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RESEARCH PAPER

Optimization and partial characterization of intracellular anticandidal protein from *Aspergillus giganteus* MTCC 8408 using taguchi DOE

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ABSTRACT

A new intracellular antifungal protein (afp) production with average molecular weight 24.3 kDa and yield of 0.65 ± 0.1 mg/gram dry cell weight (gdcw) of mycelia in submerged fermentation of *Aspergillus giganteus* MTCC 8408 was optimized. Taguchi's DOE (design of experiment) L_{27} orthogonal array (OA) was constructed using Qualitek-4 software with 8 most influensive factors namely, culture pH, temperature, slant age, inoculum volume, agitation and KH_2PO_4. Scanning electron microscopy (SEM) was used to correlate the effect of selected factors on fungal cell morphology and afp production. The crude protein purification was accomplished using pure ammonium sulfate fractionation followed by carboxymethyl cellulose (CMC) ion-exchange chromatography and sephadex G-100 gel filtration. The average molecular mass of the purified protein was figured by silver stained SDS (sodium dodecylsulphate)-PAGE (polyacryl amide gel electrophoresis). *In vitro* antifungal susceptibility assay was profiled against *Candida albicans* NCIM 3471 and minimum inhibitory concentrations (MICs) were in the range 3 to 4 μg/ml. Characterization of protein was observed with FTIR (Fourier transform infrared spectroscopy) analysis. The optimal production condition for crude afp was obtained as follows: soluble starch: 20 g/l; Corn steep liquor (CSL, 2%) + proteose peptone (PP, 1%): 30 g/l; pH: 5.8; temperature: 25°C; slant age: 3 d; inoculum size: 5% (v/v); agitation: 180 rpm; KH_2PO_4: 0.1 g/l. The validation experiments using optimized conditions confirmed an improvement in afp production by 59.4% against the expected enhancement of afp production by 61.22%. The present statistical optimization study revealed an opportunity to promote economical design at the industrial level for future scale up of effective antifungal agent against opportunistic oral and vaginal infection.

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Introduction

The exploitation of fungi for the production of biotechnologically relevant products is ancient and progressively flourished over the last few decades. Fungi are morphologically filamentous and coordinated microorganisms and possessed several structural forms throughout their life cycles.[1] In submerged culture, distinct morphological forms, from dispersed pulp like mycelial filament (low nutrients concentration gradients) to densely interwoven pellet have been reported.[2] Cell morphology induces distinguishable effects on its metabolite (intracellular) release in submerged fermentation broth. Dispersed pulp like morphology results higher mass transfer coefficients (low nutrients concentration gradients) compare with pellet type morphology and hence, fungal morphology is believed as a one of the most important qualitative tool for bioprocess optimization of its metabolite production.[3]

Extracellular antifungal protein was first reported in *Aspergillus giganteus*, isolated from the soil of a farm in Michigan (USA) and possessed beneficial physiological activities such as antitumor and capable of inhibiting germination and growth of many filamentous Ascomycetes.[4-8]

So far, only few antifungal proteins have been reported, namely AFP from *Aspergillus giganteus*, ANAFP from *Aspergillus niger*, PAF from *Penicillium chrysogenum* and NAF from *Penicillium nalgiovense*, ACLA, *Aspergillus clavatus*, ANAFP1, *Aspergillus niger* strain KCTC 2025, ANAFP2, *Aspergillus niger* strain CBS 513.88 NFAFP, *Neosartorya fischeri*,

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BFAFP, *Botryotinia fuckeliana*, GZAFP, *Gibberella zeae*, PTRAFP, *Pyrenophora tritici-repentis*.[9-15] Antifungal protein production from *A. giganteus* has been reported in submerged fermentation.[4,16] For effective improvement of afp production, it is therefore essential to optimize simultaneously the culture conditions with nutrients composition of media. To the best of our knowledge, there is not enough information regarding optimum nutritional requirements for anticandidal protein production by *Aspergillus giganteus* MTCC 8408 under submerged fermentation. Indeed, statistical optimization and growth inhibition study of this new intracellular protein against *Candida albicans* NCIM 3471 (responsible for opportunistic oral and vaginal) has been studied.

In conventional approach, an optimization process usually involves one factor at a time (OFAT),[17-19] this procedure is more time consuming and cumbersome which usually involves more experimental data sets and do not provide information about the mutual interactions of the parameters. In contrast, statistically planned Taguchi method reduces the number of experiments through developing a specific DOE, which also minimize the error in determining the values for most decisive parameters.[20,21] Although, the response surface methodology (RSM) have received much attention in previous scientific researches,[22] Taguchi method is appeared as powerful optimization techniques as it possesses some advantages, such as extraction of much quantitative information by only a few experimental trials and provides a systematic and efficient plan for performing experiments under the consideration of the interactive effects among the control factors[23] and requires half of the time than RSM,[24,25] hence, has gained much popularity in engineering as well as in the field of biotechnology applications.[26-31]

Nowadays Taguchi DOE method, based on OA design, have been appreciated in many biotechnology/bioprocess engineering,[20,21] and possesses many advantages, includes efficient and logical plan for performing experiments under the consideration of the interactive effects among the control factors.

The objective of this study was to identify the best possible condition for the production of new intracellular afp by *Aspergillus giganteus* MTCC: 8408 in submerged fermentation by applying Taguchi DOE and characterization. The experiments were designed with 8 most decisive factors including carbon and nitrogen sources, culture pH, temperature, slant age, inoculum volume, agitation and KH_2PO_4 for afp production at 3 levels with OA layout of L_{27}.

Materials and methods

Strain and culture condition

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): K_2HPO_4 1.0; yeast extract: 5.0; sucrose: 30.0; agar: 15.0; $NaNO_3$: 0.3; KCl: 0.05; MgSO4, $7H_2O$: 0.05; FeSO4, $7H_2O$: 0.001; ZnSO4, $7H_2O$: 0.001; CuSO4, $5H_2O$: 0.0005 for 7 d at 25°C at initial pH 6.0 and stored at 4°C for maximum period of 14 d. Unless otherwise stated, all chemicals used in this work were purchased from Merck and Sigma.

