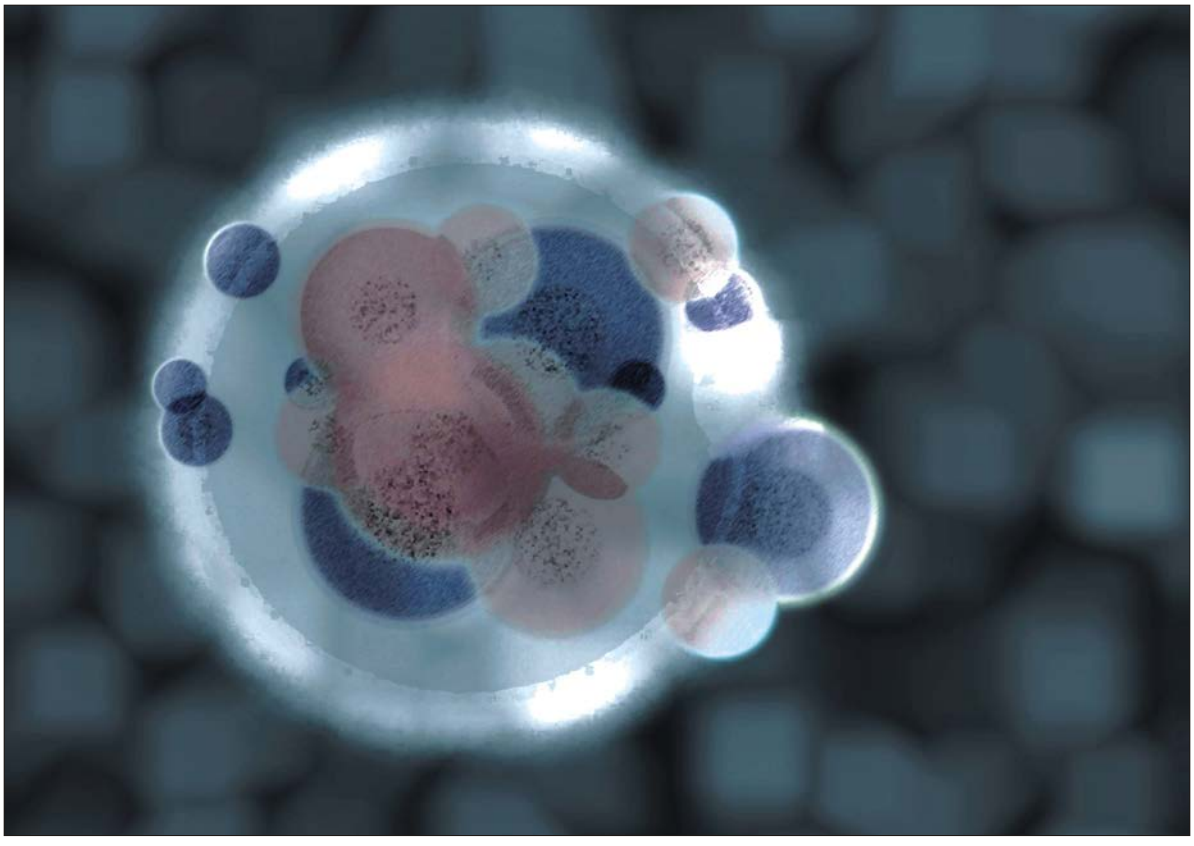

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Research Article
Potential Anticryptococcal Compound from Marine *Nocardiopsis synnemataformans*

Sudarshan Singh Rathore, Aishwarya Mathivanan, Abirami Ravindran, Ponnusami Venkatachalam and Jayapradha Ramakrishnan

Centre for Research in Infectious Diseases, School of Chemical and Biotechnology, SASTRA University, Tirumalaisamudram, Thanjavur, 613401 Tamilnadu, India

Abstract

Background and Objective: *Cryptococcus neoformans* (*C. neoformans*) is an emerging opportunistic fungal pathogen, which usually causes infection in immunocompromised hosts. Limited antifungal drugs are available to manage cryptococcal meningitis, which requires new effective class of drugs with less toxicity. The aims of the study were to enhance the production of antifungal compound from a new strain of marine *Nocardiopsis synnemataformans* AF1 against *C. neoformans* using statistical approach, to determine the molecular weight of the purified compound and to evaluate the effect of antifungal compound against the major virulence factors of *C. neoformans,* namely capsule and melanin. **Materials and Methods:** A new strain was screened and isolated from marine sediments based on antagonistic assay against *C. neoformans*. The antifungal compound was produced using Oyster shells as the substrate. The solid state cultural conditions were selected by one factor, time method and optimized by using response surface methodology. The antifungal compound was extracted using xylene and purified using HPLC. The purified fraction showing inhibitory action against the *C. neoformans* capsule growth and melanized cells were studied using MALDI-TOF MS. The minimum inhibitory concentration was determined using dilution method. Cytotoxicity was observed by HepG2 cell line. One-way ANOVA and t-test performed to test statistical significance for multiple comparisons. **Results:** The optimized condition to produce the antifungal compound from the new strain AF1 are found to be, 50% initial moisture content, 2% yeast extract, Oyster shells with the particle size of 16 and temperature at 40°C. The antifungal compound exhibits a significant reduction in *C. neoformans* cells, capsule size (30.18%) and melanized cells (99.3%). The MIC for the purified compound is estimated to be 200 μg mL^{-1}. The MALDI-TOF MS estimated the molecular weight as 242 Da. **Conclusion:** The results of this study show that the new strain *N. synnemataformans* AF1 isolated from marine environment exhibited potential antagonistic activity against *C. neoformans.*

Key words: Antifungal compound, *Cryptococcus neoformans*, capsule, melanin, optimization, oyster shells, actinomycetes

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Corresponding Author: Jayapradha Ramakrishnan, Centre for Research in Infectious Diseases (CRID), School of Chemical and Biotechnology, SASTRA University, Tirumalaisamudram, Thanjavur, 613401 Tamilnadu, India Tel: +91-9952538211

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cryptococcus meningitis is an opportunistic neurological disease in immunocompromised individuals. It has emerged as a leading cause of morbidity and mortality in HIV patients[1]. The etiologic agent, *Cryptococcus neoformans*, is an encapsulated opportunistic fungal pathogen emerging as a major threat to immunocompromised patients. However it rarely affects healthy individuals[2]. Lungs serve as a primary route for *C. neoformans* infections and cause cryptococcal meningitis when left untreated. Compared with other types of meningitis, cryptococcal meningitis accounts for 45% of deaths[3]. Recently, Centre for Disease Control estimated approximately one million new cases of cryptococcal meningitis occurring each year, resulting in 625,000 deaths worldwide. Advances in medical techniques such as organ transplantation, cancer therapies with the use of immunosuppressive drugs, diabetes mellitus and immature birth increases the incidence of cryptococcal infections [4,5]. The severity of infection is due to the presence of multiple virulence factors such as an outer coat capsule, melanization, production of phospholipase, mannitol, urease and proteinases[6]. Among these, capsule and melanin are most important virulence factors. Encapsulated *C. neoformans* resists phagocytosis and causes destructive immune responses[7]. Many of the resistance factors studied in *Cryptococcus* sp., are concerned with preventing damage by Reactive Oxygen Species (ROS). The capsule appears to be protective against ROS and other antimicrobials and protection is proportional to the size of capsule[8]. Similarly, melaninzed *C. neoformans* can potentially bind and neutralize cationic antimicrobial peptides and are most resistant to amphotericine-B and caspofungin[9,10].

