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Immobilized *Chlorella vulgaris* for efficient palm oil mill effluent treatment and heavy metals removal

Ashfaq Ahmad[a,*], A.H. Bhat[b], Azizul Buang[a,*]

[a]*Department of Chemical Engineering, Universiti Teknologi Petronas, 32610 Bandar Seri Iskandar, Perak, Malaysia, Tel. +60 5 3687627; emails: aashfaqbangash@gmail.com (A. Ahmad), azizul.buang@utp.edu.my (A. Buang)*
[b]*Department of Fundamental and Applied Sciences, Universiti Teknologi Petronas, 32610 Bandar Seri Iskandar, Perak, Malaysia, Tel. +60 5 3687674; email: aamir.bhat@utp.edu.my (A.H. Bhat)*

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ABSTRACT

In this study, a microalgae *Chlorella vulgaris* was immobilized in alginate beads for the enhancement of palm oil mill effluent (POME) remediation. After the treatment, gel beads were simply collected via filtration and thereby simplifying the harvesting process. The effect of POME composition with freshwater (1, 5, 10, 15, and 20% v/v) on *C. vulgaris* cell growth rate, lipid content, and POME remediation was investigated. The cell growth rate (0.142–0.151 d^{-1}), doubling time (4.59–4.88 d^{-1}), and lipid content (27.64%–31.67%) were achieved at 10% v/v of POME after 14 d of cultivation for both freely suspended and immobilized *C. vulgaris*, respectively. Meanwhile, cultivation of immobilized *C. vulgaris* in POME also enhanced the bioremoval of Fe(II) and Mn(II), chemical oxygen demand (95%–99.9%), biochemical oxygen demand (97%–99.9%), total nitrogen (78%–98%), and total phosphate (79%–98%). The Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy spectra of *C. vulgaris* indicated the presence of characteristic functional groups involved in the bioaccumulation process of heavy metals.

Keywords: Microalgae; *Chlorella vulgaris*; Immobilization; Palm oil mill effluent; Bioremediation

1. Introduction

Palm oil industry is one the most organized national agricultural sector of Malaysia [1,2]. Palm oil mill effluent (POME) is produced by the mill after extraction of palm oil. This wastewater has high acidity, high viscosity, but non-toxic [3,4]. In Malaysia, palm oil industry effluent is the main source of environmental pollution due to the high amount of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) [5]. In the year 2014, the production of crude palm oil touched to 19.66 million tons per ~44 million m^3 of POME. If such waste is released without proper treatment, the BOD produced would be equal to the waste produced by 75 million people which is 2.5 times the existing population in Malaysia [6,7]. POME treatment in pond system with anaerobic digestion via aerobic post-treatment is very common among the mills, due to the less apparatus budget and an easy operating mechanism to attain the standard discharge limits set by the Department of Environment (DoE), Malaysia [8,9]. POME is brownish in color because of the presence of lignin, tannin, humic acids, lipids, and fatty acids released during industrial steam extraction [1]. Nowadays, the elimination of biodegradable and non-biodegradable organic compounds from POMEs is a major concern faced by the researchers. Biological processes (aerobic and anaerobic) are generally used for wastewater remediation; however, given its complex composition, conventional biological treatments are time-consuming and usually insufficient in degrading high molecular weight fractions [10–12].

In the last decade, several treatment methods have been studied for POME treatment such as ultrasound cavitation technology [13], hybrid approach of activated carbon and ultrasound cavitation [14], coagulation by chitosan, addition of ferrous sulfate ($FeSO_4$), chitosan with hydrogen peroxide (H_2O_2) and chitosan with Fenton oxidation [15],

* Corresponding author.

anaerobic digestion, aerobic oxidation based on an activated sludge process, aerobic and anaerobic digestions, ultrafiltration membrane separation, up-flow anaerobic sludge fixed film reactor, chemical coagulation and flocculation, solvent extraction, electrocoagulation, electroflotation, adsorption, and hydrolysis [2,16–18]. POME contains different heavy metals at a high concentration such as iron (Fe), zinc (Zn), and manganese (Mn). The discharge of this polluted effluent with high amount of metal ion into the water body could cause serious problems for human health and food chain. Thereupon, the proper treatment of this effluent is crucial for a viable industry [19,20]. Conventional treatment technologies such as membrane filtration, granular activated carbon adsorption, precipitation–sedimentation, flotation, ion-exchange, and electrochemical deposition systems have been used for the removal of toxic heavy metals Cd, Pb, Fe, Mn, Cr, and As from water [21–23]. Chemical precipitation is one of the most commonly used processes for removal of heavy metals. The process involves the use of a precipitant, for example, lime which reacts with the metal ions to form an insoluble parts-per-trillion. This technology treats effluent containing heavy metal concentrations higher than 1,000 mg/L [24,25].

Microalgae for the remediation of POME pollutants have gained increased attention. The cultivation of microalgal in wastewater for treatment and biofuel production is the most economical approach. Algae are normally sensitive to the wastewaters containing imbalanced nutrient, the presence of toxic pollutants and lack of some necessary trace elements. Some algae species *Chlorella* sp. and *Scenedesmus* sp. have a high tolerance to many wastewaters. Microalgal cells are intensely reliant on growth conditions and species itself [26]. Microalgae cultivation required necessary nutrients for its growth which provides in the growth medium. Several studies have stated that POME is the most effective wastewater for the promotion of microalgae growth [27–29]. *Chlorella sorokiniana* cultivation in 75% of POME medium achieved high dried cell weight of 1,070 ± 30 mg/L, and lipid content of 156 mg/g as compared with the POME concentration of 25%–50% [30]. Cultivation of *Chlorella* sp. in POME with the addition of synthetic nutrients medium has also been reported. The maximum lipid content of 34 mg/L/d has been reported for *Chlorella* sp. cultivation in 20% of POME with 40% of synthetic nutrients containing 45 mg/L urea and 4 mg/L of triple super phosphate. The high concentration of POME inhibits the growth rate of microalgae and takes a long period to reach the stationary phase [27]. Marine algae *Isochrysis* sp. cultivation in photobioreactor and outdoor in 5% digested POME with the aid of 0.075% of NPK inorganic fertilizers has achieved 119.17 and 104.50 mg/g of fatty acids per gram of algal cells, correspondingly [28]. This shows that POME can be used as a medium for effective algal cultivation to get maximum lipid contents. Additionally, the POME and supplements required for microalgae growth differ among species, due to their living tolerance by integrating nutrients.

