A Response Surface Methodology Study for the Protein Production of *Chlorella* sp.

Esra Imamoglu, Zeliha Demirel, and Meltem Conk Dalay

***Abstract*—**The target of this study was to optimize physical parameters such as light intensity, temperature and agitation rate which might affect the cultivation of *Chlorella* sp. Ege-Macc 039 by central composite design (CCD) using response surface methodology (RSM). The cells were cultured in 250 ml Erlenmeyer flasks containing 150 ml of F/2 medium for 12 days at different light intensities, temperatures and agitation rates. A total of 18 runs were used to optimize the range and levels of the chosen variables. The optimal physical conditions were found at 28 °C under the light intensity of 55 µmolphotons $m^{-2}s^{-1}$ at the agitation rate of 168 rpm with the protein concentration of 17.92 µg/100 µL for *Chlorella* sp.. The maximum specific growth rate of 0.21 day^{-1}, which corresponded to the doubling time of 3.38 day, was obtained for *Chlorella* sp. under the determined conditions**.**

***Keywords*—** Agitation, *Chlorella* sp., Light, Protein.

I. Introduction

MICROALGAE are unique and valuable microorganisms containing chlorophyll, protein and other photosynthesis-related pigments such as carotenoids, which enable them to absorb and utilize CO_2 as principal carbon source in the growth process [1]. Algae essentially require light (energy), carbon source (CO_2 for autotrophic metabolism), growth medium (water) and nutrients (nitrogen and phosphorous) for reproduction. Optimizing the cultivation conditions required for algal growth can mitigate their production costs and significantly improve the downstream process economics [2].

Chlorella vulgaris is a photosynthetic microorganism with a fast growth rate [3]. Particularly, *Chlorella* constituting a valuable source of proteins, vitamins and other compounds for animal feed can be obtained, simultaneously with a considerable decrease of wastewater pollution [4]. Nowadays, cultivation of *Chlorella* species as engineered systems in treatment of municipal wastewater and recycling has focused on bio-fuel production, instead of algal protein feed [5]. Furthermore, an extended knowledge of the protein quality and functional properties in microalgae hydrolysates would be useful in understanding their use as potential additives for food and dietary items [6].

Response surface methodology (RSM) has been applied successfully for optimization of parameters of various processes in biotechnology [7]. RSM is a statistical method based on the multivariate non-linear model, it is useful for evaluate and understand the interactions of the various parameters affecting the process. This multivariate approach has advantages in terms of reductions in the number of experiments, improved statistical interpretation possibilities and reduced time requirements from overall analysis [8].

The aim of this study was to optimize physical parameters such as light intensity, temperature and agitation rate which might affect the cultivation of *Chlorella* sp.. For this purpose, a set of experiments were designed by central composite design using response surface methodology to statistically evaluate the findings.

II. Materials And Methods

A. Maintenance of Chlorella sp.

The microalgae of *Chlorella* sp. was isolated from Izmir, Turkey located geographically between 38°19'32" North latitude and 26°39'13" East longitude. The isolated strain of *Chlorella* sp. was joined to Ege University Microalga Culture Collection (EGE MACC) and coded with Ege-Macc 039. Stock cultures were monoalgal (non-axenic) and cultivated in F/2 medium at 22±2 °C under continuous illumination (100 µmol photons m^{-2} s^{-1}) in 2-L sterile bottle for 22 days. For the preparation of the inoculum, the cells from the stock culture were collected and concentrated by centrifugation (1160 g, 2 min) and the supernatant was removed. The collected cells were transferred, incubated aseptically in 250 mL flasks containing 100 mL of F/2 medium under the light intensity of 40 µmol photons m^{-2} s^{-1} with the agitation rate of 120 rpm at 22±2 °C for four days. Four-day-old culture of green cells was used as inoculum at 10% volume for all experiments.

B. Growth Conditions for Chlorella sp.

The microalgae strain was cultured in 250 mL flasks containing 150 ml of F/2 medium in orbital shaking incubator at different light intensities, temperatures and agitation rates for 12 days. Illumination was provided by LED down light lamp (Cata 10 W CT-5254) from the top of the orbital shaking incubator. Irradiance was measured in the center of the flask with a quantum meter (Lambda L1-185).

