Statistical optimization of anticandida metabolite production process using *Streptomyces hydrogenans* strain from mangrove soils

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Abstract

Streptomyces hydrogenans is well known to exhibit antagonistic activity against several fungal pathogens. However, improving the potency of such inhibitory activity is challenging and necessitates the manipulation of fermentation conditions and nutritional requirements of the actinomycetes. The present study is focussed on statistical optimisation of the strain cultural process for achieving ample yield of novel anti-*Candida* metabolites. Nine variables including soyabean meal, glucose, glycerol, calcium carbonate, sodium chloride, pH of the medium, strain inoculum level and incubation period were modeled by Plackett–Burman design for screening in order to predict their influence on the production of bioactive metabolites. Among them, the most critical variables that exert significant variation in the response of interest were identified to be incubation period, glucose and calcium carbonate (CaCO_3). With a goal to achieve the maximum metabolite yield/ anti-*Candida* potentiality/ or cost-cutting production, these significant factors were further optimized and validated employing a Box Behnken design. Design of experiment assisted fermentation process enhanced 1.8 fold productivity and antifungal activity at the optimal condition consisting soyabean meal (1.5%), glucose (1.030%), glycerol (0.25%), NaCl (0.5%), CaCO_3 (0.1%), sea water (50%) as medium at pH 7.0, inoculums level (10%) and incubation period of 5 days.

Keywords *Streptomyces hydrogenans* · Koringa mangrove soils · *Candida albicans* · Fermentation · Optimisation · Plackett–Burman design · Response surface methodology

1 Introduction

The rising prevalence of *Candida* infections has been significantly increased the risk of life-threatening disseminated mycoses. Nevertheless, the increase in number of immunocompromised patients is the main reason for infections. Numerous risk factors have been evidenced to be responsible for candidiasis, such as patients with HIV infections, patients undergoing treatment with cancer chemotherapy, broad-spectrum antibiotics, central venous catheters, immunosuppression therapy after organ transplantation, including uncontrolled diabetic patients and

aged population [1]. It is estimated that the *Candida* spp. secured fourth rank among the nosocomial blood stream infections with a mortality rate upto 50% [2–4]. *Candida albicans* is the most common species to cause candidemia and disseminated candidiasis in humans [5, 6]. Treatment of such mycotic infections necessitates extensive use of antifungal antibiotics. However, effective antifungals are very few and most of them develop drug resistance and side effects owing to the structural similarity of mammalian cell and fungi. Thus, the unmet need for effective and safer antifungals is the primary concern in the current pharmaceutical or biotechnology practices [7].

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Although antifungal agents are produced by several different microorganisms, actinomycete remains the greatest promising source. *S. hydrogenans* KMF-A1 isolated from mangrove soil exerts strong antagonistic activity against *C. albicans* MTCC 183. The bioactive metabolite produced by the *S. hydrogenans* KMF-A1 shows an excellent stability when exposed to variant temp and pH and has a good shelf life (3 yr) [8]. It is noteworthy to mention here that microorganisms inhabitant to extreme environment imposes improved stability while holding their molecular evolution of life. Most of the metabolic pathways are strictly regulated and triggered under defined conditions. One of the methods to activate the biosynthetic pathway for the improvement in antibiotic yield is optimizing the nutritional and physical parameters of the production medium [9].

However, production of secondary metabolites may critically vary due to a small change in cultivation parameters. Type, nature and level of medium components impact species and strains of microorganisms and lead to affect the yield and potency of antibiotics. Therefore, it is a prerequisite to optimize nutritional and environmental parameters in order to facilitate ample yield and discovery of novel antibiotics. Response surface methodology (RSM)-based multivariate approach is a preferred method of process optimization that has gained substantial consideration in recent years. Use of RSM in optimizing fermentation conditions accomplishes the best potency and yield of bioactive metabolites from the test microorganisms. Moreover, the advantages of RSM strategy have been well emphasized by many recent studies that it can efficiently identify and optimize multiple critical factors with limited experimental trials [10].

The present study deals with establishing a fit-for-purpose fermentation process for the cultivation of *S. hydrogenans* KMF-A1 isolated from mangrove soil near Kakinada region, Andhra Pradesh, India, for production of antifungal metabolites. A design of experiments approach was adopted to optimize the cultivation medium and other process parameters in order to maximize the yield and potency of the product.

