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Shelf-life enhancement of bio-inoculant formulation by optimizing the trace metals ions in the culture medium for production of DAPG using fluorescent pseudomonad R62

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ABSTRACT

Statistical experimental design was used to optimize the concentration of trace elements for production of antifungal compound, 2,4-diacetylphloroglucinol (DAPG), from fluorescent pseudomonad R62 in shake-flask cultivation. The selection of the trace metal ions, influencing DAPG production, was done using Plackett–Burman design (PBD). Only Zn^{2+}, Mn^{2+} and MoO_4^{2-} were the most significant components ($p < 0.05$). A quadratic model was used to fit the response. Application of response surface methodology (RSM) revealed that the optimum values of the salts of the trace elements Zn^{2+} ($ZnSO_4 \cdot 7H_2O$), Mn^{2+} ($MnCl_2 \cdot 4H_2O$), and MoO_4^{2-} ($Na_2MoO_4 \cdot 2H_2O$) were 83, 42 and 135 μM, respectively, to achieve 125 mg/L of DAPG, which was nearly 13-fold more compared to its production in basal synthetic medium in shake flask. The studies in 14 L bioreactor resulted in 135 mg/L of DAPG at the end of 36 h of cultivation. The culture broth containing 125 mg/L of DAPG was found to be sufficient for keeping the bio-inoculant viable in non-sterile talcum powder-based formulations (which contained 25 μg DAPG/g carrier) when stored at 28 °C for 6 months. The structure of the purified DAPG was confirmed using ^1H NMR and mass spectrometry.

1. Introduction

In rhizosphere, microbial communities can significantly influence the development of phytopathogens, nutrient acquisition, heavy metal resistance, and ecological fitness of plants [1]. Biological control of diseases and pests of crops, using microbial inoculants, is receiving increased attention as an environment-friendly alternative to the use of chemical pesticides [2].

Pseudomonas strains are one of the most active and dominant bacteria in the rhizosphere, and have been intensively investigated as biocontrol agents [1–3]. They produce several antifungal substances such as pyoluteorin, 2,4-diacetyl phloroglucinol (DAPG), phenazine-1-carboxylic acid, and hydrogen cyanide, which suppress the plant diseases [1–4]. Among the metabolites produced by *Pseudomonas* spp., the compound DAPG has been extensively studied due to its broad spectrum activity [1–5]. Many studies indicate that DAPG is one of the most important isolated antibiotics from fluorescent pseudomonads [6,7]. This compound is widely distributed in antagonistic *Pseudomonas fluorescens* strains that occur in natural disease-suppressive soil [8].

The present study was carried out on fluorescent pseudomonad strain R62 isolated from endo-rhizospheric root regime of wheat [9]. The culture broth of the strain is to be used for carrier-based formulations of bio-inoculants for agronomical purposes. Moreover, it has been found that the presence of 10 μg DAPG per gram of formulation (which also contained 54.6 μg siderophore per gram formulation) suppressed the contamination proliferation when such formulations were under shelf-storage [10]. This would mean that the fermented broth should have at least 50 mg DAPG/L when 20 mL of this broth is used for preparing 100 g bio-inoculant formulation. This level of DAPG in the broth is termed as minimum desirable level (MDL). Using un-optimized Schlegel's medium containing trace elements, it was not possible to achieve 50 mg DAPG/L in the culture broth. Although the broth can be concentrated to achieve MDL, optimization of the process for enhanced DAPG production would significantly reduce the intermediate concentration steps.

The researchers have mostly used complex medium with different nitrogen sources (organic and inorganic) and at different phosphate levels for production of enhanced DAPG [2,4,11]. It is reported that the DAPG production was enhanced when *P.*

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Table 1
Trace elements and their values used in PBD.