Inoculum preparation and submerged fermentation in shake flask culture

Aspergillus giganteus MTCC 8408 culture was grown in 250ml Erlenmeyer flasks containing 60 ml of Czapek's yeast extract broth (except agar); at temperature 25°C, orbital rotator speed 150 rpm for 24 h at initial pH 6.0, to use as inoculum ($A_{600nm} \geq 0.9$) in next phase submerged fermentation. Final phase was performed in shake flask containing broth (g/l): MgSO4, $7H_2O$: 0.6; $CaCl_2$: 0.3; NaCl: 1.0; in various combination with 8 most decisive factors at their assigned individual level (soluble starch: 20, 30, 40 g/l; CSL, 2%+PP, 1%: 30, 40, 50 g/l; pH: 5.2, 5.8, 6.4; temperature: 25, 29, 330C; slant age: 3, 7, 11 d; inoculums vol.: 2.5, 5.0, 7.5 %v/v; agitation: 120, 150, 180 rpm; KH2PO4: 0.1, 0.2, 0.3 g/l) as per Taguchi's DOE L_{27} OA (Table 1). All experiments were performed in duplicate (mean ± standard deviation of double determination).

Taguchi methodology

Taguchi method uses various types of signal-to-noise (S/N) ratios to measure the variability around the target performance.[32] 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

Table 1. Taguchi DOE L_{27} OA projection with selected factors for afp production in submerged fermentation of *A. giganteus* MTCC 8408.

Run No.	Columns 1	2	3	4	5	6	7	8	Afp* (mg/l) ± SD	S/N ratio	Mycelial* Biomass (gdcw/l ± SD)
1	1	1	1	1	1	1	1	1	16.6 ± 0.2	24.4	24.6 ± 0.35
2	1	1	1	1	2	2	2	2	14.5 ± 0.2	23.224	23.1 ± 0.48
3	1	1	1	1	3	3	3	3	12.3 ± 0.2	21.794	16.1 ± 0.78
4	1	2	2	2	1	1	1	2	10.05 ± 0.15	20.04	15.3 ± 0.65
5	1	2	2	2	2	2	2	3	10.6 ± 0.2	20.501	15.1 ± 0.8
6	1	2	2	2	3	3	3	1	12.0 ± 0.2	21.58	17.9 ± 0.54
7	1	3	3	3	1	1	1	3	7.85 ± 0.25	17.884	11.2 ± 0.42
8	1	3	3	3	2	2	2	1	8.5 ± 0.3	18.572	10.8 ± 0.23
9	1	3	3	3	3	3	3	2	8.35 ± 0.55	18.377	14.2 ± 0.44
10	2	1	2	3	1	2	3	1	6.05 ± 0.05	15.634	12.7 ± 0.21
11	2	1	2	3	2	3	1	2	5.25 ± 0.35	14.345	10.1 ± 0.88
12	2	1	2	3	3	1	2	3	4.75 ± 0.15	13.52	9.4 ± 0.58
13	2	2	3	1	1	2	3	2	7.0 ± 0.1	16.899	14.2 ± 0.87
14	2	2	3	1	2	3	1	3	6.15 ± 0.25	15.755	12.1 ± 0.41
15	2	2	3	1	3	1	2	1	4.5 ± 0.3	13.006	11.8 ± 0.77
16	2	3	1	2	1	2	3	3	4.85 ± 0.05	13.713	9.9 ± 0.43
17	2	3	1	2	2	3	1	1	4.5 ± 0.4	12.961	8.6 ± 0.9
18	2	3	1	2	3	1	2	2	4.1 ± 0.2	12.224	12.8 ± 0.12
19	3	1	3	2	1	3	2	1	8.4 ± 0.5	18.439	15.8 ± 0.39
20	3	1	3	2	2	1	3	2	7.95 ± 0.15	18.002	14.2 ± 0.29
21	3	1	3	2	3	2	1	3	7.4 ± 0.2	17.375	11.8 ± 0.32
22	3	2	1	3	1	3	2	2	5.4 ± 0.1	14.643	6.7 ± 0.66
23	3	2	1	3	2	1	3	3	5.65 ± 0.45	14.958	9.9 ± 0.21
24	3	2	1	3	3	2	1	1	6.25 ± 0.05	15.916	10.1 ± 0.45
25	3	3	2	1	1	3	2	3	9.85 ± 0.05	19.868	11.9 ± 0.12
26	3	3	2	1	2	1	3	1	10.0 ± 0.2	19.994	14.9 ± 0.38
27	3	3	2	1	3	2	1	2	8.0 ± 0.1	18.059	11.2 ± 0.67

*Mean ± SD (standard deviation) for double determination, gdcw/l = gram dry cell weight per liter of fermentation broth).

(software analysis); and validation of results. Objectives of each phase are interconnected in sequence to achieve the overall optimization process.[20,21] The performance quality "larger the better' have been used to define the optimum conditions and evaluated using Equation [1]:

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

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 upper and lower limits of each variable were chosen to encompass the range and to reflect results after a preliminary investigation of the limits. The effect of each of these factors was identified using main effect, severity index (SI) and analysis of variance (ANOVA) study.

Crude protein extraction

The harvested mycelia was filtered, washed with deionized water (conductivity <1 μS) 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, 1mM aprotinin (Sigma, USA). Sonication (UP200S, hielscher) was applied (60% amplitude and 0.5 cycles) under a constant temperature 4°C in cooling water bath.

Protein fractionation and purification

The crude proteins fractionated[33] and purified[34] 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 (9mm, benzoylated, Sigma) in the same buffer at 4°C. Each fraction 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) 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. Each purified fraction was lyophilized (24 hrs at −48°C, 0.15 mbar) and absorbance was read at 280 nm in a spectrophotometer (UV-1800, Shimadzu). Anticandidal assay was performed with each fraction and quantitative estimation of purified protein was determined by measured absorbance

(UV-1800, Shimadzu) at 545 nm using Bradford-Coomasie assay protocol.[35]

Molecular weight determination

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.[36] The bands on the gel were observed and compared with standard proteins.

MIC determination and antifungal susceptibility assay

Antifungal assay was performed according to a standardized broth micro-dilution method (Clinical and Laboratory Standards Institute (CLSI) document M27-A2). In brief, the *Candida albicans* NCIM 3471 colonies from 24-hour-old cultures were picked and resuspended in 5 mL of sterile 0.145 mol/L saline and adjusted to cell density of 10^6 cells/ml. Conventional antifungals such as amphotericin B (Sigma, USA) and fluconazole (Sigma, USA) were included. Purified proteins and the conventional antifungals were then serially diluted in RPMI 1640 growth medium buffered with morpholinepropanesulfonic acid (MOPS). 100 mL of standardized yeast suspension was then added and incubated for 48 h at 37°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited 90% growth of *Candida* species. The MICs were determined 3 times and each time in duplicate.

