J. Microbiol. Biotechnol. (2008), **18**(12), 1919–1926
doi: 10.4014/jmb.0700.743
First published online 21 October 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Statistical Optimization of the Growth Factors for *Chaetoceros neogracile* Using Fractional Factorial Design and Central Composite Design

Jeong, Sung-Eun[1], Jae-Kweon Park[1], Jeong-Dong Kim[1], In-Jeong Chang[1], Seong-Joo Hong[1], Sung-Ho Kang[2], and Choul-Gyun Lee[1]*

[1]*Institute of Industrial Biotechnology, Department of Biological Engineering, Inha University, Incheon 402-751, Korea*
[2]*Polar Environmental Research Division, Korea Ocean Polar Research Institute (KOPRI), Incheon 426-744, Korea*

Received: November 6, 2007 / Accepted: June 5, 2008

Statistical experimental designs; involving (i) a fractional factorial design (FFD) and (ii) a central composite design (CCD) were applied to optimize the culture medium constituents for production of a unique antifreeze protein by the Antartic microalgae *Chaetoceros neogracile.* The results of the FFD suggested that NaCl, KCl, $MgCl_2$, and Na_2SiO_3 were significant variables that highly influenced the growth rate and biomass production. The optimum culture medium for the production of an antifreeze protein from *C. neogracile* was found to be Kalle's artificial seawater, pH of 7.0±0.5, consisting of 28.566 g/l of NaCl, 3.887 g/l of $MgCl_2$, 1.787 g/l of $MgSO_4$, 1.308 g/l of $CaSO_4$, 0.832 g/l of K_2SO_4, 0.124 g/l of $CaCO_3$, 0.103 g/l of KBr, 0.0288 g/l of $SrSO_4$, and 0.0282 g/l of H_3BO_3. The antifreeze activity significantly increased after cells were treated with cold shock (at −5°C) for 14 h. To the best of our knowledge, this is the first report demonstrating an antifreeze-like protein of *C. neogracile.*

Keywords: *Chaetoceros neogracile*, Antarctic microalgae, antifreeze protein, fractional factorial design (FFD), central composite design (CCD)

In nature, many organisms are exposed to freezing temperatures, and this exposure may be nearly constant, occurring over the course of an Arctic winter. However, it can also be brief and intermittent, occurring at night at high elevations, even in equatorial regions. An interesting adaptation to life at subzero temperatures is the elaboration of proteins that modify ice crystal growth [3]. These glycoproteins [AF(G)P], called "antifreeze proteins", were first identified in Antarctic teleost fishes as the causative agents of serum freezing-point depression. In Antarctic fish species, the antifreeze glycoproteins (AFGP) were found to lower the freezing point by more than 1°C to match that of seawater. The antifreeze glycoproteins were immediately recognized as unusual because they caused a freezing point depression far greater than that would be predicted from colligative properties alone. In general, antifreeze glycoproteins lower the freezing point by interacting directly with the ice surface and cause a thermal hysteresis, which would account for their highly efficient noncolligative freezing-point depression. However, a precise definition of the interactions between AF(G)Ps and ice that would give rise to antifreeze activity has not been obtained to date, even though it would seem obvious that functional groups positioned to match the ice lattice on a particular plane would lead to ice binding and antifreezing activity. Although we have not succeeded in defining a biological tool in this study that shows what exactly distinguishes an antifreeze protein from a non-antifreeze protein, this is a fascinating question in protein structure, function, and evolution.

Since the first discovery of antifreeze glycoproteins in fish, numerous different AF(G)Ps have been found in plants, animals, fungi, and bacteria [15]. However, their distribution appears strictly limited to species that are exposed to cold or freezing temperature, and in many cases their occurrence within those species is limited to periods of cold exposure. Moreover, the diversity and specificity of the AF(G)Ps are complicated by the recent discovery of activities in these proteins that are distinct from ice binding and freezing-point depression. They appear to protect mammalian cell membranes from damage at temperatures above 0°C. Thus, perspectives on the distribution, diversity, and roles of AF(G)Ps are in transition [2].

C. neogracile is known as an Antarctic marine diatom. For the successful growth of algae in culture, the environment must be conditioned to meet as many of the intrinsic requirements of that organism as possible, such as light intensity, pH, and function of temperature, *etc.* [13]. In the

*Corresponding author
Phone: 82-32-860-7518; Fax: 82-32-872-4046;
E-mail: leecg@inha.ac.kr

present study, the authors have used statistical tools [3] for optimizing the growth factors for *C. neogracile*.

