

USAGE OF SECONDARY RECOVERED SEWAGE SLUDGE FOR INTRACELLULAR LIPID ACCUMULATION TO ENHANCE BIOFUEL YIELD OF MARINE MICROALGAE

Nagwa G-E Mohammady^{*a b}, Scott R. Lindell^b, Christopher M. Reddy^c,
Catherine A. Carmichael^c and Connie Pui Ling Lau^{bd}

^aBotany and Microbiology Department, Faculty of Science, Alexandria
University, Egypt,

^bMarine Resource Center, Marine Biological Laboratory, Woods Hole, MA, USA,

^cDepartment of Marine Chemistry and Geochemistry, Woods Hole
Oceanographic Institute, Woods Hole, MA, USA.

^dDepartment of Biology, The Chinese University of Hong Kong, Hong Kong,
China

Abstract

A laboratory study was carried out to enhance biofuel yield of marine microalgae *Tetraselmis chuii*, *Chaetoceros muelleri* and *Isochrysis* sp. (clone c Iso), using an aqueous extract of secondary recovered sewage sludge (SS). In this respect, we compared the intracellular lipid accumulation in cells grown on controlled to the treated with SS cultures, both quality and quantity. The gravimetric data showed an obvious increase of total lipids in the treated cells 13.16, 1.65 and 1.8 times for *T. chuii*, *C. muelleri* and *Iso.* sp. respectively. In addition, the algal oil increased 16, 1.73 and 5 times in the same order. Fatty acid methyl esters of the controlled growth cultures are dominated by C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C18:4 and C22:6. However, the treated ones showed a significant variation in the concentration of both total lipids and oil fatty acids. Moreover, some fractions are disappeared, while others are newly synthesized. The results suggest that the application of sewage sludge could enhance the accumulation of algal lipid, oil, and improve the quality of biofuel produced.

Keywords: biofuel, fatty acids, lipids, microalgae, sewage sludge, algal oil.

Introduction

The current reliance on petro-based fuels and chemicals is no more sustainable as the energy demand is projected to grow more than 50% by 2025 (Patil, 2008).

Microalgae have been suggested as very good candidates for fuel production due to high potential accumulation of intracellular lipids (McGinnis *et al.*, 1997) compared to higher plants. Theoretical fuel production yields from microalgae have been estimated to be as high as 4,000 gal/acre cultivation per year, whereas current yields of soybean oil are only about 50–60 gal/acre per year. However, production of algal oil faces many technological barriers that must be

*Corresponding author:

Phone: +203 5232 674, Cell: +2012 7431 432, Fax: +203 3911 794

E-Mail: nagwa_phyco@yahoo.com

(ISSN: 1110-8649)

overcome before these impressive theoretically maximum yields can be achieved (**Eroglu and Melis, 2009**).

The most constituent fuels extracted from algal lipids are biodiesel and different biohydrocarbons (**Chisti, 2006**). Biodiesel “bio-oils” from microalgae could be derived from the fatty acid methyl esters of accumulating triglycerides (**Sheehan *et al.*, 1998**). There are many challenges remained in biodiesel production, the biggest one is that microalgal biodiesel are not economically competitive with fossil fuels at today’s energy prices (**Deng *et al.*, 2009**). Therefore, we have to explore economical ways to maximize the potential of using microalgae to produce biofuels.

Intensive studies have shown that sewage sludge could increase algal biomass, since the waste is rich in compounds of nitrogen, phosphorus, and other nutrients (**Órpeza *et al.*, 2009**). Furthermore, microalgal biomass grown on wastes has potential for sustainable biofuel production (**Chisti, 2007; Van Harmelen and Oonk, 2006**). Due to the reasonability price of sewage sludge material, the addition of the treated waste to microalgae, as a fertilizer to enhance biofuels yield, is good economic concept. In this respect, our goal is to contribute to the development direct feasibility of producing lipids from microalgae grown on aqueous extract of secondary treated sewage sludge, as an integral component of the growth media, to simultaneously create a clean renewable energy. We compared the total lipids (as a source of hydrocarbons and jet fuel) and algal oil (as a source of biodiesel) accumulated in three microalgae (*Tetraselmis chuii*, *Chaetoceros muelleri* and *Isochrysis* sp. (clone c Iso) in response to the recovered waste.

