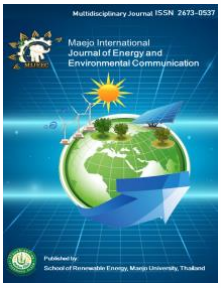
Maejo International Journal of Energy and Environmental Communication

Journal homepage: https://ph02.tci-thaijo.org/index.php/MIJEEC



ARTICLE

Autotrophic production of bio-functional proteins from freshwater microalgae using natural water medium for an economical and ecofriendly approach

David D-W. Tsai[1], Rameshprabu Ramaraj[2,3], Yuwalee Unpaprom[3,4], Prakash Bhuyar[5], Rajeswaran Ramaraj[2] and Paris Honglay Chen[1*]

[1]Department of Soil and Water Conservation, National Chung-Hsing University, 402 Taichung, Taiwan
[2]School of Renewable Energy, Maejo University, Chiang Mai, 50290, Thailand
[3]Sustainable Resources and Sustainable Engineering Research Lab, Maejo University, Chiang Mai, 50290, Thailand
[4]Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand
[5]International College, Maejo University, Chiang Mai 50290, Thailand

ARTICLEINFO

Article history:
Received 21 May 2023
Received in revised form
13 June 2023
Accepted 27 June 2023

Keywords:
Freshwater microalgae
Autotrophic cultivation
Photobioreactor
Bio-functional Proteins
Natural water medium

ABSTRACT

As natural repositories of a broad spectrum of bioactive compounds ranging from pigments and enzymes to unique fatty acids and proteins, microalgae have attracted growing interest for their potential to produce bio-functional proteins. This comprehensive study examines the autotrophic cultivation of freshwater microalgae, focusing on protein production using a photobioreactor and natural water as the growth medium. The cultivation strategy eliminated the need for supplemented nutrients, enhancing environmental sustainability and cost-effectiveness. The study achieved a biomass production rate of 0.262 mg/L/day and a notable protein yield of 27.69 mg/L/day. Our findings support the feasibility and efficacy of employing natural water as a cultivation medium for freshwater microalgae for protein production. This approach alleviates the environmental burden associated with synthetic growth media and contributes to reducing operational costs. The study thus demonstrates the potential for this methodology to pave the way for a new, eco-friendly, and economically sustainable paradigm in the production of algal bio-functional proteins. Moreover, the widespread availability of natural resources makes this approach highly adaptable and scalable for larger production systems.

1. Introduction

Microalgae consists of eukaryotic life forms and photosynthetic bacteria capable of storing an array of essential organic compounds like sugars, proteins, and lipids (Bhuyar et al., 2021; Manmai et al., 2022). They harness solar power, carbon dioxide, and other nutrients. These tiny organisms transform inorganic elements such as carbon, nitrogen, and phosphorus into organic material in various colors like green, blue-green, or brown (Nithin et al., 2020). Analysis of their chemical makeup is often conducted to provide consumers with relevant nutritional data and to observe how this composition changes under different growth conditions. It's worth noting that the chemical profile of these algae can differ widely depending on the species, the specific strain, and

* Corresponding author.
E-mail address: hlchen@dragon.nchu.edu.tw (Chen P. H)

even individual batches (Ramaraj et al., 2013, Ramaraj et al., 2016a). By altering the growth environment, one can somewhat control the composition of the algae. These algae are self-sustaining through photosynthesis, mainly utilizing sunlight and carbon dioxide for their growth (Ramaraj and Dussadee, 2015; Tsai et al., 2017). Ramaraj et al., 2016b). For uses like single-cell protein in food and feed, maintaining strict quality controls and good agricultural practices is crucial for ensuring the safety of the final product.

Expanding upon this impact, the specialized nutrient profile of microalgae is not just a supplemental addition to food and feed; it is increasingly becoming a cornerstone for innovative health and nutritional solutions (Sousa et al., 2008). Given the extensive array of nutrients — from proteins and amino acids to fatty acids and bioactive compounds — microalgae serve as a more comprehensive source of nutrition compared to many traditional crops (Ramaraj et al., 2015). The high concentrations of these beneficial compounds make microalgae particularly appealing for numerous applications in human and animal nutrition, pharmaceuticals, and cosmetics (Becker, 2007). The richness in bioactive compounds opens doors for research into potential medicinal benefits, including anti-inflammatory and anti-oxidative properties. This burgeoning field has the potential to redefine how we approach health and wellness through natural products.

