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Optimization and Elucidation of Interactions between Ammonium, Nitrate and Phosphate in *Centella asiatica* Cell Culture Using Response Surface Methodology

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Abstract The effects of macronutrients (NO_3^-, NH_4^+ and PO_4^{3-}) on cell growth and triterpenoids production in *Centella asiatica* cell suspension cultures were analyzed using the Box-Behnken response surface model experimental design. In screening and optimization experiments, PO_4^{3-} as a single factor significantly influenced cell growth where increasing the phosphate level from 0.1 to 2.4 or 2.6 mM, elevated cell growth from 3.9 to 14~16 g/L. The optimum values predicted from the response surface model are 5.05 mM NH_4^+, 15.0 mM NO_3^- and 2.6 mM PO_4^{3-}, yielding 16.0 g/L cell dry weight with 99% fitness to the experimental data. While the NH_4^+-NO_3^- interaction influenced cell growth positively in the optimization experiment, NH_4^+ and NO_3^- as single factors; and interactions of NO_3^--PO_4^{3-}, NH_4^+-PO_4^{3-} and NH_4^+-NO_3^- were all negative in the screening experiment. Cell growth and the final pH level were positively affected by PO_4^{3-}, but negatively affected by NH_4^+ and NH_4^+-PO_4^{3-} interactions. The different effects of factors and their interactions on cell growth and final pH are influenced by a broad or narrow range of macronutrient concentrations. The productions of triterpenoids however were lower than 4 mg/g cell dry weight.

Keywords: response surface methodology, *Centella asiatica*, optimization, plant cell culture, modelling, fermentation

INTRODUCTION

Centella asiatica or locally known as 'pegaga' of the *Umbellifarae* species is a well-known herb in tropical and subtropical countries. Its bioactive properties, attributable to triterpenoids (TTP) and antioxidant compounds present include antibacterial, anti-malarial, anti-proliferative and wound healing. With the success of gene cloning that encodes enzymes of terpenoid synthesis, the genetic or metabolic engineering of plant terpenoid metabolism present an avenue for studies on terpenoid-based insect repellants, pest control or plants with enhanced terpenoid vitamins [1].

Living organisms use primary metabolic pathways to provide precursor metabolites and energy to anabolic pathways that synthesize cellular constituents required for growth and maintenance [2]. There are regulatory mechanisms, which control the production of primary and secondary metabolites, in order to avoid overproduction and excretion into the environment. To improve the yield or productivity of an organism, it is necessary to regulate this control mechanism through nutritional means such as manipulating carbon, nitrogen or phosphate uptake and/or manipulating the molecular genetic level [3]. Nitrogen and phosphate are essential elements in plant growth regulators, feeding deterrents and defense compounds and for the synthesis of DNA, ATP and phospholipids [4,5]. A large part of the available carbon source is stored intracellularly, mainly as starch and sucrose (up to 40% of the biomass dry weight at the start of the stationary phase), while the entire available nitrogen source is used up (about 7% of the biomass dry weight and mainly in the form of amino acids, proteins and nucleotides) [6].

As all these compounds work in synergy, a balance must be attained to ensure smooth metabolic activities. An excessive amount of one compound over another may be detrimental to cell viability. It has been shown that nitrogen and phosphate toxicities are not only functions of both the individual and collective level of ammonium,

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nitrate and phosphate, but also the level of sucrose and medium strategies [7]. Our studies show that a high sucrose level can suppress TTP production in *Centella asiatica* cell cultures [8]. In the optimization process where time efficiency and cost-effectiveness are the goals, a statistically-designed experiment has advantages over the conventional method of varying one variable at a time while holding others constant, since it supplies the needed information about the shape of the response using a minimum amount of resources [9].

The optimization approach involves screening experiments to identify important factors and a suitable concentration range, and further optimization using a statistical experimental design such as Response Surface Methodology (RSM) within the defined factors and concentration range. A mathematical model developed from statistically designed experiments can provide insight into the interactions between dependent and independent variables, and predict the optimal level of variables.

