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Effect of different culture media on the growth and lipids of the green microalgae, *Scenedesmus obliquus* and *Micractinium reisseri* as a feedstock for biodiesel production

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Abstract : The aim of this study was to investigate the growth, lipid content and lipid productivity of the biodiesel promising microalgae *Scenedesmus obliquus* and *Micractinium reisseri* grown in batch culture using four different freshwater growth media, namely Chu-10, KC, Flory and Kuhl's medium to identify the most suitable medium for high lipid production for each species. The Results confirmed that Kuhl and KC showed the highest biomass productivity for *S. obliquus* and *M. reisseri* (0.05 and 0.07g L^{-1} d^{-1}, respectively). The Lipid content and lipid productivity were estimated at four different growth phases (early, middle, late exponential and stationary phase).The results revealed that, *M. reisseri* showed the highest lipid productivity at stationary phase (17.08 mg $L^{-1}d^{-1}$), while *S. obliquus* showed lipid productivity of 12.61 mg $L^{-1}d^{-1}$ which was insignificant with that at the late exponential phase (12.55 mg $L^{-1}d^{-1}$). The fatty acids profile of *S. obliquus* and *M. reisseri* at stationary growth phase showed that both of them were suitable as feedstock for biodiesel production because of their high saturated fatty acids content that represented by 81.3٤% and 72.51% of the total fatty content, respectively. Palmitic acid (C16:0) was represented by 15.74 and 16.06 % in the two studied species, respectively that increase the biodiesel quality for each strain. This study suggests *M. reisseri* cultivated on KC medium to provide high lipid productivity which offers a promise to be one of the sources of biodiesel.

Keywords: microalgae, Lipid productivity, growth media, *Scenedesmus obliquus*, *Micractinium reisseri*

Introduction:

In recent decades, the world has been faced an energy crisis, associated with irreversible depletion of traditional sources of fossil fuels which contribute to about 80% of global energy demand at present. However, their use as major form of energy is indeed unsustainable, further the accumulation of greenhouse gases in the atmosphere brings about global warming and causes global climate change, environmental pollution, and health problems (Singh and Gu, 2010; Chen et al., 2011). Therefore, there is a rapid need for development of new, clean, and sustainable energy sources. Among the various potential Sources of renewable energy, biofuels are of most interest, and expected to play a crucial role in the global energy infrastructure in the future (Chisti, 2007; Lo et al., 2010). Biofuels (bioethanol and biodiesel) are currently receiving much attention due to it is made from non-toxic, biodegradable and renewable resources. This also provides environmental benefits, which gives rise to decrease in harmful emissions of carbon monoxide, hydrocarbons and decrease in greenhouse effect (Campbell et al., 2011). The use of biofuels can also play an important role in avoiding the excessive dependence on fossil fuels due to high cost of petroleum and to improve the environmental sustainability (Gouveia and Oliverira, 2009). Mostly biodiesel is currently produced from animal fats, waste cooking oils and vegetable oils (Barnwal and Sharma, 2005). Use of agriculture crops, especially edible oil crops such as corn, as a feedstock for biofuel has raised concerns of food security due to the competition with arable lands that is pushing the price of edible oil to unaffordable levels (Batten and O'Connell, 2007; Mata et al., 2010).

Therefore, Microalgae were discussed to be the promising source for biodiesel due to their simple cellular structure, higher growth rate and higher lipid content than terrestrial plants, since most of microalgae can complete an entire growing cycle every few days, which results in higher oil productivity than other oil crops (Stephens et al., 2010). The yield of oil from algae is over 200 times the yield of soybean oils (Gouveia and Oliverira, 2009). Moreover, microalgae can also be grown on non-arable lands (e.g. desert, seashore, rocky and sandy lands) which do not compete with food crops and can use saline water.

