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Development of Cheap and Simple Culture Medium for the Microalgae *Nannochloropsis* sp. Based on Agricultural Grade Fertilizers Available in the Local Market of Gaza Strip (Palestine).

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Abstract:
The unicellular marine microalgae *Nannochloropsis* sp. is one of the most common live feed used in the field of aquaculture. The high cost of the culture medium which supports an optimal growth of the algae however is still one of the main problems related to the large scale culture of *Nannochloropsis* sp. In this study, an attempt was made to develop a cheap and simple medium for *Nannochloropsis* sp. based on agricultural grade fertilizers available in the local market of Gaza Strip. Five different culture media, "A", "B", "C", "D", and "E" were tested and compared to F/2 medium which is commonly used for microalgae cultivation in commercial aquaculture. Medium "A" which consists of a combination of agricultural fertilizers such as ammonium sulfate (150 mg^{-l}), urea (7.5 mg^{-l}), calcium superphospate (25 mg^{-l}), micronutrient solution (0.5 mg^{-l}) and vitamin solution (0.5 ml^{-l}) resulted in maximum average cell density of 69 $\times 10^6$ ml^{-1}. Medium "A" was also found to be highly economical, since it is about thirteen times cheaper than F/2 medium. The results of the present study suggest that agricultural fertilizers are an excellent substitute that can be used for the cultivation of microalgae.

Key words: *Nannochloropsis* sp.; cost; culture medium; agricultural fertilizer; Gaza Strip

1. INTRODUCTION

Nannochloropsis sp. is a unicellular, non motile, of about 2–5 μm cell size, golden green algae belonging to the class *Eustigmatophyceae* (Rodolfi *et al.,* 2003). This microalga is one of the most interesting phytoplankton in the field of marine biotechnology because it represents a valuable source of various natural products which have

several applications. In food industry, it is well known as a source of different valuable compounds such as vitamin E (Durmaz, 2007) and pigments; chlorophyll a, zeaxanthin, canthaxanthin and astaxanthin (Lubián *et al.,* 2000). Due to their high oil content, *Nannochloropsis* sp. was used as raw materials for biodiesel production (Luisa & Oliveira, 2009).

Furthermore, because of its high nutritional value related to the biochemical composition of essential polyunsaturated fatty acid (PUFA) such as eicosapentaenoic acid (EPA, C20:5n3) (Sukenik, 1999), it is commonly cultivated in hatcheries of marine finned fish and crustaceans. In those hatcheries, they are consumed by zooplankton e.g. rotifers (Witt *et al.,* 1981; Yúfera & Lubián, 1990), which in turn, pass these fatty acids to the newly hatched marine fish larvae. They are also used to create a ‘‘green-water effect’’ in fish larvae tanks (Fulks & Main, 1991; Lubzens *et al.,* 1995).

In recent years there was an increase interest in fish farming in Gaza Strip, both at the governmental (ministry of agriculture) and private sector levels. This interest aimed at achieving self-sufficiency in fish production, supplementing capture fishery production, and substituting the deficiency of quantities of fish sold in local markets of Gaza Strip. This is especially true after the last war on Gaza (***2008-2009***) where fishing became, in addition to the limited fishing zone (less than 3 nautical miles off the shore of Gaza Strip), a real threat to fishermen lives. Fishermen find themselves frequently subjected to arrests, seizing of boats, and shootings from the occupation navy force (IRIN, 2010).

The fish farming industry in Gaza Strip was started in 1999 with the introduction of two fresh-water fish species; Nile tilapia (*Oreochromis niloticus*) and hybrid red tilapia (*Oreochromis mossambicus* x *Oreochromis niloticus*) by the Ministry of Agriculture, after the establishment of the Palestinian Authority in 1994. In recent years, two marine fish species, the gilthead seabream (*Sparus aurata*) and the seabass (*Dicentrarchus labrax*) have been introduced to fish farming industry in Gaza Strip. Fish farmers depend mainly on purchasing the fingerlings from Egypt and rearing them in intensive, land-based seawater ponds to commercially accepted size. Some trials

were made to locally produce such fingerlings, but they mostly failed because of a variety of reasons including the fact that the staff involved had little experience or expertise in the field of aquaculture (Kennelly, 2005).

