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Enhancement of Heterotrophic Biomass Production by *Micractinium sp. ME05*

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Abstract In this study, heterotrophic growth conditions for *Micractinium sp. ME05* cells were investigated for the improvement of biomass production. Plackett Burman (PB) method was used to screen process variables, namely, pH, carbon source and yeast extract concentrations, temperature and inoculum ratio, that affect the biomass production. The Box-Behnken (BB) design of response surface methodology (RSM) was applied to evaluate the interaction effect of process variables and to optimize them. The biomass obtained from PB design was 1.07 g/L and pH, temperature and carbon source concentration were selected based on their positive effect on biomass production. Applying response optimizer tool of RSM, the highest biomass obtained was 2.08 g/L. The results revealed that a 1.9-fold increase in biomass concentration was achieved by manipulating cultivation conditions which would be valuable for large scale cost efficient industrial applications of biomass production.

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Graphical Abstract
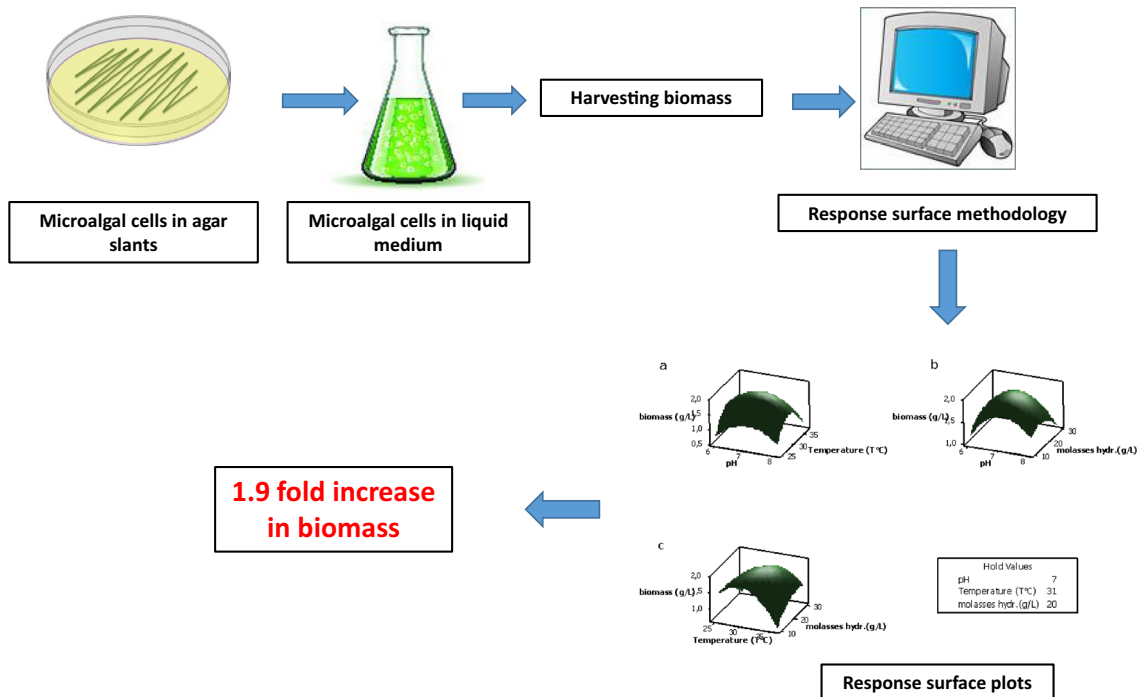
Introduction

Microalgae are procaryotic or eucaryotic photosynthetic
microorganisms that can grow rapidly and able to live in
severe conditions due to their unicellular or simple mul-
ticellular structure. Cyanobacteria (Cyanophyceae) is an
example to procaryotic microalgae wheras green algae
(Chlorophyta) and diatoms (Bacillarophyta) are examples
of eucaryotic microalgae [1].

Microalgae are useful sources for different industries
such as cosmetic, health food, chemical and pharmaceutical
industries producing high value products such as pigments,
carbohydrates, lipids, proteins and vitamins [2]. Large scale
production provides a wide variety of practical and meta-
bolic products, such as food supplements, lipids, enzymes,
biomass, polymers, toxins, pigments and 'green energy'
products. Such products were produced by cultivating
microlagae on various organic substrates such as glucose,
acetate, sugarcane baggase and synthetic or real wastewa-
ters [3, 4]. For a long time, microalgae have been known as
effective biological system to use solar energy in order to
produce biomass and different types of metabolites. Since
they have the ability to adapt to varying cultural conditions,
they can maximize the formation of targeted products [5].

The cultivation conditions of microalgae affect growth
characteristics and cellular composition of microalgae [6].
There are four major types of cultivation conditions for
microalgae; photoautotrophic, heterotrophic, mixotrophic
and photoheterotrophic cultivation [7]. In photoautotrophic
cultivation; microalgae use light (sunlight) as the energy
source and inorganic carbon such as carbondioxide in order
to produce chemical energy via photosynthesis [8]. In het-
erotrophic cultivation, organic compounds are used as
carbon and energy sources [7]. In mixotrophic cultivation
microalgae use both organic carbon and inorganic carbon
(CO_2) as a carbon source during photosythesis. In photo-
heterotrophic cultivation, microalgae require light while
using organic carbons as carbon source [9]. Microalgae can
use light efficiently but their photoautotrophic growth is
often slow because of light limitation at high cell densities
on a large scale [8, 10].

Heterotrophic cultivation can be a cost effective alterna-
tive for some microalgae that can use organic carbon sub-
stances as their sole carbon and energy source [11]. Het-
erotrophic growth of microalgae has some advantages over
photoautotrophic cultivation such as; elimination of light
requirement, enabling the control of cultivation process
and since the cell density is higher in hetetotrophic cultiva-
tion, the harvesting cost is low. In heterotrophic cultivation,
cell growth and biosythesis of products are significantly

affected by medium nutrients and environmental factors. Carbon source is one of the most important components in heterotrophic cultivation of microalgae for the production of lipids [12]. Several microalgae such as *Chlorella prothecoides* and *Crypthecodium cohnii* can uptake carbohydrates such as glucose directly and transform to lipid. Such a heterotrophic metabolism results in higher cell density and neutral lipid content [13].

