephadex G-100 column (1.6 × 36 cm^2) and eluted with the 0.1 M NaCl – 0.05 M sodium acetate buffer (pH 7.4) at the rate of 0.25 ml min^{-1}. Each purified fraction was lyophilized (24 hrs at -48^0C, 0.15 mbar) and quantitative estimation of each fraction was determined by measured absorbance read at A_{280} nm in a spectrophotometer (UV-1800, Shimadzu, bandwidth: 190-1100 nm, accuracy: ±0.002-0.006, double beam) against standard Bovine Serum Albumin (BSA) **[280].**

$$\text{Protein concentration } (\mu g\ mL^{-1}) = A_{280} / 1\ cm \times \text{Absorbance coefficient } (\epsilon_{280,\ 1\ mg\ mL^{-1}})$$

3.6 In-vitro study

3.6.1 Pathogen selection

To investigate the antifungal specificity of the protein, six fungal pathogens were tested for their susceptibility to the protein. Plant pathogenic fungi: *Fusarium oxysporum, Botrytis cinerea*; opportunistic fungal pathogen in human: *Aspergillus fumigatus (causes invasive aspergillosis), Candida albicans* NCIM 3471 *(invasive candidiasis)* and model organism: *Aspergillus niger* were selected as test organisms **[83].**

3.6.2 Antimicrobial assay

The antimicrobial activity of protein was analyzed by micro-broth dilution, disc diffusion assay **[281]**. Assay was performed in duplicate (mean ± standard deviation of double determination).

3.6.2.1 Disc diffusion study

The test was performed in sterilized Petri plates (Tarsons) impregnated with different concentration of protein on the agar surface of the plates that already inoculated with spores of selected pathogens ($A_{600} \geq 0.6$). The plates were incubated at 37^0C and examined zone of inhibition after 36 h of growth, if any clearance around the plate.

3.6.2.2 Dose response study

The test was performed with spores of selected pathogens, harvested from 36 h cultures ($A_{600} \geq 0.6$) and treated with various concentrations of protein (100 µl) in culture tube prepared by a twofold dilution method. The tubes were incubated at 37^0C and examined macroscopically after 36 h of growth. The assay was performed as per the standard method **[281].** The minimum inhibitory concentration (MIC) was elucidated at the lowest concentration that inhibited 90% growth of test pathogens. The MICs were determined three times and each time in duplicate.

Based on the results of antimicrobial assay, the active protein fraction was further investigated for molecular characterization and identification.

3.6.3 Cytotoxicity assay

3.6.3.1 Materials

Dulbecco's modified eagle medium (DMEM) were purchased from Cellclone, (Genetix Biotech Asia Pvt. Ltd, fetal bovine serum (FBS), antibiotic solution (Penicillin 1000 IU, Streptomycin 10mg ml^{-1}), trypsin and MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazoliumbromide dye) were purchased from [Himedia, India], whereas DMSO (dimethyl sulphoxide), was obtained from Merck, India. Hoechst 33342 were purchased from Sigma, USA, whereas propidium iodide (PI) was obtained from EMD Millipore-Calbiochem, USA.

3.6.3.2 Cell culture

HeLa (Human cervical cancer cell line) was maintained in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and antibiotic solution (1000 IU of Penicillin and 10mg ml^{-1} Streptomycin), at 37ºC in a humidified atmosphere of 5% CO_2.

3.6.3.3 Methods

The MTT [3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay is a colorimetric assay, which is used for assessing cell viability and proliferation. 10, 000 HeLa cells were seeded in a 96 well culture plates in complete DMEM and left to adhere for 24 h at 37°C and 5% CO_2. The cells were then treated with various concentration of antifungal peptides for 24 h at 37°C and 5% CO_2. On incubation, 10 µl MTT solutions (5mg ml^{-1}) were added into each well. The plate was then incubated for additional 2 h at 37°C to form formazan crystals. The purple formazan crystals were dissolved in 100 µl DMSO at 37°C for 30 min.

