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RESEARCH PAPER

Enhancement of Taxol Production from Endophytic Fungus *Fusarium redolens*

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Abstract The optimization of taxol production by *Fusarium redolens* by one factor at a time (OFAT) approach led to production of 70 μg/L of taxol. With sucrose and NH_4NO_3 as the carbon and nitrogen sources and medium volume (V_m) to flask volume (V_f) ratio of 0.2, a greater taxol production was attained. NH_4NO_3, $MgSO_4 \cdot 7H_2O$ and NaOAc at 6.25, 0.63, and 1.25 g/L, were the significant factors for attaining the highest taxol production. The optimization of culture variables led to the production of taxol from 66 to 198 μg/L, which is three fold higher than that in the unoptimized medium. Current study results suggested the success of Response Surface Methodology in enhancing the production of fungal taxol.

Keywords: taxol, *Fusarium redolens*, Plackett-Burman design, response surface methodology

1. Introduction

Taxol is a diterpenoid with anti-tumor activities, firstly isolated from the bark of *Taxus brevifolia* [1]. The mechanism of action of taxol is to inhibit the depolymerisation of microtubulin, thus affecting the formation of spindle, prohibiting from mitosis of tumor cell [2]. Clinically, taxol has been used successfully worldwide for the treatment of many malignant tumours [3-7]. At present, taxol is mainly extracted from the bark of yews. However, this method cannot meet the increasing demand for taxol in the market because, yews are rare, grow very slowly and endangered species. The yield of pure drug extracted from the bark is low, contributing to its high price. Many researchers throughout the globe have reported endophytic fungi isolated from the bark of yews capable of producing taxol [8-12].

Generally, optimization of medium components is regarded as the most effective measure to improve fermentation productivity of secondary metabolites *i.e.,* designing an appropriate medium and investigating the most suitable conditions such as pH, temperature, incubation time, medium-to-flask volume ratio *etc*. This operation relates to several methods of statistical experimental design. The traditional method of optimization is based on one factor at a time (OFAT) approach. Plackett-Burman design and Response surface methodology are found to be most efficient tools for process optimization. Plackett-Burman (PB) design [13] is a class of saturated orthogonal fractional two-level factorial design which reduces the number of experimental trials as it screens the most important factors influencing the productivity. Response surface methodology (RSM) [14] is also a mathematical tool based on polynomial regression fitting, significance analysis and stationary point location which finally determines the optimum concentration of selected factors affecting the desired response. This statistical tool has been widely and successfully used in optimizing the critical factors affecting secondary metabolite production in different organisms and systems [15-18]. Present study was aimed to optimize the fermentation medium by PB and RSM for the enhanced production of taxol by *Fusarium redolens.*

Taxol production by *Fusarium redolens*, isolated from the inner bark of *Taxus baccata* has been tested for its anti-tumorigenic activity using potato disc tumor induction assay [19]. In the present investigation, after optimization of fermentation conditions and medium composition, yield of taxol increased from 66 to 198 μg/L which is three fold higher than that of unoptimized medium.

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2. Materials and Methods

2.1. Start strain

Fusarium redolens isolated from the bark of *T. baccata* sub sp. *wallichiana* growing in northern Indian Himalayan region was used in this study. The fungus was inoculated in S-7 basal liquid medium (pH 6.8) [9] and incubated at 28ºC for 3 weeks under stationary condition for determining the taxol production. The putative taxol extracted from the fermentation broth and mycelium was analysed and quantified by HPLC [20] and LC-MS. Briefly, HPLC was carried out by injecting 20 µL of purified sample to HPLC (Waters Acquity HPLC, USA) equipped with a reverse phase column (RP-18 column, Waters, USA) and detected with online DAD (diode array detector) detector set at wavelength of 232 nm. Elution was carried out in an isocratic mode with mobile phase methanol-acetonitrile-water (20:40:40 v/v) at a flow rate of 1 mL/min. Purified taxol from fungal samples was subjected to LC-MS (Waters, USA) using triple quadrupole tandem LC-MS. The LC portion was eluted in an isocratic mode using acetonitrile:water (49:51) as mobile phase. The samples in 100% methanol were infused into the mass spectrometer through a reverse phase C18 column separated at a flow rate of 0.3 mL/min with column temperature of 25°C and a spray voltage of 2.2 kV by the loop injection method. The MS scanning ranged from 100 to 1,000 m/z.

