

# Use of FT–IR to Identify Enhanced Biomass Production and Biochemical Pool Shifts in the Marine Microalgae, *Chlorella ovalis*, Cultured in Media Composed of Different Ratios of Deep Seawater and Fermented Animal Wastewater

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Growth rates, photosystem II photosynthesis, and the levels of chlorophyll *a* and secondary metabolites of *Chlorella ovalis* were estimated to determine if they were enhanced by the addition of swine urine (BM) or cow compost water (EP) that had been fermented by soil bacteria to deep seawater (DSW) in an attempt to develop media that enabled batch mass culture at lower costs. Growth of *C. ovalis* in f/2, f/2–EDTA+BM60%, DSW+BM30%, and DSW+EP60% was enhanced and maintained in the log phase of growth for 16 days. The cell densities of *C. ovalis* in DSW+EP60% ( $4.1 \times 10^6$  Cells/ml) were higher than those of f/2 ( $2.9 \times 10^6$  Cells/ml), f/2–E+BM60% ( $3.7 \times 10^6$  Cells/ml), and DSW+BM30% ( $2.7 \times 10^6$  Cells/ml). The growth rate was also more favorable for *C. ovalis* cultured in DSW+EP60% ( $0.15 \text{ day}^{-1}$ ) than that of *C. ovalis* cultured in the control medium (f/2) ( $0.12 \text{ day}^{-1}$ ). Furthermore, the chlorophyll *a* concentration of *C. ovalis* cultured in DSW+EP60% (4.56 mg/l) was more than 2-fold greater than that of *C. ovalis* cultured in f/2 (2.35 mg/l). Moreover, the maximal quantum yields of photosystem II at 470 nm (Fv/Fm) were significantly higher in organisms cultured at f/2–E+BM60% (0.53) and DSW+EP60% (0.52) than in the other treatment groups. Finally, Fourier transformation infrared (FT–IR) spectroscopy revealed that *C. ovalis* grown in DSW+EP60% had more typical peaks and various biochemical pool shifts than those grown in other types of media. Taken together, the results of this study indicate that the use of DSW+EP60% to culture *C. ovalis* can reduce maintenance expenses and promote higher yields.

**Keywords:** Biomass, FT–IR spectrometer, *Chlorella ovalis*, deep seawater, fermented animal wastewater

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Approximately 10% of the micro-green algae that inhabit seawater have been extensively studied to determine if they would be of use in the production of health foods [33] fish food, or various forms of bio-energy, oil, hydrogen gas, and diesel. In addition, micro-green algae have been used in studies conducted to evaluate fundamental theories of ecophysiological fields, physiobiochemical enhancements of culture techniques, bioremediation, and CO<sub>2</sub> sequestration [20, 24].

The marine microalga *Chlorella ovalis* is one of the most important marine photosynthetic microalgae. *C. ovalis* is a phototrophic organism that contains cellulose and hemicelluloses and has a relatively rapid growth rate. The genus *Chlorella* is extremely useful as food for fish, shellfish, and zooplankton, as well as in human health foods, because greater than 40% of their cells are composed of protein [1, 3, 11, 20, 25].

Recently, many studies have been conducted in attempts to enhance the biomass and growth rates of unicellular microalgae while reducing the costs associated with their mass culture.

Shifts in the biochemical pools of microalgae can be used to evaluate the effects of changes in culture conditions such as the levels of specific nutrient elements, photoperiod, light intensity, light resources, and temperature. In addition, such shifts can be used to evaluate the effects of genetic recombination on the biomass of the algae [1, 8, 11, 14, 20, 30, 36]. For example, wastewater is a good source of nitrogen and phosphorus for microalgae that are being utilized for bioremediation. Accordingly, many studies have been conducted to evaluate the ability of microalgae such as *Chlorella kessleri*, *C. vulgaris*, *Scenedesmus obliquus*, *S. bijugatus*, and *Selenastrum capricornutum* to eliminate such nutrients through purification facilities. In addition,

the use of wastewater to provide nutrients to microalgae has been evaluated as a method of reducing culture costs.

