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Study on the optimal mixotrophic and heterotrophic conditions for lipid accumulation of *Chlorella vulgaris*

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An investigation on productivity and lipid yield of *Chlorella vulgaris*, a freshwater unicellular alga belonging to the Chlorophyceae class, in a two-stage growing – autotrophic / mixotrophic culturing with manure used as a base for culturing medias and heterotrophic culturing with application of dimethylsulfoxide to induce "lipid trigger" is presented. Combinations of nitrogen starvation, organic substrate and toxicant (dimethylsulfoxide) application are designed to turn the metabolism into an anabolic lipid-accumulating phase. To identify best strategy to induce lipid accumulation for microalgae small and high dosages were evaluated. Regimes of autotrophy and heterotrophy with different light conditions, algal concentration and culturing media composition resulted in high lipid accumulation for *Chlorella vulgaris* (22,8–61 %), different lipid composition (i.e., polar or nonpolar) accumulated upon different regimes for culture (toxicant concentration) is highlighted.

Key worlds: microalgae; renewable biomass; husbandry wastes utilization; lipid production

Изучение оптимальных миксотрофных и гетеротрофных условий для накопления липидов культурами *Chlorella vulgaris*

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Исследование эффективности и урожайности липидов *Chlorella vulgaris*, пресноводной одноклеточной водоросли, принадлежащей классу *Chlorophyceae*, при двухэтапном культивировании – автотрофном/миксотрофном с использованием жидкой части навоза молочного скота в качестве основы для среды культивирования и гетеротрофном с применением диметилсульфоксида для индуцирования "липидного метаболизма" представлено в данной статье.

Комбинации дефицита азота, органического субстрата и применения токсиканта (диметилсульфоксида) обеспечивают изменения направления метаболизма в сторону анаболического липидного накопления. Для определения наилучшей схемы культивирования и индуцирования накопления липидов для микроводорослей использованы невысокие и высокие дозы. Режимы автотрофии/миксотрофии и гетеротрофии с различными условиями освещения, концентрацией водорослей и состава среды для культивирования обеспечивали разные уровни накопления липидов для *Chlorella vulgaris*, которые варьировали в пределах (22,8–61%). Различный липидный состав (процентное соотношение полярных и неполярных липидов), был получен при разных режимах культивирования, причем высокие дозы токсиканта способствовали накоплению полярных липидов.

Ключевые слова: микроводоросли; возобновляемая биомасса; утилизация стоков животноводства; получение липидов

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Вивчення оптимальних міксотрофних та гетеротрофних умов для накопичення ліпідів культурами *Chlorella vulgaris*

