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Original

Biosynthesis of Low Molecular Weight Antifungal Protein from *Aspergillus giganteus* in Batch Fermentation and In-Vitro Assay

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In present study, Taguchi's design of experiment L_9 orthogonal array was created using Qualitek-4 software with four most critical factors namely, K_2HPO_4, $MgSO_4$, $CaCl_2$ and culture pH. Production of a new intracellular antifungal protein in submerged fermentation was optimized with yield of 0.98±0.1 mg/gram dry cell weight mycelia from *Aspergillus giganteus* MTCC 8408. The average molecular mass of the purified protein was figured as 5.122 kDa using Electro Spray Ionization-Mass Spectrometry. Scanning electron microscopy was used to correlate the effect of selected factors on fungal cell morphology and its metabolite production. In vitro antifungal susceptibility assay was profiled against *Aspergillus niger* and minimum inhibitory concentrations were in the range 0.3±0.06 µg/ml. The stronger influencing factors on afp production and mycelial biomass were noted with $CaCl_2$ and K_2HPO_4 respectively. The validation experiments using optimized conditions confirmed an improvement in afp by 3.86 times with mycelial biomass by 1.52 times, compared to the basal medium. 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 systemic aspergillosis as well as possible post harvest loss.

Key words : *Aspergillus giganteus* / Antifungal protein / Taguchi design of experiment / L_9 Orthogonal array / Analysis of variance / Scanning electron microscopy.

INTRODUCTION

Filamentous fungi are ubiquitously utilized in biotechnology, biochemical and fermentation industry for the production of enzymes, therapeutic proteins, organic acids and antibiotics (Papagianni, 2004). The widespread utilization of filamentous fungi is because of possessing an extraordinary ability to release large amount of proteins with post translational modification machinery. Cell morphology (qualitative study) has distinguishable effects on biomass production and its metabolite release in a fermentation broth. Filamentous fungal growth in submerge fermentation is usually accompanied as dispersed pulp like and pelleted morphology. Dispersed pulp like morphology results higher mass transfer coefficients (low nutrients concentration gradients) compared to pelleted morphology and thus, morphology exerts significant influence on the production kinetics (Spohr et al., 1998). At the macroscopic level biomass production is greatly affected by cell morphology and thus, the transport process, mixing or agitation and heat transfer. Hence, fungal morphology is regarded as a qualitative tool for bioprocess optimization of fungal metabolite production (Daniela et al., 2015).

During last two decades, antifungal antibiotics appeared as an urgent medical need. *Aspergillus giganteus* MDH 18894 was first reported to produce both α-sarcin and antifungal protein with beneficial physiological activities such as antitumor and later investigation on growth inhibition of many filamentous Ascomycetes was explored by many researchers (Olson and Goerner, 1965; Nakaya et al., 1990; Campos-Olivas et al., 1995; Theis et al., 2003; Theis et al., 2004; Hegedues and Marx, 2013).

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So far, no developments on statistical optimization of afp production from *Asp. giganteus* under submerged fermentation have been reported elsewhere. However, few antimicrobial proteins have been reported, viz., AFP from *Asp. giganteus* strain IfGB0203 (Wnendt et al., 1994); ANAFP1 from *Asp. niger* strain KCTC 2025; ANAFP2 from *Asp. niger* strain CBS 513.88 (Gun Lee et al., 1999); PAF from *P. chrysogenum* strain Q176 (Marx et al., 1995) ; NAF from *P. nalgiovense* (Geisen, 2000); ACLA from *Asp. clavatus* (Meyer V, 2008) ; AFP_{NN5353} from *Asp. giganteus* strain A3274 (Binder et al., 2011); PgAFP from *P. chrysogenum* strain RP42C (Rodrıguez-Mart et al., 2010); strain bubble protein (BP) from *P. brevicompactum* Dierckx (Seibold et al., 2011); AcAFP and AcAMP from *Asp. clavatus* (Skouri-Gargouri and Gargouri, 2008); NFAP from *Neosartorya fisheri* (Kovacs et al., 2011), intracellular anticandidal protein from *Asp. giganteus* MTCC 8408 (Dutta and Das, 2017a); F2 from *B. licheniformis* (Cui et al., 2012); RSM from *B. amyloliquefaciens* (Wong et al., 2008); BL21 from *E. coli* (Yadav and Mandhan, 2007); bacisubin from *B. subtilis* B-916 (Yongfeng liu et al., 2007); LA1-SCS from *Lactobacillus acidophilus* (Bernet-Camard MF et al., 1997); acrocin (*Enterobacter aerogenes*), pesticin (*Yersinia pestis*), marcescin (*Serratia marcescens*), piocin (*P. aeruginosa*); lactococcin A (*Lactobacillus lactis*), thermophilin A (*Streptococcus thermophilicus*), and bovicin (*Streptococcus bovis*) (Whitford MF et al., 2001).