Materials and Methods

Strains, Culture Media, and Conditions

The Antarctic marine diatom *Chaetoceros neogracile van Landingham* was isolated and kindly provided by the Korea Polar Research Institute (KOPRI) [6]. *C. neogracile* was subcultured in f/2 medium added to Coralife (Coralife scientific grade marine salt; Aqua Craft Inc., U.S.A.) artificial seawater (ASW). The f/2 medium consists of 75 mg/l of $NaNO_3$, 5 mg/l of $NaH_2PO{\cdot}4H_2O$, 30 mg/l of $Na_2SiO_3{\cdot}9H_2O$, 0.0013 mg/l of $FeCl_3{\cdot}6H_2O$, 0.0087 mg/l of $Na_2EDTA{\cdot}2H_2O$, 0.0098 μg/l of $CuSO_4{\cdot}5H_2O$, 0.0063 μg/l of $Na_2MoO_4{\cdot}2H_2O$, 0.022 μg/l of $ZnSO_4{\cdot}7H_2O$, 0.01 μg/l of $CoCl_2{\cdot}6H_2O$, 0.18 μg/l of $MnCl_2{\cdot}4H_2O$, 0.001 μg/l of Vitamin B_{12}, 0.001 μg/l of Biotin, and 0.2 μg/l of Thiamine-HCl. After selection of the basal artificial seawater, the strain was cultured in Kalle's artificial seawater, pH of 7.0±0.5, consisting of 28.566 g/l of NaCl, 3.887 g/l of $MgCl_2$, 1.787 g/l of $MgSO_4$, 1.308 g/l of $CaSO_4$, 0.832 g/l of K_2SO_4, 0.124 g/l of $CaCO_3$, 0.103 g/l of KBr, 0.0288 g/l of $SrSO_4$, and 0.0282 g/l of H_3BO_3. This organism was cultured at 4°C under a continuous light intensity of 15 $\mu E/m^2/s$ using white fluorescent lamps. All experiments were conducted with the seed culture during its exponential growing phase. *C. neogracile* was cultured in 250-ml Erlenmeyer flasks with 100 ml of working volume and agitated at 128 rpm in the shaking incubator. Other specific conditions are described in Table 1.

Analytical Methods

The cell numbers and the average cell size were measured with a Coulter counter (Model Z2; Coulter Electronics, Inc., Hialeah, FL, U.S.A.). Data from the Coulter counter were collected by AccuComp software and then exported to an Excel spreadsheet to calculate the cell numbers and cell size distribution as well as average cell size [7]. The total cell volume {=cell numbers (cell/ml)×average cell volume (m^3/cell)} was used to calculate the specific growth rate.

The nitrate and chlorophyll concentrations were determined using a spectrophotometer (Model HP8453B; Hewlett-Packard, Waldbronn, Germany). The nitrate concentration was measured by a standard method after removing cells by centrifugation. The chlorophyll concentration was calculated by the following equations:

Chlorophyll *a* (mg/l)=$(16.5\times A_{665})-(8.3\times A_{650})$,
Chlorophyll *b* (mg/l)=$(33.8\times A_{650})-(12.5\times A_{665})$.

Chlorophyll was extracted by using 90% methanol and the concentrations were measured after centrifugation at 13,000 rpm for 10 min according to the standard method [7, 12, 14].

Table 1. f/2 Medium stock solutions.

Quantity per liter of media	Compound	Stock concentration
1.0 ml	$NaNO_3$	75.0 g/l
1.0 ml	$NaH_2PO_4{\cdot}H_2O$	5.0 g/l
1.0 ml	$Na_2SiO_3{\cdot}9H_2O$	30.0 g/l
1.0 ml	f/2 Trace metal solution	(see recipe below)
0.5 ml	f/2 Vitamin solution	(see recipe below)
	Trace metal solution	
1.3 g	$FeCl_3{\cdot}6H_2O$	-
8.7 g	$Na_2EDTA{\cdot}2H_2O$	-
1.0 ml	$CuSo_4{\cdot}5H_2O$	98 mg/l
1.0 ml	$Na_2MoO_4{\cdot}2H_2O$	63 mg/l
1.0 ml	$ZnSO_4{\cdot}7H_2O$	0.22 g/l
1.0 ml	$CoCl_2{\cdot}6H_2O$	0.1 g/l
1.0 ml	$MnCl_2{\cdot}4H_2O$	1.8 g/l
	Vitamin solution	
1.0 ml	Vitamin B_{12}	10 mg/10 ml dH_2O
1.0 ml	Biotin	10 mg/10 ml dH_2O
200.0 mg	Thiamine-HCl	-

Statistical Analysis

Fractional factorial design (FFD) is one of the statistical tools that can be used to screen the significant factors in a medium [9]. Because of the many components contained in the medium, however, FFD would lead to a remarkable large number of designs. After selection of the best artificial seawater, FFD was performed to investigate the effect of each component on the chlorophyll concentration to optimize the culture media.

Chlorophyll concentration was measured as the response, which can be calculated using the following equation [10].

$$y=\beta_0+\sum_{i}^{k}\beta_i x_i+\sum\sum_{i<j}\beta_{ij}x_i x_j+\varepsilon \quad (1)$$

where y is the predicted response, β_i is the coefficient of the equation, β_0 is the intercept of the plane, x_i and x_j are coded levels of variables, and ε is the error term. The effects of each variable were determined by a statistical software, MINITAB (V 14, Minitab Inc., State College, PA, U.S.A.).

