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Optimization of Culture Parameters for Production of Podophyllotoxin in Suspension Culture of *Podophyllum hexandrum*

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Abstract

The root explants of the germinated seedlings of *Podophyllum hexandrum* were grown in MS medium supplemented with indole acetic acid (IAA) (2 mg/L) and activated charcoal (0.5%), and healthy callus culture was obtained after incubation for 3 wk at 20°C. The cultivation of plant cells in shake flask was associated with problems such as clumping of cells and browning of media, which were solved by the addition of pectinase and polyvinylpyrrolidone. The effect of major media components and carbon source was studied on the growth and podophyllotoxin production in suspension culture. It was found that glucose was a better carbon source than sucrose and that NH_4^+:NO_3^- ratio (total nitrogen concentration of 60 m*M*) and PO_4^{3-} did not have much effect on the growth and product formation. The relative effect of culture parameters (inoculum level, pH, IAA, glucose, NH_4^+:NO_3^- ratio, and PO_4^{3-}) on the overall growth and product response of the plant cell suspension culture was further investigated by Plackett-Burman design. This indicated that inoculum level, glucose, IAA, and pH had significant effects on growth and production of podophyllotoxin. To identify the exact optimum concentrations of these parameters on culture growth and podophyllotoxin production, central composite design experiments were formulated. The overall response equations with respect to growth and podophyllotoxin production as a function of these culture parameters were developed and used to determine the optimum concentrations of these parameters, which were pH 6.0, 1.25 mg/L of IAA, 72 g/L of glucose, and inoculum level of 8 g/L.

Index Entries: *Podophyllum hexandrum*; podophyllotoxin; Plackett-Burman design; response surface methodology; central composite design.

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Introduction

Podophyllotoxin is a raw material for the production of the anticancer drugs etoposide (VP-16-213) and teniposide (VM-26). This compound is currently being extracted from the roots of the endangered plant *Podophyllum hexandrum*, a perennial herb growing in the Northern Himalayan region. Other *Podophyllum* species also produce the compound but *P. hexandrum* of Indian origin attracted major attention because of its higher productivity in comparison with its American counterpart, *P. peltatum (1)*, and other podophyllotoxin-producing plant species such as *Linum* sp. *(2,3)* and *Juniperous chinensis (4)*. Extraction of the economically important compound podophyllotoxin from the roots of *P. hexandrum* is leading to its extinction. The plant tissue culture would provide the best solution to this problem *(5)*.

The establishment of callus culture of *P. hexandrum* has been reported by a number of investigators *(6,7)*. Propagation of *P. hexandrum* cells at the callus level, however, would not be able to meet the demand of the drug. A few studies have also been reported on the cultivation of *P. hexandrum* in suspension culture for the production of podophyllotoxin *(6,8,9)*. An advantage of suspension culture is its ability to mass propagate at a larger scale in bioreactors.

Process design of plant cell suspension cultures has been difficult to exploit industrially because of several constraints, including the instability of cell lines, the risk of microbial contamination, the intracellular nature of secondary metabolites, and the shear sensitivity of plant cells. Only a few products are being industrially produced by large-scale cultivation of plant cells.

Appropriate nutritional agents, their concentrations, and environmental factors (nutritional stress) have been able to enhance the yield and productivity of plant cell suspension cultures *(10,11)*. However, it is essential to study and quantify the effect of selected key medium components on growth as well as product accumulation and strike a perfect balance between the two to enhance the yield and productivity of plant cell suspension cultures. This is particularly important for plant secondary metabolites since conditions suitable for growth may adversely affect the product formation and vice versa.

The first important step in any bioprocess media optimization is the identification of relatively significant media components such as carbon, nitrogen, minerals, and culture conditions. The Plackett-Burman design *(12)* helps to eliminate the less important variables from the list of selected parameters. The number of experiments to be carried out for a large number of parameters is dramatically reduced by this methodology. This design has been widely used by several researchers to shorten the list to a few significant parameters for further process optimization. In microbial fermentation technology, the Plackett-Burman design has been adopted for design of media for production of alcohols *(13)*, polysaccharides *(14)*, and enzymes *(15)*.