Deep seawater is also often used to industrialize the production of marine bioresources [3, 4, 21, 22]. Indeed, central and local governments within Korea have placed an emphasis on the use of marine bio-projects as part of a master plan to industrialize deep seawater in the East Sea for the development of marine resources [21]. Deep seawater (DSW) has the potential for use as a medium to culture microalgae [16, 22]. For example, microalgae such as *Spilurina* and *Dunaliella* are cultured using deep seawater that contains a variety of minerals and then used in health foods and as food additives [22]. However, few studies have been conducted to evaluate the use of deep seawater to culture microalgae in Korea.

Biochemical screening of biomass potentials is cumbersome and costly. However, FT-IR spectroscopy is a low-cost method of rapidly screening whole cells for protein pool shifts and characterizing algal lipids [5, 7, 14]. Therefore, this study was conducted to determine if the addition of swine urine (BM) and cow compost water (EP) that had been fermented by soil bacteria [15] to DSW used to culture *C. ovalis* could enhance and sustain its vitality and enable batch mass culture at a lower cost. To accomplish this, we analyzed and compared the growth rates, photosystem II photosynthesis [6], chlorophyll *a* levels, and levels of secondary metabolites using FT-IR spectroscopy [31, 32] to evaluate *C. ovalis* grown in different culture media.

## MATERIALS AND METHODS

### Culture Conditions

The *C. ovalis* used in these experiments originated from the Korea Plankton Culture Collection for Industrialization (KPCCI). *C. ovalis* (KPCCI P-M-2) was grown in 1-l volumes in 2-l Erlenmeyer flasks in each medium for 16 days at 20°C under a light:dark photoperiod of 14 h:10 h, with a light intensity of  $180 \pm 5 \mu\text{mol m}^{-2}\text{s}^{-1}$ . The cultures were continuously shaken at 140 rpm throughout the culture period.

### Development of Media

Cultures of *C. ovalis* were grown using *f/2* as the control medium [10, 27], which contained the following components (g):  $\text{NaNO}_3$  (0.075),  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (0.006), EDTA  $\text{Na}_2$  (4.36),  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (3.15),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.01),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.02),  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.01),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.18),  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  (0.006), cyanocobalamin (0.0005),

thiamine HCl (0.1), and biotin (0.0005), per liter of filtered natural seawater. The pH was adjusted to 8.0 and the medium was then autoclaved prior to inoculation [17]. In addition, medium containing BM (Bacterial Mineral) water was utilized. BM water is produced using swine urine that originated from the Chu-Chun Farm (Table 1) and has been granted international patents based on the fermentation and bioreaction technologies that produce BM [15]. Briefly, pellets containing soil humus at a pH of 4.7 are used to culture bacteria such as actinomycetes and yeasts in swine urine. It has been established that BM water leads to enhanced cell division rates and that it acts as a substitute chelator for EDTA when added to *f/2* medium. Therefore, in this study, various ratios of BM were added to *f/2* medium [9, 12, 35]. In addition, DSW that originated from the Ullung Minerals Co. was added to BM medium at ratios ranging from 10–60% to estimate the effects of DSW and BM on the biomass of *C. ovalis* and to determine if these compounds could lead to a reduction in culture cost when compared with mass culture using *f/2*-E +BM60% (Table 2).

### Analysis of the Physicochemical Elements in DSW, BM, and EP

The concentrations of total nitrogen (T-N), total phosphorus (T-P),  $\text{NO}_3\text{-N}$ ,  $\text{PO}_4\text{-P}$ , and  $\text{SiO}_2\text{-Si}$ , as well as the weight of the suspended solids (SS) were determined using the Standard Methods [2]. DO and pH were measured using a pH/DO meter (Horiba D-55, Japan), and the turbidity was measured using a turbidimeter (DRT-15CE, U.S.A.).