Biodiesel is produced by transesterification of oils through the reaction of triacylglycerides (TAGs) with simple alcohols leading to formation of a chemical compound known as fatty acid methyl esters (FAMEs), which is known more generically as biodiesel (Fukuda et al., 2001). Successful algal biodiesel production mainly depends on choosing the good species with relevant biomass and lipid productivity. The freshwater chlorophytes *Scenedesmus obliquus* and *Micractinium reisseri* can grow in wastewaters of different origins showing good adaptation ability (Hodaifa et al., 2008; 2009). In addition, they are of the best candidates for biodiesel production among several microalgae species because of their high biomass production (Gouveia and Oliveira, 2009; Ruiz et al., 2013). Abomohra et al. (2013) screened 13 freshwater microalgae as a feedstock for biodiesel. They concluded that *Scenedesmus obliquus* was selected as a promising microalga for large-scale lipid production because of its high biomass production which resulted in high lipid and fatty acid productivities. In addition, Eldalatony (2013) screened the efficiency of wastewater isolated microalgae for biodiesel production and nominated *Micractinium reisseri* as the promising species. The present work was intended to compare the growth of both microalgae on different growth media in order to select the suitable medium and harvest time for each species.

Material and Methods:

Algal strains and growth media:

Scenedesmus obliquus (SAG276-10) was obtained from Culture Collection of Algae at Gottingen University, Germany. *Micractinium reisseri* (JN169781) isolated from agriculture drainage mixed with municipal wastewater at El-Gharbya Governorate, Egypt (Abou-Shanab et al., 2014). Four different photoautotrophic media were tested, namely KC (Kessler and Czygan, 1970), Kuhl (Kuhl and Lorenzen, 1964), Chu-10 (Stein, 1973) and 2 g L^{-1} Flory Basis Fertilizer 1 (Euflor, Germany) and 810 mg L^{-1} KNO_3; pH: 7.0 (Table 1).

Table 1. Composition of different media used for the cultivation of *Scenedesmus obliquus* and *Micractinium reisseri*.

Chemical compounds	Concentration (mg L^{-1})			
	KC	Kuhl	Chu-10	Flory
KNO_3	810	1011.1	-	-
NaCl	470	-	-	-
$NaH_2PO_4.H_2O$	470	621	-	-
$Na_2HPO_4.2H_2O$	360	89	-	-
$MgSO_4.7H_2O$	250	246.5	25	-
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.2	0.0125	-	-
$CaCl_2.2H_2O$	15	14.7	-	-
$ZnSO_4.7H_2O$	0.2	0.287	0.222	-
H_3BO_3	0.5	0.061	2.86	-
$MnCl_2.4H_2O$	0.5	-	1.81	-
NH_4NO_3	-	-	-	810
EDTA	8	-	-	-
Na- EDTA	-	-	1	-
$NaMoO_4.5H_2O$	-	-	0.390	-
$MnSO_4.H_2O$	-	0.169	-	-
$CuSO_4.5H_2O$	-	0.0025	0.079	-
$Na_2SiO_3.9H_2O$	-	-	58	-
$Ca(NO3)_2.4H_2O$	-	-	57.5	-
Na_2CO_3	-	-	20	
K_2HPO_4	-	-	10	-
$Co(NO3)_2.6H_2O$	-	-	0.049	-
Fe-EDTA complex				
$FeSO_4.7H_2O$	6	6.95	4	-
Na_2 -EDTA	-	9.3	4.16	-
$KHCO_3$	-	-	4.32	-
N-free Flory Basic Fertilizer 1	-	-	-	2000

Microalgae growth conditions:

The initial axenically cultivation of the two microalgal strains was obtained in 250 ml Erlenmeyer flasks containing 100 ml of KC medium. The cultures were incubated at 25±1 °C under continuous illumination using tubular fluorescent lamps (FL 40 T9D/38) with a light intensity of 3000 lux at the surface of the culturing vessels. A 2-weeks old culture, at vegetative cell growth phase, was used as inoculum for all experiments. For selection of the suitable medium providing best growth under photoautotrophic conditions, *S. obliquus* and *M. reisseri* were individually cultivated in the four media. All cultures were grown under sterilized conditions in 1000 ml Erlenmeyer flasks, each containing 700 ml of the growth medium, at an initial optical density (OD_{680}) of 0.01. The cultures were incubated at 25±1 °C under continuous light intensity of 3000 lux. To avoid settling, and for accelerating the growth process, continuous aeration was supplied to the culture to provide necessary CO_2 at a flow rate of 1 L min^{-1} by bubbling of filter-sterilized air

Biomass assay:

Growth curves and biomass concentrations were evaluated by measuring the optical density at 680 nm (OD_{680}) and algal cellular dry weight (CDW, g L^{-1}). Dry weight was estimated by centrifugation of 40 ml of the culture at 2000 g for 10 min, then cell pellets were washed twice using distilled water, and then oven dried at 80 °C until constant weight.