A central composite design (CCD) was used to optimize the levels of variables with a significant influence on cell growth, and the chlorophyll concentration of *C. neogracile* can be written as a function of response surface. As shown in Table 6, the CCD for four variables in a Box-Behnken design is combined with one center point and 8 axial points [11]. The responses were optimized by the following second-order model:

$$y=\beta_0+\sum_{i=1}^{k}\beta_i x_i+\sum_{i=1}^{k}\beta_{ii}x_i^2+\sum\sum_{i<j}\beta_{ij}x_i x_j+\varepsilon \quad (2)$$

The optimum values were calculated by MINITAB using the above equation [14]. To represent the statistical relevance, the culture medium components were screened at a 95% confidence level with a *p*-value smaller than 0.5.

Analysis of Antifreeze Protein Activity

Antifreeze activity of proteins was assayed by observing the morphology of ice crystals in the culture supernatant of *C. neogracile* before and after the cells were treated with cold shock (−5°C). To observe changes in ice crystal morphology, a solution was flash-frozen during the freezing stage and observed using a microscope (CSB-HP3; Sam-Won, Korea). As the temperature was lowered again, the ice crystals acquired a shape that is defined by growth along the three *a*-axes and the *c*-axis. In this assay, the antifreeze

activity was defined as absent when ice crystals formed a disc-like shape, and present (low or high) when hexagonally shaped ice crystals were created or plate-like crystals were observed under the microscope.

RESULTS

Selection of Artificial Seawater

In order to optimize the growth condition of *C. neogracile*, we first investigated the major components of the culture medium. The components of the f/2 medium stored as stock solution and used generally in the cultivation of diatoms, shown in Table 1, was added to ASW to elucidate the major components that affect cell growth. In addition, for the statistical optimization of the medium components for growth of *C. neogracile*, various types of ASW were tested; Kalle's ASW, MBL, Millero's ASW, Lyman's ASW, and Coral [4, 8, 9]. The composition of each medium is listed in Table 2. Eventually, cultures in each medium were incubated in a shaking incubator controlling at 4°C and 120 rpm, under continuous illumination by using fluorescence lamps of 20 $\mu E/m^2/s$, as described in Materials and Methods.

The concentration of chlorophyll and nitrogen in each tested medium for 18 days of incubation was measured in terms of determination of the growth rate in several known media for *C. neogracile*. As shown in Fig. 1A, the chlorophyll concentrations were 0.78 mg/l in Lyman, 1.40 mg/l in Coral, 1.58 mg/l in Kalle's ASW, 1.30 mg/l in MBL, and 1.18 mg/l in Millero's ASW, respectively. The result showed that Kalle's ASW supported higher growth of *C. neogracile* than the other of four types of artificial seawater tested. On the other hand, the concentration of nitrogen in each culture medium generally decreased corresponding to the increasing of the concentration of chlorophyll in terms of the same ratio, respectively (Fig. 1B).

Table 2. Composition of various types of artificial seawater.

Additive (g/l)	MBL	Millero's	Kalle's	Lyman's	Coral
NaCl	24.7 g	23.98 g	28.566 g	23.939	Undefined
KCl	0.66 g	0.698 g	0.667g	0.667 g	artificial
$MgCl_2 \cdot H_2O$	4.7 g	5.98 g	4.62g	6.04 g	seawater
$CaCl_2 \cdot 2H_2O$	1.9 g	1.51 g	–	1.487 g	
$MgSO_4 \cdot 7H_2O$	6.3 g	–	3.658 g	–	
$NaHCO_3$	0.18 g	–	–	0.196 g	
KBr	–	0.1 g	0.103 g	0.098 g	
H_3BO_3	–	0.0254 g	0.0282 g	0.027 g	
NaF	–	0.0029 g	–	0.003 g	
$SrCl_2$	–	0.0143 g	–	0.024 g	
Na_2SO_4	–	4.011 g	–	3.994 g	
$CaSO_4$	–	–	1.308 g	–	
K_2SO_4	–	–	0.832 g	–	
$CaCO_3$	–	–	0.124 g	–	
$SrSO_4$	–	–	0.0288 g	–	