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C.Analytical Procedure

Samples were taken at indicated times, and following growth parameters were measured immediately. The amount of protein was determined using Bradford method with Brilliant Blue G 250 dye [9]. The specific growth rate (μ) of the cells was calculated from the initial logarithmic phase of growth for at least 48 h, as $\mu = \ln X_2 - \ln X_1/dt$, where X_2 is the final cell concentration, X_1 is the initial cell concentration and dt is the time required for the increase in concentration from X_1 to X_2. Doubling time (DT) was also calculated as DT = ln 2/μ.

F/2 medium was prepared then autoclaved. Agar medium was prepared by the addition of 1.5 % agar powder to liquid media prior to autoclaving. All components (Merck Co.) were used analytical grade.

D.Experimental Design

The experimental optimization design was performed using Response Surface Methodology (RSM) with Central composite design (CCD). The experimental design was carried out using 2^3 full-factorial experiments design with six axial points (α = 1.682) and four replicates at the central point (55 μmolphotonsm^{-2}s^{-1}, 25 °C, 150 rpm), according to the Central Composite Design (CCD) by using the Design Expert software (version 7.0.0, Stat-Ease Inc., Minneapolis, MN). The range and the levels of the process variables are given in Table I. 5 different light intensities; X_1-μmol photonsm^{-2}s^{-1} (30, 40, 55, 70, 80), 5 different temperatures; X_2-°C (20, 22, 25, 28, 30) and 5 different agitation rates; X_3-rpm (100, 120, 150, 180, 200) were tested as physical variables. A total of 18 runs were used to optimize the range and levels of the chosen variables. Each run had been completed in 12 days. Protein amount (Y_1, μg/100 μL) was taken as response of the system.

The mathematical relationship of the response of these variables can be approximated by quadratic (second degree) polynomial equation;

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y represents the response variable, β_0 is model constant, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13} and β_{23} are interaction effect coefficients and β_{11}, β_{22} and β_{33} are quadratic coefficients, X_1, X_2 and X_3 are the coded levels of independent variables. The terms X_1 X_2 and X_i^2 (i = 1, 2 or 3) represent the interaction and quadratic terms, respectively. The quality of developed model was determined by the value of correlation (R^2) while analysis of variance (ANOVA) was used to evaluate the statistical significance of the model by the values of regression and mean square of residual error.

TABLE I
EXPERIMENTAL RANGE AND LEVELS OF THE INDEPENDENT VARIABLES ERROR

Independent Variables	Symbol Coded	Coded Levels				
		-α	-1	0	+1	+α
Light intensity (μmolphotonsm^{-2}s^{-1})	X_1	30	40	55	70	80
Temperature (°C)	X_2	20	22	25	28	30
Agitation rate (rpm)	X_3	100	120	150	180	200

III. RESULTS AND DISCUSSION

In this study, light intensity, temperature and agitation rate as physical factors play a dynamic role in the stimulation of protein amount of *Chlorella* sp. The experimental design and the results obtained in the experiments are given in Table II. As shown in the Table II, the protein amounts ranged from 7 to 18 μg/100 μL, depending on the conditions of experiments.

The statistical testing of the model was done by *Fisher's F* test for analysis of variance (ANOVA) as shown in Table III. The regression coefficient, R^2 of 0.9899 indicates that the regression model represented 98.99% of the experimental results, representing a good fit of the response. ANOVA showed that agitation rate is the most effective variable, followed by light intensity for protein amount of *Chlorella* sp.. On the other hand, the interaction coefficient term of X_2X_3 is insignificant ($p > 0.05$), indicating the less effect of the interaction between the two factors on protein amount. The value of $p > F$ for the model is less than 0.001, which indicate that it is highly significant and desirable model. Apart from that, the 'Lack of Fit *F*-value' of 0.54 implies that Lack of Fit is not significant relative to pure error. Hence, there is 71.95% chance that a 'Lack of Fit *F*-value this large could occur due to noise factor such as experimental errors. Non-significant lack of fit is good.