In this study, the screening and optimization of NO_3^-, NH_4^+ and PO_4^{3-} concentrations were carried out *via* the Box-Behnken RSM experimental design. The interaction between factors influencing cell growth was established and an empirical model describing the effect of the factors on cell dry weight was also developed. A test experiment was conducted using the selected levels of ammonium, nitrate and phosphate to validate the model.

MATERIALS AND METHODS

Cell Suspension Cultures

C. asiatica cell suspension culture was established in 1999 the Department of Biochemistry and Microbiology, Universiti Putra Malaysia. The cell suspension culture was initiated from callus culture derived from the leaves of *C. asiatica* cell line CA01.

Maintenance Medium and Experimental Approach

For cell culture maintenance, Murashige and Skoog medium plus Gamborg vitamin B5 was used, complimented with 1 mg/L kinetin (Sigma Co.), 2 mg/L 2-4-dichlorophenoxyacetic acid (Sigma Co.) and 3% w/v sucrose (Fisher Chemicals). Medium pH was adjusted to 5.7 to 5.75 prior to autoclaving. Cultures were shaken on 120 rpm orbital shaker and incubated at 25 ± 2°C under light intensity of 500 lux for 16 h photoperiod. 7-day-old stock cultures were used as inoculum (12.5% v/v). Environmental conditions were maintained as those of maintenance cultures. Cells were harvested on day 14.

Analytical Procedures and Statistical Analysis

The determination of cell dry weight (CDW) was done by vacuum filtration of culture and oven dried at 60°C. TTP were extracted from grinded dried cells using 100% methanol. The separation method for TTP analysis using High Pressure Liquid Chromatography (Waters) was adopted from Laugel *et al.* [10]. Chromatographic separation was performed with a C_{18} - 5 μm, 250 mm × 4.6 mm, stainless steel column (Phenomenex Luna, USA).

Response Surface Model Experimental Design

Three-level design for fitting response surfaces by combining 2^k factorials with incomplete block design was proposed by Box and Behnken [11]. The resulting design is usually very efficient in terms of the number of required runs, and they are either rotatable or nearly rotatable. The Box-Behnken design results in all points lying on a sphere of radius $\sqrt{2}$. Furthermore, the design does not contain any points at the vertices of the cubic region created by the upper and lower limits of each variable. This could be advantageous when the points on the corners of the cube represent factor-level combinations that are prohibitively expensive or impossible to test because of physical process constraints.

The experimental design and analysis of Box-Behnken design experiments were done using computer software Statgraphic® Plus Professional Version 5 (2000) (Rockville, USA). Both the screening and optimization experiment utilized the Box-Behnken design to optimize three factors at three levels with four replicates at the central point resulting in 16 runs. Central points were included in the design to improve estimates of quadratic effects and provide an additional degree of freedom for error and information about the center of the experimental region. The design is of resolution five where the two-factor interactions and quadratic effects can be estimated and are not confounded with one another [12]. A regression analysis on the data gave the quadratic model parameters as in Eq. (1) below:

$$Y=\beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 \quad \Sigma\beta_{ij} x_i x_j \tag{1}$$

Where Y is the predicted response
β_0 is the constant
β_i is the coefficient for linear
β_{ii} is the coefficient for quadratic
β_{ij} is the coefficient for interaction effects
x_i is the factor considered

The multiple coefficient of determination, R^2, explains the variability in dependent variables. The model was used to predict the optimum level and elucidate the interaction between factors within the specified range.

Pareto chart compare the relative importance of estimated effects of the factors. The chart also shows the effect of changing the factor settings. Positive effects indicate increase of the dependant variables when concentrations of independent variables increase. Negative effects show that the dependant variables decrease with the increase of independent variables.