As research continues to unveil their myriad benefits, microalgae are fast becoming a focus for sustainable and high-quality natural products that could have lasting implications for human and animal health, as well as for the sustainability of aquatic ecosystems (Sousa et al., 2008; Ramaraj et al., 2015).One standout feature of microalgae is their high protein content, which has increased recognition of these organisms as an unconventional yet valuable protein source (Spolaore et al., 2006). Extensive analyses and nutritional assessments confirm that the quality of proteins derived from algae rivals that of traditional plant-based proteins (Becker, 2007; Tsai et al., 2015).

Microalgae are crucial primary producers within aquatic ecosystems and present enormous biodiversity, ranging from unicellular microscopic forms to multicellular macroscopic varieties (Saetang and Tipnee, 2021). Notably, microalgae are exceptional biomass producers (Phukan et al., 2011) and exhibit growth rates and productivity that outpace those of terrestrial plants, forestry, and other aquatic flora (Tsai et al., 2012). Given their rapid growth, they can generate various valuable products, potentially meeting growing demands for food proteins if cultivated effectively.

The majority of previous research has utilized either artificial mediums, like chemical fertilizers (Man and Keat, 2012), or wastewater, such as domestic or industrial effluents (Hodaifa et al., 2013), for microalgae cultivation in photobioreactors. Natural water bodies offer an alternative medium that mimics environmental conditions, eliminates the need for artificial additives, and reduces both costs and potential environmental pollution (Ramaraj et al., 2013, Ramaraj et al., 2016a,b; Tsai et al., 2017). However, there is a surprising scarcity of information regarding microalgae cultivation in natural systems. In light of this gap, the primary objective of this study is to investigate the viability of using natural water media in photobioreactors for the cultivation of freshwater microalgae with the aim of protein production. By focusing on natural water as a medium, this research aims to develop a sustainable, cost-effective, and environmentally friendly approach to algal protein production.

2. Material and methods

2.1 Microalgae culture, monitoring

The mixed culture microalgae were collected from the Sustainable Resources and Sustainable Engineering Research Laboratory (SRSE-LAB), Department of Soil and Water conservation, National Chung-Hsing University (NCHU), Taichung, Taiwan. The microalgal species introduced in the filtered natural freshwater medium were initially identified microscopically and their succession and survival were monitored regularly. In the culture, the species of the genera *Anabaena*, *Chlorella*, *Oedogonium* and *Oscillatoria* were present as the numerically dominant microalgae, along with several other minor microalgae including the species of the genera *Lyngbya*, *Scenedesmus*, *Phytoconis*, *Coccochloris* and *Phormidium* and a few unidentified microalgae.

2.2 Growth medium preparation

The stream water (Natural water) was collected from the nearby Green River (24° 7'27.35"N; 120°40'22.79"E) at Fu-Te Dao temple, which was near the University (Figure 2). The collected water was filtered through a 0.45 µm filter paper and used as the medium.

Figure 2. Collection site in Green River

2.3 Growth conditions and monitoring

The algae were grown in autotrophic condition with 10 days detention time, continuously for over four and half years period with batch-fed culture in a 4L Continuously Stirred Tank Reactor (CSTR) under room temperature and illumination through fluorescent lamps. For the CO_2 source, we did not use any artificial control or extra CO_2 addition and used the open reactor to make the reactor gas exchangeable with the atmosphere. This type of design

could mimic the real conditions in the natural ecosystem best. We used three production units: P1, P2, and P3 for triplicate, and the growth system is shown in Figure 2.