### Measurement of Unicellular Growth, Chlorophyll *a*, and Photosystem II activity

The cell growth rates were evaluated *via* periodic counting using a hemocytometer, and the growth kinetics (*K*) were calculated using Guillard's formula [10], which is as follows:

$$K = \log_2 \frac{(N_1/N_0)}{t_1 - t_0}$$

where  $N_0$  = the cell number at the time of inoculation,  $N_1$  = the final cell number, and  $t_1 - t_0$  = culture time

Total pigments were extracted using a solvent system that consisted of acetone, methanol, and water at a ratio of 10:9:1 (v/v/v). Chlorophyll *a* was analyzed using the method described by Kim and Smith [19]. The maximum quantum yields of photosystem II (Fv/Fm) were measured using a pulse-amplitude-modulated fluorometer (Phyto-PAM Walz, Effeltrich, Germany) with dark-adapted samples for 5 min while stirring, and then evaluating the values using the following equation [13]:

$$Fv/Fm = (Fm' - Ft) / Fm' = dF / (Ft + dF) \quad [37]$$

where Fv/Fm = the maximal PSII quantum yield, dF = the increase in fluorescence yields during the saturation pulse, Ft = the momentary

**Table 1.** Analysis of physicochemical elements of deep seawater (DSW) and fermented animal wastewater originated from swine urine (BM) and cow compost (EP).

	pH	DO	COD	Turbidity	SS	$\text{NO}_3\text{-N}$	$\text{PO}_4\text{-P}$	$\text{SiO}_2\text{-Si}$	T-N	T-P
						(mg/l)				
DSW	7.5	9.1	1.8	-	1.0	0.6	0.1	1.0	0.1	0.1
BM	4.7	13.7	-	2.9	6.8	-	-	-	32.2	2.2
EP	7.5	10.3	-	0.1	0.7	-	-	-	1.3	0.2

**Table 2.** Biomass of *Chlorella ovalis* cultured for 16 days in media containing different composition ratios of deep seawater and fermented animal wastewater.

Media	Cells/ml ( $\times 10^6$ )	Growth rates ( $k \text{ day}^{-1}$ )	PAM	Chl <i>a</i> (g/ml)
f/2	2.9	0.12	0.42	2.35
f/2-E+BM60%	3.7	0.13	0.53	3.13
DSW+BM10%	0.8	0.04	0.35	1.86
DSW+BM20%	1.2	0.05	0.38	1.85
DSW+BM30%	2.7	0.10	0.48	2.71
DSW+BM40%	1.2	0.03	0.31	1.65
DSW+BM50%	1.2	0.05	0.38	1.89
DSW+EP60%	4.1	0.15	0.52	4.56

level of fluorescence yields prior to the saturation pulse, and  $F_m$  = the maximal fluorescence yields

#### Analysis of Lipids and Proteins Identified by FT-IR Spectroscopy

A microalgal suspension sampled during the exponential growth phase after 16 days of culture was prepared using each cell density (Table 2). Infrared measurements were then conducted using a Perkin Elmer Fourier transformation spectrophotometer (GX, U.S.A.). One volume of freeze-dried samples was mixed with 100 volume of KBr (#221864-25G; Sigma-Aldrich, U.S.A.) powder to determine the spectra in the 400–4,000  $\text{cm}^{-1}$  range. The spectra were recorded as ASCII files and the functional groups of proteins and the saturation and length of the carbon chains of fatty acids were later interpreted by comparison with the results of previously conducted studies [26, 28].

## RESULTS

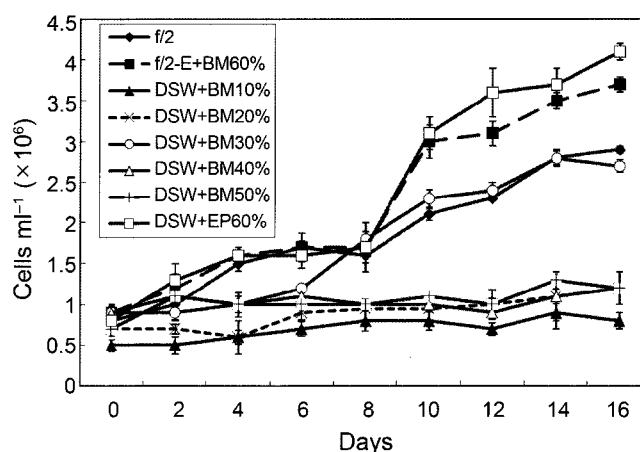
Fermented swine urine refers to original swine urine from which the inorganic nutrients have been removed by 40 days of treatment in a fermentation apparatus [17, 18]. The agricultural and floricultural efficacy of fermented swine urine has been repeatedly demonstrated since it was first evaluated in 1996 in Japan [15]. The pH of the DSW and the EP was 7.5, whereas that of the BM was 4.7 prior to mixing the media (Table 1). The DO of the BM was highest (13.7 mg/l), whereas the DO of the DSM and EP was 9.1 and 10.3, respectively. The turbidity and SS levels of the EP were 0.14 and 0.7 mg/l, respectively, whereas those of the BM were 2.9 and 6.8 mg/l, respectively. These findings indicate that the fermented EP water was cleaner than the BM water. The levels of total nitrogen and total phosphorus were also higher in the BM water (32.2 mg/l and 2.2 mg/l, respectively) than in the DSW (0.1 mg/l and 0.1 mg/l, respectively) and EP water (1.3 mg/l and 0.2 mg/l, respectively). Finally, the levels of nitrate in the DSW (0.6 mg/l) were higher than the levels of phosphate (0.1 mg/l) and silicate (0.1 mg/l).

