Original Article

Sustainable Biomass Production of the Marine Microalga *Chlorella salina* as Live Food for the Rotifer *Brachionus plicatilis*

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Abstract

Chlorella salina was selected to investigate its growth potential and feeding value for the rotifer *Brachionus plicatilis* which is extensively used as live food for larvae of economic marine fishes. *Chlorella salina* maintained the highest biomass production in Phk nutrient medium. Plackett-Burman experimental design was used for screening the component of Phk medium affecting biomass production of *C. salina*. The predicted optimum levels of the screened variables for high growth of *C. salina* were used to prepare a verification medium. The biomass production was determined as dry weight biomass after 14 days incubation period. The verification medium supported about 104% increase in dry weight (0.521 g l^{-1}) of *C. salina* compared to the control Phk medium (0.255 g l^{-1}). The results, suggested that the best composition (mg l^{-1}) of Phk medium for maximum biomass production of *C. salina* is $MgCl_2.2H_2O$ (1.33), KCl (0.924), $NaNO_3$ (0.25), NaH_2PO_4 (0.01323), $NaHCO_3$ (0.0825), $CaCl_2$ (0.125), Na_2SO_4 (4.323), $Na_2MoO_4.2H_2O$ (0.00023), Fe Citrate (0.027), Citric acid (0.081), $ZnCl_2$ (0.0011), $MnCl_2.4H_2O$ (0.00087), $CoCl_2.6H_2O$ (0.000024), Na_2EDTA (0.006), H_3BO_3 (0.0069), $FeCL_3.6H_2O$ (0.000876), $CuCl_2$ (0.00396), $Na_2SeO_3.5H_2O$ (0.00132) and soil extract (60 ml l^{-1}). Substantial increase in protein content and omega-3 and omega-6 fatty acids was recorded for *C. salina* grown in modified Phk medium and under field conditions. Field trials for culturing the rotifer *Brachionus plicatilis* were carried using living *C. salina* cultivated on modified Phk medium. A parallel rearing experiment on baker's yeast was run as control. Very high significant ($p \leq 0.001$) increase in total individual numbers and in females carrying eggs was recorded for *B. plicatilis* fed on living cells of *Chlorella*. These results may indicate the promise of *Chlorella salina* for mass production of the rotifer *B. plicatilis* that represents potential live food for larvae of marine fishes.

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1. Introduction

The ever increasing human population is creating a towering demand for high quantities of high quality animal protein. Fish is considered the most important source of animal protein as it provides about 16% of the total animal protein consumed by the world's population (FAO, 1997). Many epidemiological studies have demonstrated the role of fish consumption in the prevention of coronary heart disease, which is attributed, mainly, to the high content of polyunsaturated fatty acids (Kris-Etherton *et al.*, 2003). The development of novel technologies for fish farming to increase fish productivity and quality is urgently needed (Chullasorn *et al.*, 2011). Nutrition is one of the dominant factors influencing growth and survival of fish larvae (Marshall *et al.*, 2010). The challenge in fish nutrition is to produce live or dry feed with high levels of health-promoting long chain omega-3 fatty acids for the consumer. It has become evident that microalgae represent a potential source of human functional food and feed. The nutritional contents of algae are rapidly gaining importance as a renewable source to substitute the conventional ingredients in aquaculture/animal feed (Yaakob *et al.*, 2014). Some of the most important microalgae that have been used for aquaculture feed are the species of *Chlorella, Nannochloropsis, Isochrysis, Tetraselmis, Chaetoceros* and *Skeletonema* (Borowitzka, 1997; Muller-Feuga, 2000; Brown, 2002). The feed value of these microalgae arises from the fact that their biomass is a very rich source of high quality protein (Gatrell *et al.*, 2014), carbohydrates (Roy and Pal, 2014), vitamins (Brown et al., 1999), pigments, e.g. chlorophylls and carotenoids (Barra *et al.*, 2014), long-chain poly unsaturated fatty acids (Mourente *et al.*, 1993), in addition to the relative ease of mass culture (Ye *et al.*, 2008).

Traditionally yeasts are more economical as live food for aquatic animals than microalgae; however they contain insufficient amounts of unsaturated fatty acids, which are essential for the growth of rotifers (Kim *et al.*, 2009). Meanwhile, feeding on microalgae substantially improves the nutritional composition and value of zooplankton that are commonly used as live food for fishes, including the species of *Artemia*, rotifers and Daphnia

(Zaki and Saad, 2010; Guedes and Malcata, 2012). Therefore, the constant and feasible production of microalgae with high nutritional value is needed for successful and economic fish hatcheries (Yaakob *et al.*, 2014). One of the big challenges in mass production of microalgae is the optimization of the nutrient medium to attain high growth rates of the preferred species (Haque *et al.*, 2012). In line with this, the main objective of the present work was to evaluate the efficiency of the Plackett-Burman experimental design in screening medium components that might affect biomass production of the alga *Chlorella salina*. Also, this work aims to study the efficiency of using *Chlorella salina* biomass as live food for culturing the rotifer *Brachionus plicatilis*. The effect of *Chlorella salina*, as live food, on growth rate and egg production of *Brachionus plicatilis* is compared to Baker's yeast.

2. Materials and methods

2.1. Test alga

The microalga isolate *Chlorella salina* (Butcher, 1952) was obtained from the culture collection of Universiti Sains, Malaysia and maintained by regular subculturing on Walne's medium (Walne, 1970).

2.2. Growth characteristics of the test microalga

2.2.1. Selection of growth medium

The growth was estimated as cell count per unit volume of different nutrient media was assessed. In order to determine the most suitable medium for biomass production, the test microalga was cultured on four different nutrient media. These media were Walne's (Walne, 1970), F/2 (Guillard, 1975), Navicula medium (Starr, 1978) and Phk (Rukminasari, 2013). The chemical components of each nutrient medium were dissolved in GF/C filtered well water with 28 gl^{-1} salinity, obtained from a well located at El-Matareyya Research Station, El-Matareyya, Dakahlia, Egypt (the National institute of Oceanography and Fisheries). The culture flasks were inoculated with 5 day old cultures to obtain an initial density of 50000 cell ml^{-1} and incubated at 25±3 °C under continuous light intensity of 2.789 w/ m^2 for 16 days. Cool white fluorescent tubes were used as light

source to avoid any drastic temperature changes. During the incubation period, the average cell count was determined every 2 days, following the procedure described in APHA (2005) using a haemocytometer. The growth rates of the algal cultures were calculated according to the following equations (Guillard, 1973): $\mu = \ln X_2 - \ln X_1 / (t_2 - t_1)$; $G = 0.6931 / \mu$; where μ = growth rate, X_1 = cell count at time t_1, X_2 = cell count at time t_2 and G = Generation time.

