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Silver nanofiber assisted lipid extraction from biomass of a Louisiana *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture

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HIGHLIGHTS

- Silver nanofibers (AgNF) enhance the lipid extraction from microalgal biomass.
- 1000 μg g^{-1} of silver nanofibers increased up to 136% the microwave lipid extraction.
- The fatty acid profile of the extracted lipids meets ASTM D 6751 biodiesel standard.
- Microwave heating at 70 °C for 5 min and 1000 μg g^{-1} AgNF extracted the most lipids.

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ABSTRACT

The presence of bulk water and the resistant cell walls significantly limit the efficiency of lipid extraction from microalgal biomass paste. Current methods to rupture the cell walls (i.e. grinding after freeze-drying, osmotic shock, sonication) are energy intensive and time consuming. Due to their high surface energy concentration and high surface to volume ratio, silver nanoparticles can enhanced the cell wall rupture to increase the extraction efficiency of cell components. In this study, silver nanofibers were added as enhancers for the Folch's extraction method and microwave assisted extraction of lipids from wet biomass paste (water content of 80.9%). Nanofibers concentrations of 0–1000 μg g^{-1} were tested. Two solvent:biomass ratios were tested in the Folch's extraction method. Two temperatures (70 and 90 °C) and three treatment times (2, 5 and 10 min) were compared in the microwave assisted extraction. The results showed that the extraction efficiency increased with increased concentration of the nanofibers in the range tested. At concentrations of 1000 μg g^{-1} silver nanofibers (w/w based on the solvent and biomass solution) the efficiency of lipid extraction increased by ~30% and 50% for the Folch's and microwave assisted lipid extraction respectively. Treatment with $AgNO_3$ in the same concentration as the nanofibers did not improve the extraction compared with no silver or nanofibers addition. The extraction method affected the lipid fatty acids profile. The Folch's extraction with no silver nanofibers resulted in proportionally higher short chain saturated fatty acids, but lower lipid extraction. The microwave assisted lipid extraction provides the best results considering fatty acid profile, treatment time, solvent use and lipid extraction efficiency.

1. Introduction

Over the last several decades, microalgae have garnered significant attention as a feedstock of transportation fuels for the future primarily due to their higher growth rate than land crops, photosynthetic efficiency, lipid productivity and the ability to use non-arable lands, eliminating competition with food crops [1,2].

Microalgal biodiesel production is mainly comprised of five steps including strain selection, cultivation, biomass harvesting (dewatering), lipid extraction and transesterification [3]. Although technical issues remain in almost every of these steps for microalgal biodiesel production [4,5], life cycle analyses indicate that lipid extraction is one of the most energy intensive and thus costly processes in the chain [6]. Organic solvent based lipid extraction

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is the most commonly used method for microalgal lipid extraction [7].

Halim et al. [7] have described organic solvent-based lipid extraction from algal cells as a five-step process: (1) the solvent enters the cytoplasm by diffusion through the cell walls and membrane structure; (2) solvent and the lipid interact via van der Waals forces and (3) form a solvent–lipids complex; (4) the solvent–lipids complex diffuses through the cell wall via a concentration gradient; (5) the solvent–lipid complex passes through a static solvent film surrounding the cells and mixes with the bulk solvent. The process requires solvent to diffuse through the cell wall twice. The multilayer microalgal cell walls [8] can significantly limit the diffusion rate of both solvent and lipid. Therefore, disruption of these cell walls would drastically increase the efficiency of solvent-based lipid extraction from microalgae.

Microalgal cell walls are mainly comprised of linear and branched polysaccharides that form networks of microfibrils with strong semi-crystalline patterns [9]. Thus, the tensile strength of cell walls can be as high as 95–100 atmospheres [10]. In order to increase the lipid extraction efficiency, mechanical grinding is usually applied to break the cell wall of oil producing crops like soybean, rapeseed and canola prior to the lipid extraction process. However, the typical microalgal paste after centrifugation contains ~80% water (w/w) compared to ~10% for soybean seeds [11]. The large amount of bulk water not only generates a barrier between the solvent and the lipid/oil, it also limits the effectiveness of mechanical grinding to break the cell walls. The cells flow through the microchannels in the bulk water instead of being disrupted [12]. Many energy intensive and time-consuming methods have been utilized in an attempt to disrupt microalgal cell walls for increased lipid extraction efficiency, including sonication, manual grinding, and microwaves [7,12].

Following Soxhlet lipid extraction using sonication and microwave pretreatment of the microalgal biomass, Cravotto et al. [13] reported 4.8–25.9% improvement over Soxhlet lipid extraction without sonication. For Blight Dyer extraction, Lee et al. [14] reported less than 5% increase in lipid extraction by using sonication for 5 min as pretreatment on microalgal cells. Cooney et al. [12] obtained an increase of 45% using grinding-assisted lipid extraction in *Nannochloropsis* sp. compared to the unground freeze-dried samples. However, for the purpose of fuel production from microalgal biomass, grinding combined with freeze-drying is highly energy intensive and has little scale-up potential [7]. Microwave assisted solvent extraction changes the electric field along with the wavelength at high frequency, leading to instantaneous water heating inside the cells, causing rupture of the cell walls, facilitating a more rapid diffusion of microalgal lipids into the extracting organic solvent [7]. Lee et al. [14] reported a ~200% increase in lipid extracted from *Botryococcus* sp. for microwave extraction at 100 °C for 10 min compared to Bligh and Dyer extraction, however, the same procedure resulted in ~5% increase for *Chlorella vulgaris* and *Scenedesmus* sp.

For economic viability, an extraction method requiring less energy input to rupture cell walls in the presence of bulk water is needed. Metals such as copper, cobalt, cadmium, silver have been reported to interact with cell walls and cause structural and morphological changes within cell walls [15]. Silver and copper have been extensively studied for their antimicrobial activity, which is directly related to the interactions between the metals and cell walls [16]. This antimicrobial and cell disruption activity could be significantly enhanced by using nanostructured metals due to increased surface to volume ratio [17]. Ruparelia et al. [16] found that the antimicrobial efficiency for silver nanoparticles, which was directly related to cell wall disruption, was almost 40–50% higher than copper nanoparticles. Although these results are based on prokaryotic organisms, nanostructured silver could be a good choice for microalgal cell disruption based on the effect on cell walls of bacteria.