The current treatment strategy for cryptococcal meningitis includes amphotericin B in combination with flucytocine for a period of 2 weeks, followed by fluconozole for a minimum of 8-10 weeks. However a long term therapy leads to fluconozole resistance in *C. neoformans*, which is another emerging problem[11]. Furthermore, fluconozole intake for a prolonged period of time can lead to other yeast infection such as candidiasis. Towards the end of these antibiotics therapy, protein abnormalities may persist for years, which would cause adverse effects including nephrotoxicity[12].

Although the lipid based formulations of amphotericin B has excellent efficiency in reducing nephrotoxicity, its cost and inability to pass through the blood brain barrier are major setbacks[13]. Thus, the primary motive of this present study was to find an alternative antifungal and there are no reports to study marine bioactive compounds specifically to treat cryptococcal infections[14,15]. Present study approach in finding a specific compound that targets capsule and melanin synthesis is of great importance in treating the cryptococcal infections effectively.

Bioactive compounds are produced by both solid state and submerged fermentation. However, solid state fermentation is easily controlled and it is preferred since expenditure on media, scale up and downstream processing are less[16]. Response Surface Methodology is an efficient tool for media optimization and analysis of complexity during the interaction of various factors[17]. In the present study, used oyster shell (major marine waste product) as a novel substrate for antifungal production. This is the first report on utilizing oyster shells as substrate for the production of antifungal compounds.

This study describes the isolation and taxonomy of anticryptococcal producing strain, optimization by RSM approach, purification of bioactive molecule and determination of molecular weight by MALDI ToF MS. The compound was then evaluated for its activity against the two major virulence factors of *C. neoformans* (Capsule and melanin).

MATERIALS AND METHODS

All the studies were carried out during the 2015-2016 academic session in SASTRA University campus. All reagents were purchased commercial grade from suppliers (Himedia and SRL) and used without further purification.

Test culture: *Cryptococcus neoformans* 14116 was purchased from Microbial Culture Collection Centre, Chandigarh. The strain was maintained on potato dextrose agar slants at 4°C and 15% glycerol stocks at -80°C.

Isolation of marine actinomycetes: The marine sediment samples were collected from Pamban near Rameshwaram at Gulf of Mannar in the month of January. Actinomycetes sp., were isolated using zobell marine agar, actinomycetes isolation agar, starch agar and starch casein nitrate agar supplemented with fluconozole (50 μg mL^{-1}) and gentamycin (10 μg mL^{-1}) to inhibit the growth of fungi and bacteria, respectively[18]. The plates were incubated for 21 days at 28°C. The isolates obtained from each media were stored in 15% glycerol at -80°C.

Screening of antagonistic activity against *C. neoformans*: The antifungal activity was determined using the protocol of Ramakrishnan *et al.*[19] to select a potential strain. Briefly, the

spore suspensions of individual isolates were spot inoculated on Muller Hinton agar plates and incubated at 30°C for 3 days. The cells were killed by chloroform vapors. The plates were subsequently over laid with peptone yeast extract agar swabbed with *C. neoformans*. The resulting clear Zone of Inhibition (ZOI) was measured after 2 days of incubation. The experiment was repeated thrice. Mean diameter of ZOI and standard deviations were calculated. The strain AF1 which exhibited the maximum antagonistic activity against *C. neoformans* was selected for taxonomical investigation.

Morphology and taxonomy of antifungal producing strain: The spore morphology of AF1 was studied using Scanning Electron Microscope. The morphology was studied by examining gold-coated dehydrated specimen using the Japan make JEOL (JSM 5610 LV).

For 16S rRNA gene sequencing, the genomic DNA was isolated using the procedure described by Kimura[20]. The gene fragments were amplified by using PCR Kit (GENEI Pvt, Ltd, India) and 517 F (5'-CCA GCA GCC GCG GTA AT-3') and Act704R (5'-TCT GCG CATTTC ACC GCT AC-3')[21]. PCR was performed using Eppendorf Mastercycler pro thermal cycler 230 V/50-60 Hz with the following profile: initial denaturation at 95°C for 4 min, 30 amplification cycles of (95°C for 1 min, annealing temperature at 50°C for 60 sec, 72°C for 1 min) and a final extension step at 72°C for 4 min. The PCR product was run and excised from 1.5% agarose gel, purified with the QIAquick PCR purification kit (QIAGEN) and sequenced using the primers 8F and U1492R as previously mentioned. Sequencing was done in Chromous Biotech, Bengaluru, India using ABI 3100 sequencer (Applied Biosystems). The sequence was edited using FinchTV (Geospiza Inc.) and BioEdit (Ibis Biosciences, Abbott Labs).

Phylogenetic analysis: Sequence similarity search of 16S rRNA sequence from AF1 strain was carried out using BLAST (NCBI). Evolutionary tree was inferred by using neighbour-joining method[22]. The Clustal X program was used for multiple alignments and phylogenetic[23].

Media optimization

Substrate: Oyster shells were collected from Pamban sea shore and used as substrate for solid state fermentation. The shells were washed thoroughly with distilled water and dried at room temperature. The shells were then broken into pieces and passed through ISI meshes and fractions of mesh 4, 6, 8, 10, 12, 14, 16 were collected. Oyster shells of different particle sizes were then autoclaved at 121°C for 15 min for further experiments.

Lassical screening

One Factor A Time Method (OFAT): The process variables were selected like temperature, initial moisture level, particle size and effect of additional nutrients for antifungal production from AF1. Among the four variables, effect of additional nutrients and initial moisture level alone were optimized by OFAT method, remaining two variables are directly chosen for interaction studies by RSM approach[24].

Effect of moisture content and additional nutrients: To study the effect of initial moisture content, various moisture levels ranging from 20-70% were employed to fermentation medium by adjusting with sea water. The fermentation process was carried out at 30°C for 13 days. In addition, effect of additional nutrients such as fermentation yeast extract (1%), beef extract (1%), $(NH_4)_2SO_4$ (0.05%), NH_4Cl (0.05%) were evaluated for their effect on antifungal production from AF1 strain.

Antifungal activity was monitored by well diffusion method using *C. neoformans*. The yield was expressed as units of activity/milliliter of crude dissolved in phosphate buffer (pH 7), where one U was defined as one mm annular clear ring around the antibiotic disc[24].

