Heterotrophic and Mixotrophic Cultivation of *Chlorella vulgaris* using Chicken Waste Compost as Nutrients Source for Lipid Production

**Hong Li Tan**¹², **Man Kee Lam**¹²*, **Yoke Wang Cheng**², **Jun Wei Lim**²³, **Inn Shi Tan**⁴, **Chee Yew Henry Foo**⁴ and **Pau Loke Show**⁵

¹ Department of Chemical Engineering, Universiti Teknologi PETRONAS, 32610 Seri Iskandar, Perak, Malaysia.
² HICoE-Centre for Biofuel and Biochemical Research, Universiti Teknologi PETRONAS, 32610 Seri Iskandar, Perak, Malaysia.
³ Fundamental and Applied Sciences Department, Universiti Teknologi PETRONAS, 32610 Seri Iskandar, Perak, Malaysia.
⁴ Department of Chemical Engineering, Curtin University, Sarawak Campus CDT 250, 98009 Miri, Sarawak, Malaysia.
⁵ Department of Chemical and Environmental Engineering, Faculty of Science and Engineering, University of Nottingham Malaysia, Jalan Broga, 43500 Semenyih, Selangor, Malaysia.

Email: lam.mankee@utp.edu.my

**Abstract.** Microalgae have received global attention for the past decades as it shows promising results to be an alternative and sustainable energy resource due to their high growth rate and lipid production. In commercial microalgae cultivation, autotrophic method is always used to grow the microalgae. However, this method usually produces high biomass yield but low lipid content. One of the approaches to enhance the microalgae lipid yield is through heterotrophic and mixotrophic method, in which dark environment and organic carbon are introduced as a stress factor to induce the lipid productivity. In the present study, cultivations of microalgae were done using chicken compost and glucose under autotrophic, heterotrophic and mixotrophic conditions. The highest absorbance attained for both heterotrophic and mixotrophic cultivation conditions were 1.650 and 2.184 respectively when 1.0 g/L of glucose was used. Absorbance and biomass are correlated, therefore the absorbance in this study signifies the amount of biomass produced. Moreover, the highest lipid yield was successfully attained at 45 wt% under mixotrophic condition. Overall, the lipid yield of microalgae cultivated under mixotrophic was higher than the heterotrophic condition when compost derived from chicken waste was used as nutrients source.

1. **Introduction**

For the past decades, many research efforts have been carried out to meet the increasing energy demand, aiming at enhancing energy supply by renewable and clean sources while substituting the energy dependence on the depleting fossil fuel [1]. Moreover, finding clean and renewable energy sources appear to be the most challenging problem as consideration have to put on aspects such as economic development and prosperity, global stability, standard of life and long-term strategies [2]. Among the renewable energy, microalgae have received widespread attention to be a valuable biofuel feedstock as microalgae have great potential to produce vast amount of biomass and lipids which is needed for biodiesel production [3,4]. Besides, microalgae is a better alternative to the other
oleaginous seed plants as microalgae has higher oil yields per hectare and have benefit of lesser competition for agricultural land [5]. Moreover, microalgae can provide oil content ranging from 20-50% of dry cell weight compared to other agricultural oil crops such as soybean and oil palm which are widely being used to produce biodiesel and yet producing less than 5% of total biomass basis [5,6]. In addition, microalgae postulated to produce about 200 barrels of algal oil per hectare of land annually, which is 100-fold better than soybeans which is a feedstock being used for biodiesel in the USA [6].

Although microalgae derived biofuels have been considered a promising sustainable production, it has yet to overcome the operating cost [7]. In addition, microalgae can be cultivated under autotrophic, heterotrophic and mixotrophic conditions. Microalgae under autotrophic performs photosynthesis to grow, therefore light is essential [8]. Furthermore, thorough mixing is needed for effective light penetration in this cultivation and this would pose a problem for large-scale production as cultivating in open ponds or photobioreactor eventually becomes light limited [8,9].

On the other hand, the heterotrophic and mixotrophic cultivation can be carried out in dark condition while producing higher amount of biomass and lipid contents than autotrophic cultivation [10]. The setback is the cost required to meet the requirement of organic carbon which comprises about 80% of the total medium cost [11]. Therefore, cheaper alternatives such as sewage water, industrial wastewater or effluents have been investigated by researchers and proven to be able to provide the necessary nitrogen, phosphorus and other minerals [7,10]. Nevertheless, the nutrient in wastewater is inconsistent and therefore it is relatively difficult to maintain same nutrient concentration in every batch of cultivation.