Characterization

Characterization of the protein was performed by using FTIR (IR affinity 1-S, Shimadzu) as per standard protocol.[37] Purified Protein was dissolved in sterilized Tris-HCl buffer and stored at −20°C.

Results

Effect of carbon and nitrogen sources

The one-factor-at-a-time (OFAT) method was employed to study the effects of various carbon and nitrogen sources on production of antifungal protein in submerged fermentation of *Aspergillus giganteus* MTCC 8408. Five different carbon sources (glucose, maltose, lactose, sucrose, soluble starch) were separately provided in the nutrient broth containing (g/l): C-sources: 20; yeast extract: 30; MgSO4, $7H_2O$: 0.6; $CaCl_2$: 0.3; NaCl: 1.0; at initial pH 6.0 and temperature 25°C, initial agitation 150 rpm, inoculum level 2.5% and slant age 7 d. Among the carbon sources studied, soluble starch supported the highest mycelial growth (data not shown), with maximum afp production. Different carbon sources may have different effects of catabolic repression on the cellular secondary metabolism. In glucose and maltose, fungal cell grew as hollow mycelial pellets (due to high mass transfer limitation) with much reduced amount of afp whereas in soluble starch and lactose, it grew as dispersed phase (due to high mass transfer coefficient) pulp like growth with comparative improvement of afp.

Ten different organic and inorganic nitrogen sources were separately supplemented into the basal nutrient medium containing (g/l): N-sources: 30; soluble starch: 20; MgSO4, $7H_2O$: 0.6; $CaCl_2$: 0.3; NaCl: 1.0; at initial pH 6.0 and temperature 25°C, initial agitation 150 rpm, inoculum level 2.5% and slant age 7 d. The highest mycelial biomass and afp production were achieved with CSL (2%) + PP (1%) supplemented media (data not shown). In comparison with organic nitrogen sources, inorganic nitrogen sources resulted relatively lower mycelial biomass as well as afp production.

Fermentation factors and their interaction effect

The main effects of the fermentation factors at their individually assigned levels on afp production explained the average of obtained results (based on S/N value). Certainly, the observed variation indicates the important role of 8 most decisive factors in achieving the best possible combination for optimization.

The difference between the average value of each factor at level 2 and level 1 (L_2-L_1) predict the relative influence of the effect. The larger the difference, the stronger will be the influence. The sign of the difference (1 or 2) indicated whether the change from level 1 to level 2 increased or decreased the result. Stronger influence was observed with pH (L_2-L_1 = 1.077 mg/l) among the factors studied, followed by inoculum level (L_2-L_1 = 0.651 mg/l) in afp production (data not shown). Influence of each individual factor on afp production by *A. giganteus* MTCC: 8408 in submerged fermentation was mentioned in Fig. 1. Dispersed pulp

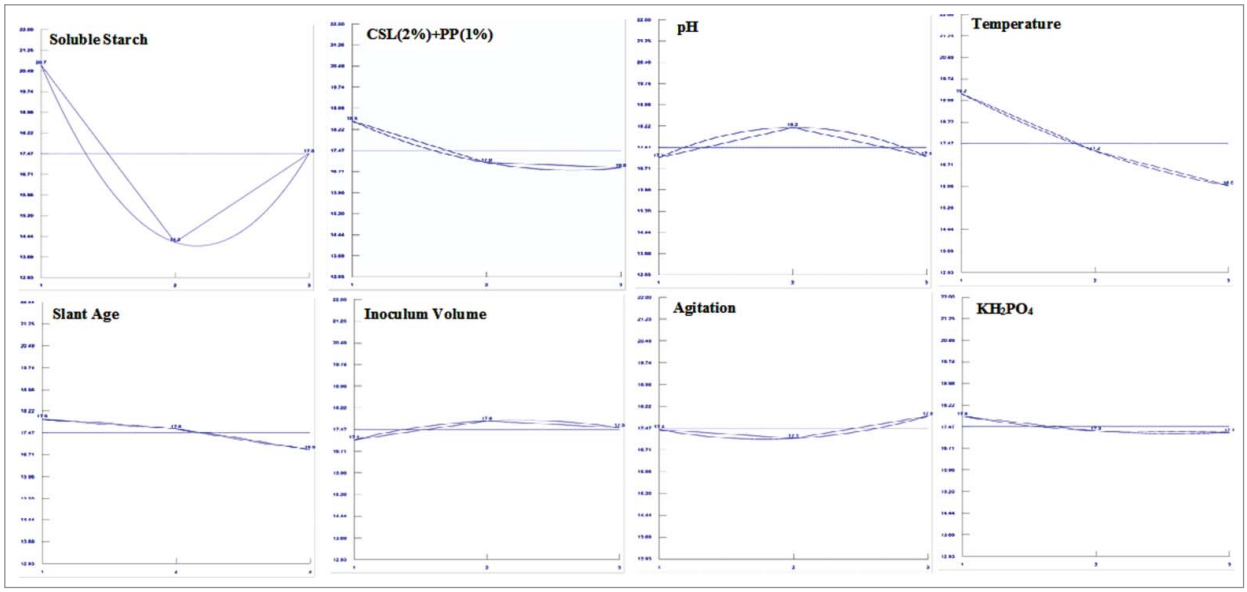

Figure 1. Impact of selected factor levels on afp production by *A. giganteus* MTCC: 8408 under submerged fermentation.

like morphology was preferred because of higher mass transfer coefficients (low nutrients concentration gradients) with improved afp production compare with pelleted morphology. Change of morphological traits of fungal mycelia at different nutrients composition at their assigned individual level was mentioned in Fig. 2.