Central Composite Design (CCD): In solid state fermentation, optimization of substrate particle size, initial moisture level and temperature are crucial factors to increase the yield of bioactive compound. The effects of yeast extract at different concentrations were also considered for experimental design. Based on OFAT method, parameters that had significant effect ($p<0.05$) on antibiotic production were selected for further experiments of Central Composite Design (CCD) and RSM. The software Minitab was used for experimental design, data analyses and quadratic model building. Experiments were carried out in 32 trials and duplicates were maintained for each run. The student's t-test and p-values were used to identify the effect of each factor on bioactive compound production. A 24 factorial composite (for 4 factors) experimental design resulting in 32 experiments as shown in Table 1. Minitab statistical software was used to optimize the screened variables grouped as temperature (X1), moisture (X2), nitrogen source (X3), particle size (X4). In developing regression equation, the test variables were coded according to the equation[25]:

$$xi = \frac{X_i - X_0}{\Delta X} \quad (1)$$

where, xi is independent variable coded value, X_i is independent variable real value on the centre point and ΔX is

Table 1: RSM study design by using four independent variables showing anticryptococcal activity in term of capsule and melanin production

Run order	T (X_1)	MC (X_2)	NS (X_3)	PS (X_4)	Reduction (%) (Melanin)	Reduction (%) (Capsule)
1	20	20	0	4	59.70	8.00
2	40	20	0	4	65.83	6.77
3	20	70	0	4	82.10	16.01
4	40	70	0	4	70.50	8.69
5	20	20	2	4	62.50	7.00
6	40	20	2	4	78.20	11.87
7	20	70	2	4	79.60	14.81
8	40	70	2	4	71.80	12.54
9	20	20	0	12	71.10	10.11
10	40	20	0	12	96.60	24.24
11	20	70	0	12	90.01	14.45
12	40	70	0	12	91.70	19.22
13	20	20	2	12	67.50	8.69
14	40	45	2	12	99.30	30.18
15	20	70	2	12	69.40	9.47
16	40	70	2	12	79.49	19.98
17	10	45	1	8	65.22	7.05
18	50	45	1	8	77.90	15.28
19	30	-5	1	8	71.80	13.70
20	30	95	1	8	77.90	12.15
21	30	45	-1	8	75.50	15.25
22	30	45	3	8	76.00	16.45
23	30	45	1	0	72.90	14.01
24	30	45	1	16	96.70	24.12
25	30	45	1	8	90.00	18.29
26	30	45	1	8	89.01	20.29
27	30	45	1	8	92.30	21.09
28	30	45	1	8	90.40	18.29
29	30	45	1	8	90.00	19.90
30	30	45	1	8	89.10	17.76
31	30	45	1	8	92.90	17.29

Where: T: Temperature, MC: Moisture content, NS: Nitrogen source, PS: Particle size

step change value. The response variable was fitted by second order model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation is:

$$Y = \beta_o + \sum \beta_i x_i + \sum \sum \beta_{ij} x_{ij} + \sum \beta_{ii} x_i^2 \quad (2)$$

where, Y is the measured response, % reduction units. βo is the intercept term, β_i, β_{ij} and β_{ii} are the measures of effect of variables xi, xij and x_i^2, respectively. The variable xij represents the first-order interaction between x_i and x_j. Fermentation was initiated by using 50 g of oyster shells of 16 mesh size and 44 mL of sea water was added to produce an initial moisture content of 45%. After autoclaving, 5 mL of seed culture was added and fermentation was carried out for 13 days at 35°C.

The statistical analyses of the model were performed in the form of analysis of variance (ANOVA). This analysis includes Fishers F-test, its associated probability, Correlation coefficient R and determination coefficient R^2 that measures the goodness of fit of the model. Response surface and 2D contour plots described by regression model are drawn to illustrate the effect of independent variables and interactive effects of each independent variable on the response variables. The supernatant obtained from each experimental trial were extracted with equal volume of xylene and checked for anticryptococcal activity. Antifungal activity was defined in terms of highest % of reduction in capsule and melaninzed cells.

Extraction and purification of antifungal compound: Five hundred mL of optimized media was sterilized and inoculated with 5% seed culture followed by incubation at 28°C for 13 days. The fermented medium was then centrifuged at 10,000 rpm for 15 min at 4°C and supernatant was extracted thrice with equal amount of xylene (optimized solvent). The crude extract was evaporated using rotary evaporator and residual material was dried at room temperature[26]. The obtained colourless compound was dissolved with phosphate buffer (pH 7) and was chromatographed using Semi-Preparatory HPLC (Agilent 1260 infinity). Reversed-phase

C18 column (10×250 mm) was used with a linear gradient from 1-60% MeOH and 9-40% water was performed over 25 min at a flow rate of 1 mL min^{-1}. The elution pattern was monitored at 225 nm. Fractions were collected and antifungal activity was performed by broth dilution method in 96 well plate.

Determination of Minimum Inhibitory Concentration (MIC): MIC of crude and purified compound was estimated by broth dilution method in 96 well plate and test tubes[27]. Five mL of PDB media added with 50 µL of *C. neoformans* culture (10^6 cells/mL). Culture was added with crude and purified bioactive compound concentration between 0-2 mg mL^{-1} with 200 µg mL^{-1} interval and 0-500 µg mL^{-1} with 50 µg mL^{-1} interval respectively, incubated for 48 h at 37°C on a rotary shaker at 120 rpm. After 24 h, 96 well plate observed in ELISA plate reader (Sunrise, Tecan, Austria GmbH) at 620 nm. Cells were plated on potato dextrose agar plates from tubes and number of cells was measured. The lowest compound concentration with more than 50% fungal inhibition was estimated to be MIC.

Cell line toxicity: The mammalian cell lines HepG2 human hepatocyte cell line exposed to bioactive compound and time interval. The cell lines maintained at 37°C in 5% CO_2 and 95% humidity incubator in DMEM media (Himedia) supplemented with 10% FBS (Himedia) and 1X antibiotic (Himedia). Cell lines were incubated with antifungal bioactive compound concentration between 0-500 µg mL^{-1} with the interval of 50 µg mL^{-1} and maximum incubation period for 24 h. After incubation cell viability was measured using MTT assay kit (CCK003, EZcount MTT cell assay kit, Himedia) following the manufacturer's protocol. The assay was performed in 96-well plates in triplicates and differences were tested for statistical significance by student's t-test[28].

Assay of antifungal compound on major virulence factors

Effect on melanin inhibition: Fifty µL of *C. neoformans* (10^6 CFU mL^{-1}) fresh culture was inoculated with crude (1 mg mL^{-1}) and pure (200 µg mL^{-1}) bioactive compound and incubated at 24 h at 30°C in minimal broth media. After incubation, 10 µL of treated culture was plated on minimal agar medium containing glucose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), thiamine (3 µM), agar (2%) with the addition of L-DOPA (1 mM) for induction of melanization[29]. Plates were incubated at 30°C for 4-7 days in dark and the number of melanized cells was counted[30]. The effect of compound on the melanised cells and non melanised cell were compared.

Effect on capsule growth: As the capsule size of *C. neoformans* is directly proportional to its virulence, the capsule was induced in Artificial cerebral spinal fluid medium[31]. *C. neoformans* with maximum capsule was used to study the effect of bioactive compound on capsule. After 48 h of induction period, 50 µL inoculated *C. neoformans* was added with 1 mg mL^{-1} and 200 µg mL^{-1} of compound and re-incubated for 24 h at 30°C. Effect of compound on capsule was evaluated by performing negative staining with India ink using Trinocular microscope (Eclipse Ci-L, Nikon). Images were taken with a camera (SLR, Nikon D5100, Camera). To calculate relative size of capsule, diameters of whole cell, including capsule (Dwc) and cell body limited by cell wall (Dcb), were measured using Image J software. The size of the capsule relative to that of the whole cell was defined, as a percentage as [(Dwc - Dcb)/Dwc]100. Fifteen cells were measured for each determination and average was calculated[32].