2 Materials and methods

2.1 Microorganisms

The antifungal compound production strain, *S. hydrogenans* KMF-A1 was isolated from mangrove soil samples. The isolate was maintained on yeast-malt extract (YEME) agar slants (composition: malt extract-1%, yeast extract-0.4%, dextrose-0.4%, sea water-50%, distilled water-50%,

agar-2%, pH adjusted to 7.0 ± 0.2 by addition of NaOH) and preserved at 4 °C throughout the study [8].

The test organism: *C. albicans* MTCC 183 was obtained from IMTECH Chandigarh, subcultured in Sabouraud agar, and incubated for 48 h at 25 °C.

2.2 Inoculum preparation

The strain, KMF-A1 was freshly inoculated on YEME agar slants and incubated for 5 days at 28 °C for sporulation. The spores were completely harvested and suspended into sterile water. Further, 5 ml of this suspension was inoculated into 45 ml of sterile YEME broth and placed in a rotary shaker at 180 rpm for 48 h.

2.3 Production of anticandida metabolite

Subsequently, the resultant inoculum each of 5–10% was transferred into selected production media (Table 1) separately and placed on a rotary shaker at 180 rpm maintained at temperature 28–30 °C, for 7 days. Further, the media were centrifuged at 7000 rpm for 20 min, and the clear supernatant was examined for antagonistic activity against *C. albicans*.

2.4 Assay of antifungal metabolite

The cup-plate method was employed to screen the growth inhibition potency of the isolate against the test organism *C. albicans* MTCC183. During the procedure, 50 ml of Sabouraud dextrose agar was inoculated with 50 µl of test strain and plated on petri dishes. After solidification wells are punched with a cork borer, 50 µl of supernatant was transferred to each well, kept for diffusion for about 1 h and incubated for 48hrs at 25 °C. The anti-Candida activity was demonstrated with a clear observation of inhibitory zone around the wells. The potency of the metabolite against the test microbe was determined by measuring the inhibition zone diameter and expressed as U/ml [11].

2.5 Study of variables affecting metabolite production

Variability in metabolite production for antifungal production is studied by varying different media composition (PM 1 to PM 7), pH (pH: 2.0, 4.0, 6.0, 8.0 and 10.0), incubation period (3, 5, 7, 9, 11 days), sea water level (20%, 40%, 50%, 60%, 80% and 100% v/v), and level of inoculums (2%, 4%, 6%, 8% and 10%v/v). The study was carried out by changing one-factor-at-a-time (OFAT), while keeping others constant at a level based on literature. Five experiments were conducted in triplicate according to the following

Table 1 Composition of production media for preliminary screening

Production media	Composition
PM-1	Soyabean meal—1.0%, corn steep liquor—0.5%, soluble starch—1.0%, dextrose—0.5%, CaCO ₃ —0.7%, sea water ^a pH adjusted to defined level ^b
PM-2	Yeast extract—0.3%, peptone—0.5%, malt extract—0.3%, Glycerol—2.0%, CaCO ₃ —0.25%, sea water ^a pH adjusted to defined level ^b
PM-3	Corn steep liquor—1.0%, soluble starch—0.5%, (NH ₄) ₂ SO ₄ —0.5%, CaCO ₃ —0.5%, sea water ^a pH adjusted to defined level ^b
PM-4	Glucose—1.0%, starch—1.0%, peptone—0.75%, meat extract—0.75%, NaCl—0.3%, sea water ^a pH adjusted to defined level ^b
PM-5	Yeast extract—0.5%, casein—0.5%, starch—2.0%, dextrose—1.0%, CaCO ₃ —0.4%, sea water ^a pH adjusted to defined level ^b
PM-6	Yeast extract—0.5%, Tryptone—0.5%, %, meat extract—0.3%, dextrose—1.0, CaCO ₃ —0.2%, sea water ^a pH adjusted to defined level ^b
PM-7	Soyabean meal—1.5%, Glucose—1.5%, glycerol—0.25%, NaCl—0.5%, CaCO ₃ —0.1%, sea water ^a pH adjusted to defined level ^b

^aSea water added in a range from 20 to 100% for optimization^bpH in a range from 2.0 to 10.0

protocol (Table 2), and an assay of antifungal metabolite was determined.