At individual level stage, the higher afp production was observed with soluble starch at level 1 (Fig. 2a)

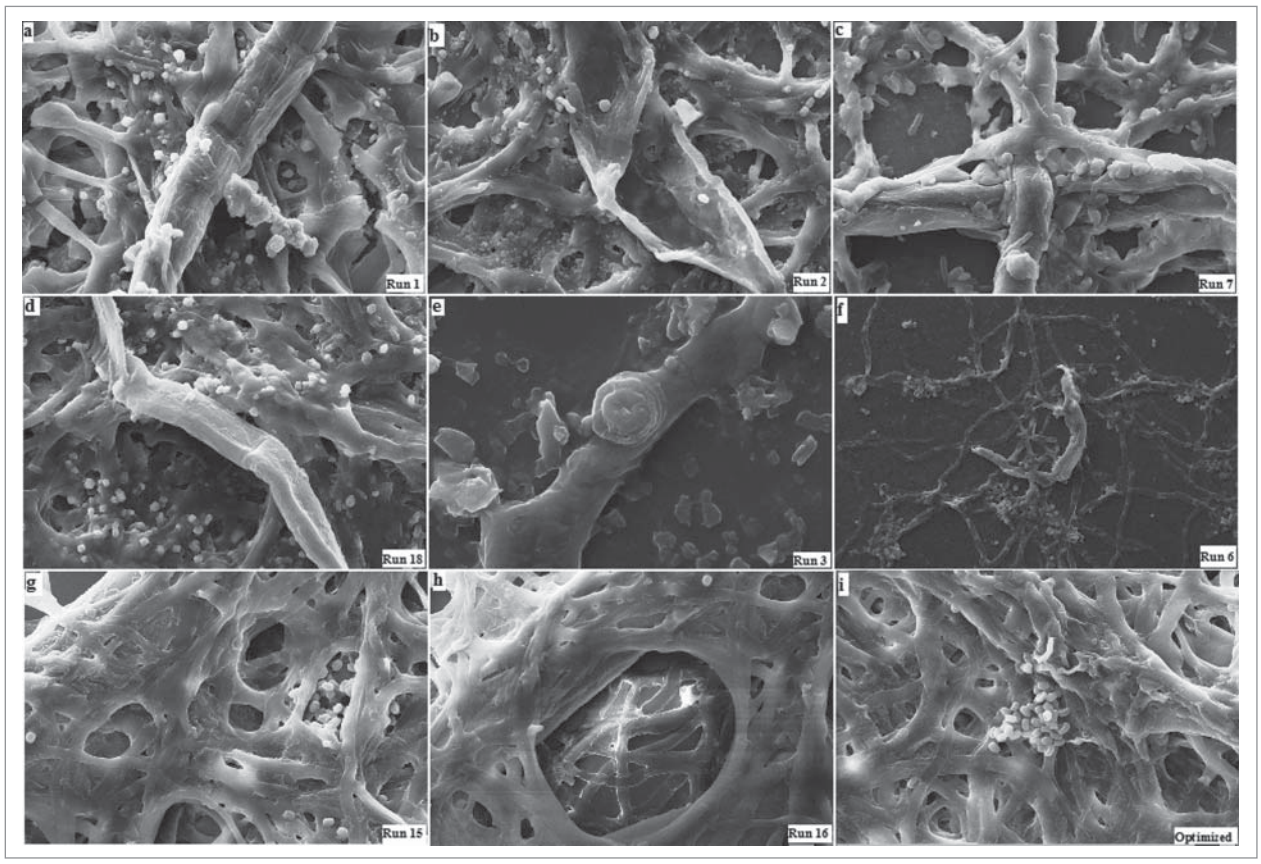

Figure 2. Morphological behavior of *Aspergillus giganteus* MTCC 8408, showing the effect of various nutrients composition on optimization. Lateral resolution: 2–10 μm; magnification: 5KX; EHT: 18.00 KV; WD: 9.5 mm.

(20.708 mg/l) followed by temperature at level 1 (Fig. 2b) (19.222 mg/l). Qualitative image analysis showed that *A. giganteus* MTCC 8408 grew as cluster of cell with better spore germination and hyphal growth. Although membrane perturbation due to the induction of intracellular oxidative stress was also noticed.

Increase in temperature enhanced reduction of afp production up to level 3 where cell grew as dispersed hyphae with dense branching and retardation of the length of the hyphae as shown in Fig. 2c (17.204 and 15.983 mg/l). At individual level stage, increase in soluble starch concentration resulted significant decrease of afp production up to level 2 where mycelia grew as swollen and short hyphae with multiple branches (Fig. 2d) (14.229 mg/l). Higher afp production (17.473 mg/l) with boisterous distribution, broken hyphal tips and concomitant cell wall immaturation at 10 μm lateral resolutions (Fig. 2e and 2f) at agitation up to level 3 (Fig. 2e) (17.883 mg/l). All other factors under this category showed variable effect on afp production, suggesting the selected factors and their levels were within the ideal average conditions. Presence of mono- and divalent cation had significant effect on cell morphology. 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 (Fig. 2a) while increase in pH had higher effect on afp production at level 2 (Fig. 2f). Higher afp production was encouraged with decrease in slant age on afp production up to level 1 (17.947 mg/l) but decreased significantly with increase in slant age up to level 3 which was clearly reflected from cell morphology (Fig. 2d) (16.872 mg/l). Inoculum level had significant effect on higher afp production up to level 2 (Fig. 2b) (17.766 mg/l) but decreased up to level 1 (17.114 mg/l) or 3 (17.529 mg/l). Increase in pH up to level 3 results procellous mycelial form of growth with swollen characteristics (Fig. 2g) while decreasing facilitate convoluted pelleted type morphology (Fig. 2h) with greater reduction of afp level.

Among the 8 most critical fermentation factors studied, the impact of factors on higher afp production was obtained as: soluble starch (at level 1) > temperature (at level 1) > CSL (2%) + proteose peptone (1%) (at level 1) > pH (at level 2) > slant age (at level 1) > agitation (at level 3) > KH_2PO_4 (at level 1) > inoculum vol. (at level 2).

To have a better perceptiveness on the overall process analysis and the possibility of presence of most interactions, severity index (SI) study was evaluated (Table 2) from Taguchi DOE L_{27} OA projection that

Table 2. Estimated interaction of severity Index (SI) for selected factors.

