Research Article

Optimization of Endophytic Fungi *Curvularia aeria* MTCC-12847 isolated from *Tribulus terrestris* L. by using RSM Technology

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ABSTRACT

A bioactive strain *Curvularia aeria* MTCC-12847 was selected for the production of secondary metabolites by optimization. For the improved production of secondary metabolites, central composite design of response surface methodology was applied in submerged fermentation. The parameters like pH, temperature, Incubation days, were optimized for the better production of secondary metabolites. Mycelial weights were weighed and the ZOI (Zone of inhibition) was calculated against gram negative bacteria (*E.coli*, MTCC-443). Hence, the optimized media produces, ZOI of 19 mm in the Temperature: 27.5, pH-6, Incubation day: 9 day and Dry mycelial weight was found to be 250 mg. Hence we concluded that optimized media produces better metabolites.

Keywords: RSM, optimization, endophytic fungi.

INTRODUCTION

Endophytes, by definition, are one which resides in the tissue beneath the epidermal cell layer and causes no apparent harm to the host [1]. Endophytic fungi are one of the most creative groups of secondary metabolite producers and represents rich biodiversity [2]. Microbial secondary metabolites have provided numerous pharmaceutical agents ranging from antibiotics to immunosuppressive compounds. Synthesis of these low molecular weight compounds is not required for normal growth of the microbe; however these compounds may provide several benefits to the organism. Fungi have the ability to produce a plethora of secondary metabolites, typically dependent on the stage of development and environmental factors ranging from nutrient concentrations to light and temperature [3-4].

Response surface methodology (RSM) is one of the most useful statistical optimization tools in biological and chemical process [5]. Response surface methodology is very beneficial tool to optimize numerous parameters of trails, to find relativeness among the factors, to find the best combination of parameters and prediction of responses. For the optimization of microbial products this method has been extensively used [6-7]. RSM is a collection of statistical and mathematical techniques useful for designing experiments, developing models and evaluating the effects of variables in which a response of interest is influenced by several variables and the objective is to optimize this response [8]. For the optimization of important fermentation parameters several types of designs are accessible, in optimization process central composite design (CCD) is one of the most useful designs [9]. RSM also provides an experimental model that predicts the correlation and interaction between a set of experimental variables and observed results, and subsequently provides optimized conditions [10].

In current study an effort has been made to produce more secondary metabolite by endophytic fungi EF1 i.e. *Curvularia aeria* MTCC 12847 using the central composite design of response surface methodology to improve the activity by optimizing various parameters in submerged fermentation.

MATERIALS AND METHODS

Isolation of endophytic fungi

The samples were washed thoroughly in running tap water before processing. Leaf samples were surface sterilized by dipping in 70% ethanol (v/v) for 1 min and 3.5% NaOCl (v/v) for 3 min, rinsed thrice with sterile water and dried. Bits of 1.0X1.0 cm size were excised with the help of a sterile blade. Two hundred segments of *Tribulus terrestris* L. leaf segments were placed on the water agar (16%) (WA) medium supplemented with Streptomycin (100 mg/l; Sigma, St. Louis, MO, USA) were used for the isolation of endophytic fungi. The Petri dishes were sealed using parafilm and The Petri dishes were incubated at 25°C-27°C till the mycelia start growing from the samples [11].

Optimization of endophytic fungi (Design of experiments; DOE)

The endophytic fungus EF1 i.e. *Curvularia aeria* MTCC 12847 was cultured in Erlenmeyer flasks containing 500 ml of optimized culture media (PDB). Response surface method: Central Composite Design (CCD) model was used to study the effect of interaction between temperature (A) in the range between 15°C to 60°C; pH (B) in the range between 3 to 11; Mycelial weight (C) 25mg to 250mg; Incubation days (D) in the range between 3 to 15 days for maximum secondary metabolite production and the response was calculated by Zone of inhibition against Gram negative bacteria (*E.coli*).