Asp. niger is common for cause of both otomycosis (fungal ear infection) in animals and postharvest fungal diseases of onions in tropical and subtropical region and causes large postharvest loss in agribusiness (Dutta and Das, 2017b). Papagianni investigated the effect of microelements on fungal cell morphology and reported as influencing factors for enhanced fungal metabolite production even at constant carbon and nitrogen sources (Papagianni and Mattey, 2006). Therefore, to improve production and promote economical design at the industrial level, it has become essential to optimize all the culture conditions (physical parameter) and composition (chemical parameter) for improved metabolite production (Roy, 2001; Dutta and Das, 2017c; Dutta and Das, 2017d). In the conventional approach, an optimization process usually involves factorial design, response surface methodology and one variable at a time approach which is time consuming and laborious for identifying various independent variables with their assigned individual effects. Taguchi method is based on orthogonal array experiments, provides quantitative elucidation and gives much reduced variance with optimal settings of control parameters (Rao et al., 2008; Dutta and Das, 2016) and appreciated as one of the powerful optimization techniques that requires lesser time than response surface methodology (Aggarwal, 2008).

Taguchi design of experiment is a fast and confers remarkable outcome in quality products with better process performance which renders high yield and better stability (Taguchi, 1986). The basic principle involved is the encompassment of large experimental data in orthogonal (unbiased) array to determine the effect of selected factors with improved efficiency and possible experimental error reduction (Byrne and Taguchi, 1987).

By employing a "larger the better" as quality attribute, Taguchi method develops desired functionality and beginning with the nominal processes, to optimize the processes by varying the control factors and make the results become reliable and reproducible. The primary goal of this study is to keep the variance in the output very low even in the presence of noise inputs and to make the process robust against all variation. Orthogonal arrays provide a set of well-balanced (minimum) experiments and signal-to-noise ratios (S/N), which are log functions of desired output, serve as objective roles for optimization, help in data analysis and prediction of optimum results (Nandal et al., 2013).

To the best of knowledge, there are no reports on effects of microelements with a physical factor on mycelial biomass and afp production using both qualitative (morphological) and quantitative approach from *Asp. giganteus* MTCC 8408 under submerged fermentation.

The aim of this study was to understand the role of cell morphology (qualitative analysis) with optimization (quantitative analysis) of afp production in L_9 OA using Taguchi DOE under submerged fermentation of *Aspergillus giganteus* MTCC 8408 in presence of three microelements viz; K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$ with a physical parameter pH at their assigned individual level.

MATERIALS AND METHODS

Strain and culture condition

Aspergillus giganteus (CBS-KNAW Fungal Biodiversity Centre, 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; $ZnSO_4$, $7H_2O$: 0.001; $CuSO_4$, $5H_2O$: 0.0005 for 7 days at initial temperature 25℃ and pH 5.5±0.05; kept at 4℃ for maximum period of 15 days. Unless otherwise stated, all chemicals used in this work were purchased from Merck and Sigma.

Inoculums preparation and submerged fermentation

Asp. giganteus MTCC 8408 culture was aseptically

TABLE 1. Taguchi DOE L_9 OA projection with selected factors for both mycelial biomass and afp production under submerged fermentation of *Asp. giganteus* MTCC 8408 in Olson medium modified with CSL and proteose peptone.

Experiment No	A	B	C	D	A (K_2HPO_4)	B ($MgSO_4$)	C ($CaCl_2$)	D (pH)	Mycelial Biomass (DCW) (g/l) ±SD*	Afp production (mg/l) ±SD**	S/N Ratio
1	1	1	1	1	2.8	0.6	0.3	5.2	7.73±0.24	2.513±0.151	8.00
2	1	2	2	2	2.8	1.2	0.9	5.8	9.06±0.39	3.084±0.165	9.78
3	1	3	3	3	2.8	1.8	1.5	6.4	15.73±0.67	5.593±0.158	14.95
4	2	1	2	3	4.8	0.6	0.9	6.4	16.4±0.28	6.417±0.151	16.14
5	2	2	3	1	4.8	1.2	1.5	5.2	24.6±0.56	2.785±0.271	8.89
6	2	3	1	2	4.8	1.8	0.3	5.8	15.2±0.77	1.774±0.250	4.97
7	3	1	3	2	6.8	0.6	1.5	5.8	13.6±0.33	5.537±0.185	14.86
8	3	2	1	3	6.8	1.2	0.3	6.4	6±0.23	1.227±0.003	6.64
9	3	3	2	1	6.8	1.8	0.9	5.2	21.47±0.45	2.914±0.340	9.28

*Mean±SD for triple determination. DCW, g/l= dry cell weight, gram per liter of fermentation broth
**The regression equation (Coomasie-Bradford Assay): Absorbance = 18.7×protein produced (mg/ml).

transferred and grown in 250ml Erlenmeyer flasks containing 60 ml of Czapek's yeast extract broth (except agar) at 150 rpm for 24 h; prior to use as inoculums ($A_{600nm} \geq 0.9$) for next phase cultivations and submerged fermentation experiments. Final phase submerged fermentation was carried out separately in shake flask containing basal Olson (Olson and Goerner, 1965) medium (g/l): beef extract: 15.0; peptone: 20; corn starch: 20.0; sodium chloride: 5.0; and in modified Olson medium (g/l): corn steep liquor: 20.0; proteose peptone: 10; soluble starch: 20.0; sodium chloride: 5.0; in various combination with K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$ and pH at their assigned individual level as per Taguchi's L_9 orthogonal array (Table 1). All experiments were performed in triplicate (mean ± standard deviation of triple determination). Total volume of fermentation broth was 3 l.

