WALAILAK JOURNAL
http://wjst.wu.ac.th Article

Factors Affecting the Biomass and Lipid Production from *Chlorella* sp. TISTR 8990 under Mixotrophic Culture

Mohammad Ariful HAQUE[1], Phuwadol BANGRAK[2], Sarote SIRISANSANEEYAKUL[3] and Wanna CHOORIT[1,*]

[1]*Division of Agro-Industry, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80161, Thailand*
[2]*Division of Biology, School of Science, Walailak University, Nakhon Si Thammarat 80161, Thailand*
[3]*Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University 90120, Thailand*

([*]Corresponding author; e-mail: cwanna35@gmail.com)

Received: 26 July 2012, Revised: 2 October 2012, Accepted: 2 November 2012

Abstract

Effects of media compositions on biomass and lipid accumulation of the isolate *Chlorella* sp. TISTR 8990 were investigated under a Plackett-Burman experimental design with mixotrophic cultivation conditions. Under this experimental design there were 15 different runs with ten factors-yeast extract, KH_2PO_4, $MgSO_4$, $FeSO_4$, $MnCl_2$, $CuSO_4$, Na_2MoO_4, H_3BO_3, $ZnSO_4$ and pH. Cultures were grown mixotrophically under 16 h light and 8 h dark regime at 30 ºC for a period of 7 days. During the light regime, the light intensity at the surface of the vessels and agitation speed were set to 67.5 µmol photons $m^{-2}s^{-1}$ and 150 rpm, respectively. Initial cell concentration was set to an absorbance (A_{540}) of 0.5. For high biomass production (2.2 g/L, run no. 6), the most effective and significant factors were yeast extract, KH_2PO_4, $FeSO_4$ and $ZnSO_4$ at concentrations 0.3 g/L, 0.3 g/L, 3 mg/L and 0.3 mg/L, respectively. Whereas for high lipid accumulation (19.59 %DCW, run no. 2), these were KH_2PO_4, pH and yeast extract, at a level of 1.7 g/L, 6.0 and 0.1 g/L, respectively. No significant factors were obtained for higher lipid content. The best treatment for biomass and lipid content was run no. 6, whose medium formula consisted of 0.3 g/L yeast extract, 1.7 g/L KH_2PO_4, 1.7 g/L $MgSO_4$, 1 mg/L $FeSO_4$, 0.9 mg/L $MnCl_2$, and pH 7.0, together with fixed concentrations of glucose, $NaHCO_3$ and KNO_3 at 5 g/L, 0.05 g/L and 0.5 g/L, respectively.

Keywords: Biomass, *Chlorella* sp., mixotroph, Plackett-Burman design, single cell oils

Introduction

Petroleum-based fuels are recognized as an unsustainable energy source due to their depleting supplies and contribution to global warming [1]. Renewable and carbon neutral biodiesel is necessary for environmental and economic sustainability. Thus there is an increasing interest in looking for new oil sources for biodiesel production. Among them microbial oils, namely single cell oils (SCOs), and lipids produced by the oleaginous microorganisms; especially microalgae are now considered as promising candidates because of their advantages of higher photosynthetic efficiency, higher biomass production and faster growth compared to other energy crops [2,3]. Generally, microalgae use CO_2 as the carbon source and light as the energy source for metabolic activity. They can also adapt to different carbon and energy sources under different environments, exhibiting four different types of metabolic patterns, photoautotrophy, heterotrophy, photoheterotrophy, and mixotrophy [4].

In a classical photoautotrophic culture, it is difficult to reach a high density of microalgal biomass since there is limited light penetration in the culture medium [5]. To overcome the limitation of light penetration, many microalgae

that are able to grow heterotrophically have been studied [6,7]. Furthermore, a mixotrophic metabolism was found to exist in some microalgae. That is, they can fix CO_2 and utilize organic carbon sources simultaneously when exposed to light [8-11].

Being a robust type microorganism *Chlorella* species can be grown in many conditions around the world; they can serve as an example of phototrophic, heterotrophic and mixotrophic growths supplied with glucose, glycerol, acetate, or other organic compounds from waste sources with zero or negative costs as a carbon source to accumulate lipids for biodiesel production. Yet, as other species are as efficient and productive as this one, the selection of the most adequate species needs to take into account other factors, for example the ability of microalgae to develop using the nutrients available under specific environmental conditions. All these parameters should be considered simultaneously in the selection of the most adequate species or strains for biodiesel production. Since *Chlorella* sp. can be grown in both fresh and marine water with a high biomass (16.8 g/L-*Chlorella protohtecoides*) [12] and lipid production (55.2 % DCW-*Chlorella protohtecoides*) [12] potentially under mixtrophism, therefore, *Chlorella* sp. were selected for this study.

Though hundreds of microalgal strains are capable of producing high lipid content have been screened [13], the most important factors that have been reported to influence biomass and lipid production of microalgae are nutrient availability, temperature, and light. There are extensive studies on the growth of *Chlorella* sp. in culture media with different concentrations of different carbon sources [14,15]. Many studies have focused on the effect of nitrogen concentration and starvation on the growth and lipid content in algae grown in autotrophic and heterotrophic bioreactors [16,17]. However, culture media also contain many elements, K, Mg, Ca, S, Fe, Cu, Mn, and Zn, which are required for the growth and enzymatic activity of microalgae [18]. For example, Mn functions with enzyme systems involved in breakdown of carbohydrates, and nitrogen metabolism. High concentrations of Cu and Zn inhibit algal growth [19]. Although variation in the elemental composition of *Chlorella* sp. under phototrophic conditions and different stages of growth have been reported [18,20] the effects of the elements, vitamins and pH level on biomass and lipid production of *Chlorella* sp. grown under mixotrophic conditions have not been investigated.