This study investigated the utilization of POME at different proportions in freshwater as a medium for microalgae *C. vulgaris* cultivation and subsequently examined the feasibility of freely suspended and immobilized algal cells for the heavy metals and POME bioremediation. The biomass and lipid productivity were estimated together with the removal of the nutrients, Fe(II), Mn(II), BOD, COD, total nitrogen (TN), and total phosphate (TP) from POME.

2. Materials and methods

2.1. POME sample collection and preparation

Samples of POME were taken from FELCRA Nasaruddin, Bota, Perak, kept in the chilled room at 4°C, and then filtered to eliminate large suspended particles by using 0.47 mm filter paper. After the filtration, the samples were then centrifuged. The POME was collected for the culturing of microalgae as it contains nutrients that suit as growth media for the algae. In order to kill the bacteria available in the sample, sterilization process was performed where it is autoclaved at 121°C for 20 min.

2.2. Microalgae, medium and cultivation condition

Freshwater microalgae *C. vulgaris* were collected from the Fisheries Research Institute (FRI), Kg Acheh, Lumut, Malaysia. The initial inoculum (39.3×10^6 cell/mL) culture was sub-cultured into 250 mL shaking flasks containing Conway media to ensure an exponential growth phase. *C. vulgaris* were cultured in sterilized freshwater enriched with Conway media, and prepared as follows (g) [31]: mineral solution in 1 L – $NaNO_3$ 100, disodium ethylenediaminetetraacetic acid 45, H_3BO_3 33.6, $NaH_2PO_4{\cdot}4H_2O$ 20, $FeCl_3{\cdot}6H_2O$ 1.30, $MnCl_2{\cdot}4H_2O$ 0.36; trace metal solution in 100 mL – $ZnCl_2$ 2.10, $CoCl_2{\cdot}6H_2O$ 2, $(NH_4)_6MO_7O_2{\cdot}4H_2O$ 0.90, $CuSO_4{\cdot}5H_2O$ 2; and vitamin solution in 1,000 mL – thiamine chlorohydrate, B1 0.2 and cyanocobalamin, B12 0.01 in 100 mL and KNO_3 116 g. Media in culture flasks were autoclaved at 121°C, for 15 min. Complete transfer of media and culture then took place in an aseptic environment in a laminar flow cabinet.

Microalgae were cultured at a control condition of 7 ppt NaCl, pH 8, constant orbital shaker at 120 rpm at ±25°C temperature and 12:12 h light:dark by using Philips Fluorescent tubes of 4,000 lux intensity for 18 d. The glasswares and media supplements were sterilized by using autoclave at 121°C for 15 min. The medium was added at sterilizing condition under a laminar flow cabinet. The experiments were run in triplicate in order to get average results for both the culture and control media.

2.3. POME medium preparation

POME was filtered to separate impurities and then centrifuged (Labogene ApS Centrifuge, Denmark). POME supernatant was diluted with freshwater at different composition of 1%, 5%, 10%, 15%, and 20% v/v for the cultivation of *C. vulgaris* and bioremediation. Free living and immobilized cells of *C. vulgaris* were cultured in 250 mL flasks under the illumination of the fluorescent tubes with light intensity of 4,000 lux under a 12 h:12 h light:dark (L:D) cycle and pH 8.

2.4. Preparation of immobilized microalgae beads

Sodium alginate solution 3% w/v was prepared and autoclaved for 20 min at 121°C. Microalgal cells were

harvested at exponential growth phase at an initial cell number of 38.4 × 10^6 cells/mL and mixed with sodium alginate solution at room temperature. The alginate–alga mixture was transferred into 50 mL burette and titrated into a 2% $CaCl_2$ (w/v) solution and stirred with a magnetic stirrer at 3,500 rpm. Alginate beads around 3–4 mm diameter were formed and stabilized for 1 h in $CaCl_2$ solution and then washed three times with distilled water to remove the remaining $CaCl_2$.

2.5. Analytical method

2.5.1. Measurement of free suspended and immobilized cells

The growth rate of freely suspended cells was measured by counting the number of cells by using hemocytometer (Hirschmann/Germany) under the biological microscope (Meiji, Mx4300L). While for the counting of immobilized cells in alginate beads, few beads were taken daily and dissolved in 1 mL of 4% $NaHCO_3$ solution for 30 min [32].

Specific growth rate and doubling time was calculated according to the following formula:

$$\mu_{max=} \frac{l_n x_2 / x_1}{t_{2-t_1}} \quad (1)$$

where μ_{max} is the specific growth rate, X_2 is the cell growth on day 14, X_1 is the cells growth on day 4.

$$t_d = \frac{\ln_2}{\mu_{max}} \quad (2)$$

where t_d is the doubling time.

2.5.2. Chemical analyses for POME characterization

COD was measured with DR5000 digested reactor and spectrophotometer by using the HACH standard method (HACH, 2008). First, the HACH DRB 200 Reactor was preheated to a temperature of 150°C before 2 mL of POME from each flask were added to high range COD digestion reagent vials. After ensuring that the contents were well mixed, the vials were placed in the reactor for 2 h. The vials were then left to cool down to room temperature before placing them in HACH DR 5000 spectrophotometer. The COD was measured by using HACH program 435 COD HR in spectrophotometer and reading was measured in mg/L COD for each vial [33].

BOD was determined by using BOD-track by conferring the HACH Standard Method [33]. On the other hand, TOC and TN were measured with TOC analyzer. Moreover, inductively coupled plasma mass spectrometry was used to analyze the heavy metals content in the treated POME. The removal efficiencies of Fe(II) and Mn(II) were computed by using the formula:

$$\text{Removal efficiency}(\%) = \frac{(C - C_i)}{C_i} \times 100 \quad (3)$$

where C_i is the initial and C_f is the final parameter concentrations.

2.5.3. Lipid extraction

The total lipid content was extracted by using Bligh and Dyer modified method. The lipids were extracted from the fresh biomass of *Chlorella vulgaris* with the solvent of methanol, chloroform, and distilled water at a final ratio of 9:10:10 [34]. The recovered lipid was transesterified into fatty acid methyl esters by using reported methods [35] and then analyzed via gas chromatography.