Palestinian fish farmers lack the experience to cultivate the microalgae such as *Nannochloropsis* sp., which is necessary for marine fish rearing, mainly during the first days of life when phytoplankton and zooplankton are the basic food source.
Another challenge facing fish farmers is the extremely high production costs of microalgae through existing formulation of conventional culture media, e.g. F/2 (Guillard, 1975). This is especially true upon large scale cultivation of microalgae. The preparation of the growth medium represents a considerable part of running cost in the process of fish culture (Zhang *et al.,* 2001). It was reported that 30-40% (max.70%) of marine hatchery operating costs can be attributed to micro-algal culture (Heasman *et al.,* 2001).

Media for the culture of marine algae generally consist of a seawater base (natural or artificial) supplemented by analytical quality nutrients (Bold & Wynne, 1985). The term 'nutrient' is generally applied to any element or compound necessary for algal growth. Analytical grade nutrients chemicals (macronutrients and micronutrients) represent the most expensive constituents in any culture medium (Molina *et al.,* 2003).

Furthermore, preparation of algal culture media is labor-intensive. Media are generally prepared from premixed stock solutions. Aliquots from these stocks are measured and added to a given volume of seawater. Micronutrient stock solution, however, must be prepared by combining aliquots of pre-prepared stock solutions and adding them to a given volume of distilled water, from which aliquots are measured and added to seawater, which may complicate the preparation processes. Precipitation of one or more component of the medium, may be encountered upon inaccurate preparation or combining the stock solutions of the culture medium (Harrison & Berges, 2005).

During the last decades, several ***studies were undertaken to develop*** various formulations of ***media for*** cultivation of ***different*** microalgal

species by replacing the analytical grade chemicals by agricultural fertilizers as nutrients (Fábregas *et al.,* 1987; Corsini & Karydis, 1990; López Ruiz *et al.,* 1995; Coutteau, 1996; Valenzuela-Espinoza *et al.,* 1999; Simental & Sánchez-Saavedra, 2003; Kanlis *et al.,* 2004). In addition to saving money upon large-scale cultivation of microalgae, media based on agricultural fertilizers are simple to prepare. Additionally, the nutritional value of algae produced is high and comparable to that of conventional, analytical grade fertilizers such as F/2 (Valenzuela-Espinoza *et al.,* 1999).

Accordingly, the aim of the present study is to formulate a cheap and simple medium, based on agricultural grade fertilizers available in the local market of Gaza Strip for cultivation of *Nannochloropsis* sp.

2. MATERIALS AND METHODS

2.1. Chemicals

The components of F/2 (Table1) medium were of analytical grade chemicals. Other chemicals (Table 2) were of agricultural grade fertilizers, they were purchased from local shops for agricultural equipments. The Multimicro fluid (AGLUKON, Heerdter Landstr. 199, D-40549 Dusseldorf-Germany) is liquid fertilizers consists of 8 micronutrients elements including copper, manganese, magnesium, iron, sulfur, boron, zinc, and molybdenum (Table 3). Its component is similar to the trace metal solution of F/2 medium. Accordingly, it is used in this study as a cheap and already prepared alternative to trace metal solution suggested by F/2 medium.

2.2. Seawater

All experiments were performed using aged Mediterranean seawater. It was collected from the coast of Gaza and filtered with Millipore (0.45 μm) filter paper. The salinity was adjusted by distilled water to 25 ‰ (Part per thousand) and kept in dark in tightly closed 10- liter glass bottles until use.