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Дослідження продуктивності та виходу ліпідів *Chlorella vulgaris*, прісноводної одноклітинної водорості, що належить до класу *Chlorophyceae*, при двостадійному культивуванні – автотрофному/міксотрофному культивуванні з коров'ячим гноєм, що використовувався як основа для культурального середовища та гетеротрофному культивуванні з застосуванням диметилсульфоксиду для індукування "ліпідного метаболізму" представлені у даній статті. Комбінації азотного голодування, наявності органічного субстрату та застосування токсиканту (диметилсульфоксиду) випробувані для спрямування метаболізму в анаболічну фазу для накопичення ліпідів. Для визначення найкращої стратегії індукування накопичення ліпідів для мікроводоростей оцінювалися малі та високі дози введення токсиканту. Режими автотрофного та гетеротрофого культивування відрізнялись умовами освітлення, стартовою концентрацією водоростей та композицією середовища для культивування, що забезпечували накопичення ліпідів клітинами *C. vulgaris* на рівні 22,8–61 %. Різний ліпідний склад (тобто полярні та неполярні ліпіди) накопичувались за різних режимів культивування та концентрацій токсиканту. Результати дослідження показали, що відсутність азоту не дозволяє підтримувати високу продуктивність культури через неможливість реалізації клітиною фундаментальних фізіологічних процесів та побудови клітинних структур. Замість цього обмеження азоту призводить до незбалансованого зростання, яке можна спостерігати при мікроскопії гетеротрофних культур – кількість клітин не збільшувалася, тоді як в обсязі з'являлися дуже великі клітини з внутрішніми краплями ліпідів. Результати цього дослідження узгоджуються з результатами інших дослідників, варіації були статистично значущими (F 4,25=3,71; p> 0,05), а отже, концентрація токсиканта впливала на накопичення ліпідів. Відповідно, мікроводорості при азотному голодуванні та дії диметилсульфоксиду збільшують культуральну біомасу, але не щільність клітин, що свідчить про наявність переходу до анаболічної активності – від синтезу білка та ДНК до накопичення ліпідів. Гетеротрофні культури демонструють незбалансований ріст та запасання ліпідів у вигляді тригліцеридів. Обмеження джерела вуглецю (гліцерину), як видається, індукує синтез неполярних ліпідів в умовах міксотрофного та гетеротрофного росту. Отримані результати показали, враховуючи продуктивність біомаси, продуктивність ліпідів, а також вміст неполярних ліпідів, для великомасштабного отримання ліпідів оптимальним є міксотрофне культивування *Chlorella vulgaris* з послідуючим гетеротрофним культивування в умовах дефіциту азоту і присутності високих доз диметилсульфоксиду.

Ключові слова: мікроводорості; відновлювана біомаса; утилізація стоків тваринництва; отримання ліпідів

Introduction

While a number of options have been proposed including grain based biofuels, cellulosic biofuels, oil shale's, natural gas to liquids, coal to liquids, etc., all have limitations on either cost effectiveness, long-term sustainability issues associated with large-scale development impacts on land and water resource availability, or overall carbon emissions (Li, Du & Liu, 2008).

Terrestrial culture are not so efficient in capturing solar energy, grow slowly, have low biomass yield and need fertilizers that generate NOx emission, reducing the greenhouse gasses saving (Ogbonna & Tanaka, 1998). This aspect, together, make unicellular algae promising feedstock for biodiesel production. In fact, when compared with superior plants microalgae show higher photosynthetic efficiency, higher biomass productivities and growth rates (Widjaja et al., 2009).

One approach being considered is the use of algae (Shnyukova & Zolotareva, 2017). Algae's biological capabilities in converting natural, renewable resources into a domestically produced liquid fuel has been well documented, all possible options for biomass conversion pathways are depicted on fig. 1.

The present investigation suggests that lipid rich algal biomass can be used as feedstock for bio- and thermo-chemical processes whereas the de-oiled cake for animal food and valuable bioactive substances extraction as well as for thermo-chemical conversion thereby serving the demand of second generation biofuels, process of production, and also can be successfully combined with wastewater treatment and greenhouse gases sequestr ation itself (llman et al., 2000).

Animal husbandry wastes can be good source of biogenic elements sufficient for growth of microalgae that can be successfully applied for controlled culturing and thus can be considered as a potential cheap source of nutrients for large scale systems (Praveen et al., 2016).

The dynamics of main biogenic elements uptake during growth of microalgae should be defined for each particular type of media based on animal wastes and each particular culture to obtain required knowledge for projection of large scale system operational parameters and potential for lipid generation when waste medias are used (Udom et al., 2013).

Investigation of best combinations of mixotrophic growth, and subsequent application of

organic substrate and toxicant dimethylsulfoxide (DMSO) application to increase lipid output through the blockage of the protein synthesis was the aim of the study.