Estimation of total lipids:

Extraction of lipids was done using chloroform: methanol (2:1) according to the method described by Folch et al. (1957). The pre-weighted glass vials containing the lipid extracts were dried at 80 °C for 30 min, cooled in a desiccator and weighed.

Productivity Calculation:

Biomass and lipid productivities were calculated according to (Andrade and Costa, 2007) and modified by Abomohra et al. (2013)

$$\text{Biomass productivity (g L}^{-1}\text{ d}^{-1}) = (CDW_L - CDW_0).(T_L - T_0)^{-1}$$

Where CDW_0 and CDW_L representing the CDW (g L^{-1}) at the starting cultivation day (T_0) and days of late exponential phase (T_L), respectively.

$$\text{Lipid productivity (mg L}^{-1}\text{ d}^{-1}) = (L_D - L_0).(T_D - T_0)^{-1}$$

Where L_0 and L_D representing the total lipid (mg L^{-1}) at the starting cultivation day (T_0) and days of the desired phase (T_D), respectively.

Fatty acid profiles:

The extracted lipids were saponified overnight with ethanolic KOH (20 %, w/v) at room temperature. Fatty acids were librated from their potassium salts by acidification with 5 N Hydrochloric acid followed by extraction using petroleum ether at 40–60 °C. The ether extract containing fatty acid methyl esters was washed three times with distilled water and dried over anhydrous sodium sulfate (Vogel 1975). Fatty acid profile was analyzed using GC–MS (Hewlett Packard HB 5890, coupled with 5989 B series mass spectrometer). The initial temperature was 70 °C and gradually accelerated to 250 °C at a rate of 10 °C per minute. The maximum peaks representing mass to charge ratio characteristics of fatty acids were compared with those in the mass spectrum library (Pandey et al., 2010). The proportion of each fatty acid (%) was calculated by the following formula,

$$Fatt\ acid\ proportion = \frac{F}{F_t} \times 100$$

Where, F is the peak area of the desired fatty acid and F_t is the total peak area for the fatty acid methyl ester.

Statistical Analysis:

Results are presented as the mean of three replicates ± standard deviation (SD). The statistical analyses were carried out using SAS (v 6.12). Data obtained were analyzed statistically to determine the degree of significance using one way analysis of variance (ANOVA) at $p \leq 0.05$.

Results:

Among the four tested media, Kuhl and KC media showed the highest growth for *S. obliquus* and *M. reisseri*, respectively (Figure 1). The duration of the exponential phase of *S. obliquus* on the four tested growth media varied between 6 to 20 days, while the exponential phase duration for *M. reisseri* varied between 14 to 22 days (Table 2). The results showed the highest biomass productivity for *M. reisseri* on KC medium (0.07 g L^{-1} d^{-1}) which was 40.8 % higher than that of *S. obliquus* on Kuhl medium. Accordingly, Lipid productivity and fatty acid profile of *S. obliquus* and *M. reisseri* were studied using the corresponding optimal medium for eac h strain.