The next step in the media optimization design is to identify the exact concentrations of selected parameters. Conventionally this has been done by single variable optimization, by maintaining other variables involved in the process at a constant level. This does not represent the combined effect of all the parameters involved. This method is also time-consuming and requires a large number of experiments to determine the optimal concentration levels, which are unreliable. These limitations of the single-parameter optimization process can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design *(16)* using response surface methodology (RSM) *(17)*. By using RSM, the relationship among the variables can be expressed mathematically in the form of a polynomial model, which gives the response as a function of relevant variables. The behavior of this response is determined when these variables are made to vary within a chosen range. The developed model(s) is used to make isoresponse contour plots, which give the variation of responses when the levels of two of the variables are changed keeping the third variable constant. These contour plots are used to determine the optimal conditions of the variables for the highest response *(18)*.

A central composite design (CCD) is usually utilized to obtain the experimental data, which will fit an empirical, full second-order polynomial model. The CCD along with a full second-order polynomial model is a very powerful tool in representing the response surfaces over a relatively broad range of parameters *(18)*. This design has been used for the optimization of nutritional constituents for the enhanced production of amylase by *Bacillus circulans (19)* and of nutritional and process parameters for increased production of xylanases by *Melanocarpus albomyces (15)*.

This powerful strategy to screen and optimize the culture and environmental parameters by statistical media design methodology has not been commonly employed in plant cell bioprocess engineering. RSM has been applied for the optimization of growth and indole alkaloid production in hairy-root cultures and ajmalicine production in cell suspension cultures of *Catharanthus roseus (20,21)*. A statistical optimization methodology was employed for the optimization of growth rates of the cell lines of *Taxus brevifolia (22)*. The present study deals with the optimization of growth of *P. hexandrum* and production of podophyllotoxin by plant cell suspension culture. The Plackett-Burman design was applied to narrow the list of the most significant parameters that influence the growth and podophyllotoxin production, and, finally, the concentrations of screened variables were optimized by RSM using CCD.

Materials and Methods

Initiation of Callus Culture

Seeds of *P. hexandrum* were surface sterilized in 0.1% mercuric chloride for 5 min and rinsed with distilled water. Decoated seeds were again treated with 0.05% mercuric chloride for 3 min followed by rinsing with

distilled water. The sterilized seeds were transferred to MS *(23)* and B5 *(24)* media containing 0.8% agar and supplemented with selected growth regulators for germination. The root explants of the germinated seeds were then inoculated on MS and B5 media, supplemented with selected growth regulators for initiation of callus. The cultures were incubated at 20°C, with a 16/8-h day/night regime.

Initiation of Suspension Culture Using Pectinase and Polyvinylpyrrolidone

The callus was transferred into MS and B5 media supplemented with phytohormones, 0.005% pectinase (prepared in MES buffer, pH 6.0) and 1% polyvinylpyrrolidone (PVP). The cultures were incubated on a gyratory shaker at 125 rpm and 20°C under complete darkness. After 3 wk, the cells were subcultured (inoculum level of 2 g dry wt/L) into fresh medium containing 0.5% PVP without pectinase.

Determination of Growth

The suspension-grown cells were harvested after 30 d. The cell suspension (15 mL) was centrifuged in conical centrifuge tubes at 1500*g* for 15 min. After centrifugation, the cells were washed with distilled water, centrifuged again, and then weighed to obtain the fresh weight. The cells were dried at 60°C for 16 h to determine the dry weight.

Extraction of Podophyllotoxin

The dried cell mass (20–200 mg) was powdered and heated with ethanol (1.5 mL) at 60°C for 30 min with constant stirring on a magnetic stirrer followed by sonication (Soniprep) for 15 min. Constant stirring helped to leach out the lignans from the cells. The supernatant was then removed after centrifugation at 6000*g* for 10 min and evaporated to dryness under vacuum. The lignan extract was redissolved in analytical-grade ethanol prior to analysis.

Thin-Layer Chromatography Analysis

The crude extract (20 µL) and podophyllotoxin (Sigma) as a reference compound (0.1 mg/mL; 20 µL) were chromatographed on a silica gel 60-F-254 plate (Merck) using chloroform:methanol (25:1 [v/v]) over a distance of 10 cm in a saturated chamber. The spots were detected under UV light (254 nm) and developed with methanol:sulfuric acid (20:1 [v/v]), followed by heating at 110°C for 10 min.