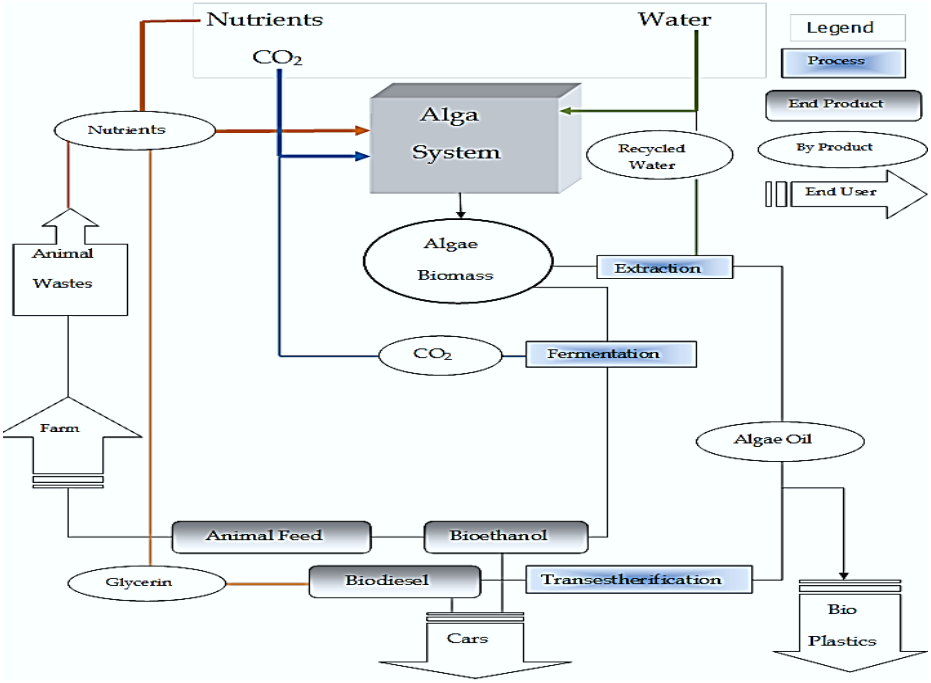

Fig. 1. Existing waste – to –biomass –to feedstock conversion pathways claimed to be promising for industry

Materials and methods

For this study species of microalgae *Chlorella vilgaris* was chosen, that claim to be able to generate lipids in certain conditions and thus promising for industrial application (Shifrin, 1984).

Chu 13 medium with following composition (per liter): 14,1 mM $NaNO_3$; 1,38 mM K_2HOP_4; 1.67 mM $MgSO_4$; 0.97 mM $CaCl_2 \cdot 2H_2O$; 0.52 mM cytric acid; 0.0001 mM $CoCl_2$; 0.0015 mM Zn_2SO_4; 0.00064 mM $CuSO_4$; 0.00035 mM Na2MoO4; was used as a baseline culturing media for biomass generation stage (Batches 3, 6, 8, 9), (Praveen et al., 2016).

Liquid part of dairy cattle manure was used as culturing media for algae as well (Batches 1, 2, 4, 5, 7, 10). Media was filtered through 3 micron nitrocellulose filter to remove colloidal organic particles and diluted to reduce turbidity (Udom et al., 2013). Diluted media was sterilized with UV to reduce bacterial activity and stabilize the media.

Inorganic nitrogen and phosphorous concentration and chemical oxygen demand for medias (Udom et al., 2013) were determined with generally accepted chemical methods recommended for waste water analysis.

Culturing of *Chlorella vulgaris* was performed in specially designed photobioreactor which duplicated natural solar conditions for culturing chambers – stable PAR on the illuminated wall of each chamber was equal to 37,8 moles per square meter per day.

Table 1
Nitrogen, phosphorous and dissolved organics concentration in medias used for *Chlorella vulgaris* culturing

Element	Concentration in liquid phase of dairy cattle manure (mg/l)	Concentration in B3 culturing media (mg/l)
N (in form of NO_3)	40	246.4
N (in form of NO_2)	23	not present
N (in form of NH_4)	37	0.42
P (in form of PO_4)	13	70.5
COD	6465	not present

High-pressure sodium vapor lamp for open and close luminaries "Planstar-T" Osram with nominal wattage 250 W was used as a light source.