2.6 Selection of fermentation process variables for screening

Identification of critical variables by risk assessment is an important criterion for the implementation of quality by design principle in biotechnology products [12]. Preliminary experimental trials by OFAT approach were executed while considering the above study protocol (Table 2). The results obtained were then analysed to select the best media composition for maximum yield and potency of the antifungal metabolite. Since fermentation is a complex process, many of the process parameters are needed to be fine-tuned so that the best yield and desired bioactivity is consistently assured [13]. Many a time, OFAT

approach is exaggerated by labor, time, cost and likely to result in unpredictable outcomes. Use of DoE approach caters to plan the experiments systematically so that all the variables' behavior and their impact on the desired response are studied and thorough process understanding is attained.

2.6.1 Media composition

Media ingredients like soyabean meal, glucose, glycerol, NaCl and CaCO₃ are essential nutrients for the cultivation and growth of microorganisms. Any change in media composition (i.e. inclusion or exclusion of components including their concentration) can affect the productivity. Apart from this, a controlled source of carbon [14], nitrogen [15], salt [16], CaCO₃ [17] is vital for cell growth and production of secondary metabolites.

Table 2 Experimental protocol for studying variability in metabolite production

Set of experiments	Fermentation condition				
	Media composition	pH	Incubation period (days)	Sea water level (% v/v)	Level of inoculums (% v/v)
1	Changing (PM 1–PM 7)	7.0	7	50	5
2	PM 7	Changing (2.0–10.0)	7	50	5
3	PM 7	7.0	Changing (3–11)	50	5
4	PM 7	7.0	7	Changing (20–100)	5
5	PM 7	7.0	7	50	Changing (2–10)

2.6.2 Sea water

The mangroves are located at the interface between land and sea of tropical and subtropical regions of the world [18]. Thus the inclusion of sea water in the composition of production medium plays a pivotal role in productivity.

2.6.3 pH of the media

The growth of actinomycetes and production of bioactive metabolites are highly pH-sensitive. Shifting the pH may trigger cell growth and antibiotic activity adversely. Hence, it is essential to maintain an optimal media pH that would provide a favorable environment for adequate biomass and potency [19].

2.6.4 Level of inoculum

The level of inoculum in the production medium also influences the antibiotic yield. Too little inoculum level may reduce the growth as well as product formation whereas high inoculum level also may reduce product formation due to reduction of dissolved oxygen, depletion of nutrients and accumulation of toxic substances [20].

2.6.5 Incubation period

Unlike primary metabolites, secondary metabolites are produced during the stationary phase of microbial growth and need more incubation time for sufficient yield [21]. However, improving the metabolite productivity with a short fermentation time is of great challenge in current biotechnology.

2.7 Implementing DoE into the fermentation bioprocess

Keeping the benefits of DoE in mind, the approach was implemented during the fermentation bioprocess with two desired objectives, i.e. maximum yield and maximum antibiotic activity. This included mathematical modeling of all the process variables so that a global optimal condition is established, where the key objectives were achieved. Moreover, DoE enables us to ascertain a statistical relationship between the process variables and their interactions with the desired responses. 'Design Expert (Version 9.0 and 12)' software, [Stat-Ease Inc., Minneapolis, USA] was used for the implementation of DoE in the fermentation process.

2.7.1 Screening of bioprocess variables

Prior to process optimization, a screening design i.e. Plackett–Burman design (PBD) [22] was employed to screen all the process variables and study their magnitude of impact on the responses. The main purpose of this screening study is to minimise the number of variables based on their criticality (impact severity) on the process performance. By this, variables with minimal to negligible effect can be skipped off during the subsequent process optimization with minimum experimental trials. A total of 9 variables (incubation period, pH, inoculum level, % sea water, concentrations of soyabean meal, glucose, glycerol, NaCl, and CaCO_3) were investigated each at two levels, high (+ 1) and low (– 1) as presented in Table 3. Additionally, 2 dummy variables (included in the spare column) are studied to find an experimental error if any. 12 experiments (Table 4) were executed as per the PBD in triplicate for 11 variables, and the mean zone of inhibition (U/ml) of each run was recorded. Subsequently, the responses obtained were subjected to analysis of variance (ANOVA) for determining the impact (effect) of each process variable.