Media composition and growth conditions influence the culture growth and thus the biomass and lipid content. Biomass and lipid content can be increased with properly optimized media compositions. Conventional optimization may involve the screening of a large number of variables. The Plackett-Burman design (PBD) provides an efficient way of sorting out a large number of variables and identifying the most important ones. Numerous reports have proved the applicability of PBD in the optimization of media components for various culture activities [21,22]. Therefore, PBD has been proposed for screening the key factors, and to determine the response of the product of interest to the factors being investigated in this study.

Materials and methods

Algal strain

Chlorella sp. TISTR 8990, a previously isolated fresh water *Chlorella* sp. from Walailak University, Thailand in February 2007 [23] was used throughout this study.

Culture media

The basal medium used to prepare starter cultures contained the following: 2 g/L glucose, 0.05 g/L sodium bicarbonate, 2 g/L KNO_3, 1 g/L KH_2PO_4, 1 g/L $MgSO_4$, 2 mg/L $FeSO_4$, 2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2$, 0.22 mg/L $ZnSO_4$, 0.08 mg/L $CuSO_4$ and 0.021 mg/L Na_2MoO_4 [24]. Initial pH was adjusted to 6.0. After autoclaving the medium, an antibiotic (ampicillin) was added at a concentration of 25 mg/L to prevent contamination from other bacteria [25]. This dose (25 mg/L) of antibiotic was suitable to prevent contamination without inhibiting algal growth in the culture (data not shown). 1.5 % agar in the basal medium except glucose and sodium bicarbonate was used for stock culture with the same pH adjustment and antibiotic concentration. The basal medium with 5 g/L glucose, 0.05 g/L $NaHCO_3$ and 0.5 g/L KNO_3 as organic/inorganic carbon and nitrogen sources, respectively were used for PBD experiments. The initial carbon/nitrogen ratios were 25.90, 23.41 and 21.43 for each experimental run at three different levels of the factors and was calculated based on the

molecular formula of glucose, KNO_3, $NaHCO_3$ and carbon and nitrogen content (N = 10.8 % and C = 38 %) of yeast extract supplied in the medium. Carbon and nitrogen contents of yeast extract were analyzed by CN analyzer (TruSpec® CN 3553, LECO Instruments Ltd., Thailand).

Growth conditions

Mixotrophically *Chlorella* sp. TISTR 8990 was grown in 100 mL of the basal medium in a 250 mL Erlenmeyer flask. The flasks were then incubated at 30 °C in a shaker equipped with six 30 W daylight cool white lights (Phillips, made in Singapore). Cultures were grown mixotrophically under 16 h light and 8 h dark regime at 30 °C for a period of 3 days for starter culture preparation, and for 7 days for PBD experiments being studied. During the light regime, the light intensity at the surface of vessels and agitation speed were set to 67.5 µmol photons $m^{-2}s^{-1}$ {for fluorescent cool white light the conversion factor for lux to µmol photons m^{-2} s^{-1} is 0.0135, i.e. multiply the lux by the conversion factor [26]} and 150 rpm, respectively. After 3 days of cultivation, the cells were harvested by centrifugation at a speed of 2,356 × *g* for 15 min and were washed twice with sterile RO (Reverse Osmosis) water. Immediately after washing them with RO water the cells were centrifuged twice so that there was no cell breakdown from osmosis. After that, the inoculums were inoculated to the vessels fixing an initial cell concentration 0.5 at OD 540 nm for 7 days batch culture in the PBD experiment. Samples were collected from each batch of cultured flask to measure the biomass concentrations and lipid contents at day 0 and 7.

Experimental design

The Plackett-Burman experimental design consisted of 10 factors (A-J). These were the concentrations of yeast extract (A), KH_2PO_4 (B), $MgSO_4$ (C), $FeSO_4$ (D), $MnCl_2$ (E), $CuSO_4$ (F), Na_2MoO_4 (G), $ZnSO_4$ (H), H_3BO_3 (I) and pH (J). Each factor was tested at three levels of low (-1), medium (0) and high (1) at concentrations shown in **Table 1**.

The effect of factors was calculated following the equation below:

$$E_{X(-1,0)} = \frac{N}{3}\left(\sum R(0) - \sum R(-1)\right) \quad (1)$$

$$E_{X(0,1)} = \frac{N}{3}\left(\sum R(1) - \sum R(0)\right) \quad (2)$$

$$E_{X(-1,1)} = \frac{N}{3}\left(\sum R(1) - \sum R(-1)\right) \quad (3)$$

where E_X is the effect on the measured response (R) for the change of a factor X from one level to another; $\sum R(i)$ (i = 0, –1, 1) is the sum of the measured values associated with level i and N is the number of design experiments.

Table 1 Factors and levels for Plackett-Burman design.

Code	Factor	Function	Level -1 (Low)	Level 0* (Medium)	Level 1 (High)
A	Yeast Extract (g/L)	Vitamin-source	0.1	0.2	0.3
B	KH_2PO_4 (g/L)	Phosphate-source	0.3	1.0	1.7
C	$MgSO_4$ (g/L)	Magnesium-source	0.3	1.0	1.7
D	$FeSO_4$ (mg/L)	Iron-source	1.0	2.0	3.0
E	$MnCl_2$ (mg/L)	Manganese-source	0.9	1.8	2.7
F	$CuSO_4$ (mg/L)	Copper-source	0.04	0.08	0.12
G	Na_2MoO_4 (mg/L)	Molybdenum-source	0.1	0.2	0.3
H	$ZnSO_4$ (mg/L)	Zinc-source	0.1	0.2	0.3
I	H_3BO_3 (mg/L)	Boron-source	0.9	1.81	2.72
J	pH	Physiological function	6.0	6.5	7.0

* The concentrations of all factors at level 0 were same concentrations as the basal medium being used by Sirisansaneeyakul *et al.* [23].