2.6. Biomass characterization

2.6.1. Fourier transform infrared spectroscopy

Algal biomass was centrifuged before and after treatment and the pellet was dried at 60°C in the oven. The algal powder was examined by using Fourier transform infrared spectrophotometer (FTIR; PerkinElmer Inc., Spectrum One/BX). FTIR studies were conducted by potassium bromide (KBr) pellet method. The infrared spectra were recorded in the range of 350–4,000 cm^{-1}.

2.6.2. Scanning electron microscopy

The dried algal biomass, before and after treatment, were coated with an ultra-thin film of gold by an ion sputter, exposed under an electron microscope at a working height of 1–50 mm with a voltage of 0.1–30 kV (Model: SUPRA 55VP, Carl Zeiss AG, Germany).

2.6.3. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) analysis was conducted with a thermo scientific, k-alpha spectrometer with monochromatized Al Kα radiation. The source was operated at 15 kV and 10 mA (1,486.6 eV). After considering the sensitivity factors, correction on elemental compositions were determined from the peak area ratios.

3. Results and discussion

3.1. Characterizations of palm oil mill effluent

An optimum pH in the range of 6.0–9.0 provides protection for the sustenance of freshwater fish and bottom dwelling invertebrates. In our study the pH value was found to be in the acidic range of 3.4–3.5 while the previously reported pH value was between 3.4 and 5.2 [12,36]. COD is the quantity of oxygen needed by the oxidizing agent to oxidize all organic matters into carbon dioxide, water, and ammonia. The COD mainly determines an amount of organic pollutants present in water [37]. In this study, the amount of COD 65,271.7 mg/L has been attained which is close to the stated results 69,500 mg/L for raw POME [38,39]. The difference indicates the diverse level of organic matter present in the sample. The standard BOD_5 value determined the amount of organic pollutants present in the POME. BOD could be used to estimate the treatment proficiency and is an indirect measure of biodegradable organic compounds present in the POME. In this study, the concentration of BOD_5 in POME was found to be 24,116.7 mg/L while the previous study range was 10,250–43,750 mg/L. However, the most common value recorded is

about 24,000–25,000 mg/L [12,36,39]. High concentration of BOD_5 suggests that the effluent has high organic matter and microorganisms will consume more oxygen to decompose the organic matter. The COD/BOD ratio can be used to assess the biodegradability of the organic matter. At high COD and low pH, raw POME could potentially inhibit or overload the process, resulting in reduced biodegradability [40]. The ammonia nitrogen value of 384.7 mg/L and TP 276.65 mg/L has been recorded. POME is also rich in mineral content, particularly phosphorus (18 mg/L), potassium (2,270 mg/L), magnesium (615 mg/L), and calcium (439 mg/L) [41]. In wastewater, nitrogen is in the form of ammonia, nitrite, and nitrate. Nitrate may be present more in sewage water, than in the new wastewater discharge. Total Kjeldahl nitrogen (TKN) is the sum of organic nitrogen, ammonia (NH_3), and ammonium ion (NH_4^+) found during the chemical analysis of soil, water, or wastewater. Nitrogen is an essential ingredient for cell growth, but too much of nitrogen and phosphorus in the environment and discharged into the waterways can contribute to massive algal bloom, leading to oxygen depletion in water with its associated problems.

POME generally contains different metals at critical levels, such as iron (Fe) and manganese (Mn). The amount of heavy metals iron (Fe(II)) of 91.4 and manganese (Mn(II)) of 52.6 mg/L was recorded in this study. Comparatively, an average value for manganese of 62 mg/L, iron 179.105 mg/L have been previously reported [44]. The input of these metals on ecosystems has an adverse effect on human health and the environment. Metals can bioaccumulate in living organisms and the soil organic layer, contaminate the ground and surface water and even cause air pollution [42]. The Department of Environment (DoE), Malaysia, has set the discharge limits of POME in the water bodies in order to inhibit the environmental pollution. The Environmental Quality Act (EQA) 1974 outlines the POME discharge limits as shown in Table 1 [7,43,44]. In 1977, the standard limitation of the palm oil effluent BOD has been announced by the DoE, Malaysia [7]. The aim of the regulation is to decrease the pollution without hampering its growth. The characteristics of POME depend on the types or age of fruit, various batches, days, and factories [45]. Processing condition, climate, cropping season, and milling process also have an influence on the quality and quantity of the effluent [44]. A huge investment in research and development has been carried out to reduce the pollution significantly. Malaysia as a pioneer has gained valued experiences for the development of upstream and downstream processing technology [46]. Unfortunately, in Malaysia, a large quantity of POME is released into natural water resources without proper treatment, such as rivers, which sources colossal negative ecological effect.

Table 1
Characteristics and standard discharge limits of raw POME [43]

Parameters	Standard discharge limits	This study
pH	6.0–9.0	3–3.5
Temperature, °C	40	80
Chemical oxygen demand (COD)	200	65,271.7
Biological oxygen demand (BOD_5)	20	24,116.6
Total phosphate (TP)	150	276.65
Total nitrogen (TN)	10	384.66
Manganese	0.20	52.6
Zinc	2.0	82.5
Iron	1.0	91.4

Note: All the units in mg/L, except temperature and pH.

3.2. Growth of freely suspended and immobilized cells

Figs. 1(a) and (b) show the effects of POME composition with freshwater on cell growth of freely suspended and immobilized *C. vulgaris* cells. A high growth rate for immobilized *C. vulgaris* of 30.47 × 10^6 cell/flask was obtained under 10% v/v POME concentration on day 14 which is slightly higher compared with freely suspended sample which registers 28.47 × 10^6 cell/flask. However, lower cell growth was observed for both freely suspended and immobilized samples when compared with the control condition, although the POME concentration was increased. Table 2 shows, for the immobilized sample, the highest cell growth rate of μ_{max} at 0.142 d^{-1}, doubling time of 4.88 d^{-1}, and lipids content of