2.7.2 Process optimization with the aid of Box-Behnken design

From the screening study, three critical variables were selected that contribute significant influence on the response. These significant process variables are considered for further optimization in order to establish a control strategy of the entire bioprocess for maximum antibiotic production and activity. Although non-critical variables do not exhibit potential influence, they cannot be completely excluded from the fermentation process. They are essential components for antifungal metabolite production and

Table 3 Variables of the fermentation bioprocess and their levels for screening by Plackett–Burman design

Factor	Coded levels	
	– 1 (Low level)	+ 1 (High level)
A: pH	6	8
B: Incubation Period (days)	5	7
C: % Sea water (v/v)	20	50
D: Inoculum level (%v/v)	4	9
E: Dummy1	–	–
F: Soyabean meal (gm)	1	2
G: Glucose (gm)	1	2
H: Glycerol (ml)	0	0.5
J: Sodium chloride (gm)	0	0.5
K: Dummy2	–	–
L: Calcium carbonate (gm)	0	0.1

Table 4 Design matrix for conducting the experiments and their responses during screening of process variables by Plackett-Burmann design

Process variables (coded levels)												Activity against <i>C. albicans</i> (U/mL) ^a
Run	A	B	C	D	E	F	G	H	J	K	L	
1	1	1	-1	-1	-1	1	-1	1	1	-1	1	640±5.6
2	1	-1	1	1	1	-1	-1	-1	1	-1	1	590±5.0
3	-1	1	1	1	-1	-1	-1	1	-1	1	1	660±5.6
4	1	-1	1	1	-1	1	1	1	-1	-1	-1	550±3.0
5	1	1	-1	1	1	1	-1	-1	-1	1	-1	600±2.0
6	-1	1	-1	1	1	-1	1	1	1	-1	-1	550±1.0
7	-1	-1	1	-1	1	1	-1	1	1	1	-1	550±1.0
8	-1	1	1	-1	1	1	1	1	-1	-1	1	610±3.0
9	1	-1	-1	-1	1	-1	1	1	-1	1	1	570±0.4
10	1	1	1	-1	-1	-1	1	-1	1	1	-1	560±1.0
11	-1	-1	-1	1	-1	1	1	-1	1	1	1	580±2.0
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	550±1.0

^a1 unit = 1.0 mm annular clearing around the cup/well [11]

may be kept constant (fixed at a defined level) while conducting experiments during the optimization study.

An RSM-based Box-Behnken design (BBD) studying 3 significant process variables at three levels low (-1), medium (0) and high (+1) was employed for execution of 17 experiments. The responses (inhibition zone diameter) were then subjected to statistical comparison by ANOVA, and a regression polynomial equation was generated (Eq. 1). The developed polynomial model enables us to predict the statistical significance of the variables' influences being studied including their interaction and quadratic terms.

$$Y = b_0 \pm b_1X_1 \pm b_2X_2 \pm b_3X_3 \pm b_{12}X_1X_2 \pm b_{13}X_1X_3 \pm b_{23}X_2X_3 \pm b_{11}X_1^2 \pm b_{22}X_2^2 \pm b_{33}X_3^2 \quad (1)$$

Where, Y = response; b_0 = intercept (average quantitative results of all the experiments); b_1 to b_{33} are regression coefficients (magnitude of variables' effect on Y); X_1 , X_2 and X_3 are the bioprocess variables selected from PBD and the terms X_1X_2 , X_1X_3 , and X_2X_3 represents the interaction of variables; X_i^2 ($i = 1-3$) represents the quadratic terms; the symbol " $\pm$ " indicates the type of variable's effect i.e., (+) synergistic or (-) antagonistic.

Further, the behavior of the variables' on the desired response was studied graphically by the use of perturbation plot and 3D response surface plots. Perturbation plots are constructed for visual presentation of individual process variable's contribution to the studied response, while keeping others constant at nominal (0) level. In perturbation, the steepest plot belongs to the most significant variable under investigation. 3D response surface plots enable us to visualize the real behavior of a process variable in presence of another variable at different levels (quantitative or qualitative) [23, 24]. In other way, we can study how

the response is being influenced due to the interaction of any two variables, while keeping the other constant.