Analytical methods

Initial cell concentrations of *Chlorella* sp. TISTR 8990 were determined at OD 540 nm to maintain a similar optical density 0.95 of the inoculums for all the runs in this design of experiment by using a UV/Visible spectrophotometer (Model TCC-240A, UV-Visible Spectrophotometer, Shimadzu, Japan) and more precisely were determined by the dry cell weight method by centrifuging the cultural broth at 2,356 × *g* for 15 min, washing twice with double distilled water and then drying the cell pellet at 105 °C until the weight was constant. For dry cell weight and oil extraction of the *Chlorella* sp. TISTR 8990, 15 and 20 mL of the culture broth respectively were used in this study.

The lipid content of *Chlorella* sp. TISTR 8990 was determined by using a modified method of Bligh and Dyer [27]. Lipids were extracted with a mixture of chloroform: methanol (1:2 v/v) until the algal cell color turned whitish. The extracted lipid was centrifuged to obtain a clear supernatant and the solvent was removed by hot air oven drying at 70 °C until a dry lipid was obtained.

The two replicate data of each experimental run in PBD were analyzed by Design-Expert®, version 7 software (Stat-Ease, Inc.).

Results and discussion

It is known that the growth rate and the biomass composition of microalgae vary under different medium composition and cultivation conditions [12,28-29]. In this work, the effects of yeast extract and pH values on biomass and lipid production were determined through PBD experiments. The initial C/N ratios of the medium were maintained at 25.9, 23.41 and 21.43 since these levels of C/N ratio can produce higher lipid content (46 %) with *Chlorella protothecoides* [12].

The microalgae *Chorella* sp. TISTR 8990 investigated in this study grew luxuriously in the basal medium at varying concentrations of the compositions (**Table 2**). The biomass was 1.61 g/L (**Table 2**) obtained from run no. 1, contained the nutrients at level 0 (**Table 1**) (0.2 g/L yeast extract, 1.0 g/L KH_2PO_4, 1.0 g/L $MgSO_4$, 2 mg/L $FeSO_4$, 1.8 mg/L $MnCl_2$, 0.08 mg/L $CuSO_4$, 0.2 mg/L Na_2MoO_4, 0.2 mg/L $ZnSO_4$, 1.81 mg/L H_3BO_3 and pH 6.5). The biomass content obtained from run no. 10 (1.39 g/L) (**Table 2**), contained the nutrients at level -1 (**Table 1**) (0.1 g/L yeast extract, 0.3 g/L KH_2PO_4, 0.3 g/L $MgSO_4$, 1 mg/L $FeSO_4$, 0.9 mg/L $MnCl_2$, 0.04 mg/L $CuSO_4$, 0.1 mg/L Na_2MoO_4, 0.1 mg/L $ZnSO_4$, 0.9 mg/L H_3BO_3 and pH 6.0). However, the lowest biomass (1.23 g/L) was found in run no. 12 (**Table 2**), in which yeast extract, iron and molybdenum were added at the lowest level (−1), but the other nutrients were at the highest level 1 (**Table 1**).

Table 2 Plackett-Burman experimental design, and Biomass & lipid produced by *Chlorella* sp. TISTR 8990.

Run no.	Code of Factors and Levels										Biomass (g DCW/L)		Error %	Total Lipid (% DCW)		Error %
	A	B	C	D	E	F	G	H	I	J	Observed	Predicted		Observed	Predicted	
1	0	0	0	0	0	0	0	0	0	0	1.61	1.68	4.16	13.56	13.82	1.88
2	-1	1	1	-1	1	1	1	-1	-1	-1	1.77	2.20	19.54	19.59	17.84	9.81
3	-1	1	-1	1	1	-1	1	1	1	-1	2.14	1.87	14.43	9.05	13.01	11.07
4	-1	1	1	1	-1	1	1	-1	1	1	1.66	1.58	5.06	13.93	12.41	12.25
5	1	1	1	-1	-1	-1	1	-1	1	1	1.87	1.77	5.64	13.01	13.51	3.70
6	1	1	1	1	-1	1	1	1	-1	-1	2.20	1.78	23.59	12.41	19.59	36.65
7	-1	1	1	1	-1	-1	-1	1	-1	1	1.63	1.87	12.83	13.52	13.82	2.17
8	1	1	-1	1	1	-1	-1	-1	1	-1	1.58	1.66	4.81	11.79	13.93	15.36
9	1	1	-1	-1	-1	1	-1	1	1	-1	1.63	1.68	2.97	14.14	13.82	4.48
10	-1	1	-1	-1	-1	-1	-1	-1	-1	-1	1.39	1.63	14.72	16.95	13.52	25.36
11	1	1	-1	1	1	1	-1	-1	-1	1	1.56	1.56	0	16.81	16.81	0
12	-1	1	1	-1	1	1	-1	1	1	1	1.23	1.63	24.53	13.51	13.51	0
13	1	-1	-1	-1	1	-1	1	1	-1	1	1.75	1.75	0	9.45	9.45	0
14	1	-1	-1	-1	1	-1	-1	1	-1	1	1.73	1.74	0.57	10.98	16.95	35.22
15	1	-1	-1	1	-1	-1	-1	1	-1	1	1.40	1.58	11.39	15.80	11.79	11.36

Among the 15 runs of the design, run no. 6 (**Table 2**) exhibited the highest biomass concentration (2.20 g/L) after 7 days of cultivation, which was higher than the previous report (1.58 - 1.72 g/L) with *Chlorella vulgaris* over the same cultivation period [30]. For run no. 6, biomass production reached the highest value due to a high dose of yeast extract, phosphorus, magnesium, iron, copper, molybdenum and zinc, and a low dose of manganese, boron and pH value. The percent error (36.65 %) for oil extraction at run no. 6 was high. This might be due to the low values of R^2 (approximately 67 %) which showed a low relationship between the experimental and predicted values.