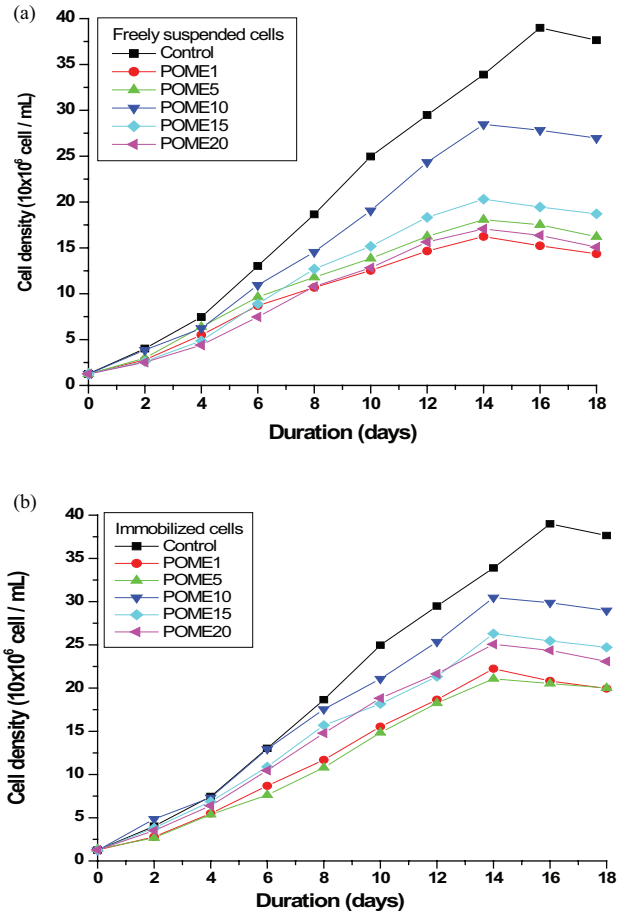

Fig. 1. (a) Freely suspended microalgal cells/flask, and (b) immobilized microalgal cells/flask, cultivation in different composition of POME with freshwater.

31.67% ± 1.50%. These were also observed under the condition of 10% v/v POME concentration. As for the freely suspended condition, the highest cell growth rate of μ_{max} at 0.151 d^{-1}, doubling time of 4.58 d^{-1}, and lipid content of 27.64% ± 1.20% was recorded for the 10% v/v POME concentration. Comparatively, *Chlamydomonas* sp. UKM 6 cultivation in POME media with distilled water at different volume ratios of 25%, 16.7%, and 12.5% achieved the highest specific growth rates of 0.715, 1.047, and 1.353 d^{-1} with biomass formation of 0.634 ± 0.042, 0.917 ± 0.026, and 0.716 ± 0.045 g/L, correspondingly [47]. Another study reported the cultivation of *Chlorella* sp. in different concentration of synthetic nutrients in POME. The high lipid productivity 34 mg/L/d of *Chlorella* sp. has been attained at 20% POME with an addition of 40% synthetic nutrient for growth medium [27]. All these findings indicate POME as a potential medium for *C. vulgaris* growth as a replacement for the synthetic nutrient.

3.3. Effects on lipids and fatty acids

The characterization of fatty acids profile in lipids from *C. vulgaris* cultivated in a commercial medium and in 10% POME medium with freshwater are shown in Table 3. The amount of total saturated fatty acids (SFAs; 71.55%),

Table 2
Kinetics of cell growth and lipid production of *Chlorella vulgaris* cultivated under control and different POME compositions in freshwater

	Media conditions	Maximum specific growth rate, μ_{max} (d^{-1})	Doubling time, t_d (d^{-1})	Lipid content (%)
Freely suspended cells	Control	0.138	5.02	30.40 ± 0.52
	1%	0.108	6.41	22.54 ± 0.32
	5%	0.104	6.64	24.63 ± 1.53
	10%	0.151	4.59	27.64 ± 1.20
	15%	0.142	4.88	25.84 ± 0.73
	20%	0.136	5.10	21.65 ± 1.12
Immobilized cells	Control	0.138	5.02	31.80 ± 0.62
	1%	0.139	4.98	27.53 ± 0.32
	5%	0.138	5.02	24.63 ± 1.53
	10%	0.142	4.88	31.67 ± 1.50
	15%	0.134	5.17	26.97 ± 0.73
	20%	0.136	5.09	23.87 ± 1.12

Table 3
Fatty acids composition of lipid from freely suspended and immobilized *Chlorella vulgaris* cultivated in 10% POME composition with freshwater

Fatty acids (%)		Microalgae		
		Control	Freely suspended cells	Immobilized cells
Saturated fatty acid				
C12:0	Lauric acid	1.32	0.61	1.52
C14:0	Tetradecanoic acid	4.83	4.98	5.21
C15:0	Pentadecanoic acid	10.2	9.43	11.3
C16:0	Palmitic acid	38.2	29.8	39.4
C17:0	Heptadecanoic acid	3.8	3.43	4.98
C18:0	Stearic acid	9.3	8.32	10.8
C20:0	Eicosanoic acid	3.9	7.32	4.23
C16:1	Palmitoleic acid	23.4	10.43	12.5
C18:1	Oleic acid	15.2	6.45	8.54
C18:2	Linoleic acid	3.2	3.65	4.54
C18:3	Linolenic acid	2.2	6.32	7.09
C20:5	Eicosapentaenoic acid (EPA)	6.8	1.34	0.96
C22:6	Docosahexaenoic acid (DHA)	4.0	2.76	2.78
Total SFA		71.55	63.89	77.44
Total MUFA		33.6	16.88	20.59
Total PUFA		16.2	14.07	15.04

monounsaturated fatty acids (MUFAs; 33.6%), and polyunsaturated fatty acids (PUFAs; 16.2%) were obtained from *C. vulgaris* cultivated in commercial Conway medium. The major components identified in *C. vulgaris* lipids were palmitic acid C16:0 (38.2%); palmitoleic acid C16:1 (23.4%); oleic acid C18:1 (15.2%); and docosahexaenoic acid DHA C22:6 (4.0%). Palmitic, stearic, oleic, and linolenic acid are the most common fatty acids in biodiesel. *Chlorella pyrenoidosa* has been reported as having C16:0, C16:2, C16:3, C18:0, C18:2, and C18:3 as the predominant fatty acids, which altogether account for 95% of the total fatty acids and therefore are suitable for the production of biofuels [48]. The highest total SFAs were found to be 77.44% in immobilized cells as compared with freely suspended cells with 63.89% and MUFA (20.59% vs. 16.88%); and PUFA (15.04% vs. 14.07%), respectively. Immobilized *C. vulgaris* cells contained high palmitic acid (C16:0) at 39.4% and palmitolic (C16:1) at 12.5%, while freely suspended cells contained palmitic acid (C16:0) at 29% and palmitolic (C16:1) at 10.43%. The similar fatty acids profile of *Chlorella lobophora* cultivated in Bold's Basal Medium has been reported [49]. Another study reported that the SFA (43.56%), MUFA (12.21%), and PUFA (36.20%) has been achieved from *C. vulgaris* cultivated in hydroponic wastewater [50]. With the highest content of C16:1, C18:1, and C14:0, these strains may be the most suitable strains isolated for the production of good quality biodiesel. The sum of PUFA (28.9%) obtained from *Chlorella* sp. was also comparable with the reported 27% level [51].