Serial No.	Factors	Columns	SI (%)	Reserved column	Level
1	Inoculum vol. × agitation	6 × 7	73.21	1	[1,1]
2	CSL+ PP × pH	2 × 3	69.66	1	[1,1]
3	Inoculum vol. × KH_2PO_4	6 × 8	64.27	14	[2,2]
4	Slant age × agitation	5 × 7	60.68	2	[1,1]
5	pH × Temperature	3 × 4	56.87	7	[1,1]
6	Slant age × KH_2PO_4	5 × 8	55.72	13	[1,1]
7	Slant age × inoculum vol.	5 × 6	53.91	3	[1,1]
8	CSL+ PP × Temperature	2 × 4	52.52	6	[1,1]
9	pH × Agitation	3 × 7	32.19	4	[2,3]
10	CSL+ PP × Inoculum vol.	2 × 6	30.39	4	[1,2]
11	CSL+ PP × KH_2PO_4	2 × 8	20.28	10	[1,1]
12	Soluble starch × pH	1 × 3	19.49	2	[1,1]
13	CSL+ PP × agitation	2 × 7	16.47	5	[1,1]
14	Soluble starch×inoculum vol.	1 × 6	15.95	7	[1,1]
15	Soluble starch × CSL+ PP	1 × 2	15.49	3	[1,1]
16	Temperature × KH_2PO_4	4 × 8	14.78	12	[1,2]
17	CSL+ PP × Slant age	2 × 5	12.91	7	[1,1]
18	Temperature × Agitation	4 × 7	12.15	3	[1,3]
19	pH × KH_2PO_4	3 × 8	10.97	11	[2,1]
20	Soluble starch × KH_2PO_4	1 × 8	10.4	9	[1,1]
21	Soluble starch × agitation	1 × 7	9.08	6	[1,1]
22	pH × Slant age	3 × 5	6.71	6	[2,1]
23	Soluble starch × Slant age	1 × 5	6.69	4	[1,1]
24	agitation × KH_2PO_4	7 × 8	6.31	15	[3,1]
25	Temperature × Slant age	4 × 5	5.48	1	[1,1]
26	pH × Inoculum vol.	3 × 6	4.58	5	[2,3]
27	Temperature×Inoculum vol.	4 × 6	2.28	2	[1,2]
28	soluble starch × temperature	1 × 4	0.87	5	[1,1]

CSL = corn steep liquor; PP = proteose peptone.

represented the influence of 2 individual factors at various levels of interaction for higher afp production.

Severity index (%SI)

SI analysis indicates relative interactions of factors on the afp production. Interaction SI 100% presents for 90° angle between the lines (factors), while 0% SI stands for parallel lines. If the interaction between the factors is reverse, that has been shown by 'Reserved column', 'Levels' indicated the level of factors desirable for the optimum conditions.

The highest interaction; SI 73.21% was appeared between inoculum level and agitation (at levels 1 and 1; reserved column 1) among the all interactions followed by SI 69.66% between CSL (2%) + proteose peptone (1%) and pH (at level 1 and 1; reserved column 1).

It was interesting to observe that soluble starch (20.708 mg/l at level 1) and temperature (19.222 mg/l at level 1) with high impact factor showed least SI 0.87% (at levels 1 and 1; reserved column 5) in combination. On the contrary, the SI interaction between high impact factors slant age (17.947 mg/l at level 1) and KH_2PO_4 (17.833 mg/l at level 1) was more than 50% (at levels 1 and 1; reserved column 13). It was apparent from the observations that the influence of individual factors on afp production had varying effects while in combination; the protein production was entirely independent of the individual influence. The relative influence of factors and interactions on afp production at chosen levels was mentioned in Fig. 3. Soluble starch has been shown to exert maximum positive impact on the production of afp production in individual cases.

Analysis of variance (ANOVA)

ANOVA was used to study the data of Taguchi DOE L_{27} OA experiment and to resolve how much variation each factor has contributed (Table 3). ANOVA Study showed that among all selected factors soluble starch contributed maximally (67.113%) on the overall afp production followed by the incubation temperature (16.672%) and CSL (2%)+PP (1%) (4.861%). All

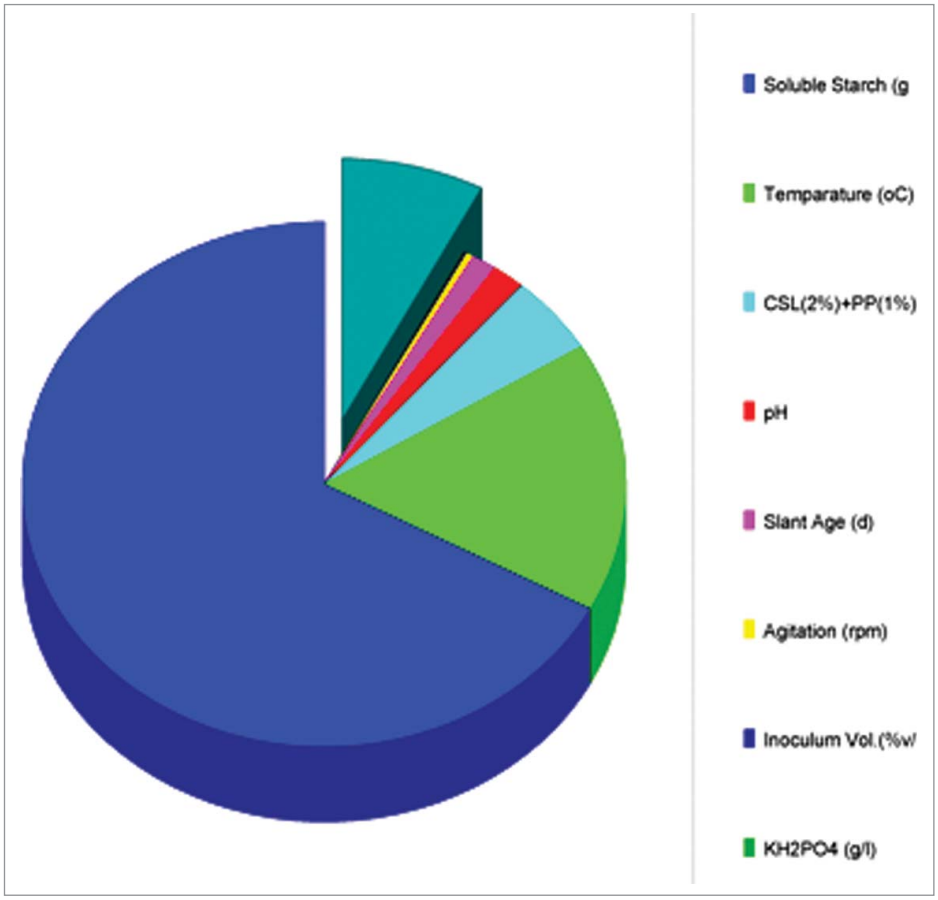

Figure 3. Relative influence of factors and interactions.