Figure 1. Photobioreactor set-up

Table 1. Chemical analysis

Parameter	Method[a]
pH	Method 423 (pH value)
alkalinity	Method 403 (alkalinity)
DO	Method 421B (azide modification)
COD	Method 508B (closed reflux, titrimetric method)
NH_4^+-N	Method 417A (preliminary distillation step) with Method 417B (nesslerization method (direct and following distillation)) for final ammonia
TKN	Method 420A (macro-Kjeldahl method) with Method 417B (nesslerization method (direct and following distillation)) for final ammonia
NO_2^--N	Method 419 (nitrogen (nitrite))
NO_3^--N	Method 418A (ultraviolet spectrophotometric screening method)
TP	Method 424E (stannous chloride method) following sulfuric acid-nitric acid digestion of Method 424C (preliminary digestion steps for total phosphorus)
TSS	Method 209C (total suspended solids dried at 103-105°C)
VSS	Method 209D (fixed and volatile solids ignited at 550°C)
FSS	Method 209D (fixed and volatile solids ignited at 550°C)

Note: [a] all processes follow the Standard Method.

2.4 Physicochemical analysis

The physicochemical parameters of pH, alkalinity, dissolved oxygen (DO), chemical oxygen demand (COD), ammonia (NH_4+-N), Kjeldahl (TKN), nitrate (NO_3-N), nitrite (NO_2-N), total nitrogen (TN) and total phosphorous (TP); and microalgal biomass of total suspended solids (TSS), volatile suspended solids (VSS), fixed suspended solids (FSS) were initially measured. Biomass production in biological processes has often been expressed as a cell dry weight (DW) by measuring the total suspended solid (TSS) concentration in cultures using standard methods. The same parameters were monitored throughout the study at regular intervals, following standard procedures (APHA-AWWA and WPCF, 2005) and details were presented in Table 1. Protein content was measured by multiplying the nitrogen content of dried algae samples by a conversion factor of 6.25 (Hunt et al., 2010). Protein was determined by Kjeldahl method. The method involves Digestion, Distillation, and Titration. The procedure was adopted from the standard method (APHA-AWWA and WPCF, 2005).

3. Results and discussion

3.1 Growth of freshwater microalgae

Microalgae are increasingly recognized as a critical bioresource for various applications, drawing significant scholarly and industrial interest (Palanisamy et al., 2022). Comprising about 50% carbon in their dry weight, these microorganisms are noteworthy for their efficiency in carbon capture and utilization (Sánchez Mirón et al., 2003). They primarily rely on CO_2 as the source of carbon and light as the energy source to drive their metabolic activities. In terms of assessing microalgal growth, it's important to note that the dry weight of the algae biomass has been commonly evaluated using TSS as an effective method for quantifying the overall biomass (Azov et al., 1982; García et al., 2006; Barthel et al., 2008). For this study, microalgal growth will be reported regarding dry weight, as depicted in Figure 3.

The increasing focus on microalgal biomass aligns well with the urgent need for sustainable alternatives in various fields. With high carbon content, microalgae serve as valuable resources for carbon capture and can also convert waste and CO_2 into valuable biomass. Dry weight, quantified through TSS, is a reliable metric for evaluating microalgal growth across different conditions and studies (García et al., 2006; Barthel et al., 2008). This standardized measure aids in developing effective cultivation strategies by helping researchers understand the influence of environmental factors on growth. In this study, biomass production varied between 0.086 to 0.262 g/L/day, averaging 0.145±0.03 g/L/day. Notably, the research utilized a natural water medium without additional chemicals or CO_2, effectively simulating an ecosystem, and yielded promising results for biomass production.