2.2. Estimation of radial growth and biomass

Mycelial disc of 5 mm cut from the edge of actively growing culture was inoculated at the centre of potato dextrose agar (PDA) in Petri plates. The plates were incubated at 28ºC for 10 days and the growth (diam.) was measured at daily intervals. To determine the biomass produced by this fungus, *F. redolens* was grown in S-7 liquid medium in 250 mL Erlenmeyer flasks and incubated at 28ºC. After 5, 10, 15, 20, 25, and 30 days of incubation, the mycelium was harvested and the fresh weight (FW) was recorded.

2.3. Optimization of medium-to-flask volume ratio (V_m/V_f ratio)

The effect of medium-to-flask volume ratio in the range of 0.01 ~ 0.03 was tested on mycelial growth and taxol production. The fungus was grown in 250 mL Erlenmeyer flasks with different volumes of S-7 medium corresponding to each volumetric ratio. Mycelium was harvested from the fermentation broth after 20 days. The fresh weight of the mycelium was recorded and taxol production was determined from culture filtrate and the mycelium.

2.4. Optimization of pH and temperature

The fungus was grown in 50 mL of S-7 liquid medium in 250 mL Erlenmeyer flasks at different pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5) and temperature (5, 15, 25, and 35ºC). After 20 days of incubation, the fresh weights of mycelium and taxol production were determined.

2.5. Experimental design

One-factor-at-a-time (OFAT) strategy was first used to determine the basic medium composition. Then, a two-level Plackett-Burman design was selected to analyse the significance of each factor. Finally, a Box-Behnken design was used to find the optimum values for the screened factors given by response prediction.

2.5.1. Effect of carbon and nitrogen sources on biomass and taxol production using OFAT

The fungus was grown in 250 mL Erlenmeyer flasks containing 50 mL of S-7 medium supplemented with different concentrations of carbon sources (4, 6, 8, 10, and 12%) such as sucrose, glucose and fructose. Similarly, liquid medium was supplemented with varying concentrations of nitrogen sources (0.4, 0.6, 0.8, and 1%) such as ammonium nitrate (NH_4NO_3), peptone and calcium nitrate [$Ca(NO_3)_2{\cdot}4H_2O$]. The cultures were incubated at 25ºC with a medium pH of 6.5 for 20 days. The fresh weight of the mycelium was recorded and taxol production was determined.

2.5.2. Plackett-Burman design (PB design)

The Plackett-Burman design was used to optimize the trace elements. PB design allows the investigation of up to N-1 variables with N experiments. This design helps to identify the most significant nutrients and their concentrations from a group for further optimization. In this investigation, eight media components (ferric chloride ($FeCl_3{\cdot}6H_2O$), potassium dihydrogen phosphate (KH_2PO_4), manganese chloride ($MnCl_2{\cdot}4H_2O$), magnesium sulphate ($MgSO_4{\cdot}7H_2O$), zinc sulphate ($ZnSO_4{\cdot}7H_2O$), sodium acetate (NaOAc), thiamine and phenylalanine) were evaluated. Trial experimental protocols were formulated using Design-Expert version 8.0.7.1 software (Stat-Ease Corporation, Minneapolis, MN). Each variable was examined at two levels, a high (+) and a low (-) level of concentration *i.e.* the higher level of the components was chosen to equal four times their lower levels (Table 1). Twelve experiments were formulated using PB design and the response was measured in terms of taxol production (Table 2). Experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of S-7 medium (optimized V_m/V_f ratio at 0.20) at 25ºC with an initial medium pH of 6.5 as still cultures in dark. After 20 days of incubation, the mycelium was harvested from the fermentation broth for biomass analysis (FW) and taxol production. The analysis of the experimental results was