2.3. Optimization of growth medium using Plackette-Burman experimental design

The Plackett-Burman experimental design was used to screen the components of Phk medium affecting biomass production of *C. Salina*. Nineteen different variables were screened and included: $MgCl_2.2H_2O$, KCl,

$NaNO_3$, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, Na_2SO_4, $Na_2MoO_4 \cdot 2H_2O$, Fe citrate, citric acid, $ZnCl_2$, $MnCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, Na_2EDTA, H_3BO_3, $FeCL_3.6H_2O$, $CuCl_2$, $Na_2SeO_3 \cdot 5H_2O$ in addition to soil extract. Each factor was tested at three levels; low (-1), medium (0) and high (+1) at concentrations shown in Table 1. The level (0) means that the studied variables were at the original concentration of the control Phk medium. The (-1) means that 50% of chemical concentration of the variables in the Phk medium was used and (+1) means that 150% of chemical concentration of the variables in the Phk medium was used. The soil extract was prepared by autoclaving 1.0 kg garden soil mixed with 2.0 l glass distilled water for 10 min at 121°C. The soil extract was then removed by filtration and re-autoclaved and kept aseptically in a refrigerator.

Table 1. The screened variables of Plackett-Burman design at their different concentrations of the Phk growth medium

Designation	Variable	Zero level (0), gl^{-1}	Low level (-), gl^{-1}	High level (+), gl^{-1}
X1	$MgCl_2.2H_2O$	2.66	1.33	3.99
X2	KCl	0.616	0.308	0.924
X3	$NaNO_3$	0.5	0.25	0.75
X4	NaH_2PO_4	0.02646	0.01323	0.03969
X5	$NaHCO_3$	0.165	0.0825	0.2475
X6	$CaCl_2$	0.25	0.125	0.375
X7	Na_2SO_4	2.882	1.441	4.323
X8	$Na_2MoO_4.2H_2O$	0.00046	0.00023	0.00069
X9	Fe Citrate	0.054	0.027	0.081
X10	Citric acid	0.054	0.027	0.081
X11	$ZnCl_2$	0.000728	0.000364	0.001092
X12	$MnCl_2.4H_2O$	0.00174	0.00087	0.00261
X13	$CoCl_2.6H_2O$	0.000048	0.000024	0.000072
X14	Na_2EDTA	0.012	0.006	0.018
X15	H_3BO_3	0.0138	0.0069	0.0207
X16	$FeCL_3.6H_2O$	0.000584	0.000292	0.000876
X17	$CuCl_2$	0.00264	0.00132	0.00396
X18	$Na_2SeO_3.5H_2O$	NC	0.00132	0.00395
X19	Soil extract	NC	20 ml	60 ml

NC= Not an original component of the medium.

Twenty different media combinations shown in Table 2 were prepared in triplicate, using the Design-Expert® Version 9 (Stat-Ease, Inc. USA). The experiments were carried out in 100 ml Erlenmeyer flasks, each containing 40 ml of each medium along with control Phk media in parallel. To avoid the precipitation of media, the stock solutions were sterilized separately and the medium combinations were prepared under aseptic conditions. Three culture flasks were used for each medium combination. Flasks were inoculated with 5 day old cultures to obtain an initial concentration of 50000 cell ml^{-1}. The culture flasks were incubated at 25±3 °C under continuous white fluorescent light (2.789 w/ m^2) for 14 days. At the end of the incubation period, the average cell count for each of the different growth media combinations was determined using a haemocytometer

(APHA, 2005). The entire experiment was repeated to make sure of the results and the mean results of both experiments were recorded.

2.4. Data analysis

The data obtained from the different combinations were analysed using the equations of Fossi *et al.* (2005) and the main effect of each variable was determined with the following equation: $E_{xi} = (\Sigma H_{xi} - \Sigma L_{xi}) / N$; where E_{xi} is the variable main effect, H_{xi} and L_{xi} are the concentration of the variable at high level and low level of the same variable, and N is the number of trials divided by 2. The main effect with a positive sign indicates that the high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum.

Table 2. Plackette-Burman experimental design with the effect of 20 different combinations of the Phk medium on growth of *Chlorella salina*.

Media combinations	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X17	X18	X19	Mean cell number (millions/ml)	% increase (+) or decrease (-) in cell number	Significance level
0 (control medium)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.907		
1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	10.29	3.89	NS
2	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	8.747	-11.712	NS
3	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	11.93	20.40	*
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	10.81	9.08	NS
5	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	9.964	0.577	NS
6	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	6.391	-35.49	**
7	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	11.07	11.702	NS
8	1	1	-1	-1	1	1	-1	-1	-1	1	1	1	1	1	-1	-1	1	-1	1	13.58	37.09	**
9	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	11.04	11.43	NS
10	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	11.02	11.25	NS
11	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	13.58	37.07	**
12	-1	1	-1	1	-1	-1	-1	1	1	1	-1	1	1	-1	-1	1	1	1	1	7.413	-25.17	*
13	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1		8.124	-17.99	*
14	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	9.493	-4.178	NS
15	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	9.156	-7.59	NS
16	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	8.778	-11.40	NS
17	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	13.78	39.07	**
18	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	1	1	1	11.2	13.05	NS
19	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	11.24	13.45	NS
20	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	16.18	63.29	***

NS= Non- significant, * = significant, ** = high significant, *** = very high significant

2.4.1. Verification experiments

In order to evaluate the accuracy of the applied Plackett-Burman design, the predicted optimum levels of the screened variables for high growth of *Chlorella Salina,* were used to prepare a verification medium. The prepared medium was inoculated with five day old fresh culture to obtain an initial concentration of 50000 cell ml^{-1} and incubated at the conditions described above. A control Phk media was run in parallel. The production was determined as dry weight biomass (gl^{-1}) after 14 days of incubation. The verification experiment was carried out in triplicate.

