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BIOTECHNOLOGY

Optimization of cell growth and bacoside-A production in suspension cultures of *Bacopa monnieri* (L.) Wettst. using response surface methodology

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Abstract Plant secondary metabolites have emerged as potential raw materials, which are used in the pharmaceutical, food, chemical, and cosmetic industries. Bacoside-A, a secondary metabolite produced by *Bacopa monnieri*, is known for its memory-facilitating properties. In recent years, various strategies have been developed to enhance biomass accumulation and synthesis of secondary compounds in cultures. In the present investigation, various factors affecting the production of biomass and bacoside-A in the cell suspension cultures of *B. monnieri* were optimized using the statistical experimental design approach. Preliminary screening by Plackett–Burman's design revealed that among the tested factors, glucose, KNO_3, KH_2PO_4, and inoculum density significantly influenced cell growth and bacoside-A production. Furthermore, using response surface methodology (RSM), glucose, KNO_3, and KH_2PO_4 at a concentration of 5.67, 0.313, and 0.29%, respectively, and an inoculum density of 0.66% in basal MS medium were found to be optimal for cell growth and bacoside-A production. After optimization, the biomass yield increased about twofold (from 5.52 to 12.58 g L^{-1} fresh cell weight) and bacoside-A production about 1.7-fold (5.56 to 9.84 mg g^{-1} dry weight). The present study results show the successful application of RSM to enhance the production of biomass and accumulation of bacoside-A content in cell suspension cultures of *B. monnieri.*

Keywords Callus · Cell suspension culture · Glucose · Inoculum · Nitrogen ratio

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Introduction

Secondary metabolites produced by plants facilitate interactions with the biotic environment and establish a defense against herbivores and pathogens (Verpoorte *et al.* 2002). Most secondary metabolites produced by plants exhibit different biological activities, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives. *Bacopa monnieri* (L.) Wettst. (Scrophulariaceae) is an important medicinal herb known for its memory-vitalizing molecules called bacosides (Singh and Dhawan 1997). The plant is well equipped with anticancer (Elangovan *et al.* 1995), antioxidant (Tripathi *et al.* 1996), memory-enhancing (Pal and Sarin 1992), and cardio-tonic properties (Mathur *et al.* 2002). The traditional Indian treatment history exhibits clear traces of its use as a vital constituent of Ayurveda. The main dynamic compounds responsible for the production of the above properties are bacosides (bacoside-A and -B), which are constituents of all parts of the plant (Mathur *et al.* 2002). Bacoside-A plays a crucial role in pharmaceutical preparations for the treatment of brain-related problems and enhancement of cognation (Singh and Dhawan 1997).

The enhanced commercial interest in bacosides has resulted in increased extraction of this herb from the wild. This has resulted in adding this herb to the list of endangered plant species (Tiwari *et al.* 2001). In addition, the production of secondary metabolites through field cultivation of plants has various disadvantages such as low yields and variations in the amounts produced due to geographical, seasonal, and environmental conditions. Thus, there is a need to develop alternative methods to produce bacosides for the pharmaceutical industry and to conserve the plant. Plant cell cultures have emerged as new alternatives for the production of secondary metabolites (Rao and Ravishankar 2002). In recent years, various strategies have been developed to enhance biomass accumulation

and the synthesis of secondary metabolites, which include strain improvement, optimization of the medium and culture environment, elicitation, nutrient and precursor feeding, immobilization, permeability, and biotransformation methods (Murthy *et al.* 2014).

The possibility of secondary metabolite production using plant cell cultures has been established and demonstrated by various researchers (Alfermann and Petersen 1995; Fowler and Scragg 1998; Rahman *et al.* 2002). However, the low productivity of secondary metabolites in cell cultures is a major constraint to scaling up the production. Several conventional approaches have been adopted to enhance the yield of secondary metabolites from cell cultures (Chattopadhyay *et al.* 2002). These methods are associated with drawbacks including that the methods are time consuming, require large sets of experiments, are not economical, and do not provide effective ways to study the interactions among the variables. To overcome these constraints, researchers apply statistical tools to enhance the metabolite production. There are several specialized designs for mixtures (mixture designs), screening factors (factorial designs), and the quantitation of factor impacts on responses (response surface methods) available for process optimization studies (Schmitz *et al.* 2016). More recently, "design of experiments" (DoE), a statistical experimental design approach, has been widely adopted as a tool for process optimization (Montgomery 2013). Using this approach, researchers can simultaneously examine the effects of multiple input parameters on a given output, cultivation process optimization in terms of biomass production, and product accumulation by considering the interaction of input parameters (Vasilev *et al.* 2013; Buyel and Fischer 2014). Many researchers have used statistical approaches for optimization of medium constituents and other fermentation variables for the production of secondary metabolites (Srivastava and Srivastava 2012; Garyali *et al.* 2014; Bansal *et al.* 2015; Abbasi *et al.* 2016).

In the present investigation, the individual and interacting effects of different factors at different concentrations were studied to optimize the medium components and conditions for enhancing bacoside-A, and cell growth in cell suspension cultures of *B. monnieri* using statistical design approaches.

Materials and Methods

Plant material, chemicals, glassware Shoot cultures of *B. monnieri* (accession BM6) used in the present study were established using terminal portions of actively growing shoots according to the method described in Aggarwal *et al.* (2013). Microshoots were subcultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 58 mM sucrose and 0.7% (*w*/*v*) agar-agar type I (basal MS medium) supplemented with 2.5 μM benzyladenine (BA), and all explants were taken from these microshoots. Unless otherwise stated, the cultures were incubated at 25 ± 1°C under white cool fluorescent lights (Crompton Greaves Consumer Electricals Limited, Mumbai, India) with light intensity of 40 μmol m^{-2} s^{-1} and a 16-h light and 8-h dark cycle. Chemicals were purchased from HiMedia® Laboratories (Mumbai, India). Unless otherwise mentioned, MS medium was used throughout the study. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min.