Materials and Methods

Characterization of sewage sludge: Secondary recovered sewage sludge (SS) was collected from the Otis sewage treatment plant on Cape Cod, MA, USA. The analysis of the waste was carried out by soil and plant tissue testing laboratory, west experiment station, University of Massachusetts, Amherst, MA, USA. This effluent had mean value of pH 6.5 and the detailed analysis is reported in Table (1). Preliminary experiments (unpublished data) have been performed to assess the appropriate dose of the sterilized aqueous sewage sludge that enhances the algal biomass. The results showed that 10% (10 ml of sterilized aqueous solution of the treatment plus 90ml growth medium) of the treatment is the perfect concentration.

Microalgae and culture conditions: Unialgal cultures of the green microalga *Tetraselmis chuii*, the diatom *Chaetoceros muelleri* and the haptophyte *Isochrysis* sp. (clone c. Iso) were batch grown in the experimental hatchery at the Marine Biological Laboratory, MA, USA. Initial inoculums were obtained from the Northeast Fisheries Science Center, Milford Laboratory, and Connecticut. Two sets of microalgae cultures were cultivated in 250 ml glass Erlenmeyer

flasks. The first set (the controlled cultures) was grown using f/2 growth medium (**Guillard, 1975**). However, 10% of autoclaved aqueous extract of sewage sludge (SS) was added to the second set (treated cultures). Algae were incubated in a climatic chamber under continuous light conditions using fluorescent light tube providing a photosynthetic active radiation expressed in photon flux of $31 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. The flasks held on an oscillating shaker (100 rpm) at $19 \pm 1^\circ\text{C}$. Cultures were harvested at the end of exponential growth phase for lipids analyses.

Extraction of algal lipids: Total lipids of freeze-dried algal biomass (10 to 50 mg) was extracted using dichloromethane/methanol (2:1v/v), according to **Gracia et al. (2008)**. The algal oil was extracted with hexane, at 55°C for 12 hours. The total lipids, along with oil, were calculated gravimetrically for different algal samples.

Transesterification reaction: The resultant lipid extract was spiked with an internal standard, ethyl nonadecanoate, and transesterified under N_2 using 10% methanolic HCl in hexane. We used ethyl nonadecanoate to check both the completeness of the transesterification reaction by monitoring the production of methyl nonadecanoate and using the latter for quantification purposes. The reaction products were extracted with hexane, reduced in volume, spiked with an external 85 standard, *n*-heptadecane, and stored until analysis by the GC-FID.

Gas Chromatography with Flame Ionization Detection (GC-FID): Fatty acid methyl esters (FAMES) were analysed in the esterified samples using a Hewlett-Packard 5890 GC-FID. Compounds were separated on a glass capillary column (J&W DB-1MS, 30m, 0.25-mm i.d., 0.25- μm film thickness) with H_2 carrier gas. FAMES were identified with standards purchased from Nu-Chek Prep (Elysian, MN) and Supelco (Bellefonte, PA, USA).

Statistical analysis: Data was statistically analyzed for three replicas, the means and the standard deviations (SD) were calculated.

Results

Aqueous secondary recovered sewage sludge: The analysis of the waste used revealed the presence of a lot of amounts of both macro and micronutrients. Both oxidized and reduced nitrogen forms are present (Table 1). It is necessary to highlight that nitrate- N_2 is the dominant macronutrient followed by ammonium- N_2 . Potassium was relatively found in a high amount (168 mg/L). Sodium content was the highest micronutrient that was estimated (182.10 mg/L), while copper represents the lowest value (4.17 mg/L).

Total lipids and algal oil content: Fig. (1) shows a significant increase of intracellular lipids extracted from the treated cells with SS compared to those grown on the controlled medium. The values increased 13.16, 1.65 and 1.8 times, for *T. chuii*, *C. muelleri* and *Iso* sp. respectively. On the other hand, the amounts

of algal oil have been increased, for the same order, by 16, 1.73 and 5 times, Fig. (2).