Statistical Analysis

All data presented are the mean values of two replicates and their significant differences were analyzed by Dun-

Table 1. Effects of NH_4^+, NO_3^- and PO_4^{3-} concentrations on cell growth and final pH: Screening experiment

Run	NH_4^+ (mM)	NO_3^- (mM)	PO_4^{2-} (mM)	DW (g/L)	pH_f
1	10	20	1.25	14.1 ± 0.52^{a}	5.15 ± 0.37^{cde}
2	10	60	1.25	13.2 ± 0.76^{ab}	7.24 ± 0.69^{f}
3	30	20	1.25	10.3 ± 0.27^{e}	3.96 ± 0.21^{ab}
4	30	60	1.25	4.53 ± 0.32^{g}	4.95 ± 0.37^{cde}
5	10	40	0.1	3.89 ± 0.07^{g}	3.81 ± 0.02^{a}
6	10	40	2.4	13.2 ± 0.46^{ab}	8.24 ± 0.25^{g}
7	30	40	0.1	4.39 ± 0.20^{g}	3.97 ± 0.04^{ab}
8	30	40	2.4	6.09 ± 0.71^{f}	4.59 ± 1.56^{abcd}
9	20	20	0.1	3.92 ± 0.06^{g}	3.74 ± 0.06^{a}
10	20	20	2.4	13.9 ± 0.45^{a}	5.18 ± 0.49^{cde}
11	20	60	0.1	4.30 ± 0.20^{g}	4.17 ± 0.03^{abc}
12	20	60	2.4	6.10 ± 0.86^{f}	4.91 ± 0.01^{bcde}
13	20	40	1.25	12.2 ± 0.96^{bcd}	5.19 ± 0.83^{cde}
14	20	40	1.25	11.7 ± 0.45^{cde}	5.80 ± 0.52^{e}
15	20	40	1.25	12.7 ± 1.57^{abc}	5.06 ± 0.51^{cde}
16	20	40	1.25	10.9 ± 0.35^{de}	5.25 ± 0.67^{de}

[a~g] Mean values with different superscripts are significantly different found using Duncan Test ($P<0.05$).

can's Multiple Range Test using SAS® System Release 6.12 (Cary, USA). Two data are significantly different if they are grouped in different superscripted alphabets.

RESULTS AND DISCUSSION

Screening Experiment

A screening experiment was carried out to determine suitable concentration ranges of ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{3-}) to be tested (Table 1). In general, concentrations of NH_4^+ around 10 to 20 mM, NO_3^- between 20 and 40 mM, and PO_4^{-3} at 1.25 to 2.4 mM produced a cell dry weight (CDW) between 10 and 14 g/L. In cases where CDWs were around 3 to 6 g/L, NH_4^+ at 40 mM, NO_3^- at 60 mM and PO_4^{-3} at 0.1 mM were noted. From a Pareto chart (not shown), PO_4^{3-} as a single factor has the most significant positive effect on CDW. In decreasing order of their effects, NH_4^+ and NO_3^- as single factors; and the interactions of NO_3^--PO_4^{3-}, NH_4^+-PO_4^{3-} and NH_4^+-NO_3^- affected CDW negatively. Based on a surface contour plot, concentrations below 15 mM for NH_4^+, below 25 mM for NO_3^- and above 1.3 mM for PO_4^{3-} were predicted to yield a CDW higher than 13 g/L. The optimum cell DW was predicted to be 16.2 g/L at 10, 20 and 2.18 mM, NH_4^+, NO_3^- and PO_4^{3-}, respectively. Based on this, the test ranges for the optimization experiment were established between 5 to 15 mM for NH_4^+, 15 to 25 mM for NO_3^- and 1.3 to 2.6 mM for PO_4^{3-}. The normal levels in MS medium are 20, 40 and 1.25 mM for NH_4^+, NO_3^- and PO_4^{3-}, respectively.

Optimization Experiment

The design and results of the optimization experiments are as tabulated in Table 2. A high CDW of 14.1 to 15.6 g/L was obtained in runs 1, 2, 4, 7, 9 and 10, and CDWs between 11.7 and 12.4 g/L was observed in runs 6, 11, 12 and 15. The response surface contour plot (Fig. 1) shows no Bull's eye pattern implying broad optimal CDWs between 15.6 and 16.2 g/L as also predicted in the screening experiment. From the Pareto chart (Fig. 2), NH_4^+-NO_3^- interactions and PO_4^{3-} as a single factor significantly influenced the cell DW positively. In contrast to the screening experiment, NH_4^+, NO_3^-, NO_3^--PO_4^{3-} and NH_4^+-PO_4^{3-} factors were insignificant. This differences may have resulted from the concentration range tested in this optimization study, which eliminated the toxic effects of high macronutrient concentrations or a low PO_4^{-3} level in the screening experiment where a CDW as low as 3.9 g/L was recorded.