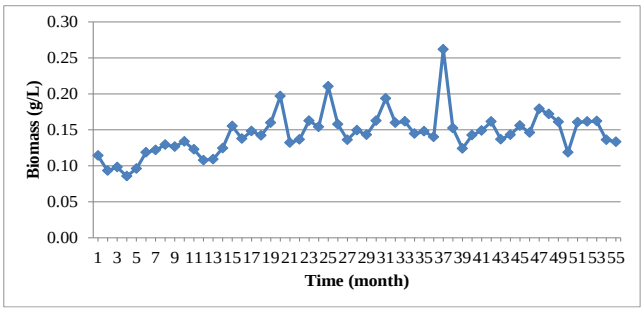


Figure 3. Algae growth and biomass yield

3.2 Microalgae growth medium and system analysis

The increased emphasis on microalgal biomass is timely, especially considering the pressing need for sustainable approaches across diverse industries. With a high concentration of carbon in their composition, microalgae stand out as an asset for initiatives aimed at carbon capture and storage. Moreover, their metabolic versatility offers them promising resources for transforming waste products and carbon dioxide into usable biomass. The TSS for assessing dry weight offers a unified and consistent metric, facilitating comparative analyses across different research endeavors and cultivation conditions (García et al., 2006; Barthel et al., 2008). This standardized methodology enhances our understanding of microalgal growth rates and their capacity for upscaling.

Such precise measurement techniques as TSS enable a nuanced understanding of how microalgae respond to varying environmental inputs, thereby shedding light on optimal conditions for cultivation. This information is crucial for devising effective strategies that maximize biomass production while minimizing resource input (Ramaraj et al., 2015). Consequently, the ongoing attention to microalgal biomass and its meticulous quantification through TSS significantly enriches our knowledge base concerning these microorganisms' ecological and commercial utility. Focusing on microalgal biomass through rigorous methodologies helps understand its growth characteristics and unravels its potential role in sustainability efforts.

The most important factors are nutrients, pH and alkalinity, light, and temperature (Unpaprom et al., 2017). The mean values of the measured physicochemical and biological parameters of the unsupplemented natural freshwater medium and growth analysis are shown in Table 2. Light intensity levels can significantly affect photosynthesis rates, directly related to an algae's growth ability. The light source was a minimum of 25 67 $\mu mol^{-1}m^{-2}$ needed (Finkel, 2001); this study has essential light intensity (27.11± 2.67 $\mu mol^{-1}m^{-2}$). The minimum microalgae use light energy to fix carbon (C) and combine the carbon with elements such as nitrogen (N) and phosphorus (P) at relatively constant stoichiometric ratios (Baird and Middleton, 2004). Temperature (28.38±2.79°C) in the reactors appears favorable for metabolic activities, which are crucial for biomass production. Moreover, the elevated pH levels in the reactors (10.13±0.34) compared to the medium (7.15±0.30), along with higher DO levels (7.36±5.36 mg/L in reactors vs. 6.02±1.20 mg/L in the medium), suggest enhanced metabolic and photosynthetic activities in the growth environment.

Notable variations also occur in alkalinity, dropping from 102.48±20.72 mg $CaCO_3$/L in the medium to 46.39±1165 mg CaCO3/L in the reactors and COD, which increased to 11.52±4.42 mg/L in the reactors from 5.54±2.61 mg/L in the medium. These changes may indicate the utilization of carbonate ions and the generation of metabolic byproducts. Nutrient concentrations such as NH_4-N (1.32±1.55 mg N/L in medium and 0.61±0.63 mg N/L in reactors) and NO_3-N (3.00±2.06 mg N/L in medium and 0.39±0.43 mg N/L in reactors) generally decreased, suggesting their incorporation into the microalgal biomass. The CNP ratio also shifts significantly from 3.56:12.14:1 in the medium to 47.47:24.35:1 in the reactors, perhaps indicating changes in nutrient utilization strategies. Final biomass metrics—TSS at 0.145±0.03 g L^{-1}, VSS at 0.083±0.02 g L^{-1}, and FSS at 0.062±0.02 g L-1—underscore the biomass generated and its significance in evaluating microalgal productivity. The analysis of these data is crucial for optimizing microalgal growth and contributes significantly to their broader sustainability potential.

Algae growth is generally equivalent to protein synthesis, where P-rich ribosomes and N-rich amino acids are the key macromolecules. The C/N/P ratios of algae are typically around 106:16:1, the Redfield ratio (Redfield et al., 1963). According to the proposed theoretical CNP ratio of algae, there was no P and N limiting problem; the results are shown in Table 2. From the data comparison between feeding and reactors, both nitrogen and phosphorous nutrients were uptaken by algae, 68.6% as TN and 84.3% as TP respectively. However, all the reactors showed that the natural water medium could provide the essential nutrients for algae growth.