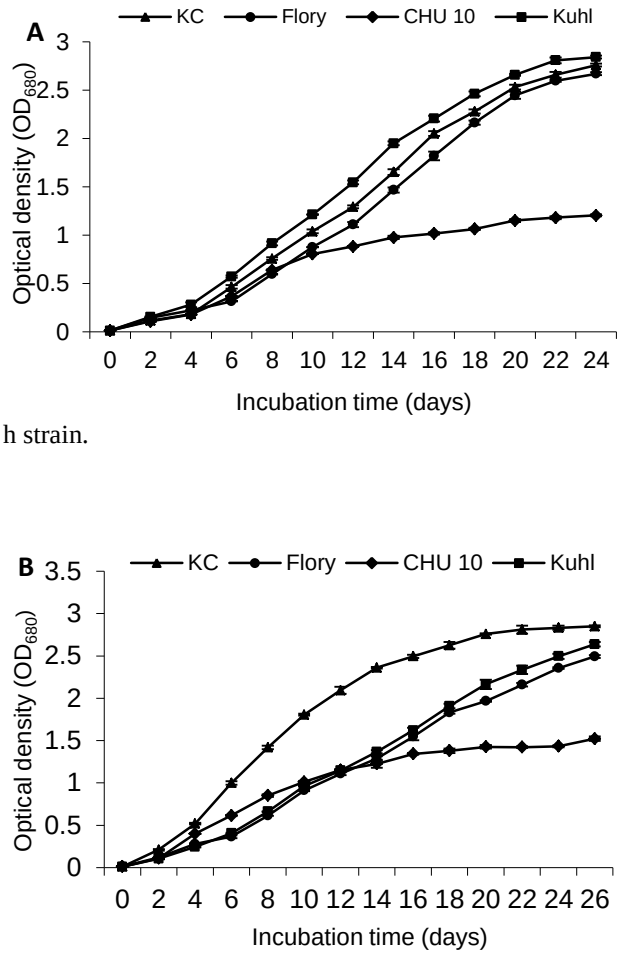

Figure 1. Growth curves of *S. obliquus* (A) and *M. reisseri* (B) cultivated on the four different growth media.

Table 2. Comparison of growth parameters of *S. obliquus* and *M. reisseri* grown in different culture media.

Microalgae	Growth media	Duration of exponential phase	CDW (g L^{-1}) at late exponential phase	Biomass productivity (g CDW L^{-1} d^{-1})
S. obliquus	KC	20	1.00 ± 0.033 [a]	0.04 ± 0.003 [ab]
	Flory	16	0.93 ± 0.033 [a]	0.04 ± 0.001 [a]
	CHU- 10	6	0.50 ±0.033 [b]	0.02 ± 0.002 [c]
	Kuhl	18	1.20 ± 0.030 [ce]	0.05 ± 0.002 [bd]
M. reisseri	KC	18	1.38 ±0.080 [d]	0.07 ± 0.004 [e]
	Flory	22	1.18 ± 0.029 [cf]	0.05 ± 0.004 [d]
	CHU- 10	14	1.10 ± 0.090 [f]	0.03 ± 0.002 [c]
	Kuhl	22	1.28 ± 0.080 [e]	0.06 ± 0.006 [f]

Each value is the mean of three readings ± standard deviation. Mean value in the same column with different letters in the superscript are significantly different ($p<0.05$)

Figure 2 shows lipid content and lipid productivity of *S. obliquus* grown in Kuhl medium at different growth phases. There was a statistically significant increase (of 86.25%) in the lipid content of *S. obliquus* from early exponential phase to stationary phase, while the increase in lipid content showed insignificant difference between late exponential phase and stationary phase. Lipid productivity of *S. obliquus* at stationary phase was 12.61 mg $L^{-1}d^{-1}$ which was insignificant with that at the late exponential phase (12.55 mg $L^{-1}d^{-1}$). Lipid content of *M. reisseri* showed the same pattern as *S. obliquus* (Figure 3). It was increased by 55.61% from early exponential to stationary phase, while the increase was insignificant from late exponential to stationary phase. However, *M. reisseri* showed higher lipid productivity at stationary phase (17.08 mg L^{-1} d^{-1}) which was 35.45 % higher than that of *S. obliquus*. In addition, lipid productivity of *M. reisseri* at stationary phase was significantly higher than that at other growth phases.