On the other hand, since the carbon source usually used in relatively higher concentrations in comparison to other medium components, it contributes to the raw material cost in high amounts. Therefore, utilization of cheaper carbon sources is strongly required. Molasses, a waste of sugar industry is one of the most favorable carbon source. It consists of approximately 45–50% (w/w) total sugars (mainly sucrose, glucose and fructose), 17–25% water and 2–5% polysachharides (dextrins, pentosans, polyuronic acid) [14]. Gaurav et al. [15] evaluated the usage of molasses to increase the biomass and lipid production of *Chlorella pyrenoidosa*. *C. pyrenoidosa* cells were maintained in 100 mL Fogg's medium that contain different sugars (10 g/L), which include glucose, sugar mixture (8 g/L sucrose, 1 g/L glucose and 1 g/L fructose), treated and untreated molasses. The biomass obtained was 0.89, 0.81, 0.52 and 1.22 g/L with glucose, sugar mixture, untreated molasses and treated molasses, respectively. By taking into consideration the increase in biomass concentration from 0.89 to 1.22 g/L and the increase in lipid content from 0.27 to 0.66 g/g using molasses instead of glucose they reported the feasibility of using molasses as a carbon source for large scale low-cost lipid production by *C.pyrenoidosa* [15]. In addition, corn powder hydrolysate [16], sweet sorghum [17] and cassava [18] are other cheap organic carbon sources that were used as carbon source for the cultivation of microalgae.

In order to maximize the industrial productivity of microalgae, there is a need for optimization of high-density cell cultivation conditions. Such an optimization is performed via repeating experiments. Instead of performing repeating experiments, response surface methology (RSM) can be applied and thus optimized values can be determined with a less number of experiments [19]. Also, interactions between operating variables can be explained by RSM and optimum conditions for algal growth are determined [20].

Several researchers have studied the optimization of cultivation conditions and medium compositions of microalgae using RSM [20, 21]. Cheng et al. [21], used the Central Composite Design of RSM to investigate the effects of C/N (carbon source/nitrogen source) and yeast extract concentration on the biomass and lipid production of *Chlorella prothecodies* [21]. Kirrolia et al. [20] used the Box-Behnken Design of RSM for medium optimization of *Chlorella spp*. during biodiesel production [20]. However no study has been found on investigation of pH, incubation temperature, carbon source and yeast extract concentration and inoculum ratio on the heterotrophic biomass production by *Micractinium sp*. cells. Thus, the aim of this study was to enhance the biomass production of *Micractinium sp. ME05* through optimization of cultivation conditions. For a cost efficient production of biomass, molasses was used as a carbon source. Different concentrations of carbon source, inoculum ratios of cells and media (basal medium and bold basal medium) were tested by one factor at a time approach and then by Plackett Burman method to screen the process variables that affect the biomass production. The interaction effect between process variables were also evaluated with Box-Benhken Design of response surface method (RSM). This study revealed the optimum heterotrophic cultivation parameters for the enhancement of biomass production of *Micractinium sp. ME05* cells with the usage of molasses as a carbon source.

Materials and Methods

Materials

The algal cells used in this study were *Micractinium sp. ME05*, which were isolated from Haymana, Ankara (latitude 39.44°N, longitude 32.48°E) geothermal flora and characterized as previously described [22]. Microalgae were maintained on agar slants that contain tris-acetate-phosphate (TAP) medium [23].

Culture Medium and Cultivation Conditions

Micractinium ME05 strain, maintained on slant agars of TAP medium, was inoculated into 100 mL of liquid TAP medium. The TAP medium was composed of 2.4 g/L of Tris (hydroxymethyl) aminomethane, 0.8 g/L of NH_4Cl, 0.1 g/L of $CaCl_2 \cdot 2H_2O$, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.3 g/L of K_2HPO_4, 0.05 g/L of $EDTA \cdot 2H_2O$, 5 mg/L of $FeSO_4 \cdot 7H_2O$, 0.022 g/L of $ZnSO_4 \cdot 7H_2O$, 5.2 mg/L of H_3BO_3, 5 mg/L of $MnCl_2 \cdot 4H_2O$, 1 mg/L of $CuCl_2 \cdot 2H_2O$, 2.6 mg/L of $Na_2 \cdot MoO \cdot 2H_2O$, and 1.6 mg/L of $CoCl_2 \cdot 6H_2O$ [23]. Cells were incubated at $25 \pm 1 °C$ with shaking at 150 rpm for 4 days in a controlled growth chamber. These cells were used as seed cells for basal cultivation medium and bold basal cultivation medium.

Micractinium ME05 cells were cultivated in 250 mL basal medium and 250 mL bold basal medium in 500 mL flasks. The components of basal culture medium were; 0.7 g/L of KH_2PO_4, 0.3 g/L of K_2HPO_4, 0.7 g/L of $MgSO_4 \cdot 7H_2O$, 3 mg/L of $FeSO_4 \cdot 7H_2O$, 0.1 g/L of glycine, 0.01 mg/L of vitamin B_1, 1 ml/L of A5 trace mineral solution (2.86 g/L of H_3BO_3, 0.04 g/L of $Na_2MoCo_4 \cdot 2H_2O$, 0.22 g/L of $ZnSO_4 \cdot 7H_2O$, 1.81 g/L of $MnCl_2 \cdot 4H_2O$,