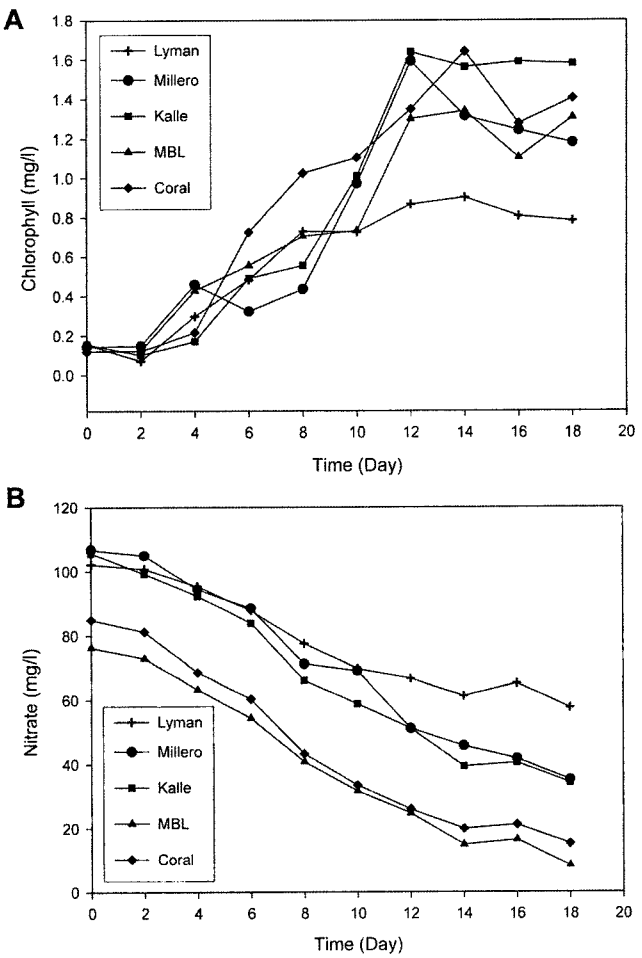

Fig. 1. Profile of chlorophyll *a* (**A**) and nitrate (**B**) concentrations for each ASW.

Hence, Kalle's ASW was selected as a base medium for statistically optimizing the growth rate and biomass productivity of *C. neogracile*, using FFD and CCD as statistical methods. These results led us to further characterize the activity of antifreeze proteins, as described in Figs. 1A and 1B.

Fractional Factorial Design

The purpose of the first optimization step was to identify which components of this defined medium had a significant effect on the growth of *C. neogracile*. Factorial design, one class of experimental designs, is very useful in identifying the important nutrients and interactions between two or more nutrients in relatively few experiments as compared with the one-factor-at-a-time technique. Factorial design requires 2^N experiments if N factors have to be investigated. In the case with 8 variables as in this investigation, a factorial design would lead to 256 experiments, which is quite a large number to run at the same time. The experimental design of this first optimization step is given in Table 3

Table 3. Eight groups of variables for screening by FFD.

Symbols	Additive	Low (−)	0	High (+)
A	NaCl	0.5	1	1.5
	KCl	0.5	1	1.5
	$MgCl_2 \cdot 6H_2O$	0.5	1	1.5
B	$MgSO_4 \cdot 7H_2O$	0.5	1	1.5
	KBr	0.5	1	1.5
	H_3BO_3	0.5	1	1.5
C	$CaSO_4 \cdot 2H_2O$	0.5	1	1.5
	K_2SO_4	0.5	1	1.5
	$CaCO_3$	0.5	1	1.5
	$SrSO_4$	0.5	1	1.5
D	$NaNO_3$	0.5	1	1.5
E	$NaH_2PO \cdot 4H_2O$	0.5	1	1.5
F	$Na_2SiO_3 \cdot 9H_2O$	0.5	1	1.5
G	f/2 Trace metal solution	0.5	1	1.5
H	f/2 Vitamin solution	0	1	1.5

with the levels in coded units. In Table 3, the (−) level means 0.5 times lower than the original medium and (+) level means 1.5 times higher than the original medium.

The number of experiments can be reduced by introducing the FFD without loss of information on the major effects of each factor. Some information about nonspecific interaction effects will be lost, unexpectedly. Then, we chose to reduce the total number of runs to 1/16 (of the original 256 experiments), resulting in 16 runs and a center point (as an internal standard) as listed in Table 4.

Moreover, the effects of each of the eight groups could be differentiated from the experimental data (summarized in Table 5). In order to achieve a 95% confidence level or higher level in each of the experiments, the groups of A, C, and F with a p-value smaller than 0.5 showed that analyzed

Table 4. Experimental design of FFD.

Run	A	B	C	D	E	F	G	H
1	−	−	−	+	+	+	−	+
2	+	+	−	+	−	−	−	+
3	−	+	−	−	+	−	+	+
4	+	−	+	+	−	+	−	−
5	+	−	+	−	+	−	−	+
6	+	+	+	+	+	+	+	+
7	+	+	+	−	−	−	+	−
8	−	+	−	+	−	+	+	−
9	−	+	+	−	−	+	−	+
10	+	−	−	+	+	−	+	−
11	+	+	−	−	+	+	−	−
12	−	−	+	+	−	−	+	+
13	+	−	−	−	−	+	+	+
14	0	0	0	0	0	0	0	0
15	−	−	−	−	−	−	−	−
16	−	−	+	−	+	+	+	−
17	−	+	+	+	+	−	−	−

Table 5. Estimated regression coefficients of FFD.

Variables	Effects	Coeff.	t-Values	p-Values
Constant	−	0.65977	13.74	0.000
A	0.08881	0.04440	0.92	0.386
B	0.04634	0.02317	0.48	0.644
C	0.10856	0.05428	1.13	0.296
D	−0.01799	−0.00900	−0.19	0.857
E	−0.01542	−0.00771	−0.16	0.877
F	0.20753	0.10376	2.16	0.068
G	0.03241	0.01620	0.34	0.746
H	−0.06002	−0.03001	−0.62	0.552

variables represent statistical relevance on cell growth under the evaluated conditions. As a result, components in groups A, C, and F showed a relatively higher influence on growth of *C. neogracile* than the other groups (Table 5).