Variables code	Independent variables	Levels (μM)	
		Low level (−1)	High level (+1)
x_1	$ZnSO_4{\cdot}7H_2O$	0.310	100
x_2	$MnCl_2{\cdot}4H_2O$	0.136	50
x_3	H_3BO_3	4.370	50
x_4	$Na_2MoO_4{\cdot}2H_2O$	0.112	100
x_5	$CoCl_2{\cdot}6H_2O$	0.757	50
x_6	$CuCl_2{\cdot}2H_2O$	0.053	100
x_7	$NiCl_2{\cdot}6H_2O$	0.075	50

fluorescens was grown in nutrient broth yeast extract medium amended with trace mineral solutions (Zn^{2+} and NH_4Mo^{2+}) [2]. However, the complex media contain un-quantifiable level of trace elements, which may lead to poor reproducibility and masking of real effects. The selection of synthetic medium is, therefore, preferred over complex medium to minimize interference from impurities. Therefore, the medium optimization with respect to trace minerals was done to maximize the DAPG production to a level higher than 50 mg/L. Thus, the current study focuses on the effect of amendment of trace elements in Schlegel's medium and their statistical optimization for improved DAPG production in shake flask cultures by the fluorescent pseudomonad R62, and on translation of the results on DAPG production in 14 L bioreactor. The effectiveness of DAPG in prolonging the shelf-life of the bio-inoculant formulation is also reported.

2. Materials and methods

2.1. Microorganism and culture conditions

Fluorescent pseudomonad strain R62 was isolated from the rhizosphere of wheat (variety UP 2338) from Badaun District, India, was used in the study [9]. This strain used in the present investigation was obtained from Dr. A.K. Sharma, Department of Biological Sciences, CBSH, GB Pant University of Agriculture and Technology (GBPUAT), Pantnagar, India. The strain has been tentatively characterized as *Pseudomonas jessenii* based on percent homology studies from amplification of 570 bp fragment of 16S rDNA at 99% [12]. The strain tested positive for phosphate solubilization, and biosynthesis of indole-3-acetic acid, siderophore (hydroxamate type) and 2,4-diacetyl phloroglucinol (DAPG), thus making it a potential plant growth promoting rhizobacteria [13]. The culture was maintained on 50% glycerol stocks at −20 °C in King's-B medium [14]. The strain was grown in 500 mL baffle-less Erlenmeyer flasks containing 100 mL modified Schlegel's medium which contained glycerol (13 g/L), succinic acid (0.5 g/L), Na_2HPO_4 (4.2 g/L), KH_2PO_4 (1.3 g/L), NH_4Cl (0.32 g/L), urea (0.35 g/L), KCl (0.35 g/L), NaCl (0.65 g/L), $MgSO_4{\cdot}7H_2O$ (0.50 g/L), ammonium ferric citrate (800 μg/L), $CaCl_2{\cdot}2H_2O$ (0.9 g/L) and trace elements solution (0.9 mL/L) [15]. The trace element solution contained $ZnSO_4{\cdot}7H_2O$ (10.0 mg), $CoCl_2{\cdot}6H_2O$ (20.0 mg), $MnCl_2{\cdot}4H_2O$ (3.0 mg), $CuCl_2{\cdot}2H_2O$ (1.0 mg), H_3BO_3 (30.0 mg), $NiCl_2{\cdot}6H_2O$ (2.0 mg), and $Na_2MoO_4{\cdot}2H_2O$ (3.0 mg) in 100 mL distilled water. All chemicals (extra pure grade) used in this study were obtained from Merck (Mumbai, India). The cultivation was carried out for 72 h at 28 °C in an orbital shaker (Scigenics Biotech, India) at 240-rev/min.

2.2. Statistical design for optimization of DAPG in shake flask

For all experiments, cultivation was done in 500 mL Erlenmeyer flasks containing 100 mL working volume. All experiments were carried out in triplicate, and the average DAPG concentration was taken as the dependent variable.

2.2.1. Identifying the significant variables using Plackett–Burman design (PBD)

The Plackett–Burman design was used to screen the trace elements which have significant impact on DAPG production. The PBD is a very useful design to screen '*n*' variables in just a minimum of '*n* + 1' number of experiments [16]. The PBD was used to identify the relative importance of various trace elements already present in Schlegel's medium. This design assesses the influence of the main factors as well as their interactions on the production of DAPG. A total of seven factors were studied as described in Table 1. A 2^5 PBD leading to 32 sets of experiments (Table 2), performed in triplicate, was used to verify the most significant factors affecting the DAPG production. The variables are coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the centre point, and ΔX_i is the step change value.

