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Growth and Quality Enhancement of *Chlorella vulgaris* Beyerinck (Beijerinck) 1890 Using Simple Cost-effective Medium

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Abstract

Microalgae are considered to be the primary food source for many aquatic life forms and play a key role in the development of aquaculture. However, mass cultivation of pure microalgae requires costly media. This study formulated a simple cost-effective medium using plant fertilisers to reduce the number of inorganic salts in the commonly used expensive conventional media for the cultivation of *Chlorella vulgaris* Beyerinck (Beijerinck) 1890. Several formulations were investigated using locally available common plant fertiliser "Serbajadi 63"containing 21:21:21 of nitrogen:phosphorus:potassium and "Serbajadi 46" containing 46 % of urea as the base formulae. NPKFM formulated from "Serbajadi 63" with addition of 0.317 $g.L^{-1}$ of $MgSO_4$ and 0.02 $g.L^{-1}$ of acidified $FeSO_4.7H_2O$, produced biomass, proteins and chlorophyll-a comparable ($P > 0.05$) to Bold's basal medium (BBM) used as a control. In addition, NPKFM produced significantly higher ($P < 0.05$) amounts of chlorophyll b, carotenoids and carbohydrates compared to BBM. The cost of formulating NPKFM was 59.09 % cheaper than BBM. This study showed that NPKFM could be a simple cost-effective medium for the cultivation of *C. vulgaris*.

Keywords: *Chlorella vulgaris*, cost-effective medium, fertiliser, microalgae

Introduction

Microalgae are the primary producer for various aquatic organisms and play a key role in the development of aquaculture. They have a higher photosynthetic efficiency and biomass

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conversion compared to terrestrial plants (Miao and Wu 2006). *Chlorella vulgaris* Beyerinck (Beijerinck) 1890 is a microalga that belongs to the phylum Chlorophyta and has been widely used in several biotechnology industries. The microalga has been used since ancient times in the Far East and is considered as a traditional food in the Orient (Ötleş and Pire 2001). *Chlorella* sp. is well known for its antitumor, anticarcinogenic, antiviral, anticataract, antiulcer and even antioxidative properties (Shibata et al. 2003). The microalga also possesses protective abilities towards viral and bacterial infections (Tanaka et al. 1986; Queiroz et al. 2003).

Cultivation of microalgae is however seen as a high cost investment and therefore a cheaper and cost effective way for its cultivation is needed (Hemaiswarya et al. 2011). The medium used for the cultivation of microalgae greatly affects its quality (Berges et al. 2001; Xin et al. 2010). Moreover, the chemicals needed for the formulation are costly and tedious to prepare due to various inorganic salts that are added to the medium (Kaladharan et al. 2002). Thus, researchers have been seeking for cost-effective media to successfully cultivate microalgae (Tuyob et al. 2007). Therefore this experiment was aimed at producing a simple cost-effective medium which is easy to prepare for the cultivation of *C. vulgaris* by reducing the amount of chemicals needed for its formulation.

Materials and Methods

Microalgae culture

The microalga *C. vulgaris* was obtained from the Aquatic Animal Health Unit, Universiti Putra Malaysia, Malaysia and maintained in Bold's basal medium (BBM) (Bischoff and Bold 1963). The medium was prepared, sterilised and left for 2 days for CO_2 equilibration before inoculating the microalga. The microalga was cultured and maintained at 25 ± 2 °C, provided continuous filtered (0.45 µm, Sartorius, Germany) aeration and light intensity of 60 ± 2 $\mu mol.m^{-2}.s^{-1}$.

Media formulation

Locally available plant fertilisers "Serbajadi 63" containing 21:21:21 of nitrogen:phosphorus:potassium and "Serbajadi 46" containing 46 % of urea were used as the base for formulating the media. Using the fertiliser dosage as recommended on the products, three basic concentrations from each fertiliser were made; i.e., 0.2, 0.4 and 0.8 $g.L^{-1}$ using "Serbajadi 63" and 0.3, 0.6 and 1.2 $g.L^{-1}$ using "Serbajadi 46". $FeSO_4$ and $MgSO_4$ were added to some of the basic concentrations to obtain 12 formulae (Table 1). The ratios of Fe:N and Mg:N in the formulations were similar to that of BBM.

Experimental design

The 12 media formulations (Table 1) were used for the experimental design. The microalga *C. vulgaris* was inoculated in an initial starter culture of 20 mL in conical flasks and was gradually scaled up to 1.5 L. The initial 20 mL culture was inoculated into 30 mL of each media in triplicates, with batch cultures scaling up in increasing volumes of 100, 250 and 500 mL.