The trace element iron with 3 mg/L contributed the best towards biomass production (run no. 6, **Table 2**). A similar iron effect was observed by Eriani *et al.* [31], where they showed an increased growth in terms of number of cells/mL. Iron uptake is strictly required for phytoplankton development, because in the absence of iron, retardation of growth, reduction of photosynthetic activity and chlorophyll content were observed [32]. High doses of magnesium induced high biomass production since magnesium is important to microalgal cell growth. Chlorophyll, the main molecule responsible for light uptake in photosynthetic plants, consists of a hydrocarbon phytol chain and a tetrapyrrole ring structure surrounding a chelated Mg^{2+} ion. This means that, for every one molecule of chlorophyll produced, one Mg^{2+} is required. Because of its importance in chlorophyll, green algae have higher magnesium contents than do red or brown microalgae.

Although Ansari and Amjad [33] found that 5 mg/L Cu (as $CuCl_2$) was the optimal dose for *Chlorella vulgaris* growth, they did not mention the biomass they obtained from their studies. They further stated that the increase in growth rate was due to the vital role of Cu in photosystems 1 and 2 and in many vitamins, enzymes, especially in multi copper proteins, i.e. blue and non-blue proteins, and in superoxide dismutase. However, 0.12 mg/L Cu as $CuSO_4$ gave the highest biomass (2.20 g/L) together with other factors in this study. Ulloa *et al*. [34] obtained the highest biomass concentration of 7.0×10^6 cells/mL using 0.12 g/L Mg in the medium with microalga *Tetraselmis suecica*, whereas it was 2.20 g/L using 0.34 g/L Mg in the present study with *Chlorella* sp., 0.3 mg/L $ZnSO_4$

together with other factors contributed towards higher biomass production. This result supports the work of Shan *et al*. [35], who stated that high Zn^{2+} concentration (1 mg/L), which exceeds those required for optimal growth, can urge the nucleic acid to degrade and suppress both NADPH formation in the chloroplast and the algae cell ATP level, however the low zinc concentration may promote the multiplication of algae. The phosphorus requirement for optimal algal growth differs considerably from species to species, even if no other external factors are limiting [36]. Higher phosphorous concentrations together with 0.5 g/L of KNO_3 in the medium triggers high biomass production (2.20 g/L) in this study, which agrees well with Fried *et al*. [37].

After 7 days of cultivation, the lipid content observed at run no. 1 was 13.56 % (**Table 2**) from medium composition that was more likely a basal medium composition (all components were added at level 0). Lipid content (13.56 %) with this medium composition was lower than from run no. 10 (16.95 %) (**Table 2**) containing low-level nutrients (level -1). Lipid content reached its lowest (9.05 % DCW) and highest (19.59 % DCW) points at run 3 and 2, respectively where the media composition of the both runs differed in Mg, Fe Cu, Zn and B levels only (**Table 2**). Illman *et al*. [16] reported that media compositions especially low levels of nitrogen per carbon drastically influenced lipid contents of *Chlorella* spp. These features imply that high biomass content may not always result in higher lipid accumulation. Xiong *et al.* [12] showed high biomass (19.8 g/L) of *Chlorella protothecoides* contained low amounts of lipid (19 %), whereas it was high (53 %) when biomass concentration was 9.1 g/L using a C/N ratio of 30 and 3, respectively. C/N ratio is an important factor for lipid accumulation in microalgae, however there was a wide variation in lipid production (**Table 2**) from an initially fixed C/N ratio of 21.4 - 25.9 in this study. Variation of lipid induction in the cultured microalga *Chlorella* sp. TISTR 8990 was probably due to imbalance of nutrient (different doses of macro and micro nutrients in each experimental run with low initial glucose concentration 5 g/L). In particular, micro nutrients such as Fe and Zn and major element such as organic carbon have a great impact on lipid production.

Low initial pH (6.0) with other nutrient combination at run no. 2 (**Table 2**) showed the highest lipid content. It is known that the major functions of plasma membrane are to regulate what comes in and goes out of cells. The status of external pH can determine complex physiological parameters such as membrane permeability and cell morphology. Therefore, a change in broth pH will affect membrane osmosis to certain ions and thus substances absorption. Many researchers have investigated the effects of broth pH values on growth kinetics of microorganisms and concluded that pH is an important environmental factor affecting cell growth and product formation [38]. For example, the activities of enzymes catalyzing all metabolic reactions were significantly affected by broth pH, thus further influencing cell growth and product synthesis [39]. Unfortunately, the effects of broth pH values on cell growth and lipid production in mixotrophic cultivation of microalgae are little known.

Ramasamy *et al*. [40] inferred that under potassium-phosphate or iron deprivation, there was no significant increase in lipid content (40.7 %) with microalga *Chlorella* sp. In this study, for 7 days batch culture of *Chlorella* sp. TISTR 8990 1.7 g/L KH_2PO_4 as phosphate together with other nutrients contributed to high lipid content.

Nevertheless, other factors, except the aforementioned ones, contributed towards biomass and lipid accumulation but were negligible under mixotrophic cultivation conditions of *Chlorella* sp. TISTR 8990.

The results on PBD experiments were calculated according to Eqs. (1-3). The linear equations for biomass and lipid production were therefore, given as follows:

Equation for biomass concentration at Day 7,

$$X(gDCW/L) = 1.68-0.17A+0.12B+0.00C-0.13D-0.04E-0.02F-0.04G-0.09H-0.05I-0.05J \quad (4)$$

Equation for lipid content at Day 7,

$$Y(\%DCW) = 13.82+1.53A+0.38B+0.82C+0.33D+1.09E-0.90F+0.88G-0.42H+0.19I+1.46J \quad (5)$$

where, A, B, C, D, E, F, G, F, H, I, and J represent yeast extract, KH_2PO_4, $MgSO_4$, $FeSO_4$, $MnCl_2$, $CuSO_4$, Na_2MoO_4, $ZnSO_4$, H_3BO_3, and pH. Intercepts for biomass and lipid in the equations were 1.68 and 13.82, respectively. In this study, the obtained model for biomass was significant with a high coefficient (R^2 = 0.9921), while for lipid content it was insignificant. The value of the adjusted determination coefficient for biomass (adjusted R^2 = 0.9423) suggested that the total variation of 94.23 % was to be attributed to the independent variables and about 5.77 % of the total variation could not be explained by the model, while for lipids the adjusted coefficient (R^2 = 0.6669) suggested that about 33.31 % of the total variation could not be explained by the model.