2.3. Micro-Algal Species

Nannochloropsis sp. was obtained from local fish farm at Dier Al-balah city, Gaza Strip. They maintained in our laboratory in sea water

at 25 ‰ salinity and enriched by F/2 medium (Table 1) for marine algae (Guillard, 1975). Except vitamin and urea which were sterilized by filtration through 0.22 µ filter, all solutions were autoclaved at 121 °C for 20 min. A stock culture of *Nannochloropsis* sp. was cultured in 0.5 l Erlenmeyer flasks containing 250 mL of medium. Stocks were kept in exponential growth phase by transferring them to fresh medium every week. The culture was kept at room temperature (spring time), and illuminated with fluorescent lamps with photoperiod 24:0 h (L:D).

Table 1: Composition of Guillard's F/2 media (Guillard, 1975).

Nutrient	**Concentration (mg. l^{-1})**
N (as $NaNO_3$)	12.353
P (as $NaH_2P_4.H_2O$)	1.125
Fe	0.6538
Zn	0.004481
Mn	0.04940
Mo	0.002485
Co	0.002972
Cu	0.002547
Vitamin solution	
Thiamine HCL	0.1000
Biotin	0.0005
Cyanocobalamin	0.0005

2.4. Growth experiments

Stock solutions of the nutrients for the different media (Table 2) were prepared in distilled water and sterilized separately.

The seawater used for preparation of the media was autoclaved at 120 °C for 20 min and then dispensed into 250-ml sterilized Erlenmeyer flasks, 100 ml each. The nutrients were aseptically added and the pH of the different media was adjusted to 8.

The flasks were supplemented with 0.5 ml/L of the micronutrient solution (Multimicro Fluid) and 0.5 ml/L of vitamin solution. The standard F/2 medium was used as reference medium in the present study. A total of three flasks were prepared for each medium.

Each flask was inoculated by 1 ml from exponentially phased culture having 19 $\times 10^6$ cells/ml. The flasks were closed with cotton enclosed by aluminum foil and arranged on cultivation shelf.

Table 2: Nutrient composition of the different culture media: A, B, C, D, and E used for culturing of marine alga *Nannochloropsis* sp. (Coutteau, 1996).

Fertilizers		Concentration (mg. l^{-1})				
	Local name	**A**	**B**	**C**	**D**	**E**
Ammonium Sulfate	أمونيات	150	100	300	-	-
Urea	يوريا	7.5	5	-	-	13
Calcium Superphosphate	سوبر	25	15	50	-	-
N:P:K* 16-20-20	16-20-20	-	-	-	13	-
N:P:K 14-14-14	14-14-14	-	-	-	-	30

***** Nitrogen: Phosphorus: Potassium

The concentrations and the composition of the different media are essentially based on previously published report (Coutteau, 1996) with some modifications.

Specifically, all culture media were supplemented with vitamin solution and Multimicro fluid solution. This is in contrast to media prescribed by Coutteau, (1996) who didn't indicate any supplementation of vitamin solution. Moreover, instead of the Multimicro fluid, the aforementioned author reported about the addition of Clewat 32 (5mg/l) to medium "B" only. Clewat 32 is a commercial name given to a formulation consists of trace metal mix powder (FAO, 1982). With some exceptions, its chemical composition is similar to that of Multimicro fluid solution. The detailed metal composition and concentration of both metal mixes are presented in Table 3.

Table 3: Metal composition and concentrations of the trace metal mixes.

Metal	Concentration	
	Multimicro fluid	**Clewat 32**

	(%)	(contents in 1 kg)
Iron	1.1	3.8 g
Manganese	1.5	7.7 g
Copper	0.5	0.07 g
Molybdenum	0.01	6.3 g
Boron	0.3	24.7 g
Cobalt	-	0.17 g
EDTA	-	a proper quantity
Zinc	1.1	-
Sulfur	5.3	-

2.5. Growth conditions

The flasks were arranged on a lighted shelf in a manner so that all flasks are received the same amount of light. Illumination was provided by four 36-watt daylight fluorescent tubes suspended at 20 cm distance above the flasks. All cultures were kept at room temperature and continuous illumination. The experiment was extended for 16 days during which the flasks were manually shaken 1-3 times every day.