Mass spectroscopy analysis: The purified compound was subjected to Nano Spray matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS) and the molecular weight was determined from the time of flight.

Statistical analysis: All experiments were performed in duplicates and the data analysis was done using Minitab 16 software and GraphPad Prism 6. One-way ANOVA and t-test performed to test statistical significance for multiple comparisons ($p<0.05$)[31]. All graphs were prepared with GraphPad Prism 6 and were expressed as Mean±Experiments done in duplicates.

RESULTS AND DISCUSSION

Isolation and taxonomy of potential strain: In this study 33 actinomycetes were isolated from marine sediments. The strain AF1 isolated using starch agar showed the maximum activity against C. neoformans. It displayed maximum activity against the test organism with 15 mm mean diameter of zone of inhibition.

The electron micrographs reveals the spores have smooth surface in oval shapes around 4-6 spores/round (Fig. 1). The 16S rRNA gene sequence of the strain AF1 and its comparison with the gene sequences against the GenBank database revealed that the organism form a distinct phylogenetic line in the *N. synnemataformans*. The isolate was closely related to the type strain of *Nocardiopsis* strain UTMC 2171, sharing a homology of 99%. The 16S rRNA sequence analysis support the classification of the isolate AF1 as a new strain of

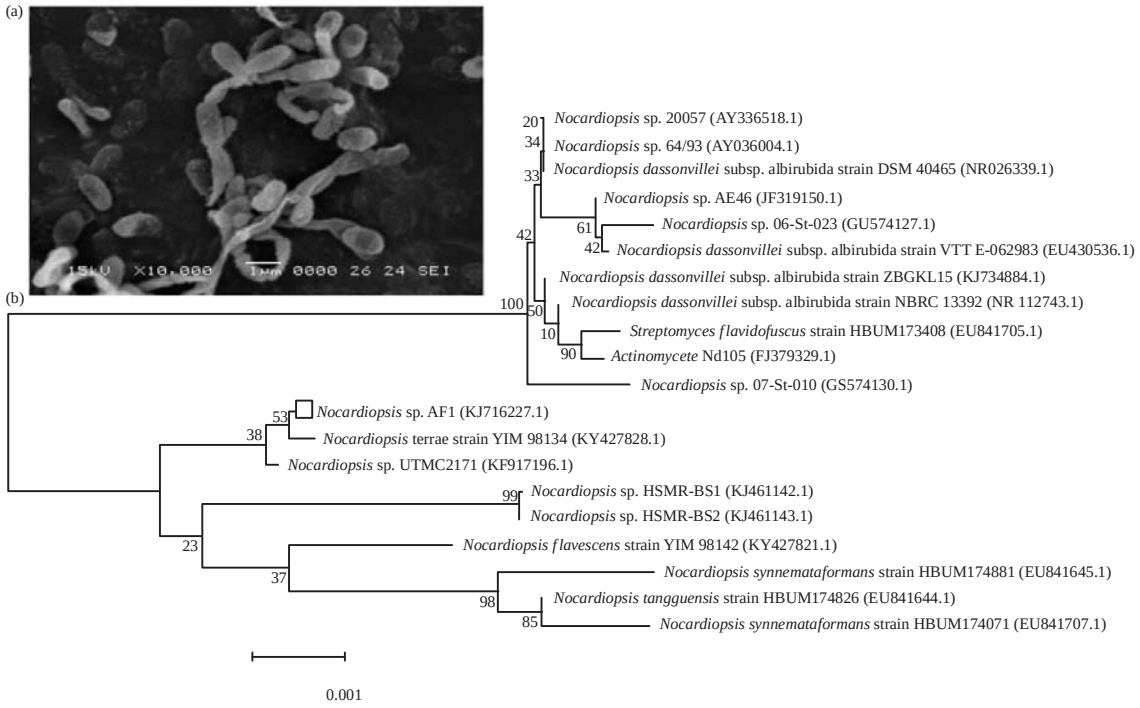

Fig. 1: (a) Scanning Electron micrograph of *Nocardiopsis synnemataformans* AF1 grown on Starch agar at 30°C for 14 days and (b) Neighbor-joining tree based on 16S rDNA gene sequences showing relationship between the strain *Nocardiopsis synnemataformans* AF1 (accession number KJ 716227) and other *Nocardiopsis* sp

Nocardiopsis UTMC 2171 (Fig. 1). The accession number KJ 716227 was obtained from NCBI. A recent review by Mayer *et al.*[33], demonstrated a slight decrease in the discovery of marine antifungal products and during the last decade, only two molecules described the novel mechanism of action. Very limited researchers are attempted to find bioactive molecules against *Cryptococcus* sp. Recently, Das *et al.*[5], identified a mangrove *Nocardiopsis* sp., demonstrating 12 mm zone of inhibition against *C. neoformans*. In the last decade some bioactive compounds, kahakamides AV, 2 neosidomycin, also a novel cyclic tetrapeptide, polyketides, thiopeptide have been discovered from marine *Nocardiopsis* strains[34]. None of these compounds have been proven to exhibit anticryptococcal activity. A thorough literature search evident the absence of reports on finding compounds against *C. neoformans*. Specific molecules are needed to treat *C. neoformans* as they are different from other pathogenic yeast on many aspects such as capsule as a shield layer, melanin production, its ability to grow at 37°C and can disseminate from lungs to central nervous[35]. Hence the bioactive compound from the marine *N. synnemataformans* AF1 would be a suitable drug candidate to treat cryptococcal infections.

Classical screening

one factor a time method

Effect of initial moisture content: The optimum initial moisture content for the production of antifungal compound using Oyster shells as the substrate was estimated. The results presented in Fig. 2 clearly indicated that the maximum antifungal production was recorded at 50% with the yield of 245 U mL^{-1}. A similar report on maximum tetracycline production from *N. synnemataformans* at 65% moisture level using pineapple peel as substrate[36]. Furthermore, maximum neomycin production from *Nocardiopsis* by solid state fermentation at 70% moisture level using agricultural wastes such as apple pomace, cotton seed meal, soy bean powder and wheat bran in a parallel study[37]. These results evident that the optimum moisture level may differ widely of the same organism growing on different substrates[38].

Oyster shells have been reported to be one of the most serious pollutants of both marine environment and soil. Recycling of oyster shells thus been recognised as necessary and they have subsequently been used in many applications including, fertilizer, sludge conditioner, eutrophication control, desulphurization sorbents[39] and waste water treatment. Our attempt in finding its relevance as cost effective medium for the cultivation of AF1 and production of its bioactive compound were achieved.

