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Research Paper

배지 성분의 통계적 최적화를 이용한 북극 미세조류 *Chlamydomonas* sp. KNM0029C의 지질 생산 증대

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Enhancing Lipid Production in the Arctic Microalga *Chlamydomonas* sp. KNM0029C by Using Statistical Optimization of Medium Components

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Abstract: A sequential statistical optimization was applied to the components of Tris-acetate-phosphate (TAP) medium, to enhance the cell density and lipid production of Arctic microalga *Chlamydomonas* sp. KNM0029C. The evaluation was preformed through an elimination process and Plackett-Burman design to select for significant factors, contributing toward cell growth. NH_4Cl, $MgSO_4 \cdot 7H_2O$, potassium phosphate, and trace elements were established as the effective components and used as variable factors in Box-Behnken design test, - a response surface methodology (RSM). The final optimized concentrations were 0.54, 0.16, 0.04, and 0.08 g/L of NH_4Cl, $MgSO_4 \cdot 7H_2O$, potassium phosphate, and trace elements, respectively. Overall, 9.9% enhanced lipid production was achieved by using optimized TAP medium at 4°C. The results of the present study could potentially contribute toward large-scale lipid production at low temperatures.

Keywords: arctic microalgae, *Chlamydomonas* sp. KNM0029C, lipid, response surface methodology, statistical optimization, tris–acetate-phosphate

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1. INTRODUCTION

Bacteria, yeast, algae, and fungi, that inhabit cold environments, have developed various adaptations to compensate for the adverse effects of low temperatures [1]. Cold-adapted microorganisms are the source of valuable products, including cold-active enzymes, and antifreeze proteins [2,3]. Recently, the microalga *Chlamydomonas* sp. KNM0029C - a species, capable of oil production, was isolated from the Arctic sea [4]. The organism which displays high total fatty acid content, exhibits promising potential as a potent biodiesel producer at low temperatures. The major benefit of *Chlamydomonas* sp. KNM0029C is its ability to grow under cold climate conditions, most prominently during the winter season. Our study focuses on investigating the effective medium composition for optimal Arctic microalga growth, and establishing the efficient lipid production conditions for this microorganism. The classical medium optimization method of changing one independent variable, while keeping

other factors constant, is both time consuming and ignores interactions among the factors - a prominent limitation. However, other statistical experimental designs, including 2-factorials and response surface methodology, have also proven effective for the optimization of culture medium [5-9]. In the present study, we have used these statistical tools to identify the most favorable medium components for *Chlamydomonas* sp. KNM0029C growth.

2. MATERIALS AND METHODS

2.1. Strain and culture media

The Arctic marine microalga *Chlamydomonas* sp. KNM0029C (KCTC 12730BP, formerly KOPRI-ArM0029C) was isolated from sea ice near the Dasan station in Ny-Alesund, Spitsbergen, Norway [4]. The culture was maintained on Tris–acetate-phosphate (TAP) medium. The unoptimized medium contained the following (g/L): Tris base, 2.42; NH_4Cl, 0.375; $MgSO_4{\cdot}7H_2O$, 0.1; $CaCl_2{\cdot}2H_2O$, 0.05; potassium phosphate, 0.432 (K_2HPO_4, 0.288 and KH_2PO_4, 0.144), plus 1 mL acetic acid, along with 1mL/L trace elements, composed of (g/L): $Na_2EDTA{\cdot}2H_2O$, 50; $ZnSO_4{\cdot}7H_2O$, 22; H_3BO_3, 11.4; $MnCl_2{\cdot}4H_2O$, 5; $FeSO_4{\cdot}7H_2O$, 5; $CoCl_2{\cdot}6H_2O$, 1.6; $CuSO_4{\cdot}5H_2O$, 1.6; $(NH_4)_6MoO_3$, 1.1.

2.2. Culture conditions

Approximately 2.0×10^5 cells mL^{-1} were inoculated in TAP medium and cultured for 2 weeks at 4°C under cool, white, fluorescent lamps (40 μmol photon m^{-2} s^{-1}) on a 16:8 light-dark cycle. For the nitrogen starvation study, cultures were grown for 2 weeks before being centrifuged at 4000 rpm for 5 min at 4C. The cell pellets were retained, washed twice with nitrogen-free TAP (TAP-N) and then resuspended in same medium for further growth and lipid production.