2.6. Determination of Cell Density

Subsamples of 1-ml were drawn almost daily from each culture medium without replacement and transferred to screw capped tube to which a few drops of lugol's solution was added in order to fix and stain the cells. The sample was shaken vigorously and counted using an improved Neubauer hemocytometer (BOECO, Germany) under binocular compound microscope at 200–400× magnification. Each time, the counting procedure from the homogenized sample was repeated six times.

2.7. Data Analysis

Cell densities were expressed as the average number of cell.ml^{-1} ± standard deviation. Growth curves for each culture media was prepared by plotting the average cell density *vs* corresponding cultivation time. Curves were prepared by using EXCEL computer program. Average maximum cell densities were compared using one-way analysis of variance (ANOVA). The significance of the results was determined at $\alpha = 0.05$.

3. RESULTS

The effects of five different media on the growth of *Nannochloropsis* sp. were simultaneously investigated. To avoid variations, the experiments were performed under the same growth conditions of temperature, light and salinity.

Growth curves for the cultures of *Nannochloropsis* sp. in the different culture media are illustrated in Figure 1. The algae generally proliferated in a characteristic pattern consisting of lag, exponential, stationary and declining phases. The growth rate was noted for 16 days, by which time the algae were found to have entered into declining and death phase in most media tested.

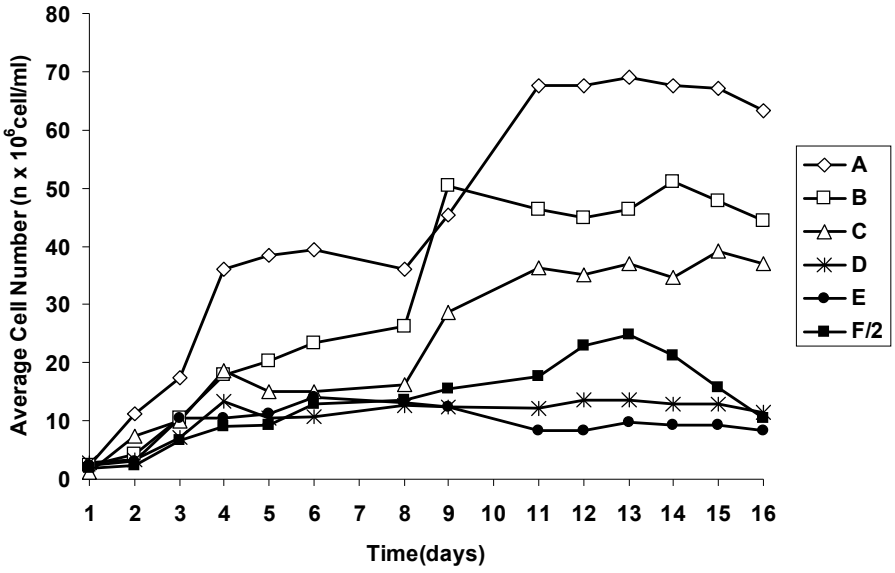

Fig. 1: Growth curves for *Nannochloropsis* sp. in A, B, C, D and E culture media in comparison with "F/2" medium.

Results represented in Figure1 indicated great variations in the algal density in the different culture media. In all culture media, the maximum cell density was attained between 13th and15th days of cultivation. While cells in media "A", F/2, "D" and "E" reached their maximum densities by day 13, the maximum densities in "B" (51 ×

10^6 cell/ml) and "C" (39×10^6 cell/ml) media were attained on the 14th and 15th days of cultivation respectively.