Establishment of cell suspension culture Callus tissue was established using leaf explants taken from microshoots of *B. monnieri* on basal MS medium supplemented with 5.0 μM α-naphthalene acetic acid (NAA) and 2.5 μM kinetin (KIN) before autoclaving. Friable callus tissue was initiated on the same medium supplemented with casein hydrolysate (1.0 g L^{-1}). Induced friable callus tissue was inoculated into 250-mL Erlenmeyer flasks containing 50 mL of liquid MS medium supplemented with NAA (5.0 μM) and KIN (2.5 μM) to establish the suspension cultures. The cultures were kept on an orbital shaker (Infors HT, Bottmingen, Switzerland) at 125 rpm in a dark chamber at 25 ± 2°C. Unless otherwise specified, cell biomass and bacoside-A levels were determined after 21 d of incubation.

Optimization procedure and experimental design screening of factors effecting cell growth and bacoside-A production Different media optimization experiments were conducted separately to select the best carbon, KNO_3/NH_4NO_3 ratio, and NAA/KIN ratio for cell growth and bacoside-A production keeping other factors constant. For testing carbon sources, sucrose or glucose (20 g L^{-1}) was added to the culture medium in separate combinations. Different ratios of KNO_3/NH_4NO_3 (60:0, 50:10, 40:20, 30:30, 20:40, 10:50, and 0:60 mM) were added to the culture medium separately to study their impact on cell growth and bacoside-A production. Varying concentrations of NAA/KIN (2.5:1.0, 2.5:2.5, 2.5:3.5, 5.0:1.0, 5.0:2.5, 5.0:3.5, 7.5:1.0, 7.5:2.5, and 7.5:3.5 μM) were also tested for their impacts on cell growth and bacoside-A content. Other parameters such as the speed of the gyratory shaker (80, 100, 120, 140, and 160 rpm), pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0), and the concentration of the MS medium (one-fourth (1/4×), one-half (1/2×), three-fourths (3/4×), full strength (1×), or double strength (2×)) were also evaluated changing one component at a time. The cell cultures were raised by inoculating *B. monnieri* cells (1.0 g L^{-1} fresh weight equivalent) in 250-mL Erlenmeyer flasks containing 50 mL of culture medium, pH 6.0. The cultures were grown for 21 d at 25 ± 2°C on a gyratory shaker (125 rpm) in a dark chamber. The growth kinetics of the cell suspension cultures was evaluated for up to

Table 1. Two levels of the selected medium components used in the Plackett–Burman design to optimize important components

Components	Symbol	Low (−1) level	High (+1) level
Glucose (%)	A	1.50	6.00
KNO_3 (%)	B	0.15	0.90
KH_2PO_4 (%)	C	0.05	0.25
$MgSO_4 \cdot 7H_2O$ (%)	D	0.02	0.08
$CaCl_2 \cdot 2H_2O$ (%)	E	0.02	0.09
Inoculum (%)	F	0.20	0.80

24 d by harvesting a portion of the cell suspension at 3-d intervals to study cell growth and bacoside-A production. Unless otherwise mentioned, the cells were harvested after 21 d of incubation for growth measurements and estimation of the bacoside-A content.

Plackett–Burman's design (Plackett and Burman 1946) was used to screen significant factors affecting cell growth and bacoside-A production by the *B. monnieri* cells. Six factors including glucose, potassium nitrate (KNO_3), potassium dihydrogen ortho-phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), and inoculum dose were chosen for this purpose which influenced the cell growth as well as bacoside-A production. (Table 1). These factors were investigated by completing eight experiments. Each factor was examined at two levels: −1 for low level and +1 for high level, and a center point was run to evaluate the linear and curvature effects of the variables. The experimental design is shown in Table 2. First-order model assumption was applied to analyze the experimental response and the coefficient value of the variables under investigation. The maximum extent of the *t* value indicates the major impact of the variable on the response, and the minimum *p* value ($p < 0.05$) indicates a more significant influence. The components with a high positive *t* value and low *p* value less than 0.05 were selected for subsequent optimization.

Response surface methodology Design-Expert® software version 8.0.7.1 (Stat Ease® Inc., Minneapolis, MN, trial version) was used to formulate 2^4 factorial central composite design (CCD), to determine the optimal level of medium constituents. Thirty combinations were generated for the four selected variables, namely glucose, KNO_3, KH_2PO_4, and inoculum. Each variable at five different concentration levels was used for optimization of cell growth and bacoside-A production. After 21 d of incubation, the cells were harvested to estimate the cell biomass and bacoside-A content. The cell growth and bacoside-A content were determined and recorded. The system behavior was interpreted using the following quadratic equation:

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

In this equation, Y indicates predicted response; β_o is the offset term; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction coefficients; and χ_i is the coded value of variables.

The significance of the equation was obtained by Fisher's *F* test, and the variance was obtained by multiple coefficients of determination (R^2). Response surface (3D) and contour plots (2D) were obtained using Design-Expert® software based on the response analysis (cell biomass and bacoside-A content) to determine the interactive effects of the most significant variables affecting cell growth and bacoside-A content.