But the carbon source has a shortage of natural water medium. The nutritional content did not reach the necessary C ratio in the water medium. There must be another carbon source to support algae growth (Unpaprom et al., 2015). The possible extra source was CO_2 in the air because this continuous study reached 4.5 years. More than 30% of the Raven et al. (2005) state that CO_2 has been emitted into the atmosphere over the last 200 years because of burning fossil fuels and anthropogenic activities. Changes in land use are stored in the oceans. Consequently, the carbon-limited medium is good practice for utilizing atmospheric CO_2 and could contribute to greenhouse reduction.

Table 2. Physicochemical and biological analysis

Parameter	Medium mean and SD[a]	Reactors mean and SD[a]
Light Intensity ($\mu mol^{-1}m^{-2}$)	-	27.02±2.23
Temperature(°C)	-	28.38±2.79
pH	7.15±0.30	10.13±0.34
DO (mg/L)	6.02±0.1.20	7.36±5.36
Alkalinity (mg $CaCO_3$/L)	102.48±20.72	46.39±1165
COD (mg/L)	5.54±2.61	11.52±4.42
NH_4^--N (mg N/L)	1.32±1.55	0.61±0.63
NO_3^--N (mg N/L)	3.00±2.06	0.39±0.43
NO_2^--N (mg N/L)	1.35±1.72	0.08±0.11
TKN (mg N/L)	2.55±2.36	1.52±1.32
TN (mg N/L)	7.06±2.03	2.22±1.65
TP (mg/L)	0.58±0.18	0.09±0.09
CNP ratio	3.56:12.14:1	47.47:24.35:1
TSS (g L^{-1})	-	0.145±0.03
VSS (g L^{-1})	-	0.083±0.02
FSS (g L^{-1})	-	0.062±0.02

3.3 Evaluating protein content and overall yield efficiency in microalgal biomass

Proteins comprise a significant fraction of the biomass of actively growing microalgae and cyanobacteria, although they are generally undervalued compared to minor products such as omega fatty acids (Wang et al., 2013). Algae are by far the best protein source, with four times the protein content of C3 macrophytes. In general, the algal proteins have a high nutritional value, being a rich source of aspartic acid, glutamic acid, and leucine, whereas threonine, lysine, tryptophan, sulfur amino acids, and histidine are limiting, but at higher levels than those found in terrestrial plants (Hernández-Carmona et al., 2009, Gressler et al., 2010).

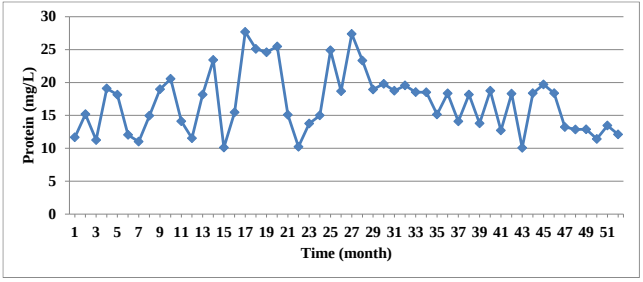


Figure 4. Time-series analysis of protein yield in microalgae

Microalgal protein production in this study would be presented in Figure 4. The protein amount ranged from 10.06 to 27.69 mg/L/day with a mean rate of 16.97±4.68 mg/L/day. The unsupplemented natural water medium could be a potential source for producing protein inexpensive method. Because of the increasing global demand for proteins in various industrial, diagnostic, and therapeutic applications, bioreactor systems are becoming more significant for producing large quantities of proteins, particularly in cases where traditional sources are limited due to cost and/or availability (Bhuyar et al., 2020; Patel et al., 2022). This study's results demonstrated the potential natural water medium and growth system (as a photo-bioreactor). Since plenty of water resources were available, this study could be a good demo for utilizing natural resources and producing valuable protein for different applications.