Five chambers were inoculated with different amount of algae culture (in two chambers culturing was performed on B3 media and in three chambers liquid phase of dairy cattle manure based media was used) in two repeats. For each chamber start volume was 700 ml.

Culturing was performed as fed – batch-a portion of culturing media was added every three hours. Such method provided more stable conditions for culturing as our previous studies showed. Added portions volumes were counted from projected growth rate data obtained during cell per-culture to support

stable cell concentration of cells in chambers during growth.

Temperature of media was maintained on average 22º C, pH was controlled to maintain stable concentration of inorganic carbon in the culture medium – once pH raised CO_2 vent was opened to keep pH in diapason 6,7–6,9 which was in compliance with concentration 12–25 mg/l of CO_2.

The volumetric output of the biomass (in millions of cells produced per milliliter of volume per hour) and add of biomass, a measures of culture productivity were controlled during culturing.

Cell counting was performed with hemacytometer. Three countings were performed for each sample and average meaning was determined after. Dry cell mass was determined as total suspended solids, samples with volume 5–20 ml were filtered through microbial cellulose filters (3 micron pore size).

Duplicate samples were collected at 12-h intervals, and dry biomass concentration, cell concentration, pH, temperature was determined.

From biomass generation (authotrophic/mixotrophic) stage biomass was harvested in the middle of exponential growth and transferred to heterotrophic stage to induce lipid accumulation in algae cells, culturing was performed without light in 3 L glass vessels within three days. Glycerin was used as carbon source for biomass during heterotrophic stage, inorganic phosphorous was added to maintain same level as for autotrophic/mixotrophic stage. To inhibit nitrogen metabolism of cells dimethylsulphoxide was added, which proved to be toxic for biomass (inhibition of photosynthesis and nitrogen metabolism). After heterotrophic stage biomass was harvested and dewatered. During dewatering volume of biomass suspension was reduced 10–20 times and drying till constant weight was performed after. Dried biomass with was immediately grinded for extraction process.

Lipid analysis consisted of extraction and gravimetric lipid quantitation. To this aim from 1 gr of the dried biomass sample lipids were continuously extracted (by sohxlet) with 100 mL 2/1/1 (v/v) dichloromethane/gasoline/methanol solution.

The resulting solution was evaporated and total lipids were determined gravimetrically (Widjaja et al., 2009).

At the end of the extraction process, which typically lasted 15 -17 hours, the flask containing the solvent and lipid is removed, the solvent is evaporated and the mass of lipid remaining is measured (M_{lipid}). The percentage of lipid in the initial sample (M_{sample}) was calculated:

$$\% \text{ Lipid} = 100 \times (M_{\text{lipid}}/M_{\text{sample}})$$

The lipids dried sample was then resuspendend in 30 mL hexane with 0.1 ml of distilled water added (to induce swallow of polar lipids), carefully shaken and settled for 24 hours. The supernatant solution was withdrawn and the solid residue treated other two times in the same manner (llman et al., 2000). All the withdrawn hexane solutions were mixed, evaporated and dried under vacuum and the remaining substance was weighed as nonpolar lipids. The solid residue after centrifugation was dried under vacuum and weighed as polar lipids.

Results and discussion

This preparatory evidence pointed out the need of light as signal for cellular duplication and the synergic effect of respiration and photosynthesis, developed during mixotrophic growth. In the adopted experimental conditions in mixotrophic regimen 1 gram of *Chlorella vulgaris* biomass produced 1,2–1,8 million cells/h, of 6–8 % of dry weight. Although nitrogen and phosphorous rates and nitrogen to phosphorous ratio were lower for dairy cattle waste based media, macroelements coming from manure could had higher biological availability than synthetic salts.

