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Aerobic and anaerobic co-cultivation of *Nannochloropsis oculata* with oil palm empty fruit bunch for enhanced biomethane production and palm oil mill effluent treatment

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ABSTRACT

This study explored co-cultivation of *Nannochloropsis oculata* microalgae with oil palm empty fruit bunch (OPEFB) for anaerobic biomethane production and palm oil mill effluent (POME) treatment. The highest specific biogas production rate (1.13–1.14 m^3 kg^{-1} COD d^{-1}) and methane yield (4606–5018 mL CH_4 L^{-1} POME d^{-1}) were achieved with co-cultivation of *N. oculata* (2 mL/mL POME) and OPEFB (0.12 g/mL POME), as similarly predicted by response surface methodology for optimum conditions. Without microalgae and OPEFB co-cultivation, the biomethane yield was 1.3-fold lower, although the specific biogas production rate remained constant at 1.13–1.16 m^3 kg^{-1} COD d^{-1}. Aerobic and anaerobic treatment of POME after 7 d with microalgae achieved higher removal efficiency of COD (90–97%), BOD (84–98%) and TOC (65–80%) as compared to without microalgae with COD (58–68%), BOD (77–86%) and TOC (58–68%).

Keywords: Anaerobic digestion; Biomethane; *Nannochloropsis oculata*; Response surface methodology; Palm oil mill effluent; Waste remediation

1. Introduction

There will be an almost 60% more worldwide energy demand in 2030, out of which 45% will be accounted for China and India [1]. Bioenergy from biological materials, such as manure, wood, straw and effluent or agricultural products, is seen as an alternative to fossil fuels and one of the key options to meet the short- and medium-term demand to mitigate greenhouse gas emissions [2]. In Malaysia, palm oil industry is estimated to generate more than 50 million tonnes of biomass in the form of mesocarp fibre, shell and empty fruit bunch (OPEFB), and liquid waste as

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palm oil mill effluent (POME). Whilst mesocarp fibre and shell are burnt in the boiler to generate steam for electricity, and the oil palm empty fruit bunch (OPEFB) is used as fertilizer or for soil mulching in the oil palm plantation, POME has not been commercially reused. POME is a dark colour viscous and acidic slurry, containing high oil and grease, and has been identified as one of the major sources of aquatic pollution in Malaysia [3]. A life-cycle assessment study on palm oil mill processes reveals that the non-recovered biomethane emission from POME contributes the highest impact towards the environment in the climate change category [4].

The high content of carbohydrates (29.6%), proteins (12.7%), nitrogenous compounds and lipids, with considerable amount of cellulose and non-toxic minerals in POME, provides good sources for microbial fermentation [5]. At present, 85% of POME treatment is based on anaerobic and facultative pond system, followed by an aerobic treatment in open tank digester with extended aeration to meet the required discharge standards [4,6,7]. An Anaerobic digestion breaks down organic material in the absence of oxygen, which produces biogas, a mixture of methane (55–75%) and carbon dioxide (25–45%) with variable trace amounts of carbon monoxide, nitrogen, hydrogen, hydrogen sulphide and oxygen [8]. Several parameters have been suggested as process indicators of the anaerobic digestion process such as biogas production, gas composition, pH, volatile solid destruction and volatile fatty acids concentrations [9]. Methane emission rate from a large-scale closed digesting tank has been reported at 5,019 kg d^{-1} or 62.5% composition, which is much higher than open pond (1043.1 kg d^{-1}) and open digesting tank (518.9 kg d^{-1}) [10]. Advanced treatment that can be implemented, but with additional cost, includes membrane technology, up-flow anaerobic filtration, up-flow anaerobic sludge blanket and up-flow anaerobic sludge fixed film bioreactor [4]. Other recent methods, such as coagulation [11], vermitechnology [12], adsorption [13] and others, are proposed, but their efficiencies in large-scale treatment of POME require more in-depth investigations.

Sustainable energy management in palm oil mill has entered a new dynamic era with the opportunity of culturing microalgae using POME [14]. Microalgae can provide different types of biofuels such as biodiesel derived from lipid, bioethanol from fermentation of biomass, biomethane by anaerobic digestion of biomass and photo-biologically produced biohydrogen [15]. Algae add an advantage to effluent treatment by increasing the performance of degradation, improving CO_2 balance and lowering energy demand for oxygen supply in aerobic treatment stage. The role of algae isto assimilate plant nutrients and to support bacteria with oxygen. Bacteria, in turn, are involved in the degradation of organic material in wastewater, the same process is utilized in activated sludge [16]. The culture of microalgae as tertiary treatment before POME is discharged is attractive due to practically low cost and high efficiency. Therefore, most of the nutrients, such as nitrate and ortho-phosphate that are not removed during anaerobic digestion, will be further treated in microalgae pond. The cultured microalgae can then be used as a diet supplement for live feed culture [17].