During the upscaling from the 500 mL conical flasks to the 2 L flask, approximately 10^4 cells.mL^{-1} were inoculated into the respective medium. Each scaling up was done during the log-phase of growth as determined by its biomass, cell density (CD) and optical density (OD). The scaling up was done in a laminar flow to prevent contamination of the culture. Each flask was hand-agitated three times a day. The growth and proximate analysis were done on samples collected from the 2 L flask.

Table 1. Composition of formulations for the culture of *Chlorella vulgaris*

Fertiliser	Formulation*
NPK 0.25	• 0.2 g.L^{-1} of fertiliser "Serbajadi 63"
NPK 0.5	• 0.4 g.L^{-1} of fertiliser "Serbajadi 63"
NPK 1.0	• 0.8 g.L^{-1} of fertiliser "Serbajadi 63"
NPKM	• 0.8 g.L^{-1} of fertiliser "Serbajadi 63" • 0.317 g.L^{-1} of $MgSO_4$
NPKF	• 0.8 g.L^{-1} of fertiliser "Serbajadi 63" • 0.02 g.L^{-1} of $FeSO_4.7H_2O$ (Acidified)
NPKFM	• 0.8 g.L^{-1} of fertiliser "Serbajadi 63" • 0.317 g.L^{-1} of $MgSO_4$ • 0.02 g.L^{-1} of $FeSO_4.7H_2O$ (Acidified)
Urea 0.25	• 0.3 g.L^{-1} of fertiliser "Serbajadi 46" • 0.12 g.L^{-1} of K_2HPO_4
Urea 0.5	• 0.6 g.L^{-1} of fertiliser "Serbajadi 46" • 0.24 g.L^{-1} of K_2HPO_4
Urea 1.0	• 1.2 g.L^{-1} of fertiliser "Serbajadi 46" • 0.48 g.L^{-1} of K_2HPO_4
UreaM	• 1.2 g.L^{-1} of fertiliser "Serbajadi 46" • 0.48 g.L^{-1} of K_2HPO_4 • 0.48 g.L^{-1} of $MgSO_4$
UreaF	• 1.2 g.L^{-1} of fertiliser "Serbajadi 46" • 0.48 g.L^{-1} of K_2HPO_4 • 0.03 g.L^{-1} of $FeSO_4.7H_2O$
UreaFM	• 1.2 g.L^{-1} of fertiliser "Serbajadi 46" • 0.48 g.L^{-1} of K_2HPO_4 • 0.0.03 g.L^{-1} of $FeSO_4.7H_2O$ • 0.48 g.L^{-1} of $MgSO_4$

Analysis of growth parameters

The growth of the microalga was determined daily by its OD, CD and biomass every 24 h at 10.30 a.m. The OD was measured with a UV spectrophotometer (Shidmazu UV 1601, Japan) at 670 nm (Zhao et al. 2015). The CD was estimated by using the Neubauer haemocytometer (Assistent, Germany).

An aliquot of well-mixed microalgae suspension was placed on a Neubauer haemocytometer, and cells that were seen in the five centre squares were counted. The number of cells was then divided with the culture volume and dilution to obtain the original cell density in each flask. The OD and CD were only used as an indicator for upscaling. It was not used as a comparative measure for the microalga growth analysis. The biomass was determined by using 3 mL microalgae samples filtered through precombusted (60 °C, 24 h) and preweighed glass fibre filters (GF/C, Whatman, UK).The filtrates were dried at 60 °C for 24 h then removed from the oven and cooled in a desiccator and weighed. The biomass was obtained by dividing the difference between the weights of the dried filter paper (after and before filtration) by the filtered volume (Lavens and Sorgeloos 1996).

Proximate composition

Proximate composition (proteins, lipids, carbohydrates) was determined at the late log-phase of its growth for each medium. Freeze dried microalga (5–6 mg) was used for the protein and carbohydrate analysis following the method of Slocombe et al. (2013) and Dubois et al. (1956), respectively. The analysis of lipid was done as reported by Marsh and Weinstein (1966) using tripalmitin as the standard.

Pigment composition

Pigment composition (chlorophyll-a and b and carotenoids) were analysed before the samples were harvested at the end of the log-phase. A sample of 2.5 mL was taken for the analysis of chlorophyll-a and b following the method of Miazek and Ledakowicz (2013). Carotenoids were analysed using 1 mL of the sample as described by Shaish et al. (1992).

Cost estimate calculations of media preparation

The total cost of each ingredient needed to formulate 1 L of each medium was summed up.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyse the collected data. Duncan multiple range test was used with a significant difference at 0.05 level of probability. Proximate and pigment percentages were arcsine transformed before doing the statistical analysis using the Statistical Package for Social Sciences (SPSS IBM version 21.0) program.

Results

Growth

Urea 0.25, Urea 0.5 and Urea 1.0 failed to support the growth of *C. vulgaris* during the upscaling, and thus were no longer considered. Urea FM and NPKFM showed no significant difference ($P > 0.05$) of biomass compared to BBM (Fig. 1).