The mechanisms through which the silver nanomaterials affect the microbial cells is not clear. Some authors have suggested that initially the silver nanoparticles attach and anchor on the surface of the cell wall [18,19]. The electrostatic forces and molecular interactions involved are believed to cause structural and morphology changes, damaging the cell wall [18,20]. The high energy concentration on the surface of silver nanoparticles caused by differential energy absorption could be another factor that attribute the ability of silver nanoparticles to rupture the cell walls [21]. Once attached to the cell wall, the nanoparticles can penetrate the cell wall through damaged areas, perforating the cell membranes and releasing intracellular materials [22]. Other authors have pointed that toxicity may be caused by reactive oxidative compounds formed by the interaction of the nanomaterials with the environment chemistry, damaging the cell wall [20]. Based on the studies above, it is likely the cell disruption activity of silver nanoparticles would significantly increase the lipid extraction efficiency from microalgal cells.

In this study, the ability of silver nanofibers to improve the lipid extraction efficiency of the Folch's [23] and microwave-assisted lipid extraction was assessed for a *C. vulgaris*/*Leptolyngbya* sp. co-culture. The fatty acid profiles of the lipid extracted by each tested method (i.e. Folch's method and microwave extraction with and without nanofibers) were analyzed to investigate the effects of the extraction methods on the fatty acid profile from this co-culture.

2. Materials and methods

The impact of silver nanofiber concentrations (0, 50, 200, 500, 1000 $\mu g\ g^{-1}$, based on the solvent–biomass mixture) on the lipid extraction efficiency of the Folch's method and microwave-assisted lipid extraction from wet paste of the Louisiana *C. vulgaris*/*Leptolyngbya* sp. co-culture was investigated. The fatty acid (FA) profiles of the lipids extracted using each method were determined. The FA profiles provided information on the impact of these extraction methods on the compositions of the biodiesel produced from this Louisiana *C. vulgaris*/*Leptolyngbya* sp. co-culture.

2.1. Strain selection and biomass production

A *C. vulgaris*/*Leptolyngbya* sp. (97:3 by cell count based on flow cytometer) co-culture was used in this research. This co-culture was isolated from the College Lake, Baton Rouge, LA. In previous work (unpublished data) it was found that the presence of the *Leptolyngbya* sp. improved the growth rate of the *C. vulgaris*. In previous studies, the lipid productivity was reported as high as 116 $g\ m^{-3}\ d^{-1}$ with 89% neutral lipids [24]. The fatty acid profile of this co-culture meet the ATSM D 6751 standards for biodiesels and does not vary with irradiance and nitrogen levels [24].

In this study, the microalgal biomass for this co-culture was produced in a hydraulically integrated serial turbid stat algal reactor (HISTAR) developed by Rusch and Malone [25]. HISTAR is comprised of two enclosed turbidostats and a series of eight open-top, continuous flow stirred-tank reactors (CFSTRs). The inoculum culture was prepared in four 10 L carboys with F/2 media and an irradiance level of 200 $\mu mol\ m^{-2}\ s^{-1}$ with 1 $L\ min^{-1}$ aeration. When the optical density (OD) measured at 664 nm of the culture reached 0.5, the co-culture in the carboys was inoculated in the turbidostats with 100 L of F/2 media. The culture grew in the turbidostats in batch mode for 3–4 days until the OD at 664 nm reached 0.6 before it was diluted to 454 L with F/2 media. The HISTAR process controller maintains the pH and inoculum

injection of the system. Each turbidostat injects high quality inoculum algal culture into the first CFSTR every 20 min, based on the turbidostats biomass concentration. Nutrients (F/2 media, Kent Marine, Inc., Franklin, WI, USA) mixed with tap water were continuously fed into the first CFSTR at the flow rate of 0.5 L min^{-1}, resulting in a system dilution rate of 0.261 day^{-1} and a local dilution rate of 2.086 day^{-1}. The biomass was harvested with a semi-continuous flow centrifuge. For the CFSTRs, illumination was provided by 400 W high pressure sodium lamps placed 26.6 cm above each CFSTR. Dual 400-W metal halide) and 400-W high pressure sodium light sources were used to provide the illumination for each turbidostat. The aeration rate for each reactor was 21.2 L min^{-1}. The pH was automatically controlled at 7–7.5 by CO_2 injection through air stones. The biomass paste was harvested daily from the centrifuge for the lipid extraction experiments. The water content of the harvested parte was 80.9%.

2.2. Preparations and characterization of silver nanofibers

The silver nanofibers were synthesized based on the protocol by Sun et al. [26], substituting the seed nanoparticles for cobalt based nanocrystals. All reagents used were reagent grade. Briefly, the procedure was as follows: 20 mL of ethylene glycol () were heated to 160 °C in a three-neck flask. One mL of $CoCl_2$ aqueous solution (1%, w/w) was slowly added into the flask. The temperature was maintained at 160 °C for 15–60 min. When the color of the mixture changed from pink to lilac, 10 mL of 4% (w/w) polyvinyl pyrrolidone (PVP) solution in ethylene glycol was slowly added into the flask, maintaining the temperature. A 10 mL volume of $AgNO_3$ solution 2% w/w in ethylene glycol was added, and the mixture was maintained at 160 °C for 1.5 h. The mixture was rinsed with acetone and then centrifuged at an RCF of 2573*g* for 10 min. The silver nanofibers were suspended in 95% ethanol to prevent agglomeration. Scanning Electron Microscopy (SEM; Quanta 3D FEG Dual Beam FIB/SEM; FEI Co. Hillsboro, Oregon, USA) was used to determine the approximate length, diameter and surface morphology of the nanofibers synthesized in the experiment. The composition of the nanofibers was determined by X-ray photoelectron spectroscopy (XPS, Kratos Axis 165 XPS/AES system, Shimadzu Co. NY, USA).

2.3. Lipid extraction

The lipid extraction experiments were conducted using Folch's and microwave assisted extraction. The effect of silver nanofibers concentration (1000, 500, 200, 50, 0 μg g^{-1}, based on the weight of biomass/solvent mixture) on the lipid yield in both methods in different conditions was explored. For the Folch's method, an additional study using $AgNO_3$ in the same Ag^+ concentrations used in the nanofibers was performed to determine if the observed effect is due to the ion or to the nanofibers. Also, two solvent concentrations (10:1 and 20:1 solvent:biomass) were tested. All experiments were done using wet biomass paste. For microwave assisted extraction, two temperatures (70 and 90 °C) and three treatment times (2, 5 and 10 min) were tested. Transmission electron microscopy (TEM; Jeol 100CX; Jeol, Peabody, MA, USA) was used to inspect the residual biomass after the extraction. All solvents used were HPLC grade.