The aim of this study was to investigate the effect of aerobic and anaerobic co-cultivation of *Nannochloropsis oculata* with the addition of OPEFB and pond sludge as inoculum for biomethane production and POME treatment. The effects of substrate, co-substrate/inocula and *N. oculata* ratio over two responses, namely biomethane and specific biogas production rate, were estimated by applying multilevel factorial design response surface methodology.

2. Materials and methods

2.1. Sample preparation

POME and OPEFB were collected from FELCRA Nasaruddin Palm Oil Mill, Bota, Perak, Malaysia. POME was stored in the chill room at 4°C to avoid microbial biodegradation activity and composition change. OPEFB was dehydrated in an oven at 105°C for about 6 h and then crushed by using electric blender to achieve practical sizes of less than 4 mm. The OPEFB powder was stored in an airtight plastic bottle at room temperature until used.

2.2. Algal strain and culture medium

Microalgal strain *N. oculata* was obtained from Fisheries Research Institute (FRI), Pulau Sayak, Kedah, Malaysia. Algal culture and maintenance was established as batch cultures at 25°C under 24 h illumination of white fluorescent light (Philips) of 90 μmol photons m^2/s intensity. For small-scale cultivation, 10 mL/mL of inocula was added into 100 mL of Conway media [18] in 250 mL Erlenmeyer flasks. After autoclaving at 121°C for 20 min, all media constituents were added aseptically from stock solutions prepared earlier into 1 L sterilized seawater (FRI) as follows (g): Mineral solution - $NaNO_3$ 100, Disodium EDTA 45, H_3BO_3 33.6, $NaH_2PO_4.4H_2O$ 0.90, $FeCl_3{\cdot}6H_2O$ 20, $MnCl_2{\cdot}4H_2O$ 1.3; Trace metal solution of 1 mL - $ZnCl_2$ 2.1, $CoCl_2{\cdot}6H_2O$ 2, $(NH_4)6MO_7O_2{\cdot}4H_2O$ 0.90, $CuSO_4.5H_2O$ 2; Vitamin solutions - Thiamine chlorhydrate, B_1 2, and Cyanocobalamin, B_{12} 0.01. Fresh medium was autoclaved at

121˚C for 20 min. All media constituents were added to the sea water aseptically, at 1 mL/L for main mineral stock solution and nitrate, and 0.1 mL/L for stock vitamin solutions. Cultures were subcultured on fortnightly basis.

2.3. Aerobic experiment

The CHALLENGE AER-200 Aerobic and Anaerobic Respirometer system was used for aerobic and anaerobic digestion experiment. The system consists of eight 500 mL serum bottles (Pyrex), MS8-300 magnetic stirring base for sample mixing, a water bath for controlling the temperature of the reaction vessels, a cell base containing eight flow measuring cells, an interface module and a computer.

Fresh POME was treated by adding dried OPEFB and one-weekold POME sludge inocula in the presence or absence of algae. Bottles were filled with 50 mL POME, 3 mL/mL POME sludge, OPEFB 0.12 g/mL POME and 2 mL/mL algae inoculated at initial density of 60.9×10^6 cells/mL and placed on magnetic stirrer set at 200 rpm, 25˚C. Initial pH of the sample was adjusted to 7.8–8 by addition of 0.025 g/L NaOH or 0.0027 g/L HCl. The experiment was conducted for 3 and 7 d.

2.4. Anaerobic digestion

The reaction vessels and related parts were cleaned using deionized water and rinsed thoroughly before autoclaving at 121˚C, 15 min, to ensure no contamination from previous experiments. The following procedures were carried out under non-sterile environment to establish the results as it would be applied in the field. The Teflon^{TM} -coated magnetic stirring bar was added, and the measured samples at designed volumes were added. Two-week-old microalgae were inoculated into reaction vessel at initial density of 63.9×10^6 cells/mL into fresh POME containing dried OPEFB and one-week-old POME sludge inocula. Each vessel was purged with nitrogen gas, and the screw cap with butyl rubber septum was quickly put on to ensure anaerobic environment. The initial pH was adjusted to 7.8–8 by addition of 0.025 g/L NaOH or 0.0027 g/L HCl. The reaction vessels were then placed on a magnetic stirring base water bath at 48˚C, the stirring rate at 300 rpm and run for 3 and 7 d.