2.2.2. Optimization of screened components by RSM

Response surface methodology (RSM) has been a popular and effective method of solving multivariate problems and optimizing several responses in many types of experimentations. Box Benkhen design (BBD) is one of the methodologies for response surface methodology. After the identification of components most significantly affecting the production of DAPG using Plackett–Burman design, BBD was employed to optimize these major variables. The three variables were designated as x_1, x_2, and x_3. A total of 17 experimental runs with different combinations of three factors were carried out. For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between independent variables and response. For the three factors this equation is

$$Y_{\text{pred}} = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y_{pred} is the predicted response (DAPG concentration), x_i and x_j are input variables which influence the response Y. β_o is the constant, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient. A quadratic polynomial, which includes all interactions, was used to fit the data obtained. The "Design-Expert" software (version 5.0, Stat-Ease Inc., Minneapolis, USA) was used for generation and evaluation of the statistical experimental design. The optimal trace element composition for DAPG production was obtained by solving the regression equation (Eq. (1)) and by analyzing the response surface contour plots using the same software.

2.3. Cultivation of pseudomonad R62 in 14-L bioreactor for DAPG production

The strain was cultivated in a stirred type 14 L bioreactor (Chemap AG, Switzerland) containing 10 L of an appropriate medium at 28 °C for 36 h. The initial aeration rate and agitation speed were kept at 0.1 vvm and 350 rpm, respectively, to achieve turbulent flow regime and uniform dispersion of air bubbles in the liquid bulk. The rpm and air supply were varied during the fermentation to maintain minimum dissolved oxygen above 25% of the saturation value for active growth of the culture. The pH was controlled at 7.0 throughout the cultivation by automatic addition of 2N KOH/2N H_3PO_4. The growth kinetics was fitted to logistic equation, whereas substrate balance model was used to fit the glycerol consumption kinetics [17].

Table 2
Experimental setup of PBD and results of the fractional factorial design.

Run	x_1	x_2	x_3	x_4	x_5	x_6	x_7	DAPG (mg L^{-1})
1	−1	1	1	−1	1	1	1	0 5.0
2	1	1	−1	−1	1	1	−1	16.0
3	1	−1	1	1	1	−1	−1	22.0
4	1	−1	1	1	−1	−1	1	20.1
5	1	1	1	−1	1	−1	−1	14.0
6	1	1	1	1	−1	1	−1	23.0
7	−1	1	1	−1	−1	1	−1	02.0
8	1	−1	−1	−1	−1	−1	−1	09.0
9	−1	−1	−1	1	−1	−1	−1	07.0
10	1	−1	−1	1	−1	1	1	15.0
11	−1	−1	1	1	−1	1	−1	07.0
12	−1	−1	−1	−1	−1	1	1	01.0
13	1	1	−1	−1	−1	1	1	09.0
14	1	1	1	1	1	1	1	13.0
15	1	−1	1	−1	1	1	1	06.0
16	1	−1	1	−1	−1	1	−1	05.0
17	−1	1	−1	1	−1	1	1	10.0
18	−1	1	1	1	−1	−1	1	11.1
19	−1	1	1	1	1	−1	−1	06.0
20	−1	1	−1	−1	1	−1	1	05.0
21	1	1	−1	1	1	−1	1	20.0
22	−1	−1	−1	1	1	−1	1	03.0
23	1	1	−1	1	−1	−1	−1	20.0
24	−1	−1	−1	−1	1	1	−1	01.0
25	−1	−1	1	1	1	1	1	03.1
26	−1	−1	1	−1	−1	−1	1	01.1
27	−1	−1	1	−1	1	−1	−1	03.0
28	1	1	1	−1	−1	−1	1	11.0
29	1	−1	−1	−1	1	−1	1	07.0
30	1	−1	−1	1	1	1	−1	14.1
31	−1	1	−1	1	1	1	−1	07.0
32	−1	1	−1	−1	−1	−1	−1	03.0

Table 3
Results of the PBD regression analysis for DAPG production.

Term	Coefficient values	*t*-value	*p*-value
x_1	4.66	10.87	<0.0001
x_2	1.59	3.72	0.0010
x_4	3.22	7.51	<0.0001
x_5	−0.28	−0.66	0.5174
x_1x_4	1.16	2.70	0.0123
x_4x_5	−1.28	−2.99	0.0062

2.4. Estimation of cell growth, glycerol and DAPG

The cell growth and DAPG were estimated according to the assays described by Saharan et al. [10]. Residual glycerol in the culture broth was estimated calorimetrically according to the method described by Bok and Demain [18].

2.5. Mass spectrometry of DAPG

Mass spectra were obtained using a Qstar hybrid electrospray ionization high-resolution mass spectrometer (Applied Biosystems, USA) equipped with quadruple and Time of Flight (TOF) mass analyzers in tandem.