2.3.1. Folch's method

A triplicate two-factorial design was used in this experiment to determine the effects of silver nanofibers on lipid extraction efficiency. Folch's method was performed with two solvent:biomass wet paste ratios (20:1 and 10:1, v/v) and five silver nanofiber concentrations (1000, 500, 200, 50, 0 μg g^{-1}, based on the weight of biomass/solvent mixture). The lipid extraction without nanofibers (0 μg g^{-1}) was the control for the experiment. Chloroform:methanol 2:1 (v/v) was mixed with biomass in a 50 mL centrifuge tube, using 3*g* biomass paste for solvent:biomass ratio of 10:1 and 1.5 g biomass paste for solvent:biomass ratio of 20:1. The moisture content of the wet biomass paste used was 80.9% (w/w). The silver nanofibers were added into the biomass/solvent matrix to achieve the desired concentrations. The mixture was agitated in an Innova™ 4340 shaker incubator at 120 rpm and 27 °C for 20 min. NaCl 0.9% w/w solution in water w/w in a proportion of 0.2 of the total solvent volume was added to separate the mixture into two phases. The chloroform/lipid phase was recovered and the chloroform was evaporated to obtain the lipid. Triplicates were done for each treatment.

Extraction by the Folch's method was tested with five concentrations (1000, 500, 200, 50, 0 μg g^{-1}) of Ag^+ in the form of $AgNO_3$ to investigate the effects of Ag^+ on the lipid extraction efficiency from the Louisiana co-culture biomass pasted. A solvent:biomass ratio of 1:10 was used. The extraction procedure followed the silver nanofiber assisted lipid extraction. The results were compared with the silver nanofibers assisted Folch's method to determine whether Ag^+ has the same effects as the metal silver nanofibers on the lipid extraction.

2.3.2. Microwave assisted lipid extraction

A three-factor design was utilized in this experiment to find the best conditions for microwave assisted lipid extraction lipid extraction using 1000 μg g^{-1} or less of silver nanofibers. Five silver nanofiber concentrations (1000, 500, 200, 50, 0 μg g^{-1}), two heating temperatures (70 and 90 °C) and three heating times (2, 5, 10 min) were tested. The experiments were run in triplicate.

The biomass paste used in this experiment contains 80.9% moisture. The co-culture biomass paste and the solvent (chloroform:methanol 2:1, v/v) were mixed in an XP-500™ vessel in a ratio of 1:10 and the silver nanofiber suspension was added. A MARS 5™ (CEM Corp., Matthews, NC, USA) laboratory microwave oven was used to heat the sample to the intended temperature (70, 90 °C). After the system cooled, 0.2 volume based on total solvent volume of 0.9% NaCl solution was added and the mixture was centrifuged at an RCF of 2573*g* for 10 min to separate it into three phases (methanol–water/biomass/chloroform–lipid). The chloroform–lipid phase was collected and the solvent was removed in a rotary evaporator. The lipid extracted (g (g dry biomass weight)$^{-1}$) was calculated for each treatment (temperature, nanofiber concentration and heating duration combination). Each treatment was performed in triplicate.

2.4. Fatty acid analysis

The fatty acids profiles of lipids extracted in four different conditions: Folch's method with 0 and 1000 μg g^{-1} silver nanofibers and microwave assisted extraction with 0 and 1000 μg g^{-1} silver nanofibers were analyzed by gas chromatography to determine the effect of extraction conditions on the fatty acids profile.

The lipids were first transesterified into fatty acid methyl esters (FAME) following the IUPAC Method II.D.19. Ten milliliters of 0.5 M NaOH methanolic solution was added to the neutral lipid sample, and the mixture was boiled for 10 min. Boron trifluoride (BF_3) methanolic solution was then added to the mixture and boiled for 2 min. After addition of 1.5 mL heptane and boiling for 1 min, 20 mL of saturated NaCl solution was slowly added resulting in a two-phase liquid system (FAME and heptane in the upper phase). About 1 mL of the upper phase was transferred into a 2 mL vial. A small amount of $NaSO_4$ was added into the vial to remove trace water. The fatty acid profile was determined in the resulting sample.

Fatty acid analysis was done in an HP 5890 Series II gas chromatography with SP™-2380 column (30 m, 0.25 mm ID, 0.20 μm film). The flow rate of the carrier gas helium was 2 mL min^{-1}. The initial oven temperature was set at 80 °C. After 1 min, the temperature was increased by 4 °C min^{-1} until a final temperature of 220 °C was achieved. This temperature was maintained for 5 min. The gas chromatography data were analyzed with Chemstation™ software, and the weight percentage of each fatty acid component was reported.

2.5. Data analysis

For each treatment, the mean and standard error of lipid extracted (g (100 g of dry biomass)$^{-1}$) was calculated. Two-way ANOVA was conducted using SAS^{TM} (v. 9.3) to determine whether the effects of silver nanofibers concentrations and solvent:biomass ratios on lipid extraction efficiency using the Folch's method were statistically significant. Three-way ANOVA was performed on the microwave assisted lipid extraction lipid content to determine whether the effects of concentrations of silver nanofibers, heating temperature and heating duration were significant. Tukey post-hoc analysis was performed for multiple comparisons. Significance was determined at $\alpha = 0.05$.

3. Results and discussion

3.1. Characterization of silver nanofibers

The silver nanofibers were predominantly >5 μm in length and around 60–100 nm in diameter based on the SEM images (Fig. 1). No attempt was made to purify the fibers. The characteristic peaks of Ag in XPS analysis (Fig. 2) at 368 eV (Ag $3d_{5/2}$) and 374 eV (Ag $3d_{3/2}$) revealed that the material synthesized with $AgNO_3$ and $CoCl_2$ were mainly composed of metal silver. The minor peak of binding energy at 780 eV (Fig. 2) suggested that there was cobalt metal present in the nanofibers. The nanofibers comprised of 98% (w/w) of silver and about 2% (w/w) of cobalt. Characteristic peaks for C_{1s} (284 eV) and O_{1s} (532.4 eV) were detected and correspond to the polyvinylpyrrolidone used in the synthesis process as capping agent.