2.8 Validation of the mathematical model and optimization

In the final stage, the mathematical model was subjected to validation by determination of percentage prediction error (PE). The most suggested solutions by the model were experimented, each in triplicate, and the observed mean response value was computed against the predicted value to find the PE (Eq. 2). The solution with least percentage of PE presents the optimal conditions to achieve the best process performance.

$$PE = \frac{\text{Observed} - \text{Predicted}}{\text{Predicted}} \times 100 \quad (2)$$

3 Results and discussion

3.1 Variables affecting fermentation bioprocess performance

3.1.1 Screening of production media for the antibiotic production

During the preliminary screening by OFAT approach, all the bioprocess variables were studied to determine their magnitude of influence (at various levels) on metabolite production and activity as illustrated in Fig. 1. Selection of a suitable production medium is essential prerequisite for microbial culture and bioactive metabolites production. Figure 1a depicts that PM-7 is the best production medium for maximum yield of anti-Candida metabolite

Fig. 1 Effect of **a** Production media, **b** Incubation period, **c** pH, **d** Seawater and **e** Inoculum level on antifungal antibiotic production

from actinomycete (KMF-A1). The antifungal activity was improved by increasing the incubation period up to a maximum of 540 U/ml after 7 days. Further increase in incubation period for more than 7 days led to decrease the bioactivity (Fig. 1b). As per Fig. 1c, maximum antifungal potency has obtained when the strain KMF-A1 was cultivated in the selected medium adjusted to neutral pH (7.0). The influence of sea water level on the production of antibiotic yield was estimated to be maximum (520U/ml) at 50% (Fig. 1d). The inoculum level for the maximum production of antifungal metabolite is found to be 5–10% (Fig. 1e).

3.2 DoE-aided fermentation process optimization

In order to accomplish the best process performance, all the possible variables were studied for their contribution

towards biomass production and antifungal activity. The responses obtained from the PBD (Table 4) were computed for variance analysis and the effect of individual variable on the response of interest was estimated. A Pareto chart (Fig. 2) was plotted to visualize the magnitude of each variable's impact on the studied antifungal activity. During the statistical analysis, it was revealed that the model fits well for the test of significance as per the calculated P (0.0002) and F (25.14) values. In this case, only three variables i.e. incubation period (B), glucose (G) and CaCO_3 (L) were found to be significant model terms with t -value limit > 2.306 . The incubation period and CaCO_3 had shown synergistic effect, while glucose was shown antagonistic effect on antifungal metabolite production. It was clearly evidenced that the quantity of CaCO_3 in the production medium exhibits the highest magnitude of effect on the response. The resulted R^2 (0.9041) value defines that the three parameters contribute

Fig. 2 Pareto chart for visual comparison of variables' contribution towards antifungal activity

significant variability in the response [25]. The adjusted R^2 (0.8681) and predicted R^2 (0.7842) values are in good agreement with each other. A higher adeq precision (14.711) value indicates an adequate signal to noise ratio and a lower coefficient of variance (2.32%) signifies a greater reliability of the experimental performance of the model.

From the PBD results, the significant variables: CaCO_3 , incubation period and glucose were selected for further optimization by BBD in order to improve the bioactive metabolite production. Variations in the three variables: ΔCaCO_3 ($0.05 \pm 0.05\%$), Δ incubation period (6 ± 1 days) and Δ glucose ($1 \pm 1\%$) were studied by BBD for 17 experiments. Table 5, depicts the observed responses obtained for each combination of the quantitative variables under study along with the predicted values. It can be noted that antifungal metabolite production values varied over a range from 580 to 760 U/mL against *C. albicans* depending on the selected variable concentration. The experimental Run-9 (glucose 1% and CaCO_3 0.1% and incubation period of 5 days) offered the highest antifungal activity (760 U/mL), while the Run-6 (glucose 2%, CaCO_3 0% and incubation period of 6 days) resulted the lowest (580 U/mL).