The test bottles were vented by briefly inserting a clean 20-gauge needle through the septum. This venting prevents gas build-up in the bottle. Reaction vessels were attached to the tubing, connected to a flow measuring cell for analysis of total gas production and its production rate and for biogas composition analysis. Plastic gas bags (SKC, Japan) were connected to each test bottle. The challenge environmental system (CES) programme was started when the temperature of water bath was stable, and no bubble was detected in the flow measuring cell. The cell counters and timer from the control screen of the computer program were reset and data acquisition initiated.

Gases produced during anaerobic digestion passed through each cell, a result of pressure build-up caused by gas production in the reaction vessel, and the cell bubbles of fixed volume were produced. These bubbles in turn were detected by the photocell and the sensor in the cell base. The signals were processed by the interface module and the computer. The CES programme automatically recorded the rate of biogas production and the total volume of biogas. The number of bubbles was measured as cumulative volume and flow rate [19]. The lowest volume of the measurement using the standard anaerobic cell was one bubble or about 0.15 mL; the upper range was 2–3 bubbles/s or about 20–25 mL/min with high-sensitivity cells having about 0.05 mL per bubble or 8–10 mL/min.

2.5. Analytical method

2.5.1. Microalgal growth analysis

Microalgae from maintenance culture were harvested every two days and the cell numbers counted using haemocytometer (Hirschmann, Germany). For fresh and dry weight determination, 100 mL sample was harvested and filtered through pre-weighed GF/F filters (934-AH, Whatman, USA). The filtered cells were washed with distilled water and dried at 80˚C in an oven until constant weight and cooled in a desiccator before weighing.

2.5.2. Chemical analyses of POME and OPEFB

The pH of POME was measured by using Mettler Toledo-320 pH probe. The biological oxygen demand (BOD_5) was analyzed using standard methods by HACH (HACH, USA). The BOD samples were incubated for 5 d at 20˚C. The COD measurement was carried out using spectrophotometer DR 5000, according to 8000-Reactor Digestion Methods [20]. The total organic carbon (TOC) was analyzed using TOC analyzer (TOC-$V_{CSH\ SHIMADZU,}$ Japan). The elemental analysis of OPEFB was performed using CHNS-932 analyzer [21].

Removal efficiencies of BOD, COD and TOC were calculated using the following equation:

$$\text{Removal efficiency } (\%) = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

where C_i and C_f, respectively, are the initial and final concentrations (mg/L).

2.5.3. Biogas composition

Biogas composition after 3 d hydraulic retention time (HRT) was determined using gas chromatography (Shimadzu, GC-2010) operated under the following conditions: Column GS-Q (J&W Scientific) of 0.32 mm diameter and 25 m length, N_2 as a carrier gas at 54 mL/min, column temperature of 60˚C, injector temperature of 150˚C, detector temperature of 200˚C, column flow rate of 0.99 mL/min and using thermal conductivity detector. Two mL of the gas sample was injected to analyze the main composition of biogas—CH_4, H_2 and CO_2.

2.6. Statistical experimental design and analyses

Experimental design, mathematical modelling and optimization were performed using Statgraphic Version 5 (Rockville, USA). Multilevel factorial design of 12 experimental runs were carried out to optimize two independent variables: *N. oculata* (mL/mL POME) (x_1) and OPEFB (g/mL) (x_2) with all possible combinations of values for each experimental factor at low ($x_1 = 0$, $x_2 = 0$), medium ($x_1 = 1$, $x_2 = 0.06$) and high ($x_1 = 2$, $x_2 = 0.12$) levels. All evaluated levels were codified and combined in different experimental runs. Each experiment was run in duplicate in order to estimate experimental error and carried out in randomized order to minimize the error. POME volume and inocula were kept constant at 50 mL and 3 mL/mL POME, respectively. The responses were the specific biogas production rate (m^3/kg COD d^{-1}) (y_1) and production of methane (mL CH_4/L POME d^{-1}) (y_2). The specific biogas production rate was calculated as follows: [22]:

$$\text{Specific biogas production rate}/(m^3/\text{kgCOD/d}) = \frac{\text{Total volume of biogas produced } (m^3)}{\text{COD load (kg)} \times \text{Time (d)}} \quad (2)$$

A second-order polynomial regression model was used to predict the optimal point for both responses:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

where y is the response variable; x_i and x_j are the independent coded variables; k is the number of independent values; ε is the random error; and β_0, β_j, β_{ii} and β_{ij} are constant coefficient, coefficient of linear, interaction regression and quadratic term, respectively. The goodness of fit of the model was evaluated by the analysis of variance (ANOVA). Pareto charts and contour surface responses evaluate the interaction that has significant effects. The synergistic effects of the two independent variables can be determined by the response contour and surface plots. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. The model terms were selected or rejected based on the p-value with 95% confidence level ($p < 0.05$). The simultaneous interaction of the two independent variables was investigated by constructing the response contour and surface plots and Standardized Pareto charts for the interactive effects of OPEFB and *N. oculata*.