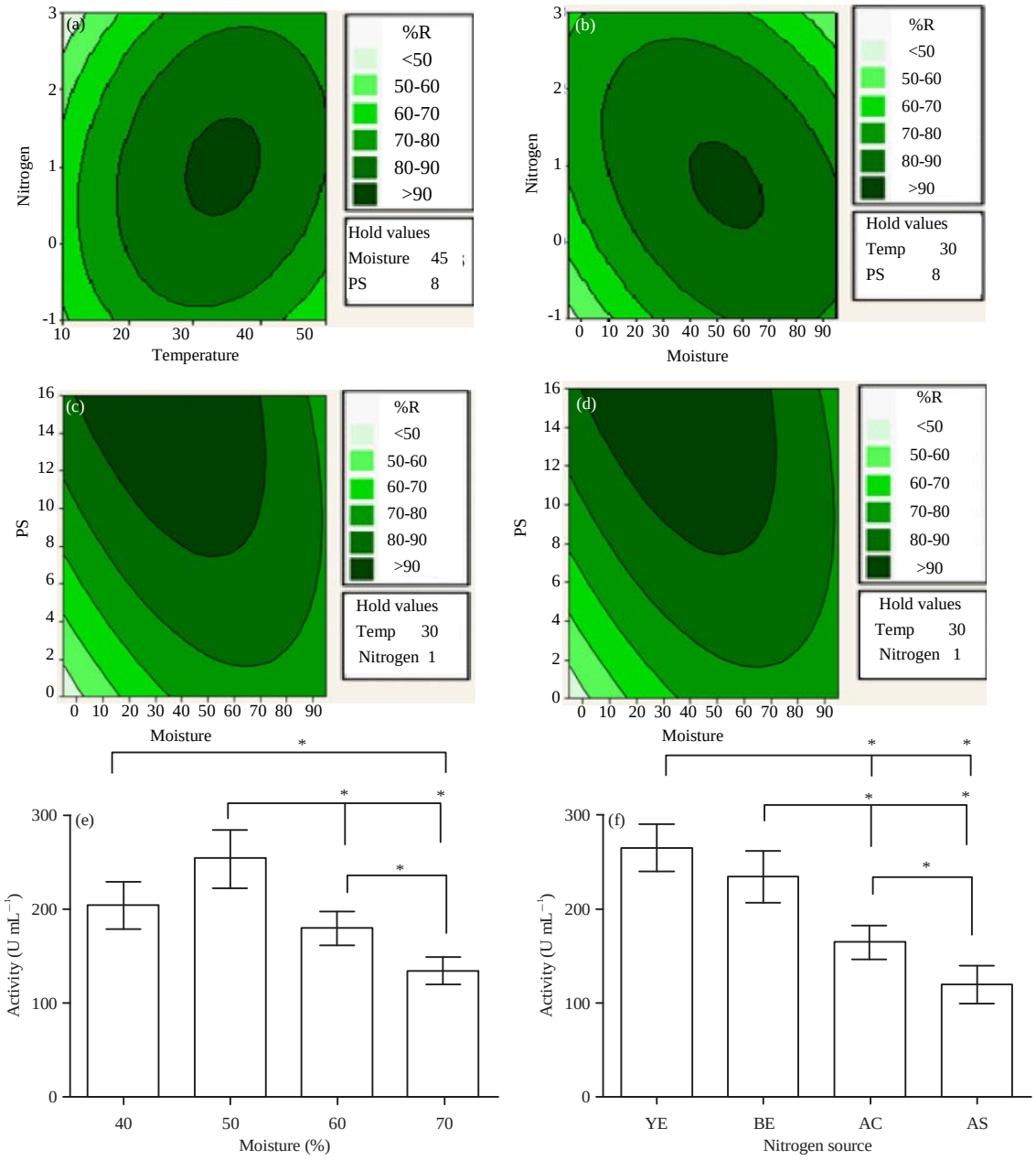

Fig. 2(a-f): Response surface plots showing interactive effects of various factors on antifungal activity (% reduction) of *Nocardiopsis synnemataformans* AF1 (a) Nitrogen source and Temperature (b) Nitrogen source and Moisture (c) Particle size and moisture (d) Particle size and Temperature. Selection of variables by OFAT method (e) Optimization of initial moisture content for antifungal production and (f) Optimization of additional nutrients for antifungal production

The experiment has done twice with duplicates at each time. Significant difference shown by *($p<0.05$). Bar represents mean and SD for triplicate samples

Effect of additional nutrients: The effect of nitrogen sources on the production of bioactive compound are shown in Fig. 2. The maximum antifungal yield (260 U mL^{-1}) was obtained with the addition of yeast extract (1% w/v). Lower yields were obtained with the addition of ammonium sulphate (130 U mL^{-1}). Thus, yeast extract was chosen as the source of nitrogen for further experiments. The fermentation without any additional nutrients yielded 240 U mL^{-1}, which confirms the significance of additional nutrients to the oyster shells fermentation medium. In general, oyster shells are rich in

calcium carbonate, other essential minerals and organic matter. For more than 3 decades till date, researchers exploit the calcium carbonate enriched medium for isolation of actinomycetes[40]. From these results, oyster shell medium enriched with yeast extract would be a suitable cost effective medium for the production of bioactive compound from *N. synnemataformans*. Moreover it can be an appropriate cultivation and production media for other actinomycetes also.

Antifungal activity of compound produced by OFAT method: While screening for the anticryptococcal activity of the crude compound obtained by the classical optimization (yeast extract -1%, moisture -50%, oyster shells -16 mesh size, temperature 35°C), the yield obtained was 260 U mL^{-1}. The effect of the compound on the Crytococcous capsule growth and on melanized cells shows the reduction of 8 and 48%, respectively.

Central composite regression design

Statistical approach: While analyzing the effects of various factors (temperature, moisture, particle size and yeast extract) and their interactions in enhancing the antibiotic production. The goodness of fit of the model based on RSM checked by applying multiple regression analysis on the experimental data, experimental results of the CCD design were fitted with second-order polynomial equation. The results of regression analyses are shown in Table 2. The student's t-test and p-values were used as a tool to check the significance of each coefficient that also indicated the interaction strength between each independent variable. The larger magnitude of t-value and smaller the p-value, more significant is the corresponding coefficient[41]. It can be seen from the degree of significance.

The R^2 value is always between 0 and 1.The closer the R^2 value to 1, stronger the model and better it predicts the response[17]. In this case coefficient of determination $R^2 = 0.9658$ and 0.9850 for capsule and melanin respectively Table 2. The R^2 also indicates only 7 and 1% of total variations was not explained by the model. The value of adjusted determination coefficient was also high (Adjusted $R^2 = 93.58\%$, 97.18% respectively, which supports that the model generated was more suitable for optimization.

ANOVA for the response surface square model suggests that the interactions between moisture and temperature had a higher influence on antifungal activity and hence the yield (Table 3).

With larger magnitude of t-value and smaller the p-value, the corresponding coefficient would be more significant. A p-value of less than 0.05 indicates that the model terms were significant and regression model having coefficient of determination R^2 value higher than 0.9 were considered as having a very high correlation[27].

From multiple regression analyses it was observed that second order polynomial equation could explain antifungal production regardless of the significant of the antifungal production.