Taguchi DOE statistical approach

Taguchi method was based on signal-to-noise ratios to measure the variability around the target performance (Cobb et al., 1994). A high value of ratio inferred the signal was much higher than the random effects of the noise factors. The noise was usually because of the uncontrollable factors that often cannot be completely cancelled. Taguchi's design of experiment has been perceived a wide acceptance as a fundamental quantitative tool for optimizing few biochemical and biotechnology process.

The quality attribute 'larger the better' parameter was used to define the optimum conditions. The S/N ratio was estimated using Equation [1]:

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

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.

In this study, four factors at three levels of variations were studied. All experiments were performed in triplicate (mean ± standard deviation of triple determination). The upper and lower limits of each variable were chosen to encompass the range and to reflect results after several preliminary investigations of the limits. The effect of these four factors was identified using the ANOVA and severity index (SI).

Crude protein extraction

The harvested mycelia was filtered through a muslin cloth, washed with deionized water (conductivity <1 µS) and resuspended in minimum volume buffer (pH 8.0±0.2) comprised 100 mM Tris-HCl, 10% ethylene glycol, 0.05 mM EDTA, 1 mM TCEP, 1mM aprotinin (Sigma, USA) and 0.1% SDS, in a constant temperature (4℃) cooling water bath.

Protein fractionation and purification

The crude proteins were fractionated (0-30%, 30-50%, 50-70% and 70-90%) and purified using pure solid ammonium sulphate precipitation (nomogram chart) protocol (Scopes, 1994). Each fraction was pooled in minimum volume buffer (pH 7.4±0.2) comprised 100 mM Tris-HCl, 1 mM TCEP, 1mM aprotinin (Sigma, USA) and dialyzed overnight (9mm, benzoylated, Sigma) in

the same buffer at constant 4℃ in cooling water bath. Each fraction was purified using cation-exchange resin carboxymethyl cellulose column (2.8×20 cm^2) and eluted in a stepwise gradient on NaCl (0-1.0 M) at a flow rate of 0.5 ml/min. Fractions of 2 ml were collected, and the absorbance was read at 280 nm in a spectrophotometer (UV-1800, Shimadzu). Each fraction was lyophilized (24 h at -48℃, 0.15 mbar). The concentrated sample was passed through a 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. Antifungal susceptibility assay and MIC determination was performed against *Asp. niger*. Quantitative estimation of protein was determined (UV-1800, Shimadzu) at 545 nm using Bradford-Coomasie assay protocol (Peterson, 1983).

Molecular weight determination

Average molecular weight of the protein was determined using electro spray ionization-mass spectrometry (Trauger et al., 2002). 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 Mass Spectrometry Software ESIProt 1.0.

MIC determination and antifungal susceptibility assay

Three major filamentous fungi responsible for bulbs rots, namely, *Fusarium oxysporum*, *Aspergillus niger and Saccharomyces cerevisiae* were investigated. Antifungal assay was carried out according to a standardized broth micro-dilution method (Clinical and Laboratory Standards Institute document M38-A). 100 mL of standardized *Asp. niger* suspension was then added and incubated for 48 hours at 28℃. The MIC was defined as the lowest concentration that inhibited 90% growth of species. The MICs were determined three times and each time in duplicate.

Kinetics of growth and cell morphology

Typical time course of substrate consumption vs. mycelial biomass against afp production in submerge fermentation by *Asp. giganteus* was observed. Minimum and maximum dry cell weight and afp production were observed under the L9 orthogonal array. Scanning electron microscopy was employed to image cells in a lyophilized state. It helps in development of significant high-resolution images of fungi (Read and Christopher, 1991). Cell pellet was suspended in 0.1 M PBS (pH 7.4) and centrifuged. Pellet was lyophilized, and imaged with gold coating.

RESULTS AND DISCUSSION

Fermentation factors and their interaction effect

Minimum and maximum mycelial dry cell weight and afp production were 6±0.23 g/l to 24.6±0.56 g/l and 1.227±0.003 mg/l to 6.417±0.151 mg/l respectively under the L_9 OA projection (Table 1), showing the present experimental efficiency. Certainly, the observed variation suggests the important role of three microelements and a physical factor in achieving the best possible combination of four factors. Microelements $CaCl_2$ and $MgSO_4$, exert stronger influence for higher afp production, while microelements $CaCl_2$ and K_2HPO_4 exert stronger influence for higher mycelial biomass production because of lower rank and higher delta value (Table 2).