The coefficient estimates of Eq. (4) and Eq. (5) and the corresponding *P*-values are shown in **Table 3**. On the basis of *P*-value, variable A (yeast extract), B (KH_2PO_4), D ($FeSO_4$) and H ($ZnSO_4$) showed a significant influence on biomass (*P*-value = 0.0124, 0.0231, 0.0193 and 0.0373, respectively). The significance of each coefficient was determined by probability values, which are listed in **Table 3**. Other factors did not significantly affect the biomass production in this study. In contrast to biomass, there were no factors significantly affecting the lipid production of this algae species.

Table 4 shows the ANOVA of the Plackett-Burman design. The ANOVA of the linear model Eq. (4) and Eq. (5) demonstrates that the model was significant for biomass as can be seen with low probability values (0.0478). For lipid content, however, it was insignificant with a probability value of 0.2513. The soundness of the model can be checked by the determination of the coefficient, R^2 and the adjusted R^2.

Table 3 Coefficients and *P*-values of biomass and lipid production by *Chlorella* sp. TISTR 8990, obtained from the analysis of the results that are listed in **Table 2**.

Variable	Biomass		Lipid Content	
	Coefficient	***P*-value**	**Coefficient**	***P*-value**
Intercept	1.68	0.0478*	13.82	0.2513
A	-0.17	0.0124*	1.53	0.0874
B	0.12	0.0231*	0.38	0.5142
C	1.66 E-003	0.9366	0.82	0.2327
D	-0.13	0.0193*	0.32	0.5740
E	-0.042	0.1539	1.09	0.1550
F	-0.023	0.3356	-0.90	0.2039
G	-0.043	0.1447	0.88	0.2122
H	-0.093	0.0373*	-0.42	0.4753
I	-0.050	0.1146	0.19	0.7269
J	-0.048	0.1212	1.46	0.0951

Biomass: R^2 = 0.9911, Adjusted R^2 = 0.9423; Lipid: R^2 = 0.9487; Adjusted R^2 = 0.6669
*Significance level at 95 %

Actual biomass and lipid content results with their predicted values from the design are plotted in **Figures 1** and **2**, indicating the accuracy of the experiments graphically under this design.

The effects of factors on biomass and lipid accumulation were calculated from the coded values of each factor and from measured responses (**Table 2**). These calculated effects (**Table 5**), which show the magnitude of the influence of the change of a factor, can be visualized in the effect-plot (**Figures 3** and **4**), where the behavior of the response is estimated as a function of the factor levels [41]. It can be remarked that only two of the three above effects are independent.

Table 4 ANOVA for biomass concentration and lipid content at Day 7.

Code	df		SS		MS		F-ratio		*P*-value	
	Biomass	Lipid	Biomass	Lipid	Biomass	Lipid	Biomass	Lipid	Biomass	Lipid
A	1	1	0.33	28.21	0.33	28.21	79.04	9.97	0.01*	0.09
B	1	1	0.17	1.75	0.17	1.75	41.81	0.62	0.02*	0.51
C	1	1	3.333E-005	8.10	0.00	8.10	8.065E-003	2.86	0.94	0.23
D	1	1	0.21	1.25	0.21	1.25	50.33	0.44	0.02*	0.57
E	1	1	0.02	14.13	0.02	14.13	5.04	4.99	0.15	0.16
F	1	1	6.533E-003	9.79	0.01	9.79	1.58	3.46	0.34	0.20
G	1	1	0.02	9.26	0.02	9.26	5.45	3.27	0.14	0.21
H.	1	1	0.10	2.15	0.10	2.15	25.29	0.76	0.04*	0.48
I	1	1	0.03	0.46	0.03	0.46	7.26	0.16	0.11	0.73
J	1	1	0.028	25.58	0.03	25.58	6.78	9.04	0.12	0.10
Error	2	2	8.267E-003	5.66	4.133E-003	2.83				
Corrected	14	14	0.93	112.48						

Table 5 Calculated effects of the factors on biomass and lipid production[a].

Code	Factors	Effects					
		$E_{X(-1,0)}$		$E_{X(0,1)}$		$E_{X(-1,1)}$	
		Biomass	Lipid	Biomass	Lipid	Biomass	Lipid
A	Yeast Extract	-1.642	-11.812	2.422	18.166	0.78	6.354
B	KH_2PO_4	-0.654	-4.534	**3.41**	**28.23**	**2.756**	**23.696**
C	$MgSO_4$	-2.314	-18.282	1.75	14.482	-0.564	-3.8
D	$FeSO_4$	-2.	-17	2.11	16.06	0.16	-0.9
E	$MnCl_2$	-2	-17.41	2	16	-0	-2
F	$CuSO_4$	**-2.376**	-17.398	1.688	15.366	-0.688	-2.032
G	$NaMoO_4$	-2	-17	1.7	15	-1	-2
H	$ZnSO_4$	-2.07	-20	2	13	-0	-7
I	H_3BO_3	-2.344	-16	2.42	17.1	0.78	1.36
J	pH	-1.82	**-20.39**	1.7	12.374	-0.664	-8.016

[a]Bold numbers indicate most important effects

The third effect is mainly the sum of the first two towards biomass and lipid content of *Chlorella* sp TISTR 8990.