Table 1. The two levels of medium components used in the Plackett-Burman design

Code	Factors	Low level (-1) (g/L)	High level (+1) (g/L)
A	$FeCl_3$	0.001	0.004
B	KH_2PO_4	0.2	0.8
C	$MnCl_2$	0.001	0.004
D	$MgSO_4$	0.25	1
E	$ZnSO_4$	0.001	0.004
F	NaOAc	0.5	2
G	Thiamine (Vitamin B1)	0.05	0.2
H	Phenylalanine	0.002	0.008

performed based on the first-order model assumption to calculate the coefficient value of each selected constituent. A high positive coefficient value (*t* value) indicates that the analysed factor has a major impact on response and a low *p* value of variables ($p < 0.05$) indicates a significant effect. The components giving high positive *t* value and *p* value less than 0.05 were selected for subsequent concentration optimization.

2.5.3. Response surface methodology (RSM)

Selected relevant factors *i.e*. those having high *t* value and low *p* value, were subjected to RSM for determining their optimum concentrations while the rest of the medium components were kept at constant level. A three coded level Box-Behnken design (BB design) using the Design-Expert software was employed for determining the optimal concentrations of the screened factors in PB design. Seventeen experiments were formulated using BB design for the three independent variables [NH_4NO_3 (conc. range 2.5 ~ 10 gm/L), $MgSO_4 \cdot 7H_2O$ (conc. range 0.25 ~ 1 gm/L) and NaOAc (conc. range 0.5 ~ 2 gm/L)] each at three levels of concentrations (Table 3) used to optimize the taxol production. After 20 days of incubation, the biomass (FW) was harvested and taxol production was determined. The data on taxol production obtained from RSM was subjected to analysis of variance (ANOVA). The behaviour of the system was explained by the following quadratic equation:

$$Y = \beta_o + \Sigma\beta_i\chi_i + \Sigma\beta_{ij}\chi_i\chi_j + \Sigma\beta_{ii}\chi_i^2$$

where Y is the predicted response, β_o the offset term, β_i the linear offset, β_{ii} the squared offset, β_{ij} the interaction effect and χ_i is the dimensionless coded value of X_i. The statistical significance of the above model equation was determined by Fisher's *F*-test value and the proportion of variance explained by the model was given by the multiple coefficient of determination, R^2 value. Contour plots (2D) were generated by the statistical Design-Expert software on the basis of the response (taxol production) analysis to visualize the interactive effect of the significant factors on taxol production. Unless otherwise mentioned all experiments were performed in triplicates.

2.6. Analytical methods

After 20 days of incubation, the mycelium was harvested and blotted on a filter paper to remove excess of medium and weighed for fresh weight (FW) estimation. Taxol production was determined from culture filtrate and mycelium as described in Garyali *et al*. [19].

3. Results

3.1. Standardization of basic parameters

Maximum radial growth of *F. redolens* on surface of PDA media was observed on 7th day of culturing. The mycelial growth and bioactive metabolite production was higher at

Table 2. Experimental design matrix with response obtained (taxol produced) for the experimental protocols in Plackett-Burman design for medium optimization

Run order	A	B	C	D	E	F	G	H	Taxol (μg/L)
1	1	1	-1	1	1	1	-1	-1	156.7
2	-1	1	1	-1	1	1	1	-1	143.3
3	1	-1	1	1	-1	1	1	1	189.1
4	-1	1	-1	1	1	-1	1	1	106.3
5	-1	-1	1	-1	1	1	-1	1	129.3
6	-1	-1	-1	1	-1	1	1	-1	173.6
7	1	-1	-1	-1	1	-1	1	1	75.2
8	1	1	-1	-1	-1	1	-1	1	158.8
9	1	1	1	-1	-1	-1	1	-1	124.7
10	-1	1	1	1	-1	-1	-1	1	118.0
11	1	-1	1	1	1	-1	-1	-1	89.4
12	-1	-1	-1	-1	-1	-1	-1	-1	81.6

Table 3. Experimental recipe and response in Box-Behnken experimental design protocol for medium optimization