Applying regression analysis to the experimental data results in a second order polynomial model fit as shown in Eq. (2).

$$DW=29.2-1.35y_1-0.73y_2-2.5y_3-0.0037y_1^2+0.054y_1y_2+0.142y_1y_3+0.004y_2^2-0.011y_2y_3+0.695y_3^2 \quad (2)$$

This model, however, is only 71.2% fit to the experimental data, which is moderately good for model fitness. The optimum CDW was calculated as 16.0 g/L at 5.05 mM NH_4^+, 15.0 mM NO_3^- and 2.6 mM PO_4^{-3}. Table 3 shows data from the test experiment to validate the model as in Eq. (2). The concentrations of NH_4^+, NO_3^- and PO_4^{-3} were selected using the data where a high CDW

Table 2. Effects of NH_4^+, NO_3^- and PO_4^{3-} concentrations on cell growth: Optimization experiment

Run	NH_4^+, y_1 (mM)	NO_3^-, y_2 (mM)	PO_4^{3-}, y_3 (mM)	NH_4^+:NO_3^-, x_1	Total N, x_2 (mM)	Dry cell weight (g/L)	
						Experimental	Predicted
1	5	20	2.60	0.25	25	14.5 ± 0.55^{abc}	19.8
2	15	20	2.60	0.75	305	15.3 ± 0.01^{ab}	16.3
3	10	20	1.95	0.50	30	13.7 ± 0.29^{cde}	16.0
4	5	15	1.95	0.33	20	15.6 ± 0.01^{a}	18.0
5	10	20	1.95	0.50	30	13.6 ± 0.24^{cdef}	16.0
6	10	20	1.95	0.50	30	12.0 ± 1.31^{gh}	16.0
7	10	15	2.60	0.67	25	14.4 ± 0.25^{abcd}	16.7
8	5	20	1.30	0.25	25	12.8 ± 0.04^{efgh}	16.1
9	10	20	1.95	0.50	30	14.3 ± 0.57^{bcd}	16.0
10	10	25	2.60	0.40	35	14.1 ± 1.19^{bcd}	19.8
11	5	25	1.95	0.20	30	12.4 ± 0.10^{fgh}	17.5
12	15	15	1.95	1.00	30	11.8 ± 0.08^{h}	11.8
13	10	25	1.30	0.40	35	13.2 ± 0.10^{defg}	15.3
14	10	15	1.30	0.67	25	13.4 ± 0.33^{cdef}	19.8
15	15	20	1.30	0.75	35	11.7 ± 0.56^{h}	16.3
16	15	25	1.95	0.60	40	13.9 ± 0.16^{cde}	16.0

$^{a-g}$ Mean values with different superscripts are significantly different found using Duncan Test ($P<0.05$).

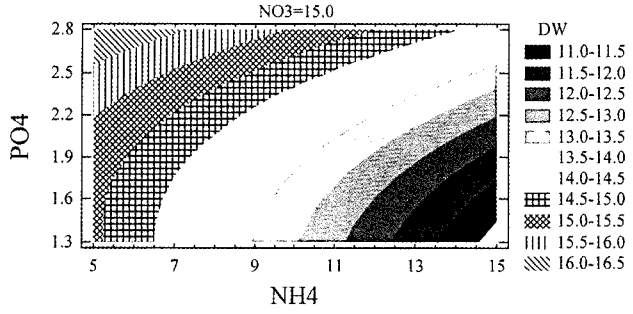

Fig. 1. Response surface contour plot for cell dry weight with NH_4^+ and PO_4^{3-} as independent variables at 15 mM NO_3^-.