Table 2 (Fig.1) shows the interaction with factor's average effect at their individual assigned levels on higher afp production and mycelial biomass growth. 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. Microelements $CaCl_2$ (for higher afp production, 6.835 mg/l) and K_2HPO_4 (for higher mycelial biomass growth, 4.975 g/l) has been found to exert stronger influence in submerge fermentation.

At individual level stage, the higher afp production was followed with decrease in concentration of $MgSO_4$ at level 1 (12.993 mg/l) while higher mycelial biomass was followed with increase in concentration of K_2HPO_4 at level 2 (25.236 g/l). Increase in concentration of $CaCl_2$ ensued both the higher afp production and mycelial biomass at level 3 (12.88 and 24.795 mg/l). At individual level stage, decrease in pH had the significant impact on maximum mycelial biomass production at level 1 (24.064 g/l) while it resulted maximum afp production with increase in pH at level 3 (10.954 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. Among the four factors studied, the effect of microelements and pH on higher afp production was followed as $MgSO_4$ > $CaCl_2$ > pH > K_2HPO_4. While for higher mycelial biomass production effect of microelements and pH was followed as K_2HPO_4 > $CaCl_2$ > $MgSO_4$ > pH.

Decrease in concentration of K_2HPO_4 and $MgSO_4$ caused higher afp production at level 1 but not at level 3 while increase in concentration of $CaCl_2$ and pH had higher effects on afp production at level 3. Varying afp production and mycelial biomass was not constant with the factors, yet significant difference was noticed with K_2HPO_4, $MgSO_4$ and pH factors for higher mycelial biomass growth and converse effect was followed (Table 2).

TABLE 2. Factor's average effects on mycelial growth and afp production

Mycelial growth (g/l)						Afp production (mg/l)					
Factors	Levels				Delta/ Rank	Factors	Levels				Delta/ Rank
	L_1	L_2	L_3	L_2-L_1			L_1	L_2	L_3	L_2-L_1	
K_2HPO_4	20.26	25.236	21.612	4.975	4.9/2	K_2HPO_4	10.897	9.942	8.61	-0.955	2.287/3
$MgSO_4$	21.569	20.824	24.715	-0.745	3.89/3	$MgSO_4$	12.993	6.79	9.665	-6.203	6.204/2
$CaCl_2$	18.966	23.347	24.795	4.381	5.83/1	$CaCl_2$	4.867	11.702	12.88	6.835	8.012/1
pH	24.064	21.794	21.249	-2.27	2.81/4	pH	8.671	9.823	10.954	1.152	2.284/4

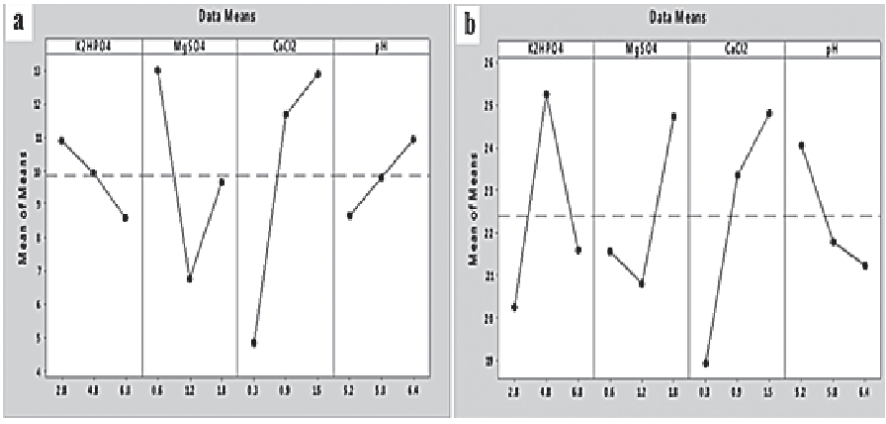

FIG. 1. Impact of selected factor levels and relative influence on metabolite release by *Asp. giganteus* MTCC: 8408 in submerged fermentation.

Higher mycelial biomass formation was encouraged with increase in concentration of K_2HPO_4 and $MgSO_4$ at level 2 and at level 3 respectively, while decrease in pH favors better mycelial development at level 1. $CaCl_2$ showed almost similar effects on both the afp production and mycelial biomass growth.

To have a better perceptiveness on the overall process analysis and the possibility of presence of most interactions, SI study was assessed (Table 3) from Taguchi DOE that represents the influence of two individual factors at various levels of interaction for higher afp production as well as for higher mycelial biomass growth.