0.074 g/L of CuSO$_4$·5H$_2$O) [24]. The components of bold basal culture medium were ; 0.175 g/L of K$_2$HPO$_4$, 0.025 g/L of CaCI$_2$·2H$_2$O, 0.075 g/L of MgSO$_4$7H$_2$O, 0. 25 g/L of NaNO$_3$, 0.075 g/L of K$_2$HPO$_4$, 25 g/L of NaCI, 11.42 mg/L of H$_3$BO3, 1 ml/L of trace metal solution consisting of 8.82 g/L of ZnSO$_4$·7H$_2$O, 1.44 g/L of MnCI$_2$·4H$_2$O, 0.71 g/L of MoO$_3$, 1.57 g/L of CuSO$_4$·5H$_2$O, 0.49 g/L of Co(NO$_3$)$_2$·6H$_2$O, alkaline EDTA stock solution (1 ml/L) consisting of 50 g/L Na$_2$EDTA and 31 g/L of KOH, and acidified iron stock solution (1 ml/L) consisting of 4.98 g/L of FeSO$_4$ and 1 mL of H$_2$SO$_4$ [25].

All media and cultivation apparatus were sterilized by autoclaving at 121 °C for 15 min. After sterilization, 30 g/L glucose and 2 g/L yeast extract [26] were added to 250 mL basal medium in 500mL flasks by filtering through a milipore filter. *Micractinium sp. ME05* in exponential phase was inoculated (0.2 and 5%, v/v) into basal cultivation medium.10 and 30 g/L glucose were added to 250 mL bold basal medium separately by filtering through a milipore filter. *Micractinium sp. ME05* in exponential phase was inoculated (10%, v/v) into bold basal cultivation medium. Heterotrophic cultivation was carried out with air flowing (~ 0.5 L min) in the dark at 25 ± 1 °C for 5 days in a controlled growth chamber.

Pretreatment of Molasses

The molasses used in this study was obtained from Konya Sugar Factory, Konya, Turkey. Acidic pretreatment was applied to molasses. For this purpose 10 mL of molasses was mixed with 90 mL of 1% (w/v) and 3% (w/v) of H$_2$SO$_4$ seperately. The solutions were autoclaved at 121 °C for 40 min. The reducing sugar content was determined using 3,5-dinitrosalicylic acid (DNS) method [27].

Cell Growth and Biomass Measurement

Cell growth was monitored by optical density measurements at 680 nm using Shimadzu UV-1800 spectrophotometer. Microalgae biomass was harvested in stationary phase by centrifugation at 3000 g for 10 min at 4 °C [22]. The harvested biomass was washed with distilled water and then dried at 70 °C in a hot air oven. The biomass was determined gravimetrically [28].

Experimental Design

One Factor at a Time Approach

The approximate medium composition and inoculum ratio for the heterotrophic cultivation of *Micractinium sp. ME05* was determined by varying one factor at a time while keeping all other factors constant. For this purpose

two different inoculum ratios 0.2 and 5% (v/v) of *Micractinium sp. ME05* cells were evaluated in terms of biomass production. Basal medium was used as a cultivation medium for these inoculum ratios. Bold basal medium was supplemented with 10 and 30 g/L glucose separately and 10% (v/v) of *Micractinium sp. ME05* cells were inoculated into these media.

Screening of Process Variables by Plackett Burman Method

Plackett-Burman Design was used to screen factors that significantly affect biomass production. pH, temperature, glucose, yeast extract and inoculum size were selected as variables (Table 1). All experiments were conducted in triplicate. The independent variables; pH, temperature, glucose, yeast extract and inoculum size were coded as X_1, X_2, X_3, X_4 and X_5 respectively. The predicted response ($\hat{Y}$) was written as follows:

$$\hat{Y} = \beta_0 + \beta_1 X + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 \quad (1)$$

where $\hat{Y}$ is the predicted response (biomass concentration), β_0, β_1, β_2, β_3, β_4 and β_5 are regression coefficients [29].

The statistical software MINITAB 16.0 (Minitab Inc., State College, PA, USA) was used to analyze the experimental design. A 24-run Plackett-Burman Design was used to screen 5 factors.

Box-Behnken Design

The interaction effect between process variables were studied using the Box-Behnken Design (BBD) with a quadratic model using MINITAB 16.0 (Minitab Inc., State College, PA, USA). Based on the outcome of Plackett-Burman Design experiments, BBD matrix was created using pH (X_1), temperature (X_2) and molasses hydrolysate concentration (g/L)(X_3). The ranges of process variables were 6–8 for pH, 25–37 °C for temperature and

Table 1 Coded and uncoded variables at different levels used in Plackett-Burman Design

Variable	Symbol	Value		
		−1	0	+1
pH	X_1	6	7	8
Temperature	X_2	25	31	37
Inoculum (v/v %)	X_3	5	7.5	10
Yeast Extract (g/L)	X_4	0	2	4
Glucose (g/L)	X_5	10	15	30

10–30 g/L for glucose concentration. A set of 45 experiments were carried out. A second order polynomial equation was related to biomass production as follows:

$$\hat{Y} = \beta_0 + \beta_1 X + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 \\ + \beta_6 X_3^2 + \beta_7 X_1 X_2 + \beta_8 X_1 X_3 + \beta_9 X_2 X_3 \tag{2}$$

where $\hat{Y}$ is the predicted response (biomass concentration) and β_0 the intercept, β_1, β_2 and β_3 are linear coefficients, β_4, β_5 and β_6 squared coefficients, β_7, β_8 and β_9 interaction coefficients and X_1, X_2 and X_3 independent variables used in this study. The optimum conditions for maximizing biomass production was determined by using Response Optimiser Tool in MINITAB16.0 (Minitab Inc., State College, PA, USA) .