Table (1): Analysis of aqueous secondary recovered sewage sludge.

Macronutrients	mg/L	Micronutrients	mg/L
Nitrate-N ₂	340	Zinc	8.92
Ammonium-N ₂	250	Boron	4.73
Phosphorus	39	Manganese	5.45
Potassium	168	Copper	4.17
Calcium	17	Iron	61.25
Magnesium	9	Sodium	182.10
Sulfur	5		

These results demonstrated that under control conditions, the diatom *C. muelleri* is the best source for biodiesel and other biofuels among the examined strains. However, the response of the two other strains (*T. chuii* and *Iso sp.*) to the waste was more rapidly. Moreover, the treated *T. chuii* with SS is highlighted to be the most promising examined microalga for different biofuels production.

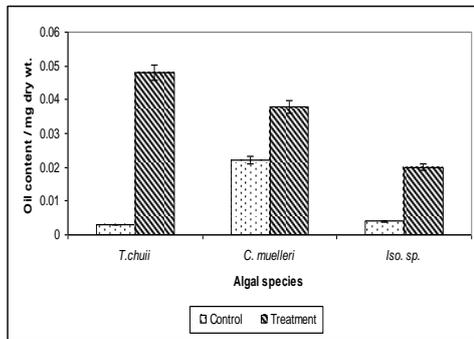


Figure (1): Total lipid content of the examined algae strains grown on controlled and 10% SS.

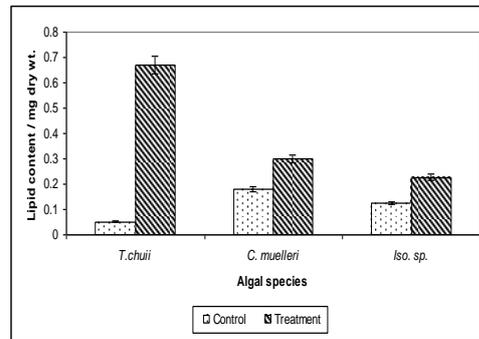


Figure (2): Oil content of the examined algae strains grown on controlled and 10% SS.

Fatty acid composition of total lipids: Table (2) shows a significant positive effect of SS on FAMES of the investigated spp. In *T. chuii* grown on the controlled growth medium, the fatty acid pool is composed of C16:0, C18:1, C18:2, C18:3. However, after adding SS, newly synthesized profiles (C16:1, and C18:0) were detected. In *C. muelleri*; C18:1, C18:2 and C18:3 are appeared in cultures integrated with SS. However, the amounts of already found profiles: C14:0, C16:1, C18:0, and C18:4, are increased by 55, 87, 88 and 44% respectively, after addition of SS. A negative effect of the treatment on the amounts of C16:0 and an unknown fraction were also observed.

In *Iso sp.*, the newly synthesized fatty acids appeared in cells grown with SS are: C16:1, C18:2, C18:3 and C22:6. The treatment with SS increased the

percent of C18:1 by more than 55%. However, C14:0, C16:0, 18:4 were decreased after the addition of the treatment.

Table (2): Total lipid-FAMES % of the examined strains grown on controlled medium and 10% SS.

Fatty acids	<i>Tetraselmis chuii</i>	<i>Tetraselmis chuii</i> + ss	<i>Chaetoceros muelleri</i>	<i>Chaetoceros muelleri</i> + ss	<i>Isochrysis</i> sp. (clone c Iso)	<i>Isochrysis</i> sp. (clone cIso) + ss
C 14:0	-	-	11.11	20.00	40.00	15.79
C 16:1	-	2.13	37.78	50.00	-	5.26
C 16:0	42.86	40.43	35.56	7.50	20.00	15.79
C 18:4	-	-	2.22	5.00	20.00	10.53
C 18:2&3	14.29	12.77	-	2.50	-	5.26
C 18:1	42.86	42.55	-	2.50	20.00	36.84
C 18:0	-	2.13	2.22	2.50	-	-
Unknown	-	-	11.11	10.00	-	-
C 22:6	-	-	-	-	-	10.53

Fatty acid composition of algal oil: In Table (3), the newly synthesized profiles in the treated cells of *T. chuii* are C16:1 and C18:0. While neither new profiles were appeared for *C. muelleri* nor *Iso* sp. after addition of SS. The treatment showed a variable effect on the fatty acid concentration of the two strains.