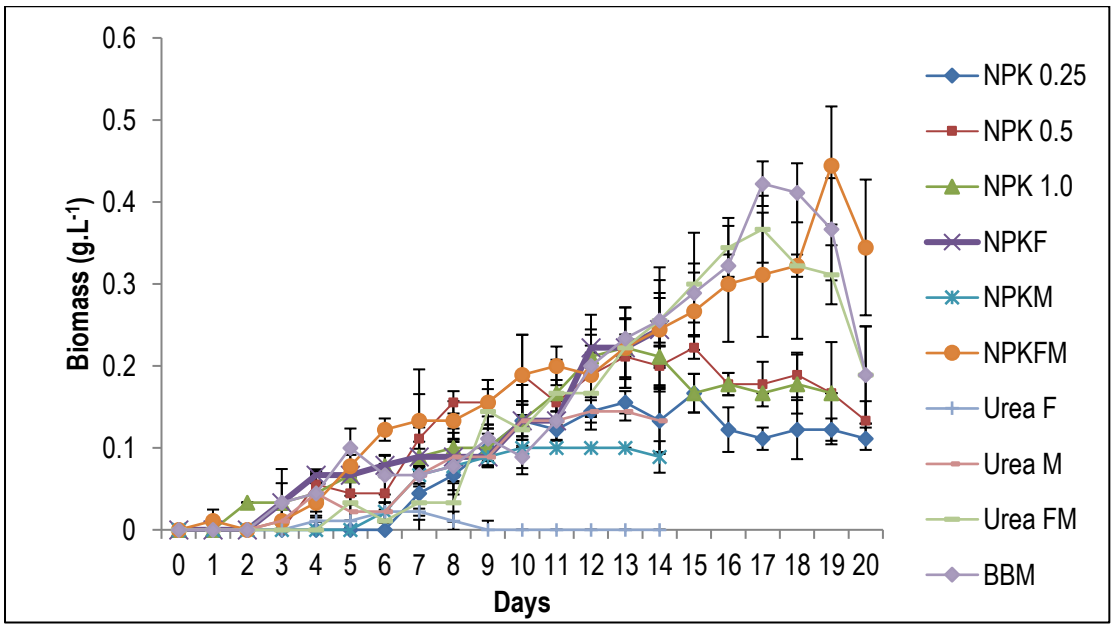

Fig. 1. Biomass of *Chlorella vulgaris* cultivated in different media. Values are mean ± standard error (n=3)

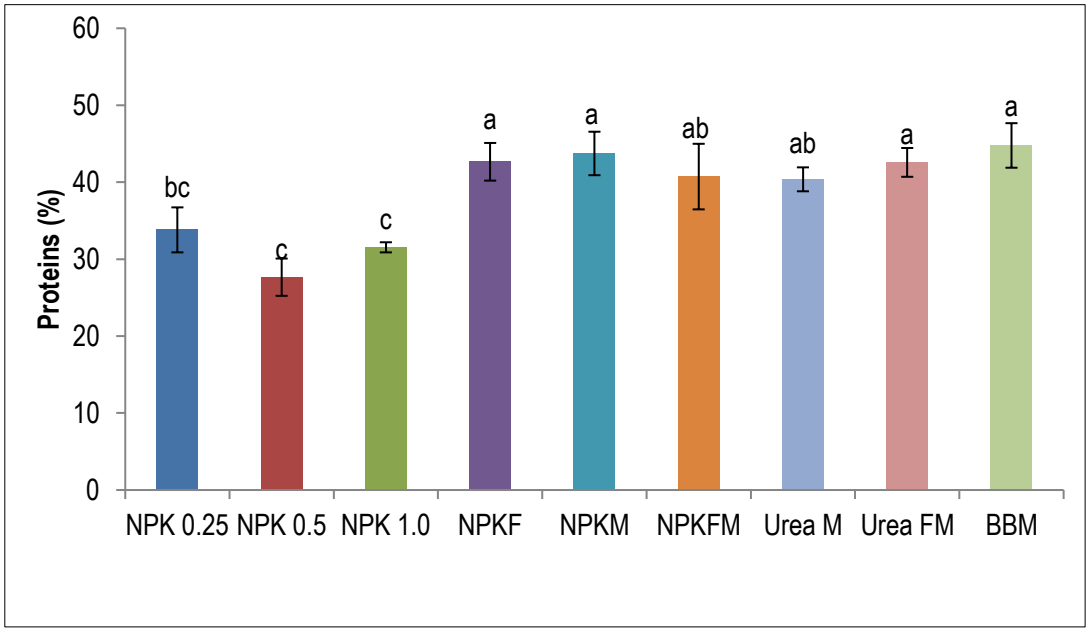

Fig. 2. Protein compositions of *Chlorella vulgaris* cultivated in different media. Values are mean ± standard error (n=3). Different letters on error bars represent significant difference (One way ANOVA, Duncan's test, $P < 0.05$)