2.6. NMR spectroscopy of DAPG

The spectra were recorded on a 300-MHz NMR spectrometer (Brucker GmbH, Rheinstetten, Germany), equipped with a 5-mm, triple gradient TXI probe, at 300 K. The samples were dissolved in $CDCl_3$, contained in 5-mm NMR tube. All experiments were conducted at 25 °C and no shift relaxation agents were employed. The ^{1}H NMR chemical shift values were reported on the δ scale in ppm.

2.7. Preparation of talcum powder-based bio-inoculant formulations and determination of its shelf-life

Non-sterile talcum powder was used for making bio-inoculant formulations of the pseudomonad strain with two types of broths, one having 125 mg/L of DAPG (produced in culture broth of optimized medium) and the other containing 9 mg/L of DAPG (produced in culture broth of un-optimized medium). Both the broths, however, contained 1.5–2.0 × 10^{10} cfu/mL and were used for the preparation of formulations. To 98 mL of culture broth, 1 mL glycerol (final concentration – 1% v/v) and 1 mL of 10 g/L carboxymethylcellulose (final concentration – 0.1 g/L) were added as additives. Glycerol served as a carbon source and humectant for keeping the cells viable while CMC acted as an adhesive. The broth containing additives was mixed uniformly on a vortex mixer. To make 100 g of inorganic carrier-based formulations 80 g of the talcum powder and 20 mL of culture broth with additives were mixed following the method of Vidhyasekaran and Muthamilan [19]. Thus the two formulations contained 25 μg DAPG/g carrier and 1.8 μg DAPG/g carrier, respectively.The product was shade-dried to reduce the moisture content to ~18% and then packed in UV sterilized polythene bags and sealed. To study the shelf-life of the formulations, the formulated product was kept at 28 °C for 6 months. The numbers of surviving bacteria (cfu) were counted on King's B agar plates [14]. The initial counts of bacteria were 2.5 × 10^9 cfu/g before storing the formulations for shelf-life studies. Aseptic conditions were maintained throughout the process.

3. Results and discussion

3.1. Screening of important factors by Plackett–Burman design for DAPG production

Out of seven trace elements likely to affect DAPG production, responses for only three trace elements ($ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, and $Na_2MoO_4{\cdot}2H_2O$) were significant ($p < 0.05$) as per a 2-level Plackett–Burman design for DAPG production (Table 3). The predicted regression equation for the response Y (DAPG in mg/L) as a function of the coded values of critical variables was as follows:

$$Y = 9.34 + 4.66x_1 + 1.59x_2 + 3.22x_4 - 0.28x_5 + 1.16x_1x_4 - 1.28x_4x_5 \quad (2)$$

The results of *t*-test for variance between average of observation of two-level experiment and centre point showed that the difference was significant ($p < 0.01$). This result indicated that optimum point was in the domain of our experiment.

Table 4
Coded levels of major variables used in RSM.

Factor	Name	Units	−1 Level	0 Level	+1 Level
x_1	$ZnSO_4{\cdot}7H_2O$	μM	0.30	100.15	200
x_2	$MnCl_2{\cdot}4H_2O$	μM	0.13	50.06	100
x_3	$Na_2MoO_4{\cdot}2H_2O$	μM	0.11	100.05	200

Table 5
Response from Box Behnken design experiment (trace concentration in μM).

Run	x_1	x_2	x_3	DAPG (mg L^{-1})
1	1	1	0	83.5
2	0	0	0	118.5
3	−1	0	1	64.9
4	0	1	−1	48.1
5	−1	1	0	46.5
6	0	−1	1	91.3
7	0	0	0	123.9
8	0	1	1	72.0
9	−1	0	−1	47.3
10	1	0	−1	21.6
11	1	0	1	35.4
12	0	0	0	117.5
13	0	0	0	118.6
14	0	0	0	115.3
15	0	−1	−1	48.1
16	1	−1	0	48.1
17	−1	−1	0	81.9