3.4. Biological oxygen demand and chemical oxygen demand

Biological oxygen demand measurement indicates the amount of substances that could be degraded biologically by utilizing dissolved oxygen during effluent treatment. The maximum pre-treatment BOD of 3,012 mg/L and minimum 194 mg/L was obtained for media conditions of 20% and 1% v/v of POME, correspondingly. In this study, as shown in Table 4, the COD level was reduced by 96% from 5,887 to 235.48 mg/L and BOD level saw a reduction of 90% (from 1,702 to 170.2 mg/L) by immobilized *C. vulgaris* with a retention period of 8 d, which is superior to freely suspended microalgae. The COD and BOD removal were enhanced when the POME level and incubation periods were increased. The highest removal of COD reached to 99.9% at which the final concentration of 0.63–11.187 mg/L was achieved. Meanwhile the BOD removal reached to 99.9% and obtained the final concentration of 0.194–3.012 mg/L for all the range of 1%–20% v/v of POME. Such results could meet the standard requirements for effluent discharge set by the Department of Environment (DoE) under the EQA in 1974 before discharging the POME into the river. This suggested that microalgae consumed the organic carbon within POME as a source of energy. When the concentration of POME was increased to 20% v/v, the efficiency got lower as well as growth rate was inhibited. This may be due to the fact that microalgae need more time to consume organic carbon or excess of carbon could be toxic to its growth. Immobilization of *C. vulgaris* could significantly enhance the abilities of nutrient removal from POME and contributed an effective, economical technology for the improvement of the environment. The high removal of COD and BOD is due to microalgae consumed nutrients within POME for survival, growth, and replication. During tertiary treatment, microalgae would produce oxygen that contributed to the oxidation of organic matters (waste), therefore, reducing the level of BOD and COD. The reduction of COD has been improved and reached up to COD 70% at optimum light illumination and aeration rate [52]. High removal efficiency of inorganic nutrients from domestic effluents was achieved with *Scenedesmus* sp., while the removal efficiency of *C. vulgaris* has been reported in the range of 88%–89.60% of COD and BOD [53,54]. *Chlamydomonas incerta* cultivation in POME under the light intensity of ±15 μmol/m^2/s at room temperature removed organic carbon in the ratio of 100:7, 100:13, and 100:31 on the second day of cultivation, correspondingly. The optimum removal of COD 67.35% has been reported from POME concentrations of 250 mg/L at 28 d of treatment [55].

3.5. Total nitrogen and phosphate removal

Microalgae required nitrogen as a nutrient source to be fixed into ammonia, nitrates, and similar molecules. Nitrogen predominant in POME is normally in the form of ammonia and nitrate, which are naturally consumed by microalgal cells. Nitrate is usually utilized after in vivo transformation to nitrite or ammonia by microalgae through an assimilation process [56]. The maximum total nitrogen of 99.3 mg/L and total phosphate of 164.2 mg/L was achieved in 20% v/v POME used for this study. The maximum removal efficiency of TN of 59% (58.2 reduced to 23.86 mg/L) was attained in 10% v/v POME after 8 d incubation with immobilized *C. vulgaris* as shown in Table 4. The removal efficiency of TN was enhanced up to 90%–98% (with final concentration of between 0.127 and 9.93 mg/L), as the POME concentration increased, after 16 d of treatment with immobilized microalgae. These results are higher as compared with the freely suspended microalgae condition. It seems that immobilized *C. vulgaris* assimilated TN at extremely high concentration. Nitrogen source which normally appears in nitrate form is crucial for the microalgae cell growth and the concentration of nitrate required in the range of 200–400 mg/L for efficient microalgae cultivation [57]. The amount of total nitrogen and phosphorus in POME is high and do not meet the standard discharge limit for industrial wastewater, according to the Department of Environment and Malaysian Palm Oil Board (MPOB). Thus, microalgae cultivation in POME offers an economical alternative conventional tertiary wastewater treatment and in the meantime, consuming nitrogen, and phosphorus compound in POME for algal biomass generations. The maximum removal of NH_3–N of 82.1% and TP of 88.3% has been reported with *C. vulgaris* cultures in the sterilized POME after 10 d of cultivation. While the cultivation of *C. vulgaris* in the filtered and raw POME has removed up to 90% of NH_3–N and TP within two to three time cycles [58]. Another study reported the TN removal of 76%–83%, total phosphorus content of 63%–75%, and COD value of 27.4%–38.4% with *Chlorella* sp. from diluted digested dairy manure (10, 15, 20, and 25 times) [59]. The removal of TKN by 36%, N (NH_4–N) 18%, (NO_3–N) 22%, and (NO_2–N) 57.8% has been observed from an algae-based sewage treatment plant using algae euglenoides and chlorophycean species [60].

Table 4
Removal efficiency of freely suspended and immobilized *Chlorella vulgaris* at different compositions of POME with freshwater

	POME level	Removal efficiency											
		Raw POME (mg/L)				After 8 d treatment				After 16 d treatment			
		COD	BOD	TN	TP	COD	BOD	TN	TP	COD	BOD	TN	TP
Freely suspended cells	1%	630	194	6.34	21.2	365.4 (42%)	110.6 (43%)	3.55 (44%)	10.81 (49%)	75.6 (88%)	32.98 (83%)	2.03 (68%)	5.72 (73%)
	5%	3,014	887	30.5	75.8	1,416.6 (53%)	478.98 (46%)	19.212 (37%)	43.96 (42%)	271.3 (91%)	44.35 (95%)	9.15 (70%)	25.04 (67%)
	10%	5,887	1,702	58.2	112.4	2,590.28 (56%)	782.92 (54%)	29.1 (50%)	56.2 (50%)	235.48 (96%)	34.04 (98%)	12.80 (78%)	23.60 (79%)
	15%	8,923	2,532	83.8	152.3	4,372.27 (51%)	1,316.6 (48%)	46.09 (45%)	83.77 (45%)	713.84 (92%)	101.28 (96%)	22.63 (73%)	44.17 (71%)
	20%	11,187	3,012	99.3	164.2	5,705.4 (49%)	1,506 (50%)	57.59 (42%)	96.99 (41%)	783.09 (93%)	150.6 (95%)	25.82 (74%)	49.32 (70%)
Immobilized cells	1%	630	194	6.34	21.2	63 (90%)	31.04 (84%)	2.66 (58%)	9.33 (56%)	0.63 (99.9%)	0.194 (99.9%)	0.127 (98%)	0.424 (98%)
	5%	3,014	887	30.5	75.8	210.98 (93%)	124.18 (86%)	14.03 (54%)	31.08 (59%)	3.014 (99.9%)	8.87 (99.9%)	1.53 (95%)	4.55 (94%)
	10%	5,887	1,702	58.2	112.4	235.48 (96%)	170.2 (90%)	23.86 (59%)	35.97 (68%)	5.887 (99.9%)	1.702 (99.9%)	1.164 (98%)	2.25 (98%)
	15%	8,923	2,532	83.8	152.3	713.84 (92%)	430.33 (83%)	39.39 (53%)	71.58 (53%)	8.923 (99.9%)	2.54 (99.9%)	6.704 (92%)	13.71 (91%)
	20%	11,187	3,012	99.3	164.2	1,118.7 (90%)	301.2 (90%)	49.65 (50%)	82.2 (50%)	11.187 (99.9%)	3.012 (99.9%)	9.93 (90%)	24.66 (85%)