Validation of response surface model In order to verify the predicted results obtained using an optimization function in the RSM, an experiment was conducted in 250-mL Erlenmeyer flasks containing 5.67% glucose, 0.313% KNO_3, 0.291% KH_2PO_4, and 0.66% inoculum in addition to standard MS medium constituents. The cell growth and bacoside-A content were determined to record the response.

Table 2. Experimental design matrix with response obtained (cell growth as fresh cell weight (FCW) and bacoside-A (BacA) production for experimental protocols in Plackett–Burman design for culture medium optimization

Run order	A	B	C	D	E	F	Responses	
	Glucose	KNO_3	KH_2PO_4	$MgSO_4$	$CaCl_2$	Inoculum	FCW (g L^{-1})	BacA (mg g^{-1} DW)
1	−1	1	−1	−1	1	−1	7.02	7.32
2	−1	−1	−1	1	1	1	4.82	6.32
3	1	−1	−1	−1	−1	1	7.86	7.01
4	1	1	1	1	1	1	12.78	7.75
5	−1	−1	1	−1	−1	1	7.17	7.23
6	1	−1	1	−1	1	−1	12.32	7.67
7	1	1	−1	1	−1	−1	12.74	7.53
8	−1	−1	1	1	−1	−1	9.67	7.08

DW dry weight, *FCW* fresh cell weight

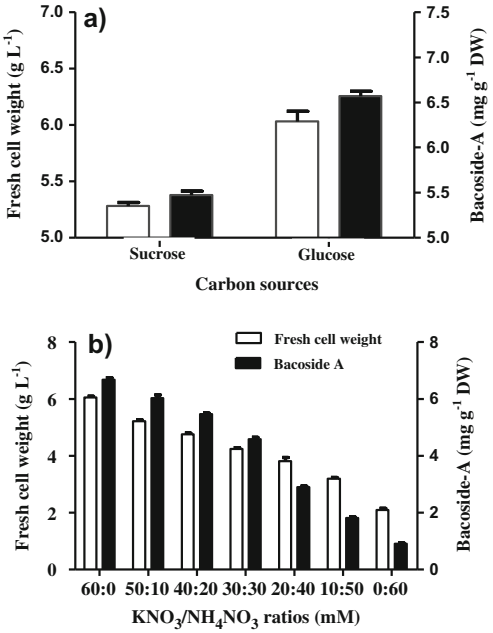

Fig. 1. The effect of (*a*) carbon sources (glucose and sucrose) and (*b*) nitrogen sources on cell growth and bacoside-A levels in cell suspension cultures of *B. monnieri*. *Bars* represent mean ± SD ($n = 3$).

Analytical methods Cell suspensions were filtered through Whatman filter paper grade no. 1 (11 μm) (GE Healthcare Life Sciences, New Delhi, India) to harvest the cells. The cells in the filter were washed with distilled water to remove the medium, and the fresh cell weight (FCW) was recorded. The cells were then dried to constant weight at 60°C in a hot air oven (Narang Scientific Works Pvt. Ltd., New Delhi, India) and the dry weight (DW) was recorded. The increase in the cell weight was determined by dividing the dry weight of the biomass harvested by the weight of the inoculum. The dried cell samples were powdered using a mortar and pestle, and powdered samples (1.0 g each in triplicate) were extracted and purified for the estimation of the bacoside-A content using the method described by Phrompittayarat *et al.* (2007). In brief, powdered samples (1.0 g each in triplicate) were soaked in 10.0 mL water for 24 h. These were filtered through glass wool and the filtrates were discarded. The residues were extracted with 20 mL of aqueous 95% (*v*/*v*) ethanol for 3 d. These were then filtered through glass wool. The extraction of residue was repeated three times (×20 mL) with 95% (*v*/*v*) ethanol. Filtrates from the three extractions were pooled and dried *in*

Table 3. The effects of different concentrations of NAA and KIN on cell growth, based on dry weight (DW) and bacoside-A content in cell suspension cultures of *B. monnieri*

NAA (μM)	KIN	DW (g L^{-1})	Bacoside-A (mg g^{-1} DW)
2.5	1.0	5.01 ± 0.37a	2.52 ± 0.32c
2.5	2.5	4.14 ± 0.31b	2.47 ± 0.26c
2.5	3.5	4.80 ± 0.28ab	3.89 ± 0.11b
5.0	1.0	5.52 ± 0.37a	5.56 ± 0.27a
5.0	2.5	4.08 ± 0.16bc	2.28 ± 0.27c
5.0	3.5	3.52 ± 0.26c	1.76 ± 0.16 cd
7.5	1.0	3.31 ± 0.17c	1.52 ± 0.13d
7.5	2.5	5.09 ± 0.22a	2.63 ± 0.22c
7.5	3.5	4.12 ± 0.26b	2.61 ± 0.11c

Values sharing a common letter within the columns are not significant at $p < 0.05$. Values are mean ± SE ($n = 3$)