To determine which concentrations of BM, EP, and DSW were most favorable for the growth of *C. ovalis*, cultures were prepared in DSW containing BM 10%, 20%, 30%, 40%, and 50% or EP 60% (v/v). All samples were

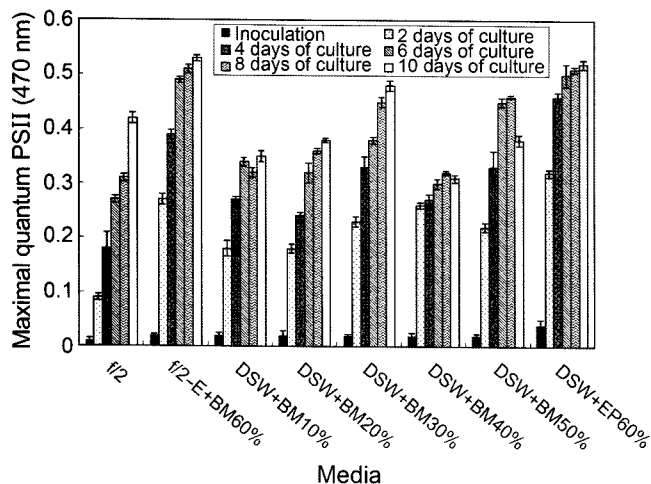
prepared in batch culture rather than continuous culture to reduce maintenance costs.

The growth of *C. ovalis* in f/2, f/2-E+BM60%, DSW+BM30%, and DSW+EP60% was maintained in the log phase for 16 days, whereas the growth of *C. ovalis* cultured in DSW+BM10%, DSW+BM40%, and DSW+BM50% remained stationary throughout the experiments. The densities of *C. ovalis* cultured in f/2, f/2-E+BM60%, DSW+BM30%, and DSW+EP60% were  $2.9 \times 10^6$  cells/ml,  $3.7 \times 10^6$  cells/ml,  $2.7 \times 10^6$  cells/ml, and  $4.1 \times 10^6$  cells/ml, respectively. The highest cell densities and growth rate of *C. ovalis* were obtained in the DSW+EP60% medium (Table 2 and Fig. 1). In addition, growth rate ( $0.15 \text{ day}^{-1}$ ) was more favorable for *C. ovalis* cultured in DSW+EP60% than for *C. ovalis* cultured in f/2 ( $0.12 \text{ day}^{-1}$ ). Furthermore, the concentration of chlorophyll *a* in *C. ovalis* cultured in DSW+EP60% (4.56 mg/l) was 2-fold greater than that of *C. ovalis* cultured in f/2 (2.35 mg/l).

The maximal quantum yields of photosystem II at 470 nm ( $F_v/F_m$ ) for *C. ovalis* varied significantly for each medium according to the culture period (Fig. 2); however, the greatest yields were obtained when *C. ovalis* was cultured in f/2-E+BM60% and DSW+EP60%. In addition, the  $F_v/F_m$



**Fig. 1.** Cell growth of *Chlorella ovalis* cultured in media containing different ratios of deep seawater (DSW) and fermented animal wastewater that originated from swine urine (BM) and cow compost (EP). Values are shown as means  $\pm$  SD ( $n=3$ ).