3.2. Extraction by Folch's method

The results of the two-way ANOVA indicated that both silver nanofiber concentrations ($p < 0.0001$) and solvent:biomass ratios ($p < 0.0001$) had significant effects on the lipid extraction efficiency from the biomass of the Louisiana *C. vulgaris*/*Leptolyngbya* sp. co-culture by the Folch's method. The highest lipid content extracted (g (100 g dry biomass)$^{-1}$ w/w) was obtained for the 1000 μg g^{-1} silver nanofibers and solvent:biomass ratio of 20:1 (v/v) (Table 1). The operation parameters including nitrogen level and dilution rate were optimized for biomass production other than lipid productivity, thus, the lipid content (g (100 g dry biomass)$^{-1}$) reported in this work were lower than the previous studies conducted in batch mode on the same co-culture [24]. The results of non-linear regression indicated that the lipid extracted and silver nanofiber concentrations followed an exponential growth relationship for both 20:1 and 10:1 solvent:biomass ratios (Fig. 3). The equations in Fig. 3 could provide a predictive tool to estimate the lipid extraction efficiency for different silver nanofiber concentrations. However, extrapolation for the lipid extracted with silver nanofiber concentrations greater than 1000 μg g^{-1} is not suggested since the actual lipid content in the microalgal cells is limited, and lipid extraction efficiency will reach a maximum value. The lipid from biomass samples extracted with 0 μg g^{-1} silver nanofibers, used as experimental controls were 10.15 and 14.14% g (100 g dry biomass)$^{-1}$ for solvent:biomass ratio of 10:1 and 20:1 (w/w) respectively (Table 1). For extraction with the solvent:biomass ratio of 10:1, 50 and 200 μg g^{-1} nanofibers had no significant effect on the lipid extraction efficiency compared to the control. The lipid content extracted from the microalgal paste increased about 40% with the 1000 μg g^{-1} silver nanofibers and 30% with the 500 μg g^{-1} nanofibers at solvent:biomass ratio 10:1. As the solvent doubled to 20:1, solvent:biomass ratio, w/w, the lipid content of the microalgal biomass for each nanofiber concentration increased. A positive correlation of 0.93 and 0.97 of the lipid extracted with nanofiber concentration was obtained for solvent:biomass ratios of 10:1 and 20:1 respectively. However, individual comparisons showed that as the concentration of silver nanofibers increased, there was no statistically significant increase ($p > 0.05$) in lipid extracted until the nanofiber concentration reached 1000 μg g^{-1}. Approximately 22.14 g of lipid (100 g dry biomass)$^{-1}$ was attained for 1000 μg g^{-1} silver nanofibers, an almost 57% increase compared to the control (Table 1).

The increase of lipid extraction efficiency can be attributed to disruptive effects of silver nanofibers on cell walls as reported by Chwalibog et al. and Nel et al. [18–20]. These researchers provided evidence that silver nanomaterials anchor and attach to the surface of the cell wall of bacteria and fungi. The electrostatic forces and molecular interaction between the silver nanomaterials and the

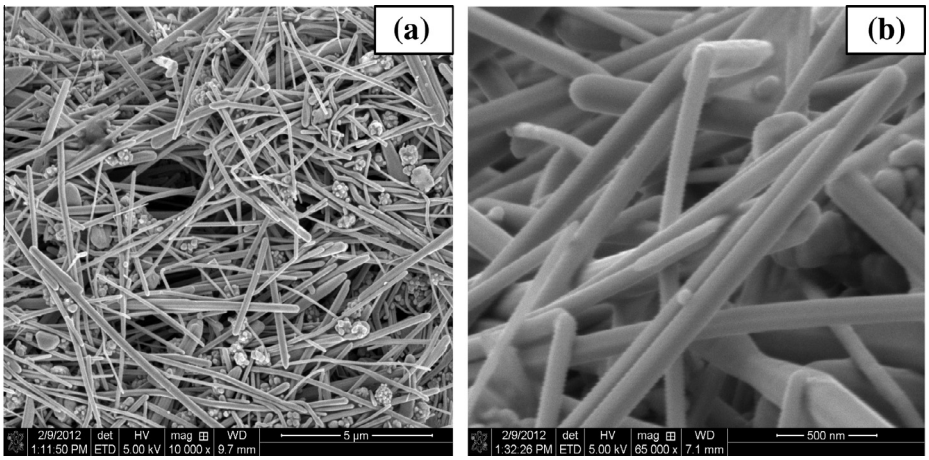

Fig. 1. (a) SEM image (10,000*x*) showing the overall dimensions (length and diameter) silver nanofibers and (b) SEM image (65,000*x*) showing detailed the surface morphology of the silver nanofibers.