3.2. Optimization of selected trace elements

Based on the identification of variables by the 2-level Plackett–Burman design, a 3-level Box Behnken design (BBD) was used for optimization of DAPG production. The limits for the three-screened variables $ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, and $Na_2MoO_4{\cdot}2H_2O$ are shown in Table 4. The BBD of these three variables in coded format is presented along with DAPG concentrations as responses in Table 5. In order to determine the maximum DAPG production corresponding to the optimum levels of the variables, a second-degree polynomial model was used to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, a second-degree polynomial model in coded unit (Eq. (3)) explains the significance of each variable and their second-order interactions in producing DAPG:

$$Y_{pred} = 34.92 + 0.53x_1 + 0.32x_2 + 0.95x_3 - 3.830E-03x_1^2 - 6.255E-03x_2^2 - 3.838E-03x_3^2 + 3.553E-03x_1x_2 - 9.470E-05x_1x_3 - 9.678E-04x_2x_3 \quad (3)$$

where Y_{pred} is the predicted value of DAPG concentration and x_1, x_2 and x_3 are the concentrations of $ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, and $Na_2MoO_4{\cdot}2H_2O$, respectively.

The first order main effects of $ZnSO_4{\cdot}7H_2O$ ($p = 0.1025$) and $Na_2MoO_4{\cdot}2H_2O$ ($p = 0.0093$) were less significant than their quadratic main effects ($p < 0.001$) on DAPG production (Table 6). The quadratic interaction effect of zinc sulphate and manganese

Table 6
Significance of quadratic model coefficients for DAPG production.

Factor	Coefficient values	*t*-values	*p*-value
x_1	−6.51	−1.88	0.1025
x_2	−2.41	−0.69	0.5098
x_3	12.33	3.56	0.0093
x_1^2	−38.19	−7.99	<0.0001
x_2^2	−15.60	−3.26	0.0138
x_3^2	−38.34	−8.02	<0.0001
x_1x_2	17.72	3.61	0.0086
x_1x_3	−0.95	0.19	0.8526
x_2x_3	−4.83	−0.99	0.3574