2.5. Outdoor mass production of *Chlorella Salina*

Porcelain basins (2.4 x 0.7 x 0.7 m) were used for cultivation of *C. Salina*. The functional volume of algal culture was approximately 0.5 m^3. The functional surface area was 1.68 m^2. Glass containers (0.70 x 0.30 x 0.20 m) were used for preparation of inoculum from *C. Salina*, for inoculation of the porcelain basin. The alga was cultured in Phk medium modified by Plackett-Burman design. The well water used for mass culturing was sterilized by filtration through 50 μm mesh filter, chlorinated at the rate of 5 ppm chlorine for 48 h and then dechlorinated by an equivalent molar solution of sodium thiosulfate. At the beginning of the biomass production process, a volume of 100 l medium was added to the basins then inoculated to obtain an initial cell density of approximately 50000 cell ml^{-1} and incubated under field conditions of temperature 28.57±5.3 °C and light energy of 74.13±16.18 w/m^2. The modified Phk medium was gradually added every two weeks until the culture volume reached 0.5 m^3. To ensure mixing of the culture, a continuous air current was introduced into the culture. After 14 days, about 80% of the culture volume was drained to settling tanks and then the original culture volume of basins was restored using fresh modified Phk medium. The algal biomass was harvested by raising the pH to 11 using 0.5 M NaOH, left to settle for 2 hours (Vandamme *et al.,* 2012). The supernatant was then decanted and algal biomass slurry was washed several times with chlorine sterilized well water, until pH 7 – 8. Then, the fresh algal biomass was

collected and stored in the refrigerator at 4 °C until use for rotifer feeding experiments. The algal biomass harvesting process was repeated every 14 day and the dry weight of biomass production was recorded. The mass production of *C. salina* was continued for four months started from September 01, 2014 to December 30, 2014.

2.6. Mass production of the rotifer *Brachionus plicatilis*

Trials for growing the rotifer *Brachionus plicatilis* under field conditions (22 ± 5°C) were done using living cell of *Chlorella salina* cultivated on the modified Phk medium, a parallel rearing experiment on Baker's yeast was used as control. The effect of living cells of *C. salina* and Baker's yeast as feed on growth and egg production of *B. plicatilis* were compared.

2.6.1. Stock culture of rotifer

The stock culture of the rotifer *B. plicatilis* was obtained from El-Matareyya Research Station and was stocked in 60 L aquaria at density of 30 individual ml^{-1} on Baker's yeast. Baker's yeast was obtained from The Egyptian Starch Yeast and Detergents Co., Alexandria, Egypt. *B. plicatilis* was cultivated in semi-continuous cultures for one month (October 15, 2014 to December 15, 2014) using chlorine sterilized well water (28 gl^{-1} salinity) at a temperature of 27±2 °C and continuous light of 1.46 w/ m^2 intensity. The cultures were aerated and maintained in suspension by air bubbling. During the cultivation period, the water pH was regularly monitored and kept between 7.0 and 7.8 using 0.5 M NaOH.

2.7. Determination of the nutritional value of *Chlorella Salina* and Baker's yeast

The nutritional value of the biomass of Baker's yeast and *C. Salina* cultivated on control and modified Phk medium were determined by means of water content, dry matter, ash, lipids, carbohydrates, protein, crude fiber and ash content in addition to fatty acid composition. Water content, dry matter and ash were determined according to the standard official methods of analysis (1990), lipid content was determined by the exhaustive

soxhlet method with (dichloromethane: methanol) (2:1; v/v) (Folch *et al.*, 1957), protein content by Bradford (1976), total carbohydrates by anthrone method (Hedge and Hofreiter, 1962) and crude fibers (Maynard, 1970). The fatty acid composition of the total lipids extracted from *C. Salina* and Baker's yeast were determined using gas chromatography- mass spectroscopy (GC-MS). For analysis of the fatty acid profile, lipids were transesterified using the method of Mc Donough *et al.* (1999) to convert fatty acids to fatty acids methyl esters (FAME) and analysed by GC-MS.

2.8. Effect of feeding with *C. salina* on *B. plicatilis* growth rate and egg production

The rotifer *B. plicatilis* was cultivated in 60 l aquaria containing 10 l chlorine sterilized well water. The initial density of rotifers was 32 ± 3 individuals ml^{-1}. The initial cell density of *C. salina* and Baker's yeast was 5 x 106 cell ml^{-1}. The cultivation period was for 10 days. Water temperature was adjusted to 22 ± 2 °C and pH to 7.5 - 7.8. Three aquaria replicates were used for each feed type including *C. salina* and Baker's yeast. A volume of 1.0 l culture was filtered from each culture on a daily basis and 0.1 ml sub-samples were used for counting of rotifer population density and eggs ratio under a stereo microscope. Also, the remaining cells of *C. salina* and Baker's yeast were daily counted. The growth rate (μ) and generation time (G) in days of the *B. plicatilis* cultivated on *C. salina* and Baker's yeast was determined according to the Guillard (1973) equations mentioned before. The egg ratio (ER; eggs indv.$^{-1}$) was calculated according to Kobayashi (2009) equation; ER =

Table 3. Effect of different growth media on specific growth rate (μ) and generation time (G) of *Chlorella salina*. Results were obtained after 16 days and are the average ± SD (n = 3).

Growth medium	μ	G
Phk medium	0.380 ± 0.046	1.82 ± 0.195
Walne's medium	0.269 ± 0.095	2.57 ± 0.209
F/2 medium	0.275 ± 0.097	2.52 ± 0.891
Navicula medium	0.366 ± 0.134	1.89 ± 0.248

eggs (ml^{-1})/ rotifers (ml^{-1}).

2.9. Statistical analysis of data

Values of each measurement represent the mean of three replicates ± SD (Standard Deviation). All data obtained were analysed using STATGRAPHICS (STSC, ver. 5.1) program.

3. Results and Discussion

Chlorella is the most cultivated eukaryotic green microalga for commercial purposes in the food, feed, aquaculture, pharmaceutical and cosmetics industries (Sharma *et al.*, 2011). In the present study, the growth characteristics of *Chlorella salina* in four different nutrient media were studied (Figure 1 and Table 3). *Chlorella salina* was able to grow well and to build-up biomass in different growth media, with the highest cell count (10.25×10^6 cell ml^{-1}) recorded after 14 days of culture in Phk medium (Figure 1). The highest growth rate (μ=0.38) was also recorded for *C. salina* cultured in Phk medium (Table 3). The production of microalgae biomass for aquaculture must be economically feasible and cost competitive with traditional feed. It is well known that, media composition (Haque *et al.*, 2012) and growth conditions (Liang, 2011) influence the growth and thus biomass production of microalgae. In this context, the Plackett-Burman design was employed for screening the nutritional variables of the Phk medium affecting biomass production of *Chlorella salina*. Most of the experimental combinations maintained non-significant to significant ($P \leq 0.05$) increase or decrease in cell count, however, the experimental combinations ≠8, ≠11 and ≠17 maintained high significant ($P \leq 0.01$) increase in cell count (Table 2). Meanwhile, only, the combination ≠20 showed very high significant ($P \leq 0.001$) increase in cell count. In general, the cell count in different media combinations fluctuated between 6.39×10^6 cell/ml (combination ≠6) and 16.18×10^6 cell/ml (combination ≠20) (Table 2). The cell count of the control Phk medium was 9.91 x 106 cell ml^{-1} (Table 2).