Proximate composition

Protein compositions were similar ($P > 0.05$) for NPKF, NPKM, NPKFM, Urea M, Urea FM and BBM (Fig. 2). Figure 3 showed that BBM had the most amounts of lipids. However, among all the formulations, the highest amount of lipids (35.74 ± 1.63 %) was found in NPKFM. All formulations showed no significant difference ($P > 0.05$) of carbohydrate production to BBM (Fig. 4).

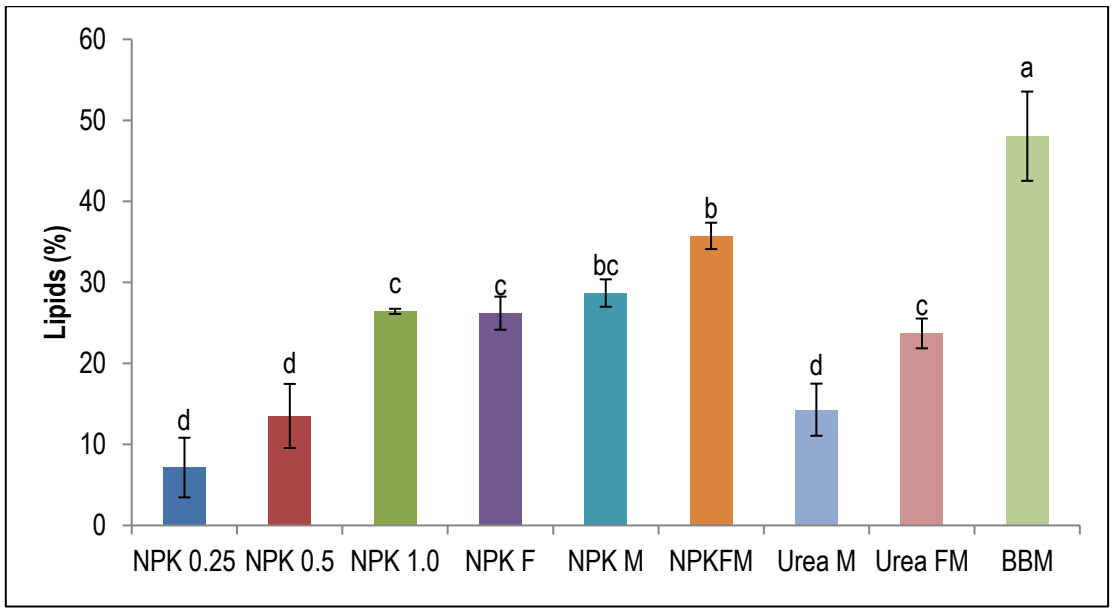

Fig. 3. Lipid compositions of *Chlorella vulgaris* cultivated in different media. Values are mean ± standard error (n=3). Different letters on error bars represent significant difference (One way ANOVA, Duncan's test, $P < 0.05$)