Medium "A" had the highest cell count compared to F/2 and other media, reaching approximately 69×10^6 cell/ml by day 13 (Table 4). At the same period of cultivation, medium "A" was followed by medium "B" (46×10^6 cell/ml), then medium "C" (36×10^6 cell/ml), then F/2 medium (25×10^6 cell/ml), followed by "D" (14×10^6 cell/ml) and finally "E" (10×10^6 cell/ml).

Table 4: Summary of maximum cell density in different media after 13 days of cultivation.

Medium	Cell density mean ± S.D. (Cells × 10^6 /ml)
A	$69^{a*} \pm 8.6$
B	$46^{b} \pm 1.4$
C	$36^{bc} \pm 7.7$
F/2	$25^{cd} \pm 2.6$
D	$14^{cd} \pm 1.9$
E	$10^{d} \pm 3.0$

* Different letters indicate significant ($\alpha = 0.05$) differences (one-way ANOVA)

When compared statistically to the significance level of 5% ($P<0.05$), significant differences were found between medium "A" and F/2 medium. These differences were also registered between medium "B" and F/2 medium (Table 4).

The cost estimate for preparing 1000 L ($1m^3$) of conventional F/2 and other culture media based on the local prices is given in Table 5. Preparation of 1000 L of culture medium "A" costs 1.60 NIS. In comparison, production of 1000 l of F/2 medium involves a cost of 20.76 NIS. Hence, medium "A" was found to be 12.92 times cheaper than the conventional medium. The prices of other media were almost as the same as "A" medium. However, after taking into account the high cell density achieved in this medium, medium "A" can be considered as cheap alternative to F/2 medium

Table 5: Comparative costing for producing 1000 L of conventional (F/2 medium) and other five non-conventional media

Culture media	Cost in NIS*	Net difference in cost between F/2 and other media

		(in ratio)
F/2	20.76	
A	1.60	1 : 12.92
B	1.51	1 : 13.70
C	1.86	1 : 11.19
D	1.37	1 : 15.10
E	1.45	1 : 14.35

*: New Sheqalim

4. DISCUSSION

The results of the present study indicated great variation in cell density of *Nannochloropsis* sp. in the different media. Since nitrogen is a major nutrient for microalgal cultivation and marine environment is nitrogen limited, this difference could be attributed to availability and variability of nitrogen sources in these media.

Although the components of medium "A" and "B" were the same (but with different concentration), the maximum cell density in "A" was 1.35 times higher than "B", this is because medium "A" has higher nutrients concentrations than "B".

The enhancement of algal density by nutrients concentrations observed in this study is consistent with results reported for *Dunaliella* sp, in which a positive correlation was found between nutrient concentration and cell density, with a high cell density with richer medium (Becerra-Dórame *et al.,* 2010).

Similar results were also obtained with *Nannochloropsis gaditana* where addition of considerably extra concentrations of nitrogen sources to F/2 medium led to increase the growth rate of this microalgae (Rocha *et al.,* 2003). Similarly, **Converti *et al.***, (2009) observed a gradual increase in the growth rate of *Nannochloropsis oculata* as a result of increase of $NaNO_3$ concentration from 0.075 to 0.150 to 0.300 g/l.

The growth of *Nannochloropsis* sp. in medium "A" and "B" was significantly higher than F/2 media, this may be because the nitrogen sources in the former media were $NH4^+$ (as ammonium sulfate) and urea while that of the later medium was NO_3.

The superiority of ammonium over other forms of nitrogen sources such as nitrate was previously reviewed (Dortch, 1990) and attributed

to its reduced form (Wheeler, 1983) i.e. it does not have to be reduced prior to amino acid synthesis. The favorable effect of ammonium on algal growth was documented and confirmed with different algal species. Ammonium was found to be consumed by *Isochrysis* aff. *Galbana* eight times faster than nitrate when they added together in the culture medium and that nitrate uptake ceased in the presence of ammonium due to the inactive nitrate reductase when the ammonium concentration is present as the main source of nitrogen (Valenzuela-Espinoza *et al.,* 1999). ***It was also observed that,*** the presence of ammonium in the culture media would prevent the uptake of nitrate by *Navicula ostrearia* , *Nitzschia ovalis*, and *Amphora coffeaeformis* (Maestrini *et al.,* 1986).