Regarding the contribution of the tested variables to biomass production, some variables exhibited positive
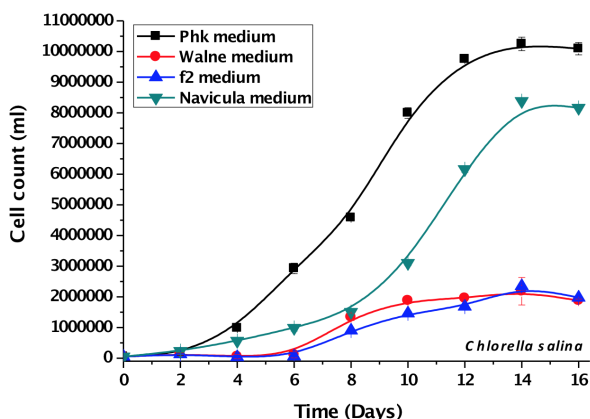
Fig. 1. Growth curves of Chlorella salina grown in different growth media.

and others negative effects on growth of *Chlorella salina* (Figure 2). The positive main effect of the variables; KCl, Na_2SO_4, citric acid, $ZnCl_2$, $FeCL_3.6H_2O$, $CuCl_2$ and soil extract indicated that the maximum biomass production of *C. salina* requires 1.5 fold concentrations of these components compared to that of control standard Phk medium. The negative main effect of $MgCl_2.2H_2O$, Na_2NO_3, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, $Na_2MoO_4.2H_2O$, Fe Citrate, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, Na_2EDTA, H_3BO_3 and $Na_2SeO_3.5H_2O$ indicated that the tested alga required half concentrations of the control standard Phk medium, for attaining the highest biomass production (Figure 2). These results agreed with many studies which reported that some nutrients greatly affect biomass production of microalgae such as nitrogen limitation (Illman *et al.*, 2000), phosphate limitation (Reitan *et al.*, 1994) and iron content (Liu *et al.*, 2008).

In order to evaluate the accuracy of the applied Plackett-Burman design, a verification media for high biomass production was applied to compare the predicted optimum levels of the screened variables. As seen from Figure 3, the verification Phk medium supported about 104% increase in dry weight biomass (0.521 gl^{-1}) of *C. salina* compared to that of control medium (0.255 gl^{-1}). The results, suggested that the best composition of Phk medium for maximum biomass production of *C. salina* is as follows (gl^{-1}): $MgCl_2.2H_2O$

(1.33), KCl (0.924), $NaNO_3$ (0.25), NaH_2PO_4 (0.01323), $NaHCO_3$ (0.0825), $CaCl_2$ (0.125), Na_2SO_4 (4.323), $Na_2MoO_4.2H_2O$ (0.00023), Fe Citrate (0.027), Citric acid (0.081), $ZnCl_2$ (0.0011), $MnCl_2.4H_2O$ (0.00087), $CoCl_2.6H_2O$ (0.000024), Na_2EDTA (0.006), H_3BO_3 (0.0069), $FeCL_3.6H_2O$ (0.000876), $CuCl_2$ (0.00396), $Na_2SeO_3. 5H_2O$ (0.00132) and soil extract (60 ml).
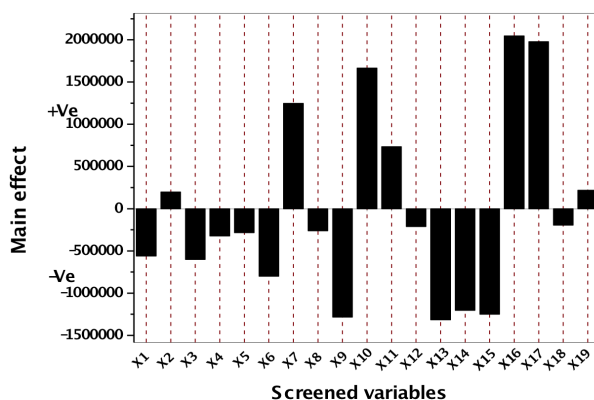

Fig. 2. Elucidation of the main effect of the secerned medium variables on growth of *Chlorella salina*.
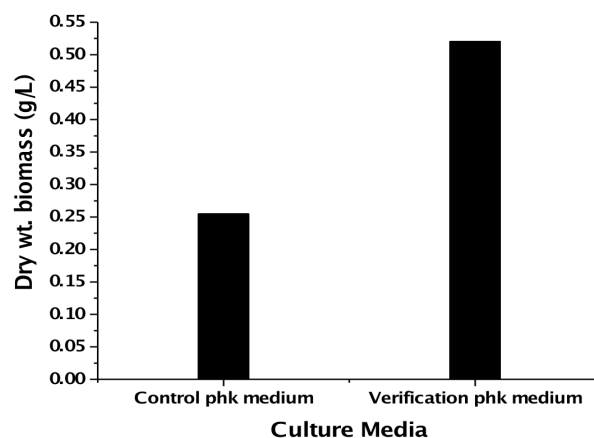

Fig. 3. Dry weight biomass of *Chlorella salina* grown for 10 days in control and verification Phk nutrient medium .

It has been widely reported that the biochemical composition of microalgae influences their nutritional value (De Pauw and Persoone, 1988; Fernandez-Reiriz *et al.*, 1989, Dhert *et al.*, 2001). In the present study, the modified Phk medium was used for semi-continuous outdoor mass production of *Chlorella salina* for four

months. As seen in Figure 4, the general main biochemical composition of *Chlorella salina* cultivated on control and modified Phk medium is comparable and only slight non-significant ($P \leq 0.05$) changes in lipids and protein content were detected. However, a very high significant ($P \leq 0.001$) increase in biomass production from 0.60 to 0.79 gl^{-1} dry weight biomass was recorded for the outdoor cultures grown in control and modified Phk media respectively. This result may indicate the feasibility of using the modified Phk medium for large scale mass production of *Chlorella salina* to be used as live food in aquaculture. The major chemical composition (total proteins, lipids, carbohydrates, ash content and crude fibers) of *Chlorella salina* grown in standard and Plackett-Burman - selected media are illustrated in Figure 4A and Figure 4B, respectively. The major chemical composition of Baker's yeast, which is traditionally used for feeding rotifers in aquaculture, is included (Figure 4C) for comparison.