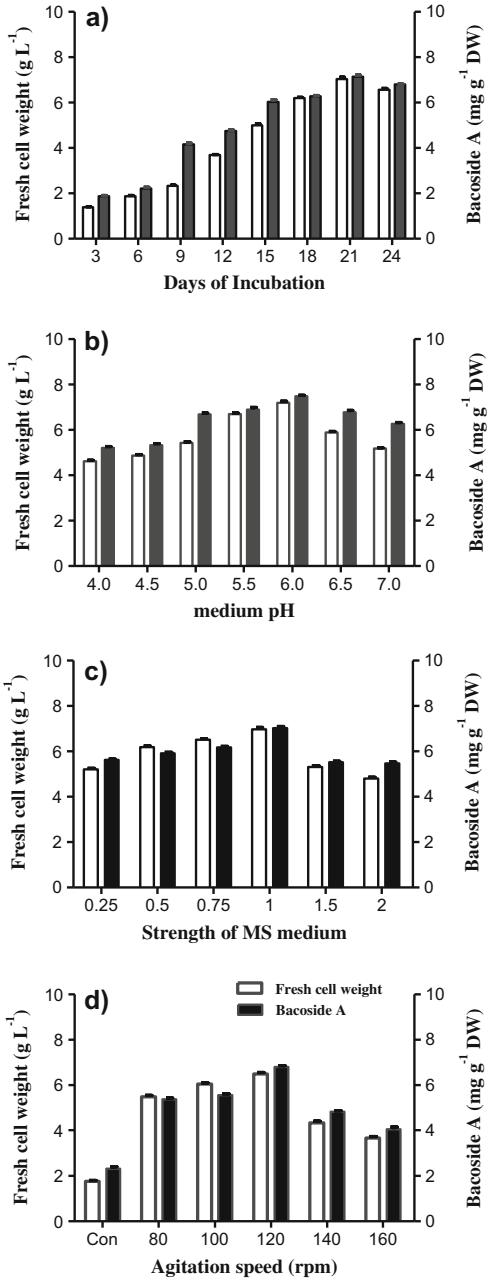

Fig. 2. The effect of (*a*) days of incubation, (*b*) medium pH, (*c*) strength of MS medium, and (*d*) agitation speed on cell growth and bacoside-A content by cell suspension cultures of *B. monnieri*. *Bars* represents mean ± SD ($n = 3$).

Table 4. ANOVA table for the determination of variables with a highly significant role for cell growth based on fresh cell weight (FCW) and bacoside-A (BacA) production using the Plackett–Burman design

Factors	*t* coefficient		Studied effect		Contribution (%)		Prob > *F* *p* value	
	FCW	BacA	FCW	BacA	FCW	BacA	FCW	BacA
A: glucose	+29.72	+20.78	4.32	0.55	45.24	25.62	0.0220	0.0424
B: KNO_3	+17.87	+12.62	2.27	0.48	12.51	19.94	0.0418	0.0480
C: KH_2PO_4	+25.87	+15.78	3.81	0.72	35.19	44.76	0.0249	0.0321
D: $MgSO_4{\cdot}7H_2O$	−0.78	−0.94	−0.85	−0.27	1.73	2.39	0.1113	0.1369
E: $CaCl_2{\cdot}2H_2O$	−0.25	+0.71	−0.060	0.097	8.76	0.81	0.7567	0.2290
F: inoculum	+9.23	−9.46	1.47	−0.17	5.27	6.38	0.0442	0.0346
Model	–	–	–	–	–	–	0.0039	0.0055

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

vacuo (30°C) in a rotatory film evaporator (Yamato Scientific Co., Ltd., Tokyo, Japan). Residues were reconstituted in 1.0 mL methanol and filtered through 0.45-μm pore size filters (Millipore, Sigma-Aldrich®, Carrigtwohill, Ireland) prior to quantification using high-performance liquid chromatography (HPLC). Quantification of the bacoside-A content in the purified extracts was conducted using reverse-phase HPLC (Waters Corporation, Milford, MA) equipped with a high-pressure binary pump system (515), diode array detector (2998), and Rheodyne® injector with 20 μL sample loop. Samples (20 μL) were injected through the injector into a Sunfire™ C18 column, 250 mm × 4.6 mm, particle size 5.0 μm (Waters Corporation), and elution was carried out in an isocratic mode with a mobile phase consisting of aqueous acetonitrile (65:35 *v*/*v*) containing 0.2% (*v*/*v*) phosphoric acid (pH 3.0) at a flow rate of 1.0 mL min^{-1}. Column eluates were monitored with an online photodiode array detector (Waters Corporation) set at 205 nm. Calculations were completed using external standard curves, which were plotted using known quantities of standard compounds (individually bacoside A3, bacopaside II, bacosaponin C, and bacopaside X) (Sigma-Aldrich® Company, St Louis, MO).

Statistical analysis Preliminary screening experiments conducted for cell growth and bacoside-A production were performed in triplicate, and values were expressed as mean ± SE. These data were analyzed using analysis of variance (ANOVA), and the means were compared by Tukey's honestly significant difference test. All the analyses were performed by using GraphPad Prism 5.1 software (GraphPad Software Inc., La Jolla, CA).

Results

Factors effecting cell growth and bacoside-A production Callus was established from leaf explants taken from actively growing 15–20-d-old microshoots of *B. monnieri* on basal MS medium supplemented with 5.0 μM NAA and 2.5 μM KIN. Initially, the callus tissue obtained was compact, green, and morphogenetic, which became friable and non-morphogenetic after three to four subcultures on medium supplemented with casein hydrolysate.

Cell growth and bacoside-A content varied significantly in cultures in the presence of sucrose, glucose, and KNO_3/NH_4NO_3. Glucose significantly increased the cell growth and bacoside-A content compared to sucrose (Fig. 1*a*). Among the different concentration ratios of KNO_3/NH_4NO_3 tested, cell growth and bacoside-A content increased significantly when the concentration of KNO_3 was higher than NH_4NO_3 in the medium (Fig. 1*b*). Maximum cell growth and bacoside-A levels were recorded in medium containing KNO_3 as the only source of nitrogen, and minimum cell growth and bacoside-A levels were noted when ammonium nitrate was used as sole source of nitrogen (Fig. 1*b*).