Table (3): Algal oil-FAMES % of the examined strains grown on controlled medium and 10% SS.

Fatty acids	<i>Tetraselmis chuii</i>	<i>Tetraselmis chuii</i> + ss	<i>Chaetoceros muelleri</i>	<i>Chaetoceros muelleri</i> + ss	<i>Isochrysis</i> sp. (clone c Iso).	<i>Isochrysis</i> sp. (clone c Iso)
C 14:0	-	-	10.23	19.73	38.75	14.04
C 16:1	-	1.53	38.35	51.17	7.23	4.82
C 16:0	39.78	40.06	35.23	6.69	14.46	16.23
C 18:4	2.58	0.76	2.56	5.35	14.46	11.84
C 18:2&3	16.34	13.76	1.14	0.13	10.24	6.14
C 18:1	41.29	41.74	0.57	3.68	13.25	36.84
C 18:0	-	2.14	1.42	2.34	-	-
Unknown	-	-	10.23	9.70	-	-
C 22:6	-	-	0.28	-	3.01	10.09

Discussion

In relation to the characterization of the aqueous sewage sludge used, it is generally accepted that algae can use a wide variety of nitrogenous compounds, both organic and inorganic forms, as nitrogen sources for the synthesis of amino acids (Przytocka-Jusiak *et al.*, 1984 and Maestrini *et al.*, 1986).

In this paper, we demonstrated the possibility of using secondarily treated sewage as an integrated medium to grow microalgae to enhance intracellular lipid accumulation. A result agrees with **Órpez *et al.* (2009)**, who demonstrated a notable amount of lipids accumulated in *Botryococcus braunii* when cultivated in secondarily treated sewage, since the waste is rich in compounds of nitrogen, phosphorus and many micronutrients. Indeed, the utilization of nutrients from aqueous waste for algal growth and accumulation of intracellular lipids demonstrate the potential cost savings and economic significant for such purposes (**Martinez *et al.*, 2000**).

In this study, both total lipids and algal oil are increased significantly for examined strains by adding SS. Although *C. muelleri* is the most biofuels (total lipids and biodiesel) producer strain among the investigated microalgae in the controlled conditions, the treated cells of *T. chuii* with SS showed a great response towards biofuels production. A result makes this strain to be the most recommended for such purposes. A similar result was demonstrated for the species of fresh water microalgae *Scenedesmus* and *Chlorella* suggesting that even higher lipid percentages could be achieved using the treatment without optimizing cultivation (**Thomas *et al.*, 1984**).

In this paper, the analysis of fatty acid methyl esters revealed the presence of saturated, mono- and polyunsaturated long chain fatty acids for both total lipids and biodiesel. There is no doubt that upon the addition of SS the profiles of total lipids mostly increased. However, In case of biodiesel, C22:6 is disappeared for *C. muelleri* and no change was noticed for fatty acid composition of *Iso* sp., except for the concentrations. This indicates that the treatment has an obvious effect on the fatty acid pathway in the algal cells. Generally, fatty acids quantities and types influence the quality of the biofuel produced, particularly biodiesel (**Knothe, 2005**).

Shorter saturated fatty acids have greater storage stability and are less likely to polymerize during combustion (**Sheehan *et al.*, 1998**), but they have poorer cold temperature properties (e.g., cloud point) (**Sharma *et al.*, 2008**). Longer polyunsaturated fatty acids can be oxidized during storage (**Sheehan *et al.*, 1998**). Several researchers reported that reducing the saturated fatty acid content of plant oil can improve the cold temperature low properties of the biodiesel derived from it because long-chain saturated fatty esters significantly increase the cloud point and the pour point of biodiesel (**Serdari *et al.*, 1999**; **Stournas *et al.*, 1995**). In this work, fatty acid composition is mostly composed of the unsaturated fractions: C16:1, C18:1, C18:2, 18:3 C18:4, and C22:6. Besides, the saturated fractions 16:0 and 18:0. As postulated by **Deng *et al.* (2009)**, biodiesel from highly unsaturated sources oxidizes more rapidly than conventional diesel, resulting in forming insoluble sediments to interfere with engine performance. Therefore, the proper percentage of saturated and