The present study gave considerable attention to fatty acid composition of *C. salina* grown in standard and modified Phk medium (Table 4). It became evident that the increase in polyunsaturated fatty acids (PUFAs), and in particular omega-3 and omega-6 fatty acids, substantially increases the food and feed value of algal cells (Simopoulos, 2002; Adarme-Vega e*t al.,* 2012). Although marginal increase (only 2.59 wt %) of total lipids of *C. salina* culture grown in modified Phk medium (Figure 4), substantial increase in weight percent of PUFAs from 29.75% (control culture) to 56.97% (modified Phk culture) was recorded (Table 4). The fatty acid methyl esters (FAMEs) of *C. salina* cultivated on the modified Phk were dominated by the polyunsaturated fatty acid α–linolenic acid methyl ester (18:3, n-3) (24.96%), 7,10,13-Hexadecatrienoic acid methyl ester (16:3, n-3) (17.46%), linoleic acid methyl ester (18:2, n-6) (7.93%) and 7,10-hexadecadienoic acid methyl ester (16:2, n-6) (6.63%). It is perhaps relevant to mention that the PUFAs comprised only 5.47% of the total lipids of Baker's yeast (Table 4). The FAMEs of Baker's yeast were mainly represented by methyl oleate (18:1, n-9) (32.68%), 11(Z)- hexadecenoic acid, methyl ester
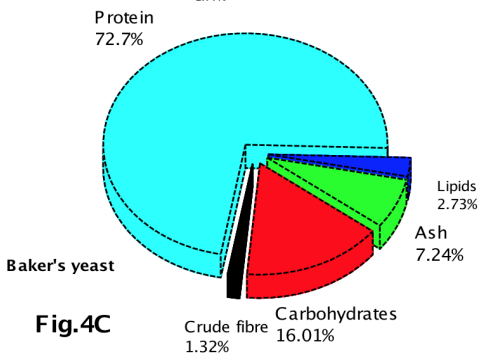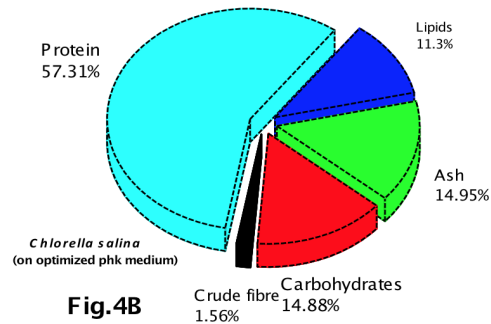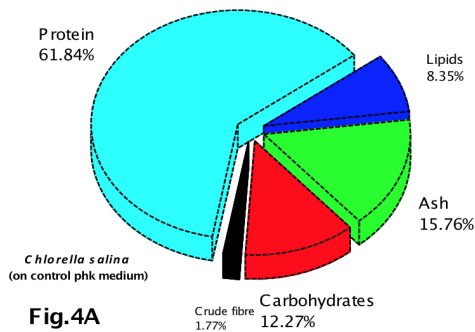

Fig. 4. Biochemical composition of *Chlorella salina* grown in control (Fig.4A) and in modified (Fig.4B) Phk medium. The biochemical composition of Baker's yeast (Fig.4C) was included for comparison.

(31.36%), palmitic acid, methyl ester (13.43%), 7, 10, 13-hexadecatrienoic acid, methyl ester (16:3, n-3) (4.7%) and 5,8,11,14,17-eicosapentaenoic acid, methyl ester (EPA, 20:5, n-3) (0.12%). The results obtained clearly indicated that the lipids of *C. salina* grown in modified Phk medium are substantially enriched in omega-3 and omega-6 fatty acids (Table 4). Accordingly, the biomass of this alga may represent potential feed-

stock for feeding rotifers that are extensively used as live food for economic fish larvae.

The results of feeding *Brachionus plicatilis* on *Chlorella salina* culture grown in modified Phk and Baker's yeast are illustrated in Figure 5. It is clear from the figure that the cell count of *C. salina* (Figure 5A) and Baker's yeast (Figure 5B) declined substantially with increasing feeding time. This result is expected due to

Table 4. Fatty acids profile of Baker's yeast and *Chlorella salina* grown in control and modified Phk media.

Identified pounds	Peak area %		
	Baker's yeast	*Chlorella salina* (on control Phk)	*Chlorella salina* (on modified Phk)
Tetradecanoic acid, methyl ester (Myristic acid, methyl ester) (14:0)	ND	3.494	0.378
Pentadecanoic acid, methyl ester (15:0)	ND	1.012	0.302
Hexadecanoic acid, methyl ester (Palmitic acid, methyl ester) (16:0)	13.42	14.218	23.73
9-hexadecenoic acid, methyl ester (Palmitoleic acid, methyl ester (16:1, n-7)	0.294	ND	ND
11(Z)-Hexadecenoic acid methyl ester (Palmitvaccenic acid) (16:1)	31.35	ND	ND
7,10-hexadecadienoic acid, methyl ester (16:2, n-6)	ND	0.241	6.625
7,10,13-Hexadecatrienoic acid, methyl ester (16:3, n-3)	4.7	5.241	17.46
9-Octadecenoic acid, (E)- (trans-Elaidic acid) (18:1, n-9)	1.078	ND	ND
Cis-9-Octadecenoic acid, methyl ester (Methyl oleate (18:1, n-9)	32.68	ND	0.388
9,12-Octadecadienoic acid, methyl ester (Linoleic acid, methyl ester) (18:2, n-6)	ND	ND	7.931
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (α-linolenic acid, methyl ester) (18:3, n-3)	0.637	24.65	24.96
Octadecanoic acid, methyl ester (Stearic acid, methyl ester) (19:0)	2.499	0.988	ND
Eicosanoic acid, methyl ester (Arachidic acid, methyl ester) (20:0)	ND	7.289	1.5
5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)- (EPA) (20:5, n-3)	0.12	ND	ND
Sum. of fatty acids	86.78	57.68	83.27
Other lipid compounds other than fatty acids	13.22	42.32	16.73
∑Saturated fatty acids	15.92	26.88	25.91
∑Monounsaturated fatty acids	65.41	1.048	0.388
∑Polyunsaturated fatty acids	5.457	29.75	56.97
∑n-3	5.457	29.5	42.42
∑n-6	ND	0.241	14.56
∑n-7	0.294	1.048	ND
∑n-9	33.758	ND	0.388

