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Effects of nitrogen source on enhancing growth conditions of green algae to produce higher lipid

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ABSTRACT

Microalgae represent a potential source of biological material to produce biodiesel. This study focused on the effects of nitrogen concentration to enhance lipid content from *Chlorella sorokiniana* and their potential use to serve as a raw material for biofuel production. Light intensity and different nitrogen concentrations were used to determine the optimum cultivation environment for the fresh water microalgae, *C. sorokiniana*. The effects of various nitrogen sources were examined in order to determine the optimum lipid content produced by the microalgae. It was found that the optimum cultivation of microalgae growth has caused the biomass growth and has led to higher lipid production. The growth rate and lipid content were determined by measuring the optical density at 620 nm and fluorescence intensity using Nile red method. Microalgae of 10% (v/v) concentration was found to be the optimum inoculum concentration with higher growth rate obtained when compared to 50% (v/v). The NH_4NO_3 nitrogen concentration showed greater lipid production compared to $NaNO_3$ cells cultivated with final lipid content. The 0.2 M of NH_4NO_3 nitrogen concentration produced highest lipid (3.138 a.u), when compared to two different nitrogen sources: NH_4NO_3 and $NaNO_3$ with different concentrations.

Keywords: Microalgae; *Chlorella sorokiniana*; Nitrogen source; Lipid productivity

1. Introduction

Natural resources are not only renewable resources which have attracted the researchers and scientists to work on other alternatives to meet the growing demand of fuel. In the current list of fuel production, biofuel has been added. The overall biodiesel production in the world for the past 10 years was appraised to be around 1.8 billion L [1]. Biodiesel can be produced using vegetable oils, such as soybean, sunflower, and palm oils. According to Guan and his researchers [2], biodiesel can be produced from microalgae and it is gaining importance mainly because the algal lipid content can serve as a raw material for biofuel production. Biofuel is also made from nontoxic, biodegradable, and renewable resources. These

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provide environmental benefits, which in turn decreases the harmful emissions of carbon monoxide and hydrocarbons, thereby resulting in the decrease of greenhouse effect [3]. The use of biofuels can also play an important role in avoiding the excessive dependence on fossil fuels and this will certainly help to improve the environmental sustainability [4]. Microalgae are microscopic photosynthetic organisms which can be found in both marine and fresh water environments [5]. Microalgae are eukaryotic cells that consist of organelles, including nuclei and plastids. Proteins, carbohydrates, and lipid (natural oil) are included in the biomass. The percentage of chemical composition varies with the type of algae [6]. There are many varieties of microalgae and each species has a different proportion of lipids (fats), starch, and proteins. Depending on the proportion, the microalgae can be used to produce oil for bio crude or if, the variant contains more carbohydrates and less oil it can be fermented to make ethanol or biogas. It is interesting to note however that some microalgae strains or variants contain up to 50% lipids making them very suitable for production of liquid fuels [7]. Other oil crops, such as sunflower and soybean oil are different compared to microalgae which can grow rapidly.

The potential use of microalgae for biofuel production is promising because of its higher growth rate and capability to accumulate more amount of lipids [8] when compared to conventional oil crops [9]. The significant advantage of microalgae over plants is mainly due to their metabolic flexibility which means that variation in the biochemical composition of biomass towards higher (lipid, carbohydrates, or protein accumulation) can be regulated by varying the cultivation conditions [10] and therefore the yield of oil obtained per hectare from microalgae can exceed the yield from oil plants like rapeseed and palm. The use of residual nutrient sources and nutrient recycling is one of the key elements for obtaining sustainable production of biodiesel from microalgae.

Lipids are made up of functionally-diverse group of compounds [11]. For example, triacylglyceroles and some other neutral lipids associated with energy and carbon storage may be synthesized, preferentially, when the nutrients are presented in limiting conditions [12] and [13]. In contrast, many polar lipids are associated with various types of membranes and are likely to dominate the total lipid pool when light is limiting and/or nutrients are sufficient to support rapid growth [12].

Triacylglycerides (TAGs) generally serve as energy storage in microalgae and can be extracted and converted into biodiesel through transesterification reactions [7] and [14]. Transesterification displaces glycerol with small alcohols (e.g. methanol).