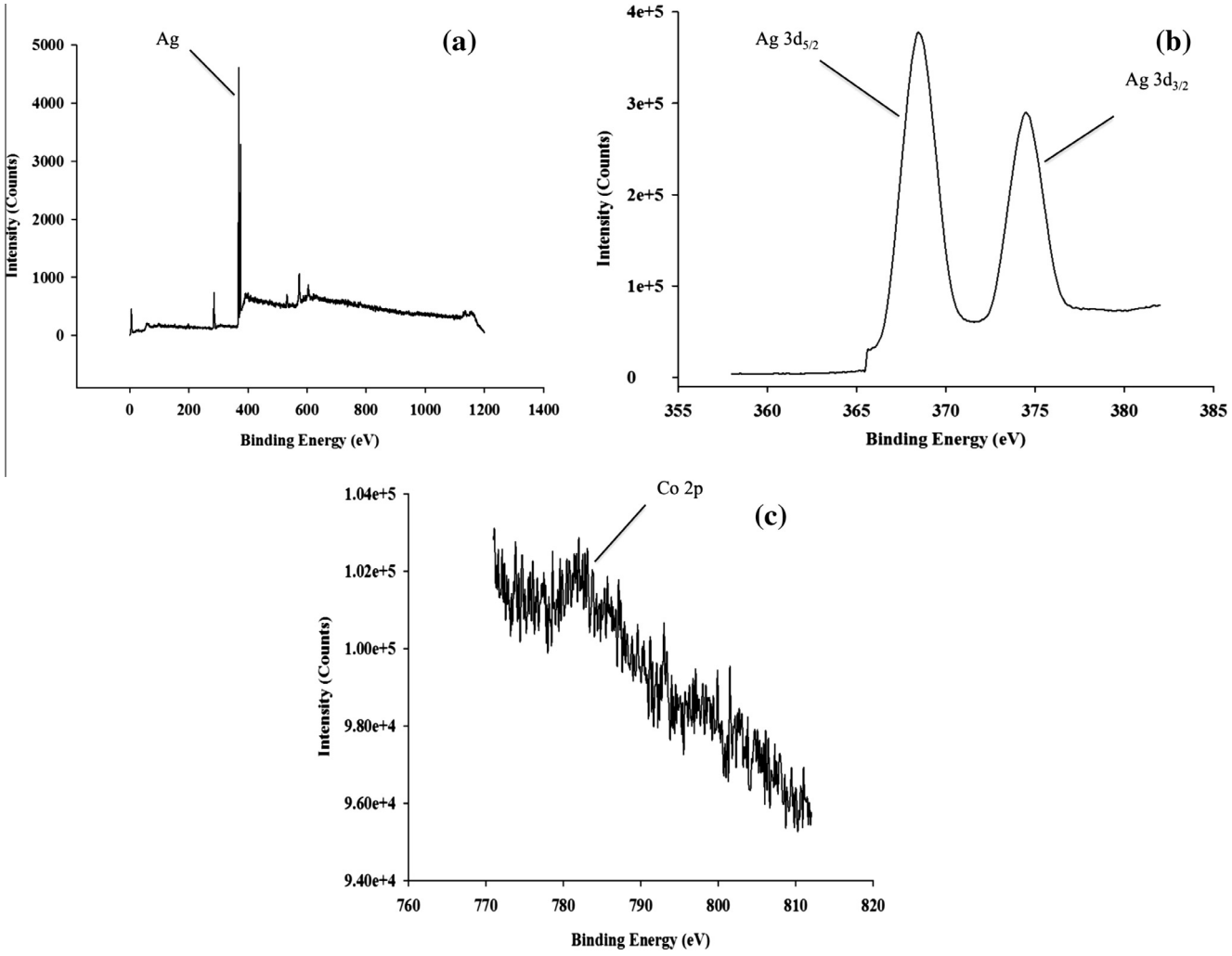

Fig. 2. (a) XPS survey for silver nanofiber synthesized in the work, (b) sputter XPS Ag (3d5/2) and Ag (3d3/2) spectra and (c) sputter XPS Co (2p5/2) spectra.

Table 1
Results of lipid extraction (g (100 g dry biomass)$^{-1}$) using the Folch's method with the addition of silver nanofibers (0, 50, 200, 500, 1000 μg g^{-1} based on the biomass/solvent mixture) and biomass:solvent concentration of 1:10 and 1:20 v/v.

Biomass:solvent (v/v)	Nanofiber concentration (μg g^{-1})				
	0	50	200	500	1000
1:10	10.2 ± 0.23^{e}	11.4 ± 0.93^{d,e}	11.9 ± 0.19^{c,d,e}	12.8 ± 0.97^{b,c,d}	14.6 ± 0.57^{b}
1:20	14.1 ± 0.86^{b,c}	14.4 ± 0.22^{b,c}	15.2 ± 1.60^{b}	14.4 ± 0.71^{b,c}	22.1 ± 1.19^{a}

Mean ± standard error (%) of triplicate samples.
$^{a-e}$ Values with different superscript letters indicate a significant difference ($p < 0.05$) between treatments, while values share same superscript letters indicate no significant difference.

cell wall caused the damages on the cell wall [18,19]. The nanomaterials then penetrate the cell wall through the damaged areas, perforating the cell membranes and releasing the intracellular materials [20]. 'Although most studies of antimicrobial action of silver nanomaterials have been done in bacteria and fungi, the same interactions may be occurring between the microalgal cells and the silver nanofibers. Once the cell wall integrity is compromised, the lipids could directly interact with solvent without first having to diffuse through the cell wall, increasing the lipid extraction efficiency. The morphologic differences between microalgal cells extracted with and without silver nanofibers addition (Fig. 4) show a complete disruption of the cell wall in the treated sample. The *C. vulgaris* cell lost part of its intracellular content after lipid extraction with 1000 μg g^{-1} silver nanofibers (Fig. 4a) indicating efficient cell wall disruption, while for the extraction conducted without silver nanofibers, the cell wall was almost intact (Fig. 4b).

The lipid content (14.61 g (100 g dry biomass)$^{-1}$) of the 1000 μg g^{-1} sample and 10:1 solvent:biomass ratio was almost equal to the lipid content (14.14 g (100 g dry biomass)$^{-1}$) of the sample treated with twice the volume of solvent and no silver nanofibers. This result implies that the addition of 1000 μg g^{-1} silver nanofibers could save half of the solvent for the lipid extraction by using Folch's method. For the commercial scale lipid extraction process, although the solvent is partly recovered, the energy consumption for the recovering process is significant. The addition

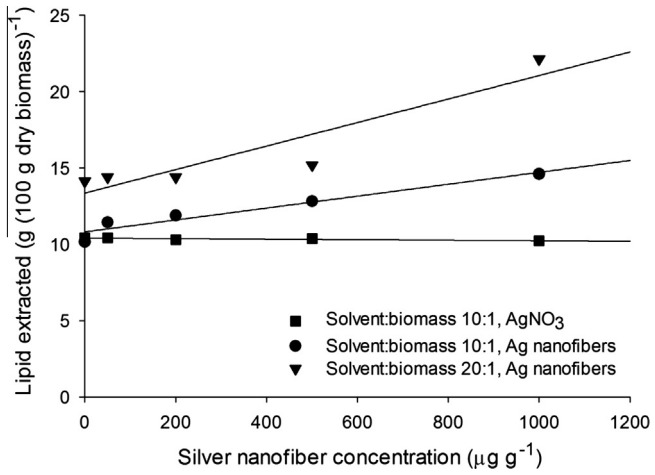

Fig. 3. The exponential relationships between the Ag nanofiber concentration (x, μg g^{-1} based on the solvent and biomass solution weight) and lipid extracted (y, g (100 g dry biomass)$^{-1}$ w/w) for the Folch's extraction method. (1) Solvent:biomass 10:1, $AgNO_3$, $y = 10.40 + 0.0x$ ($R^2 = 0.0613$); (3) Solvent:biomass 10:1, $y = 10.82 + 0.0004x$ ($R^2 = 0.91$); (2) Solvent:biomass 20:1, $y = 13.36 + 0.0008x$ ($R^2 = 0.81$).

of the silver nanofibers could lower the energy consumption in the lipid extraction process.

The lipid extractions conducted with the addition of Ag^+ instead of silver nanofibers had no significant effects on the lipid extraction efficiency at any of the concentrations tested (0, 50, 200, 500, 1000 μg g^{-1}) (Table 2). This result may indicate that the mechanical interactions of the silver nanofibers play a role on the disruption of the cells. The high energy concentration on the surface of nanostructured metal silver [21] not found for the Ag^+ in the nitrate could also be another factor inducing strong interaction between the silver nanofibers and the cell wall, inducing more damages. It is highly likely that the mechanical strength of the nanostructured metal silver is required to physically pierce through the strong semi-crystalline structures of the cell wall [9].