The names in parenthesis represent the common names of the identified compounds; ND, not detectable.
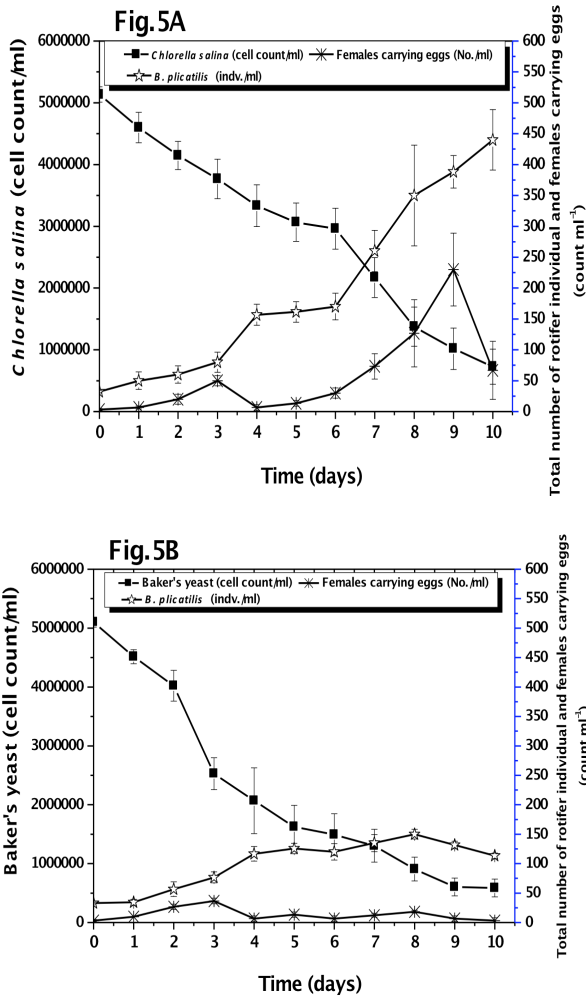
Fig. 5. Semi-continuous culture of B. plicatilis fed on *Chlorella salina* grown in modified Phk medium (Fig.5A) and Baker's yeast (Fig.5B).

feeding activities of B. plicatilis in a constant population size of live cells of Chlorella and Baker's yeast. The most pronounced and elegant results were the very high significant (p ≤ 0.001) increase in total individual numbers and females carrying eggs of B. plicatilis fed on living cells of Chlorella (Figure 5A) compared to those fed on Baker's yeast (Figure 5B). After 9 days of feeding experiments, the density (total individuals number ml^{-1}) reached 440±60 ml^{-1} and 132±8 ml^{-1} and the number of fertile females (carrying eggs) 225 ml^{-1} and 18ml^{-1} of cultures fed on *C. salina* (Figure 5A) and Baker's yeast (Figure 5B) respectively. Figure 6 indicates that the egg

ratio (number of fertile females/ number of total rotifer individuals) was substantially higher for rotifer fed on C. salina (56%) compared to those fed on Baker's yeast (5%). Moreover, the rotifer females fed on Chlorella were much more fertile (mostly carrying 6 eggs) compared to females fed on Baker's yeast (Photo 1). The microscopic examinations revealed the number of eggs carried by rotifer females fed on Baker's yeast never exceed 3 eggs per single female. In concise quantitative terms, Table 5 indicated that after 10 days feeding experiments, the average   rotifer densities were 440±60 ml^{-1} and 132±8 ml^{-1} for cultures fed on *C. salina* and
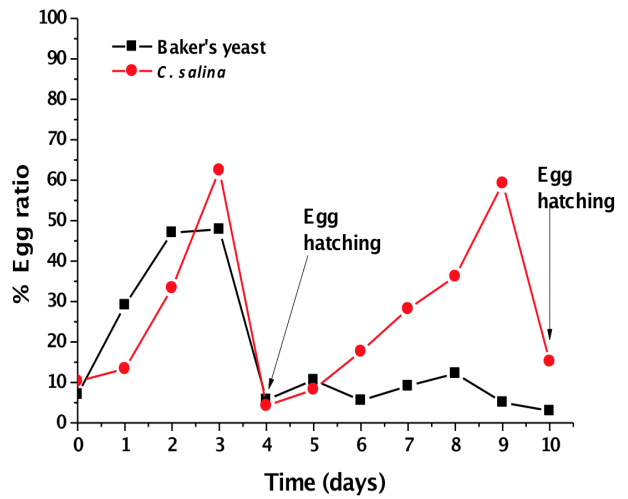

Fig. 6. Effect of feeding on cells of *Chlorella salina* and Baker's yeast on egg ratio of *B. plicatilis*.
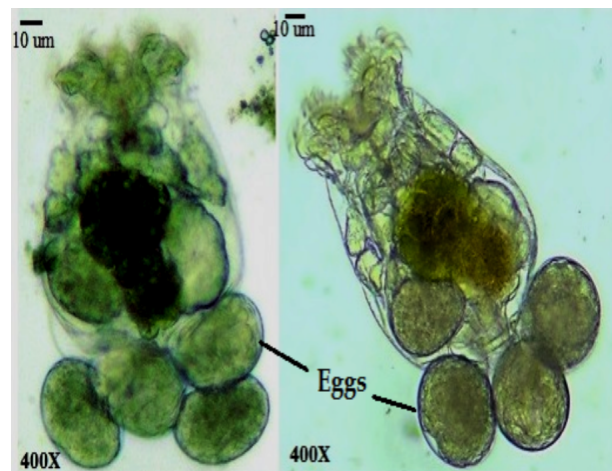

Photo 1. Egg production of a female individual of *B. plicatilis* fed on the living cells of *Chlorella salina*.

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Baker's yeast, respectively. The substantial growth of rotifer fed on *C. salina* was further assessed by higher specific growth rate (0.261 ± 0.011) and lower generation time (2.66 ± 0.114 days) (Table 5).

Table 5. Specific growth rate (μ), generation time (G) and density (ind./ml) of B. plicatilis fed for 10 days either on Baker's yeast or *Chlorella salina*. Results are the average ± SD (n=3).