On the contrary, chicken manure which is rich in nitrogen, phosphorus and potassium is also an alternative for organic carbon [12]. However, chicken manure consists of high amount of ammonia, bacteria and pathogens that potentially contaminate microalgae culture. Although both heterotrophic and mixotrophic cultivation conditions seem promising by providing higher amount of lipid contents, but microalgae are very sensitive and vulnerable to contamination [13]. Hence, composted chicken manure will be a better choice.

A large-scale microalgae farming infrastructure which requires high capital cost and intensive care compared to conventional agricultural farm is the major hurdle for commercialization of microalgae biofuels [14]. In addition, chicken compost may significantly enhance the overall cost effectiveness while being environmentally friendly by reducing the abundant amount of livestock waste. Moreover, the study of cultivating in medium made of waste nutrient like composted chicken manure together with an organic carbon is still lacking in literature. Therefore, this present work was aimed to study the effect of mixotrophic and heterotrophic cultivation conditions with respect to their growth performance and lipid yield when chicken compost was used as nutrient source.

2. Methods

2.1. Microalgae strain and culture conditions
Freshwater C. vulgaris was obtained from Prof. Lee Keat Teong (School of Chemical Engineering, Universiti Sains Malaysia). The strain was cultivated in Bold's Basal Medium (BBM) [15]. The initial pH of the medium was adjusted to the range of 3.0–3.5. The strain was cultured and maintained in a 5 L Duran bottle at 25 ± 3 °C under aeration with atmospheric pressure and cool-white fluorescent tubes with constant lighting (60 - 70 μmol m⁻² s⁻¹).

2.2. Nutrient preparation
Chicken compost was purchased from the local market. 10 g of chicken compost was added into 600 mL of tap water in a 1 L beaker and stirred for 24 hours continuously at 700 rpm using a magnetic stirrer under room temperature. After the stirring process, the solution was centrifuged to remove the excess non-soluble particulate solids at 4000 rpm.

2.3. Experimental design
Chicken compost was used as the main source of nutrient in this study to explore the effect of heterotrophic and mixotrophic cultivation conditions towards growth and lipid yield on microalgae. Growth under autotrophic cultivation was set as the control. Furthermore, 1 L cultures were grown under autotrophic cultivation (40 mL of chicken compost, aerated with atmospheric pressure under cool-white fluorescent tubes with light intensity of 60 - 70 μmol m$^{-2}$s$^{-1}$); heterotrophic cultivation (40 mL of chicken compost, 1.0 g of glucose, placed in fully covered incubator shaker under continuous shaking of 170 rpm at room temperature); and mixotrophic cultivation (40 mL of chicken compost, 1.0 g of glucose under dark/light cycle of 16/8 hours, during dark cycle same as heterotrophic, during light cycle same as autotrophic).

2.4. Analytic methods

2.4.1. pH measurement. The pH of each culture was measured using pH meter (OHAUS Starter 3100) and adjusted every day if necessary. It was to ensure that the pH value was within the pH 3 – 3.5. Furthermore, 1 M sulfuric acid and 1 M sodium hydroxide were used to decrease and increase the pH to determined value respectively using a dropper. Distilled water was used to rinse the pH sensor before taking measurement each time.

2.4.2. Absorbance measurement. The absorbance of each microalgae culture sample was measured at 688 nm by using UV-VIS Spectrophotometer (SHIMADZU UV-1280) to monitor the growth of microalgae every day. The blank sample was distilled water. Furthermore, each sample was measured in triplicates to obtain the average result. Distilled water was used to rinse and clean the cuvettes before starting and after finishing each test.

2.4.3. Biomass measurement. For biomass analysis, settled microalgae were harvested into a container and dried in the oven at 105.5 ºC for 24 hours. The weight of the container before and after drying were measured. The biomass of culture was calculated as

\[
DW_f - DW_i
\]

Where \( DW_f \) is final drying weight and \( DW_i \) is initial drying weight.

2.4.4. Lipid contents. The extraction of lipids from microalgae biomasses were performed using the Bligh and Dyer (1959) method with modification [16]. 0.2 g of the grinded biomass was placed into a 250 mL beaker containing 60 mL mixture of methanol : chloroform (2:1, v/v). The mixture was then stirred for 24 hours at room temperature using magnetic stirrer. After stirring, the mixture was filtered into a vial using filter paper and the vial was purged by air for 12 hours in the fume hood. Further drying of the filtrate was continued in the oven at 105.5 ºC for 24 hours. The weight of the vial before and after drying were measured and quantified gravimetrically as lipid percentage on a dry weight basis.

3. Results

3.1. Microalgae growth

Figure 1. shows the growth curve of microalgae throughout 14 days of cultivation under autotrophic (control), heterotrophic and mixotrophic conditions. The growth of \( C. vulgaris \) was strongly affected by different type cultivation conditions. Besides, it can be observed that \( C. vulgaris \) growth was almost linear when it was cultivated under autotrophic condition. Moreover, the highest absorbance was attained on day 14th for all cultivation conditions by the control (2.212), but the absorbance under mixotrophic cultivation (2.184) was significantly higher than heterotrophic (1.650).