Although the concentration of Ammonium Sulfate in "C" is double than that of "A" medium, the results indicated higher cell density in "A" rather than "C". This may be attributed to the presence of urea as additional nitrogen source in "A" but not in "C".

The positive effect of urea over different nitrogen sources other than ammonia such as KNO_3, $NaNO_3$, $Ca(NO_3)_2$-$4H_2O$ and glycine on the growth rate of *Nannochloropsis gaditana* was confirmed (Rocha *et al.,* 2003). Another interpretation of the finding could be also attributed to the very high concentration of ammonium sulfate itself.
In aqueous solution, ammonium ion ($NH4^+$) exists in equilibrium with the toxic, un-ionised or free ammonia (NH_3) according to the dissociation equation

$$NH_3 + H_2O \leftrightarrows NH^+_4 + OH^-$$

Because of the equilibrium, increasing one of them automatically increases the other. Furthermore, this equilibrium mainly depends on pH. In the present study, no pH control was maintained during the experiment, thus as the algal density increases the pH in the medium will increase due to carbon dioxide assimilation. These conditions will force the reaction to move to the left, thus increasing the concentration of ammonia (NH_3) which has a toxic effect on alga growth and consequently, decreasing the algal density in C medium.

Some results achieved in this study showed limited growth of *Nannochloropsis* sp. especially when it cultivated in media based

essentially on NPK fertilizers such as media "D" (14 x 10^6 cell/ml) and "E" (10.0 x 10^6 cell/ml). These results are consistent with previous study of **Kanlis *et al*.,** (2004) who indicated that, the components of the NPK fertilizers are mostly suitable for crop (land) agriculture and provide only the barest essentials for the growth of algae.

The present study indicated a superiority of agricultural grade fertilizer in term of money saving over conventional F/2 medium. Similar results has been documented previously by **Salas *et al*.,** (1992) who reported a 98% saving by using agricultural fertilizers compared to the F/2 culture medium. Other authors have indicated that their agricultural fertilizer media were eight times cheaper than the F/2 medium (Valenzuela-Espinoza *et al.,* 1999; Simental & Sánchez-Saavedra, 2003).
In addition to saving money, this culture medium could be easily prepared, as their components such as the micronutrient solution (Multimicro fluid) which was used as an alternative to trace metal solution of F/2 medium is already prepared and thus it is ready to use.

The results of the present study agree with most of the literature concerning the use of agricultural fertilizers on the fact that the commercial fertilizer formulations can be as effective as analytical grade reagent for algal cultivation. It is well known however, that the composition of culture medium not only affects the cell productivity, but also affects cell composition and yield of specific products (Lourenço *et al.,* 1997; Sánchez *et al.,* 2000; Imamoglu *et al.,* 2007). Analysis of cell composition of the microalgae however was beyond the scope of the present study which focused only on cell density (quantity) but not on cell composition (quality).

In conclusion, of all culture media tested, best growth of *Nannochloropsis* sp. was achieved in medium "A", since the cell densities attained in this medium was greater than those recorded in any other culture medium. This may indicate that such recipe which consists of a combination of agricultural fertilizers such as urea, calcium superphospate, ammonium sulfate, micronutrient and vitamin solutions strongly support the growth of *Nannochloropsis* sp. and

confirm that using agricultural grade fertilizers can substitute the F/2 media which commonly used for culture in commercial aquaculture.
Finally, the field of fish farming in Gaza Strip is a relatively new, accordingly there is no escape from going into basic research in order to understand and solve practical issues. It is also important that, the research in this field has to cover all technological aspects from the test tube to the commercial scale cultivation, whenever possible.

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