3. Results and discussion

3.1. Microalgal growth, lipid content and OPEFB analyses

Fig. 1(a) and (b) show the cell density, dry weight and lipid content of *N. oculata*. The highest cell density of 63.9×10^6 cells/mL, dry weight of 0.5 g/L and lipid content of 23.5% was obtained after two weeks. The decline phase was observed thereafter with reduction of cell number to 58.35×10^6 cells/mL, dry weight to 0.35 g/L and lipid content to 20.8%. These results are comparable to the biomass (1.10–2.32 g/L) of *N. oculata* and variation of total lipid content (10%–30% dry weight) [23–25]. The elemental composition of carbon, hydrogen, nitrogen and sulphur of OPEFB (Table 1) were 40.1, 5.3, 1.4 and 0.29%, respectively. The C:N ratio of 29:1 suggests the presence of nutrients and minerals required for bacterial growth, which are comparable to previously reported values [22,26].

3.2. Aerobic and anaerobic POME treatment with and without microalgae

The average characteristics of raw POME are as shown in Table 2. The pH was 3.5–5 containing COD of 65771.7 mg/L, BOD of 24116.7 mg/L, TOC of 4745.8 mg/L, TSS of 68,367 mg/L and oil and grease of 3,546 mg/L, indicating high amount of organic matter. At high COD and low pH, raw POME could potentially

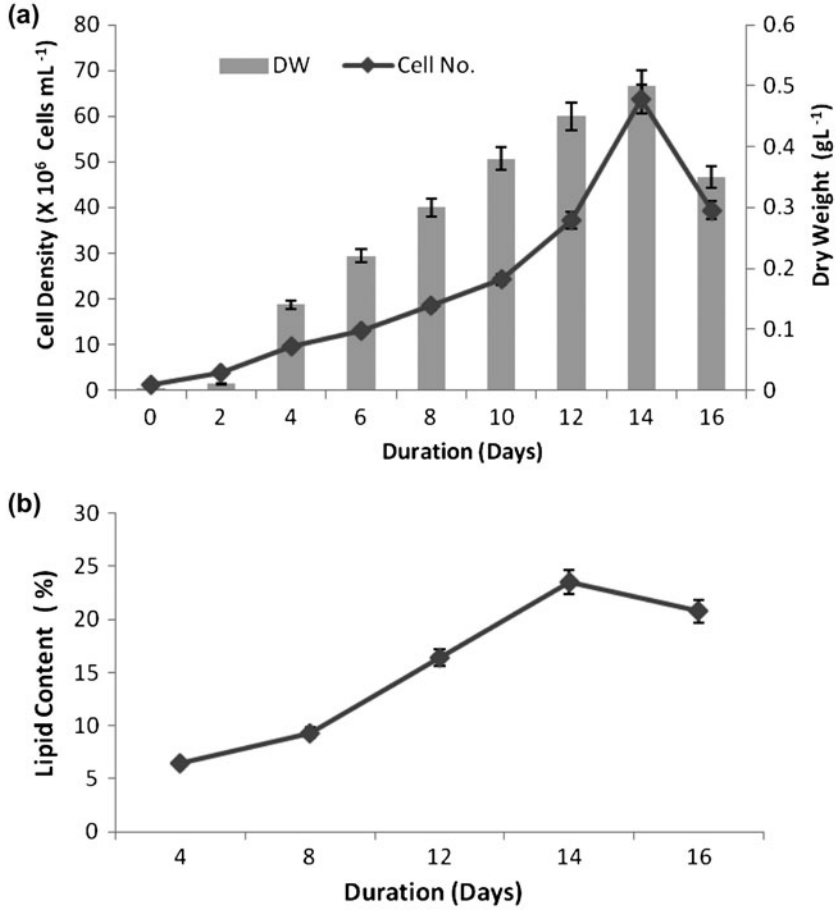

Fig. 1. Profile of (a) cell density and dry weight and (b) lipid contents of *N. oculata*.

Table 1
Elemental composition of oil palm fibres [17,21]

Parameters	OPEFB	Palm kernel	Fibre (%)	Shell (%)	OPEFB (This study)
Carbon (%)	45.5	56.3	47.3	52.2	40.1
Hydrogen (%)	6.1	9.0	6.0	6.3	5.3
Nitrogen (%)	1.7	1.7	1.4	0.6	1.4
Sulphur (%)	0.14	0.18	0.3	0.2	0.29
C/N ratio	27:1	33:1	34:1	87:1	29:1

inhibit or overload the process resulting in reduced biodegradability [27]. The anaerobic treatment with *N. oculata* after 7 d HRT achieved the highest removal efficiency of COD, BOD and TOC of 97, 98 and 80%, as compared to 90, 84 and 65%, respectively, under aerobic treatment with microalgae. These are superior to that reported for batch culture of *Chlorella sp*. which reportedly removes COD, ammonia, total nitrogen and total phosphorus by 90.8, 93.9, 89.1 and 80.9%, respectively, after 14 d [28]. Short HRT of 2.5 d has also been reported in the presence of microalgal biofilms for removal of nitrogen, phosphorus and COD from wastewater [29].