Trials	Coded values and actual values (g/L)			Taxol (µg/L)	
	NH_4NO_3	$MgSO_4$	NaOAc	Experimental	Predicted
1	-1 (2.5)	0 (0.63)	-1 (0.5)	145.7	150.76
2	-1 (2.5)	0 (0.63)	1 (2)	179.8	178.79
3	1 (10)	0 (0.63)	-1 (0.5)	172.8	173.81
4	1 (10)	0 (0.63)	1 (2)	143.0	137.94
5	0 (6.25)	-1 (0.25)	-1 (0.5)	178.1	173.30
6	0 (6.25)	-1 (0.25)	1 (2)	174.8	176.07
7	0 (6.25)	1 (1)	-1 (0.5)	160.9	159.62
8	0 (6.25)	1 (1)	1 (2)	144.2	149.00
9	-1 (2.5)	-1 (0.25)	0 (1.25)	166.9	166.64
10	1 (10)	-1 (0.25)	0 (1.25)	156.9	160.69
11	-1 (2.5)	1 (1)	0 (1.25)	153.0	149.21
12	1 (10)	1 (1)	0 (1.25)	137.0	137.36
13	0 (6.25)	0 (0.63)	0 (1.25)	198.1	195.54
14	0 (6.25)	0 (0.63)	0 (1.25)	192.9	195.54
15	0 (6.25)	0 (0.63)	0 (1.25)	196.6	195.54
16	0 (6.25)	0 (0.63)	0 (1.25)	192.3	195.54
17	0 (6.25)	0 (0.63)	0 (1.25)	197.8	195.54

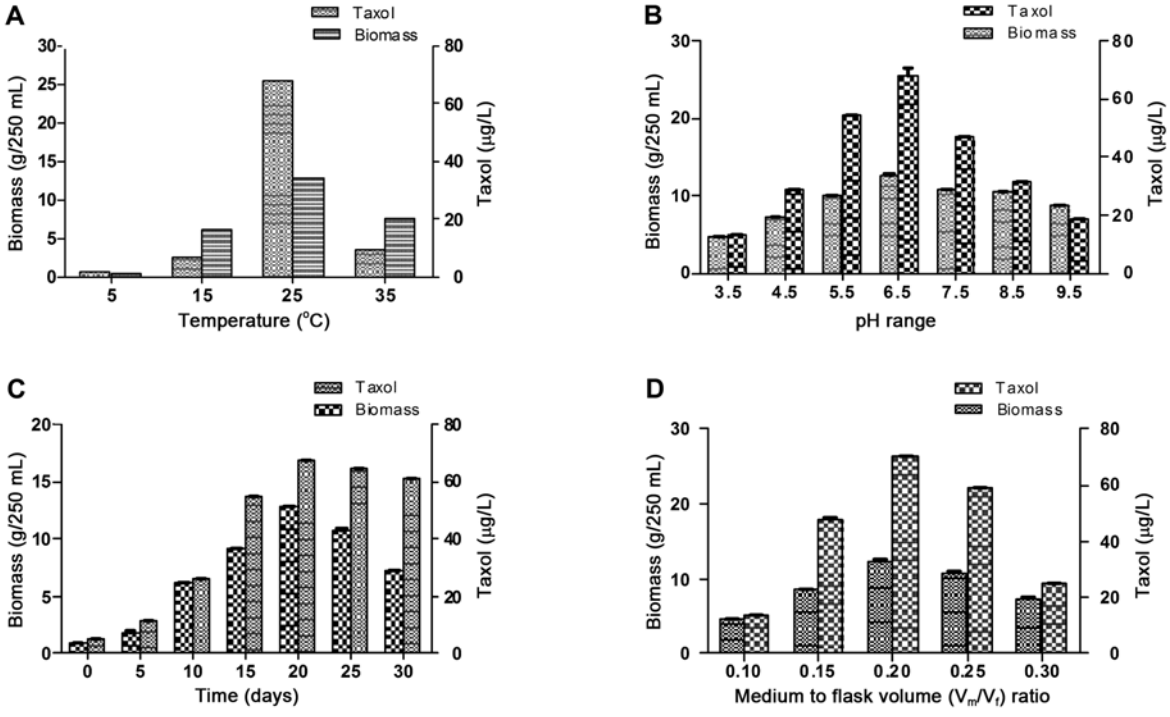

Fig. 1. Determination of optimum (A) Temperature; (B) pH for the growth of *F. redolens* in S-7 medium; (C) Estimation of fungal biomass; (D) Optimization of medium-to-flask volume ratio.