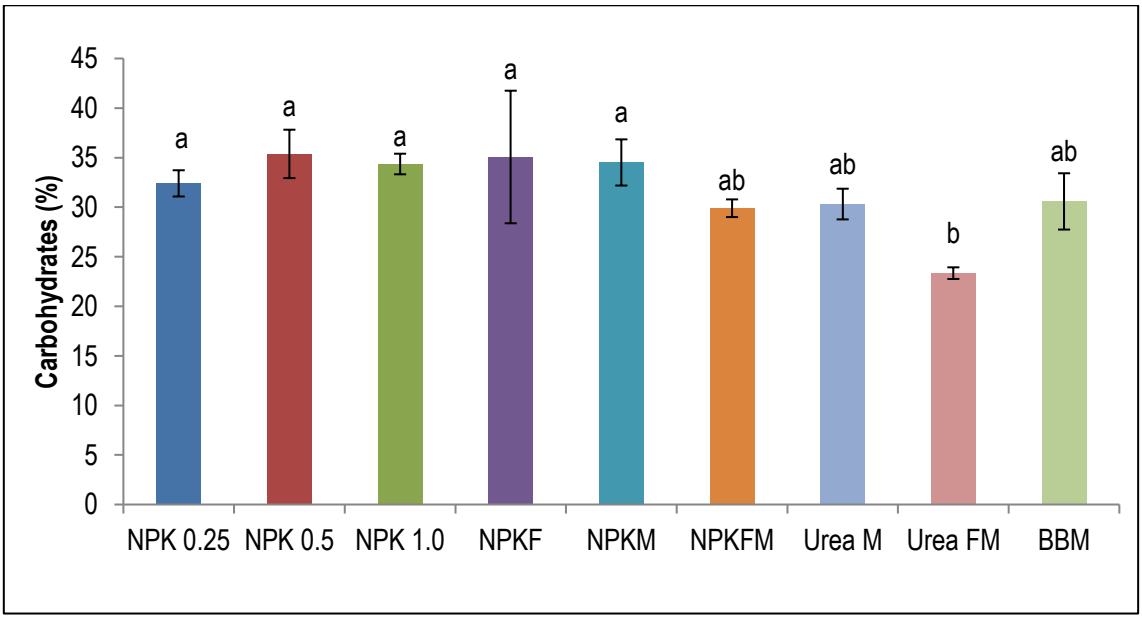

Fig. 4. Carbohydrate compositions of *Chlorella vulgaris* cultivated in different media. Values are mean ± standard error (n=3). Different letters on error bars represent significant difference (One way ANOVA, Duncan's test, $P < 0.05$)

Pigment composition

The highest significant ($P < 0.05$) producer of chlorophyll-a was in Urea FM (Fig. 5a). NPKFM produced the most chlorophyll-b (Fig. 5b); however, it was not significantly different ($P > 0.05$) from NPK 1.0, NPKF and NPKM. BBM had significantly lower ($P < 0.05$) carotenoids compared to NPK 1.0, NPKF, NPKM, NPKFM and Urea FM (Fig. 5c).