Model Verification

The accuracy of the applied model was determined by comparing the results of the experiments with the results of the predicted biomass calculations. In order to evaluate the performance of the model a statistical difference measure test was performed. Root mean square error (RMSE) and mean absolute error (MAE) was calculated as follows:

$$RMSE = \left(\frac{1}{N} \sum_{i=1}^{N} \left(X_{pred,i} - X_{exp,i} \right)^2 \right)^{0.5} \tag{3}$$

$$MAE = \frac{1}{N} \sum_{i=1}^{N} \left| X_{pred,i} - X_{exp,i} \right| \tag{4}$$

where $X_{pred,i}$ and $X_{exp,i}$ are predicted and experimental biomass concentrations, respectively; N is the number of data points [29].

Results and Discussion

One factor at a Time Approach

In order to determine the approximate medium composition and inoculum ratio for the heterotrophic cultivation of *Micractinium sp. ME05* cells, two different inoculum ratios (0.2 and 5% v/v) of *Micractinium sp. ME05* cells into basal medium were evaluated while keeping all other factors constant. When basal medium that contain 30 g/L glucose, was inoculated with 0.2% (v/v) cells, the biomass obtained was 1.33 g/L. With a 5% (v/v) inoculum, the biomass increased to 2.69 g/L. In bold basal medium containing 10 g/L glucose, the biomass obtained was 0.96 g/L, whereas at 30 g/L glucose, the biomass increased to 2.23 g/L (Table 2). These biomass results indicated that, basal medium inoculated with higher inoculum resulted in higher biomass. Also for bold basal medium, higher concentration of carbon source gave higher biomass results, which agreed well to other studies.

In the heterotrophic cultivation of *Chlorella prothecoides* using basal medium that contain 10 g/L glucose as carbon source and inoculum ratio of 10% (v/v), the biomass obtained was reported as 3.74 g/L after 144 h of incubation [16]. In a study of Yan et al. (2011), basal medium that contain 30 g/L glucose and 2 g/L yeast extract was inoculated with 10% (v/v) *Chlorella prothecoides* cells. After 120 h of incubation at 28 °C, the biomass reported as 12.8 g/L [26]. Wei et al. [18] evaluated cassava starch hydrolysate (CSH) instead of glucose to increase biomass and reduce the cost of heterotrophic cultivation of *C. prothecoides*. In order to compare glucose and CSH, different levels of glucose concentration (10, 20, 30, 40 and 50 g/L) were used in a glucose based medium and the same glucose concentrations were used in the CSH based medium by adding equivalent amount of CSH to basal medium. These media were inoculated with 5% (v/v) *C. prothecoides* cells. After incubation at 28 °C for 240 h under dark conditions, they reported that high glucose concentrations increased biomass but decreased the lipid content. On the other hand low glucose concentration decreased the biomass concentration and increased the lipid content. In terms of cassava starch hydrolysate, they reported that higher or lower glucose concentration in CSH inhibited the growth of *Chlorella*. As a result, a medium with 30 g/L glucose in CSH was preferred for the heterotrophic culture of *Chlorella* [18].

In our study, during heterotrophic cultivation of *Micractinium sp. ME05*, different inoculum ratios and glucose concentrations gave rise to different biomass results. In order to screen the important variables that affect biomass concentration, a 2-level Plackett-Burman Design was used prior to response surface optimization.

Table 2 Biomass results of *Micractinium sp. ME05* cells in different medium composition and different inoculum ratios

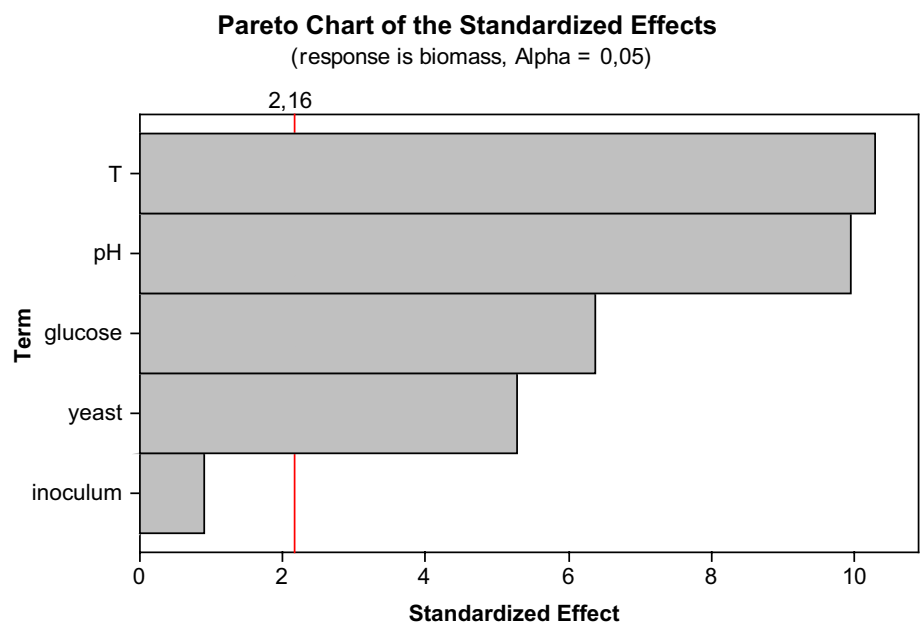| Cultivation medium	Inoculation ratio (v/v %)	Biomass (g/L)
Basal medium + 30 g/L glucose + 2 g/L yeast ext	0.2	1.33
Basal medium + 30 g/L glucose + 2 g/L yeast ext	5	2.69
Bold basal medium + 10 g/L glucose	10	0.96
Bold basal medium + 30 g/L glucose	10	2.23

Table 3 The Plackett–Burman experimental design and corresponding results

Run	pH	T °C	Inoc. (v/v %)	Yeast ext. (g/L)	Glucose (g/L)	Biomass (g/L)
1	8	25	5	0	30	1.12
2	6	37	10	4	10	1.25
3	6	37	10	0	30	1.24
4	6	25	10	4	30	1.78
5	6	37	5	0	10	1.69
6	8	25	10	4	10	1.25
7	8	37	5	4	30	1.67
8	6	25	5	0	10	1.07
9	6	25	5	4	30	2.38
10	8	25	10	0	10	1.66
11	8	37	10	0	30	1.92
12	8	37	5	4	10	2.05