Central Composite Design

Based on the results of the FFD, variables of NaCl, KCl, $MgCl_2$, and Na_2SiO_3 were subsequently employed to determine the independent significance on cell growth. The next experiment was performed to find the factor values more precisely in order to produce a desired response. The CCD is very useful to acquire data to fit a polynomial. CCD was generated for the four variables (concentrations of Na_2SiO_3, NaCl, KCl, and $MgCl_2$) with 25 factorial points, two center points (running in duplicate), and 8 axial points where four factors were set at their center point level (Table 6). The result of central composite design for the four factors is given in Table 7.

The three-dimensional graph obtained from the calculated response surface is shown in Fig. 2. Three-dimensional response surface plots of NaCl, KCl, $MgCl_2$, and Na_2SiO_3 against the concentration of chlorophyll *a* can further explain the results of the statistical and mathematical analyses. The probability is based on the assumption that the random error associated with the model is normally distributed. A small p-value suggests that the coefficient is a large signal in comparison with the noise, because it is too large to have arisen by chance alone. Coefficients with small p-values are significantly greater than zero; that is, they identify effects that appear to be truly important. For instance, $p<0.1$ suggests significance at the 0.10 level ($\alpha=0.10$). This corresponds to a 90% confidence level for a test of the hypothesis that the coefficient in question is equal to zero. Therefore, small p-values are associated with large t-values because they imply that the coefficient is much greater than its standard error (SE). This is a confirmation that the fitted surface has maximum points, which are 24.57 g/l NaCl, 0.667 g/l KCl, 0.16 g/l $MgCl_2$,

Table 6. Central composite design for four variables.

Run order	NaCl	KCl	$MgCl_2$	Na_2SiO_3	Run order	NaCl	KCl	$MgCl_2$	Na_2SiO_3
1	+	+	+	+	13	+	+	–	–
2	+	–	–	–	14	–	–	–	–
3	–	+	+	–	15	–	–	+	–
4	0	++	0	0	16	– –	0	0	0
5	–	–	–	+	17	0	0	0	– –
6	0	0	– –	0	18	–	+	–	+
7	–	+	+	+	19	+	–	+	+
8	+	+	–	+	20	+	–	+	–
9	++	0	0	0	21	0	0	0	++
10	0	– –	0	0	22	+	+	+	–
11	–	–	+	+	23	0	0	0	0
12	+	–	–	+	24	–	+	–	–
					25	0	0	++	0

and 0.18 g/l Na_2SiO_3. Maximum cell numbers on this point using the model employed in this study can be calculated in $7.016 \times E^6$ cells/ml (Fig 2). The significance of each regression coefficient of variables was validated and indicated in *p*-values, as shown in Table 8. This implies that a *p*-value less than 0.01 for Na_2SiO_3 showed the highest significant effect on growth of *C. neogracile*.

These results indicated that the four variables NaCl, KCl, $MgCl_2$, and Na_2SiO_3, especially Na_2SiO_3, may act as a major or limiting component in nutrients of culture medium, and small variations in their components can alter the growth rate and/or production of antifreeze protein from *C. neogracile*. As expected, this is not surprising because $Na_2SiO_3 \cdot 9H_2O$ (in group F) is well known as an essential nutrient for composition of the diatom's cell wall, and salts in group A (NaCl, KCl, and $MgCl_2$) are related to salinity and osmolarity.

Validation of CCD

C. neogracile was cultivated in the optimized and non-optimized media to compare the growth rate and the biomass productivity (Table 9). *C. neogracile* grew rapidly and reached the maximum after 12 days of cultivation (Fig. 3A). The growth was stably maintained and the culture pH determined during the cultivation ranged between 7 and 8. During fermentation, the concentration of chlorophyll *a* and/or *b* in the optimized medium increased rapidly to about 3 times higher than the non-optimized medium. Moreover, in the non-optimized medium, the nitrate concentration decreased very slowly and did not fall off to zero (Fig. 3B). In conclusion, the maximum cell numbers of *C. neogracile* increased up to 300% after the

Table 7. Central composite design for cell growth.

Factors	–2	–1	0	1	2
NaCl	0	1.4283	2.8566	5.7132	11.4264
KCl	0	0.03335	0.0667	0.1334	0.2668
$MgCl_2 \cdot 6H_2O$	0	0.414	0.828	1.656	3.312
$Na_2SiO_3 \cdot 9H_2O$	0	0.003	0.006	0.012	0.024