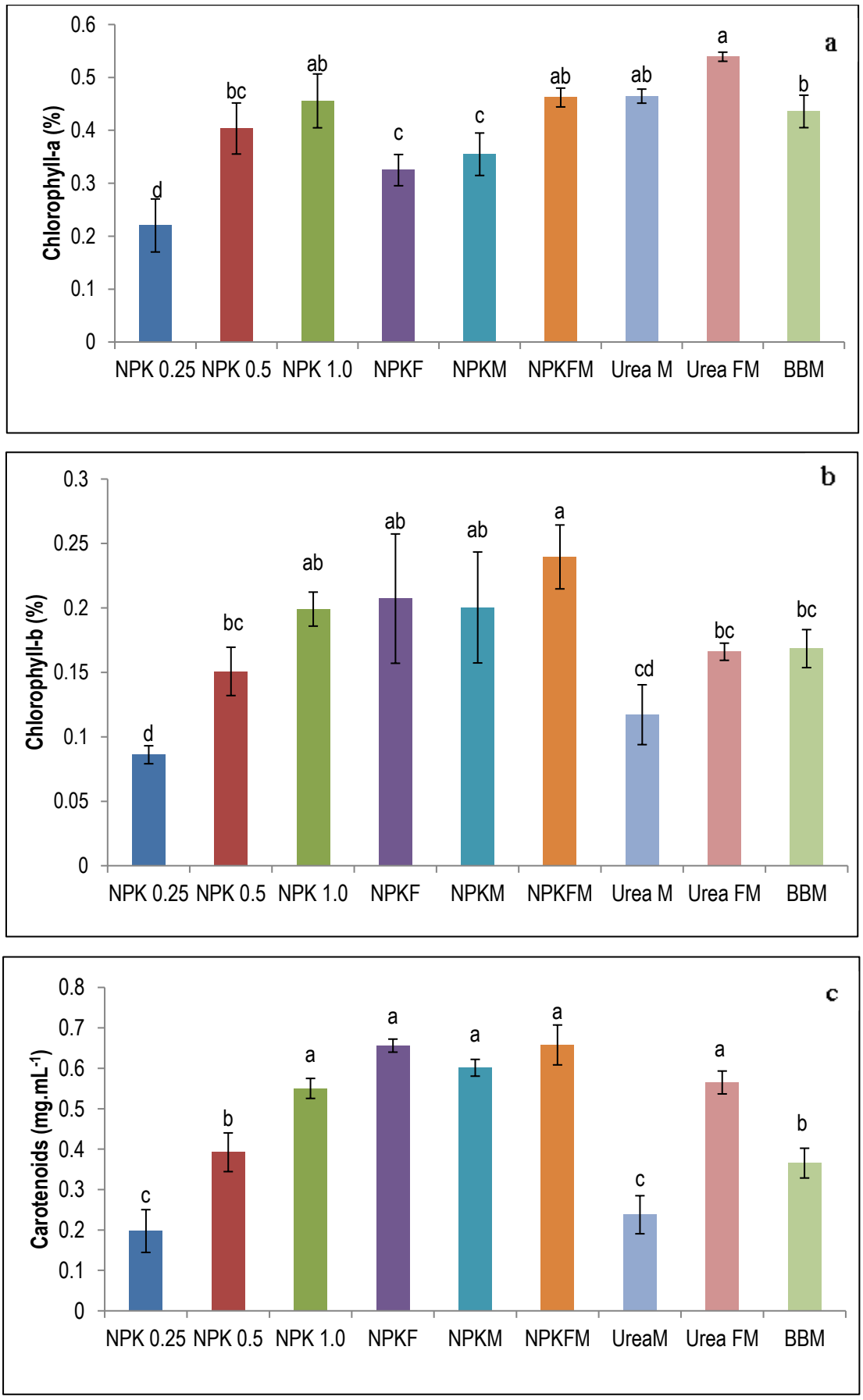

Fig. 5. Pigment compositions (a) Chlorophyll-a, (b) Chlorophyll-b and (c) Carotenoids of *Chlorella vulgaris* in different media. Values are mean ± standard error (n=3). Different letters on error bars represent significant difference (One way ANOVA, Duncan's test, $P < 0.05$)

Cost estimate of media preparation

The cost estimate for the preparation of 1 L of each medium is stated in Table 2. BBM was used as a reference to show the difference (%) in cost of each medium. NPKFM was 59.09 % cheaper than BBM.

Table 2. Comparative cost for producing 1 L of each medium

Formulations	Cost (US$)*	Cost difference compared to BBM (%)
BBM	0.055	-
NPK 0.25	0.001	-97.95
NPK 0.5	0.002	-95.95
NPK 1.0	0.004	-91.91
NPKF	0.022	-59.36
NPKM	0.011	-80.36
NPKFM	0.023	-59.09
Urea 0.25	0.003	-96.96
Urea 0.5	0.003	-93.93
Urea 1.0	0.007	-87.86
Urea F	0.041	-24.72
Urea M	0.025	-55.32
Urea FM	0.042	-22.77

* 1US$=RM 3.87 (as on 29.03.2018)

Discussion

The main objective of the current study was to formulate a cost-effective and easy to prepare medium for the cultivation of *C. vulgaris*. In the current experiment, two inorganic salts, $FeSO_4$ and $MgSO_4$ were added to the fertilisers to enhance their properties for the growth of *C. vulgaris*. The new NPKFM formulation was 59.09 % cheaper and comparable or better in certain parameters than BBM that consists of many chemicals. Another study to formulate economical medium was reported by El Nabris (2012) by combining different plant fertilisers which was 13 times cheaper compared to f/2 medium and was an excellent substitute for the cultivation of *Nannochloropsis* sp. Tuyob et al. (2007) cultured *Chlorella ellipsoidea* Gerneck 1907 successfully in factory effluent; however, the growth and production of the microalgae in the effluent was not at par or better than BBM. The present formulations, NPKFM and Urea FM, produced good amount of biomass, proteins, carbohydrates, chlorophyll-a, chlorophyll-b and carotenoids which were at par or even better than BBM, that was used as the control medium.

The growth and quality of the microalgae are influenced by the inorganic chemicals used in the medium. Mg and Fe were some of the chemicals reported to enhance the growth of microalgae (Kulshreshtha and Singh 2013). Furthermore, Mg in its salt form ($MgSO_4$ and $MgCl_2$) is important for all algal species because Mg is the central atom of the chlorophyll molecule (Desouky et al. 2011; Kulshreshtha and Singh 2013).

In addition, Fujimoto and Sakamoto (1955) discovered that the amount of Mg present in the media not only influences the synthesis of cell materials but has a great effect on cell multiplication. Small quantities of Fe in the microalgae's environment were discovered to increase the chlorophylls in each cell (Coale et al. 1996) due to Fe being directly involved in the enzymatic reactions of photosystem I (PSI) and II (PSII) (Sun et al. 2014). Furthermore, addition of Fe into the media also extends the exponential phase of the microalgae hence increasing the final cell density (Liu et al. 2008). In the present study, similar findings of maximum growth were noted in Urea FM and NPKFM containing both Mg and Fe. Among all the formulations, the growth of *C. vulgaris* in NPKFM and Urea FM were at par with BBM.

Protein, lipid and carbohydrate composition for every microalga varies according to the species (Brown et al. 1997). Protein yield in the present study was significantly higher in medium containing either Mg or Fe or both and was comparable to BBM. The amount of proteins produced was approximately 40–50 % which was similar to the yield reported in a review by Safi et al. (2014) for *C. vulgaris.*

According to Desouky et al. (2011) and Liu and Wang (2014), Mg and Fe have been proven to not only increase the growth of microalgae but also the lipid yields. Thus, when NPKFM and Urea FM consisting of both Mg and Fe were used, the production of lipids was significantly higher compared to the formulations containing one or none of these elements. The lipid production in NPKFM (35.74 ± 1.63 %) far exceeded the average amount of lipids produced by microalgae as reported by Brown et al. (1997) which should be around 7–23 %.