Table 4 Estimated effects and coefficients from screening process variables by Plackett–Burman method (coded units)

Term	Effect	Coefficient	SE coefficient	t value	P value
Constant		1.2883	0.02894	44.51	0.000
pH	0.6883	0.3442	0.02736	12.58	0.000
T °C	0.7068	0.3534	0.02894	12.21	0.000
Inoculum (v/v %)	−0.0769	−0.0385	0.02194	−1.75	0.103
Yeast extract (g/L)	−0.3650	−0.1825	0.02736	−6.67	0.000
Glucose (g/L)	−0.4218	−0.2109	0.02894	−7.29	0.000

Result is signficant when $P < 0.05$ and $R^2 = 0.9442$, R^2 (adj) = 0.8751% and R^2(pred) = 0.9227

Screening of Process Variables by Plackett Burman Method

A 12 run Plackett-Burman design was used to screen five factors. All experiments were conducted in two replicates and the response was the average biomass concentration (g/L) after 120 h of incubation. For Plackett-Burman Design, experimental response (Y) and predicted response (Ŷ) are given in Table 3. The first order equation expressed in coded units for biomass production is as follows:

$$\hat{Y} = 1.2883 + 0.3442X_1 + 0.3534X_2 - 0.0385X_3 - 0.1825X_4 - 0.02109X_5 \quad (5)$$

where X_1, X_2, X_3, X_4 and X_5 are coded variables for pH, temperature, inoculum ratio (v/v %), yeast extract

Fig. 1 Pareto chart for results of Plackett–Burman Design

Pareto Chart of the Standardized Effects
(response is biomass, Alpha = 0,05)

Table 5 The Box-Behnken Design, experimental and predicted values for biomass production

Run	pH	T °C	Molasses hydrolysate (g/L)	Y (g/L)[a]	Ŷ (g/L)[b]
1	7	25	10	1.32	1.41
2	6	31	30	1.31	1.32
3	7	37	10	0.62	0.68
4	8	37	20	0.70	0.64
5	7	31	20	1.88	1.95
6	8	31	30	0.95	1.01
7	8	25	20	0.82	0.82
8	7	25	30	0.76	0.69
9	6	37	20	0.79	0.78
10	6	31	10	1.22	1.15
11	7	31	20	1.92	1.95
12	7	31	20	2.05	1.95
13	6	25	20	0.73	0.78
14	7	37	30	1.25	1.24
15	8	31	10	1.36	1.35

Inoculum ratio and yeast extract concentration were used at center point levels

[a]Y(g/L) is the experimental biomass concentration

[b]Ŷ (g/L) is the predicted biomass concentration

concentration (g/L) and glucose concentration (g/L), respectively.

Estimated effects and coefficients for each variable is given in Table 4. Pareto chart of design variables is given in Fig. 1. According to the results, pH and temperature indicated positive effects on biomass production, whereas inoculum ratio, yeast extract concentration and glucose concentration had negative effects on biomass production. On the other hand, the variables pH, temperature, yeast extract concentration and glucose concentration had significant (p < 0.05) effects on biomass production at confidence level of 95%. The variable, inoculum ratio was found to be insignificant (p > 0.05). Due to their negative effects on biomass production, in subsequent optimization study, the inoculum ratio and yeast extract concentration were used at the center point levels. Although glucose concentration also showed a negative effect on biomass production, it was included in the optimization part since it was the major raw material of this study.

Li et al. (2011) used a similar approach including Box-Behnken Design to optimize carbon, nitrogen and phosphorus concentration in the medium of *Chlorella minutissima* UTEX2341 and to increase the biomass. Carbon, nitrogen and phosphorus concentration were selected as variables with the maximum and minimum values 3.12–25 g/L, 0.78–3.12 g/L and 0.03 g/L and 0.52 g/L for carbon, nitrogen and phosphorus concentration, respectively. They reported that, carbon and nitrogen concentration

significantly affected biomass production on the other hand phosophorus concentration had no significant efffect on biomass. The optimized carbon, nitrogen and phosphorus concentrations were 26.37, 2.61 and 0.03 g/L, respectively [30].

Box-Behnken Design

The results of Box-Behnken experiments to study the effects of pH, temperature and molasses hydrolysate concentration are presented in Table 5. The regression coefficients for biomass production is presented in Table 6. The coefficient of determination (R^2) for biomass production was 0.94. As the R^2 value gets close to 1.0, the model gets stronger [31]. The R^2 value obtained here demonstrated that the model adequately fitted to the data. The insignificant lack of fit (P = 0.131 > 0.05) also demonstrated that the model fitted well to the experimental data.

Among the variables pH, temperature and molasses hydrolysate concentration showed insignificant effects (p > 0.05) under the tested range. Although their linear effects were insignifcant, the quadratic effects of each variable showed significant effects (p < 0.05). The interaction between pH and molasses hydrolysate and temperature and molasses hydrolysate were significant (p < 0.05), whereas the interaction between pH and temperature was insignifcant (p > 0.05). The highest biomass concentration of 2.05 g/L was observed at a pH of 7, at a temperature of 31 °C with 20 g/L molasses hydrolysate. The second order polynomial equation for the prediction of biomass was:

$$\hat{Y} = 1.95000 - 0.02708X_1 - 0.03333X_2 - 0.03375X_3$$
$$- 0.48452X_1^2 - 0.70792X_2^2 - 0.25375X_3^2$$
$$- 0.004500X_1X_2 - 0.012417X_1X_3 + 0.29833 X_2X_3$$
$$(6)$$