Since the absorbance and biomass concentration are correlated, therefore the trend for biomass growth should be the same as the absorbance [15]. The biomass yield at the end of this experiment by autotrophic, mixotrophic, and heterotrophic conditions were 0.59 g/L, 0.45 g/L and 0.43 g/L respectively. The higher growth under mixotrophic compared to heterotrophic condition was mainly
due to the ability of photosynthesis and dark respiration during the light and dark cycle respectively [17]. Not all strain of microalgae can be grown and adapted in total darkness rapidly [18]. However, this experiment shows that this strain of *C. vulgaris* can grow in the mixture of chicken compost and organic carbon while being cultivated in both dark and light conditions.

![Figure 1. Growth of *Chlorella vulgaris* under different cultivation method.](image)

### 3.2. Lipid yield
Percentage of lipid yield at different cultivation conditions is shown in Table 1. From the table, it can be clearly observed that the highest percentage of lipid yield by *C. vulgaris* was under mixotrophic condition where it obtained 45 wt% whereas heterotrophic and autotrophic conditions obtained only 30 wt% and 16 wt% respectively. In addition, Ge et al. had studied the biomass and lipid production from cultivation of *C. vulgaris* with 1.8 L of wastewater and different doses of glycerol under heterotrophic and mixotrophic cultivation conditions in photobioreactors [19]. From the study, the lipid yield from using single-dose initial glycerol (2.86 ml) feeding in both mixotrophic and heterotrophic cultivations were 22.7 ± 2.5 wt% and 10.8 ± 0.8 wt% respectively [19]. Moreover, the highest lipid yield attained was 29.4 ± 4.9 wt% under mixotrophic condition with single-dose exponential glycerol feeding [19]. Besides, Liang et al. had investigated the biomass and lipid productivity of *C. vulgaris* under heterotrophic and mixotrophic growth conditions using 1 g/L of glucose as organic carbon [20]. The lipid yield through heterotrophic and mixotrophic conditions were 23 ± 2 wt% and 21 ± 1 wt% respectively [20]. In summary, the lipid contents obtained through both mixotrophic and heterotrophic methods from this experiment were higher compared to the studies assessed. Furthermore, the overall lipid yield achieved under mixotrophic (0.20 g/L) and heterotrophic (0.13 g/L) in this experiment were significantly higher than the control (0.10 g/L). This indicates that under mixotrophic or heterotrophic conditions, this strain of *C. vulgaris* can produce higher amount of lipid compared to the autotrophic condition. This is because microalgae use aerobic glycolysis process to breakdown glucose and only Embden-Meyerhof pathway (EM pathway) and the Pentose Phosphate pathway (PP pathway) were found in microalgae [21]. In addition, these metabolic pathways were used for assimilation of carbon and production of energy, the EM and PP pathways are used mainly with and without light respectively. Moreover, the Calvin cycle shows that with the assimilation of glucose, more carbon source or energy is being funnelled into lipid biosynthesis [21, 22].
photosynthesis during the day, more carbon is produced, leading to more lipid production. Therefore, the overall lipid yield in mixotrophic growth is higher than heterotrophic growth.

Table 1. Percentage of lipid yield of microalgae cultivated under different cultivation method.

| Cultivation Conditions | Percentage of Lipid Yield (wt %) |
|------------------------|----------------------------------|
| Autotrophic            | 16                               |
| Heterotrophic          | 30                               |
| Mixotrophic            | 45                               |

4. Conclusion
The findings from this study demonstrated that this strain of *C. vulgaris* was able to utilize chicken compost as nutrient source for its growth. Mixing chicken compost and organic carbon for cultivation under heterotrophic and mixotrophic can increase the efficiency of the production of biodiesel from microalgae. In this study, mixotrophic cultivation produce higher absorbance and biomass than heterotrophic cultivation, 2.184 and 0.45 g/L respectively. The results were acceptable as the lipid yield of both heterotrophic (30 wt%) and mixotrophic (45 wt%) growth conditions were higher compared to Liang et al. where theirs were 23 ± 2 wt% and 21 ± 1 wt% respectively while cultivating *C. vulgaris* using 1 g/L of glucose as organic carbon. Although cultivating under autotrophic (control) attained the highest biomass yield at 0.59 g/L, nevertheless the highest overall lipid yield was 0.20 g/L under mixotrophic condition. Lastly, chicken compost may very well be the solution for a cost effective and environmentally friendly large-scale production of microalgae biodiesel.