Table 2: Analysis of variance for the model regression representing reduction of capsular growth and melanized cells

	Co_{ef}		SE Co_{ef}		T		P	
Terms	C_c	C_m	C_c	C_m	C_c	C_m	C_c	C_m
Constant	18.9871	90.53	0.5449	0.7113	34.847	127.27	0	0
T	2.5588	4.0363	0.2943	0.3842	8.695	10.507	0	0
MC	0.2171	1.9196	0.2943	0.3842	0.738	4.997	0.471	0
NS	0.3937	-0.7812	0.2943	0.3842	1.338	-2.034	0.2	0.059
PS	2.9529	5.9363	0.2943	0.3842	10.035	15.453	0	0
T×T	-2.1013	-4.6645	0.2696	0.3519	-7.794	-13.254	0	0
MC×MC	-1.6613	-3.842	0.2696	0.3519	-6.162	-10.917	0	0
NS×NS	-0.930	-3.617	0.2696	0.3519	-3.45	-10.277	0.003	0
PS×PS	-0.1263	-1.3545	0.2696	0.3519	-0.468	-3.849	0.646	0.001
T×MC	-2.0981	-5.4219	0.3604	0.4705	-5.822	-11.524	0	0
T×NS	1.5156	1.7544	0.3604	0.4705	4.205	3.729	0.001	0.002
T×PS	3.5531	4.1656	0.3604	0.4705	9.859	8.854	0	0
MC×NS	-0.6369	-3.0181	0.3604	0.4705	-1.767	-6.415	0.096	0
MC×PS	-1.7819	-2.6044	0.3604	0.4705	-4.944	-5.535	0	0
NS×PS	-0.4031	-2.9806	0.3604	0.4705	-1.119	-6.335	0.28	0

T: Temperature, MC: Moisture content, NS: Nitrogen source, PS: Particle size, C_c: *C. neoformans* (capsule) and C_m: *C. neoformans* (melanin), *C. neoformans* (capsule) S = 1.44160 PRESS = 137.374, R-Sq = 96.58% R-Sq(pred) = 85.86% R-Sq(adj) = 93.58%, *C. neoformans* (melanin), S = 1.88198 PRESS = 266.224, R-Sq = 98.50% R-Sq(pred) = 92.93% R-Sq(adj) = 97.18%

Table 3: ANOVA for Significant Interactions between moisture and temperature

	Seq SS		Adj SS		Adj MS		F		P	
Sources	C_c	C_m	C_c	C_m	C_c	C_m	C_c	C_m	C_c	C_m
Regression	938.199	3710.61	938.199	3710.61	67.014	265.043	32.25	74.83	0	0
Linear	371.258	1339.81	371.258	1339.81	92.814	334.953	44.66	94.57	0	0
T	157.133	390.99	157.133	390.99	157.133	390.992	75.61	110.39	0	0
MC	1.131	88.44	1.131	88.44	1.131	88.435	0.54	24.97	0.471	0
NS	3.721	14.65	3.721	14.65	3.721	14.648	1.79	4.14	0.2	0.059
PS	209.273	845.74	209.273	845.74	209.273	845.738	100.70	238.78	0	0
Square	197.867	1177.15	197.867	1177.15	49.467	294.286	23.8	83.09	0	0
T×T	102.095	445.49	126.259	622.17	126.259	622.168	60.75	175.66	0	0
MC×MC	71.017	330.35	78.919	422.10	78.919	422.096	37.97	119.17	0	0
NS×NS	24.299	348.84	24.733	374.10	24.733	374.105	11.90	105.62	0.003	0
PS×PS	0.456	52.46	0.456	52.46	0.456	52.462	0.22	14.81	0.646	0.001
Interaction	369.074	1193.65	369.074	1193.65	61.512	198.941	29.60	56.17	0	0
T×MC	70.434	470.35	70.434	470.35	70.434	470.348	33.89	132.80	0	0
T×NS	36.754	49.25	36.754	49.25	36.754	49.245	17.69	13.90	0.001	0.002
T×PS	201.995	277.64	201.995	277.64	201.995	277.639	97.20	78.39	0	0
MC×NS	6.490	145.75	6.490	145.75	6.490	145.745	3.12	41.15	0.096	0
MC×PS	50.801	108.52	50.801	108.52	50.801	108.524	24.44	30.64	0	0
NS×PS	2.600	142.15	2.600	142.15	2.600	142.146	1.25	40.13	0.28	0
Residual error	33.251	56.67	33.251	56.67	2.078	3.542				
Lack-of-fit	20.941	42.99	20.941	42.99	2.094	4.299	1.02	1.88	0.514	0.226
Pure error	12.311	13.68	12.311	13.68	2.052	2.281				
Total	971.450	3767.27								

$$Y_c = \begin{bmatrix} 17.6338+3.1856X_1+0.1444X_2+0.6894X_3+3.4956X_4- \\ 1.8381X_1*X_2+1.2556X_1*X_3+3.2931X_1*X_4-0.3769X_2* \\ X_3-1.52192X_2*X_4-0.6631X_3*X_4-4.8898X_1^2-3.1298X_2^2- \\ 0.2048X_3^2 32-4.0102X_4^2 \end{bmatrix} \quad (3)$$

$$Y_m = \begin{bmatrix} 90.53+4.0363X_1+4.0363X_2-0.7812X_3+5.9363X_4- \\ 5.4219X_1*X_2+1.7544X_1*X_3+4.1656X_1*X_4-3.0181X_2*X_3- \\ 2.6044X_2*X_4-2.9806X_3*X_4-4.6645X1_2-3.8420X_2^2- \\ 3.6170X_3^2-1.3545X_4^2 \end{bmatrix} (4)$$

where, Y_c and Y_m correspond to the effects on the capsule and melanized cells, respectively.

The interaction studies from 2D contour plot shown in Fig. 2 reveal that all factors (moisture, temperature, yeast extract, particle size), had significant effects ($p<0.05$) on the antibiotic production. The best interactions are between moisture of 40% temperature of 40°C, yeast extract of 2% and Oyster shells with the particle size of 16. The smaller particle size of oyster shell provides large surface area which helps to mix the substrate with the microorganisms, other nutrients and uniform distribution of temperature that supports the microbes to enhance the production of antibiotics. Ellaiah *et al.*[42] convey that the increase in higher substrate moisture in solid state fermentation results in sub optimal product formation due to reduced mass transfer process such as diffusion of solutes and gas to cell during fermentation. Also, the decrease in moisture results in reduced solubility minimizes heat exchange, oxygen transfer and low availability of nutrients to the culture, leading to decrease in productivity[42]. This supports our results at 45% initial moisture level using oyster shell.

Further validation experiments were also carried out to verify the adequacy and accuracy of the model on the result suggested that the predicted value agreed with experimental values. The enhanced antifungal activity was obtained with statistically optimized production medium exhibiting 20.18 and 99.3% reduction of capsule growth and melanized cells, respectively. Figure 3 clearly reveals the significant reduction ($p<0.05$) in capsule growth and melanized cells, respectively. The antifungal compound production yield was increased by 60 U mL^{-1} in statistically optimized condition when compared to conventional method.