unsaturated fatty acid is very important to microalgae as a biodiesel feedstock. In this study, the integrated cultures with SS showed some newly synthesized oil fatty acids, such as C16:1 and C18:0 in *T. chuii*. In addition, a lot of newly synthesized total lipid- fatty acids, were appeared for the three investigated strains. Therefore, we suggest extraction of biodiesel from a mixture of mass cultivation of the examined species. This will support the idea that blending oil from various feedstocks offers advantages in fuel performance over the neat form.

Conclusions

Secondary treated sewage sludge is a better substrate as integrated microalgae culture for intracellular lipid accumulation to enhance biofuel yield.

The response of the examined species to the aqueous extract of secondary recovered sewage sludge (SS) showed high yield lipid production, which makes it of economical interest. *T. chuii* showed highly response towards the treatment with SS among the examined strains, makes it the most promising species for such purposes.

Finally, biofuel (particularly biodiesel) production from algae grown on SS, as an integral component of the growth medium, has the potential to address two important societal needs: increasing development of renewable clean energy and management of wastes to protect our environments as well.

Acknowledgement

The authors wish to acknowledge the Lucy B. Lemann research grant provided by Marine Biological Laboratory, MA, USA, for financially supporting this research.

References

- Chisti, Y.** (2007). Biodiesel from microalgae. *Biotechnol. Adv.*, **25:294-306**.
- Chisti, Y.** (2006). Microalgae as sustainable cell factories. *Environmental Engineering and Management Journal*, **5: 261-274**.
- Deng, X.; Li Y. and Fei, X.** (2009). Microalgae: A promising feedstock for biodiesel. *African Journal of Microbiology Research*, **3: 1008-1014**.
- Eroglu, E. and Melis, A.** (2009). “Density Equilibrium” Method for the Quantitative and Rapid In Situ Determination of Lipid, Hydrocarbon, or Biopolymer Content in Microorganisms. *Biotechnology and Bioengineering*. **102: 1406-1415**.
- Garcia, A. S.; Parrish, C. C.; Brown, J. A.; Johnson, S. C. and Leadbeater, S.** (2008). Use of differently enriched rotifers, *Brachionus plicatilis*, during larviculture of haddock,

- Melanogrammus aeglefinus*: effects on early growth, survival and body lipid composition. *Aquaculture Nutrition*, **14**: 431-444.
- Guillard, R. L.** (1975). Culture of phytoplankton for feeding marine invertebrates. Culture of marine invertebrate animal, p. 29-60.
- Knothe, G.** (2005). Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology*. **86(10):1059-1070**.
- Martínez, M. E.; Sánchez, S.; Jiménez, J. M.; El Yousfi, F. and Muñoz, L.** (2000). Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. *Bioresource Technol.*, **73(3): 263–272**.
- Maestrini, S. Y.; Robert, J. M.; Leftley, J. W. and Collos, Y.** (1986). Ammonium threshold for simultaneous uptake of ammonium and nitrate by oyster pond algae, *J. Exp. Mar. Biol. Ecol.*, **102: 75-98**.
- McGinnis, K. M.; Dempster, T. A. and Sommerfeld, M. R.** (1997). Characterization of the growth and lipid content of the diatom *Chaetoceros muelleri*. *J. Appl. Phycol.*, **9:19-24**.
- Órpez, R.; Martínez, M.; Hodaifac, G.; El Yousfi, F.; Jbarib, N.; Sánchez, S.** (2009). Growth of the microalga *Botryococcus braunii* in secondarily treated sewage. *Desalination*, **246: 625-630**.
- Patil, V.** (2008). The relevance of biofuels. *Curr. Sci.*, **92: 707**.
- Przytocka-Jusiak, M.; Duszota, M.; Matusiak, K. and Mycielski, R.** (1984). Intensive culture of *Chlorella vulgaris*/AA as the second stage of biological purification of nitrogen industry wastewaters, *Water Res*, **18:1-7**.
- Serdari, A; Lois, E. and Stournas, S.** (1999). Impact of esters of mono- and dicarboxylic acids on diesel fuel quality. *Ind. Eng. Chem. Res.*, **38: 3543-3548**.
- Sharma, Y.C.; Singh, B.; and Upadhyay, S. N.** (2008). Advancements in development and characterization of biodiesel: A review. *Fuel*, **87(12): 2355-2373**.
- Sheehan, J.; Dunahay, T.; Benemann, J. et al.** (1998). A Look Back at the U.S. Department of Energy's Aquatic Species Program - Biodiesel from Algae. National Renewable Energy Laboratory. Golden, CO, USA. **325 p**.
- Stournas, S. ; Lois, E. ; Serdari, A.** (1995). Effects of fatty-acid derivatives on the ignition quality and cold flow of diesel fuel. *J. Am. Oil Chem.*, **72: 433-437**.