Different combinations of NAA and KIN significantly affected the cell growth and bacoside-A content. Maximum cell growth (5.52 g L^{-1} FCW) and bacoside-A levels (5.56 mg g^{-1} DW) were recorded in a medium supplemented with 5.0 μM NAA and 1.0 μM KIN followed by 2.5 μM NAA and 3.5 μM KIN (Table 3).

With an increase in the incubation time, cell growth and bacoside-A content increased for up to 21 d and decreased thereafter. Maximum cell growth and bacoside-A content were observed at 21 d of culture (Fig. 2*a*). The pH of the medium also affected cell growth and bacoside-A content. The maximum cell growth and bacoside-A content were recorded when cultures were grown in the medium pH of 6.0. No significant differences were observed between pH 5.5 and 6.0 relative to cell growth, and for bacoside-A production, no significant differences were observed between pH 5.0 and 6.0 (Fig. 2*b*). Among the different basal MS media strengths tested, suspension cultures grown on full-strength basal MS medium showed the maximum cell biomass compared to other media formulations (Fig. 2*c*). Bacoside-A levels increased from 5.62 mg g^{-1} DW to 7.03 mg g^{-1} DW with an increase

Table 5. Central composite experimental design matrix with experimental and predicted values for media optimization for cell growth based on fresh cell weight (FCW) and bacoside-A (BacA) production: milligrams per gram dry weight (DW)

Trials	Actual values (%)				Responses			
	Glucose (A)	KNO_3 (B)	KH_2PO_4 (C)	Inoculum (F)	Experimental		Predicted	
					FCW ($g\ L^{-1}$)	BacA ($mg\ g^{-1}$)	FCW ($g\ L^{-1}$)	BacA ($mg\ g^{-1}$ DW)
1	6.0	0.15	0.3	0.8	10.08	8.57	8.03	7.99
2	1.5	0.15	0.3	0.2	3.95	2.08	4.76	2.81
3	3.75	0.52	0.2	0.5	10.42	6.53	10.06	8.39
4	6.0	0.9	0.3	0.2	4.08	6.02	5.45	6.76
5	1.5	0.15	0.1	0.2	1.95	1.08	2.24	2.09
6	3.75	0.52	0.2	0.5	10.26	8.73	10.06	7.39
7	1.5	0.15	0.3	0.8	9.08	8.37	9.73	8.25
8	6.0	0.9	0.1	0.2	3.78	7.83	2.93	7.72
9	3.75	0.52	0.2	0.5	11.08	6.01	8.06	8.39
10	1.5	0.9	0.1	0.2	3.08	7.28	2.66	7.75
11	6.0	0.15	0.3	0.2	4.85	6.51	2.62	6.17
12	1.5	0.9	0.1	0.8	7.04	7.73	8.07	7.84
13	1.5	0.9	0.3	0.8	9.67	7.03	9.74	8.53
14	3.75	0.52	0.2	0.5	10.46	7.98	10.06	8.39
15	1.5	0.15	0.1	0.8	10.98	4.73	9.13	4.88
16	6.0	0.9	0.1	0.8	11.07	7.03	10.79	7.19
17	1.5	0.9	0.3	0.2	8.09	6.81	7.26	5.79
18	6.0	0.15	0.1	0.8	7.88	5.82	7.51	6.61
19	6.0	0.15	0.1	0.2	1.73	5.05	2.18	4.44
20	6.0	0.9	0.3	0.8	13.02	9.84	12.58	9.79
21	3.75	0.52	0.2	1.1	9.38	8.03	10.70	7.45
22	3.75	0.52	0.2	0.06	3.38	6.23	3.84	6.07
23	3.75	0.52	0.2	0.5	8.99	9.59	10.06	8.39
24	3.75	0.33	0.2	0.5	8.18	7.73	8.67	6.56
25	8.25	0.52	0.2	0.5	4.88	7.02	5.73	7.32
26	0.125	0.52	0.2	0.5	5.38	7.02	5.08	5.58
27	3.75	0.52	0.2	0.5	9.78	9.1	10.06	8.39
28	3.75	0.52	0	0.5	9.38	8.87	9.95	4.33
29	3.75	1.27	0.2	0.5	4.12	9.09	4.52	8.97
30	3.75	0.52	0.4	0.5	11.51	6.98	12.06	6.73

in medium strength from 1/4× to 1× basal MS medium. Among the different agitation speeds tested, the maximum cell growth and bacoside-A content were recorded in cell suspension cultures kept at 120 rpm on a shaker, whereas cell suspension cultures in static medium (control) showed the minimum cell biomass and accumulated lower levels of bacoside-A (Fig. 2*d*).

Plackett-Burman (PB) design Experimental results of the PB design showed significant variations in cell growth and bacoside-A content (Table 4). In the present study, *t* values for glucose, KNO_3, KH_2PO_4, and inoculum were significant factors that affected the cell growth and bacoside-A production. Table 4 shows the *t* value (*t* coefficient), studied effect, contribution (%), and *p* value of the PB analysis. Glucose, KNO_3, and KH_2PO_4 showed positive influences, while $CaCl_2$ and $MgSO_4$ did not show significant influences on cell growth and bacoside-A yield (Table 4). The media component quantities of glucose, KNO_3, KH_2PO_4, and inoculum which demonstrated significant impacts as determined by the PB design experiments were then modeled using RSM.