Another macronutrient that is essential for growth is phosphorus, which is absorbed by algae as inorganic orthophosphate (PO_4^{3-}). The uptake of orthophosphate is an active process that requires energy. Phosphorus is assimilated into the cells in the form of polyphosphate granules by microalgae [61]. The maximum removal of TP was between 67% and 98% (with final concentration between 49.32 down to 2.25 mg/L) with immobilized *C. vulgaris* for 1–20% v/v of POME concentration, as shown in Table 4. The observed TP removal rate was higher compared with the reported literature, which stated that approximately 55% of TP removal was achieved by *C. vulgaris* in seepage effluent wastewater [62]. Another study, demonstrated by using two-stage treatment of dairy effluent using immobilized *C. pyrenoidosa*, reported the complete removal of NH_4^+–N and a 98% removal of PO_4^{3-}–P was achieved within 96 h of two-stage purification processes [63]. Consequently, these results suggested that POME from secondary treatment pond could significantly be used as a medium for the cultivation of microalgae particularly *C. vulgaris*. Microalgae are able to eliminate excessive nutrients in POME that might lead to eutrophication in the waterways.

3.6. Bioremoval of heavy metals

The bioremoval of heavy metals characteristics of *C. vulgaris* were examined with regard to Fe(II) and Mn(II) ions removal from POME. The residual concentrations of the respective metals after 8 and 16 d of incubation period with freely suspended and immobilized microalgae are given in Table 5. The removal capacity of Fe(II) and Mn(II) by immobilized *C. vulgaris* was higher compared with the freely suspended cells at different POME composition. The highest bioremoval capacity of Fe(II) between 71% and 99.9% and Mn(II) up to 98%–99.9% was achieved with immobilized *C. vulgaris* at a different concentration of POME. This suggested that immobilized microalgae offer some protection against toxic metals which can enhance the removal efficiency. Some other minerals such as Fe, Zn, P, Mg, Ca, and K that are required for microalgal growth are also available in POME. The bioaccumulation of heavy metals in microalgae occurs by different mechanisms. Microalgae required many trace nutrient metals, as they are part of the active sites of essential enzymes. Hence, they have evolved highly efficient mechanisms for recovering metals such as Mg, Cu, Zn, and Mn at very low concentrations, by active biological transport and accumulation inside the cell. Heavy metals enter microalgae cells via micronutrient transporters [64]. The removal of Fe up to 99.73%, Mn (99.6%) and Zn (81.53%) from 5 mg/L initial metal concentration by using *Spirogyra* sp. and *Spirulina* sp. has been reported [65]. Living algal biomass has been used for removal of heavy metal from contaminated wastewaters, due to its ability to remove such contaminants, either by adsorption onto the cell surface or by incorporation into the cells themselves [66]. The high content of organic materials and nutrients in POME makes a potentially low-cost substrate for cultivating microalgae. Microalgae utilize the carbon and nutrient sources in POME for its growth, thus removing these contaminants. Microalgae are able to remove up to 93% of the organic material and nutrient contents from POME [67]. In addition, the microalgae biomass could be harvested and used for various valuable purposes, such as biofertilizer, animal feed, high value-products, and biofuel production. The utilization of microalgae for POME treatment could reduce the operating cost, enhance the treatment efficiency, shorten the treatment period, and add value to the POME treatment.

3.7. Biomass characterization

3.7.1. Fourier transform infrared spectroscopy analysis

FTIR study was carried out to identify the functional groups present in the microalgae in the range of 4,000–350 cm^{-1}. The FTIR spectra of pure microalgal biomass were compared with the spectra obtained after bioaccumulation of metals by freely suspended and immobilized microalgae to analyze the observed changes which are due to the

Table 5
Heavy metals removal with freely suspended and immobilized *Chlorella vulgaris* cultivated under different POME compositions in freshwater

	POME level	Removal efficiency (mg/L)					
		Before treatment (mg/L)		After 8 d treatment		After 16 d treatment	
		Fe(II)	Mn(II)	Fe(II)	Mn(II)	Fe(II)	Mn(II)
Freely suspended cells	1%	3.43	0.60	1.99	0.18	0.04	ND
	5%	3.83	0.52	0.81	0.15	0.07	ND
	10%	11.9	1.06	2.38	0.80	0.88	ND
	15%	14.2	1.32	3.45	0.91	1.05	ND
	20%	15.2	1.74	3.54	0.98	1.44	ND
Immobilized cells	1%	3.43	0.60	0.97	0.01	ND	ND
	5%	3.83	0.52	0.12	0.03	ND	ND
	10%	11.9	1.06	0.15	0.07	ND	ND
	15%	14.2	1.32	0.32	0.11	ND	ND
	20%	15.2	1.74	0.10	0.15	ND	ND

ND, not detected.

interaction of functional groups with metal ions, as shown in Fig. 2. The absorption peaks were tabulated in Table 6 for pure biomass and heavy metals loaded algal biomass.