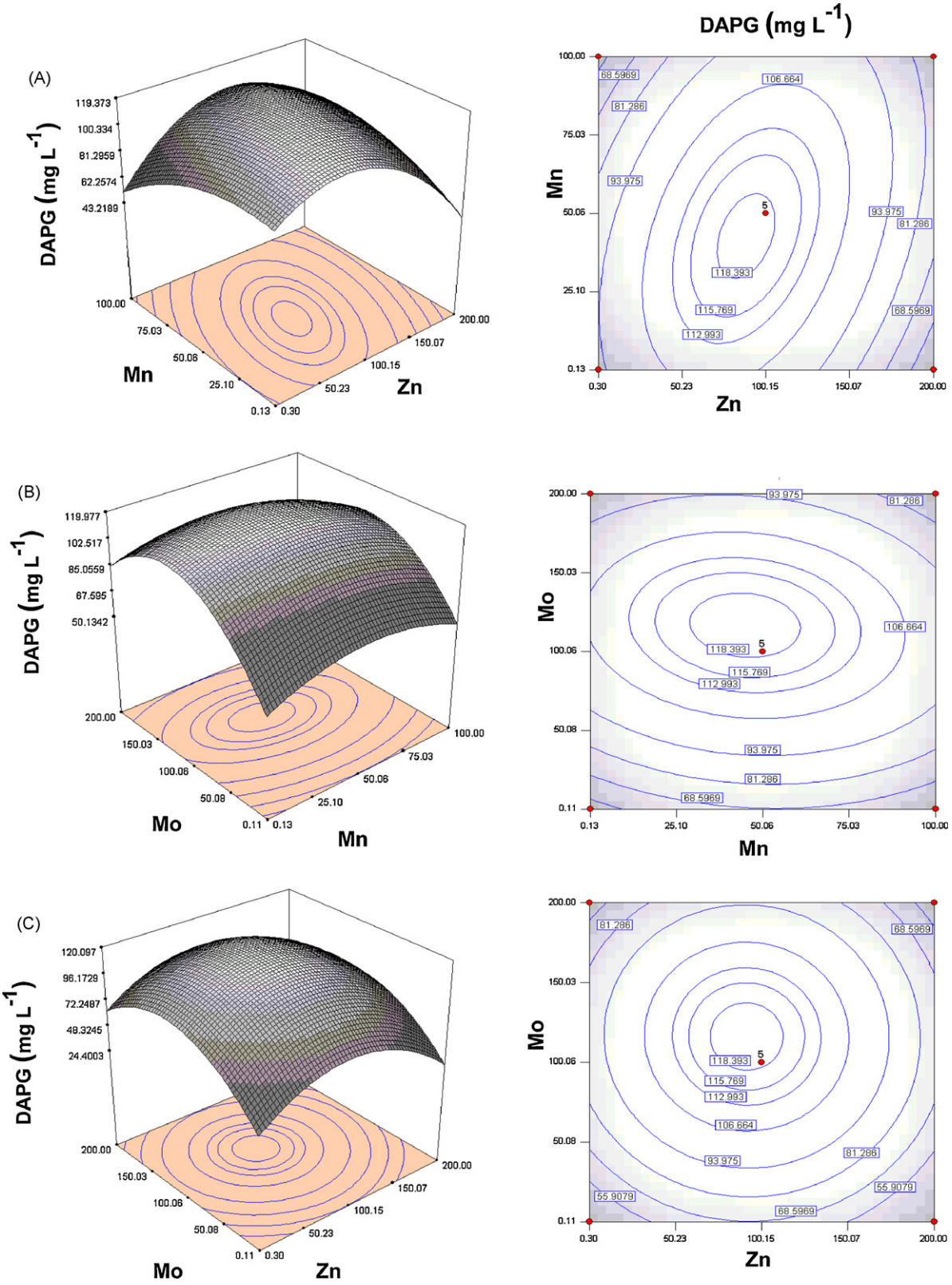

Fig. 1. Response surface and contour plots for the effects of (A) $ZnSO_4{\cdot}7H_2O$ (x_1) and $MnCl_2{\cdot}4H_2O$ (x_2), (B) $MnCl_2{\cdot}4H_2O$ (x_2) and $Na_2MoO_4{\cdot}2H_2O$ (x_3), and (C) $ZnSO_4{\cdot}7H_2O$ (x_1) and $Na_2MoO_4{\cdot}2H_2O$ (x_3) on DAPG (mg/L) production.

chloride ($p = 0.0086$) was quite significant among all the interactions. Thus, larger the magnitude of t-test and smaller the p-value, the more significant is the corresponding coefficient. The fitness of the model was examined by determination coefficient ($R^2 = 0.9632$), which implied that the sample variation of more than 96% was attributed to the variables, and only less than 4% of the total variance could not be explained by the model (Table 7). The generated response surfaces and contour plots developed using the fitted quadratic polynomial equation obtained from regression analysis are shown in Fig. 1. The effect of manganese chloride and zinc sul-

Table 7
ANOVA for response surface quadratic model obtained for DAPG production.

Term	DAPG
F-values	2.76
$p > F$[a]	0.0003
DF	16
R^2	0.9632
RMSE	981
Dep. mean	75.47
C.V.	12.99
PRESS	10186.50
Adj R^2	0.9160
Adeq precision[b]	12.553
Model sum of squares	17636.38
Residual sum of squares	673.26
Model mean square	1959.60
Residual mean square	96.18
F-value	20.37

[a] Value of "$p > F$" less than 0.05 indicate model terms are significant.
[b] The value >4 indicates adequate precision in the model.

phate on the DAPG production at a fixed sodium molybdate level is shown in Fig. 1A. Similarly, Fig. 1B and C represents the effect of two variables on the production of DAPG, while third variable was held at zero level. In all the three cases, a clear optimal convergence was observed to find the optimal levels of all the three independent variables on DAPG production. On the basis of optimization, the quadratic model predicted that the maximum production of 125 mg/L of DAPG was possible when $ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$ and $Na_2MoO_4{\cdot}2H_2O$ were at 83, 42 and 135 μM, respectively. To verify the predicted results an experiment was performed in triplicate in shake flasks and the response was compared with the predicted value from the model. The average concentration of DAPG in the broth was 119 mg/L, which suggests the accuracy of the model was 95.96%. Whereas the predicted value for DAPG by the model was 125 mg/L which translates into a deviation of only 4.20%. From earlier shake-flask studies it was observed that in unoptimized medium only 9 mg/L of DAPG was produced, therefore with this optimized levels of trace elements there was a 13-fold increase in DAPG production.