Growth rate determined in this study is an integrated value thus equal value of growth rate for *Chlorella vulgaris* with cattle manure based media also could be results of more stable nitrogen and phosphorus concentrations in the media provided by chelating effect of dissolved organic matter contained in cattle manure (fig. 2, 3).

All the four nutrient limiting conditions performed in mixotrophy were analyzed for cellular growth and biomass and lipid productivity. As shown in Table 2 the highest dosage of glycerol and toxicant provided the highest algal lipid proliferation (Widjaja, Chien и Ju 2009). On the contrary, simple nitrogen deprivation was also inducing lipid generation but overall lipid productivity was significantly lower. The combined nitrogen starvation, carbon substrate and toxicant (dimethylsulfoxide) resulted in the more than 41–61 % lipid yield.

The biomass productivity shows how the complete deprivation of nitrogen or phosphorus do not support high productivity, reasonably for the impossibility to develop a variety of fundamental physiological processes and cellular structures. The double limitation instead give the highest productivity, showing an unbalanced growth, which could be observed during microscopy – cells number was not increasing while very big cells with lipid droplets inside were appearing in the volume. The results of this study may agree more with the findings of other group that significance of change was slight but variations were statistically significant ($F\ 4{,}25 = 3{,}71$; $p > 0{,}05$), which means concentration of toxicant did affected lipid accumulation.