Experimental designs were performed using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, ver. 11.0.0). A total of 57 runs were employed in CCD to estimate curvature and interaction effects of selected variables, and finally, significance of the obtained model was checked by F-test (calculated p-value) and goodness of fit by multiple correlation R as well as determination R^2 coefficients. Zone of inhibition (mm) was the measured experimental response.

Statistical Analysis: Analysis of variance (ANOVA) was used to estimate the statistical parameters for optimization of culture conditions. A probability value of P value <0.05 was used as the criterion for statistical significance.

RESULTS

For the optimization, Response surface methodology was used. After running the CCD (Table 1), the experimental results were statistically analyzed using analysis of variance; ANOVA (Table 2). The Model F-value of 174.21 implies the model were significant and there was only a 0.01% chance that a ‘‘Model F-Value” this large could occur due to noise. The model showed that A, B, AB, AC, A^2, B^2, C^2 were significant model terms.

The ‘‘Predicted R-Squared” of 0.9690 which was in reasonable agreement with the ‘‘Adjusted R-Squared” of 0.9774. The adequate Precision – measures the signal to noise ratio and a ratio greater than 4 was desirable – equals to 38.401. The R^2 value 0.9831 which indicates that the model was reliable (Table 3). Accordingly, this model can be used to navigate the ZOI design space. The interactions between factors (Fig.1-6) and the actual and predicted results of the model runs (Table 4) were based on the final equation of the model shown below. The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. The model in terms of actual variables for the predicted response was based on following equation:

ZOI against *E.coli* (mm) for EF1 = + 2.25522 + 2.10264 * Temperature - 4.55518* pH - 0.030984* Mycelial weight - 2.09537* Incubation days - 0.266008* Temperature * PH - 0.000855* Temperature * Mycelial weight - 0.022776*Temperature * Incubation days - 0.005890* PH * Mycelial weight + 0.324269 * PH * Incubation days + 0.005491* Mycelial weight * Incubation days + 0.001380* Temperature² + 0.835395 * PH² + 0.000275 * Mycelial weight² - 0.013412* Incubation days²

Table 1: Central composite design (CCD) used for optimization of secondary metabolite production (organized in triplicate runs) in case of EF1

Run numbers	Temperature(°C)	pH	Mycelial weight(mg)	Incubation days(days)	ZOI against *E.coli*
1	15	3	40	6	6
2	15	3	40	6	6
3	15	3	40	6	6
4	17.5	4	60	6	7
5	17.5	4	60	6	7
6	17.5	4	60	6	7
7	20	5	80	6	8
8	20	5	80	6	8
9	20	5	80	6	8
10	22.5	5	108	6	11.5
11	22.5	5	108	6	11.5
12	22.5	5	108	6	11.5
13	25	5	190	6	13.5
14	25	5	190	6	13.5
15	25	5	190	6	13.5
16	**27.5**	**6**	**250**	**9**	**19**
17	27.5	6	249	9	19
18	27.5	6	250	9	19
19	30	6	240	9	18.5
20	30	6	240	9	18.5
21	30	6	240	9	18.5
22	32.5	6	230	9	18
23	32.5	6	230	9	18
24	32.5	6	230	9	18
25	35	6	199	9	17
26	35	6	199	9	17

27	35	6	199	9	17
28	37.5	7	190	9	15
29	37.5	7	190	9	15
30	37.5	7	190	9	15
31	40	7	175	12	13
32	40	7	175	12	13
33	40	7	175	12	13
34	42.5	7	150	12	12.5
35	42.5	7	150	12	12.5
36	42.5	7	150	12	12.5
37	45	8	100	12	11
38	45	8	100	12	11
39	45	8	100	12	11
40	47.5	8	90	12	9
41	47.5	8	90	12	9
42	47.5	8	90	12	9
43	50	9	80	12	8
44	50	9	80	12	8
45	50	9	80	12	8
46	52.5	9	70	15	7.5
47	52.5	9	70	15	7.5
48	52.5	9	70	15	7.5
49	55	10	59	3	7
50	55	10	59	3	7
51	55	10	59	3	7
52	57.5	10	55	6	6.5
53	57.5	10	55	6	6.5
54	57.5	10	55	6	6.5
55	60	11	32	9	6
56	60	11	31	9	6
57	60	11	32	9	6