3.2.1 ANOVA analysis for determination of variables' effect on the response

The adequacy of the model was investigated by multiple regression analyses of the observed antifungal activity

Table 5 Box–Behnken design for the study of three process variables for the antibiotic production by the isolate KMF-A1

Run	Incubation period in (Days)	Glucose (%)	CaCO_3 (%)	Antifungal activity (U/mL)	
				Observed	Predicted
1	6	1	0.05	680	675.647
2	7	1	0	640	643.897
3	6	0	0	666	686.147
4	6	2	0.1	740	738.147
5	5	0	0.05	700	696.397
6	6	2	0	580	586.647
7	6	1	0.05	680	675.647
8	6	0	0.1	680	691.647
9	5	1	0.1	760	757.397
10	6	1	0.05	680	675.647
11	7	0	0.05	700	681.397
12	5	2	0.05	680	689.897
13	6	1	0.05	680	675.647
14	5	1	0	640	628.897
15	7	1	0.1	660	672.397
16	7	2	0.05	640	634.897
17	6	1	0.05	680	675.647

so that the effects of individual variables along with their interactions can be estimated. A second-order regression model (Eq. 3) was constructed to express the behavior of

the three process variables and their interactions for the metabolite production and activity. The (–) sign prefix to the coefficient value revealed that the incubation period and glucose exert an antagonistic effect, while the (+) sign indicates a synergistic effect by CaCO_3 . Where, the coefficient value prefix to individual variable denotes the magnitude of its effect. It is clear to mention here that CaCO_3 exhibited the highest magnitude of effect on the antifungal activity.

$$\text{Antifungal activity} = 675.65 - 17.50A - 13.25B + 39.25C - 10.00AB - 25.00AC + 36.50BC \quad (3)$$

where, A , B and C were incubation period, glucose and CaCO_3 respectively.

The significance of the model is expressed by the F and P values obtained from the ANOVA table (Table 6) [26, 27]. The model is considered to be significant where we got a larger value of F (27.75) and a smaller value of P (< 0.0001). The coefficient of determination (R^2) of 0.9433 indicates that the statistical model fits well for better prediction of response variability. A reasonable agreement between the predicted R^2 (0.7236) and the adjusted R^2 (0.9094) was obtained. A relatively low value of CV (2.44%) expressed that the method is precise and reliable and a higher adeq precision (21.978) value indicates an adequate signal to noise ratio [28].

Diagnostic plots were constructed to investigate the adequacy of the model and thereby clarifying any sign of experimental problems. The data were statistically analyzed, and the correlation between observed and predicted responses was drawn (Fig. 3a). All the data points were observed to be distributed closely to a straight line ($R^2 = 0.9433$) concluding an excellent correlation between the predicted and experimental values of the response. Normal probability plots for studentized residuals exhibited a linear pattern (Fig. 3b) indicating their normality behavior. The (Fig. 3c) depicted that the

studentized residuals were randomly scattered against the predicted values showing a constant variance of the original observation. Perturbation plot (Fig. 3 d) represents the influence of process variables on the response studied. The steepest factorial plot; as in this case- C (CaCO_3), demonstrates to exhibit significant effect on the response.

3.2.2 3D response surface plots

The three-dimensional response surface plots as drawn in Fig. 4 depicted that the estimated influence of all the variables were significantly linear with no curvature. From this, we can conclude that all the factors have the sole contribution towards antifungal metabolite production and activity. Figure 4a revealed that the interaction between glucose and incubation period has a negligible effect on the response. Therefore, increasing or decreasing their levels could not result in any variation in the response. From Fig. 4b, it is clearly noticed that a significant rise in antifungal activity achieved with increasing the level of CaCO_3 , while the influence is little with the incubation period. Figure 4c depicts the influence of interaction between CaCO_3 and glucose, where the response varies in a linearly ascending order with increasing level of CaCO_3 and linearly descending order with increasing concentration of glucose. The overall analysis of 3D response surface plots indicates that the antifungal activity increases with increase in CaCO_3 and decrease in glucose concentration. Hence, it can be concluded that CaCO_3 and glucose significantly contribute to maximum productivity of bioactive metabolites and thereby improving the antifungal potency.