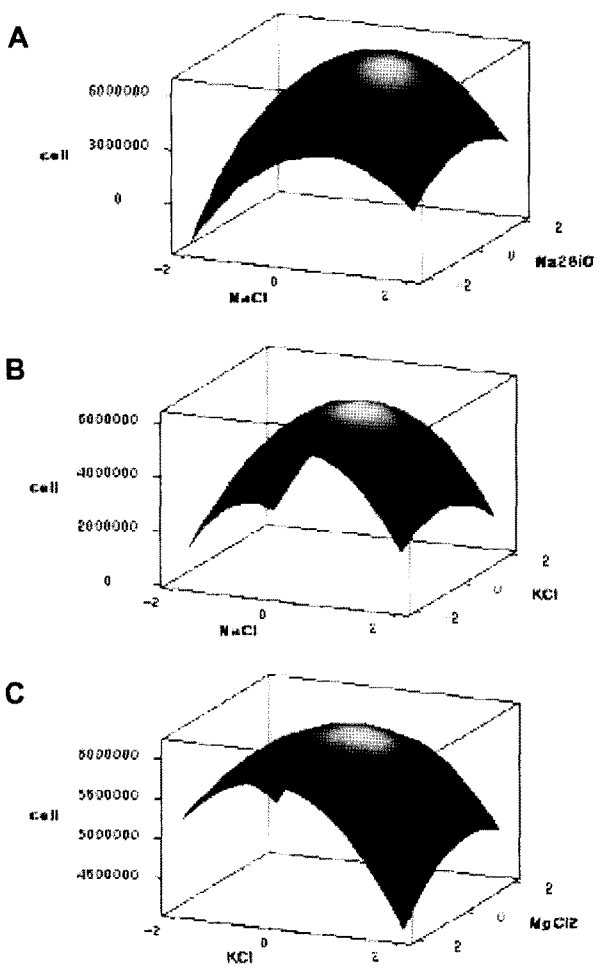

Fig. 2. Response surface plot and contour plot of cell growth. (**A**) $Na_2SiO_3 \cdot 9H_2O$ *vs.* NaCl, (**B**) NaCl *vs.* KCl, and (**C**) $MgCl_2$ *vs.* KCl.

Table 8. Estimated regression coefficients of CCD.

Variables	Coef.	*t*-Values	*p*-Values	Optimum value (g/l)
Constant	6,120,000	6.112	0.000	-
NaCl	254,765	1.247	0.241	2.5618
KCl	-131,694	-0.644	0.534	0.0667
$MgCl_2 \cdot 6H_2O$	8,752	0.043	0.967	0.0375
$Na_2SiO_3 \cdot 9H_2O$	696,757	3.409	0.007	0.0104

optimization of the culture medium was compared with the control in the validation experiment (Fig. 3A). These results indicated the importance of the optimization of components in the culture media for growth of *C. neogracile,* which could be applied to industrial processing for the production of biologically valuable materials.

Characterization of Antifreeze Protein

In this study, we not only optimized the culture medium for growth rate and biomass productivity, but we also investigated the production of antifreeze-like protein from *C. neogracile*. Thus, upon the optimization of the medium using statistical methods such as FFD and CCD, we further attempted to assess whether or not *C. neogracile* produces an antifreeze-like protein. We collected the supernatant from the culture medium and conducted an assay to obtain the antifreeze protein activity. To observe changes in ice crystal morphology, the supernatant was flash-frozen during the freezing stage and observed using a microscope. As the temperature lowered again, the ice crystal acquired a shape that was defined by growth along the three *a*-axes and the *c*-axis. Practically, in water or in a solution of substances that do not interact with ice, ice was grown as a round and flat crystal. In a similar manner as can be seen in Fig. 4A, the supernatant from the optimized culture medium after 18 days of cultivation without cold shock did not show a hexagonal shape. However, after cold-shock treatment to the cell for about 14 h, we were able to observe that most proteins in the supernatant were bound to the prism face of the ice, creating hexagonally shaped crystals, as shown in the Fig. 4B. These results strongly suggest that *C. neogracile* produces antifreeze-like protein(s) into the culture medium after the cell is subjected to the cold shock.

Table 9. Components of the original and modified medium.

Stock	Additive	Original medium	Modified medium
1	NaCl	28.57	24.57
	KCl	0.67	0.67
	$MgCl_2 \cdot 6H_2O$	6.32	0.16
2	$MgSO_4 \cdot 7H_2O$	6.05	6.05
	KBr	0.10	0.10
	H_3BO_3	0.03	0.03
3	$CaSO_4 \cdot 2H_2O$	1.15	1.15
	K_2SO_4	0.83	0.83
	$CaCO_3$	0.12	0.12
	$SrSO_4$	0.03	0.03
4	$NaNO_3$	0.075	0.075
5	$NaH_2PO \cdot 4H_2O$	0.005	0.005
6	$NaSiO_3 \cdot 9H_2O$	0.03	0.18
7	f/2 Trace metal solution	-	-
8	f/2 Vitamin solution	-	-

Discussion

Interestingly, in an Antarctic environment, although many organisms are exposed to freezing temperatures that are thought to be unconducive to life, these organisms still survive. Among the adaptations of microbial organisms at subzero temperatures is the elaboration of proteins that have an ability to bind to ice and modify the normal formation of ice crystals. However, for most bioprocess