5. References
[1] Baldisserotto C, Popovich C, Giovanardi M, Sabia A, Ferroni L, Constenla D, et al. Photosynthetic aspects and lipid profiles in the mixotrophic alga Neochloris oleoabundans as useful parameters for biodiesel production. *Algal Research*. 2016;16:255-65.
[2] Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*. 2010;14(1):217-32.
[3] Borowitzka MA, Moheimani NR. Sustainable biofuels from algae. *Mitigation and Adaptation Strategies for Global Change*. 2013;18(1):13-25.
[4] Ho S-H, Chen C-Y, Chang J-S. Effect of light intensity and nitrogen starvation on CO₂ fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N. *Bioresource Technology*. 2012;113:244-52.
[5] Chisti Y. Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology*. 2008;26(3):126-31.
[6] Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, et al. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal*. 2008;54(4):621-39.
[7] Lowrey J, Brooks M, McGinn P. Heterotrophic and mixotrophic cultivation of microalgae for biodiesel production in agricultural wastewaters and associated challenges - A critical review. *Journal of Applied Phychology*. 2014;27.
[8] Dickinson S, Mientus M, Frey D, Amini-Hajibashi A, Ozturk S, Shaikh F, et al. A review of biodiesel production from microalgae. *Clean Technologies and Environmental Policy*. 2016;19.
[9] Chini Zittelli G, Biondi N, Rodolfi L, Tredici M. Photobioreactors for Mass Production of Microalgae. 2013. p. 225-66.
[10] Ebrahimian A, Kariminia H-R, Vosoughi M. Lipid production in mixotrophic cultivation of Chlorella vulgaris in a mixture of primary and secondary municipal wastewater. *Renewable Energy*. 2014;71:502-8.
[11] Engin İK, Cekmecelioglu D, Yücel AM, Oktém HA. Evaluation of heterotrophic and mixotrophic cultivation of novel Micractinium sp. ME05 on vinasse and its scale up for
biodiesel production. *Bioresource Technology*. 2018;251:128-34.

[12] Tan XB, Uemura Y, Lim JW, Lam MK. Cultivation of Chlorella vulgaris in photobioreactor by using compost as a nutrient source for biomass production. *Journal of Fundamental and Applied Sciences* 9 (6S). 2017;288-297

[13] Chen C-Y, Yeh K-L, Aisyah R, Lee D-J, Chang J-S. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology*. 2011;102(1):71-81.

[14] Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N. Biofuels from Microalgae. *Biotechnology Progress*. 2008;24(4):815-20.

[15] Lam MK, Lee KT. Potential of using organic fertilizer to cultivate Chlorella vulgaris for biodiesel production. *Applied Energy*. 2012;94:303-8.

[16] Bligh EG, Dyer WJ. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology*. 1959;37(8):911-7.

[17] Bassi A, Saxena P, Aguirre A-M. Mixotrophic Algae Cultivation for Energy Production and Other Applications. In: Baijai R, Prokop A, Zappi M, editors. *Algal Biorefineries: Volume 1: Cultivation of Cells and Products*. Dordrecht: Springer Netherlands; 2014. p. 177-202.

[18] Lage S, Kudahettige NP, Ferro L, Matsakas L, Funk C, Rova U, et al. Microalgae Cultivation for the Biotransformation of Birch Wood Hydrolysate and Dairy Effluent. *Catalysts*. 2019;9(2):150.

[19] Ge S, Qiu S, Tremblay D, Viner K, Champagne P, Jessop PG. Centrate wastewater treatment with Chlorella vulgaris: Simultaneous enhancement of nutrient removal, biomass and lipid production. *Chemical Engineering Journal*. 2018;342:310-20.

[20] Liang Y, Sarkany N, Cui Y. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters*. 2009;31(7):1043-9.

[21] Bashan Y, Perez-Garcia O. Microalgal Heterotrophic and Mixotrophic Culturing for Bio-refining: From Metabolic Routes to Techno-economics. 22015. p. 61-131.

[22] Chen H, Zheng Y, Zhan J, He C, Wang Q. Comparative metabolic profiling of the lipid-producing green microalga Chlorella reveals that nitrogen and carbon metabolic pathways contribute to lipid metabolism. *Biotechnology for Biofuels*. 2017;10(1):153.

**Acknowledgments**

The authors would like to acknowledge the financial support received from Universiti Teknologi PETRONAS (YUTP-FRG with cost centre 015LC0-192). Financial support from Ministry of Higher Education Malaysia through HICoE award to CBBR (cost centre 015MA0-052) and Fundamental Research Grant Scheme (FRGS/1/2019/TK02/CURTIN/03/2) are duly acknowledged.