High-Performance Liquid Chromatography Analysis

High-performance liquid chromatography (HPLC) analysis of the extract was carried out using a Nova Pak C_{18} column (250 × 4.6 mm) in an

Table 1
Range of Variables Selected to Screen Most Important Parameters by Plackett-Burman Design

Level	IAA (mg/L)	NH_4^+:NO_3^- ratio (total N = 60 m*M*)	PO_4^{3-} (m*M*)	Glucose (g/L)	Inoculum (g/L)	pH
Level –1	0.0	1:5	0.0	20.0	2.0	5.0
Level +1	5.0	5:1	2.5	100.0	10.0	6.0

HPLC system (Waters) connected to a UV detector. Acetonitrile:water: methanol (37:58:5) was used as a mobile phase at a flow rate of 1.5 mL/min. The extract (20 µL) was injected into the column for analysis. Podophyllotoxin was detected at 280 nm. Podophyllotoxin (0.1 mg/mL) was used as a standard for calculating podophyllotoxin content in the samples on the basis of total area under the peak obtained from integrator.

Experimental Design

Plackett-Burman Design

Six variables (as shown in Table 1) were selected on the basis of existing literature reports and previous experiments to investigate their influence on the growth of *P. hexandrum* cells in suspension and production of podophyllotoxin using the methodology of Plackett-Burman. Each independent variable was tested at two levels: a high (+) and a low (–) level, as shown in Table 1. Eight experiments were formulated using these six parameters. The experimental design protocol was developed using Design-Expert version 5.0.9 software (Stat-Ease) and is represented in Table 2. The inoculum used for the experiments was 3-wk-old dark-grown culture of *P. hexandrum*. The cultures were incubated on a gyratory shaker (125 rpm) at 20°C under complete darkness. The flasks were harvested at the end of 30 d. The cell dry weight and podophyllotoxin content were analyzed for each flask.

Central Composite Design

Once the effective parameters were selected from the Plackett-Burman design, CCD was used to determine the actual level of the parameters for optimal growth and podophyllotoxin production. A 2^4-factorial CCD leading to a total of 30 sets per experiment was formulated to optimize the initial inoculum level, glucose concentration, initial pH, and indole acetic acid (IAA) level for the growth of *P. hexandrum* cells and podophyllotoxin production in suspension culture. The CCD experimental protocol was also developed using Design-Expert version 5.0.9 software (Stat-Ease) and is represented in Table 3. The incubation conditions were the same for these experiments as for the Plackett-Burman design.

Table 2
Design of Experiments by Plackett-Burman Methodology Using Design-Expert Software and Responses

Run	IAA (mg/L)	NH_4^+:NO_3^- ratio	PO_4^{3-} (m*M*)	Glucose (g/L)	Inoculum (g/L)	pH	Response	
							Dry wt (g/L)	Podophyllotoxin (%, dry wt)
1	5.00	0.20	0.00	20.00	2.00	6.00	6.8	0.078
2	0.00	5.00	2.50	20.00	2.00	6.00	8.8	0.043
3	0.00	0.20	2.50	100.00	2.00	5.00	11.2	0.021
4	0.00	0.20	0.00	100.00	10.00	6.00	30.4	0.015
5	5.00	5.00	0.00	100.00	2.00	5.00	5.2	0.01
6	5.00	5.00	2.50	100.00	10.00	6.00	21.6	0.021
7	5.00	0.20	2.50	20.00	10.00	5.00	16.0	0.027
8	0.00	5.00	0.00	20.00	10.00	5.00	16.0	0.055

Table 3
Design Matrix of Independent Variables by CCD Using Design-Expert Software and Responses