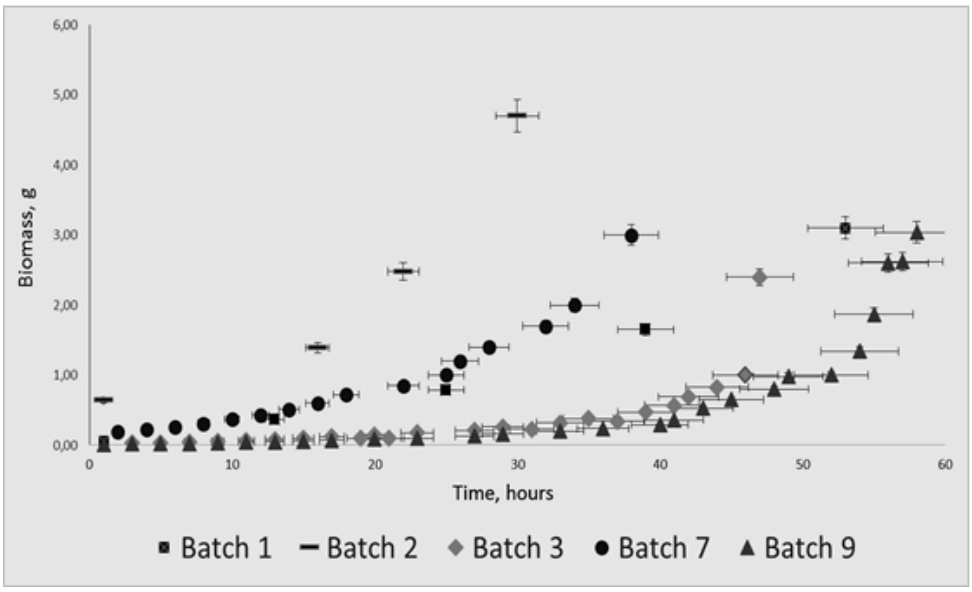

Fig. 2. *Chlorella vulgaris* autotrophic/mixotrophic growth

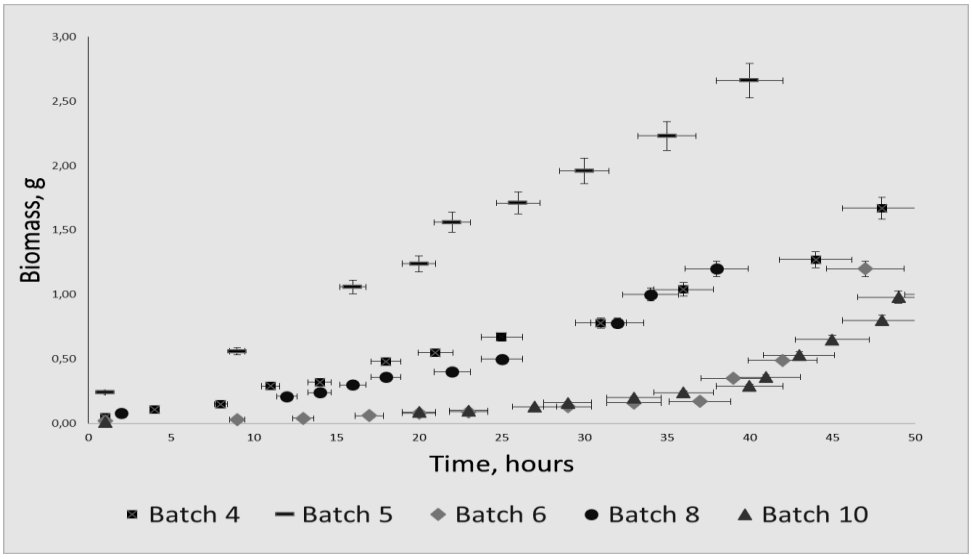

Fig. 3. *Chlorella vulgaris* autotrophic/mixotrophic growth

Table 2
Heterotrophic growth conditions and lipid yield from *Chlorella vulgaris* cultures

Batch	Growth rate of culture	Biomass weight (for extraction) (g)	Lipid content %	Autotrophic/ Mixotrophic stage substrate	Heterotrophic stage duration (days)	Concentration of carbon substrate and toxicant in heterotrophic stage culturing medium
1	0,03	3.082	46	Dairy manure	1	Glycerin: 12 g/L; Dimethylsulfoxide: 12 g/L
2	0,02	4.63	47	Dairy cattle manure	1	Glycerin: 12 g/L; Dimethylsulfoxide: 12 g/L
3	0,017	2.53	41	B3	1	Glycerin: 12 g/L; Dimethylsulfoxide: 12 g/L
4	0,01	1.2	37	Dairy cattle manure	1	Glycerin: 4 g/L; Dimethylsulfoxide: 0,1 g/L
5	0,02	1.9	41.3	Dairy cattle manure	1	Glycerin: 4 g/L; Dimethylsulfoxide: 0,1 g/L
6	0,02	1.18	22.8	B3	2	Glycerin: 4 g/L; Dimethylsulfoxide: 0,1 g/L
7	0,008	2.77	46.9	Dairy cattle manure	2	Glycerin: 12 g/L; Dimethylsulfoxide: 12 g/L
8	0,016	2.73	30.4	B3	2	Glycerin: 4 g/L; Dimethylsulfoxide: 0,1 g/L
9	0,033	2.19	41	B3	2	Glycerin: 12 g/L; Dimethylsulfoxide: 12 g/L
10	0,027	1.72	61	Dairy cattle manure	3	Glycerin: 12 g/L; Dimethylsulfoxide: 12 g/L

Table 3

Lipids characterization for different culturing conditions

A	Glycerin: 12 g/L; DSMO: 12 g/L	Glycerin: 4 g/L; DSMO: 0,1 g/L	Gl: 4 g/L; DSMO: 0,1 g/L	Glycerin: 12 g/L; DSMO: 12 g/L	A – Autotrophy H - Heterotrophy	
T, %	41	22,8	30,4	41	T - Total lipids;	
P, %	30,7	6,6	20,1	29,9	P – Polar lipids;	
N, %	10,3	14,2	10,3	11,1	N- Nonpolar lipids.	
H	Glycerin: 12 g/L; DSMO: 12 g/L	Glycerin: 12 g/L; DSMO: 12 g/L	Glycerin: 4 g/L; DSMO: 0,1 g/L	Glycerin: 4 g/L; DSMO: 0,1 g/L	Glycerin: 12 g/L; DSMO: 12 g/L	Glycerin: 12 g/L; DSMO: 12 g/L
T, %	46	47,3	37,2	41,3	46,9	61
P, %	36,1	13,8	13,5	16,6	35,3	36,9
N, %	10,9	33,5	24,7	24,7	11,6	24,1

That means microalgae were accumulating substrates that increase the culture biomass but not the cells density, evidencing the possibility to switch the anabolic activity from the protein and DNA synthesis to the lipid accumulation.