Table 6 Regression analysis results for biomass production

Term	Coef	SE Coef	T	P
Lack of fit				0.131
Constant	1.95000	0.04064	47.986	0.000
pH	−0.02708	0.02488	−1.088	0.284
T °C	−0.03333	0.02488	−1.340	0.189
Molasses**	−0.03375	0.02488	−1.356	0.184
pH × pH	−0.48452	0.03663	−13.252	0.000
T °C × T °C	−0.70792	0.03663	−19.326	0.000
Molasses × molasses	−0.25375	0.03663	−6.927	0.000
pH × T °C	−0.04500	0.03519	−1.279	0.209
pH × molasses	−0.12417	0.03519	−3.528	0.001
T °C × molasses	0.29833	0.03519	8.477	0.000

*Result is signficant when P < 0,05 and R^2 = 0.9470, R^2 (adj) = 0.9333% and R^2(pred) = 0.9121

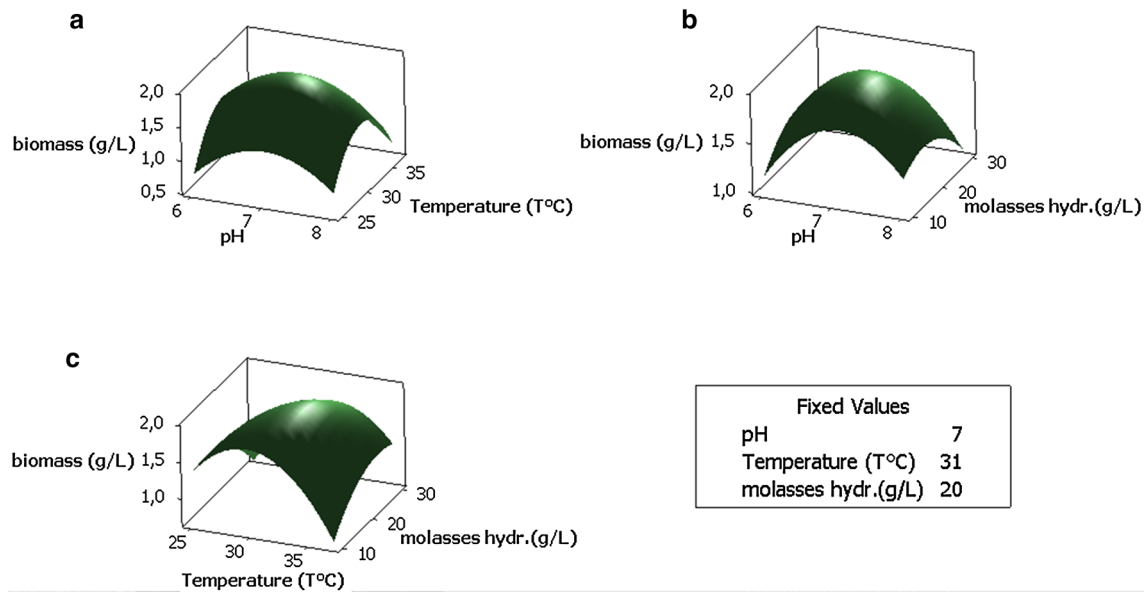**Molasses is the molasses hydrolysate concentration

Fig. 2 Response surface plots showing the effect of **a** temperature and pH, **b** molasses hydrolysate concentration and pH, **c** temperature and molasses hydrolysate concentration on biomass production. The third factor, in all cases was held constant at the middle level (i.e. pH:7, temperature 31 °C and molasses hydrolysate: 20 g/L)

where $\hat{Y}$ is the predicted biomass concentration, X_1, X_2 and X_3 are uncoded variables for pH, temperature and molasses hydrolysate concentration, respectively. The response surface plots described by the regression model were plotted for better understanding (Fig. 2). All surface plots demonstrated that as the level of variables increased, biomass production increased up to a certain level. In Fig. 2a biomass concentration demostated a non-linear effect with the increase of pH from 6 to 8, and with the increase of temperature from 25 to 35 °C under constant molasses hydrolysate concentration. In Fig. 2b surface plots demonstrated that both high and low temperatures had negative effect on biomass production. Similarily high and low concentrations of molasses hydrolysate had negative effect on biomass production. The effects of temperature and carbon source concentration in our study are in accordance with the findings of Lakshmikandan et al. (2016), who reported that high and low concentrations of subtsrate and high and low values of temperature had negative effect on cell growth and biohydrogen production [32]. Such a behaviour may result from the fact that under sub-optimal temperature conditions, cytoplasmic viscosity of the cells change and carbon utilization becomes less efficient. On the other hand, increasing the temperature beyond the optimum reduces protein sythesis and as a result reduces cell growth rate [33]. An increase in pH with a simultaneous increase in molasses hydrolysate concentration (temperature constant) led to an increase in biomass production until they reached their optimal values (Fig. 2b). Such a behaviour was expected

since pH determines the solubility of essential nutrients and maximum algal growth occurs around neutral pH [33]. When pH was held constant, an increase in temperature about to 30 °C and an increase in molasses hydrolysate concentration to 20 g/L caused an increase in biomass concentration, as denoted by the significant interaction term in Table 6. In Fig. 2c, the molasses hydrolysate concentration showed a non-linear effect on biomass production. As molasses hydrolysate concentration was increased to about 20 g/L, biomass concentration increased to about 1.5 g/L. An incrase in biomass concentration with an incrase in molasses hydrolysate concentration was due to the fact that biomass is mainly controlled by the carbon source and is essential for building up biomass [20].