25ºC (Fig. 1A) compared to low (15ºC) and high (35ºC) temperatures. The growth was almost ceased at < 10ºC and > 40ºC. The pH of 6.5 of S-7 liquid medium was found to be optimal for growth and bioactive metabolite production (Fig. 1B). Growth was not observed at pH <3.0 and pH >11.0. The incubation time of 20 days was optimum for maximum biomass and metabolite production (Fig. 1C). The medium volume (V_m) to flask volume (V_f) ratio of 0.2 showed maximum biomass compared to 0.1 and 0.3 ratio (Fig. 1D). Taxol production continuously increased from 14 to 70 µg/L with an increase in V_m/V_f from 0.10 to 0.20. Hence, the optimal value of V_m/V_f for the maximum biomass

and taxol production was selected as 0.20.

3.2. Medium optimization

3.2.1. Effect of carbon and nitrogen sources on biomass and taxol production

Higher biomass and taxol production was observed in sucrose amended medium (80 gm/L) compared to other carbon sources. Maximum taxol yield was observed when the concentration of sucrose was 8% (w/v). The biomass increased with the increasing concentration of sucrose, suggesting that high concentration of sucrose did not affect the growth of the fungus (Fig. 2A). Among the different nitrogen sources, NH_4NO_3 (8 gm/L) enhanced the biomass and taxol production compared to other nitrogen sources (Fig. 2B).

3.2.2. Plackett-Burman design

Plackett-Burman design results showed that KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, NaOAc and vitamin B1 are significant model terms. NaOAc, $MgSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ were shown as the important components which gave main contribution on taxol production, while, $ZnSO_4$ had a negative effect. The same effect for the three micronutrients (NaOAc, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$) was confirmed from the high positive *t* values (or regression coefficients) calculated for these components in the ANOVA test by Design-Expert version 8.0.7.1 software (Stat-Ease Corporation, Minneapolis, MN). Among the eight micronutrients studied under PB design, $ZnSO_4$ had a negative effect on taxol production (as indicated by the negative *t* value and studied effect) and factors with "Prob > *F*" (*p* value) more than 0.1 *i.e.* $FeCl_3$, $MnCl_2$ and phenylalanine did not have a significant effect on taxol production. These four micronutrients were included at a fixed concentration level in the optimized medium. Although the PB design could be successfully used for the reasonable prediction of the significance level of the different variables (micronutrients) affecting the response (taxol production), some of the significant interactive effects of the chosen variables (two-factor interaction) were confounded in its complex structure. Because of this reason, the actual main effects of these variables may have been influenced. Hence, the significant components NaOAc and $MgSO_4 \cdot 7H_2O$ whose interactive effects were demonstrated by PB design were further modelled more precisely by RSM.