The absorbance was then spectrophotometrically measured in a plate reader (MicroScan MS5608A) at 570 nm (Mosmann, 1983). All experiments were carried out in three replicates and the inhibitory concentration (IC_{50} value) of the extract was estimated using the formula,

$$\% \text{ inhibition} = \frac{(\text{Control absorbance} - \text{sample absorbance}) \times 100}{\text{Control absorbance}}$$

3.6.4 Cell microscopy

Cells morphology of *Aspergillus giganteus* MTCC 8408 at various nutrients composition was visualized by scanning electron microscopy (SEM). Cell lysis of target pathogen (in-vitro) at various antimicrobial protein concentrations was visualized by confocal microscopy, SEM and atomic force microscopy (AFM). Apoptotic detection (cytotoxicity) in HeLa cells was visualized by inverted fluorescence microscopy (EVOS FL).

3.7 Molecular characterization

3.7.1 SDS-PAGE

Silver stained SDS-PAGE on a 12% w/v acryl amide gel-0.1% SDS was performed for the determination of average molecular weight of the purified protein in accordance with the procedure of a standardized method described by Chevallet et al., **[282].**

3.7.2 ESI-MS study

Precise molecular weight of the protein was determined using electro spray ionization-mass spectrometry **[283].** These spectra are characterized by assigned intensities around the base peak and increased spacing between their peaks with increasing value of signal to noise ratio. Data was analyzed using ESI-MS Software ESIProt 1.0.

3.7.3 FTIR study

Characterization of the protein was performed by using FTIR (IR affinity 1-S, Shimadzu) as per standard protocol **[284].** Purified Protein was dissolved in sterilized Tris-HCl buffer and stored at -20^0C.

3.7.4 MALDI-MS study

In-gel protein digests of active fraction were prepared as described **[285]**. The purified band from the SDS-PAGE gel was sliced out. After washing with distilled water, the gel slices were incubated for 20 min in wash solution (50% acetonitrile in 50 mM NH_4HCO_3) to remove the staining dye. Gel pieces were treated with 150 ml 10 mM DTT in 100 mM NH_4HCO_3 for 10 min and incubated in 5% acetonitrile for 1 h at 55^0C. The gel pieces were dehydrated in 100 ml 100 mM NH_4HCO_3 and 100% acetonitrile for 10 and 20 min, respectively. For alkylation, 100 ml 50 mM iodoacetamide in 100 mM NH_4HCO_3 was added to the gel pieces and incubated in the dark at room temperature for 30 min.

The gel pieces were washed with 100 ml 100 mM NH_4HCO_3 for 10 min and then dried in a vacuum centrifuge for 15 min. Subsequently, 20 ml trypsin solution (10 ng ml^{-1} in 50 mM NH_4HCO_3) was added to tubes having gel pieces and incubated at 37^0C for 16 h. Digested peptides were extracted with buffer (5% trifluoroacetic acid in 60% acetonitrile) and concentrated by cold centrifugation for 3 h. The lyophilized peptides were resolubilized in a resuspension solution (1% trifluoroacetic acid in 50% acetonitrile) and then centrifuged for 2 min to obtain the supernatant. The peptide solution was mixed with matrix sinapinic acid (3,5-dimethoxy-4-hydroxy cinnamic acid) in a ratio of 1:1 and subjected to analysis by MALDI-TOF MS (Bruker TOF/TOF).

3.8 Identification

3.8.1 MASCOT analysis

The N-terminal peptide sequences along with their masses acquired from MALDI-TOF MS were analyzed by the Mascot search of Matrix Science to find out the peptide matches present in the MSDB protein database. Multiple alignments of sequences were performed using NCBI BLAST and Uni-Prot data base search to elucidate sequence homology and peptide matches **[286]**.

3.8.2 In Silico investigation

The SignalP1 4.1 server was used to predict the cleavage site of the signal sequence **[287].** The molecular weight, pI, grand average of hydropathy (GRAVY) value, total net charge, and disulfide bridge pattern of the were predicted by ExPAsy ProtParam tool **[288],** Protein Calculator v3.4 server (The Scripps Research Institute; http://www. scripps.edu/~cdputnam/protcalc.html), and DISULFIND Cysteines Disulfide Bonding State and Connectivity Predictor server **[289]**, respectively. Structure of protein using artificial neural network was investigated using PSIPRED workbench.