The highest biomass result obtained with BB Design experiments using 19 g/L molasses hydrolysate is 2.05 g/L and the biomass productivity is 0.27 g/L/day. Such a biomass productivity is higher than those obtained in the literature that were concerned with the optimization of biomass production from *Micractinium* species. For example, Karpagam et al. [28] focused on increasing biomass and lipid production of *Coelastrella sp. M-60* and *Micractinium sp. M-13* using sugarcane industry effluent (0.625, 1.2 and 2.5 mL/L), citric acid (10, 20, 30 mg/L) glucose (0.05, 0,1, 0,15 and 0.2%)and vitamin B_{12} (0.001, 0.002 and 0.003%) with BG-11 medium. Medium optimization studies were performed with Central Composite Design (CCD) and it was reported that the growth of *Micractinium sp. M-13* was maximum for BG-11 medium containing 25 mg/L citric

acid and 1.25 ml/L sugarcane industry effluent with a biomass productivity of 0.0615 g/L/day [28]. Beside this, there are different studies that are concerned with the enhancement of microalgal biomass production by using Response Surface Methdology and Multi-Objective Optimization [21, 34]. Kanaga et al. [34] focused on maximizing lipid and biomass production of *Chlorella pyrenoidosa* NCIM 2738 by using multi objective optimization strategy. Glucose, $NaNO_3$ and K_2HPO_4 concentration were selected as variables and were investigated with Central Composite Design. It was reported that the optimal level of $NaNO_3$, K_2HPO_4 and glucose concentration to maximize biomass concentration were 23.63 mM, 286.4 μM and 4.18% (w/w), respectively. Under such conditions the biomass obtained was 1.395 g/L [34]. Different biomass quantitites observed in the previous studies and in this study is due to the fact that between various species the lipid content, lipid and biomass productivites show significant differences [35].

In comparison with one factor at a time approach in this study, the decrease in the amount of carbon source concentration from 30 g/L glucose to 19 g/L waste molasses hydrolysate can remarkably reduce the cost of cultivation since the cost of glucose is estimated to be 80% of the total medium cost [36]. On the other hand, alternatively cheap carbon sources have been suggested to replace glucose for lowering culture costs [15, 18].

Model Verification Results

The optimum conditions for biomass production predicted by the model were found as temperature of 30.9 °C, a pH of 6.9 and molasses hydrolysate concentration of 19 g/L. Under these conditions the biomass was measured as 2.08 g/L. The biomass obtained was very close to corresponding predicted value of 1.95 g/L with a very low standart deviation (0.09) and very low coefficient of variation (0.04 or 4%). These results indicated that the model adequately estimates the biomass produced. The constructed model was also checked using error analysis. The RMSE and MAE values were calculated as 0.10 and 0.08, respectively, which both indicate low error and high accuracy of prediction.

Conclusion

This study presented optimization of heterotrophic cultivation conditions for *Micractinium sp. ME05* to improve biomass production. Process variables were screened by PB method. The PB method gave 1.07 g/L biomass and 0.15 biomass yield ($Y_{x/s}$) with 7.18 g/L glucose consumption. Response surface optimization resulted in 2.08 g/L biomass and 0.21 biomass yield ($Y_{x/s}$) with 9.82 g/L molasses

hyrdolysate consumption. The results demonstrated that cultivation conditions can be manipulated with RSM to increase the biomass about to 1.9-fold. Since molasses hydrolysate is a low cost substrate compared to glucose, this study provides useful information for further studies regarding cost efficient large scale production of biomass for industrial applications.

References

1. Brennan, L., Owende, P.: Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. **14**, 557–577 (2010). doi:10.1016/j.rser.2009.10.009
2. Suali, E., Sarbatly, R.: Conversion of microalgae to biofuel., Renew. Sustain. Energy Rev. **16**, 4316–4342 (2012). doi:10.1016/j.rser.2012.03.047
3. Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y.: Heterotrophic cultures of microalgae: Metabolism and potential products. Water Res. **45**, 11–36 (2011). doi:10.1016/j.watres.2010.08.037
4. Rattanapoltee, P., Kaewkannetra, P.: Utilization of agricultural residues of pineapple peels and sugarcane bagasse as cost-saving raw materials in Scenedesmus acutus for lipid accumulation and biodiesel production. Appl. Biochem. Biotechnol. **173**, 1495–1510 (2014). doi:10.1007/s12010-014-0949-4
5. Agwa, O.K., Ibe S.N., Abu, G.O.: Heterotrophic cultivation of *Chlorella* sp. using different waste extracts. Int. J. Biochem. Biotechnol. **2**, 289–297 (2013)
6. Sonmez, C., Elcin, E., Akın, D., Avni, H., Yucel, M.: Bioresource technology evaluation of novel thermo-resistant *Micractinium* and *Scenedesmus* sp. for efficient biomass and lipid production under different temperature and nutrient regimes. Bioresour. Technol. **211**, 422–428 (2016). doi:10.1016/j.biortech.2016.03.125
7. Chojnacka, K., Marquez-Rocha, F.-J.: Kinetic and Stoichiometric Relationships of the Energy and Carbon Metabolism in the Culture of Microalgae. Biotechnology **3**, 21–34 (2004). doi:10.3923/biotech.2004.21.34
8. Huang, G., Chen, F., Wei, D., Zhang, X., Chen, G.: Biodiesel production by microalgal biotechnology. Appl. Energy. **87**, 38–46 (2010). doi:10.1016/j.apenergy.2009.06.016
9. Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., Chang, J.-S.: Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. Bioresour. Technol. **102**, 71–81 (2011). doi:10.1016/j.biortech.2010.06.159
10. Wen, Z.-Y., Chen, F.: Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnol. Adv. **21**, 273–294 (2003). doi:10.1016/S0734-9750(03)00051-X
11. Chen, F.: High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol. **14**, 421–426 (1996). doi:10.1016/0167-7799(96)10060-3

12. Liu, J., Sun, Z., Zhong, Y., Gerken, H., Huang, J., Chen, F.: Utilization of cane molasses towards cost-saving astaxanthin production by a Chlorella zofingiensis mutant. J. Appl. Phycol. **25**, 1447–1456 (2013). doi:10.1007/s10811-013-9974-x

13. Miao, X., Wu, Q.: Biodiesel production from heterotrophic microalgal oil. Bioresour. Technol. **97**, 841–846 (2006). doi:10.1016/j.biortech.2005.04.008