2.3. Statistical optimization

The Plackett-Burman design [10] was used to identify the essential components, influencing the growth of KNM0029C. Based on its results, seven elements of the TAP medium were isolated and examined at two levels, low (−) and high (+), producing a first-order model, $Y = b_0 + Sb_iX_i$, where Y is the predicted response (number of cells), b_0 is the model intercept, $_i$ - the linear coefficient, and X_i is - the level of the independent variable.

To optimize the units of each component, the Box- Behnken surface response method [11], was applied. The individual amounts were coded into three levels: (−), (0), and (+) for low, intermediate, and high concentrations, respectively. A second-order polynomial model was designed to describe the relationship between the independent variables (components) and the predicted response: $Y = b_0 + Sb_iX_i + Sb_{ij}X_iX_j + Sb_{ii}X_i^2$, where Y is the response (number of cells), b_0, b_i, b_{ij}, and b_{ii} are the constant and regression coefficients of the model, and X_i and X_j represent the independent nutrient component.

2.4. Cell growth monitoring and assay of lipid

The growth of KNM0029C was monitored by visualizing the number of cells using a hemocytometer and an optical microscope (Zeiss Axio Imager.A2, Germany). Total lipids were extracted from 20 mg of freeze-dried samples, and fatty acid methyl esters (FAMEs) were prepared and analyzed as described by Kim et al [4]. Fatty acids were quantified against the internal standard (1 mg of C16:0 in hexane), and the values were summed and expressed as milligrams of FAME per gram of dry cell weight (DCW). In general, 1.0×10^6 cells corresponded to 0.09 ± 0.002 mg DCW.

3. RESULTS AND DISCUSSION

3.1. Components selection

To eliminate unnecessary medium constituents, each of the seven elements was individually tested. Table 1 summarizes the test levels, effects, *t*-statistics, and *P*-values. Results with a *P*-value of <0.05 were accepted as significantly affecting the KNM0029C growth. $CaCl_2{\cdot}2H_2O$ displayed a result >0.05, indicating that this compound was not a significant factor (Fig. 1). The other components, (Tris base, NH_4Cl, $MgSO_4{\cdot}7H_2O$, potassium phosphate, trace elements, and acetic acid), all exhibited *P*-values of <0.05, and were therefore retested using

Table 1. Statistical analysis of medium components, using the initial Plackett–Burman experiment

Variable	Medium component	+ value (g/L)	− value (g/L)	Effect	*t*-statistic	*P*-value
X_1	Tris base	0	2.42	564.6	17.19	0.000
X_2	NH_4Cl	0	0.375	296.7	9.03	0.000
X_3	$MgSO_4{\cdot}7H_2O$	0	0.1	211.3	6.43	0.000
X_4	$CaCl_2{\cdot}2H_2O$	0	0.05	−13.7	−0.42	0.683
X_5	Potassium phosphate	0	0.432	111.7	3.40	0.004
X_6	Trace elements	0	0.0977	75.0	2.28	0.036
X_7	Acetic acid	0	1.0	570.3	17.36	0.000