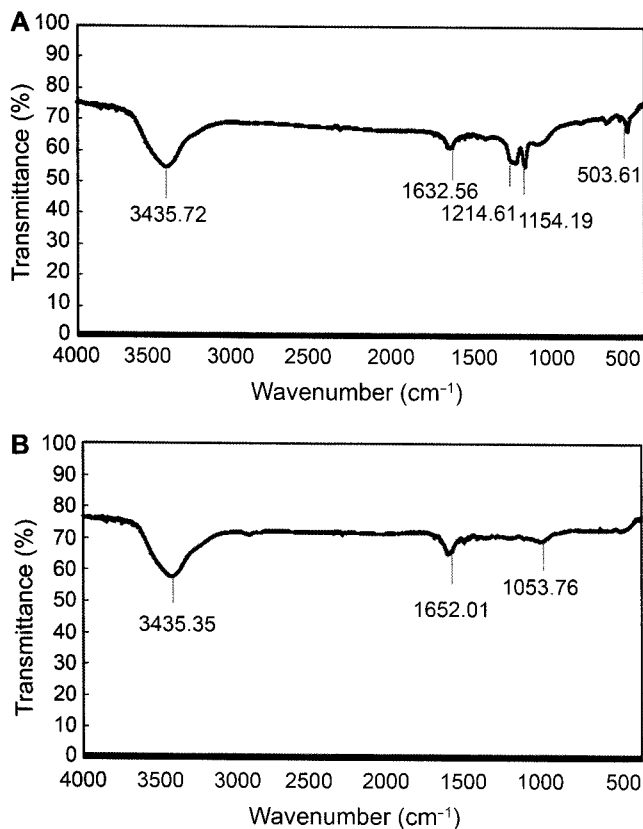


**Fig. 2.** Variations in the maximal quantum PSII (470 nm) of *Chlorella ovalis* cultured in media containing different ratios of deep seawater and fermented animal wastewater. Values are shown as means $\pm$ SD (n=3).

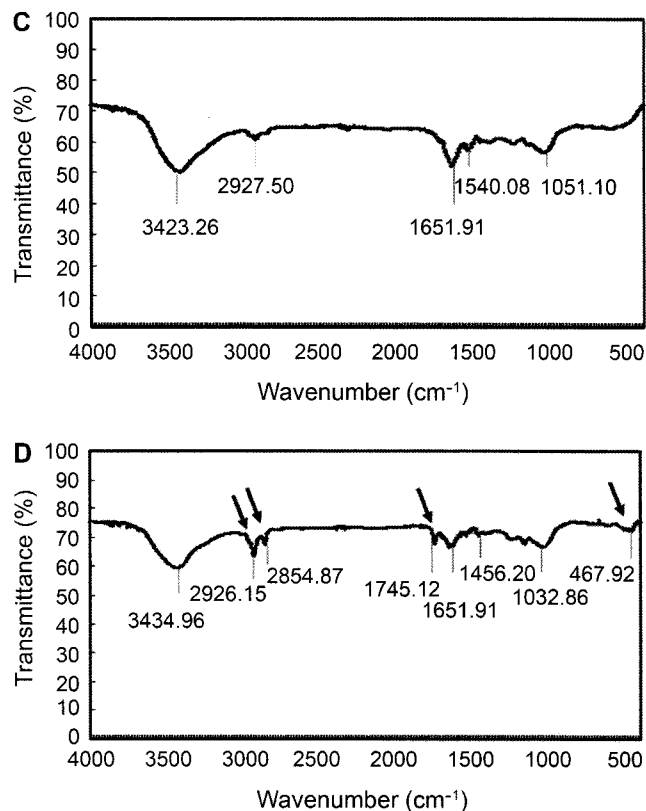
of *C. ovalis* cultured in f/2-E+BM60% and in DSW+EP60% were highest (0.53 and 0.52, respectively), whereas those of *C. ovalis* cultured in DSW+BM40% were lowest (0.31). However, despite the similar Fv/Fm values, the chlorophyll *a* concentration of *C. ovalis* cultured in DSW+EP60%

(4.56 mg/l) was higher than that of *C. ovalis* cultured in f/2-E+BM60% (3.13 mg/l) (Table 2).