Figure 3 implies that from medium to high concentration of the factors the effect of factors B (KH_2PO_4) and A (yeast extract) are prominent for biomass production, while from medium to low doses the effect of factors F ($CuSO_4$), I (H_3BO_3), C ($MgSO_4$), H ($ZnSO_4$) were higher towards biomass. In contrast to biomass, the medium to higher doses of factor B (KH_2PO_4) and A (yeast extract) and for medium to low doses factor J (pH), H ($ZnSO_4$) and C ($MgSO_4$) (**Figure 4**, **Table 5**) exhibited a considerable effect on lipid accumulation. Interestingly, the effect of medium concentration of each factor was low both for biomass and lipid content of *Chlorella* sp. TISTR 8990 in this study.

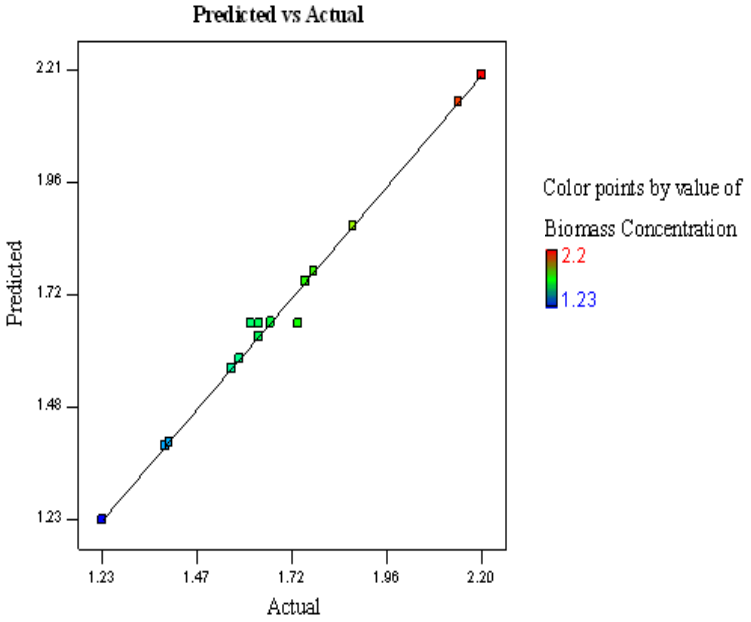

Figure1 Predicted vs actual values for biomass concentration from the experimental design.

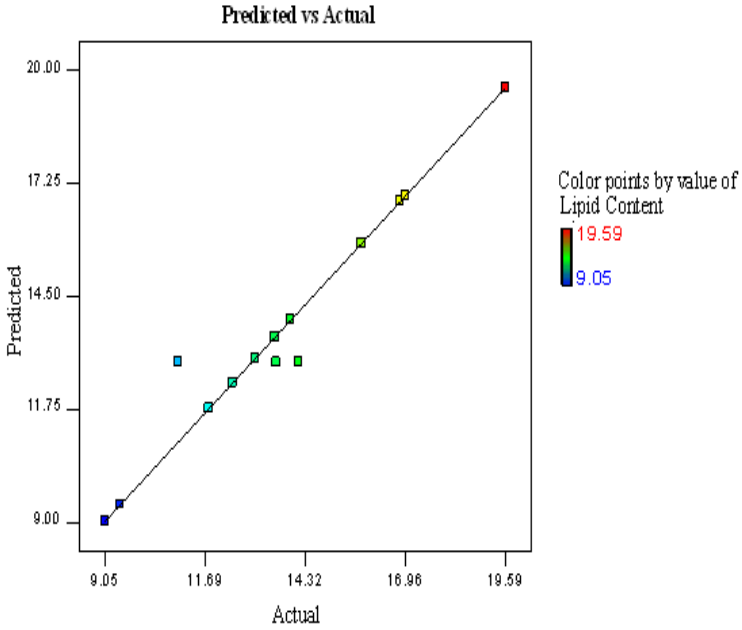

Figure 2 Predicted vs actual for the Lipid Content from the experimental design.

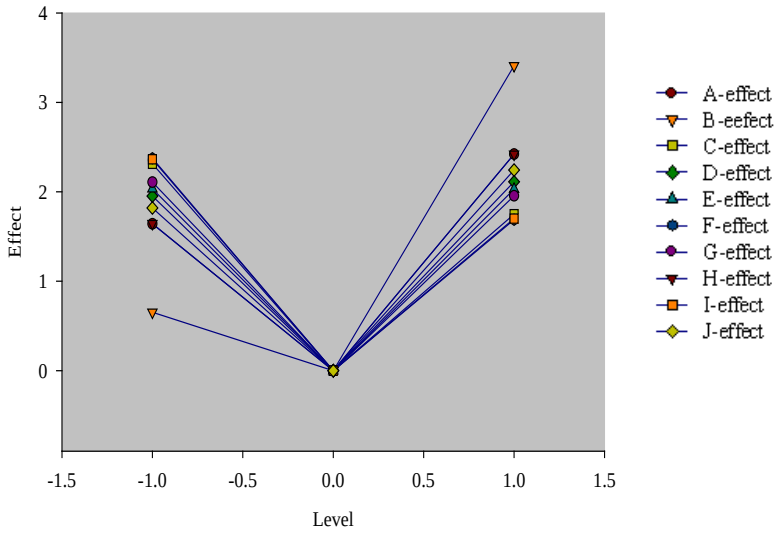

Figure 3 Effect of factors on biomass; where = level vs Yeast extract; = level vs KH_2PO_4; = level vs $MgSO_4$; = level vs $FeSO_4$; = level vs $MnCl_2$; = level vs $CuSO_4$; = level vs $NaMoO_4$; = level vs $ZnSO_4$; = level vs H_3BO_3 and = level vs pH.