Response surface methodology The four variables (glucose, KNO_3, KH_2PO_4, and inoculum) were further evaluated using response surface methodology to determine their optimal concentrations and interactions. Table 5 shows various media

Table 6. Regression coefficients and significance for response surface model

Source	Sum of squares		Df		Mean squares		*F* value		Prob > *F*	
	FCW	BacA	FCW	BacA	FCW	BacA	FCW	BacA	FCW	BacA
Model	635.00	109.69	14	14	45.36	7.83	35.38	4.34	<0.0001	<0.0001
A	9.26	10.22	1	1	9.28	10.22	7.24	5.67	0.0168	0.0310
B	6.46	16.40	1	1	6.46	16.40	5.04	9.09	0.0403	0.0087
C	5.22	6.78	1	1	5.22	6.78	4.07	3.67	0.0620	0.0717
F	443.2	33.61	1	1	443.22	33.61	345.8	18.63	<0.0001	0.0001
AB	0.11	5.70	1	1	0.11	5.70	0.089	3.16	0.7697	0.0958
AC	4.32	1.01	1	1	4.32	1.01	3.37	0.56	0.0864	0.4670
AF	19.74	0.39	1	1	19.74	0.39	15.40	0.21	0.0014	0.6497
BC	4.34	7.22	1	1	4.34	7.22	3.38	4.00	0.857	0.0639
BF	30.00	7.30	1	1	30.00	7.30	23.41	4.05	0.0002	0.0626
CF	8.54	7.01	1	1	8.54	7.01	6.66	3.88	0.0209	0.0675
A^2	43.13	8.00	1	1	43.13	8.00	33.65	4.43	<0.0001	0.0525
B^2	22.72	1.67	1	1	22.72	1.67	17.72	0.93	0.0008	0.3512
C^2	0.91	8.35	1	1	0.91	8.35	0.71	4.63	0.4134	0.0482
F^2	4.11	0.22	1	1	4.11	0.22	3.21	0.12	0.0936	0.7341
Lack of fit	16.70	16.67	10	10	1.67	1.67	3.30	0.80	0.0996	0.6426

A glucose, *B* KNO_3, *C* KH_2PO_4, *F* inoculum, *FCW* fresh cell weight, *Bac A* bacoside-A production, milligrams per gram dry weight (DW)

combinations used, cell growth, and production of bacoside-A (experimental and predicted). Experimental data were subjected to regression and graphical analyses. The interactions between media components for biomass accumulation (model 1) and production of bacoside-A (model 2) were obtained by the following equations:

$$\begin{aligned} \textit{Cell biomass}\left(\mathrm{g\,L^{-1}}\right) = {} & +10.06 + 0.64\,\mathrm{A} - 0.56\,\mathrm{B} + 0.53\,\mathrm{C} \\ & +4.46\,\mathrm{F} + 0.084\,\mathrm{AB} - 0.52\,\mathrm{AC} + 1.11\,\mathrm{AF} \\ & +0.52\,\mathrm{BC} - 1.37\,\mathrm{BF} - 0.73\,\mathrm{CF} - 1.40\,\mathrm{A2} \\ & -1.11\,\mathrm{B2} + 0.24\,\mathrm{C2} + 0.43\,\mathrm{F2} \end{aligned} \quad (\text{model1})$$

$$\begin{aligned} \textit{Bacoside}\text{-A}\left(\mathrm{mg\,g^{-1}DW}\right) = {} & +8.39 + 0.68\,\mathrm{A} + 0.89\,\mathrm{B} + 0.60\,\mathrm{C} \\ & +1.23\,\mathrm{F} - 0.60\,\mathrm{AB} + 0.25\,\mathrm{AC} \\ & -0.16\,\mathrm{AF} - 0.67\,\mathrm{BC} - 0.68\,\mathrm{BF} + 0.66\,\mathrm{CF} \\ & -0.61\,\mathrm{A2} - 0.30\,\mathrm{B2} - 0.71\,\mathrm{C2} - 0.099\,\mathrm{F2} \end{aligned} \quad (\text{model2})$$

where *A* is glucose, *B* is potassium nitrate, *C* is potassium dihydrogen ortho-phosphate, and *F* is inoculum.

The data were analyzed by ANOVA, and the ANOVA model equation indicated that the model was highly significant, with a very low probability value "Model Prob > F" 0.0001 (Table 6). In this case, *A*, *B*, and *F* are significant model terms. An R^2 value greater than 0.9 indicated a highly significant association. The R^2 values for cell biomass and bacoside-A production were 0.97 and 0.93, respectively, which indicated adequate model fitness. The adjusted determination coefficient (adjusted R^2 = 0.94 and 0.71 for cell biomass and bacoside-A content, respectively) also demonstrated a high significance of the model. A predicted R^2 value of 0.82 indicated acceptable reasonable predictability of the model for bacoside-A content. The comparatively lower value of the coefficient of variation (12.94 cell growth and 18.66 production of bacoside-A) with acceptable precision values of 23.13 and 9.37 indicated an adequate precision and accuracy of the models.

Response surface curves for cell growth and bacoside-A production that show interaction between % inoculum and glucose, and % inoculum and KNO_3, respectively, are presented in Fig. 3. Among the factors studied (*A*: glucose, *B*: KNO_3, *C*: KH_2PO_4, and *F*: inoculum), the largest effect by far was observed with the inoculum dose compared to other factors. For cell growth, maximum interactive effect was observed between inoculum dose and glucose, while for bacoside-A, it was between inoculum dose and KNO_3 (Table 6). A slope or curvature in Fig. 3 shows that cell growth and bacoside-A production were sensitive to these factors.