The fatty acid composition of *S. obliquus* and *M. reisseri* grown on the two best media (Kuhl and KC, respictively) were studied at stationary growth phase and represented in Table 3. The result showed that the two studied species are characterized by high percentage of saturated fatty acid 81.34 and 72.51% for *S. obliquus* and *M. reisseri*, respectively. The highest percentage of SFA in *S. obliquus* grown on Kuhl medium was due to the presence of Tridecylic (C13:0), Palmitic (C16:0) , Stearic (C18:0), Lauric (C12:0) which represented by 19.53%, 15.74%, 10.05% and 7.34% of the total fatty acids, respectively. On the other hand, the highest content of monounsaturated fatty acids (MUFAS) constituted 27.49 % was recorded in *M. reisseri* which due to the presence of Myristoleic (C14:1) and Pentadecenoic acid (C15:1). Finally, the total polyunsaturated fatty acids (PUFAs) constituted 2.93 % of the total fatty acids in *S. obliquus* due to the presence of Linoleic (C18:2c). (Table3).

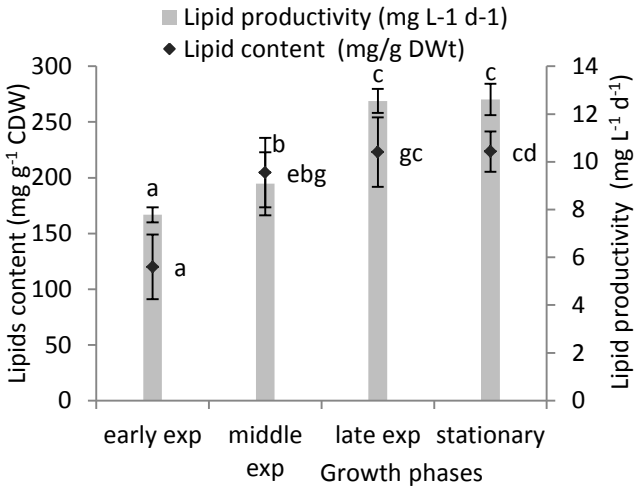

Figure 2. Variation of lipid content and lipid productivity of *S. obliquus* cultivated in kuhl medium at different growth phases. Error bars show the SD for three measurements; the significance of differences is denoted by different letters ($p<0.05$)

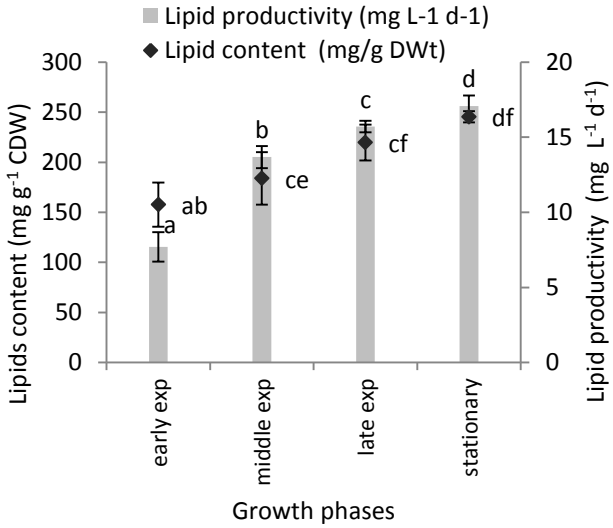

Figure 3. Variation of lipid content and lipid productivity of *M. reisseri* cultivated in KC medium at different growth phases. Error bars show the SD for three measurements;

the significance of differences is denoted by different letters ($p<0.05$).

Table 3. Fatty acid profiles of *S. obliquus* and *M. reisseri* measured at stationary growth phase. Values are given as percent (%) of total fatty acids. SFA Saturated fatty acid, UFA unsaturated fatty acid

Fatty acids	*S. obliquus*	*M. reisseri*
SFAs		
Caprylic (C8:0)	*nd*	0.47
Capric(C10:0)	1.8۲	0.70
Undecylic(C11:0)	3.62	*nd*
Lauric(C12:0)	7.34	5.20
Tridecylic(C13:0)	19.53	12.03
Myristic(C14:0)	13.87	20.28
Pentadecylic(C15:0)	9.37	16.68
Palmitic(C16:0)	15.74	16.06
Stearic(C18:0)	10.05	1.09
Sum SFAs	81.34	72.51
MUFAs		
Myristoleic(C14:1)	7.79	12.51
Pentadecenoic acid (C15:1)	*nd*	14.98
Oleic(C18:1c)	7.94	*nd*
Sum NUFAs	15.73	27.49
PUFAs		
Linoleic(C18:2c)	2.93	*nd*
Sum PUFAs	2.93	*nd*

nd means not detected

Discussion:

The present study mainly focused on freshwater microalgae which can be found in the local freshwater ponds and also be able to grow easily under laboratory conditions. *S. obliquus* and *M. reisseri* were selected as good strains for this work based on literature review (Abomohra et al., 2013; Eldalatony, 2013; Abou-Shanab et al., 2014; Onay et al., 2014). The selection of these strains was based on high growth rate and/or high lipid content which results in high lipid productivity. The selection of the suitable medium for high biomass and lipid production depends on the growth requirements of the algae, how the constituents of the medium may affect final product quality and the components cost (Borowitzka, 2005).

The highest biomass productivity for *M. reisseri* was recorded on KC medium which was 25 % higher than that recorded by Abou-Shanab et al. (2014) for *M. reisseri* grown on bold basal medium for 20 days. The higher biomass productivity for *M. reisseri* grown in KC medium is correlated to its chemical composition, which containing all growth requirements for *M. reisseri* such as higher concentration of $Na_2HPO_4.2H_2O$, $MgSO_4.7H_2O$ and $(NH_4)_6Mo_7O_{24}.4H_2O$ as a source of phosphorus, Mg^{2+} and Mo^{2+}, respectively, compared to other media. In addition, the presence of NaCl in KC medium may change the salinity of water that affects the growth, metabolism and photosynthesis of microalgae (Moisander et al., 2002; Lartigue et al., 2003).

The higher biomass productivity of *S. obliquus* grown in Kuhl medium might be related to higher concentration of KNO_3, $FeSO_4.7H_2O$ and $ZnSO_4$ as a source of nitrogen, iron and Zn^{2+}, respectively, compared to other tested media, which are an essential constituents of all structural and functional components of the algal cells (Terry and Abadía, 1986; Chu et al., 2007). In addition, the presence of ($CuSO_4.5H_2O$) as a source of cupper in low concentration compared to its high concentration in Chu-10 medium or its absence in KC medium. Cupper is one of the essential micronutrients that required for microalgal growth and involved in numerous metabolic processes, including photosynthesis and energy storage (Liu et al., 2008; Chen et al., 2011).

Growth phase of the culture also affects lipid content and fatty acid composition. In many algal species the increase in lipid content is often observed during stationary phase. Therefore, the highest lipid content of *S. obliquus* and *M. reisseri* in stationary phase cultures might be due to the shift in lipid metabolism from membrane lipid synthesis to the storage of neutral lipids. That shift in lipid metabolism because of the depletion of essential nutrients, including nitrogenous compounds that are necessary for protein synthesis, and phosphate-containing compounds which are needed for the formation of phospholipids that are major structural components of cell-membranes (Dunstan et al., 1993; Brown et al., 1996). In accordance to the present results, Bigogno et al. (2002) found that total fatty acid portion forming triacylglycerols (TAGs) in the chlorophyte *Parietochloris incise* increased from 43% in the logarithmic phase to 77% in the stationary phase.

The continuous increase in the lipid content of *S. obliquus and M. reisseri* from early exponential phase to stationary phase, in addition to the increase of biomass productivity resulted in increase in lipid productivity for both strains from early exponential phase to stationary phase. Lipid productivity of *M. reisseri* showed significant differences a long side different growth phases. However, the increase in lipid productivity of *S. obliquus* from late exponential phase to stationary phase showed insignificant difference which suggests the optimum harvest times for *M. reisseri* and *S. obliquus* at stationary phase and late exponential phase, respectively. The obtained results showed increase in lipid productivity of *M. reisseri* due to its higher biomass productivity which makes this microorganism desirable for further studies on optimization of lipid production and on large-scale cultivation.