Table 3. Analysis of Variance (ANOVA).

Columns	Factors	DOF	Sum of squares	Variance	F-ratio	Pure sum	%contribution
1	Soluble Starch	2	188.918	94.459	114.928	187.274	67.113
2	CSL+PP	2	15.210	7.605	9.253	13.566	4.861
3	pH	2	6.655	3.327	4.048	5.011	1.796
4	Temperature	2	48.167	24.083	29.302	46.523	16.672
5	Slant age	2	5.388	2.694	3.277	3.744	1.341
6	Inoculum vol.	2	1.957	0.978	1.191	0.314	0.112
7	Agitation	2	2.725	1.362	1.658	1.082	0.387
8	KH_2PO_4	2	1.796	0.898	1.092	0.152	0.054
	Other/Error	10	8.218	0.821		7.664	
	Total	26	279.039			100.000%	

CSL = corn steep liquor; PP = proteose peptone.

factors and interactions considered in Taguchi DOE are statically significant ($p < 0.05$), indicating that the variability of experimental data explained in terms of significant effects. pH and slant age also showed contribution of 1.796 and 1.341%, respectively while KH_2PO_4 showed the least impact (0.054%) at the individual level on overall production of afp under the aforementioned fermentation conditions, even though it has major impact on mass transfer of nutrients during fungal growth. Negligible contribution was observed with agitation and inoculum volume (0.387% and 0.112%). This study revealed that overall 83.785% contribution was noticed with only 2 selected parameters (soluble starch and incubation temperature) and rest 8.551% (with negligible error, 7.664%) by other 6 selected factors. From ANOVA evaluation, the impact of factors on the overall afp production was observed as: soluble starch > temperature > CSL+ proteose peptone > pH > slant age > agitation > inoculum vol. > KH_2PO_4 at their assigned individual level under the Taguchi DOE L_{27} OA projection.

Statistically optimized conditions and validation

The optimum conditions and their contribution are shown in Table 4. Based on detailed analysis, optimized condition for maximum afp production could be obtained at soluble starch: 20 g/l; CSL (2%) +PP (1%): 30 g/l; pH: 5.8; temperature: 25°C; slant age: 3 d; inoculum size: 5% (v/v); agitation: 180 rpm; KH_2PO_4: 0.1 g/l.

The expected afp production at optimum conditions was 19.42 mg/l (based on SN = 25.765 mg/l) with total contribution from all the factors was 2.64 mg/l (based on SN = 8.294 mg/l) with grand average performance of 7.53 mg/l (based on SN = 17.47 mg/l). The observed 86.4% grand average performance of the fungal strain and 61.22% contribution of all fermentation factors revealed the potential of the fermentation factors concentration and their interaction for afp production by the fungus, A. giganteus MTCC: 8408. Performance distribution of current condition versus improved condition was mentioned in Fig. 4. It is evident from the observations that upon considering the optimum culture condition from the Taguchi DOE L_{27} OA, the overall enhancement of afp production of 61.22% i.e. from 7.53 mg/l to 19.42 mg/l

Table 4. Optimum culture conditions and their contribution.

Columns	Factors	Level description	Level	Contribution
1	Soluble Starch	20	1	3.238
2	CSL+ PP	30	1	1.056
3	pH	5.8	2	0.701
4	Temperature	25	1	1.752
5	Slant age	3	1	0.476
6	Inoculum vol.	5.0	2	0.296
7	Agitation	180	3	0.413
8	KH_2PO_4	0.1	1	0.363
	Total contribution from all	factors		8.294
	Current grand average	performance		17.470
	Expected result at optimum	condition		25.765

CSL = corn steep liquor; PP = proteose peptone.

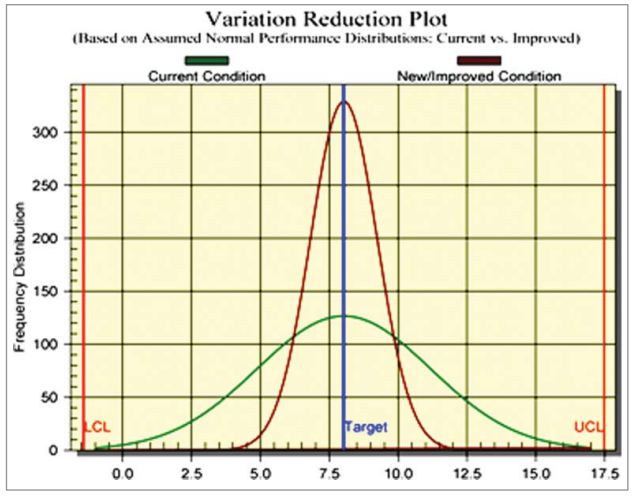

Figure 4. Performance distribution plot: current vs. improved condition.

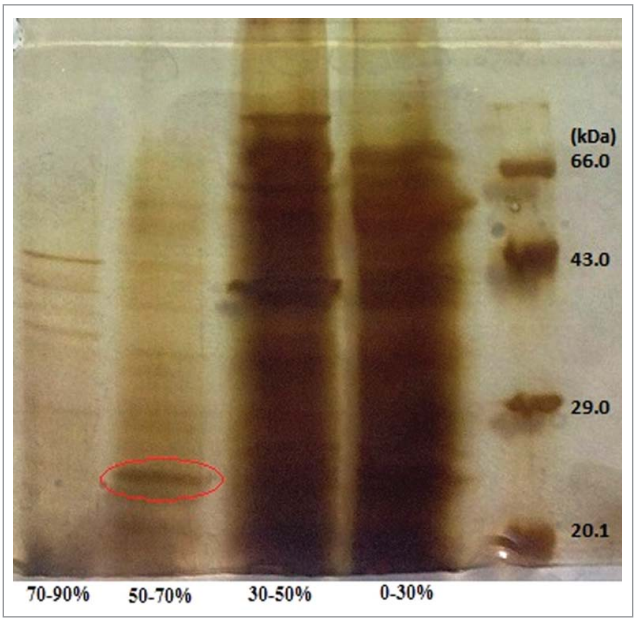

Figure 5. Silver stained SDS-PAGE of *Aspergillus giganteus* MTCC 8408 intracellular protein taken at 4 different fractionations (0–30%, 30–50%, 50–70%, 70–90%).

may be achieved. Further to validate, experiments were performed for afp production by employing the optimized culture conditions (Table 4). The experimental data showed an enhancement of afp yield by 59.4% i.e., from 7.53 mg/l (based on SN = 17.47 mg/l) to 18.54 mg/l (SN = 25.362 mg/l) with mycelial biomass 28.2 ± 0.9 gdcw/l, therefore; proving the validity of the method with the new modified culture conditions (Fig. 2i).