The highest interaction for higher afp production; SI 33.09% was followed between K_2HPO_4 and $CaCl_2$ (at levels 2 and 2; reserved column 2) while for mycelial biomass; SI 55.45% was followed between $MgSO_4$ and pH (at level 2 and 1; reserved column 6). The SI 54.47% was noticed between $CaCl_2$ and pH for higher mycelial biomass suggesting influence of individual $CaCl_2$ (large L_2-L_1 difference) and mycelial biomass production dependent of the individual influence. On the contrary, the SI interaction between stronger influencing factors $CaCl_2$ and pH (large L_2-L_1 difference) for higher afp production was 12.85% and for higher mycelial biomass the SI interaction between stronger influencing factors K_2HPO_4 and $CaCl_2$ was 2.76%. While with lesser influencing factors K_2HPO_4 and $MgSO_4$ (small L_2-L_1 difference) the SI interaction was 31.63% and for higher mycelial biomass the SI interaction between lesser influencing factors $MgSO_4$ and pH was 55.45%. From these observations it revealed that influence of individual factors on afp production had varying effects while in combination; and the production was solely independent of the individual influence. $MgSO_4$ was followed to exert maximum positive impact on the afp production in individual cases.

ANOVA

To figure out the impact of each individual factor for a successful fermentation process, ANOVA was used to elucidate the effect of four factors at three levels based on L_9 OA projection data and contribution of each factor on variations (Table 4). All the factors and interactions considered in the experimental design were statistically significant with 90% of confident limit. ANOVA study showed that among all selected factors, percent contribution (%P) was followed as $CaCl_2$ (60.424%) > $MgSO_4$ (31.112%) > K_2HPO_4 (4.257%) > pH (4.206 %) at their assigned individual level on overall afp production under the L_9 OA projection. ANOVA investi-

TABLE 3. Estimated interaction of severity index (% SI) for different factors.

Mycelial growth					Afp production				
Interacting Factor pairs	Column	S.I (%)	Reserved Column	Levels	Interacting Factor pairs	Column	S.I (%)	Reserved Column	Levels
$MgSO_4 \times pH$	2×4	55.45	6	[2,1]	$K_2HPO_4 \times CaCl_2$	1×3	33.09	2	[2,2]
$CaCl_2 \times pH$	3×4	54.47	7	[3,1]	$K_2HPO_4 \times MgSO_4$	1×2	31.63	3	[2,1]
$K_2HPO_4 \times pH$	1×4	22.72	5	[2,1]	$MgSO_4 \times pH$	2×4	20.69	6	[1,3]
$MgSO_4 \times CaCl_2$	2×3	12.09	1	[2,3]	$K_2HPO_4 \times pH$	1×4	20.07	5	[2,3]
$K_2HPO_4 \times MgSO_4$	1×2	8.76	3	[2,2]	$CaCl_2 \times pH$	3×4	12.85	7	[2,3]
$K_2HPO_4 \times CaCl_2$	1×3	2.76	2	[2,3]	$MgSO_4 \times CaCl_2$	2×3	6.0	1	[1,2]

TABLE 4. Analysis of Variance (ANOVA)

Col # / Factor	DOF (f)	Sum of Square (S)	Variance (V)	F-Ratio (F)	Pure Sum (S')	Percent P (%)
K_2HPO_4	2	7.912	3.956	8520.30	7.912	4.257
$MgSO_4$	2	57.824	28.912	57130.95	57.824	31.112
$CaCl_2$	2	112.303	56.151	112289.89	112.303	60.424
pH	2	7.817	3.908	78178.56	7.817	4.206
Other/Error	8	185.858				100.00

gation brought out overall 91.536% contribution, noticed with only two selected parameters ($CaCl_2$ and $MgSO_4$) while rest 8.464% by other two selected factors.

Evaluation of bioprocess optimization

Based on detailed investigation on Taguchi DOE L_9 OA projection, statistically optimized value of K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$ and pH, for maximum afp production (Table 3) was K_2HPO_4: 2.8 g/l, $MgSO_4$: 0.6 g/l, $CaCl_2$: 1.5 g/l, and pH 6.4 and for maximum mycelial biomass growth was K_2HPO_4: 4.8 g/l, $MgSO_4$: 1.8 g/l, $CaCl_2$: 1.5 g/l, and pH 5.2; by *Asp. giganteus* MTCC: 8408 in submerge fermentation using 10% (v/v) inoculums, 2.0% soluble starch, 2.0% CSL, 1.0% PP, 0.5% NaCl, at 25℃, orbital rotator speed 120 rpm and incubation period 144 h.