The band peak at 3,411.71 and 3,245.40 cm^{-1}, for freely suspended and immobilized algae, respectively, is assigned to the binding –OH and binding –NH groups and the peak is moved to the 3,412.39 and 3,431.11 cm^{-1} indicating the association of these groups in the biosorption process. Other peaks shift in 1,640.47 cm^{-1} were also detected to lower frequencies 1,634.18 cm^{-1} after metal adsorption, in the case of freely suspended samples. This may be due to heavier metal atom attachment with active functional groups causing in lower vibration frequency. The significant change in the wave number reveals the involvement of the carboxylic acid

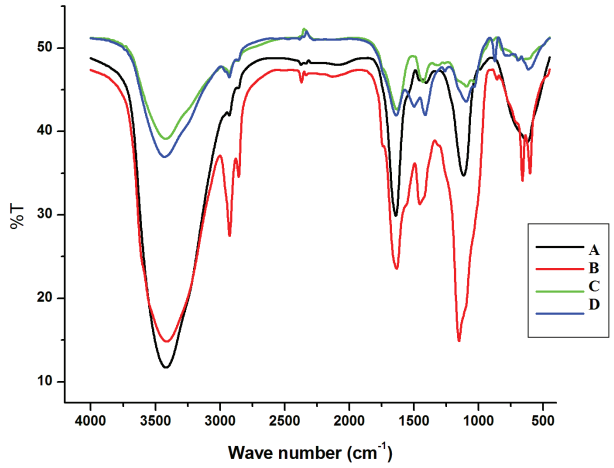

Fig. 2. FTIR spectrum of *C. vulgaris* (a) freely suspended cells before treatment, (b) after treatment, (c) immobilized cells before treatment, and (d) after treatment.

in the ion-exchange process. These peaks show the association of the functional groups in heavy metals adsorption. The peaks observed confirmed the presence of carboxylic acids, hydroxyl ions, and carbonyl groups among others on the algal surface has been reported [68,69].

The characteristic absorption peaks detected in the freely suspended cells at 2,927.5 cm^{-1} representing CH_2 stretching vibrations, C≡N in the polyacrylonitrile and amide (N–H) were shifted to 2,930.9 cm^{-1} after adsorption. A change in metal-loaded biomass can conclude that N–H involvement in metal binding and the same correlation was reported for *Pleurotus ostreatus* for biosorption [70]. The bands 816.2, 823.5, and 874.9 cm^{-1} are fingerprint zones of phosphate and sulfur functional groups and N-containing bioligands. The important alterations in specific peaks wavelength hydroxyl, amide, bounded –OH, bounded –NH, C≡O stretching vibrations, –C–O benzene ring stretching groups could be involved in the heavy metals biosorption and comparable with the results reported for algal species biosorption of heavy metals [71].

3.7.2. Scanning electron microscopy

The morphology of freely and immobilized *C. vulgaris* surface was examined by scanning electron microscopy (SEM) before and after POME treatment, as shown in Figs. 3(a)(d). SEM provides images of the sample surface by scanning it with a high-energy beam of electrons. These electrons interact with the sample atoms and produced signals that comprise evidence about topography, morphology, and surface composition [72]. SEM images of freely suspended and immobilized cells before treatment were smooth and had certain dimensions as shown in Figs. 3(a) and (c). After treatment, cells were destroyed, became swollen and their surface becomes meanders and porous with different pore diameters and areas as shown in Fig. 3(d). Consequently,

Table 6
Band positions of freely suspended and immobilized *C. vulgaris* before and after heavy metals removal

Main peak (cm^{-1})				Description
A	B	C	D	
3,411.71	3,412.39	3,425.40	3,431.11	Water ν(O–H) stretching protein ν(N–H) stretching (amide A) or –OH, –NH
2,927.5	2,926.38	2,930.9	2,927.5	Lipid – carbohydrate mainly vas(CH_2) and vs(CH_2) stretching or C–H (stretching vibration)
–	2,855.5	–	2,862.4	Carbohydrate ν(C–O–C) of polysaccharides
1,640.47	1,634.18	1,632.10	1,637.73	Protein amide I band mainly ν(C=O) stretching or C=O (stretching of typical amide) or carboxylic acids
–	1,455.33	1,433.4	1,498.6	C–N (amide)
1,406	–	–	1,412.9	
–	–	–	1,262.1	P=O (phosphate ester)
1,115.47	1,151.43	–	1,094.2	Sulfoxides
–	816.2	823.5	874.9	S=O and C–S–O bands, from ester sulfonate
–	660.61	686.44	693.3	N containing bioligands
619.87	602.13	652.17	617.9	N containing bioligands

Note: FTIR bands of *C. vulgaris* A – freely suspended cells before treatment, B – after treatment, C – immobilized cells before treatment, D – after treatment.

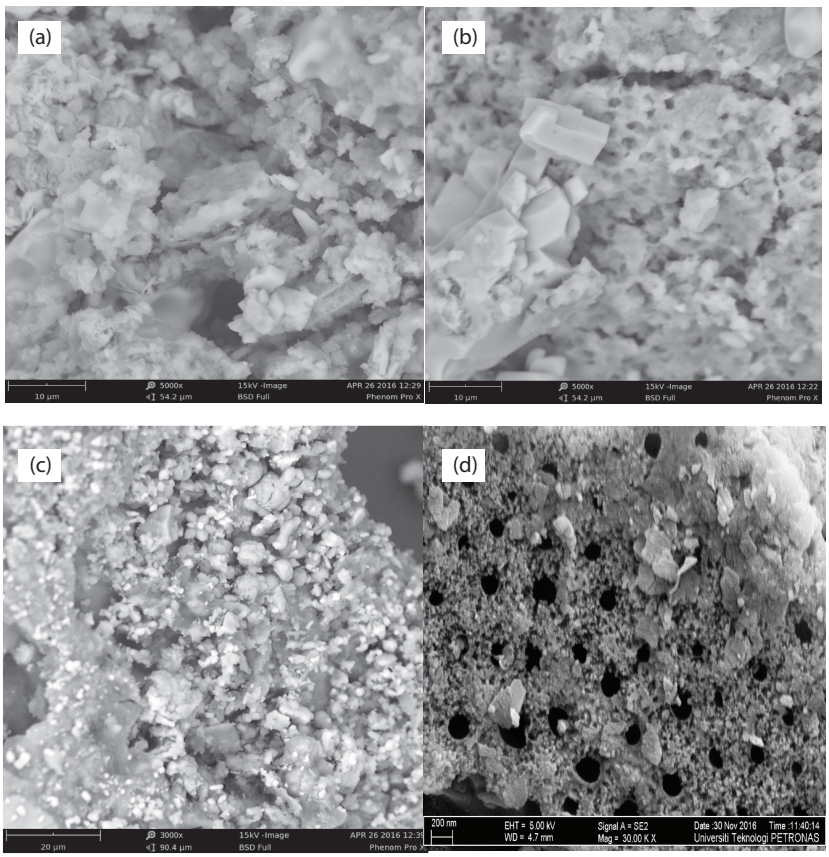

Fig. 3. SEM images of *C. vulgaris* (a) freely suspended cells before treatment, (b) after treatment, (c) immobilized cells before treatment, and (d) after treatment.