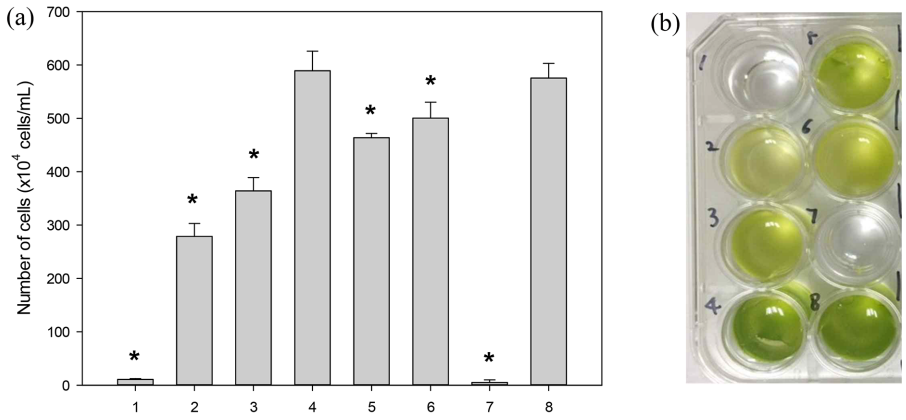

Fig. 1 Effect of medium components on the KNM0029C cell growth. (a) Number of cells (b) Image of cell growth in the absence of 1, Tris base; 2, NH_4Cl; 3, $MgSO_4·7H_2O$; 4, $CaCl_2·2H_2O$; 5, Potassium phosphate; 6, Trace elements; 7, Acetic acid; 8, Control (no absence). *Asterisks* denote $P < 0.05$.

a secondary Plackett-Burman design. Potassium phosphate and trace elements were identified as negatively effective while NH_4Cl and $MgSO_4·7H_2O$ were found to be positively correlated with KNM0029C growth (Table 2).

3.2. Optimization of medium components for KNM0029C growth

The effects of the six established elements were further studied, using a Box-Behnken design. The amounts of potassium

Table 2. Statistical analysis of selected medium components, using the secondary Plackett–Burman design

Variable	Medium component	− value (g/L)	+ value (g/L)	Effect	*t*-statistic	*P*-value
X_1	Tris base	0.484	4.84	−22.0	−1.11	0.279
X_2	NH_4Cl	0.075	0.75	57.4	2.90	0.009
X_3	$MgSO_4·7H_2O$	0.02	0.2	57.4	2.90	0.009
X_4	Potassium phosphate	0.0864	0.864	−47.0	−2.37	0.028
X_5	Trace elements	0.01954	0.1954	−320.3	−16.18	0.000
X_6	Acetic acid	0.2	2.0	−23.1	−1.17	0.257

Table 3. Box-Behnken optimization of selected significant medium components

Variable	Medium component	− value (g/L)	0 value (g/L)	+ value (g/L)
X_1	NH_4Cl	0.075	0.4125	0.75
X_2	$MgSO_4·7H_2O$	0.02	0.11	0.2
X_3	Potassium phosphate	0.0432	0.2376	0.432
X_4	Trace elements	0.00977	0.053735	0.0977

Table 4. Response surface regression for selected medium component, using Box-Behnken design

Term	Coefficient	SE coefficient	T	P
Constant	542.3	27.46	19.749	0.000
X_1 (NH_4Cl)	40.0	13.73	2.913	0.015
X_2 ($MgSO_4$)	26.4	13.73	1.922	0.084
X_3 (Potassium phosphate)	−15.1	13.73	−1.103	0.296
X_4 (Trace elements)	19.6	13.73	1.426	0.184
X_1^2	−128.6	20.60	−6.244	0.000
X_2^2	−33.5	20.60	−1.627	0.135
X_3^2	−5.4	20.60	−0.262	0.799
X_4^2	−57.5	20.60	−2.791	0.019
X_1X_2	11.1	23.78	0.466	0.651
X_1X_3	−35.9	23.78	−1.510	0.162
X_1X_4	36.3	23.78	1.528	0.158
X_2X_3	−5.2	23.78	−0.217	0.832
X_2X_4	−5.4	23.78	−0.228	0.824
X_3X_4	−29.5	23.78	−1.240	0.243

R^2=88.9%, R^2(adj)=71.1%

phosphate and trace elements, were reduced to 50%, while the positive components remained unchanged. The Box-Behnken optimization values, were calculated by linear multiple regression using Minitab software (ver. 14; Minitab Inc., USA) (Table 3). Estimated coefficients and related statistical terms are shown in Table 4. The main effect and the specific interactions between the four factors are displayed in Fig. 2. The expected number of KNM0029C cells was determined to be 5.87×10^6 cells/mL at the optimal NH_4Cl, $MgSO_4{\cdot}7H_2O$, potassium phosphate, and trace elements concentrations of 0.545, 0.155, 0.043, and 0.077 g/L, respectively.