Time killing assay

Intracellular protein with anticandidal activity was fractionated in 50–70% ammonium sulfate precipitation and purified by cation-exchange (CMC) chromatography and gel filtration. The average molecular weight of purified protein appeared approximately 24.3 kDa (Fig. 5) in silver stained SDS-PAGE. MICs and susceptibility assay were performed as per standard protocol (CLSI document M27-A2). Anticandidal activity of the intracellular proteins was further studied by considering their time killing profile on C. albicans strain at concentrations MIC value including amphotericin B and fluconazole as standard antifungal. Time killing profile (data not shown) showed that antifungal protein caused a mean maximum decrease of A_{490} 0.57 to 0.42 (>90% killing) within 5 h of investigation while amphotericin B and fluconazole decreased the number of viable yeast cell with mean maximum of A_{490} 0.58 to 0.33 and 0.57 to 0.3 respectively (>99% killing) within 5 h. Fluconazole demonstrated the better killing profile by eradicating the fungal cells rapidly within 2.5 h with 0.6 to 0.52 A_{490} reduction (>90% killing) while amphotericin B displayed a slight reduction in yeast growth showing fungistatic activity against the tested strain.

FTIR analysis

Fourier transform infrared (FTIR) spectroscopy is an established tool for the structural characterization of proteins.[38] FTIR data was analyzed using EssentialFTIR 2.0 (Madison, USA) with ATR correction (Fig 6). Out Of all the amide bands, the most intense and useful band for the analysis of the secondary structure of proteins was amide I band, which represents primarily the C=O stretching vibration of the amide groups (though coupled to in-plane bending of the N–H and stretching of the C–N bonds) and occurs in the region 1600–1700 cm^{-1} as observed (Fig 6). The most useful IR band for the direct measurement of secondary structure of protein is a broad band found between 1660 and 1639 cm^{-1}. Two sharp bands were observed at 1654.9241 cm^{-1}, characteristic of proteins with predominantly α-helical secondary structures exhibited by amide I band (1650–1658 cm^{-1}) and 1635.6360 cm^{-1} (1625–1640 cm^{-1}) may be considered as evidence of the presence of a significant amount of β-sheet secondary structures. The absorption suggestive of β-sheet secondary structures in fact most likely arises from turns within the protein. Two bands at 1624.0631 and 1674.2123 cm^{-1} affirms the presence of an antiparallel β-sheet structure. Presence of ester-, keto-, and acetyl C=O modes at the periphery of the protein molecules, which can form hydrogen bonds to suitable partners in the protein site were also analyzed with IR study. Investigation revealed the presence of keto C=O (1715–1650 cm^{-1}) and acetyl C=O group (1650 and 1620 cm^{-1} but no ester C=O group (1750–1710 cm^{-1}). An essentially symmetric and weak hydrogen bonding was also observed from C=O stretches at approximately either 1654.9241 or 1670.3547 cm^{-1}. In particular, absorptions arising from turn structures are difficult to assign, due to the different hydrogen bonding characteristics of the various types of turn. In some points, β-turns may give rise to a number of amide absorptions and a

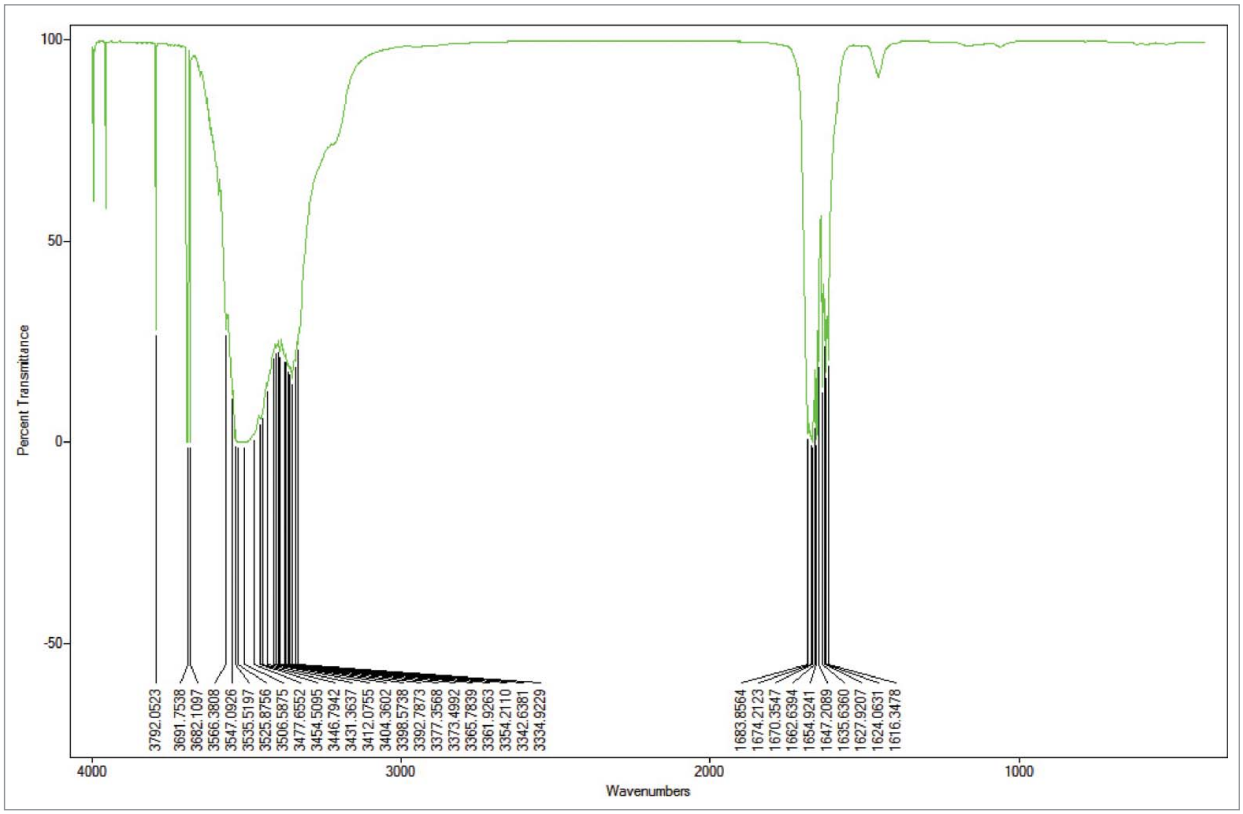

Figure 6. FTIR analysis of *Aspergillus giganteus* MTCC 8408 intracellular protein.