these pores facilitate the good possibility of heavy metal ions sorption. At 500× magnifications, a rough surface texture with lots of asymmetrical surface layout was detected after treatment. After absorption, the particles have granular, complex, uneven, and porous surface textures that were not found in the natural biomass of *C. vulgaris*. This may be due to metal ions precipitated around the cell surface and linked with their functional groups. These changes are mostly due to samples exposure to the heavy metal solution; the metal ions replaced some of the cations initially present in the cell wall matrix and created stronger crosslinking. Due to the ion-exchange mechanism, the heavy metal ions occupied the available free binding sites [73].

3.7.3. X-ray photoelectron spectroscopy

Binding energy (BE) profile of iron (Fe 2p), manganese (Mn 2p), carbon (C 1s), and oxygen atom (O 1s) in *C. vulgaris* biomass-pure and heavy metal-loaded samples has been illustrated in Figs. 4(a), (b) and (c). The peak for Fe 2p and Mn 2p was not present in the spectrum of pure algal cells; while after the treatment, a wide scan visibly showed a small peak around 712.57–714.25 eV for Fe(II) and 642.86–643.4 eV for Mn(II) in both freely suspended and immobilized *C. Vulgaris*, indicating the accumulation of heavy metals. Fig. 4 shows that the peak area of Fe 2p and Mn 2p in the immobilized cells is larger than the freely suspended cells, reflecting the higher accumulation capacity of the immobilized cells. Figs. 4(a) and (c) show the changes of the BE of C 1s spectra before and after the bioaccumulation of metals. Such results and peak area ratio (%) have been further summarized in Table 7. The deconvolution spectra observed were comprised of three peaks with a BE of 284.19, 286.45, and 288.74 eV and can be assigned to C–C, C–O, O–C–O, and O=C–O bonds for alcohol, ether, and carboxylate groups, respectively. The carbon atoms of these respective organic functional groups of the seaweeds are typical in algal polysaccharides and have different electron densities. The O 1s deconvolution spectra contained peak of metal-bound, pristine, and protonated biomass. BE peaks of 532.83 C=O, 533.21 eV alcohol, hydroxyl and 534.7 eV can be assigned to metal oxide, and C–O (carboxyl and ether) [74].

4. Conclusion

This study demonstrated that *C. vulgaris* has a high potential to be a beneficial microalgae for the enhancement of POME treatment, as it cultivates well in different concentration of POME with freshwater. High lipid content for *C. vulgaris* was observed at 10% v/v POME suggested a potential wastewater medium for microalgae growth as a replacement for synthetic commercial medium. The maximum removal proficiencies of heavy metals, COD, BOD, TN, and TP were attained with immobilized *C. vulgaris* at a different concentration of POME. The bioremoval mechanism of *C. vulgaris* illustrated by FTIR and XPS showed bands conforming to C–N, –OH, COO^-, –CH, C=C, C=S,

Table 7
Results of the deconvolution of C 1s and O 1s spectra for *C. vulgaris* before and after treatment

Microalgae	C 1s		O 1s	
	Peak position (eV)	Peak area ratio (%)	Peak position (eV)	Peak area ratio (%)
Pure cells	286.45	45.78	532.83	35.64
Freely suspended cells	288.74	43.97	534.7	39.08
Immobilized cells	287.19	35.64	533.21	41.14

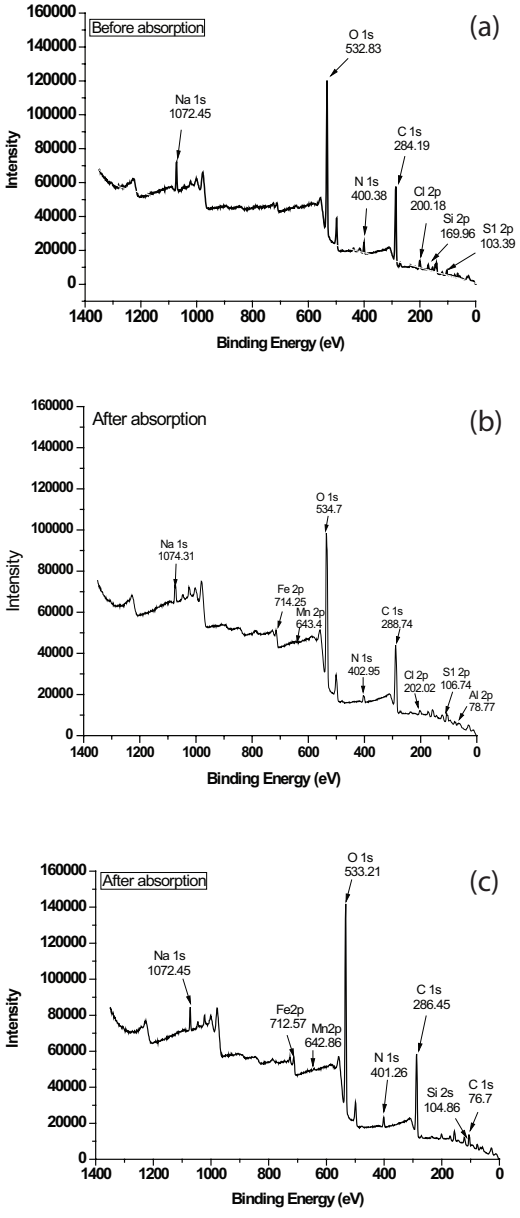

Fig. 4. (a) XPS spectrum of pure biomass of *C. vulgaris*, (b) freely suspended *C. vulgaris* after treatment, and (c) immobilized *C. vulgaris* after treatment.

and –C– groups are closely related to the binding of heavy metals. The SEM showed porous morphology, which greatly helps in the biosorption of heavy metals. Immobilization technology could simplify the harvesting of biomass from wastewater via common filtration method without utilizing a high amount of energy.

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