3.3. Comparison of KNM0029C growth and lipid production in original vs optimized

To confirm our results KNM0029C growth was evaluated in different media via the Box-Behnken design. Figure 3 illustrates the number of microalga cells per unit of time in identical culture conditions, but in the two different TAP-environments. Maximal concentrations of 5.28 and 5.37×10^6 cells/mL were obtained in original and optimized- medium, respectively, indicating slightly enhanced cell growth in the latter conditions and the adequacy of our mathematical model. To evaluate the production of lipids, a nitrogen starvation study was performed. KNM0029C cells were cultivated for 2 weeks at 4°C in original and optimized TAP medium, before being deprived of viable nitrogen sources- NH_4Cl and trace elements were also not added. After an additional two-week period, the cells were harvested and analyzed (Fig. 4). Our data indicate higher lipid production of 120.1 mg FAME/g DCW and a 9.9% yield increase in the optimized TAP medium.

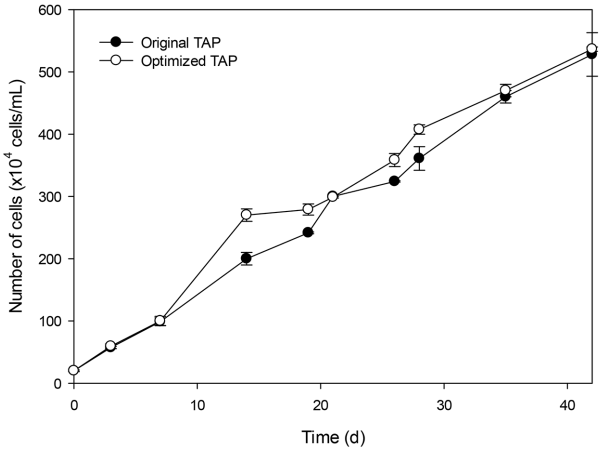

Fig. 3. Time profile of KNM0029C cell growth. Solid circle conveys original and open circle represents optimized TAP conditions.