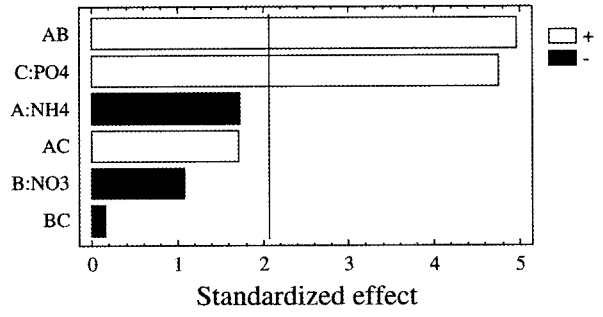

Fig. 2. Pareto chart for standardized effects of NH_4^+, NO_3^- and PO_4^{3-} on cell dry weight. Standardized effect values passed the blue line indicate significance of the effect. White colored bar indicate positive effect of the factor (Increasing the factor resulted in increasing response). Black colored bar indicate negative effect the factor (Increasing the factor will resulted in decreasing response). Two alphabets together (*e.g.* AB) means the two factors are interacting with each other.

was predicted in the screening and optimization experiments. For the control experiment, the model developed from the screening experiment was used as concentrations of NH_4^+, NO_3^- and PO_4^{-3} for the control concentrations are out of range to those established for the optimization experiment. The model predicts the experimental data well with above 87% accuracy, thus validating Eq. (2) as a predictive model within the concentration range of optimization experiment. In addition, Eq. (2) is only valid for *C. asiatica* cell culture in shake flask. Nevertheless, the fitted model can be used to estimate initial values to be used in a bioreactor system later.

The TTP level in all experiments was below 4 mg/g CDW (data not shown). The madecassoside (MO) content in the control (macronutrients as in standard MS medium) was highest at 4.2 mg/g CDW. In the optimization experiment MO and asiaticoside (AO) contents were detected below 3 mg/g CDW but AO was not detected in the control. Madecassic acid (MA) was only detected in runs 10 and 11 at less than 3 mg/g CDW and was ab sent in the control sample. Asiatic acid (AA) was detected at less than 2 mg/g CDW as compared to 2.0 mg/g CDW of AA in the control. The regressed experimental data attained R^2 values between 28 to 60%; therefore, the interactive effects of NH_4^+, NO_3^- and PO_4^{3-} on TTP could not be predicted.

Effect of Macronutrient Concentrations on Cell Growth

In both the screening and optimization experiment, PO_4^{-3} as a single factor positively affected CDW. The surface contour plot in Fig. 2 supports this result where PO_4^{-3} concentrations above 1.3 mM yielded a CDW above 15 g/L. The optimum PO_4^{-3} concentrations predicted for the screening and optimization experiments were 2.17 and 2.6 mM, respectively. At lower PO_4^{3-} con-

Table 3. Experimental run to test and validate the predictability of DW fitted to the model as in Eq. (2)

Run	NH_4^+, y_1 (mM)	NO_3^-, y_2 (mM)	PO_4^{3-}, y_3 (mM)	Dry cell weight (g/L)		
				Exp	Predict	% diff
1	20	40	1.25	11.0 ± 0.02[c]	11.9*	6.96
2	5	15	2.60	15.9 ± 0.18[a]	16.0	0.39
3	10	20	2.17	15.4 ± 0.24[a]	13.6	13.1
4	5	15	1.95	14.4 ± 0.53[b]	15.2	4.90
5	10	20	1.25	13.8 ± 0.28[b]	12.6	9.86

[a–c] Mean values with different superscripts are significantly different ($P<0.05$).
Predicted value was calculated from the model derived from the screening experiment.

centrations, cell growth was limited. This observation probably resulted from limited sucrose utilization [13] and limited phosphorylated intermediates necessary for sugar degradation and respiratory pathways [14]. An insufficient supply of PO_4^{3-} causes lower ATP generation; ATP is needed for the diversion of a carbon skeleton to synthesize large molecules [15]. On the other hand, a high phosphate concentration could lead to excess intake of inorganic phosphate, as reported for a *Catharantus roseus* culture, resulting in cell swelling, loss of starch granules in the cytoplasm and eventually cell rupture [16].