Before treatment, the sample pH was adjusted to 7.4 for aerobic and 8 for anaerobic treatment. After 3 and 7 d, the final pH of aerobically treated sample was stable at 7.7–7.9, as compared to anaerobic treatment which dropped to 5.6–6.3. The pH drop can be attributed to the accumulation of high volatile fatty acid concentration and ammonia, and this could influence anaerobic digestion by affecting acetate-utilizing methanogenic archaea, hydrogen-utilizing

Table 2
Physico-chemical characterization of aerobic and anaerobic treatment of POME with and without microalgae

Parameters	Raw POME (mg/L)	*Removal efficiencies (%)							
		Aerobic treatment				Anaerobic treatment			
		Without algae		With algae		Without algae		With algae	
		3 d	7 d	3 d	7 d	3 d	7 d	3 d	7 d
pH	3.5–5	7.8	7.7	7.9	7.8	6	5.7	6.3	5.6
COD	65,771.7	53	62	65	90	69	96	83	97
BOD	24,116.7	73	77	82	84	83	86	90	98
TOC	4,745.8	49	58	56	65	59	68	63	80

*All values indicate removal efficiencies except for raw POME and pH.

methanogens and syntrophic bacteria, and subsequently may inhibit anaerobic bacteria and reduce methanogenesis [30–32]. Optimal loading rates and HRTs must be achieved for optimal conversion of organic matter. The type and composition of the algal substrate must be assessed, as the accessibility of the microalgal intracellular content to the anaerobic microflora may be limited by the resistance of the microalgal cell wall to hydrolysis when the cells are directly introduced into the anaerobic process [33,34].

Co-digestion is beneficial because potential toxic NH_4 is diluted which allows improved loading rate and enhanced biogas yield [35]. Addition of microalgae enhances the buffering capacity and minimize acidifications. The better result of anaerobic treatment with microalgae may suggest that co-digestion allows microalgae to consume CO_2 whilst providing O_2 to facultative anaerobic microbes to digest POME, thus working synergistically to sustain volatile acid production and stabilize the pH. In the presence of light, microalgal photosystems capture light energy and store it in the chemical bonds of ATP and NADPH. The reactions of the Calvin cycle use the energy stored to reduce CO_2 to carbohydrates, and these may occur in the dark. During anaerobic cultivation, the thick POME and mass culture may prevent penetration of light allowing Calvin cycle reactions, although the energy conversion efficiency may be reduced due to limitation of light [36].

3.3. Response surface methodology

The RSM experiments and the responses are shown in Table 3. The POME volume and sludge inoculum were set constant at 50 mL and 150 mL, respectively. After 3 d HRT, the highest biomethane yield of 4,606–5,018 mL CH_4/L POME d^{-1} and the highest specific biogas production rate of 1.13–1.14 m^3/kg COD d^{-1} were achieved with co-digestion of *N. oculata* (2 mL/mL POME) and OPEFB (0.12 g/mL POME). These were much higher than the reported biomethane production of (3900.8 mL CH_4/L POME d^{-1}) and specific biogas production rate (0.1162 m^3/kg COD d^{-1}) from co-digestion of *T. suecica* (2 mL/mL POME) and OPEFB (0.12 g/mL POME) [37]. By applying multiple regression analysis, second-order polynomial equation can be developed to represent the specific biogas production rate (Eq. (4)) and biomethane production (Eq. (5)):

$$\text{Specific biogas production rate}/(m^3/\text{kg COD/d}) = 0.943 - 0.229x_1 + 0.736x_2 + 0.121x_2^2 - 0.092x_1x_2 + 8.703x_2^2 \quad (4)$$

$$\text{Biomethane production}/(\text{mL } CH_4/\text{L POME d}^{-1}) = 2730.3 - 311.3x_1 + 4969.2x_2 + 453.4x_2^2 - 32.5x_1x_2 + 27463.0x_2^2 \quad (5)$$

The optimum values were predicted at 0.12 g/mL POME with *N. oculata* of 2 mL/mL POME to obtain cumulative biomethane yield of 4,912 mL CH_4/L POME d^{-1} and the specific biogas production rate of about 1.17 m^3/kg COD d^{-1}. The r^2 of 92.5% with mean absolute error percentage between experimental and predicted values of 0.025% for specific biogas production rate, r^2 of 91.8% and error of 8.03% for biomethane suggest a good agreement between experimental and predicted values as shown in Table 4.