TABLE II
EXPERIMENTAL DESIGN MATRIX AND THE RESULTS OF *CHLORELLA* SP

Runs	X_1	X_2	X_3	Protein (μg/100 μL)
1	30	25	150	7.603
2	55	25	150	16.436
3	40	22	120	9.028
4	70	22	120	10.417
5	55	25	150	17.242
6	40	28	180	14.961
7	55	20	150	16.091
8	70	28	120	7.044
9	40	22	180	12.441
10	70	22	180	15.536
11	55	25	200	15.163
12	70	28	180	15.496
13	55	25	100	7.516
14	80	25	150	8.135
15	55	30	150	17.123
16	55	25	150	17.659
17	40	28	120	8.353
18	55	25	150	16.071

The adjusted determination coefficient (AdjR^2 = 0.9755) is also high, implying that the model has high significance. A very high degree of precision and a good deal of reliability of the experimental values are indicated by a low value of the coefficient of variation (C.V. = 4.85%).

The final estimative response model equation (based on the coded value) for protein amount of *Chlorella* sp. was estimated was as follows:

$$Y = 16.85 + 0.34X_1 + 0.012X_2 + 2.27X_3 - 0.66X_1X_2 + 0.44X_1X_3 + 0.82X_2X_3 - 3.17X_1^2 - 0.082X_2^2 - 1.94X_3^2 \quad (2)$$

where Y is the predicted response, i.e. protein (μg/100 μL), and X_1, X_2 and X_3 are the coded values of the test variables,

light intensity (µmolphotons $m^{-2}s^{-1}$), temperature (°C) and agitation rate (rpm), respectively. As shown in "(2)", protein amount have linear and quadratic effect by the three process variables.

TABLE III
ANALYSIS OF VARIANCE FOR RESPONSE SURFACE OPTIMIZATION OF PHYSICAL CONDITIONS FOR PHOTOTROPHIC CULTIVATION OF *CHLORELLA* SP. ON PROTEIN AMOUNT

Source	*SS	*DF	*MS	*F*-value	*p* > *F*
Model	268.48	10	26.85	68.66	< 0.0001
Light Int. (X_1)	1.55	1	1.55	3.97	0.086
Temp. (X_2)	$2.1*10^{-3}$	1	$2.06*10^{-3}$	$5.26*10^{-3}$	0.9442
Agitation r. (X_3)	29.24	1	29.24	74.77	<0.0001
X_1X_2	3.46	1	3.46	8.84	0.0207
X_1X_3	1.58	1	1.58	4.03	0.0847
X_2X_3	5.33	1	5.33	13.62	0.0077
X_1^2	127.26	1	127.26	325.45	<0.0001
X_2^2	0.086	1	0.086	0.22	0.6532
X_3^2	47.84	1	47.84	122.36	<0.0001
Residual	2.74	7	0.39		
Lack of fit	1.15	4	0.29	0.54	0.7195
Pure error	1.59	3	0.53		
Core total	271.22	17			
Std. dev.	0.63	R-squared		0.9899	
Mean	12.91	Adj R-squared		0.9755	
C.V. %	4.85	Pred R-squared		0.9507	
Press	13.37	Adeq precision		19.875	

*SS, sum of squares; DF, degrees of freedom; MS, mean square

The effect of interaction between light intensity and temperature (varying from 30-80 µmolphotons $m^{-2}s^{-1}$ and 20-30 °C, respectively) on the protein amount of *Chlorella* sp.. was shown in Fig. 1. It is important to note that concave response surface was found. A weak effect on the response was observed for both the lowest levels of light intensity and temperature. The protein amount increased with increasing the light intensity from 40 to 55 µmolphotons $m^{-2}s^{-1}$ within the studied range of temperature. As predicted by the model, the maximum protein amount of 17.12 µg/100 µL was occurred when the temperature was 24 °C at 55 µmolphotons $m^{-2}s^{-1}$, while the agitation rate was kept at 174 rpm.

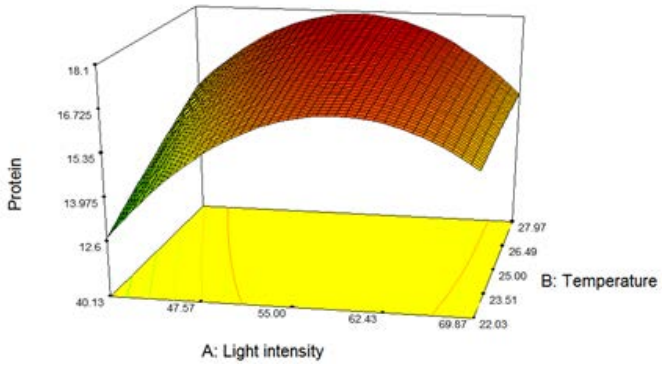

Fig. 1 3D response surface plot of central composite design showing the mutual effects of light intensity (µmolphotons$m^{-2}s^{-1}$) and temperature (°C) on protein amount (µg/100 µL) of *Chlorella* sp. Ege-Macc 039