Compared to the results of several cell disruption methods on *C. vulgaris* [12,14], the increase in lipid extracted by the Folch's method with the addition of 1000 μg g^{-1} silver nanofibers is similar to 15 min of sonication pre-treatment, which is much more efficient than beads beating, osmotic shock and microwave (100 °C for 5 min). Nevertheless, cell disruption by silver nanofibers does not require extra energy and time for pretreatment, which could reduce significant amount of cost for lipid extraction process in an industrial size lipid extraction system.

3.3. Effects of silver nanofibers in microwave assisted lipid extraction

The highest lipid content (24.43 g (100 g dry biomass)$^{-1}$,) was achieved with 1000 μg g^{-1} of silver nanofibers, 70 °C and 5 min of heating time. The addition of 1000 μg g^{-1} silver nanofibers improved the lipid extracted by 67% at 70 °C with 5 min of treatment time compared to the same treatment with no nanofibers. Based on the results obtained with the Folch's method (without silver nanofibers), the lipid extracted (g (100 g dry biomass)$^{-1}$, w/w) increased by more than 100%. These results indicate that both the microwave treatment and the silver nanofibers have an effect on the efficiency of the extraction.

Three-way ANOVA analysis showed that temperature ($p = 0.2871$) had no significant effects on the lipid content of the microalgal biomass. However, both heating time ($p < 0.0001$) and silver nanofiber concentration ($p < 0.0001$) had significant effects on the lipid content for microwave assisted lipid extraction from the Louisiana *C. vulgaris*/*Leptolyngbya* sp. co-culture. Although only the nanofibers concentration of 1000 μg g^{-1} resulted in statistically significant differences in lipid extraction efficiency, an

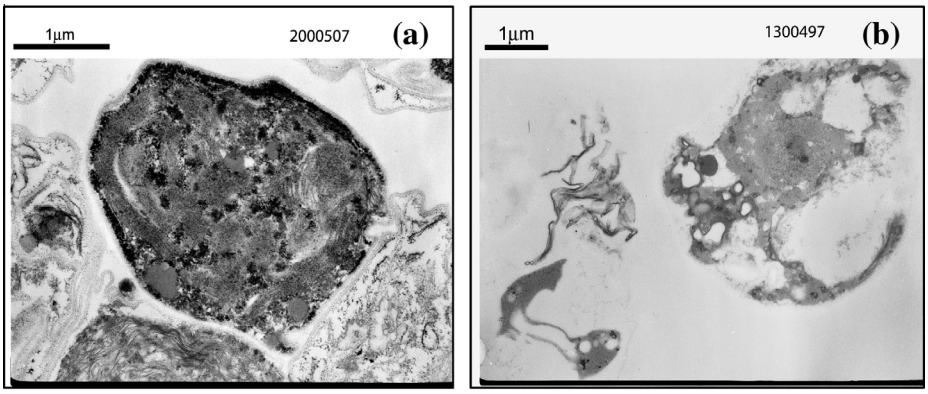

Fig. 4. TEM photos of the microalgal cells from the *Chlorella vulgaris/Leptolyngbya* sp. co-culture after lipid extraction with Folch's method. (a) Folch's method without Ag nanofibers and (b) Folch's method with Ag nanofibers.

Table 2
Results of lipid extraction (g (100 g dry biomass)$^{-1}$) using Folch's method with the addition of Ag^+ in the form of $AgNO_3$ (0, 50, 200, 500, 1000 mg g^{-1} based on the biomass:solvent mixture). The biomass:solvent ratio was 10:1 v/v.

Biomass:solvent (v/v)	Ag^+ concentration (μg g^{-1})				
	0	50	200	500	1000
1:10	10.4 ± 1.29^a	10.4 ± 0.60^a	10.3 ± 0.47^a	10.4 ± 1.20^a	10.2 ± 0.25^a

Mean ± standard error (%) of triplicate samples.
$^{a-e}$Values with different superscript letters indicate a significant difference ($p < 0.05$) between extraction conditions; while values share the same superscript letters indicate no significant difference.

Table 3
Results of lipid extraction (g (100 g dry biomass)$^{-1}$) using microwave assisted extraction method with the addition of silver nanofibers (0, 50, 200, 500, 1000 μg g^{-1}) at two temperatures (70 and 90 °C) with 3 heating times (2, 5 and 10 min). The solvent biomass ratio was 10:1 (v/v) for all the samples.

Nanofibers concentration (μg g^{-1})						
T/(°C)	Heating time (min)	0	50	200	500	1000
70	2	9.68 ± 0.24^{i}	10.3 ± 0.27^{h,i}	12.1 ± 0.79^{g,h,i}	13.9 ± 0.56^{e,f,g,h,i}	22.9 ± 3.89^{a,b}
	5	15.6 ± 2.50^{c,d,e,f,g,h,i}	17.3 ± 0.77^{b,c,d,e,f,g}	19.8 ± 2.48^{a,b,c,d,e}	21.4 ± 2.89^{a,b,c}	24.4 ± 3.60^{a}
	10	11.4 ± 3.13^{g,h,i}	12.3 ± 1.88^{g,h,i}	12.5 ± 2.19^{f,g,h,i}	13.6 ± 1.56^{f,g,h,i}	20.6 ± 0.78^{a,b,c,d}
90	2	12.5 ± 1.02^{g,h,i}	13.3 ± 1.59^{f,g,h,i}	14.2 ± 1.21^{e,f,g,h,i}	15.9 ± 2.22^{c,d,e,f,g,h}	18.6 ± 1.07^{a,b,c,d,e,f}
	5	12.9 ± 0.64^{f,g,h,i}	12.6 ± 0.51^{f,g,h,i}	14.6 ± 1.66^{d,e,f,g,h,i}	15.8 ± 2.34^{c,d,e,f,g,h,i}	21.3 ± 0.40^{a,b,c}
	10	13.8 ± 0.46^{e,f,g,h,i}	13.4 ± 0.78^{f,g,h,i}	14.6 ± 1.85^{d,e,f,g,h,i}	16.0 ± 1.52^{c,d,e,f,g,h}	20.3 ± 3.64^{a,b,c,d}

Mean ± standard error (%) of triplicate samples.
$^{a-i}$Values with different superscript letters indicate a significant difference ($p < 0.05$) between treatments, while values share the same superscript letters indicate no significant difference.

efficiency increase was observed with increasing silver nanofiber concentration (Table 3) at all tested temperatures and heating durations. Positive correlations >0.95 (p > 0001) were obtained for the 70 °C treatments at all treatment times and >98% ($p > 0001$) for the treatments at 90 °C. As higher concentrations of nanofibers were added in the biomass/solvent mixture more frequent contact occur between the cell wall and silver nanofibers. Thus, the damage caused on the cell wall by silver nanofibers through electrostatic forces, molecular interaction and reactive species [20] should increase. The diffusion rate of solvent and lipid would be expected to increase with the more damaged cell wall, resulting higher lipid extraction efficiency. The lipid content and the silver nanofiber concentrations follow exponential growth relationships under each heating temperature and duration (Figs. 5 and 6). These relationships enable the prediction of lipid extracted by applying the silver nanofibers within the range tested in this work (0–1000 μg g^{-1}).