In the current study, every formulation including BBM managed to produce 30–35 % carbohydrate except for Urea FM. Whilst a review by Safi et al. (2014) indicated that *C. vulgaris* produces carbohydrates in the range of 12–55 %. However, there are not many reports related to the production of carbohydrates with the addition of different chemicals.

Iron and magnesium play an important role in microalgae photosynthesis. Addition of Mg is supposed to increase yield of chlorophyll in the cell (Finkle and Appleman 1953; Wang et al. 2008). However, the present study showed that for some media, addition of either Fe or Mg decreased the amount of chlorophylls produced. NPKF and NPKM produced less chlorophyll-a compared to NPK 1.0. This could be because certain elements are only beneficial to microalgae at a certain concentration; high concentrations can cause negative effects such as the impairment of photosynthetic mechanism, blockage of cell division or inhibition of enzyme activity in microalgae cells (Monteiro et al. 2012). Urea FM with the combination of both Fe and Mg produced significantly higher amounts of chlorophyll-a and b compared to Urea M. NPKFM and Urea FM seemed very promising as cost-effective media for the production of chlorophyll-a and b in *C. vulgaris.* Present study showed that formulations with either the addition of Fe or Mg or both, significantly increased ($P < 0.05$) the amount of carotenoids produced. Urea FM consisting of Fe showed higher amount of carotenoids compared to Urea M that was devoid of Fe. Cai et al. (2009) reported a relationship between Fe and carotenoid production and stated that a high concentration of $FeC_6H_5O_7$ (36 μmol.L^{-1}) produced 7-fold more carotenoids than without Fe. However, there is a lack of information on the association of Mg to carotenoid production.

In the present experiment, cultivation of *C. vulgaris* using some of the formulations showed encouraging results in terms of quality and quantity which were comparable with the control medium BBM. However, BBM was seen to perform better for lipid production. In terms of quality, Urea FM gave comparable or better results than BBM for the production of proteins, chlorophyll-a and carotenoids whereas, NPKFM for proteins, carbohydrates, chlorophyll-a, chlorophyll-b and carotenoids.

Conclusion

The high demand for microalgae biomass and the fact that media is one of the major constraints, requires investigation on the use of locally available, cost-effective ingredients for microalgae culture. In this study fewer chemicals in the formulation of NPKFM lowered the cost by 59.09 % compared to the control to mass culture *C. vulgaris*. The low-cost NPKFM made with plant fertilisers compared to conventional media showed comparable or better growth, pigment production as well as enhanced the proximate composition. The addition of only two elements, Fe and Mg, to the fertilisers was relatively easy to prepare compared to conventional media.

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References

Berges, J., D. Franklin and P. Harrison. 2001. Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the last two decades. Journal of Phycology 37:1138–1145.

Bischoff, H.W. and H.C. Bold. 1963. Phycological studies IV. Some soil algae from Enchanted Rock and related algal species. University of Texas Publication 6318:1–95.

Brown, M.R., S.W. Jeffery, J.K. Volkman and G.A. Dunstan. 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151:315–331.

Cai, M., Z. Li and A. Qi. 2009. Effects of iron electrovalence and species on growth and astaxanthin production of *Haematococcus pluvialis*. Chinese Journal of Oceanology and Limnology 27:370–375.

Coale, K.H., K.S. Johnson, S.E. Fitzwater, R.M. Gordon, S. Tanner, F.P Chavez, L. Ferioli, C. Sakamoto, P. Rogers, F. Millero, P. Steinberg, P. Nightingale, D. Cooper, W.P. Cochlan, M.R. Landry, J. Constantinou, G. Rollwagen, A. Trasvina and R. Kudela. 1996. A massive phytoplankton bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean. Nature 383:495–501.

Desouky, S.A., M.A. Usama and W.A. Ahmed, 2011. Interaction effects of magnesium and vitamins on *Chlorella vulgaris* Beijer cultures. Journal of Environmental Studies 7:1–8.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350–356.

El Nabris, K.J.A., 2012. Development of cheap and simple culture medium for the microalgae *Nannochloropsis* sp. based on agricultural grade fertilisers available in the local market of Gaza Strip (Palestine). Journal of Al Azhar University-Gaza (Natural Sciences) 14:61–76.

Finkle, B.J. and D. Appleman. 1953. The effect of magnesium concentration on chlorophyll and catalase development in *Chlorella*. Plant Physiology 28:652–663.

Fujimoto, Y. and J. Sakamoto. 1955. The influence of magnesium deficiency on the growth of *Chlorella*. Bulletin of the Chemical Society of Japan 19:253–257.