Fig. 2 demonstrates the response surface 3D plots indicating the mutual effect of light intensity and agitation rate on the protein amount of *Chlorella* sp., while holding the other parameter (temperature) at its center point. The optimum for maximum protein amount lies near the central point of the light intensity. It can be noticed that by increasing the agitation rate, the protein amount increased. In addition, higher and lower levels of light intensity did not result in higher protein amount when the agitation rate was the minimum level.

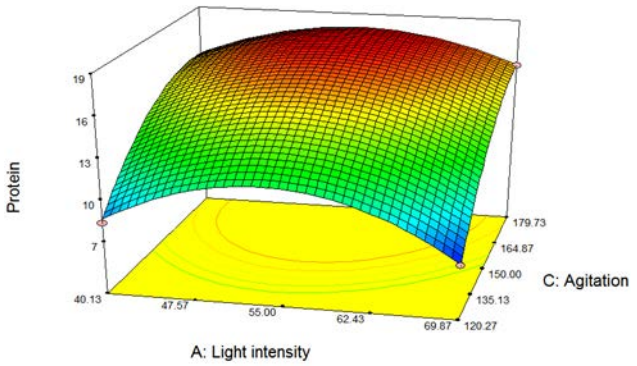

Fig. 2 3D response surface plot of central composite design showing the mutual effects of light intensity (µmolphotonsm^{-2}s^{-1}) and agitation rate (rpm) on protein amount (µg/100 µL) of *Chlorella* sp. Ege-Macc 039

It can be seen that both terms follow an almost linear trend (Fig. 3). At the lowest level of temperature, an increase in the agitation rate enhanced the protein amount. The maximum protein amount was occurred when the temperature was at the maximum level with the agitation rate of 180 rpm, while the light intensity was kept at the middle level. The observed phenomenon occurred as increasing the agitation rate tended to induce protein formation.

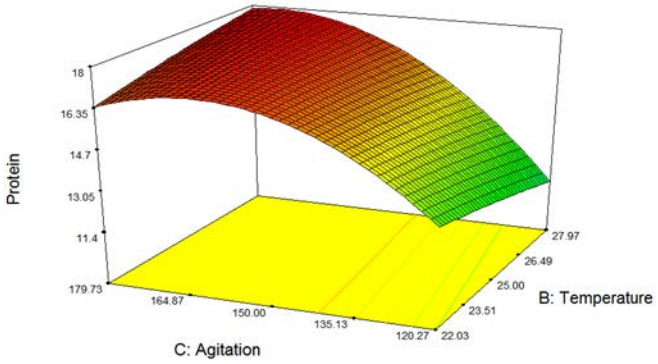

Fig. 3 3D response surface plot of central composite design showing the mutual effects of temperature (°C) and agitation rate (rpm) on protein amount (µg/100 µL) of *Chlorella* sp. Ege-Macc 039

In the optimization stage, the physical process variables (light intensity, temperature and agitation rate) were set within the range between low (-1) and high (+1) and the response was set to the maximum value. The optimization solution of *Chlorella* sp. (approximately at 28 °C with the agitation rate of 168 rpm under the light intensity of 55 µmolphotons $m^{-2}s^{-1}$) was selected because it resulted in the highest predicted response with the highest desirability.

The protein content of *C. vulgaris* was 550 ± 30.0 mg g^{-1} of the harvested biomass which was rich in eight kinds of essential amino acids (around 44.5% of the total) [5]. Agitation in the shaking flask cultures is directly connected to the level of aeration, and it is very important in the microalgae cultures because it helps to maintain a uniform temperature, to

provide light, CO_2 and other nutrients, and to distribute oxygen and other metabolic products in the culture [10,11].

Light is an essential environmental parameter in photosynthetic organisms where light energy is converted to chemical energy via photosynthesis and cellular respiration [12, 13]. It is also important to underline that light has profound quantitative and qualitative effects on protein formation. To our knowledge, this is the first report describing the correlations of light intensity, temperature and agitation rate and their effect on the protein production for *Chlorella* sp.

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