3.3. DAPG production in 14 L bioreactor

The pseudomonad R62 was cultivated in 14 L bioreactor containing 10 L Schlegel's medium with optimized levels of the three trace elements as mentioned above for the production of DAPG. The glycerol consumption was very rapid up to 18 h during which 4.3 g/L of dry cell weight was produced (Fig. 2). Higher biomass was achieved in bioreactor in comparison to shake-flask cultivation due to better aeration and agitation conditions. The dry cell weight leveled off to a maximum value of 4.5 g/L after 21 h indicating the onset of stationary phase. During this period, residual glycerol was observed to be less than 1.5 g/L. Once the culture entered the stationary phase, DAPG production was triggered and a maximum of 135 mg/L of DAPG was obtained at the end of 36 h amounting to a productivity of 3.75 mg/L h. The level of DAPG obtained in bioreactor was thus about the same as in shake flask, when optimized medium was used. It is pertinent to mention here that under these optimized medium conditions the pseudomonad R62 produced only 54 mg/L of the siderophore.

The DAPG concentration obtained in the current study was compared with other published data. It has been reported that a maximum level of 100 mg/L DAPG was achieved with *P. fluorescens* strain pf-5 using complex growth medium in 72 h [11]. In another study, 0.63 mg/L DAPG was produced in complex medium containing malt and yeast extracts by *P. fluorescens* Q2-87 [20]. Sucrose and Fe^{3+} increased DAPG production to 5.5 mg/L in *P. fluorescens* F113 when cultivated for 48 h [2]. However, using *P. fluorescens* S272 grown on 2% ethanol with complex nitrogen sources, a maximum value of 500 mg/L DAPG was reported in 72 h, but the levels of trace minerals were not discussed [4]. Micronutrients zinc (0.7 mM $ZnSO_4{\cdot}7H_2O$), copper (0.7 mM $CuSO_4$) and molybdenum (1 mM $Mo_7(NH_4)6O_{24}{\cdot}4H_2O$) amendment individually to the complex medium along with 1% glycerol increased the DAPG production to 6.6, 4.7, and 1.8 mg/L, respectively, in *P. fluorescens* CHAO when cultivated for 48 h [2]. In the current study, it was observed that zinc, manganese and molybdenum influenced the DAPG production, thus indicating that its production was strain specific.

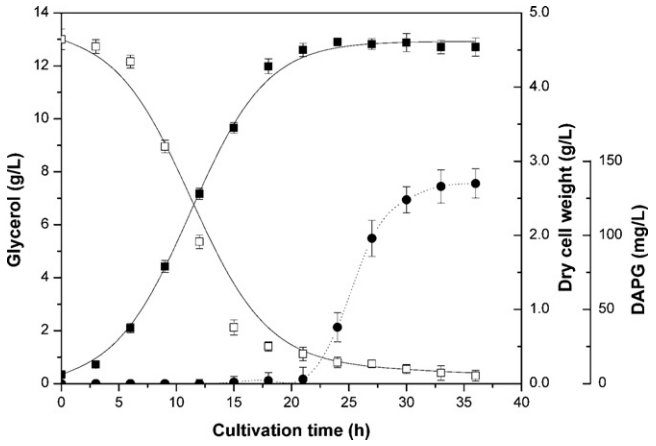

Fig. 2. The batch kinetic profiles of glycerol consumption (□), dry cell weight (■), and DAPG production (●) during growth of fluorescent pseudomonad strain R62 in 14 L bioreactor. The solid lines represent the model predictions. Data is shown as mean ± standard error ($n = 2$).

3.4. Identification of DAPG using ^{1}H NMR and mass spectrometry

The positive-ion FAB spectrum of the DAPG showed three ions related to the M_r, *m/z* 211 $(M+H)^+$, *m/z* 233 $(M+Na)^+$ and *m/z* 249 $(M+K)^+$. The ^{1}H NMR spectrum showed the presence of aromatic hydrogen and a singlet signal corresponding to aliphatic hydrogens. The chemical shifts with multiplicity and integration is provided in Table 8 for the DAPG structure shown in Fig. 3, and the results were compared to that of Zakrzewski et al. [21]. For a singlet aromatic proton, there is singlet acetyl proton with integration factor of 6, indicating the characteristic and confirmation of DAPG.