4. Conclusion

This study develops a novel strategy employing freshwater mixed microalgal species grown in an autotrophic cultivation platform to produce protein biomass as desired. The utilization of natural water medium which came from the water body directly without any extra nutrition addition, demonstrated the potential to adopt the algal function. Our results supported the feasibility of cultivating freshwater microalgae by using stream water as a water source and a nutrient source. The atmospheric air CO_2 is the extra carbon source for algae growth. This study demonstrated high biomass algae production and our results showed that proper operation could promote the production obviously despite natural water medium without any extra nutrition addition. Moreover, the natural water medium is a promising method to produce algal protein. Consequently, this study delivered a cost-efficient and environmentally sustainable way for biomass production in microalgae containing a reasonable protein content.

Conflict of Interest Declaration

The authors assert that no conflicts or personal affiliations might be construed as impacting the outcomes shared in this research.

References

APHA, AWWA, WEF., (2005). Standard methods for the examination of water and wastewater. 21st ed. Washington DC: American Public Health Association/American Water Works Association/Water Environment Federation.

Baird, M. E., & Middleton, J. H. (2004). On relating physical limits to the carbon: nitrogen ratio of unicellular algae and benthic plants. Journal of Marine Systems, 49(1-4), 169-175.

Barthel, L., Oliveira, P. A. V. D., & Costa, R. H. R. D. (2008). Plankton biomass in secondary ponds treating piggery waste. Brazilian archives of biology and technology, 51, 1287-1298.

Becker, E. W. (2007). Micro-algae as a source of protein. Biotechnology Advances, 25(2), 207-210.

Bhuyar, P., Rahim, M. H. A., Maniam, G. P., Ramaraj, R., & Govindan, N. (2020). Exploration of bioactive compounds and antibacterial activity of marine blue-green microalgae (*Oscillatoria* sp.) isolated from coastal region of west Malaysia. SN Applied Sciences, 2, 1-10.

Bhuyar, P., Trejo, M., Dussadee, N., Unpaprom, Y., Ramaraj, R., & Whangchai, K. (2021). Microalgae cultivation in wastewater effluent from tilapia culture pond for enhanced bioethanol production. Water Science and Technology, 84(10-11), 2686-2694.

García, J., Green, B. F., Lundquist, T., Mujeriego, R., Hernández-Mariné, M., & Oswald, W. J. (2006). Long term diurnal variations in contaminant removal in high rate ponds treating urban wastewater. Bioresource Technology, 97(14), 1709-1715.

Gressler, V., Yokoya, N. S., Fujii, M. T., Colepicolo, P., Mancini Filho, J., Torres, R. P., & Pinto, E. (2010). Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algae species. Food Chemistry, 120(2), 585-590.

Hernández-Carmona, G., Carrillo-Domínguez, S., Arvizu-Higuera, D. L., Rodríguez-Montesinos, Y. E., Murillo-Álvarez, J. I., Muñoz-Ochoa, M., & Castillo-Domínguez, R. M. (2009). Monthly variation in the chemical composition of *Eisenia arborea* JE Areschoug. Journal of Applied Phycology, 21, 607-616.

Hodaifa, G., Sánchez, S., Martínez, M. E., & Órpez, R. (2013). Biomass production of Scenedesmus obliquus from mixtures of urban and olive-oil mill wastewaters used as culture medium. Applied Energy, 104, 345-352.

Hunt, R. W., Chinnasamy, S., Bhatnagar, A., & Das, K. C. (2010). Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*. Applied Biochemistry and Biotechnology, 162, 2400-2414.

Lam, M. K., & Lee, K. T. (2012). Potential of using organic fertilizer to cultivate *Chlorella vulgaris* for biodiesel production. Applied Energy, 94, 303-308.

Wang, L., Li, Y., Sommerfeld, M., & Hu, Q. (2013). A flexible culture process for production of the green microalga *Scenedesmus dimorphus* rich in protein, carbohydrate or lipid. Bioresource Technology, 129, 289-295.

Manmai, N., Balakrishnan, D., Obey, G., Ito, N., Ramaraj, R., Unpaprom, Y., & Velu, G. (2022). Alkali pretreatment method of dairy wastewater based grown *Arthrospira platensis* for enzymatic degradation and bioethanol production. Fuel, 330, 125534.