Validation of response surface model Based on the regression equation, the optimum levels of test variables for maximum cell growth and bacoside-A production were predicted as 5.67% glucose, 0.313% KNO_3, 0.291% KH_2PO_4, and 0.66% inoculum. In order to verify the predicted results, an experiment was performed under the optimized conditions. The developed model predicted a cell biomass of 12.58 g L^{-1} FCW and bacoside-A level of 9.79 mg g^{-1} DW, which was

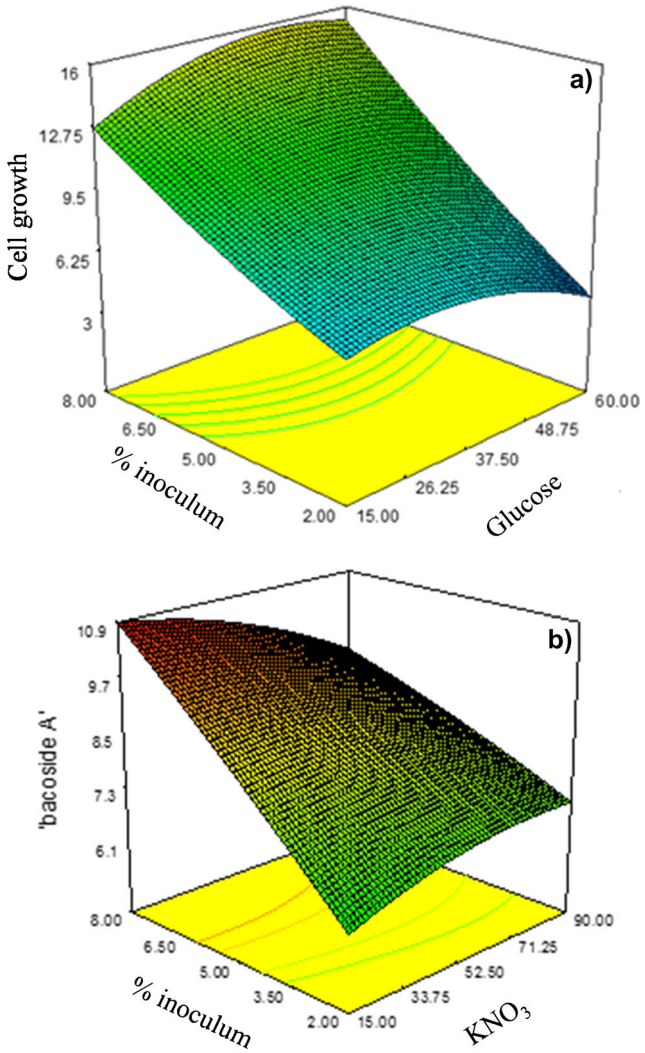

Fig. 3. Response surface curves showing (*a*) interactive effect between % inoculum and glucose for cell growth and (*b*) interactive effect between % inoculum and KNO_3 for bacoside-A production.

experimentally verified and yielded a cell biomass of 13.02 g L^{-1} FCW and bacoside-A level of 9.84 mg g^{-1} DW. These results suggested that experimental and predicted values for cell growth and bacoside-A production were in good agreement.

Discussion

The present study was conducted to optimize the medium and conditions for enhancing bacoside-A production from cell suspension cultures of *B. monnieri*. In this investigation, initially, the medium was standardized by testing one factor at a time followed by the identification of medium components and other factors, which significantly influence the growth of cells and accumulation of bacoside-A in suspension cultures. This was accomplished by designing experiments using a statistical tool called Plackett–Burman (PB) design. The components identified to have significant impacts on cell growth and bacoside-A accumulation were further optimized by response surface methodology (RSM) (Box and Behnken 1960). The RSM has been used to optimize media components and culture conditions for the growth and secondary metabolite production from different types of cultures (Das *et al.* 2001; Prakash and Srivastava 2005; Singh and Chaturvedi 2012a; Bansal *et al.* 2015; Heng *et al.* 2015). Previously, Rahman *et al.* (2002) investigated *B. monnieri* cell suspension cultures for the production of bacoside-A using a traditional approach.

In the present investigation, calli were induced from leaf explants on MS medium supplemented with 5.0 μM NAA and 2.5 μM KIN. The friable callus tissue required for establishment of cell suspension culture was obtained on a medium that was supplemented with casein hydrolysate. The inclusion of casein hydrolysate in the callus culture medium has been found to be beneficial for transforming compact callus into friable callus. In the present study, supplementation of 1.0 g L^{-1} casein hydrolysate in the MS medium resulted in transforming compact and morphogenetic callus into friable callus. Similar results were reported previously, in which MS medium supplemented with 1.0 g L^{-1} casein hydrolysate supported the active proliferation of friable green callus in *B. monnieri* (Rahman *et al.* 2002) and *Rosa damascene* (Kumar *et al.* 2000). Llamoca-Z'arate *et al.* (1999) also reported the requirement of 400 mg L^{-1} casein hydrolysate for producing friable callus in *Opuntia ficus-indica.*

Incorporation of NAA and KIN in the medium altered the growth and bacoside-A levels in cell suspension culture. In the present study, maximum cell growth and bacoside-A levels were achieved on the medium supplemented with 5.0 μM NAA and 1.0 μM KIN. Auxin and cytokinin are known to influence secondary metabolite yield including bioactive compounds in cell and callus cultures of many plant species (Pavlov *et al.* 2000; Zhao *et al.* 2001; Lian *et al.* 2002; Nagella and Murthy 2010).