low-frequency absorption (1645 to 1630 cm^{-1}) attributed to C=O groups may be involved in hydrogen bonds which may stabilize the turn. Although a high frequency absorption (1660 to 1680 cm^{-1}) may be from C=O groups that sterically constrained and are not hydrogen bonded. It was evident from the Fig 6 that a sharp shoulder at 1635.6360 cm^{-1} indicates C=O groups involved in hydrogen bonding that stabilized the turn and presence of non hydrogen bonded amide C=O group at 1670. 3547 cm^{-1} (1666–1670 cm^{-1}) and antiparallel β-sheet structures (1674.2123 and 1683.8564 cm^{-1}) indicates that protein can be assigned to a high-frequency β-sheet component that arises from transition dipole coupling. Primary amides ($-NH_2$) band near 3350 cm^{-1} (3354.2110 cm^{-1}) indicates that protein can also be assigned to a high asymmetric N-H stretch and Secondary amides have one band (-NH) near 3300 cm^{-1} (3334.9229 cm^{-1}).

Discussion

The increasing incidence of immune-compromised hosts like AIDS patient, burn patient; antibiotic resistant plant pathogen like Fusarium species and *Magnaporthe grisea*.[39,40]; animal pathogen like *Aspergillus fumigatus*, *Candida albicans* (invasive *Aspergillosis* and *Candidiasis*) and the yeast *Cryptococcus neoformans* (meningitis) [41,42] have motivated the development of alternative antifungal agents. Proteins sequence determination and cell cytotoxicity measurement is under progress. Physicochemical parameters such as cationicity, hydrophobicity and amphipathicity are also taken into consideration for antifungal protein design with potent putative moiety.

Among all statistical methods, Taguchi DOE are preferred for optimizing biotechnological processes[43,44] Soluble starch was excellent growth substrate for mycelial biomass production due to its linear, soluble fraction and amylopectin fractions. CSL is a complex substrate produced in the corn-processing industry and comprised of peptides, sugars, lactic acids, vitamins and metallic ions. CSL supplies both nitrogen and carbon sources, and improves fungal growth during early stages of colonization.[45,46] In presence of excess phosphate (KH_2PO_4), CSL favors increased growth rates with reduced pellet formation. The elemental phosphorous is essential to living organisms because it is the part of the backbone of DNA, the carrier and transmitter of genetic

information in cells. The optimum phosphorous content not only supported robust growth, but also helped in production of higher afp yield. Mg^{2+} and Ca^{2+} plays a key role to increase in afp production by altering cell membrane permeability, exocytosis, hyphal sheathe desorption, protein stabilization.[47] Among several physiological properties, the slant age and inoculum volume (%v/v) may play an important role in fungal development. Temperature is one of the most important factors affecting submerged fermentation and the optimal temperature generally lies in the range of 18–35°C [48,49] where many kinds of ascomycetes have relatively low temperature optima in their submerged cultures. The pH of the medium is also one of the most detrimental environmental factors affecting the mycelial growth and metabolite production and the transport of various components across the cell membrane. However, the interaction between pH and temperature accounted higher SI 56.87%.

Declaration

The application of Taguchi DOE L_{27} OA helped in logical studying the interactive effects of selected factors at their individually assigned level for better revelation of the bioprocess strategies and improvement of its metabolite yield. Combinatorial approach of one factor at a time (OFAT) and Taguchi L_{27} orthogonal array methods with qualitative investigation of cell morphology (SEM analysis) helped in reaching optimal solution and critically analyzing the interactive effects of most decisive parameters on anticandidal protein production by *Aspergillus giganteus* MTCC 8408 in submerged fermentation. Among eight most decisive factors, soluble starch contributed maximum (67.113%) followed by temperature (16.672%). It clearly indicated that carbon source and physical factor are vital for intracellular protein production by by *Aspergillus giganteus* MTCC 8408 in submerged fermentation. Growth inhibitory effect on *Candida albicans* NCIM 3471 resulted MIC of antifungal protein of 3–4 μg/ml. Time killing kinetics study showed that intracellular antifungal protein caused 90% killing within 5 hours of investigated period of time while amphotericin B and fluconazole showed 99% eradication of yeast cell. FTIR analysis of proteins indicated the strong presence of antiparallel β-sheets structure with keto and acetyl group possessed both symmetric and weak hydrogen bonding. FTIR study also indicated that C=O groups in hydrogen bonding stabilized the β-turn and presence of non hydrogen bonded amide C=O group with antiparallel β-sheet structures indicated the existence of transition dipole coupling. The present study revealed that statistical optimization may promote economical design at the industrial level for future scale up and for better revelation of the bioprocess strategies with improved microbial metabolite yield.

Ethical statement/approval

"This article does not contain any studies with human participants or animals performed by any of the authors."

Abbreviations

Afp	antifungal protein
ANOVA	Analysis of variance
CMC	carboxymethyl cellulose
CSL	Corn steep liquor
DOE	design of experiment
FTIR	Fourier transform infrared
gdcw	gram dry cell weight
kDa	kilo-Dalton
MTCC	microbial type culture collection
MIC	minimum inhibitory concentration
NCIM	national collection of industrial microorganisms
OA	orthogonal array
PP	proteose peptone
SDS-PAGE	sodium dodecyl sulfate-poly-acryl amide gel electrophoresis
SEM	Scanning electron microscopy
S/N	Signal to noise
μS	micro Siemens

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

This paper represents the results of the knowledge-driven submerged fermentation of *A. giganteus* MTCC: 8408. The scientific responsibility rests with its authors. The authors gratefully acknowledge R. K. Roy, Nutek Inc., USA, for providing the Qualitek-4 software.

Notes on contributors

Entire planning and design of experiment using Taguchi DOE L_{27} OA: DD; Experimentation and characterization: DD;

Result analysis: DD, MD; Manuscript drafting: DD, MD; all the authors have contributed in the final approval of the manuscript; all the authors have read and approved the manuscript.

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