Extraction and Purification of antifungal compound: The supernatant which were obtained by growing the strain AF1 in the optimized medium for a period of 13 days were extracted with xylene and the extract was evaporated to dryness resulting in a yellow oily residue (2.6 g). The crude extract was purified by using semi preparatory HPLC. The HPLC analysis yield 20 fractions and the compounds corresponding to the first peak starts from 3.5-14.4 min (Fig. 4). The active peak was identified by performing antifungal assay by disc diffusion method. A zone of clearance of 26 mm for HPLC peak 10th with a RT of 8.7-8.9 min was observed on assay plates. However, the other peaks shows no antifungal activity. Also the purified compound showed

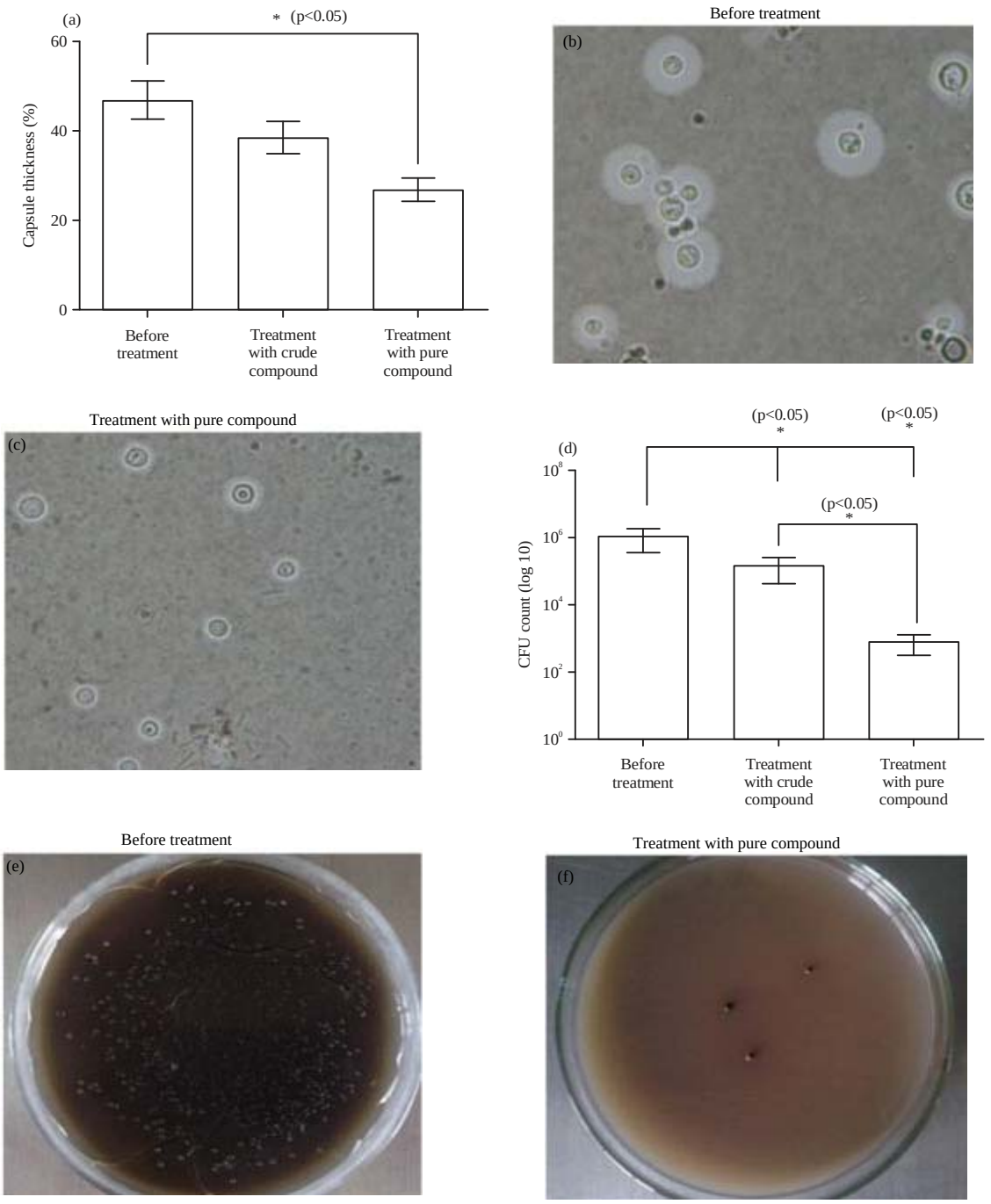

Fig. 3(a-f): Effect of bioactive molecule from *Nocardiopsis synnemataformans* AF1 on capsule growth and melanized cells. India ink analysis of crytptococcal yeast cells was done for capsule size: (I) Capsule inhibition: (a) Effect of crude (1 mg mL^{-1}) and pure (200 µg mL^{-1}) compound on capsule thickness (%), (b) Induction of capsule in MOPS medium, (c) Reduction in capsule size-cells treated with 200 µg mL^{-1} of compound. (II) Melanin inhibition, (d) Effect of crude (1 mg mL^{-1}) and pure (200 µg mL^{-1}) compound melanin producing cells, results analyzed by CFU mL^{-1} count, (e) Growth of cryptococcal cells producing dark pigmentation of colonies-without treatment and (f) Growth of cryptococcal cells in the treated with bioactive molecule with the addition of 200 µg mL^{-1} showing significant reduction of melanin production. The experiment has done twice with duplicates at each time

Significant difference shown by *p<0.05. Bar represents mean and SD for triplicate samples

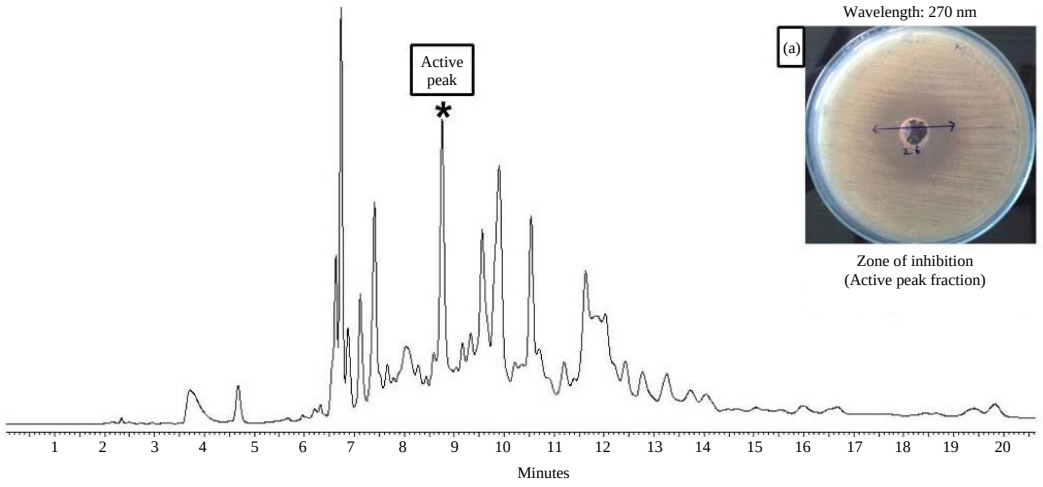

Fig. 4: HPLC profile of Nocardiopsis synnemataformans AF1
(*) represents active peak shows anticryptococcal activity; (A) Zone of inhibition (26 mm) of active peak on Mueller Hinton Agar