14. Najafpour, G.D., Poi Shan, C.: Enzymatic hydrolysis of molasses. Bioresour. Technol. **86**, 91–94 (2003). doi:10.1016/S0960-8524(02)00103-7

15. Gaurav, K., Srivastava, R., Sharma, J.G., Singh, R., Singh, V.: Molasses based growth and lipid production by *Chlorella pyrenoidosa*: A potential feedstock for biodiesel. Int. J. Green Energy. **5075**, 150122092222001 (2015). doi:10.1080/15435075.2014.966268

16. Xu, H., Miao, X., Wu, Q.: High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. J. Biotechnol. **126**, 499–507 (2006). doi:10.1016/j.jbiotec.2006.05.002

17. Gao, C., Zhai, Y., Ding, Y., Wu, Q.: Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Appl. Energy. **87**, 756–761 (2010). doi:10.1016/j.apenergy.2009.09.006

18. Wei, A., Zhang, X., Wei, D., Chen, G., Wu, Q., Yang, S.-T.: Effects of cassava starch hydrolysate on cell growth and lipid accumulation of the heterotrophic microalgae *Chlorella protothecoides*. J. Ind. Microbiol. Biotechnol. **36**, 1383–1389 (2009). doi:10.1007/s10295-009-0624-x

19. Kim, W., Park, J.M., Gim, G.H., Jeong, S.-H., Kang, C.M., Kim, D.-J., Kim, S.W.: Optimization of culture conditions and comparison of biomass productivity of three green algae. Bioprocess Biosyst. Eng. **35**, 19–27 (2012). doi:10.1007/s00449-011-0612-1

20. Kirrolia, A., Bishnoi, N.R., Singh, R.: Response surface methodology as a decision-making tool for optimization of culture conditions of green microalgae *Chlorella* spp. for biodiesel production. Ann. Microbiol. **64**, 1133–1147 (2014). doi:10.1007/s13213-013-0752-4

21. Cheng, Y., Lu, Y., Gao, C., Wu, Q.: Alga-based biodiesel production and optimization using sugar cane as the feedstock. Energy Fuels. **23**, 4166–4173 (2009). doi:10.1021/ef9003818

22. Onay, M., Sonmez, C., Oktem, H.A., Yucel, A.M.: Thermoresistant green microalgae for effective biodiesel production: Isolation and characterization of unialgal species from geothermal flora of Central Anatolia. Bioresour. Technol. **169**, 62–71 (2014). doi:10.1016/j.biortech.2014.06.078

23. Gorman, D.S., Levine, R.P.: Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. Proc. Natl. Acad. Sci. USA. **54**, 1665–1669 (1965). doi: 10.1073/pnas.54.6.1665

24. Xiong, W., Li, X., Xiang, J., Wu, Q.: High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Appl. Microbiol. Biotechnol. **78**, 29–36 (2008). doi:10.1007/s00253-007-1285-1

25. Abou-shanab, R.A., Raghavulu, S.V., Hassanin, N.M., Kim, S., Kim, Y.J., Oh, S.U., Oh, Y., Jeon, B.: Manipulating nutrient composition of microalgal growth media to improve biomass yield and lipid content of Micractinium pusillum, Afr. J. Biotechnol. **11**, 16270–16276 (2012). doi:10.5897/AJB12.2628

26. Yan, D., Lu, Y., Chen, Y.F., Wu, Q.: Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. Bioresour. Technol. **102**, 6487–6493 (2011). doi:10.1016/j.biortech.2011.03.036

27. Miller, G.L.: Use of dinitrosalicyclic acid reagent for determination of reducing sugar. Anal. Chem. **31**, 426–428 (1959). doi:10.1021/ac60147a030

28. Karpagam, R., Raj, K.J., Ashokkumar, B., Varalakshmi, P.: Characterization and fatty acid profiling in two fresh water microalgae for biodiesel production: Lipid enhancement methods and media optimization using response surface methodology. Bioresour. Technol. **188**, 177–184 (2015). doi:10.1016/j.biortech.2015.01.053

29. Uncu, O.N., Cekmecelioglu, D.: Cost-effective approach to ethanol production and optimization by response surface methodology. Waste Manag. **31**, 636–643 (2011). doi:10.1016/j.wasman.2010.12.007

30. Li, Z., Yuan, H., Yang, J., Li, B.: Optimization of the biomass production of oil algae Chlorella minutissima UTEX2341., Bioresour. Technol. **102**, 9128–9134 (2011). doi:10.1016/j.biortech.2011.07.004

31. Gurkok, S., Cekmecelioglu, D., Ogel, Z.B.: Optimization of culture conditions for *Aspergillus sojae* expressing an *Aspergillus fumigatus* α-galactosidase. Bioresour. Technol. **102**, 4925–4929 (2011). doi:10.1016/j.biortech.2011.01.036

32. Lakshmikandan, M., Murugesan, A.G.: Enhancement of growth and biohydrogen production potential of Chlorella vulgaris MSU-AGM 14 by utilizing seaweed aqueous extract of Valoniopsis pachynema. Renew. Energy. **96**, 390–399 (2016). doi:10.1016/j.renene.2016.04.097

33. Juneja, A., Ceballos, R., Murthy, G.: Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: A Review. Energies **6**, 4607–4638 (2013). doi:10.3390/en6094607

34. Kanaga, K., Pandey, A., Kumar, S., Geetanjali: Multi-objective optimization of media nutrients for enhanced production of algae biomass and fatty acid biosynthesis from *Chlorella pyrenoidosa* NCIM 2738., Bioresour. Technol. **200**, 940–950 (2016). doi:10.1016/j.biortech.2015.11.017

35. Mata, T.M., Martins, A.A., Caetano, N.S.: Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy Rev. **14**, 217–232 (2010). doi:10.1016/j.rser.2009.07.020

36. Xiufeng, W.Q. L., Han, X., Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnol. Bioeng. (2007). doi:10.1002/bit.