The expected mycelial biomass growth and afp production at optimized conditions were 38.45 g/l (SN ratio: 31.699 g/l) and 8.2 mg/l (SN ratio: 18.273 mg/l), respectively with total contribution were 2.92 g/l and 2.64 mg/l (SN ratio: 9.33 g/l and 8.456 mg/l) respectively. Fig.2 depicts the performance distribution of current condition along with improved condition. It was evident from the observations that grand average performance of 13.14 g/l (SN ratio: 22.369 g/l) for mycelial biomass growth and 3.1 mg/l (SN ratio: 9.816 mg/l) afp production were observed at optimized culture condition, respectively. Detailed investigation demonstrated 92.4% grand average performance and

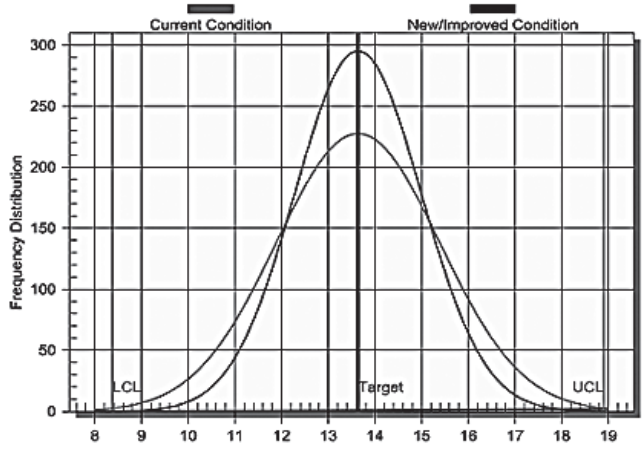

FIG. 2. Performance distribution plot: current vs. improved condition

65.82% contribution of all fermentation factors for mycelial growth while 67.8% grand average performance and 62.2% contribution of all fermentation factors for afp production which indicates the potential effect of microelements with a physical factor and their interaction for mycelial biomass and afp production by the *Asp. giganteus* MTCC 8408.

The validation experiment with statistically optimized culture condition, K_2HPO_4: 2.8 g/l, $MgSO_4$: 0.6 g/l, $CaCl_2$: 1.5 g/l, and pH 6.4; resulted afp production improved by 2.91 times (from expected 8.2 mg/l to 23.9±0.21 mg/l). Compared to basal Olson medium,

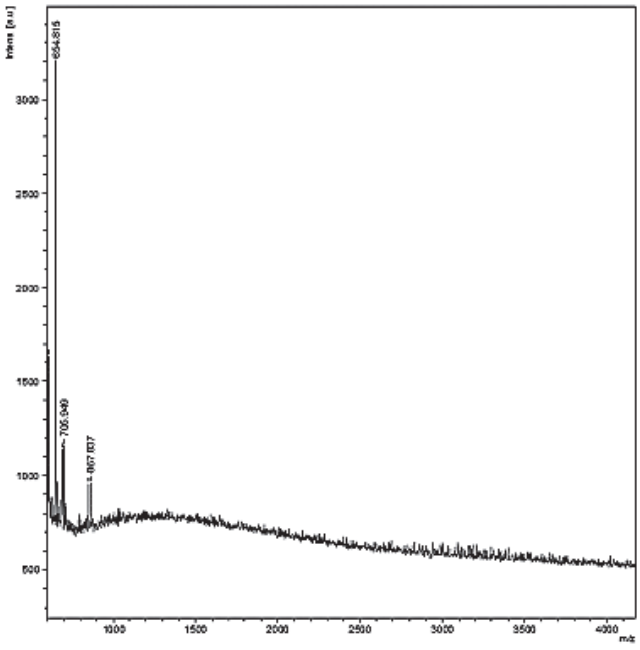

FIG. 3. ESI-MS plot for determination of average molecular weight of intracellular protein

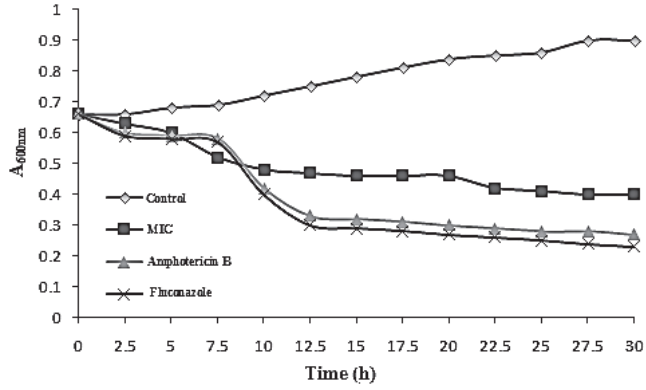

FIG. 4. Time killing assay of intracellular protein against *Asp. niger*

statistically Optimized Culture condition showed an enhancement of 3.86 times afp level (from 6.18 mg/l± 0.29 mg/l to 23.9±0.21 mg/l) with an enhancement of 1.52 times mycelial biomass (from 15.9±0.34 g/l to 24.2±0.65 g/l). The validation experiment with statistically optimized culture condition, K_2HPO_4: 4.8 g/l, $MgSO_4$: 1.8 g/l, $CaCl_2$: 1.5 g/l, and pH 5.2; showed biomass improved by 8.45% (from 38.45 g/l to 42.0± 0.26 g/l). Compared to basal Olson medium, statistically optimized culture condition for biomass production showed an enhancement of 2.64 times biomass level (from 15.9±0.34 g/l to 42.0±0.26 g/l) with an improvement of 1.93 times afp level (from 6.18 mg/l± 0.29 mg/l to 11.98±0.21 mg/l).