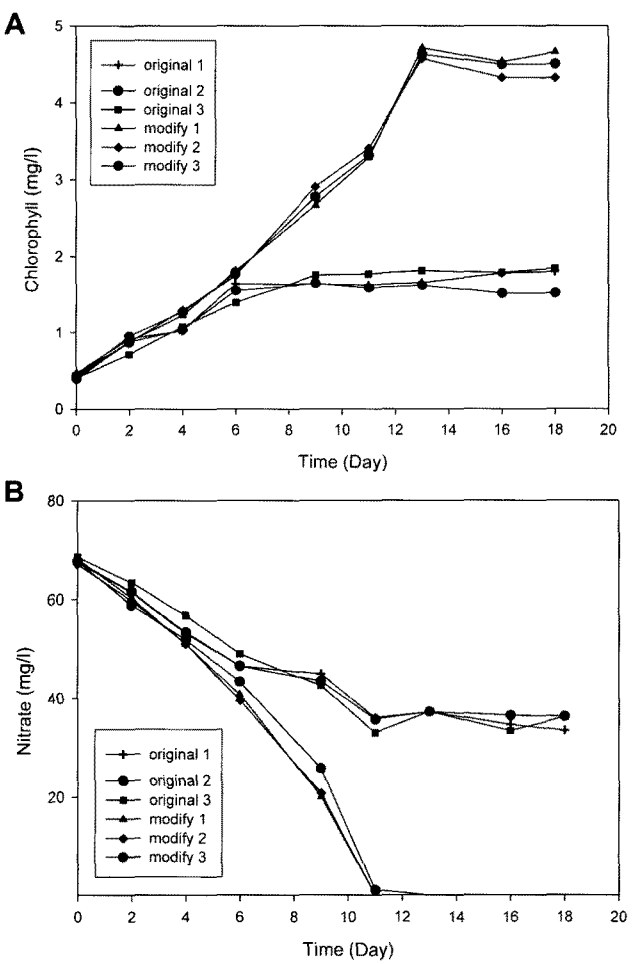

Fig. 3. Profile of chlorophyll *a* (**A**) and nitrate (**B**) concentrations for the validation experiment.

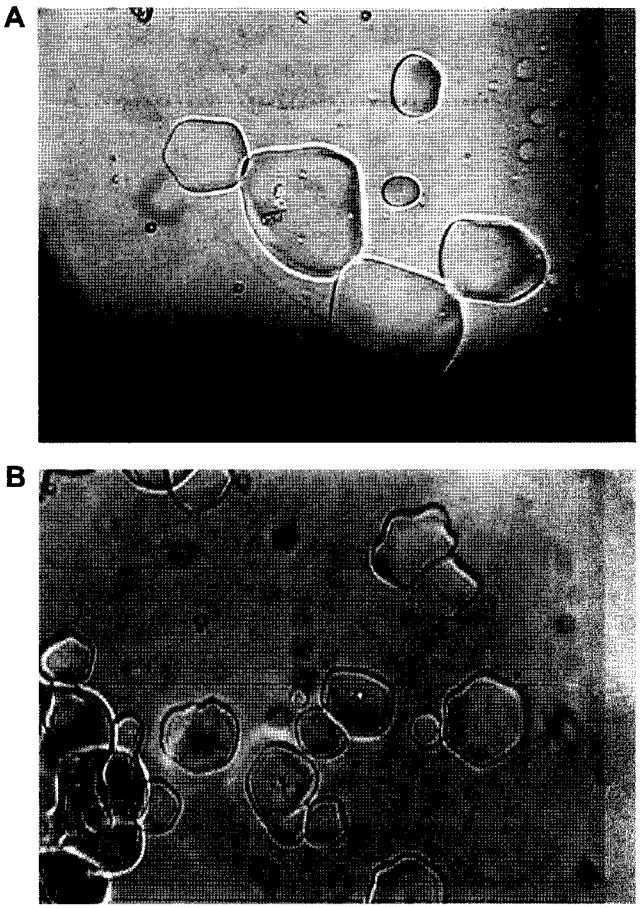

Fig. 4. Assays of antifreeze protein activity by ice crystal morphology.
A. Supernatant of optimized medium after 18 days of cultivation **B**. After cultivation, treatment of cold shock to the cell for 14 h.

technologies, there are no theoretical models that can be used to explain the process' performance. Owing to lack of reports on the statistical optimization for the production of AFP and biomass *C. neogracile* was isolated as an Antartic diatom from the Antarctic Ocean by the Korea Polar Research Institute [6]. In the present study, we optimized the culture medium components using the statistical methods of FFD and CCD, which can provide a set of powerful tools to optimize the efficiency and productivity of empirical problem solving. With the use of the statistical optimization method, there was a significant (55.3%) increase in lactic acid production by *Lactobacillus* sp. [13].