The highest biomethane and specific biogas production rate without microalgae were 3,538–3,760 mL CH_4/L POME d^{-1} and 1.13–1.16 m^3/kg COD d^{-1},

Table 3
Multilevel factorial design and responses

Independent variables				Responses					
				Specific biogas production rate, y_1 (m^3/kg COD d^{-1})		Methane, y_2 (mL CH_4/L POME d^{-1})		CO_2 (mL CO_2/L POME d^{-1})	Hydrogen (mL H_2/L POME d^{-1})
Run	X_1	X_2	POME sludge inoculum (mL/mL POME)	Experimental value	Predicted value	Experimental value	Predicted value	Experimental value	Experimental value
1	1	0.12	3	1.08	1.04	4,200.0	3,893.0	2,350.4	ND
2	2	0.06	3	1.08	1.03	4,500.0	4,347.2	2,806.0	ND
3	0	0.12	3	1.13	1.15	3,538.0	3,754.9	3,534.4	ND
4	1	0.06	3	0.85	0.90	3,160.0	3,300.3	2,583.4	ND
5	0	0	3	0.94	0.94	2,540.6	2,763.1	1,674.8	ND
6	0	0.06	3	1.04	1.02	3,024.0	3,160.2	1,838.6	ND
7	2	0.12	3	1.13	1.16	5,018.0	4,938.0	2,789.4	ND
8	1	0	3	0.86	0.83	2,830.0	2,905.2	2,236.0	ND
9	2	0	3	0.97	0.97	3,966.0	3,954.0	2,211.8	ND
10	1	0	3	0.85	0.84	3,060.0	2,839.6	2,209.2	ND
11	0	0.12	3	1.16	1.16	3,760.0	3,689.3	2,044.2	ND
12	2	0	3	0.96	0.97	3,782.0	3,888.4	2,734.2	ND
13	1	0.06	3	0.86	0.90	2,900.0	3,234.6	1,385.6	ND
14	2	0.06	3	1.08	1.04	4,400.0	4,281.6	2,748.0	ND
15	1	0.12	3	1.09	1.04	3,840.0	3,827.5	3,318.2	ND
16	0	0.06	3	1.03	1.02	3,424.0	3,094.6	2,313.2	ND
17	0	0	3	0.96	0.95	2,866.0	2,697.5	2,534.2	ND
18	2	0.12	3	1.14	1.17	4,606.0	4,872.4	3,228.2	ND
19	0	0	0	0.1	0.13	ND	ND	217	81.2
20	0	0.6	0	0.13	0.27	ND	ND	290.6	119.6
21	0	0.12	0	0.13	0.16	ND	ND	300	124.4
22	0	0	0	0.001	0.02	ND	ND	86	47.6
23	0	0.6	0	0.12	0.16	ND	ND	108	38.4
24	0	0.12	0	0.13	0.50	ND	ND	80.0	32.0

Note: X_1 = *Nannochloropsis oculata:* 0, 1, 2 mL/mL POME; X_2 = OPEFB: 0, 0.06, 0.12 g/mL POME; ND = Not detected.

Table 4
ANOVA for Specific biogas production rate and biomethane production

Source	Sum of squares		*F*-ratio		*p*-value	
	Biogas	Biomethane	Biogas	Biomethane	Biogas	Biomethane
A: *N. oculata*	0.00000954	4.22691E6	0.15	73.27	0.4988	0.0001
B:OPEFB	0.001534	2.92764E6	22.41	50.75	0.0001	0.0001
AA	0.000713	822,165	10.43	14.25	0.0001	0.0031
AB	0.0000014	30.42	0.04	0.00	0.7020	0.9821
BB	0.0000245	39,098.5	0.72	0.68	0.1354	0.4278
Total error	0.0012561	634,546				
Total (corrected)	0.0000051	5.75501E6				
R^2	92.5%	91.8%				
R^2 adjusted	95%	95%				
Standard error of est.	0.0058473	240.179				
Mean absolute error	0.0027481	158.472				