Saturated fatty acid contents and chain length of algae lipids would cause noticeable changes in the biodiesel properties. Saturated fatty acids have significantly higher melting points than unsaturated fatty acids. Therefore, biodiesel fuels derived from fats or oils with significant amounts of saturated fatty compounds display poor cold flow properties (Dunn, 2005). In accordance to the present results, Knothe (2009) reported that the most common fatty acids methyl esters (FAMEs) present in biodiesel with good burning qualities are palmitic acid (C16:0) and oleic acid (C18:1n9c), which were also the major fatty acids in both studied miceoalgae in the present study.. The high percentage of saturated fatty acids, e.g. palmitic acid (C16:0), increases the biodiesel quality due to increasing of cetane number which decreased by increasing the degree of unsaturation (Kinoshita et al., 2006).

On the other hand, Zhang et al. (2008) concluded that higher content of polyunsaturated fatty acid in the feedstock oil causes deterioration in the quality of biodiesel upon storage, due to oxidation, or even the structural features of the fatty acids. Although the fatty acid profile of both studied microalgae showed superior characteristics to be used as feedstock for biodiesel production, the higher lipid productivity of *M. reisseri* makes it one of the best candidates for biodiesel synthesis.

In conclusion, the effect of different culture media on the growth and lipid productivity of green microalgal strains *S. obliquus* and *M. reisseri* suggested *M. reisseri* grown in KC medium is the most suitable microalga for biodiesel production because it showed higher biomass and lipid productivities.

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تأثير أنواع مختلفة من الأوساط الغذائية على نمو الطحالب الخضراء سينيدزمس اوبليكس وميكراكتينيوم ريزيرا كمصدر لانتاج الديزل الحيوى

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هدفت هذه الدراسة الى قياس النمو، المحتوى الدهنى وإنتاجية الدهون لطحلبى سينيدزمس أوبليكس وميكراكتينيوم ريزيرا لاعتبارهما من الطحالب الدقيقة الواعدة فى إنتاج الديزل الحيوي والتى تم تنميتهما على أوساط غذائية مختلفة. تم استخدام أربعة أوساط غذائية من المياه العذبة وهى KC, flory, Kuhl Chu-10 لاختيار الوسط الغذائى المناسب للحصول على أعلى إنتاجية من الدهون. أكدت النتائج ان الوسطين الغذائيين Kuhl وKC أظهرا أعلى إنتاجية للكتلة الحيوية لطحلبى سينيدزمس أوبليكس وميكراكتينيوم ريزيرا بقيم 0.05 و0.07 جرام/ لتر/ يوم على التوالى. بالإضافة الى ذلك تم قياس المحتوى الدهنى وإنتاجية الدهون فى أربعة مراحل مختلفة من النمو وهى اول ووسط واخر طور النمو اللوغارتمى وطور النمو الثابت. أظهرت النتائج ان إنتاجية الدهون لطحلب الميكراكتينيوم ريزيرا كانت الأعلي بقيمة 17.08 مللى جرام/ لتر/ يوم خلال طور النمو الثابت فى حين كانت إنتاجية الدهون لطحلب سينيدزمس أوبليكس 12.61 مللى جرام /لتر/ يوم خلال نفس طور النمو بفارق غير معنوى مع انتاجية الدهون فى نهاية طور النمو الوغارتمى (12.55 مللى جرام/ لتر/ يوم). علاوة على ذلك أظهرت دراسة أنواع الاحماض الدهنية لطحلبى سينيدزمس أوبليكس وميكراكتينيوم ريزيرا فى طور النمو الثابت ان كل من الطحلبين مناسب كمصدر خام لانتاج الديزل الحيوى بسبب محتواهم العالى من الاحماض الدهنية المشبعة والتى تمثل ٨١.٣٤% و٧٢.٥١% من المحتوى الكلى للدهون على التوالى. وكانت نسب حمض البالمتيك فى الطحلبين ١٥.٧٤% و١٦.٠٦% على التوالى والتى تحسن من جودة الديزل الحيوى. ختاما، تشير هذة الدراسة إلى أن تنمية الميكراكتينيوم ريزيرا على الوسط الغذائى KC تقدم مقترحا واعداً لاستخدامها كواحدة من مصادر الديزل الحيوى.