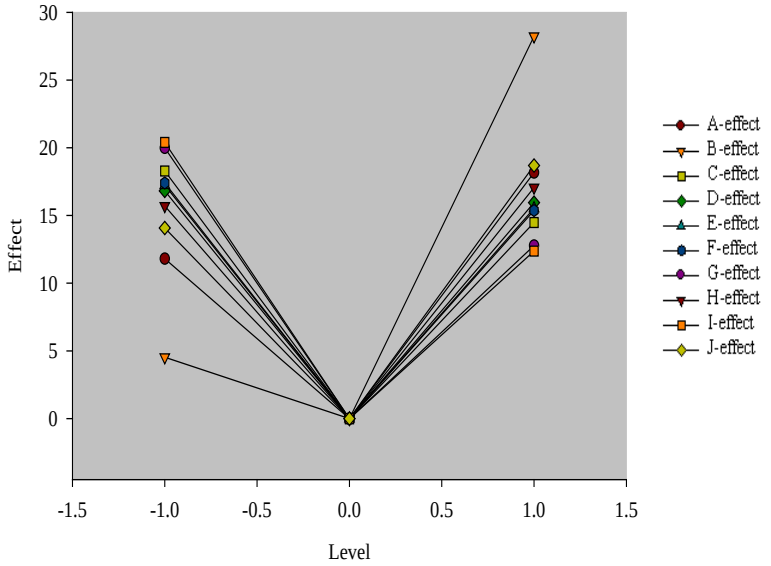

Figure 4 Effect of factors on lipid accumulation; where = level vs Yeast extract; = level vs KH_2PO_4; = level vs $MgSO_4$; = level vs $FeSO_4$; = level vs $MnCl_2$; = level vs $CuSO_4$; = level vs $NaMoO_4$; = level vs $ZnSO_4$; = level vs H_3BO_3 and = level vs pH.

Conclusions

In conclusion, KH_2PO_4, yeast extract, $CuSO_4$ and $ZnSO_4$ contributed greatly towards biomass, while for lipid content, the most important parameters were pH, KH_2PO_4 and yeast extract. However, among all runs for biomass and lipid accumulation, the most effective was run no. 6 under this experimental design. The optimal conditions for the selected isolate *Chlorella* sp. TISTR 8990 for high biomass and lipid content were 0.3 g/L yeast extract, 1.7 g/L KH_2PO_4, 1.7 g/L $MgSO_4$, 1 mg/L $FeSO_4$, 0.9 mg/L $MnCl_2$, and pH 7.0 together with a fixed concentration of glucose, $NaHCO_3$ and KNO_3 at 5 g/L, 0.05 g/L and 0.5 g/L, respectively.

Acknowledgements

This work was supported by Walailak University Fund.

References

[1] Y Chisti. Biodiesel from microalgae beats bioethanol. *Trends Biotechnol*. 2008; **26**, 126-31.

[2] GH Huang, F Chen, D Wei, XW Zhang and G Chen. Biodiesel production by microalgal biotechnology. *Appl. Energy* 2010; **87**, 38-46.

[3] Y Chisti. Biodiesel from microalgae. *Biotechnol. Adv*. 2007; **25**, 294-306.

[4] MA Borowitzka. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *J. Biotechnol.* 1999; **70**, 313-21.

[5] F Chen and M Johns. A strategy for high cell density culture of heterotrophic microalgae with inhibitory substrates. *J. Appl. Phycol*. 1995; **7**, 43-6.

[6] XL Miao and QY Wu. Biodiesel production from heterotrophic microalgal oil. *Bioresour. Technol*. 2006; **97**, 841-6.

[7] AL Wei, XW Zhang, D Wei, G Chen, QY Wu and ST Yang. Effects of cassava starch hydrolysate on cell growth and lipid accumulation of the heterotrophic microalgae *Chlorella protothecoides*. *J. Ind. Microbiol. Biotechnol*. 2009; **6**, 1383-9.

[8] XW Zhang, YM Zhang and F Chen. Kinetic models for phycocyanin production by high cell density mixotrophic culture of the microalga *Spirulina platensis*. *J. Ind. Microbiol. Biotechnol*. 1998; **21**, 283-8.

[9] MCC García, JMF Sevilla, FGA Fernandez, EM Grima and FG Camacho. Mixotrophic growth of *Phaeodactylum tricornutum* on glycerol growth rate and fatty acid profile. *J. Appl. Phycol*. 2000; **12**, 239-48.

[10] PF Ip, KH Wong and F Chen. Enhanced production of astaxanthin by the green microalga *Chlorella zofingiensis* in mixotrophic culture. *Process Biochem*. 2004; **39**, 1761-6.

[11] MCC García, FG Camacho, AS Miron, JMF Sevilla, Y Chisti and EM Grima. Mixotrophic production of marine microalga *Phaeodactylum tricornutum* on various carbon sources. *J. Microbiol. Biotechnol*. 2006; **16**, 689-94.

[12] W Xiong, XF Li, JY Xiang and QY Wu. High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. *Appl. Microbiol. Biotechnol*. 2008; **78**, 29-36.

[13] ME Huntley and DG Redalje. CO_2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. *Mitigation and Adaptation Strategies for Global Change* 2007; **12**, 573-608.

[14] R Jayasankar and KK Vasala. Influence of different concentrations of sodium bicarbonate on growth rate and chlorophyll content of *Chlorella salina*. *J. Mar. Biol. Assoc. India*. 2008; **50**, 74-8.

[15] M Sostaric, J Golob, M Bricelj, D Klinar and A Pivec. Studies on the growth of *Chlorella vulgaris* in culture media with different carbon sources. *Chem. Biochem. Eng*. 2009; **4**, 471-7.

[16] AM Illman, AH Scragg and SW Shales. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme Microb. Technol*. 2000; **27**, 631-5.