The hike in petroleum price and the need to reduce greenhouse gas emission has driven us for going towards the production of environmentally friendly product, such as biodiesel [15]. Based on the above-mentioned facts, the consideration of optimization growth condition to enhance the lipid production in *Chlorella sorokiniana* was conducted in this study. In order to achieve the objective of this study, different nitrogen sources, such as $NaNO_3$ and NH_4NO_3 have been used in the experiment to produce more lipid from the microalgae.

2. Materials and methods

2.1. Algal cultures

C. sorokiniana was obtained from Algaetech Ltd. Kuala Lumpur, Malaysia. The algae species were maintained in modified Bristol's medium (devoid of organic matter). The stock of *C. sorokiniana* was cultured in Proteose medium. Proteose medium contains sufficient amount of carbon, vitamins, salts, and other nutrients (nitrogen and phosphorous) which are vital for microalgal growth [16]. This medium was prepared according to the formulations available via the Culture Collection of Algae at The University of Texas at Austin. A volume of 10 ml *C. sorokiniana* was inoculated into 100-ml fresh medium (0.1% v/v) and was then cultured and expanded up to 5 L.

2.2. Growth condition

The growth rate of *C. sorokiniana* was compared using Proteose medium and BBM containing $NaNO_3$ as nitrogen source of different ratio (0.3, 0.9, 1.5, 3, 6, and 11 M) and NH_4NO_3 (0.2, 0.7, 1.5, 3.0, and 6.0 M). The cultures were maintained under fluorescent lamp with light intensity of 22.25 μmol m^{-2} S^{-1} for 16 h light: 8 h dark period which were setup via relay controller for all experiments carried out in this study. The pH of the medium was 7 and the temperature was 27˚C which was room temperature with manual swirling applied once a day.

2.3. Effect of nitrogen concentrations

Cultivation was also carried out in various different nitrogen concentrations by using sources of nitrogen, $NaNO_3$ and NH_4NO_3. The concentration of $NaNO_3$ varies, such as, 0.3, 0.9, 1.5, 3.0, 6.0, and 11.0 and the concentration of NH_4NO_3 varies, such as, 0.2,

0.7, 1.5, 3.0, and 6.0. Both nitrogen sources were initially cultivated with 300 ml of 10% (v/v) of microalgal inoculums and were added in 2,700 ml of Proteose medium as preparation for 3,000 ml of microalgal culture. The cultures were cultivated with fluorescence light at room temperature and agitated manually 3 times a day. The optical density (OD) was taken at 620 nm [17] and lipid content was analyzed by using the Nile red (NR) method every 3 days for 30 days.

2.4. Kinetic parameters

The calculation of oil productivity in microalgae is estimated by the mass of oil produced per unit volume of the microalgal broth per day. It is based on the microalgal growth rate and the oil content of the biomass. High oil productivities of microalgae are desired for producing biodiesel [7]. The specific growth rate (μ) was determined by using the following equation:

$$\mu = \frac{1}{t}\ln\left(X_m/X_0\right) \quad (1)$$

where X_m = concentration of biomass at the end of batch run; X_0 = concentration of biomass at the beginning of batch run, t = duration of batch run (h/d).

2.5. Measurement of lipid content-NR method

A fluorescence dye NR was used to stain the lipid found in the culture. The quantification of the cellular neutral lipid was carried out with fluorescent spectrophotometer (Hitachi F-4500) according to Griffiths et al. [18]. First, the NR stock was prepared by dissolving 0.5 mg of NR in 1 ml of acetone. Second, the phosphate buffer saline was prepared by dissolving 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate, and 0.24 g of potassium hydrogen phosphate in 1,000 ml of distilled water and autoclaved at 121℃ for 15 min. The pH of the solution was adjusted to 7.4.

3. Results and discussion

3.1. Selection of suitable species

This study mainly looked for fresh water microalgae which is found in the local fresh water ponds and also be able to grow easily in laboratory conditions. Lipid content was also one of the key factors of this study considered for selecting the species. *C. sorokiniana* was selected as a good species for this research work based on the published literature [19].

3.2. Growth curve of C. sorokiniana

For examining optimum stock culture, 10% (v/v) microalgal culture was used and it gave higher growth of microalgae, which was observed in terms of morphology—greener in color—and also from the result shown in Fig. 1, moreover 10% (v/v) of sample was found to be more suitable during the preliminary study (data not shown) to be used as the starting culture for high lipid production.