The contents and functional groups of the biochemical pool shifts identified using FT-IR spectroscopy were observed between 400 and 4,000  $\text{cm}^{-1}$  (Figs. 3 and 4). Equal FT-IR peaks were observed for *C. ovalis* cultured in all types of media at 3,435  $\text{cm}^{-1}$ , which indicates the presence of an OH group of carbohydrates, proteins, and lipids (Table 3) [28]. In addition, peaks were observed at 1,154  $\text{cm}^{-1}$  and 1,215  $\text{cm}^{-1}$ , as well as at 1,633  $\text{cm}^{-1}$  when *C. ovalis* cultured in f/2 was evaluated, which indicates the presence of carbohydrates [43] and proteins, respectively [8, 28, 34]. Unique peaks were observed at 1,054  $\text{cm}^{-1}$  when *C. ovalis* cultured in f/2-E+BM60% was evaluated, which indicates the presence of a P=O group of phospholipids, DNA, and RNA in carbohydrates [28]. *C. ovalis* cultured in DSW+BM60% showed peaks of 2,928  $\text{cm}^{-1}$ , which were representative of lipids and proteins, as well as peaks at 1,540  $\text{cm}^{-1}$  that represented the N-H group of amides for proteins. *C. ovalis* cultured in DSW+EP60% produced unique peaks at 2,926 and 2,854  $\text{cm}^{-1}$  that represented the CH<sub>2</sub> group of lipids and proteins, as well as peaks that represented the C=O functional group for lipids and fatty acids at 1,745  $\text{cm}^{-1}$ , and peaks at 1,456  $\text{cm}^{-1}$  that represented the CH<sub>3</sub> and CH<sub>2</sub> group of proteins.



**Fig. 3.** FT-IR spectra of *Chlorella ovalis* cultured in f/2 (A) and f/2-E+BM60% (B).



**Fig. 4.** FT-IR spectra of *Chlorella ovalis* cultured in DSW+BM60% (C) and DSW+EP60% (D) according to the ratios of deep seawater and fermented animal wastewater.

**Table 3.** Assignment of principal infrared bands identified using FT-IR spectroscopy to evaluate microalgae.

Wavelength (cm <sup>-1</sup> )	Groups	Biomolecules	References
~3,460	OH stretching	Carbohydrates, proteins, and lipids	[28]
~2,950	CH <sub>3</sub> stretching	Lipids and proteins	[28]
~2,850	CH <sub>2</sub> stretching	Lipids and proteins	[28]
~1,730	C=O stretching	Lipids (esters of fatty acids)	[28, 34]
~1,650	C=O stretching	Proteins	[8, 28, 34]
~1,540	N-H	Amides of proteins	[8, 34]
~1,455	CH <sub>3</sub> and CH <sub>2</sub>	Proteins	[8, 34]
~1,398	CH <sub>3</sub> and CH <sub>2</sub>	Proteins	[8, 34]
~1,275	C-O-H	Carbohydrates, proteins, DNA, and RNA	[28]
~1,240	P=O	Phospholipids, DNA, and RNA	[28, 34]
1,200–1,160	C-O-C	Carbohydrates as polysaccharides	[28]
~1,075	Si-O	Silicate	[34]
1,020–1,085	P=O	Phospholipids, DNA and RNA	[28]
~830	C=N (ring)	Nucleotides and chlorophyll pigments	[28]
697–753	CH <sub>2</sub> bending	Carbohydrates, proteins, and lipids (sterols and fatty acids)	[28]

## DISCUSSION

Recently, many studies of microalgae have focused on developing methods to enhance biomass and reduce the cost of culture associated with industrial applications. To enhance unicellular growth rates, microalgal cultures are subjected to various modifications to control the mass culture scale. These modifications include changes in raceway pond and bioreactor culture conditions, variations in the concentration of specific elements (N, P, Si), the addition of wastewater, and changes in the light intensity, light source, and photoperiod [11, 23, 36]. The modification of medium promotes the formation of the pyrenoids, chlorophyll *a*, chlorophyll *c*, and carotenoids, which helps maintain photosynthetic activity during batch culture [17, 18]. In addition, microalgae grown using modified media can be applied as bioresources for the biotechnological, health food, cosmetics, and feed industries.