[17] Y Li, B Wang, N Wu and CQ Lan. Effects of nitrogen sources on cell growth and lipid production of *Neochloris oleoabundans*. *Appl. Microbiol. Biotechnol*. 2008; **81**, 629-36.

[18] T Oh-Hama and S Miyachi. *Chlorella In:* Borowitzka MA and Borowitzka LJ (eds.). Microalgal Biotechnology. Cambridge University Press, Cambridge, 1988, p. 3-26.

[19] J Ivanova, T Toncheva-Panova, G Chernev and B Samuneva. Effect of Ag^{+}, Cu^{2+} and Zn^{2+} containing hybrid nanomatrixes on the green algae *Cholrella keissleri*. *Gen. Appl. Plant Physiol.* 2008; **34**, 339-46.

[20] PJ Harrison, PA Thompson and GS Calderwood. Effect of nutrient and light limitation on the biochemical composition of phytoplankton. *J. Appl. Phycol*. 1990; **2**, 45-56.

[21] RMA Abedin and HM Taha. Antibacterial and antifungal activity of cyanobacteria and green microalgae. Evaluation of media components by Plackett-Burman design for antimicrobial activity of *Spirulina platensis. Global J. Biotechnol. Biochem*. 2008; **3**, 22-31.

[22] Y Li, Z Liu, F Cui and Z Liu. Application of Plackett-Burman experimental design and Doehlert design to evaluate nutritional requirements for xylanase production by *Alternaria mali* ND-16. *J. Appl. Microbiol. Biotechnol*. 2007; **77**, 285-91.

[23] S Sirisansaneeyakul, S Singhasuwan, W Choorit, N Phoopat, JL Garcia and Y Chisti. Photoautotrophic production of lipids by some *Chlorella* strains. *Mar. Biotechnol.* 2011; **13**, 928-41.

[24] T Horikoshi, A Nakajima and T Sakaguchi. Accumulation of uranium by *Chlorella* cells grown under autotrophic, heterotrophic and mixotrophic culture conditions. *Agric. Biol. Chem*. 1981; **454**, 781-3.

[25] PA Thompson. Algal Cell Culture. *Encyclopedia of Life Support System* (EOLSS), Biotechnology Vol 1, Available at: http://www.eolss.net/sample-chapters/c17/E6-58-01-02.pdf, accessed May 2012.

[26] RW Thimijan, W Richard and RD Heins. Photometric, radiometric, and quantum light units of measure: A review of procedures for interconversion. *Hortic. Sci*. 1982; **18**, 818-22.

[27] EG Bligh and WJ Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys*. 1959; **37**, 911-7.

[28] CY Chen, KL Yeh, R Aisyah, DJ Lee and JS Chang. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. *Bioresour. Technol*. 2011; **102**, 71-81.

[29] G Liang, Y Mo, J Tang and Q Zhou. Improve lipid production by pH shifted-strategy in batch culture of *Chlorella protothecoides*. *Afr. J. Microbiol. Res*. 2011; **5**, 5030-8.

[30] Y Feng, C Li and D Zhang. Lipid production of *Chlorella vulgaris* cultured in artificial waste water medium. *Bioresour. Technol*. 2011; **102**, 101-5.

[31] D Eriani, O Suriyaphan and N Chaiyanate. Effect of iron concentration on growth, protein content and total phenolic content of *Chlorella* sp. cultured in basal medium. *Sains Malaysiana* 2011; **40**, 353-8.

[32] W Wiesnner. *Inorganic Micronutrients In:* Lewin RA (ed.). Physiology and Biochemistry of Algae. Academic Press, New York, 1962, p. 267-86.

[33] ZA Ansari and M Amjad. Copper toxicity to algae (*Chlorella vulgaris*). *Pak. J. Sci*. 2008; **60**, 1-2.

[34] G Ulloa, A Otero, M Sanchez, J Sineiro, MJ Nunez and J Fabregas. Effect of Mg, Si, and Sr on growth and antioxidant activity of the marine microalga *Tetraselmis suecica*. *J. Appl. Phycol*. 2011, DOI: 10.1007/s10811-011-9764-2. Available at: http://www.springerlink.com/content/k8p668q19205422m/fulltext.pdf.

[35] W Shan, S Jingwei and H Yueheng. Experimental study of Zn^{2+} on growth of *Chlorella* in reclaimed water for waterscape recharge. Available at: http://zt.cast.org.cn/n12603275/n12603434/12991298.html, accessed June 2011.

[36] A Kuhl. *Phosphorus In*: Stewart WDP (ed.). Algal Physiology and Biochemistry, Botanical Monographs. California Press, 1974, p. 636-54.

[37] S Fried, B Mackie and E Nothwehr. Nitrate and phosphate levels positively affect the growth of algae species found in Perry Pond. *Tillers*. 2003; **4**, 21-4.

[38] A Amanullah, CM McFarlane, AN Emery and AW Nienow. Scale down model to simulate spatial pH variations in large-scale bioreactors. *Biotechnol. Bioeng*. 2001; **73**, 390-9.

[39] M Elibol. Product shifting by controlling medium pH in immobilized *Streptomyces coelicolor* A3 (2) culture. *Process Biochem*. 2002; **37**, 1381-6.

[40] P Ramasamy, S Kalifulla, M Gopalakrishnan, AA Mohammmad and T Nooruddin. Influence of nutrient deprivations on lipid accumulation in a dominant indigenous microalga *Chlorella* sp., BUM11008: Evaluation for biodiesel production. *Biomass Bioenergy* 2012; **37**, 60-6.

[41] S Boonkerd, MR Detaevernier, Y Vander Heyden, J Vindevogel and Y Michotte. Determination of the enantiomeric purity of dexfenfluramine by capillary electrophoresis: use of a Plackett-Burman design for the optimization of the separation *J. Chromatogr. A* 1996; **736**, 281-9.