Run	Inoculum (*A*) (g/L)	Glucose (*B*) (g/L)	pH (*C*)	IAA (*D*) (mg/L)	Response	
					Dry wt (g/L)	Podophyllotoxin (%, dry wt)
1	2.00 (–2)	60.00 (0)	6.00 (0)	2.50 (0)	9.41	0.074
2	6.00 (0)	20.00 (–2)	6.00 (0)	2.50 (0)	6.89	0.045
3	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16	0.072
4	6.00 (0)	60.00 (0)	5.00 (–2)	2.50 (0)	16	0.096
5	8.00 (+1)	80.00 (+1)	6.50 (+1)	1.25 (–1)	23.2	0.1
6	6.00 (0)	60.00 (0)	7.00 (+2)	2.50 (0)	17.1	0.043
7	4.00 (–1)	80.00 (+1)	6.50 (+1)	1.25 (–1)	19.26	0.048
8	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	17.3	0.08
9	4.00 (–1)	40.00 (–1)	5.50 (–1)	1.25 (–1)	15.4	0.054
10	6.00 (0)	60.00 (0)	6.00 (0)	5.00 (+2)	17.9	0.13
11	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
12	4.00 (–1)	80.00 (+1)	5.50 (–1)	3.75 (+1)	11.85	0.073
13	6.00 (0)	60.00 (0)	6.00 (0)	0.00 (–2)	18.21	0.02
14	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
15	8.00 (+1)	80.00 (+1)	5.50 (–1)	3.75 (+1)	19.1	0.06
16	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
17	8.00 (+1)	80.00 (+1)	6.50 (+1)	3.75 (+1)	24.4	0.011
18	6.00 (0)	60.00 (0)	6.00 (0)	2.50 (0)	16.5	0.076
19	8.00 (+1)	40.00 (–1)	6.50 (+1)	1.25 (–1)	11.45	0.068
20	8.00 (+1)	80.00 (+1)	5.50 (–1)	1.25 (–1)	24.9	0.082
21	4.00 (–1)	80.00 (+1)	5.50 (–1)	1.25 (–1)	16.3	0.06
22	8.00 (+1)	40.00 (–1)	6.50 (+1)	3.75 (+1)	17.9	0.032
23	4.00 (+1)	40.00 (–1)	6.50 (+1)	3.75 (+1)	9.78	0.031
24	6.00 (0)	100.00 (+2)	6.00 (0)	2.50 (0)	14.62	0.027
25	8.00 (+1)	40.00 (–1)	5.50 (–1)	1.25 (–1)	18.79	0.03
26	4.00 (–1)	40.00 (–1)	5.50 (–1)	3.75 (+1)	12.74	0.025
27	10.00 (+2)	60.00 (0)	6.00 (0)	2.50 (0)	24	0.041
28	4.00 (–1)	80.00 (0)	6.50 (+1)	3.75 (+1)	8.3	0.01
29	8.00 (+1)	40.00 (0)	5.50 (–1)	3.75 (+1)	15.85	0.032
30	4.00 (–1)	40.00 (0)	6.50 (+1)	1.25 (–1)	8.6	0.022

[a]The values shown in parentheses are the coded values of the parameters.

Results and Discussion

Development of Callus

Several defined nutrient media (MS and B5, supplemented with growth regulators) were used for the initiation of callus from the root explants of the germinated seedlings. Among the various media combinations tested for this purpose, MS + IAA (2 mg/L) + activated charcoal (0.5%) demonstrated the best response at 20°C and 16/8-h light/dark regime in terms of growth and texture of the callus. The activated charcoal was used to adsorb the brown-colored compounds leached out of the cells. The browning was probably associated with the oxidation of the phenolic compounds produced by the plant cells. Activated charcoal could also prevent tissue necrosis caused by browning *(25)*. The callus was maintained on MS media supplemented with only 0.5% activated charcoal in the absence of growth regulators. Regular subculturing was done every 3 wk to maintain the stock callus culture.

Development of Suspension Culture

The suspension culture was initiated on MS and B5 media supplemented with different growth regulators either alone or in combination. Among the different media combinations tested, B5 + coconut milk (5%) produced a maximum of 4 g/L of biomass (dry wt basis) with an inoculum level of 2 g/L (dry wt). A few problems associated with the growth of the cells in the suspension culture were clumping of the cells, browning of the culture media, and drop in pH during culture growth. These problems were addressed with the inclusion of pectinase and PVP in the media. The use of pectinase helped to release the cells from the clumps into the suspension, and PVP helped to prevent the browning, presumably by arresting the oxidation of the phenolic compounds. The use of PVP in *Cinchona ledgeriana* suspension culture has also been reported to prevent phenolic oxidation *(25)*. This improved the magnitude of growth of *P. hexandrum* cells in suspension culture. The best growth response was observed in MS + IAA (2 mg/L) + PVP (1%). Pectinase was used at a very low concentration (0.005%) for initiation of suspension culture from the callus and was withdrawn in subsequent subcultures. PVP level was reduced to 0.5% during subsequent subcultures. It was also found that glucose was a better carbon source than sucrose for the suspension cultures of *P. hexandrum*. The highest cell mass of 8 g/L (dry wt) was observed in MS + glucose (3%) + PVP (0.5%) medium without any growth regulator.