respectively, but at high OPEFB (0.12 g/mL POME). With reduced amount of OPEFB (0.06 g/mL POME) and high *N. oculata* (2 mL/mL POME), methane yield remained high (4,400–4,500 mL CH_4/L POME d^{-1}), but the specific biogas production rate was lower at 1.07–1.08 m^3/kg COD d^{-1}. At lower amount of *N. oculata* (1 mL/mL and high OPEFB (0.12 g/mL), biomethane yield (3,840–4,200 mL CH_4/L POME d^{-1}) and the specific biogas production rate (1.08–1.09 m^3/kg COD d^{-1}) were reduced. Without both *N. oculata* and sludge inocula, no biomethane was detected, and the specific biogas production rate (0.13 m^3/kg COD d^{-1}) and CO_2 (300–80.2 mL CO_2/L POME d^{-1}) were much lower, although some biohydrogen (32–124 mL H_2/L POME d^{-1}) were detected. The production of some amount of hydrogen at runs 19–24 could be attributed to the presence of acidogens within POME which degrade the constituent carbohydrates and fatty acids into carbon dioxide and hydrogen. The absence of sludge inocula as a bacterial source, high pH and temperature appear to inhibit hygrogen production which is agreeable with the previous finding [38].

3.4. *Effects of* N. oculata *and OPEFB*

Pareto charts as shown in Fig. 2 describe relative importance of the factor and the estimated effects of factor setting adjustment, from the most influencing factor to the least one. Response surface and contour plots constructed from quadratic regression model (Fig. 3) further show optimal level at high concentration of OPEFB and microalgae. At confidence levels above 95%, OPEFB ($p < 0.0001$) has the most significant positive effect on specific biogas production rate, and *N. oculata* ($p < 0.0001$) is most significant on biomethane production. Based on the regression ANOVA, the model represents the experimental values well within the defined experimental range.

Anaerobic decomposition of organic matter involves the action of several different metabolic

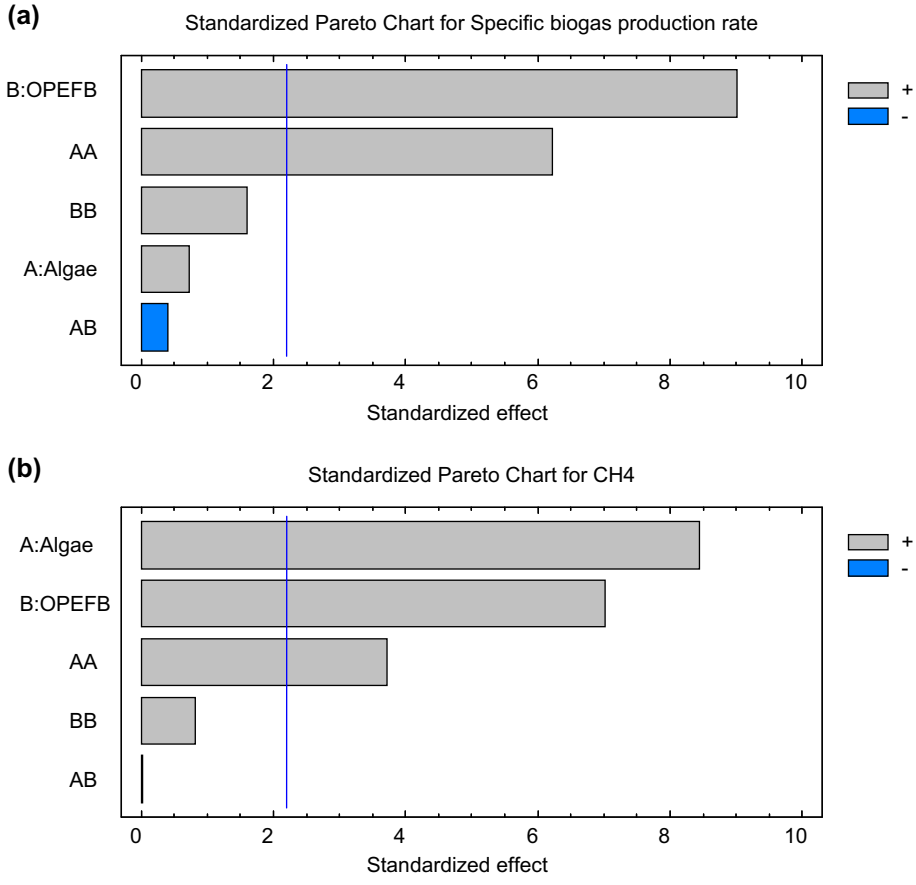

Fig. 2. Standardized Pareto chart for (a) specific biogas production rate and (b) biomethane production for anaerobic co-digestion of *N. oculata* and OPEFB.