The lipid extraction efficiency response to the dose of nanofibers change with temperatures and heating times (Table 3). For example, at 70 °C and 2 min heating time, lipid extraction efficiency went from 9.68% for 0 μg g^{-1}, to 22.87 for 1000 μg g^{-1}, an increase of more than 136% but at 70 °C and 5 min, the lipid content increased by 56% and 80% for 10 min treatment. At 90 °C, the effect of the nanofibers was lower (48%, 65% and 47% increase for 2, 5 and 10 min treatment respectively. Among the three heating time (2, 5, 10 min), 5 min proved to be the optimum for any given heating temperature and silver nanofiber concentration. For 2 min heating, the diffusion rate of solvent and lipids was limited and cell rupture caused by both microwave and silver nanofibers was reduced compared to 5 min heating, constraining the lipid extraction efficiency. The lower lipid extraction efficiency for 10 min heating duration was most likely attributed to the thermal oxidation of the lipids, which was caused by exposure to high temperature for prolonged heating duration [27]. Additionally, silver nanoparticles have been reported to have oxidative effects, especially under high temperatures [28]. In the oxidation process, unsaturated fatty acids reacted with oxygen in the ambient air and primarily converted to lipid hydroperoxide [29]. Pokorny et al. [30] found that oxidized lipid could form chloroform-insoluble compounds with cellulose, protein and other cell components. Thus, the lipid extraction efficiency at 10 min heating duration was lower than 5 min.

When the temperature was increased from 70 °C to 90 °C, both the cell disruption and diffusion rate would be expected to improve significantly. However, similar to the effects of prolonged heating duration, the increase in lipids extracted at 90 °C was most likely eliminated due to the oxidative effects of microwave and silver nanofibers at higher temperature. Therefore, the lipid extraction efficiency had no significant difference between 70 °C and 90 °C ($p > 0.05$).

The highest lipid extracted for microwave and silver nanofibers assisted extraction in this work was 1000 μg g^{-1} of silver

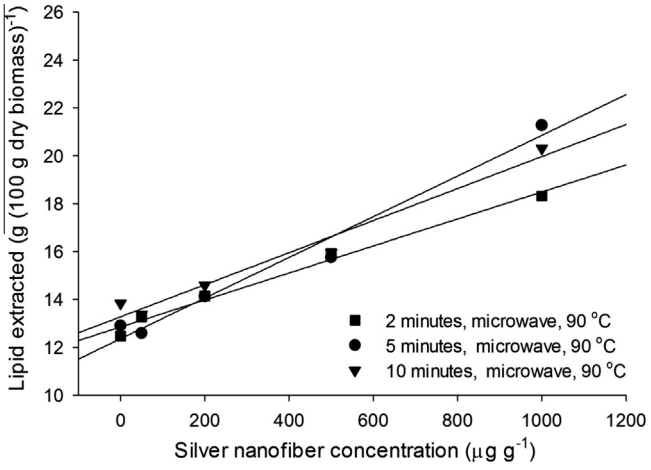

Fig. 5. The exponential growth relationships between Ag nanofiber concentration (x, μg g^{-1} based on the solvent and biomass solution weight) and lipid extracted (y, g (100 g dry biomass)$^{-1}$ w/w) with microwave assisted extraction at 90 °C under different heating durations. (1) 2 min $y = 12.85 + 0.0006x$ ($R^2 = 0.98$); (2) 5 min $y = 12.36 + 0.0008x$ ($R^2 = 0.97$) and (3) 10 min $y = 13.27 + 0.0007x$ ($R^2 = 0.960$).

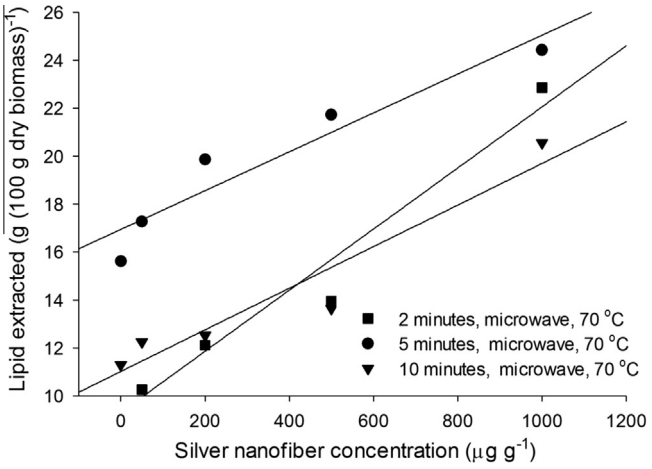

Fig. 6. The exponential relationships between Ag nanofiber concentration (x, μg g^{-1} based on the solvent and biomass solution weight) and lipid extracted (y, g (100 g dry biomass)$^{-1}$ w/w) with microwave assisted extraction at 70 °C under different heating durations. (1) 2 min $y = 9.309 + 0.0013x$ ($R^2 = 0.95$). (2) 5 min $y = 16.90 + 0.0008x$ ($R^2 = 0.882$) and (3) 10 min $y = 11.014 + 0.0009x$ ($R^2 = 0.893$).

nanofibers, at 70 °C and 5 min of heating, although no statistically significant differences were obtained for concentrations between 200 and 1000 μg g^{-1}. The lipid extracted under these extraction conditions was higher (~24 g (100 g dry biomass)$^{-1}$) than those obtained by Lee et al. [14] with for microwave assisted lipid extraction on *C. vulgaris* (10%).

Although the cost of producing silver nanofibers by the method used in this work could be high due to the high temperature and reagent cost (e.g. $AgNO_3$) needed for the silver nanofibers synthesis, strategies to reuse the silver nanofibers and faster production methods could be developed to minimize the cost. One of the possible strategies involves taking advantage of the magnetic properties of the cobalt in the nanofibers to recover the material. Another possible way to recover the silver nanofibers is to use silver nanoparticles fixed on bulk substrate (i.e. wafer, metal plates or tubes). Although the surface area of silver nanoparticles fixed on a bulk substrate could be relatively limited, the recover and reuse of the silver nanoparticles could be significantly improved compared to the silver nanoparticle suspension. Other methods such as density gradient separation and photoreactivity can also be explored in future work.