3.5. Shelf-life of the bio-inoculant formulation containing different concentrations of DAPG

The assessment of storage period on the viability of the pseudomonad strain R62 containing different concentrations of DAPG in non-sterile talc-based formulations was carried out at 28 °C. The formulation containing 25 μg DAPG/g carrier lowered the cfu/g from 2.5×10^9 to 1.3×10^8 and for the formulation containing 1.8 μg DAPG/g carrier the value decreased drastically to 3.2×10^4 over a period of 6 months (Fig. 4). It is worth mentioning that the cfu count should be more than 1.0×10^7 for making a formulation [22]. This indicates that to retain the viability of cells during storage, the level of DAPG played an important role probably by

Table 8
^{1}H NMR data for DAPG.

Proton (s)	Chemical shift (δ) (multiplicity, integration)	
	This study	Zakrzewski et al. [21]
OH	15.09 (s, 1H)	16.37 (s, br, 1H)
H-6	5.78 (s, 1H)	5.90 (s, 1H)
H-2′, H-4′	2.89 (s, 6H)	2.64 (s, 6H)

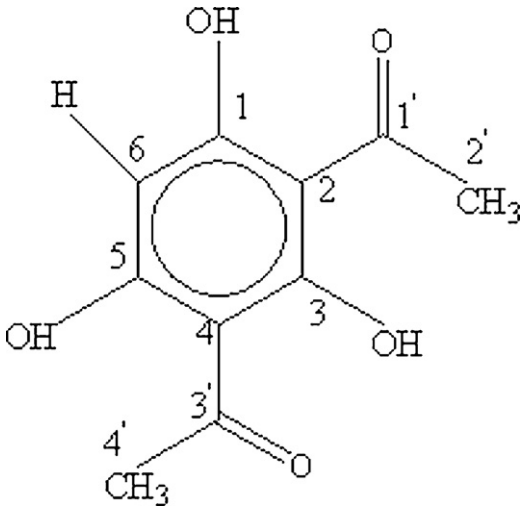

Fig. 3. The structure of 2,4-diacetylphloroglucinol (DAPG) with ^{1}H positions labeled.

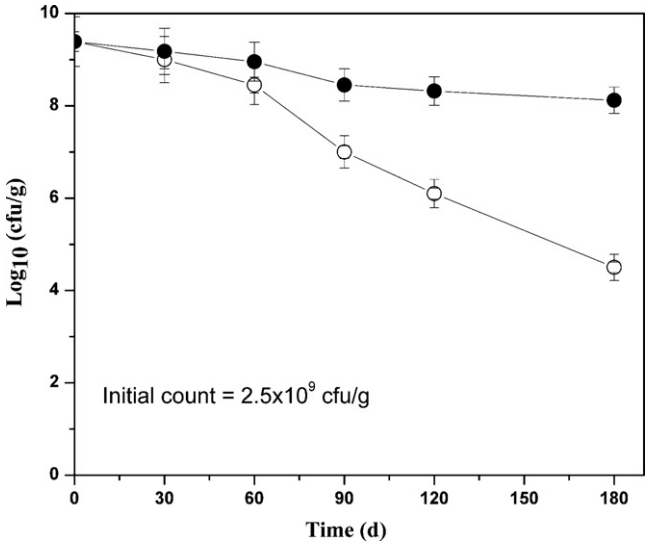

Fig. 4. The $\log_{10}$ (CFU/g) profiles over a period of 6 months storage time of bio-inoculant formulations containing 25 μg DAPG/g carrier (●) and 1.8 μg DAPG/g carrier (○). Data is shown as mean ± standard deviation (n = 3).

protecting the cells from contaminants [10]. Thus, the culture broth of the optimized medium, which contained 125 mg/L DAPG was effective in making a formulation containing 25 μg DAPG/g carrier such that the bio-inoculant could be stored for about 6 months as it still contained sufficient number of viable cells to be used in making bio-inoculant formulations. Even though the formulation contained only 10.8 μg siderophore/g carrier, the DAPG level in the formulation was able to improve the effectiveness of bio-inoculant in terms of shelf-life.

4. Conclusion

The screening and optimization of trace elements in the Schlegel's medium resulted in production of 125 mg/L of DAPG in shake flask and 135 mg/L in bioreactor by pseudomonad R62 when $ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$ and $Na_2MoO_4{\cdot}2H_2O$ were at 83, 42 and 135 μM, respectively, using response surface methodology. The optimized medium increased the DAPG production by 13-fold as compared to its level in un-optimized medium. Further, the culture broth containing 125 mg/L DAPG when used for preparing talc powder-based bio-inoculant formulations was able to significantly retain the viability of the cells for a period of 6 months at 28 °C.

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