Table 6 Analysis of Variance for the BBD model for antifungal metabolite production by the isolate KMF-A1 against *C. albicans*

Source	Sum of Squares	df	Mean Square	F value	p-value Prob > F	Remarks
Model	24,408.00	6	4068.00	27.75	< 0.0001	Significant
A Incubation Period	2450.00	1	2450.00	16.71	0.0022	
B-glucose	1404.50	1	1404.50	9.58	0.0113	
C-calcium carbonate	12,324.50	1	12,324.50	84.08	< 0.0001	
AB	400.00	1	400.00	2.73	0.1296	
AC	2500.00	1	2500.00	17.05	0.0020	
BC	5329.00	1	5329.00	36.35	0.0001	
Residual	1465.88	10	146.59			
Lack of Fit	1465.88	6	244.31			
Pure Error	0.000	4	0.000			
Cor Total	25,873.88	16	4068.00

Fig. 3 Residual diagnostic plots showing **a** Observed versus predicted, **b** Normal % probability plot of the studentized residuals, **c** Externally studentized residuals versus Predicted and **d** Perturbation plot

3.3 Numerical validation of the optimization process

The optimization process was validated by assessing the predictability of the model mathematically [29]. Experiments for eight check point solutions from the model were executed, and PE was calculated to verify that the observed response values are in close agreement with the predicted values (Table 7). A good correlation between the predicted and observed values confirms the existence of an optimal point and the validity of the RSM model. A response with least PE was concluded as the best desired response. The results demonstrated that the condition of solution-1 with least PE value of 0.09413 is considered as the optimal to obtain the desired antifungal activity (759.1 U/mL). Therefore, the optimal condition of the fermentation process for maximum yield and antifungal potency consists of soyabean meal (1.5%), glucose (1.030%), glycerol (0.25%), NaCl (0.5%), CaCO_3 (0.1%), sea water (50%), pH of the medium

adjusted to 7.0, inoculums level (10%) and incubation period of 5 days.

The summary of the study revealed that the rapidity of secondary metabolite biosynthesis is directly related to the incubation time essential for the inoculum to reach the idiophase. An incubation period of 5 days is reasonably good for maximum yield and desired activity. From the literature, it was evidenced that at molecular level the carbon source may influence the catabolite repression or prevents transcriptional activation of genes that are responsible for antibiotic production. As an essential source of carbon and energy, glucose has been reported to augment biosynthesis of secondary metabolites [16, 30]. However, studies also available to reveal that such rapidly metabolized carbon source have markedly suppressed the production of antibiotics such as neomycin, bacitracin etc.[31–33]. Glucose has a negative influence on the production of nystatin by *S. noursei*, whereas glycerol improved productivity [33]. In our case too, we found that a decrease

Fig. 4 Response surface plots showing the interaction effects of **a** Incubation period and glucose, **b** Calcium carbonate and incubation period, **c** Glucose and calcium carbonate for the antifungal metabolite production by the isolate KMF-A1

Table 7 Numerical optimization and assessment of predictability

Solution	Incubation period (days)	Glucose (%)	CaCO ₃ (%)	Antifungal activity (U/mL)		PE (%)
				Predicted value	Observed value	
1*	5.000	1.030	0.100	758.387	759.1	0.094132413
2	6.081	2.000	0.100	733.916	755.1	2.934610282
3	5.023	1.286	0.100	765.526	717.2	6.30751213
4	5.307	1.621	0.098	760.236	760.2	5.892670422
5	5.393	1.967	0.097	763.040	732.3	4.02456971
6	5.104	1.333	0.098	760.076	765.7	0.748898822
7	5.215	1.575	0.097	761.688	740.2	2.828039137
8	5.491	0.884	0.100	733.243	725.9	5.48709353

in glucose concentration in the production medium enhanced the antifungal activity. CaCO₃ had the most noteworthy effect on antifungal antibiotic production. It is determined to have synergistic effect on the yield

of antibiotic production. Calcium plays an important role in the activation of several enzymes involved in the secondary metabolism that directs the biosynthesis of secondary metabolites [17, 34].

4 Conclusion

A fermentation process for culture of *S. hydrogenans* KMF-A1 isolated from mangrove soil for production of antifungal metabolites was statistically optimized with the aid of DoE. The optimal bioprocess condition presented the most promising antifungal activity against *C. albicans*. Application of DoE in the optimization suggested the three major process variables (incubation period, glucose and CaCO_3) to be significant and require sensible monitoring during the fermentation. The DOE approach resulted in 1.8 fold increase in antifungal metabolite production at optimal conditions consisting of soyabean meal (1.5%), glucose (1.030%), glycerol (0.25%), NaCl (0.5%), CaCO_3 (0.1%), sea water (50%), pH of the medium adjusted to 7.0, inoculums level (10%) and incubation period of 5 days. The process is rapid and robust to improve the productivity and potency.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

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