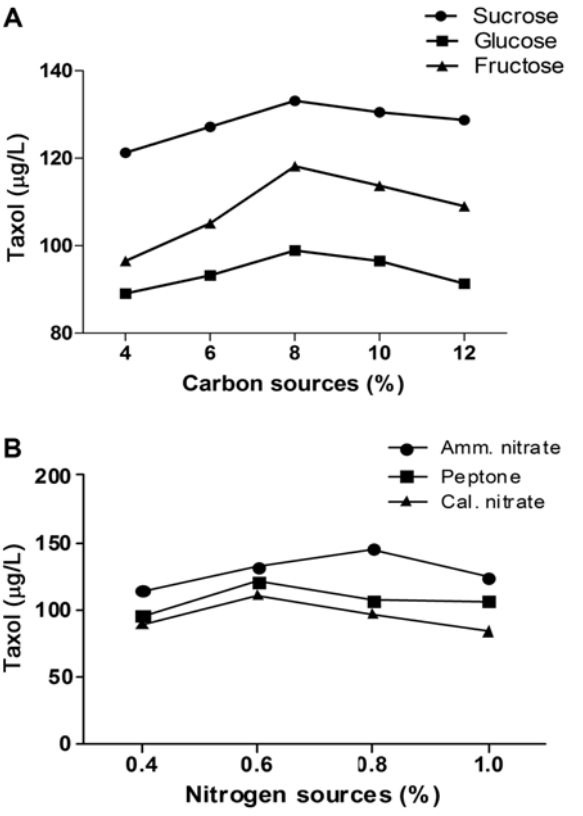

Fig. 2. Determination of suitable (A) Carbon substrate; (B) nitrogen source for the growth of *F. redolens* in S-7 medium.

Table 4. Statistical data for the determination of variable significance in the Plackett-Burman design experiment

Symbol	Components	*t* Coefficient	Studied effect	Contribution (%)	Prob > *F* (*p*-value)*#
A	$FeCl_3$	2.9	6.97	0.98	0.1358
B	KH_2PO_4	3.3	11.6	2.72	0.0433
C	$MnCl_2$	2.97	6.93	0.97	0.1371
D	$MgSO_4$	6.7	20.03	8.12	0.0101
E	$ZnSO_4$	-7.3	-24.27	11.92	0.0058
F	NaOAc	17.24	59.27	71.08	0.0004
G	Thiamine (Vit. B1)	4.2	13.07	3.46	0.0320
H	Phenylalanine	0.95	1.23	0.031	0.7436
	Model	-	-	-	0.0039

*Values of "Prob > *F*" less than 0.0500 indicate model terms are significant.
#Values greater than 0.1000 indicate the model terms are not significant.

3.2.3. Response surface methodology

Various combinations of the components were used and corresponding taxol yields (experimental and predicted) were recorded. The amounts of remaining components in all assemblies were the same as those in basal medium. The response for taxol production were analysed by linear multiple regression and graphical analysis using the Design-Expert version 8.0.7.1 software (Stat-Ease Corporation, Minneapolis, MN). The mathematical models incorporating the interactive effect of these nutrients were proposed for taxol production as:

$$\text{Taxol} = +195.54 - 4.45A - 10.19B - 1.96C - 1.48AB - 15.98AC - 3.35BC - 23.12A^2 - 18.94B^2 - 12.10C^2$$

where, A, B and C are the symbols of concentration of NH_4NO_3, $MgSO_4{\cdot}7H_2O$ and NaOAc, respectively.

ANOVA of linear regression model equation demonstrated that the model equation was highly significant, as evident from value of "Model Prob > *F*" less than 0.0001 (Table 5). In this case A, B, AC, A^2, B^2, and C^2 are significant model terms. The "Lack of Fit Prob > *F*-value" of 0.0614 implies that the lack of fit is insignificant. The goodness of fit of model was tested by the determination coefficient (R^2). In the present study, R^2 value of this model is 0.9772, so it is reasonable to use the regression to analyse the trends in the responses.

The 3D response surface and 2D contour plots generated during data analysis are graphical representation of regression equation. The 2D contour plots in Fig. 3 illustrate the interactions of the concentrations of most significant effectors; NH_4NO_3, $MgSO_4{\cdot}7H_2O$ and NaOAc on taxol production. From the study of these response surface contour plots, maximum taxol production was obtained when the concentration of NH_4NO_3, $MgSO_4{\cdot}7H_2O$ and NaOAc were 6.25, 0.63 and 1.25 g/L, respectively.

In order to verify the RSM predicted results, an experiment was performed under the optimized nutrients levels, and the experimental and predicted values were compared. The predicted response in Box-Behnken experimental design for taxol production gave a value of 195 μg/L, while the actual experimental value of taxol production was 198 μg/L, which was three folds higher than taxol production in un-optimized medium (66 μg/L), suggesting experimental and predicted values are in good agreement.