3.3. The various concentration effect of $NaNO_3$ on lipid content

The production of fatty acids by photosynthetic microalgae cultures has been studied by many previous researchers and it has been demonstrated that yields are sensitive to a number of environmental factors, including temperature, nitrogen concentrate, and light intensity [20–23]. This research was conducted to confer the importance of two different types of nitrogen composition for the growth of microalgae, *C. sorokiniana* using NR method. This is an important parameter as nitrogen limiting conditions proved to significantly increase the lipid content in many microalgae, especially *Chlorella* [24]. A total of six nitrogen concentrations using $NaNO_3$ as the source of *N* and the readings taken for OD at 620 nm and fluorescent spectrophotometer reading taken at 580 nm were employed: 0.3, 0.9, 1.5, 3, 6, and 11 M.

Fig. 2 and Table 1 illustrate that 0.3 M of nitrogen concentration was found with best growth on the 7th day in producing high biomass. From Fig. 2, it was

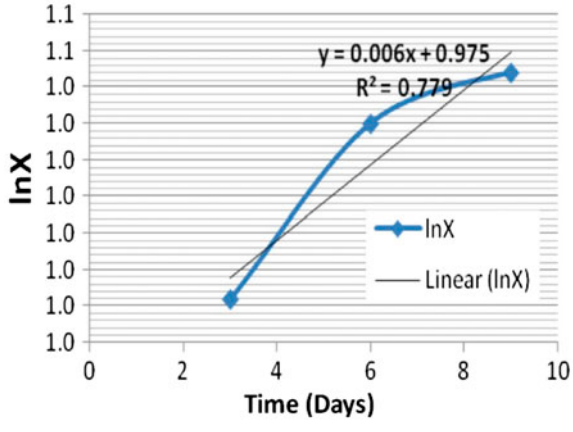

Fig. 1. Growth rate of microalgae.

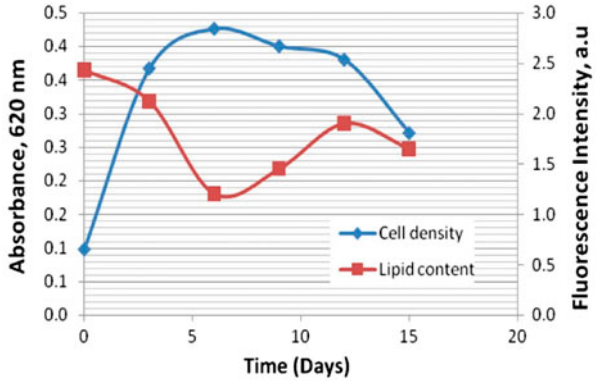

Fig. 2. The cell density and lipid content of *C. sorokiniana* at various time intervals for 0.3 M $NaNO_3$ taken.

Table 1
The specific growth rate for various nitrogen concentrations in $NaNO_3$

Nitrogen concentration (M)	Specific growth rate, μ	Highest lipid produced (a.u)
0.3	0.022	2.432
0.9	0.011	2.238
1.5	0.006	2.132
3.0	0.175	2.211
6.0	0.128	2.108
11.0	0.003	2.111

observed that 3.0 M of nitrogen concentration produced higher biomass compared to other nitrogen concentrations (0.3, 0.9, 1.5, 3, 6, and 11 M). The microalgae grew best when there was limited concentration of nitrogen which triggers high amount of carbohydrate and lipid per cell since protein biosynthesis was limited [25]. The culture in 0.3 M nitrogen concentration produced highest peak (2.432 a.u) on the first day compared to other nitrogen concentrations. Under nutrient limitation, the microalgae used most of their energy to make lipids as storage products for survival [6]. Hence, the lipid production was shown to increase at this period. The traditional gravimetric method to detect lipid in microalgae takes about 3–4 days and needs at least 10–15 mg wet weight of cells [26]. However, with fluorescent measurement by NR, the process of lipid measurement was more simple, rapid, and sensitive [27,28]. Lipid measurement was previously proposed using NR, a lipid-soluble fluorescent probe that possesses specific interaction with hydrophobic molecules [29]. The method has been used to estimate the lipid concentration in microalgae [30].

The specific growth rate of the culture was studied using different concentrations of $NaNO_3$ (0.3, 0.9, 1.5, 3, 6, and 11 M) and highest lipid produced (2.432 a.u) for 0.3 M nitrogen concentration as shown in Table 1.