The nitrogen deprivation, or limitation in mixotrophic growth conditions, causes an accumulation of polar lipids not suitable for biodiesel production. The same application of the toxicant dimethylsulfoxide during heterotrophic growth causes a more balanced distribution of lipids. In mixotrophic conditions, in fact, the cultures show lower cellular density with cells of little size, due to lower concentration of biogens elements thus volumetric output is lower, but higher growth rate. Heterotrophic cultures instead show a not balanced growth and are formed by a few big cells, in which the lipids are stocked as triglycerides.

Conclusions

Dairy cattle manure, as study demonstrated, can be used as a source of nutrients for biomass generation in microalgae lipid production chain. Main biogens from dairy wastes are more biologically available than synthetic salts.

The carbon source (glycerol) limitation, appear to induce the synthesis of nonpolar lipids in both mixotrophic and heterotrophic growth conditions.

The obtained results clearly show, considering both biomass and lipid productivity and lipid nonpolar content that, for larger scale lipid production from *Chlorella vulgaris* cultures the best option appears to be mixotrophic nitrogen limited growth conditions with application of dimethylsulfoxide.

References

Honda, R., Rukapan, W., Komura, H., Teraoka, Y., Noguchi, M., & Hoek, E. M. V. (2015). Effects of membrane orientation on fouling characteristics of forward osmosis membrane in concentration of microalgae culture. *Bioresource Technology*, 197, 429–433.

Larronde-Larretche, M., & Jin, X. (2017). Microalgal biomass dewatering using forward osmosis membrane: Influence of microalgae species and carbohydrates composition. *Algal Research*, 23, 12–19.

Li, Q., Du, W., & Liu, D. (2008). Perspectives of microbial oils for biodiesel production. *Applied Microbiology and Biotechnology*, 80(5), 749–756.

Li, Y., Horsman, M., Wang, B., Wu, N., & Lan, C. Q. (2008). Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. *Applied Microbiology and Biotechnology*, 81(4), 629–636.

Illman, A., Scragg, A., & Shales, S. (2000). Increase in Chlorella strains calorific values when grown in low nitrogen medium. *Enzyme and Microbial Technology*, 27(8), 631–635.

Ogbonna, J. C., & Tanaka, H. (1998). Cyclic autotrophic/heterotrophic cultivation of photosynthetic cells: A method of achieving continuous cell growth under light/dark cycles. *Bioresource Technology*, 65(1-2), 65–72.

Praveen, P., Heng, J. Y. P., & Loh, K.-C. (2016). Tertiary wastewater treatment in membrane photobioreactor using microalgae: Comparison of forward osmosis & microfiltration. *Bioresource Technology*, 222, 448–457.

Shnyukova, E. I., & Zolotareva, E. K. (2017). Ecological Role of Exopolysaccharides of Bacillariophyta: A Review. *International Journal on Algae*, 19(1), 5–24.

Udom, I., Zaribaf, B. H., Halfhide, T., Gillie, B., Dalrymple, O., Zhang, Q., & Ergas, S. J. (2013). Harvesting microalgae grown on wastewater. *Bioresource Technology*, 139, 101–106.

Widjaja, A., Chien, C.-C., & Ju, Y.-H. (2009). Study of increasing lipid production from fresh water microalgae Chlorella vulgaris. *Journal of the Taiwan Institute of Chemical Engineers*, 40(1), 13–20.