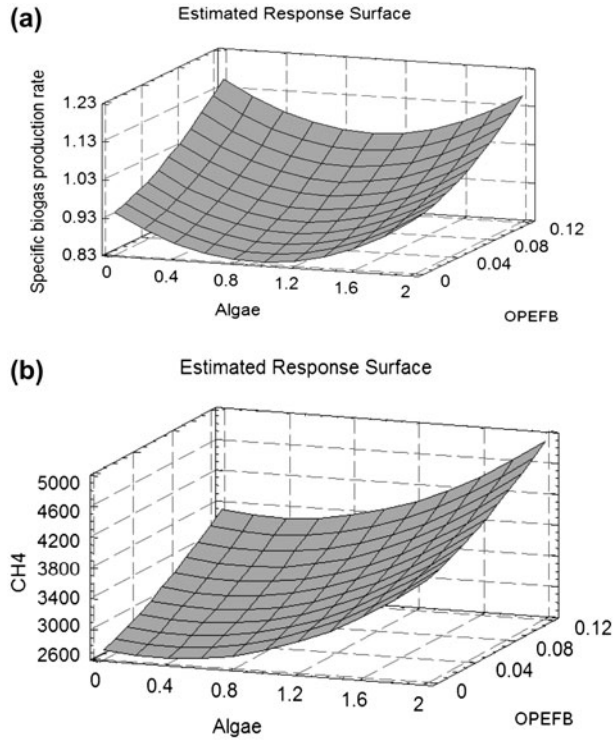

Fig. 3. Estimated response surface plot of (a) specific biogas production rate and (b) biomethane production for anaerobic co-digestion of *N. oculata* and OPEFB.

groups of microorganisms to produce biogas. POME is degraded into methane, carbon dioxide and water, as a result of sequence of reactions involving hydrolysis, acidogenesis (including acetogenesis) and methanogenesis. The complex molecules (i.e. carbohydrates, lipids and proteins) are hydrolyzed into sugars, fatty acids and amino acids by fermentative bacteria during hydrolysis. These sugars, fatty acids and amino acids are then converted during acidogenesis into organic acids by acidogenic bacteria, before being converted further by acetogens to acetate together with CO_2 and hydrogen. Hydrogen is utilized by hydrogenotrophic methanogens while acetic acid and CO_2 utilized by acetoclastic methanogens to methane [39].

In general, low or absence of microalgae and OPEFB will reduce specific biogas production rate and methane production. With excess volatile acids, the acidogens grow rapidly and produce more volatile acids to further reduce the pH. In such conditions, methanogenesis cannot occur as methanogenesis requires a pH around 6.5–7.5. The methanogens may not be able to keep up with this change and degrade acids as fast as they are generated, and these lead to low methane production [22]. The pre-treatment and addition of OPEFB improve anaerobic digestion of POME. The C:N ratio (29:1) (Table 1) is within the range of C/N ratio for anaerobic digestion 25–30:1 from lignocellulosic materials [40] for high CH_4 yield. The small size (~ 4 mm) of pressed-shredded OPEFB, as compared to that reported earlier [41], may have increased the surface area for microbial consumption.

High microalgae and OPEFB co-digestion with POME in the presence of POME sludge inocula maximize methane yield, as microalgal biomass typically has high lipids, protein and carbohydrate content and break open microalgal cells. Algal biomass containing between 2 and 22% lipid produces methane yield ranging from 0.47 to 0.80 m^3 CH_4/kg VS in anaerobic digestion [28,42]. The higher the lipid content of the cell, the higher will be the potential methane yield, as these can serve as nutrients for bacteria, and microalgae may work in tandem with bacteria to breakdown the OPEFB and POME. As discussed earlier, microalgae may also consume CO_2 to give out O_2 to facultative anaerobic bacteria to further assist more efficient anaerobic digestion.

4. Conclusion

Microalgal biomass was successfully co-cultivated for aerobic and anaerobic treatment of POME and was used as a co-substrate with POME in anaerobic digestion to enhance methane production. Highest specific biogas production rate (1.13–1.14 m^3/kg COD d^{-1}) and methane yield (4,606–5,018 mL CH_4/L POME d^{-1}) were achieved with co-digestion of *N. oculata* (2 mL/mL POME) and OPEFB (0.12 g/mL POME). Response surface methodology predicted optimum conditions well and showed good agreement with experimental values with mean error less than 10%. Without co-digestion of microalgae and OPEFB, the methane yield was 1.3-fold lower, although the specific biogas production rate remained the same at 1.13–1.16 m^3/kg COD d^{-1}. OPEFB has the most significant positive effect on the specific biogas production rate, and *N. oculata* addition is most significant on the methane yield. Anaerobic treatment of POME with microalgae achieved high removal efficiency of COD, BOD and TOC after 3 and 7 d HRT at 65–90%, 82–84%, 56–65% and 83–97%, 90–98%, 63–80%, respectively, much better than previously reported values.

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Abbreviations

POME	—	palm oil mill effluent
OPEFB	—	oil palm empty fruit bunch
COD	—	chemical oxygen demand
BOD	—	biological oxygen demand
TOC	—	total organic compound
DW	—	dry weight

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