3.4. The various concentration effects of NH_4NO_3 on lipid content

This research was conducted to confer the importance of nitrogen composition to the growth of microalgae, *C. sorokiniana*. This was an important parameter as nitrogen limiting conditions were proven to significantly increase the lipid content in many microalgae, especially *Chlorella* [24]. A total of five nitrogen concentrations using NH_4NO_3 as the source of N were tested: 0.2, 0.7, 1.5, 3.0, and 6.0 M. The lipid content was analyzed using NR method.

Table 2 shows that 0.7 M of nitrogen concentration has lowest growth rate with 0.002 μ. Although the growth was slow, the lipid content produced for 0.7 M was (3.077 a.u) in comparison with the highest lipid content produced (3.138 a.u) for 0.2 M NH_4NO_3. The slow growth could have been due to the cells adapting themselves to nutrient deficient environment. This condition could have been caused by the nitrogen starvation theory. As there is excess nitrogen concentration, the lipid production was being inhibited. Hence, they can grow as usual by using the nutrients in medium. According to Sheehan et al. [31], an increase in biomass concentration does not always increase the lipid content in microalgae. As the nitrogen limited stress condition leads to the inhibition of cell division, the oil production decreases slowly. The growth rate of microalgae in Fig. 3 shows that 6.0 M of nitrogen concentration was the highest. Huang et al. [2], proposed the fact that nitrogen sufficient medium enhances microalgal growth. As *C. sorokiniana* is a fast-growing and productive strain, these cells enhance high biomass concentration. The lipid content of animal cells and micro-organisms, such as mammalian cells is frequently evaluated by NR, a

Table 2
The specific growth rate and highest lipid amount for various nitrogen concentrations in NH_4NO_3

NH_4NO_3 concentration (M/l)	Specific growth rate, μ	Highest lipid produced (a.u)
0.2	0.011	3.138
0.7	0.002	3.077
1.5	0.020	3.000
3.0	0.034	2.771
6.0	0.088	2.430

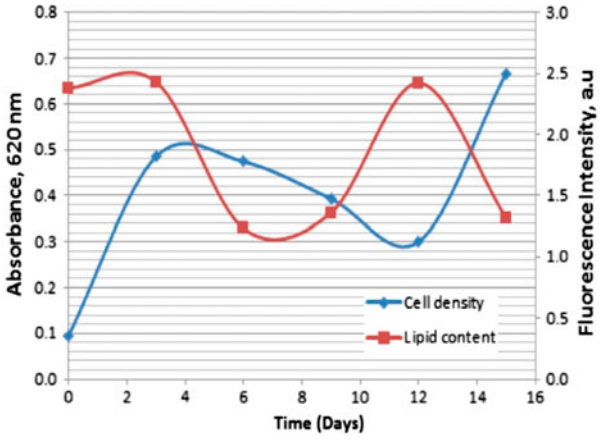

Fig. 3. The cell density and lipid content of *C. sorokiniana* at various time intervals for 6.0 M NH_4NO_3 taken.

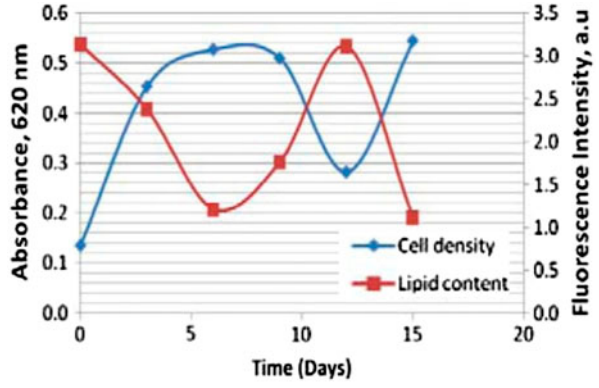

Fig. 4. The cell density and lipid content of *C. sorokiniana* at various time intervals for 0.2 M NH_4NO_3 taken.

lipid soluble fluorescent dye [32], which include other cells, such as bacteria [33], yeasts [30,34], zooplankton [35], and microalgae [36–38]. NR assay was usually carried out in individual cuvettes, using a UV spectrophotometer [39]. Hence, it can be concluded from the findings that the higher biomass do not contribute to high lipid concentration on a particular cell.

From Table 2 and Fig. 4, it is observed that 0.2 M of NH_4NO_3 concentration produced higher lipid content compared to different nitrogen concentrations which includes $NaNO_3$. The culture in 0.2 M nitrogen concentration produced highest peak (lipid content) at first and 12th day as compared to other nitrogen concentrations. The algae grew best when there was limited concentration of nitrogen which triggers high amount of carbohydrate and lipid per cell since protein biosynthesis was limited [25]. Under nutrient limitation, the algae will use most of their energy to make lipids as storage products for survival [6]. Hence, the lipid production was shown to increase at this period.