Initially, the chlorophyll *a* and/or *b* concentrations after 18 days of incubation was measured to determine what type of artificial seawater was best for the propagation of *C. neogracile*. These results showed the biomass productivity measured as the concentration of chlorophyll *a* and/or *b*: 0.78 mg/l in Lyman, 1.40 mg/l in Coral, 1.58 mg/l in Kalle's ASW, 1.30 mg/l in MBL, 1.18 mg/l in Millero's ASW. Therefore, for further study, Kalle's ASW was selected as the best culture medium for the growth of *C. neogracile*. In the present study, fractional factorial design and central composite design, known as powerful tools for optimization of medium components, were carried out to maximize the growth rate and biomass production. The results of fractional factorial design showed that NaCl, KCl, $MgCl_2$, and Na_2SiO_3 were significant factors (see Table 8). In CCD, the central values of the NaCl, KCl, $MgCl_2$, and Na_2SiO_3 concentrations were 24.57 g/l, 0.667 g/l, 0.16 g/l, and 0.18 g/l, respectively. Corresponding to the results of statistical optimization, the maximum cell numbers of *C. neogracile* increased up to 300% after the optimization of the culture medium, when compared with the control duing the validation experiment (Fig. 3A).

In addition, as shown in Fig. 4B, we also were able to observe that *C. neogracile* produces an antifreeze-like protein in the supernatant of the culture medium. Although growth conditions for *C. neogracile* were optimized, it has not been established how the activity of antifreeze protein could be obtained, because even though cell numbers increased, no significant antifreeze protein activity from the culture supernatant was obtained based on the results shown in Fig. 4A. However, upon stimulating the cells by cold shock for 14 h at −5°C, we could certainly observe antifreeze-like protein activity, reflected by the creation of hexagonally shaped crystals (Fig. 4B).

Taken together, we optimized the culture medium for growth of *C. neogracile* using the statistical analysis models FFD and CCD, and introduced a possibility for the production of antifreeze-like protein produced from *C. neogracile* under cold-shock conditions. To the best of our knowledge, this is the first report describing the statistical optimization of the culture medium for growth of *C. neogracile*, with the indication of its possible antifreeze-like protein production. Owing to a lack of detailed structural information, further kinetic studies are now in progress for the purification as well as the determination of the consensus sequence of the ice-binding domain of antifreeze protein(s) from *C. neogracile*.

Acknowledgment

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (R01-2006-000-10856-0), for which the authors are grateful.

REFERENCES

1. Chauhan, K., U. Trivedi, and K. C. Patel. 2006. Application of response surface methodology for optimization of lactic acid production using date juice. *J. Microbiol. Biotechnol.* **16:** 1410–1415.

2. Ewart, K. V., Q. Lin, and C. L. Hew. 1999. Structure, function and evolution of antifreeze proteins. *Cell. Mol. Life Sci.* **55:** 271–283.
3. Griffith, M. and K. V. Ewart. 1995. Antifreeze proteins and their potential use in frozen foods. *Biotechnol Adv.* **13:** 375–402.
4. Joshi, S., S. Yadav, A. Nerurka, and A. J. Desai. 2007. Statistical optimization of medium components for the production of biosurfactant by *Bacillus licheniformis* K51. *J. Microbiol. Biotechnol.* **17:** 313–319.
5. Kalle, K. 1945. *In*: *Probleme der Kosnishen Physik*, 2nd Ed.
6. Kang, J.-S., S.-H. Kang, Y.-H. Lee, J.-H. Shim, and S. H. Lee. 2003. UV-B effects on growth and nitrate dynamics in Antarctic marine diatoms *Chaetoceros neogracile* and *Stellarima microtrias*. *Algae* **18:** 13–20.
7. Lee, C.-G. and B. Ø. Palsson. 1994. High-density algal photobioreactors using light-emitting diodes. *Biotechnol. Bioeng.* **44:** 1161–1167.
8. Lyman, J. and R. H. Fleming. 1940. Composition of sea water. *J. Marine Res.* **3:** 134–146.
9. Millero, F. J. 1996. *Chemical Oceanography.* CRC Press, Boca Raton, FL, U.S.A.
10. Montgomery, D. C. 1997. *Design and Analysis of Experiments*. John Wiley & Sons, Inc., NY, U.S.A.
11. Ooijkaas, L. P., E. C. Wilkinson, J. Tramper, and R. M. Buitelaar. 1999. Medium optimization for spore production of *Coniothyrium minitans* using statistically-based experimental designs. *Biotechnol. Bioeng.* **64:** 92–100.
12. Park, E.-K. and C.-G. Lee. 2001. Astaxanthin production by *Haematococcus pluvialis* under various light intensities and wavelengths. *J. Microbiol. Biotechnol.* **11:** 1024–1030.
13. Park, S. Y., K. Y. Seo, and S. D. Ha. 2007. A response surface model based on absorbance data for the growth rates of *Salmonella enterica* serovar Typhimurium as a function of temperature, NaCl, and pH. *J. Microbiol. Biotechnol.* **17:** 644–649.
14. Richmond, A. 2004. *Handbook of Microalgal Culture; Biotechnology and Applied Phycology.* Blackwell Science.
15. Wen, Z. Y. and F. Chen. 2001. Application of statistically-based experimental designs for the optimization of eicosapentaenoic acid production by the diatom *Nitzschia laevis*. *Biotechnol. Bioeng.* **75:** 159–169.