Table 2: ANOVA for Quadratic model **for ZOI against *E.coli* (response) in case of EF1**

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	1088.80	14	77.77	174.21	< 0.0001	Significant
A-Temperature	0.0120	1	0.0120	0.0269	0.8705	
B-PH	0.8941	1	0.8941	2.00	0.1644	
C-Mycelial weight	2.36	1	2.36	5.28	0.0266	
D-Incubation days	0.1055	1	0.1055	0.2362	0.6295	
AB	0.4101	1	0.4101	0.9186	0.3433	
AC	0.1683	1	0.1683	0.3770	0.5425	
AD	0.0877	1	0.0877	0.1964	0.6599	
BC	0.4345	1	0.4345	0.9733	0.3295	
BD	0.3998	1	0.3998	0.8955	0.3494	
CD	0.2994	1	0.2994	0.6707	0.4174	
A^2	0.0019	1	0.0019	0.0042	0.9483	
B^2	0.4254	1	0.4254	0.9529	0.3346	
C^2	3.18	1	3.18	7.12	0.0108	
D^2	0.2033	1	0.2033	0.4555	0.5034	
Residual	18.75	42	0.4464			
Lack of Fit	18.75	6	3.12			
Pure Error	0.0000	36	0.0000			
Cor Total	1107.55	56				

The Model F-value of 174.21 implies the model was significant. There was only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms were significant.

Table 3: Fit Statistics for EF1 for response against *E.coli*

Std. Dev.	0.6681	R^2	0.9831
Mean	11.26	**Adjusted R^2**	0.9774
C.V. %	5.93	**Predicted R^2**	0.9690
		Adeq Precision	38.4011

The **Predicted R^2** of 0.9690 was in reasonable agreement with the **Adjusted R^2** of 0.9774; i.e. the difference was less than 0.2. **Adeq Precision** measures the signal to noise ratio. A ratio greater than 4 was desirable. Our model ratio of 38.401 indicates an adequate signal. Hence, this model can be used to navigate the design space.

Table 4: Central composite design for runs with actual and predicted response values for EF1

Run Number	ZOI against *E.coli*		
	Actual value	Predicted value	Residuals
1	6.00	6.02	-0.0180
2	6.00	6.02	-0.0180
3	6.00	6.02	-0.0180
4	7.00	7.13	-0.1301
5	7.00	7.13	-0.1301
6	7.00	7.13	-0.1301
7	8.00	8.50	-0.4990
8	8.00	8.50	-0.4990
9	8.00	8.50	-0.4990
10	11.50	10.20	1.30
11	11.50	10.20	1.30
12	11.50	10.20	1.30
13	13.50	14.43	-0.9328
14	13.50	14.43	-0.9328
15	13.50	14.43	-0.9328
16	19.00	18.97	0.0301
17	19.00	18.87	0.1268
18	19.00	18.97	0.0301
19	18.50	18.47	0.0332
20	18.50	18.47	0.0332
21	18.50	18.47	0.0332
22	18.00	18.08	-0.0787
23	18.00	18.08	-0.0787
24	18.00	18.08	-0.0787
25	17.00	16.37	0.6288
26	17.00	16.37	0.6288
27	17.00	16.37	0.6288
28	15.00	14.56	0.4425
29	15.00	14.56	0.4425
30	15.00	14.56	0.4425
31	13.00	13.69	-0.6863
32	13.00	13.69	-0.6863
33	13.00	13.69	-0.6863
34	12.50	12.35	0.1509
35	12.50	12.35	0.1509
36	12.50	12.35	0.1509
37	11.00	10.36	0.6380
38	11.00	10.36	0.6380
39	11.00	10.36	0.6380
40	9.00	9.73	-0.7269
41	9.00	9.73	-0.7269
42	9.00	9.73	-0.7269
43	8.00	8.97	-0.9729
44	8.00	8.97	-0.9729
45	8.00	8.97	-0.9729
46	7.50	6.91	0.5897
47	7.50	6.91	0.5897
48	7.50	6.91	0.5897
49	7.00	7.17	-0.1720
50	7.00	7.17	-0.1720
51	7.00	7.17	-0.1720
52	6.50	6.29	0.2084
53	6.50	6.29	0.2084
54	6.50	6.29	0.2084
55	6.00	5.81	0.1920
56	6.00	5.89	0.1116
57	6.00	5.81	0.1920