Molecular weight determination and time killing assay

The intracellular protein produced by *Asp. giganteus* MTCC 8408 with antifungal activity was found in 70-90% ammonium sulfate fractionation. The purified afp appeared as a sharp peak in ESI-MS plot (Fig.3) with average molecular weight 5.122 kDa. Antifungal assay of the intracellular fractionated and purified protein was studied by considering their time killing profile on *Asp. niger* strain at concentrations MIC value including amphotericin B and fluconazole as standard antifungal. Time killing profile (Fig.4) showed that antifungal protein of average molecular weight 5.122 kDa caused a mean maximum decrease of A_{600} 0.6 to 0.48 (>90% killing) within 5 hours of investigated period while amphotericin B and fluconazole decreased the number of viable cell with mean maximum of A_{600} 0.58 to 0.33 and 0.57 to 0.3 respectively (>99% killing) within 5 hours. Fluconazole demonstrated the better killing profile by eradicating the fungal cells rapidly within first 2.5 hours with mean maximum reduction 0.66 to 0.59 A_{600} (>90% killing) while amphotericin B displayed the fungistatic activity against the tested strain. Two strains were unaffected at the protein concentrations tested: *Fusarium oxysporum* and *Saccharomyces cerevisiae* were insensitive when concentrations up to 180 µg/ml were used.

Fungal morphology and kinetics of growth

Fig.5 showed the morphological characterization of fungal mycelia at different nutrients composition at their assigned individual level. Qualitative investigation showed that *Asp. giganteus* grew as cluster of cell in Fig.5a with K_2HPO_4: 4.8 g/l, $MgSO_4$: 1.8 g/l, $CaCl_2$: 1.5 g/l, and pH 5.2 (optimized for mycelial biomass); while with K_2HPO_4: 2.8 g/l, $MgSO_4$: 0.6 g/l, $CaCl_2$: 1.5 g/l, and pH 6.4 (optimized for afp production); cell grew as dispersed hyphae with dense branching in Fig.5b. Boisterous distribution with concomitant cell wall immaturation with 10 µm lateral resolution was observed in Fig.5c when *Asp. giganteus* grown in basal Olson medium.

Surface became progressively smoother with regular hyphal network and branching pattern in Fig.5d when cell was grown in Olson medium modified with CSL and PP. Thus, it was possible to predict the effect of microelements with physical factor pH on morphological changes that accompanied cell wall maturation. Analysis also showed that under various physiological and environmental conditions, morphology changes from dormancy to germination proved that filamentous fungi were capable of remodeling hyphal as well as branching network.

A typical time course graphical presentation (Fig.6)

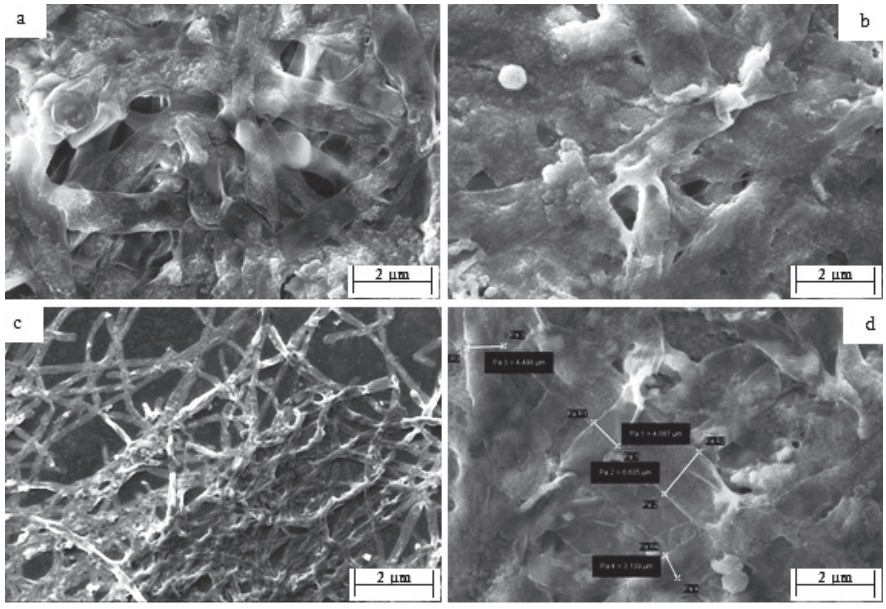

FIG. 5. Effect of various nutrients composition on morphological analysis (SEM study) of *Asp. giganteus* MTCC 8408, under submerged fermentation (a) optimized condition for mycelial biomass; (b) optimized condition for afp production; (c) grown in basal Olson medium; (d) grown in Olson medium modified with CSL and PP. Lateral resolution: 2 µm; magnification: 5KX; EHT: 18.00 KV; WD: 9.5 mm