High CDWs were observed in both experiments at low NH_4^+ with high PO_4^{3-} concentrations. The Pareto chart analysis suggests a significant negative effect of NH_4^+ and NO_3^- as single factors in the screening experiment. While the NH_4^+-NO_3^- interaction was negative on cell growth in the screening experiment, the effect was insignificant in a narrow concentration range as in the optimization experiment. However, factors expressed as the NH_4^+ to NO_3^- ratio and total nitrogen (N) did not give significant effects. A high NH_4^+ level has been reported to depress calcium, magnesium and potassium uptake, which could in turn restrict the nutrient flow into the cells and lower cell yield [17]. Our study predicted optimum levels at 6.7 mM NH_4^+, 13.3 mM NO_3^- and 20 mM total N suggesting the need to adjust the ratio towards lower NH_4^+ and NO_3^- levels with reasonable total N. The surface contour plot (Figure not shown) shows the optimum range of the NH_4^+:NO_3^- ratio between 0.45 and 0.85 for cell growth can be enhanced by increasing PO_4^{3-} concentrations. This is consistent with the report that regardless of nitrogen sources availability; a phosphorus deficiency will depress the uptake of NH_4^+ and NO_3^-, leading to the disturbance of nitrogen metabolism [13,18].

Correlation between Cell Growth, Macronutrients and Final pH

In the screening experiment (Pareto chart not shown), final pH (pH_f) was affected positively by PO_4^{3-}, followed by NO_3^- as single factors, but NH_4^+ as a single factor and NH_4^+-PO_4^{3-} interactions affected pH_f negatively. In the optimization experiment, only NO_3^- appreciably affected pH_f positively while NO_3^--PO_4^{3-} interactions affected pH_f negatively. In terms of the effects of the NH_4^+ to NO_3^- ratio and total N, only total N affected pH_f positively while NH_4^+:NO_3^- affected negatively.

A variation in culture pH is linked to an influx and efflux of NH_4^+ and NO_3^- with its corresponding anions and cations. In both the screening and optimization experiment, the positive significant effects of PO_4^{3-} on cell growth are consistent with their positive effects on pH_f. In the optimization experiment, the NH_4^+-NO_3^- interaction had a positive effect on cell growth but the NH_4^+:NO_3^- ratio affected pH_f negatively. An increase in NO_3^- is associated with the release of OH^-, which should increase pH_f as observed in the screening and optimization experiments for NO_3^- as a single factor. The effect of NO_3^-, nonetheless, could be offset by NH_4^+, which has shown a consistent negative effect on cell growth and pH_f in the form of NH_4^+ as a single factor and the NH_4^+-PO_4^{3-} interaction. We propose that the relationship between cell growth and the pH_f level is influenced by PO_4^{3-} for positive effects, and NH_4^+ and the NH_4^+-PO_4^{3-} interaction for negative effects. The effects of other factors and interactions on cell growth and pH_f are influenced by a broad or narrow range of macronutrient concentrations.

The consistent effect of NH_4^+ as compared to NO_3^- on cell growth and pH_f can be explained in terms of the preference of cells towards NH_4^+ when both ions are present in the medium [19]. In a medium with higher NH_4^+, the uptake of cations is reduced [20] and replaced instead by the uptake of an anion such as phosphorus with an accompanying release of an H^+ equivalent [21], which could lead to a more acidic pH [22]. Hence, a high level of NH_4^+ can be toxic to cells.

CONCLUSION

Cell growth of *C. asiatica* was influenced positively by PO_4^{3-} and the NH_4^+-NO_3^- interaction with a CDW above 14 g/L in both the screening and optimization experiment. While NH_4^+ and NO_3^- as single factors, and the NO_3^--PO_4^{3-}, NH_4^+-PO_4^{3-} and NH_4^+-NO_3^- interactions influenced CDW negatively in the screening experiment they were insignificant in optimization experiments. This shows the influence of a concentration range in determining the outcome of RSM experiments. The optimum values predicted by the model generated from the RSM experimental design are 5.05 mM NH_4^+, 15.0 mM NO_3^- and 2.6 mM PO_4^{3-} with a biomass yield of 16.5 g/L.

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