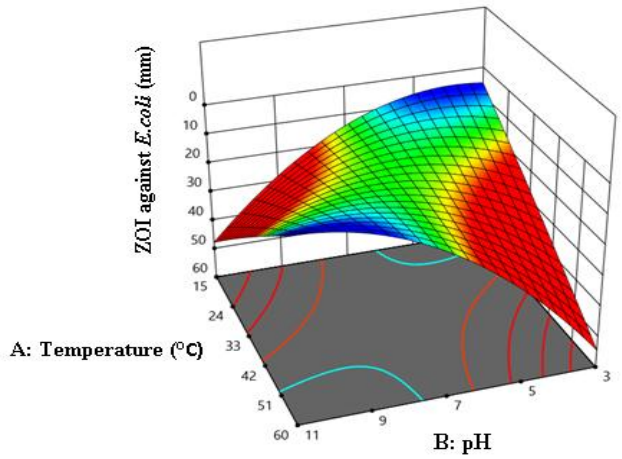

Figure 1: 3D plot of the effect of Temperature and pH on ZOI against *E.coli* by EF1 under Submerged fermentation

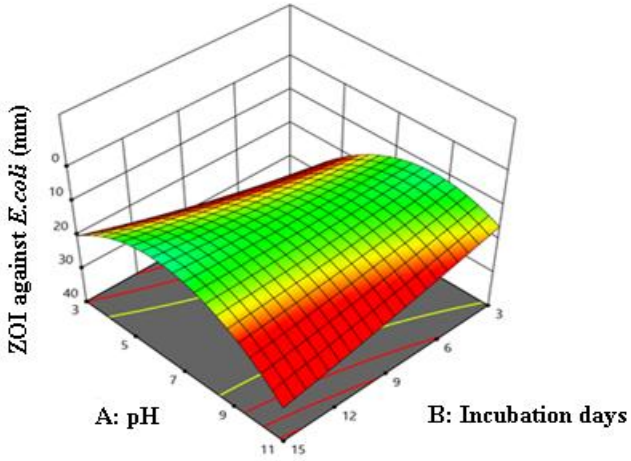

Figure 2: 3D plot of the effect of pH and Incubation days on ZOI against *E.coli* by EF1 under Submerged fermentation

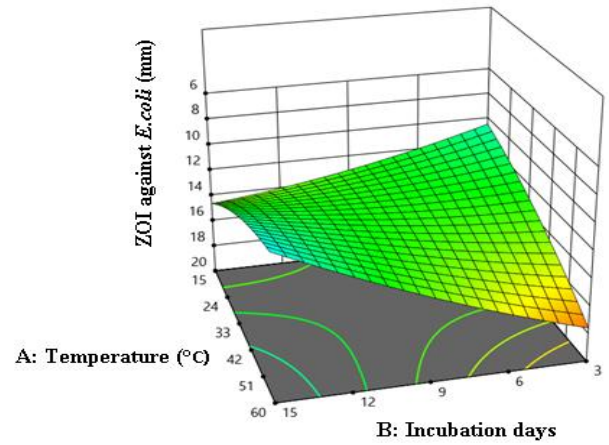

Figure 3: 3D plot of the effect of Temperature and Incubation days on ZOI against E.coli by EF1 under Submerged fermentation