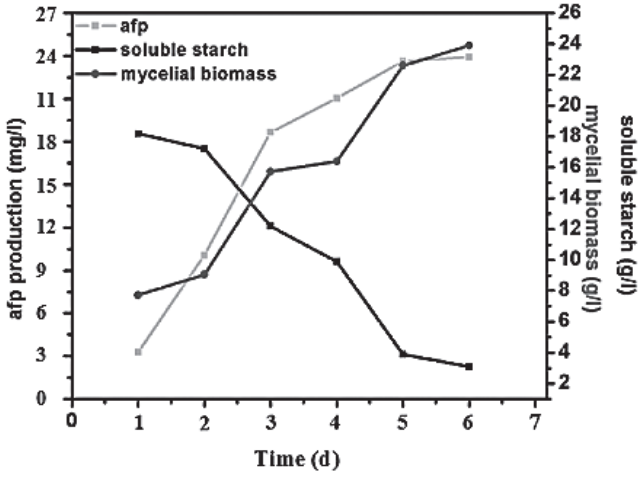

FIG. 6. Growth dynamics of mycelial biomass against substrate consumption under statistically optimized condition for improved afp production during 6 days submerged fermentation

of substrate consumption vs. mycelial biomass against afp production in submerge fermentation by *Asp. giganteus*, showed that cells grew as dispersed pulp like form and reached a maximum mycelial biomass of 23.2± 0.65 g/l with growth associated afp produced at the end of the fermentation (11.98±0.21 mg/l).

Effect of three microelements with a physical factor pH had pronounced impact on morphology due to cell wall immaturation and branching pattern of hyphae. Productivity was increased by controlling fungal morphology properly as shown in Fig.5b.

Main effect study showed $CaCl_2$ contributed higher influence at individual level followed by pH on afp production whereas K_2HPO_4 accounted for higher influence followed by $CaCl_2$ on mycelial growth. SI study showed that K_2HPO_4 accounted for higher SI with $CaCl_2$ on afp production while $MgSO_4$ contributed higher SI with pH at their assigned level. ANOVA investigation showed that among all selected factors $CaCl_2$ contributed maximally on the overall afp production followed by $MgSO_4$. The validation experiments using optimized conditions confirmed an improvement in both afp production and mycelial growth against the expected response indicates embraced Taguchi DOE.

The experiment with statistically Optimized Culture condition for afp production showed an improvement by 2.91 times (from expected 8.2 mg/l to 23.9±0.21 mg/l) and hence validated.

Compared to basal Olson medium (Olson and Goerner, 1965), statistically Optimized Culture condition for afp production showed an better enhancement of both afp and biomass production while statistically Optimized Culture condition for biomass production showed rapid fall in afp level. This may be because of decrease in pH which stimulated pellet type morphology and thereby enhanced mass transfer limitation.

Many investigators have preferred soluble starch as carbon source for the growth of *Asp. giganteus* and afp production (Olson and Goerner, 1965; Theis et al., 2003; Theis et al., 2004). Its linear, soluble fraction (amylose) and amylopectin fractions served as carbon and investigated as excellent growth substrates for mycelial biomass production. CSL a byproduct of corn-processing industry 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 (Levin et al., 2010). Under optimized condition for improved afp production, the sharp increase was due to CSL and proteose peptone that provide increased growth rates including excess phosphate (K_2HPO_4) concentrations and has been shown to increase hyphal length and branching frequency (Fig.5b). Mg^{2+} played a key role to increase in afp production by altering cell membrane permeability, exocytosis, hyphal sheathe desorption, protein stabilization (Schmit and Brody, 1976) Ca^{2+} played significant role in fungal growth by changing internal Ca^{2+} that controls the cytoplasmic Ca^{2+} gradient, ensued better yield of fungal metabolite and improve cell membrane permeability interactions (Kim et al., 2005), both maximum mycelial biomass growth and afp yield were followed at 25℃, which was comparable to many kinds of ascomycetes that have fairly low temperature optima in their submerged cultures (Kim et al., 2005). The increasing incidence of immune-compromised hosts like AIDS patient, burn patient, antibiotic resistant plant pathogen like *Fusarium* species and *Magnaporthe grisea* (Papavizas, 1985; Talbot, 2003) animal pathogen like *Asp. fumigatus*, *Candida albicans* (invasive Aspergillosis and Candidiasis) and the yeast *Cryptococcus neoformans* (meningitis) (Gupte et al., 2002; Edwards, 2004), have motivated the development of alternative antifungal antibiotics.

CONCLUSIONS

Mycelial microorganisms are exploited extensively in the commercial production of a wide range of secondary metabolites (Braun and Vecht-Lifshitz, 1991; Daniela et al., 2015) Statistical approach using Taguchi L_9 OA with qualitative investigation of cell morphology helped in unfolding Taguchi DOE and in logically analyzing the interactive effects of most influential factors at their individually assigned level on mycelial biomass and associated influence on afp production by *Asp. giganteus* MTCC: 8408 for better revelation of the bioprocess strategies and improving the microbial metabolite productivity. The present study revealed that statistical optimization may promote economical design at the industrial level for future scale up.

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