Effect of Inoculum Level, Glucose Concentration, Initial pH, IAA Concentration, NH_4^+:NO_3^- Ratio, and PO_4^{3-} Concentration of Media on Growth of P. hexandrum Cells and Production of Podophyllotoxin

The results of data analysis for the effect of six selected parameters on the growth of *P. hexandrum* and production of podophyllotoxin were

expressed in terms of regression coefficients and *t* values, which were calculated using Design-Expert version 5.0.9 software. From the coefficient and *t* values, it was clear that IAA, NH_4^+:NO_3^- ratio, and PO_4^{3-} concentration had no significant effect on the growth of *P. hexandrum* cells, whereas initial inoculum level, glucose concentration, and initial pH of the media had a positive effect on the growth of *P. hexandrum*. Inoculum level had the highest positive influence on the growth of *P. hexandrum*, followed by glucose concentration and pH. On the other hand, the production of podophyllotoxin was influenced by IAA level and initial pH of the culture media, since the regression coefficients and *t* values were positive for these two parameters only (values not shown).

From the values of the responses shown in Table 2, it can be seen that a threefold increase in biomass was observed in experiment 4, where 30.4 g/L of biomass (dry wt) was obtained at the inoculum level of 10 g/L (level +1). However, the increase in biomass was more than fivefold at the inoculum level of 2 g/L (level –1) in experiment 3. This indicates that cell-cell contact (high at inoculum level of 10 g/L) was not the only contributing factor for an increase in biomass in *P. hexandrum*, and, therefore, the relative contribution of glucose and initial pH of the medium needs to be examined carefully. It can be seen from this observation that for both experiments 3 and 4, the glucose level was 100 g/L (level +1), indicating that a higher glucose concentration resulted in more biomass production. It was also observed that biomass increase was more than threefold when the pH of the culture media was 6.0 (level +1), except in experiment 6, where it was about twofold. Note that in experiment 6, there were a relatively high (level +1) IAA concentration, NH_4^+:NO_3^- ratio, and PO_4^{3-} concentration, which had a negative effect on the growth of *P. hexandrum*. The highest podophyllotoxin (0.078%, dry wt) was obtained at the lowest inoculum level and glucose concentration (levels –1) in experiment 1. It is pertinent to mention that these are only some indications concerning the effect of these parameters on growth and that their optimum values for growth as well as podophyllotoxin production can be ascertained by employing CCD methodology.

Central Composite Design

From the Plackett-Burman design it was established that inoculum level, glucose concentration, and initial pH of the culture media had a positive response toward the growth of *P. hexandrum* cells in suspension, whereas the production of podophyllotoxin was influenced by initial pH and IAA concentration of the culture media. Thus, these four parameters were finally identified from the original list of six to determine their exact optimum levels by using CCD. The experimental design matrix was formulated using Design-Expert software, as shown in Table 3. The responses in the form of growth of *P. hexandrum* and production of podophyllotoxin were experimentally determined; these are also shown in the experimental design matrix (Table 3).

Proposed Model for Growth and Podophyllotoxin Production

The growth of *P. hexandrum* could be expressed in terms of the four selected variables (in actual factor level) by the following quadratic equation:

$$\text{Growth} = 100.24 - 4.66A - 0.20B - 19.05C - 9.44D + 0.035A^2 - 0.0033B^2 + 0.40C^2 + 0.30D^2 + 0.029AB + 0.54AC + 0.39AD + 0.11BC - 0.055BD + 1.37CD$$

The production of podophyllotoxin could also be expressed in terms of the four selected variables (in actual factor level) by the following equation:

$$\text{Product} = -0.83 - 0.016A + 0.0085B + 0.20C + 0.11D - 0.0017A^2 - 0.003B^2 - 0.015C^2 - 0.0016D^2 + 0.005AB + 0.0067AC - 0.0025AD - 0.0074BC - 0.002BD - 0.012CD$$

The model parameters of these model equations were determined with the help of 30 experimental responses of growth and podophyllotoxin production, as shown in Table 3, using the Design-Expert computer program.