4. Conclusions

In the production of lipid by microalgae, *C. sorokiniana* was studied in this research. Regardless of the illumination scheme, the highest lipid content in microalgae cells was observed at 0.2 M of NH_4NO_3 concentration as compared to other cells cultivated in different nitrogen concentrations. As expected, these experiments indicated that N-deficient cultures will develop higher percentage lipid content than the N-sufficient cultures. These results proved that the growth of microalgal cells was influenced by variation of nitrogen sources and concentrations which were $NaNO_3$ and NH_4NO_3. The research was conducted at constant temperature and photoperiod. From the study, it was proven that nitrogen varieties could play a crucial role in lipid accumulation in microalgal cells. For future research, it may be considered as appropriate microalgae species for wastewater treatment such as palm oil mill effluent and also the output of this study maybe use as a by-product for lipid production purpose because microalgae can easily grow in palm oil mill effluent and is abundantly present throughout the year in Malaysia.

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References

[1] L. Fulton, Biomass and Agriculture Sustainability, Markets And Policies, International Energy Agency (IEA) Biofuels Study–Interim Report: Result and Key Messages So Far, IEA, France, OECD Publication Service, Washington, DC, 2004, pp. 105–112.
[2] G.H. Huang, F. Chen, D. Wei, X.W. Zhang, G. Chen, Biodiesel production by microalgal biotechnology, Appl. Energy 87 (2010) 38–46.
[3] P.K. Campbell, T. Beer, D. Batten, Life cycle assessment of biodiesel production from microalgae in ponds, Bioresour. Technol. 102(1) (2011) 50–56.
[4] L. Gouveia, C.A. Oliverira, Micro algae as a raw material for biofuel production, J. Ind. Microbiol. Biotechnol. 36 (2009) 269–274.
[5] H.C. Greenwell, L.M.L. Laurens, R.J. Shields, R.W. Lovitt, K.J. Flynn, Placing microalgae on the