Food type	rotifer density (ind./ml)	μ	G (days)
Baker's yeast	132 ± 8	0.126 ± 0.004	5.49 ± 0.174
Chlorella salina	440 ± 60	0.261 ± 0.011	2.66 ± 0.114

In conclusion, the results of this study clearly indicated possible feasible biomass production of the microalga *Chlorella salina* through Plackett-Burman screening design. The selected modified Phk media supported not only a substantial increase in Chlorella biomass but also significant increases in the omega-3 and omega-6 contents. In addition, significant increase in total individual count and fertile females of the rotifer *Brachionus plicatilis* was only recorded for cultures fed on *Chlorella salina*. These results indicated the promise of cells of *Chlorella salina* as live food for mass production of the rotifer *B. plicatilis*, which represents an excellent live food resource for the larvae of the economic fish. This, of course, will maintain positive consequences in production of economic fishes to a level that cope with the ever-increasing demand for animal protein.

References

Adarme-Vega, T.C.; Lim, D.K.; Timmins, M.; Vernen, F.; Li, Y. and Schenk, P.M. (2012): Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. *Microbial Cell Factories*, 11(1): 96-106.

American public health association (APHA) (2005): Standard methods for the examination of water and wastewater. 21st ed. American public health association, 800 I Street, NW, Washington, DC 20001-3710.

Barra, L.; Chandrasekaran, R.; Corato, F. and Brunet, C. (2014): The challenge of ecophysiological biodiversity for biotechnological applications of marine microalgae. *Marine drugs*, 12(3): 1641-1675.

Borowitzka, M.A. (1997): Microalgae for aquaculture: opportunities and constraints. *Journal of Applied Phycology*, 9: 393-401.

Bradford, M.M. (1976): Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248–254.

Brown, M.R. (2002): Nutritional value and use of microalgae in aquaculture. Avances en Nutrición Acuícola VI Memorias del VI Simposium Internacional de Nutrición Acuícola, 3: 281-292.

Brown, M.R.; Mular, M.; Miller, I.; Farmer, C. and Trenerry, C. (1999): The vitamin content of microalgae used in aquaculture. *Journal of Applied Phycology*, 11: 247-255.

Butcher, R.W. (1952): Contributions to knowledge of the smaller marine algae. *Journal of the Marine Biological Association of the United Kingdom,* 31: 175-191.

Chullasorn, S.; Ivanenko, V.N.; Dahms, H.U.; Kangtia, P. and Yang, W., X. (2011): A new species of *Tigriopus* (Copepoda, Harpacticoida, Harpacticidae) from Thailand with the description of its naupliar development. *Helgoland Marine Research,* 66: 139–151.

De Pauw, N. and Persoone, G. (1988): Micro-algae for aquaculture. In: Borowitzka, M.A. and Borowitzka, L.J. (Eds.) Micro-algal Biotechnology. Cambridge University Press, Cambridge, U.K., pp. 197-221.

Dhert, P.; Rombaut, G.; Suantika, G. and Sorgeloos, P. (2001): Advancement of rotifer culture and manipulation techniques in Europe. *Aquaculture,* 201:129-146.

Folch, J.; Lees, M., Sloane-Stanley, G.M. (1957): A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, 226:497-509.

Fossi, B.T.; Tavea, F.; Ndjouenkeu, R. (2005): Production and partial characterization of a thermostable

amylase from ascomycetes yeast strain isolated form starchy soil. *African Journal of Biotechnology*, 4(1): 14-18.

Gatrell, S.; Lum, K.; Kim, J. and Lei, X.G. (2014): Non-ruminant nutrition symposium: Potential of defatted microalgae from the biofuel industry as an ingredient to replace corn and soybean meal in swine and poultry diets. *Journal of Animal Science,* 92 (4): 1306-1314.

Guedes, A.C. and Malcata, F.X. (2012): Nutritional Value and Uses of Microalgae in Aquaculture. In: Muchlisin, Z.A. (Ed.): Aquaculture. InTech Open Acess Publisher. DOI: 10.5772/30576. Available from:http://www.intechopen.com/books/aquaculture/nutritional-value-and-uses-of-microalgae-in-aquaculture

Guillard, R.R.L. (1973): Division rates. In: Stein, J.R. (Ed.) Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, Cambridge, 1: 289–312.

Guillard, R.R.L. (1975): Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L. and Chanley, H.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York, pp. 29-60.

Haque, M.A.; Bangrak, P.; Sirisansaneeyakul, S. and Choorit, W. (2012): Factors affecting the biomass and lipid production from *Chlorella* sp. TISTR 8990 under mixotrophic culture. *Walailak Journal of Science and Technology (WJST),* 9(4): 347-359.

Hedge, J.E. and Hofreiter, B.T. (1962): Methods. In: Whistler, R.L. and BeMiller, J.N. (Eds.) Carbohydrate Chemistry. Academic Press, New York, 17: 420.

Illman, A.M.; Scragg, A.H. and Shales, S.W. (2000): Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme* and *Microbial Technology*, 27: 631-635.

Kim, H.Y., Kim, J.K. and Hur, S.B. (2009): Dietary value of Candida utilis for *Artemia nauplii* and *Mytilus edulis* larvae. *J Aquac*, 22: 68-73.

Kobayashi, T.; Nagase, T.; Hino, A. and Takeuchi, T. (2009): Influence of photoperiod on the eicosapen-taenoic acid concentration of *Nannochloropsis*, and fatty acid composition of S-type rotifer *Brachionus* in a continuous culture system. *Aquaculture Science (Japan),* 57(1):133-139.

Kris-Etherton, P.; Harris, W. and Appel, L. (2003): Fish con¬sumption, fish oil, omega-3 fatty acids, and cardiovascu¬lar disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 23: 20–30.

Liang, G.; Mo, Y.; Tang, J.; and Zhou, Q. (2011): Improve lipid production by pH shifted-strategy in batch culture of *Chlorella protothecoides*. *African Journal of Microbiology Research, 5*(28): 5030-5038.

Liu, Z.Y.; Wang, G.C. and Zhou, B.C. (2008): Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresource Technology*, 99: 4717-4722.

Marshall, R.; McKinley, S. and Pearce, C.M. (2010): Effects of nutrition on larval growth and survival in bivalves. *Reviews in Aquaculture*, 2: 33–55.

Maynard, A.J. (1970): Methods in food analysis. Academic Press, New York, London, 176.

Mourente, G.; Rodriguez, A.; Tocher, D.R. and Sargent, J.R. (1993): Effects of dietary docosahexaenoic acid (DHA; 22:6n-3) on lipid and fatty acid compositions and growth in gilthead sea bream (*Sparus aurata* L.) larvae during first feeding. *Aquaculture*, 112: 79–98.