Medium f/2, which is commonly used for the growth of microalgae, contains various chemical substances that facilitate their growth; however, this medium is expensive and cumbersome to prepare. In addition, it is not a convenient or efficient medium for use in industrial scale cultures. Therefore, it is necessary to develop a novel medium for the growth of microalgae.

In the first study of recycled animal wastewater, BM could be used as a medium for the culture of microalgae [17, 18]. In a previous study [12], fermented animal wastewater was found to be a natural substitute chelator for EDTA (ethylenediaminetetraacetic acid). In the present study, the use of media produced from a combination of natural deep seawater containing various minerals and animal wastewater (DSW+EP60%) to culture *C. ovalis* was found to provide nutrients that facilitated cell division at a reduced cost. Indeed, of the different types of media evaluated in this study, DSW+EP60% was found to be the most suitable

medium for the culture of *C. ovalis*, based on the maximal quantum yields of photosystem II at 470 nm and the chlorophyll *a* levels.

To screen for shifts in the biochemical pools of microalgal cells cultured under different conditions, we can measure the energy flow from the absorbed quanta of solar energy (light reaction of photosynthesis) to the biochemical and metabolic reactions (dark reaction of photosynthesis). The nutrient compositions of each medium result in changes in the proportion of proteins, lipids, and carbohydrates present in the culture [34]. The energy flow absorbed into newly formed biomass depends on the photosynthetic performance as well as the metabolic efficiency at which the quanta are converted into biomass [31]. The metabolic costs of carbon production are dependent on factors such as incident light climate, nutrient availability, and temperature. In addition, it has been shown that the amount of absorbed quanta is related to gas exchange rates and the conversion of newly formed biomass into proteins, carbohydrates, lipids, and nucleic acids [34].

In this study, photosynthetic metabolites were identified using FT-IR spectroscopy to observe the cells at wavelengths between 400 and 4,000 cm<sup>-1</sup>. The carbon chains of fatty acids were then analyzed and found to contain peaks attributable to C-H (3,015 cm<sup>-1</sup>), CH<sub>2</sub> (2,920 cm<sup>-1</sup>), and CH<sub>3</sub> (2,975 cm<sup>-1</sup>) (Table 3) [31]. Furthermore, the length of the carbon chains of fatty acids in the lipid pool, the amide groups in the protein pool, and the polysaccharides in the carbohydrate pool were shifted according to the different compositions of the media.

It is interesting to note that *C. ovalis* cultured in DSW+EP60% contained more typical peaks and biochemical pool shifts than *C. ovalis* cultured in other types of media. For example, the results revealed the presence of a CH<sub>2</sub> group of lipids and proteins, a C=O belonging to an ester functional group for lipids and fatty acids, and CH<sub>3</sub> and

CH<sub>2</sub> groups in proteins produced by *C. ovalis* grown in DSW+EP60%. These findings indicate that the DSW+EP60% medium contained more typical compositions that lead to the stimulation of cell division than other media, which suggests that the addition of cow compost (EP) medium to DSW more efficiently shifted the metabolite pool and induced the growth of cells than the addition of swine urine (BM) medium.

The FT-IR spectra of *C. ovalis* grown in DSW+EP60% contained the most variable peaks, which indicates that this medium was composed of the most suitable ratio of both types of water.

The use of DSW+EP60% may reduce maintenance expenses and promote higher yields of *C. ovalis* without the need for additional nutrients. Therefore, the use of DSW+EP60% could lead to the enhanced growth of *C. ovalis*, thereby facilitating the production of fish food, health food supplements, and bioenergy resources produced using this microalga. In addition, the use of this novel medium should improve the cost efficiency of industrial production using a large-scale system while decreasing the negative impacts on the environment associated with the production of *C. ovalis*. Overall, the results of this study indicate that DSW+EP60% will be applicable at an industrial scale, which will enable the production of *C. ovalis* at levels sufficient to meet the demands of the local market.

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