biofuels priority list: A review of the technological challenges, J. R. Soc. Interface 36 (2010) 269–274.
[6] E.W. Becker, Microalgae. Biotechnology and Microbiology, Cambridge University Press, Cambridge, 1994.
[7] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. 25 (2007) 294–306.
[8] P.M. Schenk, S.R. Thomas-Hall, E. Stephens, U.C. Marx, J.H. Mussgnug, C. Posten, O. Kruse, B. Hankamer, Second generation biofuels: High-efficiency microalgae for biodiesel production, Bioenergy Res. 1 (2008) 20–43.
[9] H.M. Amaro, A.C. Guedes, F.X. Malcata, Advances and perspectives in using microalgae to produce biodiesel, Appl. Energy 88(10) (2011) 3402–3410.
[10] M.R. Tredici, Photobiology of microalgae mass cultures: Understanding the tools for the next green revolution, Biofuels 1(1) (2010) 143–162.
[11] M.I. Gur, Lipid Biochemistry. Chapman & Hall, New York, NY, 1991.
[12] C.C. Parish, P.J. Wangersky, Particulate and dissolved lipid classes in cultures of *Phaeodactylum tricornutum* grown in cage culture turbidostats with a range of nitrogen supply rates, Mar. Ecol. Prog. Ser. 35 (1987) 119–128.
[13] A.T. Lombardi, P.J. Wangersky, Influence of phosphorous and silicon on lipid class production by the marine diatom *Chaetoceros gracilis* grown in turbidostat cage cultures, Mar. Ecol. Prog. Ser. 77 (1991) 39–47.
[14] H. Fukuda, A. Kondo, H. Noda, Biodiesel fuelproduction by transesterification of oils, J. Biosci. Bioeng. 92 (2001) 405–416.
[15] Y.F. Chen, Production of Biodiesel from Algal Biomass: Current Perspectives and Future, Academic Press, Waltham, MA, 2011, p. 399.
[16] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications, Renew. Sustain. Energy Rev. 14 (2010) 217–232.
[17] S.J. Lee, B.D. Yoon, H.M. Oh, Rapid method for the determination of lipid from the green alga *Botryococcus braunii*, Biotechnol. Technol. 12 (1998) 553–556.
[18] M. Griffiths, S.T.L. Harrison, Lipid productivity as a key characteristic for choosing algal species for biodiesel production, J. Appl. Phycol. 21 (2009) 493–507.
[19] B.F. Cordero, I. Obraztsova, I. Couso, R. Leon, M.A. Vargas, H. Rodriguez, Enhancement of lutein production in *Chlorella sorokiniana* (Chorophyta) by Improvement of culture conditions and random mutagenesis, Mar. Drugs 9 (2011) 1607–1624, doi:10.3390/md9091607.
[20] M. Piorreck, K. Baasch, P. Pohl, Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes, Phytochemistry 2 (1984) 207–216.
[21] P. Pohl, H. Wagner, Control of fatty acid and lipid biosynthesis in *Eugleun agracilis* by ammonia, light and DCMU, Z. Naturforsch. 27 (1972) 53–61.
[22] Z. Cohen, A. Vonshak, A. Richmond, Effect of environ-mental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: Correlation to growth rate, J. Phycol. 24 (1988) 328–332.
[23] Y. Lee, H.M. Tan, Effect of temperature, light intensity and dilution rate on the cellular composition of the red alga *Porphyridium cruentum* in light-limited chemostat cultures, J. Appl. Microbiol. Biotechnol. 4 (1988) 231–237.
[24] A.M. Illman, A.H. Scragg, S.W. Shales, Increase in Chlorella strains calorific values when grown in low nitrogen medium, Enzyme Microb. Technol. 27 (2000) 631–635.
[25] Y. Suen, J.S. Hubbard, G. Holzer, T.G. Tornabene, Total lipid production of the green algae *Nannochloris sp*., Ql1 under different nitrogen regimes, J. Phycol. 23 (1987) 289–296.
[26] E.G. Bligh, W.J. Dyer, A rapid method for total lipid extraction and purification, Biochem. Physiol. 37 (1959) 911–917.
[27] K.E. Cooksey, J.B. Guckert, S. Williams, P.R. Callis, Fluorometric determination of the neutral lipid content of microalgal cells using Nile red, J. Microbiol. Methods 6 (1987) 333–345.
[28] J.C. Priscu, L.R. Priscu, A.C. Palmisano, C.W. Sullivan, Estimation of neutral lipid levels in Antarctic sea ice microalgae by Nile red fluorescence, Antarct. Sci. 2 (1990) 149–155.
[29] P. Greenspan, E.P. Mayer, S.D. Fowler, Nile red: A selective fluorescent stain for intracellular lipid droplets, J. Cell Biol. 100(3) (1985) 965.
[30] K. Kimura, M. Yamaoka, Y. Kamisaka, Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence, Microbiol. Methods 56 (2004) 331–338.
[31] J. Sheehan, T. Dunahay, J. Benemann, P. Roessler, A look back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae. Close-out report. National Renewable Energy Lab, Department of Energy, Golden, Colorado, USA, 1998.
[32] G. Genicot, J.L.M.R. Leroy, A. Van Soom, The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes, Theriogenology 63 (2005) 1181–1194.
[33] J. Izard, R.J. Limberger, Rapid screening method for quantitation of bacterial cell lipids from whole cells, Microbiol. Methods 55 (2003) 411–418.
[34] C.T. Evans, C. Ratledge, S.C.A. Gilbert, Rapid screening method for lipid accumulating yeast using a replica-printing technique, Microbiol. Methods 4 (1985) 203–210.
[35] Y. Kamisaka, N. Noda, T. Sakai, K. Kawasaki, Lipid bodies and lipid body formation in an oleaginous fungus, *Mortierella ramanniana* var. angulispora, Biochim. Biophys. Acta 1438 (1999) 185–198.
[36] K.M. McGinnis, T.A. Dempster, M.R. Sommerfeld, Characterization of the growth and lipid content of the diatom chaetoceros muelleri, Appl. Phycol. 9 (1997) 19–24.
[37] M.L. Eltgroth, R.L. Watwood, G.V. Wolfe, Production and cellular localization of neutral long-chain lipids in the haptophyte algae *Isochrysis galbana* and *Emiliania huxleyi*, Phycology 41 (2005) 1000–1009.
[38] D. Elsey, D. Jameson, B. Raleigh, M.J. Cooney, Fluorescent measurement of microalgal neutral lipids, Microbiol. Methods 68 (2007) 639–642.
[39] W. Chen, C. Zhang, L. Song, M. Sommerfeld, Q. Hu, A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae, Microbiol. Methods 77 (2009) 41–47.