To determine the most adequate operating conditions and analyze the process of the growth and product formation, the response surfaces were studied in detail for all the possible contributions by keeping two parameters constant at a time. Since simultaneous analysis of so many plots is a complex task, the predicted responses of the two given equations were studied by a particular programming feature of the Design-Expert software, which allows study of the responses of these model equations by independent variation of one parameter at a time. It has been practically observed that podophyllotoxin production was better at pH 6.0 and when the concentration of IAA was 1.25 mg/L. The same was also predicted by the model equations. Therefore, pH and concentration of IAA were kept constant at 6.0 and 1.25 mg/L, respectively, and contour plots were made for growth and product formation to study critically the interactions between the glucose concentration and inoculum level. Figures 1 and 2 represent the two-dimensional (2D) and three-dimensional (3D) contour plots for growth and podophyllotoxin production, respectively, with variation in glucose and inoculum level at the constant values of IAA (1.25 mg/L) and initial pH (6.0). It was observed that a glucose concentration of 72 g/L and an inoculum level of 8 g/L produced the best result in terms of growth of *P. hexandrum* (22.7 g/L, dry wt) and production of podophyllotoxin (0.075%, dry wt). A maximum of 20.2 g/L (dry wt) of biomass and 0.071% podophyllotoxin (dry wt) was produced when *P. hexandrum* was cultivated under statistically optimized culture conditions, representing 89 and 94.7% validity of the predicted models for growth and podophyllotoxin production, respectively.

A significant increase in culture response has been reported for *Panax ginseng*, in which cell growth and ginseng production were stimulated by increasing inoculum level and sugar concentration to 6 g/L (dry wt) and

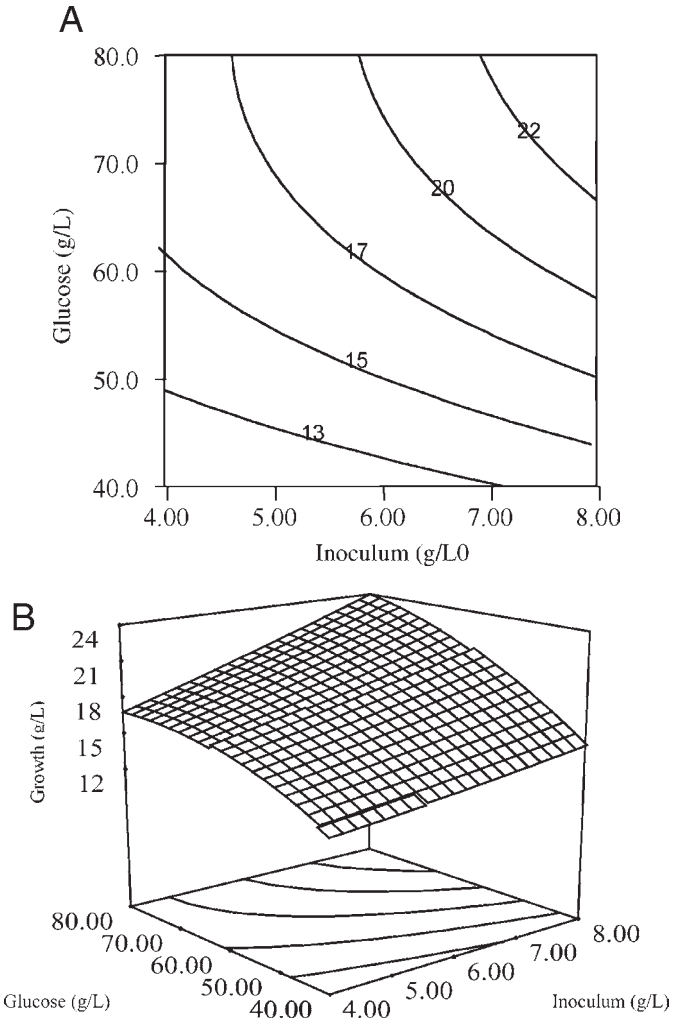

Fig. 1. **(A)** Isoresponse contour (2D) of growth of *P. hexandrum* between inoculum level and glucose concentration (IAA = 1.25 mg/L; pH = 6.00); **(B)** isoresponse contour (3D) of growth of *P. hexandrum* between inoculum level and glucose concentration (IAA = 1.25 mg/L; pH = 6.00).