4. Discussion

The basic parameters optimized for biomass and taxol production by *F. redolens* was observed when the culture was grown at 25ºC for 7 days with the initial pH of 6.5. The growth and taxol production remain almost static after 20 days of incubation. Huang *et al*. [21] also reported the antitumour and antifungal activity of endophytic fungi when the cultures were incubated at 25ºC for 7 days.

Taxol is a secondary metabolite whose synthesis is regulated by carbon and nitrogen sources, phosphate, trace elements and precursors [22]. In the present study, sucrose served as best carbon source for taxol production compared to other carbon sources tested. Contrary, Feng *et al.* [15] reported glucose as best carbon source for taxol production by *Fusarium maire*. Ammonium nitrate served as good source of nitrogen in this study. Strobel *et al.* [11] reported ammonium nitrate and peptone as good nitrogen sources for taxol production by *Pestalotiopsis microspora*. NH_4NO_3

Table 5. Regression coefficients and their significance for response surface model

Source	Sum of squares	df	Mean square	*F*-value	Prob > *F*
Model	6943.04	9	771.45	33.39	<0.0001
A	158.42	1	158.42	6.86	0.0345
B	830.28	1	830.28	35.94	0.0005
C	30.81	1	30.81	1.33	0.2861
A^2	8.70	1	8.70	0.38	<0.0001
B^2	1020.80	1	1020.80	44.19	<0.0001
C^2	44.89	1	44.89	1.94	0.0013
AB	2250.67	1	2250.67	97.42	0.5588
AC	1511.21	1	1511.21	65.41	0.0003
BC	615.95	1	615.95	26.66	0.2060
Lack of fit	131.50	3	43.82	5.79	0.0614

A-NH_4NO_3; B-$MgSO_4$; C-NaOAc.
The model *F*-value of 33.39 implies the model is significant.
Values of "probability > *F*" less than 0.0500 indicate model terms are significant.
In this case A, B, AC, A^2, B^2, C^2 are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