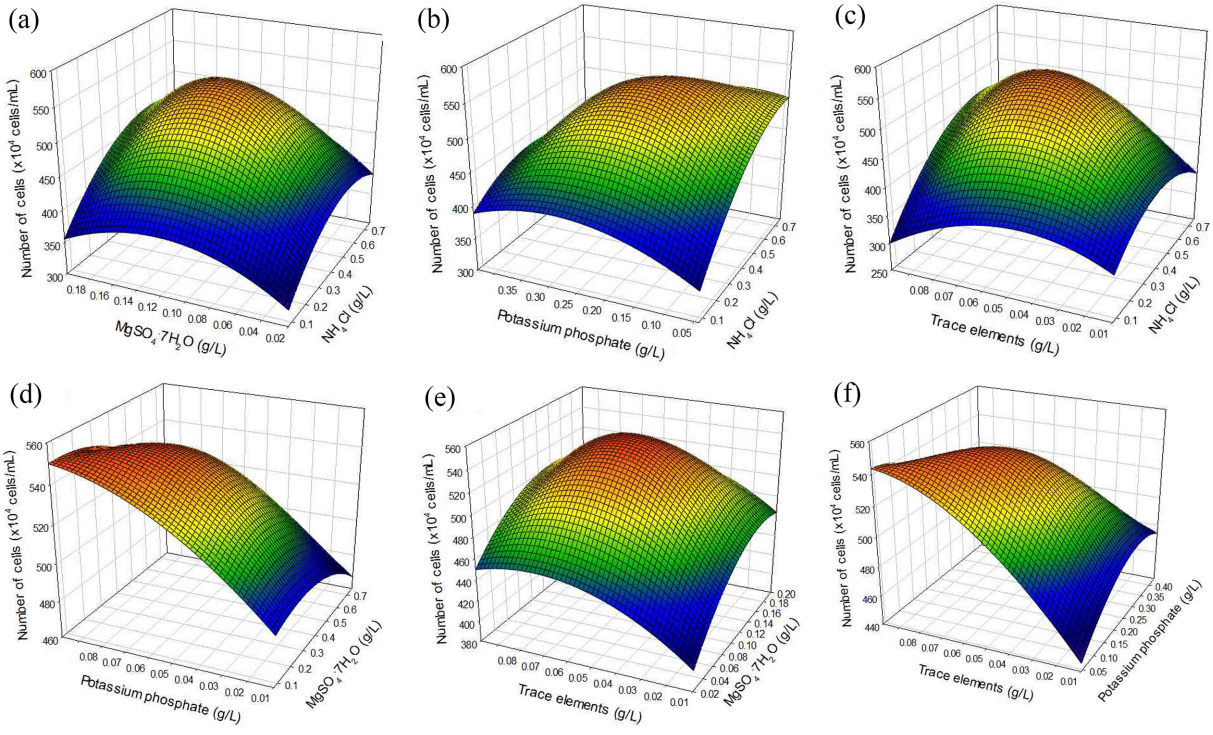

Fig. 2. Three-dimensional response plot showing the effect of (a) NH_4Cl and $MgSO_4{\cdot}7H_2O$, (b) NH_4Cl and potassium phosphate, (c) NH_4Cl and trace elements, (d) $MgSO_4{\cdot}7H_2O$ and potassium phosphate, (e) $MgSO_4{\cdot}7H_2O$ and trace elements, and (f) Potassium phosphate and trace elements.

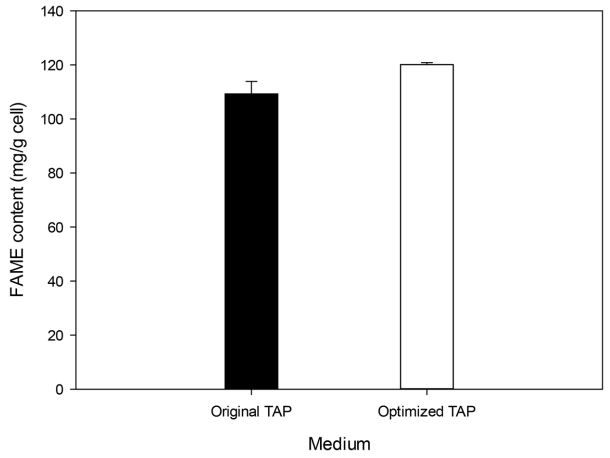

Fig. 4. Comparison of KNM0029C lipid production in different conditions, where solid bars illustrate cells cultured in original TAP and open bars represent those grown into the optimized medium.

Table 5. Comparison of medium composition of original TAP medium and optimized TAP medium

Component	Original TAP (g/L)	Optimized TAP (g/L)
Tris base	2.42	2.42
NH_4Cl	0.375	0.545
$MgSO_4 \cdot 7H_2O$	0.1	0.155
$CaCl_2$	0.05	0.05
K_2HPO_4	0.288	0.029
KH_2PO_4	0.144	0.014
Trace	0.097	0.077
AcOH	1.0 (mL)	1.0 (mL)

4. CONCLUSION

To enhance the growth of Arctic microalga *Chlamydomonas* sp. KNM0029C, medium components were statistically optimized via the Plackett-Burman and Box-Behnken designs. The final composition was as follows (g/L): Tris, 2.42; NH_4Cl, 0.545; $MgSO_4 \cdot 7H_2O$, 0.155; $CaCl_2$, 0.05; K_2HPO_4, 0.029; KH_2PO_4, 0.014; trace, 0.077; and one mL of AcOH (Table 5). The amounts of negatively effective components, like K_2HPO_4, KH_2PO_4, and trace were diminished, whereas those, correlated with growth, including NH_4Cl and $MgSO_4 \cdot 7H_2O$ were elevated. Overall, 9.9% increase in lipid production was obtained by a sequential statistical analysis.

Until recently, no practical information was available on the optimal environmental conditions of Arctic microalgae growth. The present study provides a suitable medium composition for raising and improving lipid production by *Chlamydomonas* sp. KNM0029C.

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