60 g/L, respectively *(26)*. The production of catharanthine by immobilized *Catharanthus roseus* cells was doubled when the inoculum density was doubled to 100 g/L (fresh wt) *(27)*.

Conclusion

The Plackett-Burman design indicated that concentration of glucose, inoculum level, concentration of IAA, and pH were the four parameters that produced positive responses on the growth of and podophyllotoxin production by *P. hexandrum*. The Plackett-Burman design also suggested

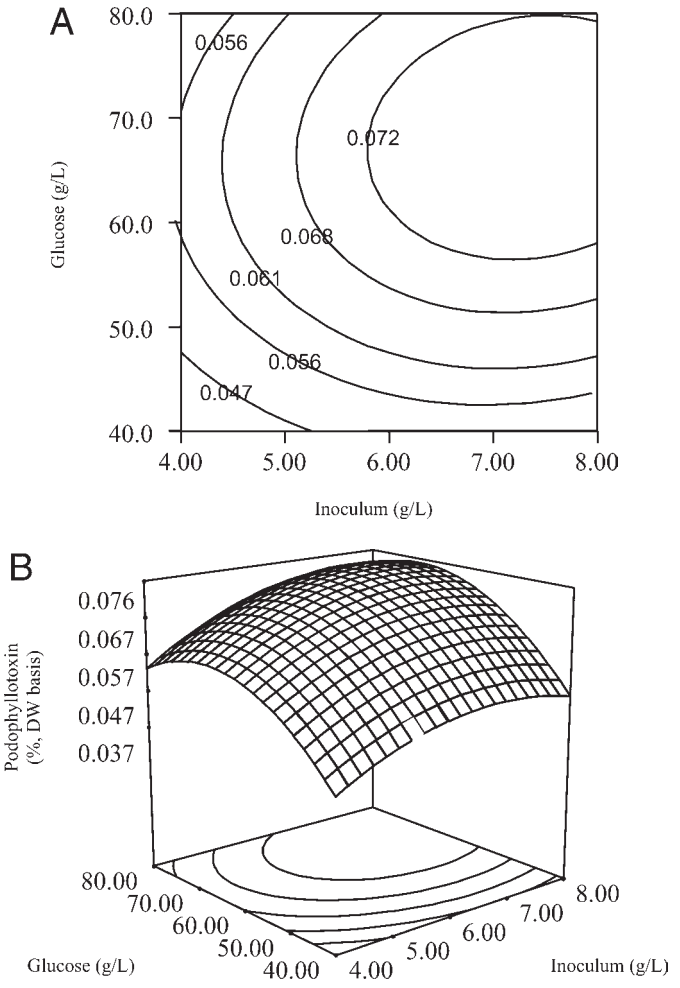

Fig. 2. **(A)** Isoresponse contour (2D) of production of podophyllotoxin between inoculum level and glucose concentration (IAA = 1.25 mg/L; pH = 6.00); **(B)** isoresponse contour (3D) of production of podophyllotoxin between inoculum level and glucose concentration (IAA = 1.25 mg/L; pH = 6.00). DW, dry weight.

that the ratio of NH_4^+:NO_3^- and concentration of PO_4^{3-} did not have any significant effect on both growth and product formation. CCD was utilized to develop the second-order polynomial model for the growth and product responses. The model parameters were determined using the responses of the designed experimental protocols. The response of the model was studied by making the isoresponse curves, which gave optimal growth (22.7 g/L, dry wt) and product formation (0.075%, dry wt) conditions at pH 6.0, 1.25 mg/L of IAA, 72 g/L of glucose, and inoculum level of 8 g/L. The experimental values of growth and podophyllotoxin production at these conditions confirmed the validity of the predicted models.

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