Nithin, B. R., Bhuyar, P., Trejo, M., Rahim, M. H. A., Maniam, G. P., & Govindan, N. (2020). Culturing of green photosynthetic microalgae (*Chlorella* sp.) using palm oil mill effluent (POME) for future biodiesel production. Maejo International Journal of Energy and Environmental Communication, 2(1), 1-8.

Palanisamy, K. M., Bhat, O. A., Oteikwu, M. O., Govindan, N., Maniam, G. P., Ramaraj, R., & Unpaprom, Y. (2022). Production of biofuel from microalgae grown in wastewater-A review: Microalgae. Maejo International Journal of Energy and Environmental Communication, 4(3), 16-26.

Patel, A., Krikigianni, E., Rova, U., Christakopoulos, P., & Matsakas, L. (2022). Bioprocessing of volatile fatty acids by oleaginous freshwater microalgae and their potential for biofuel and protein production. Chemical Engineering Journal, 438, 135529.

Phukan, M. M., Chutia, R. S., Konwar, B. K., & Kataki, R. (2011). Microalgae *Chlorella* as a potential bio-energy feedstock. Applied Energy, 88(10), 3307-3312.

Ramaraj, R., Tsai, D. D., & Chen, P. H. (2013). Chlorophyll is not accurate measurement for algal biomass. Chiang Mai Journal of Science, 40(4), 547-555.

Ramaraj, R., & Dussadee, N. (2015). Biological purification processes for biogas using algae cultures: a review. International Journal of Sustainable and Green Energy, 4(1), 20-32.

Ramaraj, R., Tsai, D. D. W., & Chen, P. H. (2015). Carbon dioxide fixation of freshwater microalgae growth on natural water medium. Ecological Engineering, 75, 86-92.

Ramaraj, R., Unpaprom, Y., & Dussadee, N. (2016a). Cultivation of green microalga, *Chlorella vulgaris* for biogas purification. International Journal of New Technology and Research, 2(3), 117-122.

Ramaraj, R., Unpaprom, Y., & Dussadee, N. (2016b). Potential evaluation of biogas production and upgrading through algae. International Journal of New Technology and Research, 2(3), 263567.

Raven, J., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., Riebesell, U., Shepherd, J., Turley, C., & Watson, A. (2005). Ocean acidification due to increasing atmospheric carbon dioxide. The Royal Society.

Redfield, A. C., Ketchum, B. H., & Richards, F. A. (1963). The influence of organisms on the composition of seawater. The Sea, 2, 26-77.

Saetang, N., & Tipnee, S. (2021). Towards a sustainable approach for the development of biodiesel microalgae, *Closterium* sp. Maejo International Journal of Energy and Environmental Communication, 3(1), 25-29.

Sousa, I., Gouveia, L., Batista, A. P., Raymundo, A., & Bandarra, N. M. (2008). Microalgae in novel food products. Food Chemistry Research Developments, 75-112.

Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. Journal of Bioscience and Bioengineering, 101(2), 87-96.

Tsai, D. D. W., Ramaraj, R., & Chen, P. H. (2012). Growth condition study of algae function in ecosystem for CO_2 bio-fixation. Journal of Photochemistry and Photobiology B: Biology, 107, 27-34.

Tsai, D. D. W., Chen, P. H., Chou, C. M. J., Hsu, C. F., & Ramaraj, R. (2015). Carbon sequestration by alga ecosystems. Ecological Engineering, 84, 386-389.

Tsai, D. D. W., Chen, P. H., & Ramaraj, R. (2017). The potential of carbon dioxide capture and sequestration with algae. Ecological Engineering, 98, 17-23.

Unpaprom, Y., Tipnee, S., & Ramaraj, R. (2015). Biodiesel from green alga *Scenedesmus acuminatus*. International Journal of Sustainable and Green Energy, 4(1), 1-6.

Unpaprom, Y., Ramaraj, R., & Whangchai, K. (2017). A newly isolated green alga, *Scenedesmus acuminatus*, from Thailand with efficient hydrogen production. Chiang Mai Journal of Science, 44, 1270-1278.