Hemaiswarya, S., R. Raja, R. Ravi Kumar, V. Ganesan and C. Anbazhagan. 2011. Microalgae: a sustainable feed source for aquaculture. World Journal of Microbiology and Biotechnology 27:1737–1746.

Kaladharan, P., R. Gireesh and K.S. Smitha. 2002. Cost effective medium for the laboratory culture of live feed micro algae. Seaweed Research and Utilisation 24:35–40.

Kulshreshtha, J. and G.P. Singh. 2013. Evaluation of various inorganic media for growth and biopigments of *Dunaliella salina*. International Journal of Pharma and Bio Sciences 4:1083–1089.

Lavens, P. and P. Sorgeloos. 1996. Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper. No. 361. FAO, Rome. 295 pp.

Liu, Z. and G. Wang. 2014. Effect of Fe^{3+} on the growth and lipid content of *Isochrysis galbana*. Chinese Journal of Oceanology and Limnology 32:47–53.

Liu, Z., G. Wang and B. Zhou. 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technology 99:4717–4722.

Marsh, J.B. and D.B. Weinstein. 1966. Simple charring method for determination of lipids. Journal of Lipid Research 7:574–576.

Miao, X. and Q. Wu. 2006. Biodiesel production from heterotrophic microalgal oil. Bioresource Technology 97:841–846.

Miazek, K. and S. Ledakowicz. 2013. Chlorophyll extraction from leaves, needles and microalgae: A kinetic approach. International Journal of Agricultural and Biological Engineering 6:107–115.

Monteiro, C.M., P.M.L. Castro and F.X. Malcata. 2012. Metal uptake by microalgae: Underlying mechanisms and practical applications. Biotechnology Progress 28:299–311.

Ötleş, S. and R. Pire. 2001. Fatty acid composition of *Chlorella* and *Spirulina* microalgae species. Journal of AOAC International 84:1708–1714.

Queiroz, M.L.S., A.P.O. Rodrigues, C. Bincoletto, C.A.V. Figueirêdo and S. Malacrida. 2003. Protective effects of *Chlorella vulgaris* in lead-exposed mice infected with *Listeria monocytogenes*. International Immunopharmacology 3:889–900.

Safi, C., B. Zebib, O. Merah, P-.Y. Pontalier and C. Vaca-Garcia. 2014. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. Renewable and Sustainable Energy Reviews 35:265–278.

Shaish, A., A. Ben-Amotz and M. Avron. 1992. Biosynthesis of β-carotene in *Dunaliella.* Methods in Enzymology 213:439–444.

Shibata, S., Y. Natori, N.T. Ishihara, K. Tomisaka, K. Matsumoto, H. Sansawa and V.C. Nguyen. 2003. Antioxidant and anti-cataract effects of *Chlorella* on rats with streptozotocin induced diabetes. Journal of Nutritional Science and Vitaminology (Tokyo) 49:334–339.

Slocombe, S.P., M. Ross, N. Thomas, S. McNeill and M.S. Stanley. 2013. A rapid and general method for measurement of protein in micro-algal biomass. Bioresource Technology 129:51–57.

Sun, X., Y. Cao, H. Xu, Y. Liu, J. Sun, D. Qiao and Y. Cao. 2014. Effect of nitrogen starvation, light intensity and iron on triacylglyceride/carbohydrate production and fatty acid profile of *Neochloris oleoabundans* HK-129 by a two-stage process. Bioresource Technology 155:204–212.

Tanaka, K., T. Koga, F. Konishi, M. Nakamura, M. Mitsuyama, K. Himeno and K. Nomoto. 1986. Augmentation of host defense by a unicellular green alga, *Chlorella vulgaris*, to *Escherichia coli* infection. Infection and Immunity. 53:267–271.

Tuyob, M.A, S.R. Ahmed, M.I. Miah and M.A.B. Habib. 2007. Growth performance and nutritional value of *Chlorella ellipsoidea* in fertilizer factory effluent media. Asian Fisheries Science 20:65–79.

Wang, B., Y. Li, N. Wu and C.Q. Lan. 2008. CO_2 bio-mitigation using microalgae. Applied Microbiology and Biotechnology 79:707–718.

Wang, W., F. Han, Y. Li, Y. Wu, J. Wang, R. Pan and G. Shen. 2014. Medium screening and optimization for photoautotrophic culture of *Chlorella pyrenoidosa* with high lipid productivity indoors and outdoors. Bioresource Technology 170:395–403.

Xin, L., H. Hong-ying, G. Ke, S. Ying-xue. 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology 101:5494–5500.

Zhao, Z., J. Pan, L.Wu, W. Zhang, J. Xu, Y. Chen and Z. Fu. 2015. Determination of the concentration of *Chlorella vulgaris* Beij by visible spectrophotometer. Agricultural Biotechnology 4:54–55, 58.

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