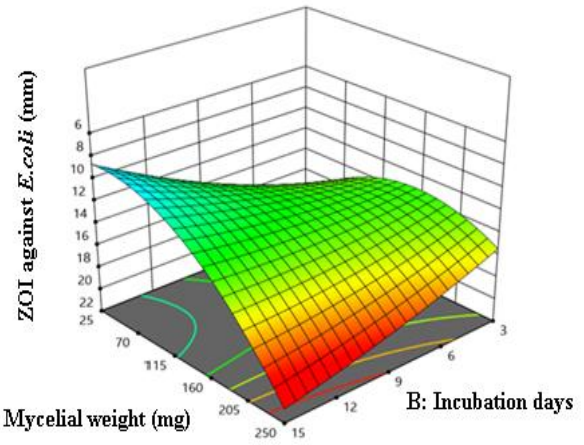

Figure 4: 3D plot of the effect of Mycelial weight and Incubation days on ZOI against E.coli by EF1 under Submerged fermentation

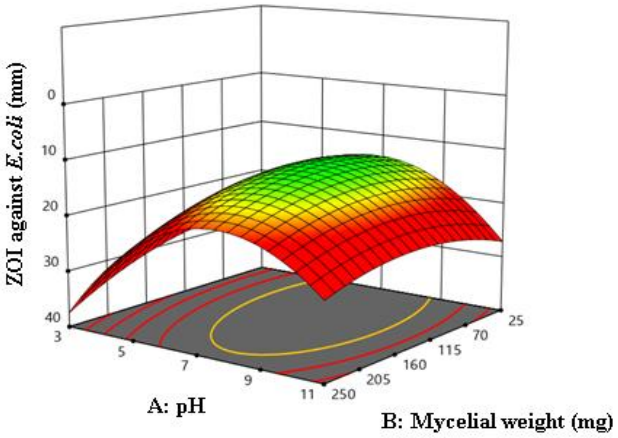

Figure 5: 3D plot of the effect of pH and Mycelial weight on ZOI against E.coli by EF1 under Submerged fermentation

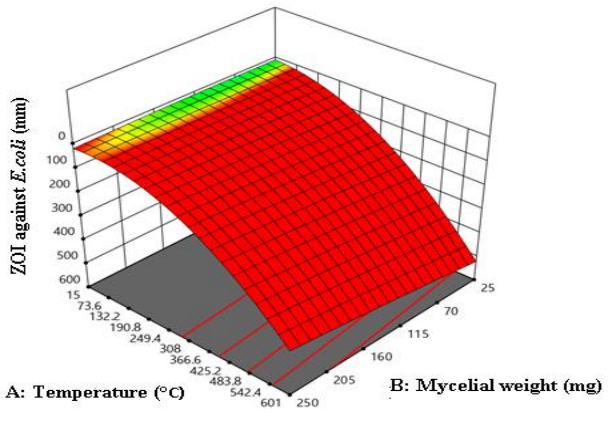

Figure 6: 3D plot of the effect of Temperature and Mycelial weight on ZOI against E.coli by EF1 under Submerged fermentation

DISCUSSION

In several, metabolite biosynthesis in microbes are tightly controlled by regulatory mechanisms to avoid over production; yet, these regulatory mechanisms often sometimes process to undesirably low levels. The yield of bioactive compounds can sometimes be substantially increased by the optimization of physical (temperature, salinity, pH and light) and chemical factors (media components, precursors, and inhibitors) for the growth of microbes [12-13]. Nowadays, before going to microbial fermentation, RSM-based optimization is widely accepted due to its higher efficiency, reasonable design for integrated analysis of variants, and mathematical modelling with recommended values of most possible optimum conditions, along with corresponding product yield. The approach is techno-economically more precise and viable than univariate strategies, and more acceptable industrially [14].

CONCLUSION

In this study, statistically based experimental designs were proven to be valuable tools for optimizing medium to produce Secondary metabolites. The predicted values were in excellent agreement with the experimental values in validation experiments, which confirmed the accuracy of the model. Hence, RSM can be used for the optimization of Culture condition for the better production of secondary metabolites.

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