In order to identify better carbon and nitrogen substrates, different commonly used carbon sources such as sucrose and glucose, and nitrogen sources such as nitrate and ammonia were used as nutrients. The present study results indicated that both cell growth and bacoside-A production increased in the presence of glucose as a carbon source and potassium nitrate as a nitrogen source. As sucrose is the translocatable sugar in plants, it has been found to be mostly beneficial for intact organ cultures (Fuentes *et al.* 2000). However, the beneficial effect of glucose in cell suspension cultures in this study could be due to the lack of requirement for translocation. Similar findings were reported by Prakash and Srivastava (2005) in cell suspension cultures of *Azadirachta indica*. For production of artermisinin by hairy roots of *Artemisia annua*, glucose has also been reported to be superior to other sugars (Weathers *et al.* 2004). The

cell biomass and bacoside-A production were significantly improved in a medium with a high KNO_3/NH_4NO_3 ratio. Although both salts provide inorganic nitrogen, NH_4^+ provides a reduced form of nitrogen, which is known to affect the growth, morphogenesis, and secondary metabolite production in plant cultures (Hyndman *et al.* 1982; Prakash and Srivastava 2005; Naik *et al.* 2011). Higher cell growth and bacoside-A production was achieved when potassium nitrate was used as the sole nitrogen source. A high concentration of ammonium had an inhibitory effect on growth and product formation. It was also observed that with ammonium as the sole nitrogen source, cell growth was significantly reduced and bacoside-A production was also suppressed. Similar findings were observed in which ammonium ion inhibition was reported in the production of saponin and polysaccharide by *Panax quinquefolium* (Zhong and Wang 1998) and azadirachtin production in cell cultures of *Azadirachta indica* (Prakash and Srivastava 2005). These results also supported the findings of Naik *et al.* (2011), which stressed the requirement for higher concentrations of the NO_3^- form of nitrogen for growth and production of bacoside-A in shoot cultures of *B. monnieri*.

A pH of 6.0 was found to be the most conducive for higher cell growth and bacoside-A content. The impact of pH on the production of secondary metabolites in cell cultures of *Withania somnifera* has been reported previously (Nagella and Murthy 2010). An acidic pH (4.5) of the culture medium was also reported to result in higher biomass and bacoside-A levels in microshoots of *B. monnieri* (Naik *et al.* 2010). These differential responses of plants to pH can be attributed to different requirements of shoot cultures compared to cell suspension cultures. Furthermore, nutrient availability for growing plants is also known to influence secondary metabolite production and growth (McDonald and Jackman 1989; Leifert *et al.*1992). The present study also revealed the dependency of growth and bacoside-A content on the strength of the medium. Maximum levels of bacoside-A and cell growth were observed on basal MS medium. These results are in agreement with earlier reports of Nagella and Murthy (2010) with cell suspension cultures of *Withania somnifera* and Lian *et al.* (2002) in *Panax ginseng*.

In addition to the MS medium strength, the agitation speed during incubation was also found to affect bacoside-A content. Maximum bacoside production was observed in suspension cultures agitated at a speed of 120 rpm. The effect of the agitation speed could be due to appropriate aeration of the cultures and adequate nutrient transfer between cells (Chattopadhyay *et al.* 2002). Results were found to confirm the earlier reports with *Spilanthes acmella* (Singh and Chaturvedi 2012b) and *Withania somnifera* (Chattopadhyay *et al.* 2002).

The role of nutrient levels on growth and secondary metabolite production is also well established. To achieve maximum production of secondary metabolites, it is essential to optimize each component of the culture medium, as there is a correlation between the concentration of different medium components and biosynthesis of secondary metabolites (Zhang and Greasham 1999). In order to provide complete nourishment, prerequisites for metabolite production, PB design was employed to investigate the significance of the medium constituents and inoculum density. Previously, a similar strategy was employed in various systems aimed to enhance secondary metabolite production (Garyali *et al.* 2014; Bansal *et al.* 2015). Therefore, the importance of the components in the culture medium and inoculum density was investigated by PB design. Four factors reflected by high t values and low p values, namely, glucose, KNO_3, KH_2PO_4, and inoculum, were selected as the most important variables affecting cell growth and bacoside-A productivity. Bansal *et al.* (2015) previously used the PB design to identify variables effecting growth of hairy roots and bacoside-A accumulation in *B. monnieri*.

In the present study, after optimization of the medium, both biomass accumulation and bacoside-A levels increased about twofold compared to the levels in a medium that was not optimized. In this study, the values predicted by the model generated were in agreement with the experimentally observed values. This suggested that the model developed using RSM had utility in process optimization. The utility of statistical packages such as RSM for optimizing the culture medium and other factors to improve productivity of secondary metabolite production in plants has been documented (Das *et al.* 2001; Prakash and Srivastava 2005; Bansal *et al.* 2015). Lu *et al.* (2008) reported a threefold enhancement in the lycopene yield after optimization of the culture medium using RSM. Similar findings were recorded by Pavlov *et al.* (2000), who reported enhancement of rosmarinic acid by 27-fold in *Lavandula vera* cell cultures.

Conclusion

The present study demonstrated that RSM was effective for optimizing the medium components and other factors to increase the cell growth and bacoside-A yield in *B. monnieri* cell suspension cultures. Statistical analysis using PB design experiments showed glucose, KNO_3, KH_2PO_4, and inoculum were the important medium components affecting bacoside-A production. Further optimization using RSM increased bacoside-A content by about 1.7-fold and cell growth by 2-fold. The reported protocol can be effectively utilized for the large-scale production of bacosides in the bioreactor.

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Compliance with ethical standards

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