- Thomas, W. H.; Tornabene, T. G. and Weissman, J.** (1984). Screening for lipid yielding microalgae: activities for 1983. *SERI/STR-231-2207*. p. 31.
- Van Harmelen, T. and Oonk, H.** (2006). Microalgae Biofixation Processes: Applications and Potential Contributions to Greenhouse Gas Mitigation Options. Built Environment and Geosciences, Netherlands Organisation for Applied Scientific Research (TNO). Apeldoorn, The Netherlands. 44.

استخدام حمأة الصرف الصحي المعالجة ثانويا في تراكم الدهن الخلوى فى الطحالب البحرية لتعزيز انتاج الوقود الحيوى.

¹نجوى جمال الدين محمدى، ²سكوت لينديل، ³كريستوفر ريدي ، ³كاترين كارميشيل و⁴كوني لاو بوى

- 1- قسم النبات والأحياء الدقيقة ، كلية العلوم ، جامعة الاسكندرية ، مصر ،
2- مركز الموارد البحرية ، المختبر البيولوجي ، وودز هول، الولايات المتحدة الأمريكية ،
3- الكيمياء البحرية الجيولوجية ، معهد علوم المحيطات ، وودز هول ، الولايات المتحدة الأمريكية .
4- قسم الأحياء، الجامعة الصينية في هونغ كونغ ، هونغ كونغ ، الصين

لقد أجريت دراسة مخبرية لتعزيز انتاج الوقود الحيوي على الطحالب البحرية الدقيقة: التيتراسيلماس تشاى، كيتوسيروس ميليرى و ايزوكريزيس جنس (ت) وذلك باستخدام المستخلص المائي لحمأة الصرف الصحي المعالجة ثانويا. وفي هذا الصدد فقد تمت دراسة مقارنة بين كمية ونوع الدهن المتراكم في الخلايا المزروعة تحت الظروف المنضبطة والمعالجة باستخدام الحمأة. وقد أظهرت النتائج زيادة واضحة فى إجمالي كمية الدهن في الخلايا المعالجة 13.16 ، 1.65 و 1.8 مرة فى التيتراسيلماس تشاى، كيتوسيروس ميليرى و ايزوكريزيس جنس (ت) على التوالي. بالإضافة إلى ذلك، زادت الزيوت 16.73 و 5 مرات بنفس الترتيب. وقد سادت الأحماض الدهنية: C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C18:4, C22:6 فى الخلايا المنضبطة. كذلك أظهرت النتائج تفاوتاً واضحاً فى الخلايا المعالجة فى تركيز كل من كمية الدهون الكلية وانواع الأحماض الدهنية، فقد اختلفت بعض الأنواع فى حين تم بناء أنواع اخرى. وتشير النتائج إلى أن تطبيق حمأة الصرف الصحي على نمو الطحالب الدقيقة يمكن أن يعزز تراكم الدهن فى الطحالب البحرية وتحسين نوعية الوقود الحيوي المنتجة.