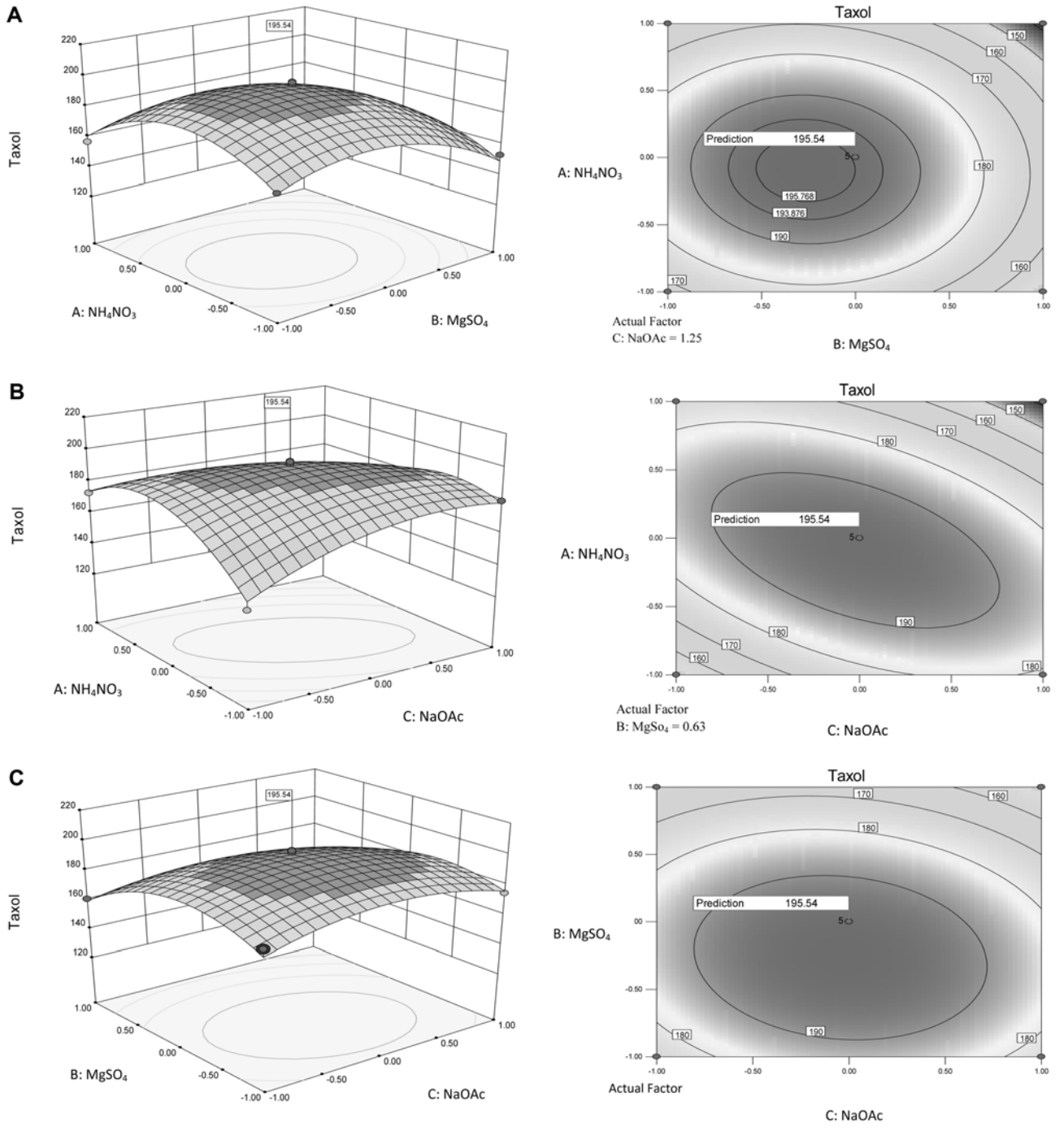

Fig. 3. 3D surface and contour plots showing the effect of different variables on taxol production. (A) Effect of NH_4NO_3 and $MgSO_4 \cdot 7H_2O$ on taxol production. (B) Effect of NH_4NO_3 and NaOAc on taxol production. (C) Effect of $MgSO_4 \cdot 7H_2O$ and NaOAc on taxol production.

was also found to have significant effect on taxol production in case of *F. maire* [15].

Nutrition plays an important role in the onset and intensity of secondary metabolism. To achieve high product yield, it is prerequisite to design proper production medium as there is relationship between media composition and biosynthesis of secondary metabolites [18]. NaOAc, $MgSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ were shown as important components for taxol production in this study. Sodium acetate as an activator was also reported with *Taxomyces andreanae* [9]. Similar results were also reported by Feng *et al.* [15] who found enhancement of taxol yield from 20 to 225 μg/L in mutant

strain of *F. maire*. Zhao *et al*. [23] found enhancement of taxol production from 397 to 456 μg/L while Feng *et al*. [15] reported 31% increase in taxol production upon media optimization. Luo and He [16] also found 2 times higher production of Paclitaxel upon optimizing the concentration of elicitors and precursors. The present study led to enhancement of fungal taxol up to three folds which are encouraging in terms of product yield for scale up studies and commercial exploitation of fungal taxol.

In conclusion, response surface methodology allowed a rapid screening of most significant factors affecting the production of taxol in *Fusarium redolens.* Statistical analysis of coefficients in Plackett Burman design experiments demonstrated that NH_4NO_3, $MgSO_4 \cdot 7H_2O$ and NaOAc are the major factors influencing the production of taxol. With the application of RSM, production of fungal taxol increased 3 folds compared to unoptimized medium. Our work has proved the effectiveness of statistical tools in bioprocess optimization for exploring scale up feasibility, augmenting the economic viability of the process. Further improvement in taxol production can be achieved by improvement of the fungal strains through genetic manipulations and/ or mutagenesis or by augmentation of elicitor and precursor molecules to this fungal strain.

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