Muller-Feuga, A. (2000): The role of microalgae in aquaculture: situation and trends. *Journal of Applied Phycology,* 12: 527-534.

Official Methods of Analysis (1990): Determination of moisture, dry matter and ash, 15th Edition, Association of Official Analytical Chemists (AOAC), 2200 Wilson Boulevard Arlington Virginia 22201 USA.

Reitan, K.I.; Rainuzzo, J.R. and Olsen, Y. (1994): Effect of nutrient limitation of fatty acid and lipid content of marine microalgae. *Journal of Phycology,* 30: 972-979.

Roy, S.S. and Pal, R. (2014): Microalgae in Aquaculture: A Review with Special References to Nutritional Value and Fish Dietetics. Proceedings of the Zoological Society, pp. 1103-1107.

Sharma, R.; Singh, G.P. and Sharma, V.K. (2011): Comparison of different media formulations on growth, morphology and chlorophyll content of green alga, chlorella vulgaris. *International Journal of Pharmacy and Biological Sciences*, 2(2): B509-B516.

Simopoulos, A.P. (2002): Omega-3 fatty acids in inflammation and autoimmune diseases. *Journal of the American College of Nutrition,* 21(6): 495-505.

Starr, R. C. (1978): The culture collection of algae at the University of Texas at Austin. *Journal of Phycology,* 14: 92–96.

Vandamme, D.; Foubert, I.; Fraeye, I.; Meesschaert, B. and Muylaert, K. (2012): Flocculation of *Chlorella vulgaris* induced by high pH: role of magnesium and calcium and practical implications. *Bioresource technology,* 105: 114-119.

Walne, P.R. (1970): Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera Ostrea, *Crassostrea, Mercenaria*, and *Mytilis*.

Fish. Invest, 26: 162.

Watanabe, T.; Kitajima, C. and Fujita, S. (1983): Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture* 34, 115-143.

Yaakob, Z.; Ali, E.; Zainal, A.; Mohamad, M. and Takriff, M.S. (2014): An overview: biomolecules from microalgae for animal feed and aquaculture. *Journal of Biological Research*, 21:6.

Ye, Z.W.; Jiang, J.G. and Wu, G.H. (2008): Biosynthesis and regulation of carotenoids in Dunaliella: Progresses and prospects. *Biotechnology Advances*, 26: 352-360.

Zaki, M.I. and Saad, H. (2010): Comparative study on growth and survival of larval and juvenile Dicentrarchus labrax rearing on rotifer and Artemia enriched with four different microalgae species. *African Journal of Biotechnology*, 9(24): 3676-3688.

الملخص العربى

الإنتاج المستدام للكتلة الحيوية من الطحلب البحري الدقيق كلوريلا سالينا (*Chlorella salina*) واستخدامها كغذاء حي للروتيفر *Brachionus plicatilis*.

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في هذه الدراسة تم اختيار طحلب *Chlorella salina* لدراسة خصائص نموه وقيمته كغذاء حي لنوع من أنواع الروتيفرز وهو *Brachionus plicatilis* والذي يستخدم علي نطاق واسع كغذاء حي ليرقات الأسماك البحرية. وجد أن الوسط الغذائي Phk يحقق أعلي انتاجية للكتلة الحيوية من الطحلب *Chlorella salina*. و لذلك تم إستخدام تصميم بلاكت برمان لدراسة تاثير العناصر الغذائـة المكونة للوسط Phk علي إنتاج الكتلـة الحويـة في طحلب *Chlorella salina* . ومن أجل تقييم مدى دقة تطبيق تصميم بلاكت برمان، تم تحديد المستويات المثلى المتوقعة من العناصر الغذائية لتحقيق اعلي انتاج من الكتلة الحيويـة للطحلب وتحضير وسط غذائي تحقيقي. تم تعيين الكتلة الحيوية بعد 14 يوم فترة تحضين، ولوحظ ان الوسط الغذائي التحقيقي أدي الي زيادة بنسبة 104% في انتاج الكتلة الحيوية (0.521 جرام/ لتر) مقارنة بالكنترول (0.255 جرام/ لتر). من النتائج السابقة يتضح أن تركيب الوسط الغذائي المعدل phk لأنتاج اقصي انتاج من الكتلة الحيويـة كالتالي (جرام/لتر): (0.924) KCl ,(1.33) $MgCl_2.2H_2O$ Fe (0.25) $NaNO_3$ (0.01323) NaH_2PO_4 (0.0825) $NaHCO_3$ (0.125) $CaCl_2$ (4.323) Na_2SO_4 (0.00023) $Na_2MoO_4.2H_2O$ Citrate (0.027) citric acid (0.081) $ZnCl_2$ (0.0011) $MnCl_2.4H2O$ (0.00087) $CoCl_2.6H_2O$ (0.000024) Na_2EDTA (0.006) (0.00132) $Na_2SeO_3.5H_2O$ (0.00396) $CuCl_2$ (0.000876) $FeCL_3.6H_2O$ (0.0069) H_3BO_3 و مستخلص التربة (60 مللي/لتر). وأشارت النتائج إلى احتواء الكلوريلا المزروعة علي وسط Phk الغذائي المعدل تحت الظروف الحقلية علي نسبة عالية من البروتين ومن الدهون التي تحتوي علي نسبة عالية من أوميغا 3 وأوميغا 6 . ثم اجريت بعض التجارب الحقلية لتنمية الروتيفر *Brachionus plicatilis* علي الخلايا الحية لطحلب *Chlorella salina* والتي تم تنميتها علي وسط Phk الغذائي المعدل. اجريت أيضا تجربة موازية لتغذية *Brachionus plicatilis* علي خلايا الخميرة الحية ككنترول. أشارت النتائج الي وجود زيادة مؤثرة جدا (p ≤ 0.001) في العدد الكلي وعدد الأناث الحاملة للبيض للروتيفر المُغذي علي خلايا الكلوريلا الحية. ومن هذه النتائج يتضح الأهميـة الإقتصادية لإستخدام طحلب *Chlorella salina* للأنتاج الكتلي للـروتيفر *Brachionus plicatilis* والذي يعد من أهم انواع الروتيفرز الذي يستخدم علي نطاق واسع كغذاء حي ليرقات الأسماك البحرية.

Sustainable Biomass Production of the Marine Microalga *Chlorella salina* as Live Food for the Rotifer *Brachionus plicatilis*

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