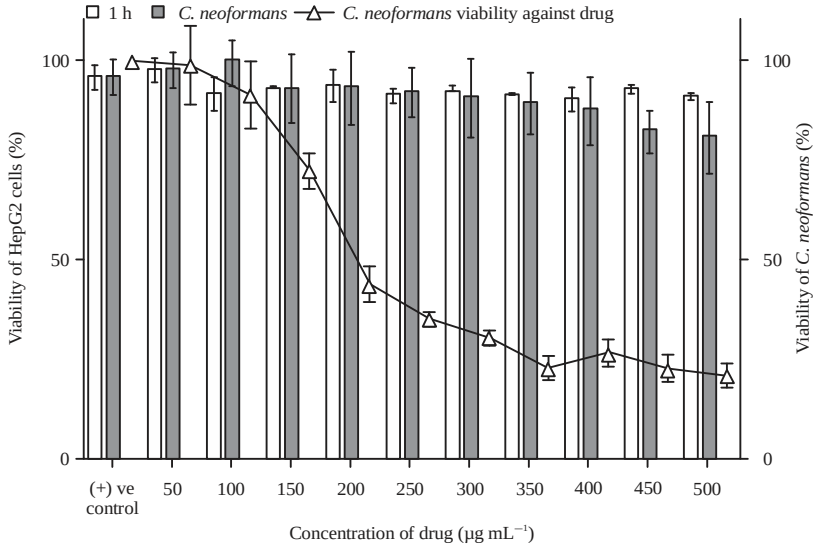

Fig. 5: AF1 antifungal compound minimum inhibitory concentration and cytotoxicity study against HepG2 cell line. Antifungal AF1 compound MIC50 was observed at 200 µg mL against *C. neoformans* and more than 75% viability of HepG2 cells was observed up to 500 µg mL^{-1}
Bar represents mean and SD for triplicate samples

significant reduction ($p<0.05$) in capsule growth and melanin pigment production. Thus, the compound produced during the study would be a potential molecule against *C. neoformans*.

MIC determination: The crude and purified compound at a final concentration of 1 mg mL^{-1} (data not shown) and 200 µg mL^{-1} was found to be the MIC_{50}, respectively. MIC was confirmed by the absence of growth in agar plate and 96 well plate at OD_{600} was comparable with positive and negative control, results shown in Fig. 5.

Cell line toxicity: Cytotoxicity of antifungal bioactive compound on HepG2 human hepatocyte cell linehas evaluated by MTT assay. The multiple concentrations of antifungal compound used, results shown in Fig. 5. There was no cytotoxicity was observed for compound in HepG2 up to concentration of 500 µg mL^{-1}, more than 80% viability

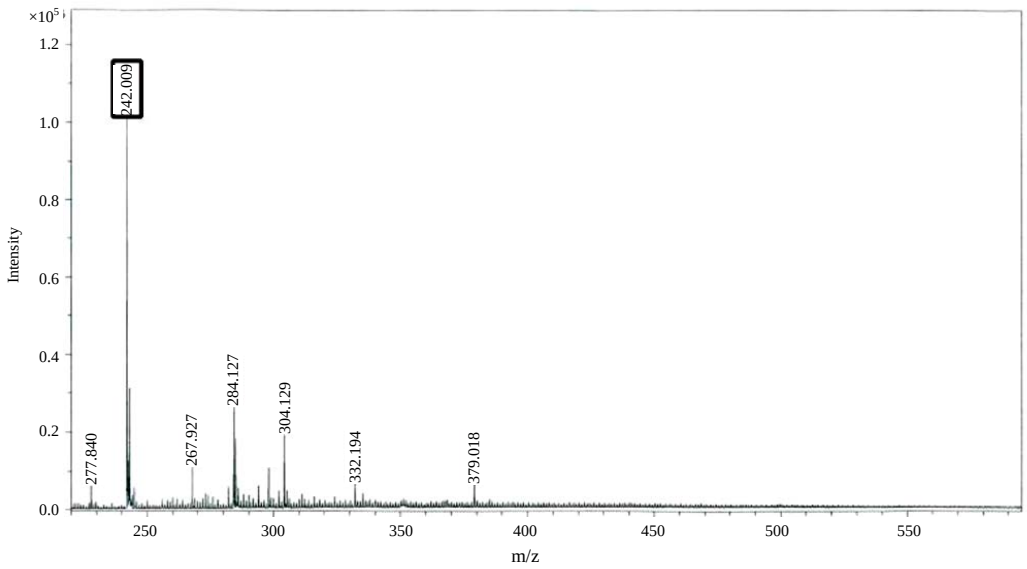

Fig. 6: Mass spectroscopy analysis of HPLC purified antifungal compound from *Nocardiopsis synnemataformans* AF1

observed with maximum of 24 h incubation for the cell lines with antifungal compound. As per test parametric statistical analysis, no significant difference was observed for 1 and 24 h compound treatment to HepG2 cells.

MALDI-TOF analysis: The MALDI-TOF MS spectrum confirms the molecular weight of the purified antifungal compound to be 242.1 Da Fig. 6. Amphotericine B is the standard initial therapy to treat cryptococcal infections. The larger encapsulated strains are less likely to respond to the current therapy (AMB plus flucytocine). Although AMB function as immunomodulator, its effectiveness are insignificant to control the cryptococcal infections in severely immune-compromised host. Amphotericine B is derived from Streptomyces sp which has the molecular weight of 924 Da[43]. Molecular size is one of the important criteria to traverse the drugs to the CNS tissue barrier[44]. Hence, the isolated molecule with low molecular size from a new strain of *Nocardiopsis synnemataformans* might be a novel and potential antifungal molecule which requires the complete structural elucidation studies.

CONCLUSION

The results of this study show that the strain AF1 isolated from marine environment exhibited potential antagonistic activity against *C. neoformans*. The solid state cultural conditions were optimized by using RSM approach. The enhancement of antifungal production and antifungal activity were achieved at initial moisture level of 50%, temperature at 40°C, yeast extract of 2% and Oyster shells with the particle size of 16. The potentiality of the purified antifungal molecule from AF1 was demonstrated by its significant influence on reduction of capsule growth and melanization. At the same time, the low molecular weight of the molecule was found to be a good promise as a potential drug candidate. Also there no toxicity was observed, cell viability was more than 80% up to 500 μg mL^{-1} concentration in HepG2 cell line. The complete structural elucidations are in the laboratory pipeline.

SIGNIFICANCE STATEMENTS

This study discovered the potential application of oystershell as a suitable substrate to produce antifungal compound. The study highlights the use of response surface methodology an efficient tool for media optimizations and analysis of complexity during the interactions of various factors. The present study describes the isolation and taxonomy of anticryptococcal producing strain, optimization by RSM approach, purification of bioactive molecule and determination of molecular weight by MALDI-ToF MS. This is the first study to identify and produce anticryptococcal compound from marine *Nocardiopsis* sp. The present study will help the researchers to explore marine habitat as the potential source to discover antifungal compound.

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