3.4. Fatty acid analysis

In biodiesel production, chain lengths close to those found in petrol diesel (C12) is preferred since the viscosity and gelling points of biodiesel are dependent on the chain lengths of the fatty acids. However, the dominant compositions of biodiesel are C16 and C18 [31]. Saturated fatty acids are desirable because saturated fatty acids give higher cetane numbers and higher oxidative stability than unsaturated fatty acids [32]. Additionally, unsaturated content is more corrosive to metals, impairing the integration of biodiesel to the existing infrastructure (i.e. transportation pipe, tank, engine) [33]. Therefore, knowledge about the effects of extraction methods on the fatty acids profiles is imperative for microalgal based biodiesel production.

There were 14 fatty acids detected in the lipids samples from this co-culture (Table 4). The majority of fatty acids were C16 and C18, similar to the fatty acids profiles of biodiesel [31]. However, the fatty acids profiles varied with extraction technique tested.

The fatty acid profiles with the Folch's method without the addition of silver nanofibers, generated the highest content of short chain fatty acids (C14 and C16) and saturated fatty acids than other conditions tested (Table 4). The cetane number (CN) calculated by the method described by Tong et al. [34] for the lipid extracted by the Folch's method was 48.98. This value is higher than the minimum value of 47.00 indicated by the ASTM standard D 6751. However, considering lipid extraction efficiency and fatty acid profiles, microwave assisted lipid extraction with silver nanoparticles resulted in similar or higher extraction of the target fatty acids due to the higher total extraction.

For the Folch's method, the application of silver nanofibers increased the percentage (based on total fatty acids) of C18 fatty acids ($p < 0.05$). The solubility of fatty acids in chloroform decreases with an increase in the chain length [35]. Most likely, C18 fatty acids were not completely extracted when the lipid extraction efficiency was relatively low. In the samples with the addition of silver nanofibers, more microalgal cell walls were ruptured and lipid extraction efficiency significantly increased. Therefore, the ratio of the less soluble C18 fatty acids to the total fatty acids increased. Since most of the C18 fatty acids of the lipid extracted from the Louisiana co-culture were unsaturated fatty acids, an increase in C18 fatty acid content resulted in a higher portion of unsaturated fatty acids. However, the increase of C18 fatty acids and unsaturated fatty acids was not dramatic (difference <10%).

For microwave assisted lipid extraction, the addition of the silver nanofibers lowered the C18 fatty acids content while simultaneously increasing overall lipid extraction efficiency. The majority of the C18 fatty acids in the co-culture were unsaturated. The silver nanofibers most likely enhanced the thermal oxidation effects on the lipid [28], resulting in more C18 unsaturated fatty acids being oxidized. Therefore, the sample with silver nanofibers had less C18 fatty acids and more saturated shorter chain fatty acids. The cetane number (CN) for the oil extracted with using nanofibers and microwave, based on the fatty acids profile was 49.23. This number fit the ASTM D 6751 standard for biodiesel. The addition of silver nanofibers for microwave assisted lipid extraction not only increased the lipid extraction efficiency but also improved the quality of the biodiesel. When silver nanofibers were not added, the microwave assisted extraction led to higher concentration of C18 fatty acids and unsaturated fatty acids than using Folch's method. Although microwaves may oxidize some C18 fatty acids, more C18 fatty acids were extracted due to improved lipid extraction efficiency resulting in a net increase in C18 fatty acids. For the microwave extraction with silver nanofibers, lower percentages of C18

Table 4
Fatty acids profiles under four lipid extraction conditions: Microwave assisted lipid extraction with silver nanofibers; Microwave assisted lipid extraction without silver nanofibers; Folch's extraction with silver nanofibers; Folch's extraction without silver nanofibers.

Fatty acids	Microwave with AgNF	Microwave without AgNF	Folch with AgNF	Folch without AgNF
C14:0	17.4 ± 0.62	11.8 ± 0.66	15.1 ± 2.89	21.1 ± 0.56
C16:0	31.7 ± 2.78	20.1 ± 1.40	26.9 ± 1.74	30.5 ± 1.49
C16:1n7	0.56 ± 0.09	0.63 ± 0.13	0.69 ± 0.09	0.52 ± 0.03
C16:1n9	1.81 ± 0.38	2.53 ± 0.73	2.71 ± 0.31	2.12 ± 0.20
C16:2n10	2.39 ± 0.65	3.70 ± 0.77	2.80 ± 0.20	2.15 ± 0.11
C18:0	5.70 ± 1.59	1.67 ± 0.24	2.21 ± 0.30	3.30 ± 0.56
C18:3n9	4.69 ± 1.04	7.44 ± 1.02	5.78 ± 0.50	4.62 ± 0.21
C18:1n9c	4.51 ± 1.34	5.37 ± 1.85	5.95 ± 0.28	5.20 ± 0.16
C18:1n9t	1.21 ± 0.06	1.17 ± 0.18	1.29 ± 0.08	1.10 ± 0.06
C18:2	10.2 ± 1.48	14.7 ± 0.76	11.3 ± 0.80	9.52 ± 0.47
C20:0	1.55 ± 0.19	1.98 ± 0.14	2.07 ± 0.33	1.57 ± 0.11
C18:3n3	17.0 ± 2.54	28.4 ± 1.01	22.2 ± 2.18	17.1 ± 1.35
C20:1	0.66 ± 0.31	0.42 ± 0.04	0.61 ± 0.09	0.72 ± 0.13
C22:0	0.59 ± 0.14	0.17 ± 0.02	0.38 ± 0.08	0.43 ± 0.08
Saturated FA	56.9 ± 5.32	35.7 ± 2.46	46.7 ± 5.34	56.9 ± 2.80
FA ⩽ C16	53.9 ± 4.52	38.8 ± 3.69	48.2 ± 5.32	56.4 ± 2.39

Mean ± standard error (%) of triplicate samples.
AgNF stands for silver nanofibers.
FA stands for fatty acids; FA ⩽ C16 stands for fatty acids that have less than 16 carbons.

fatty acids and unsaturated fatty acids were obtained. This is most likely because silver nanofibers could enhance the thermal oxidative effects of microwave.

Overall the fatty acid profiles of the lipid met the criteria of ASTM D 6751 for all tested extraction methods, although there were some variations caused by microwave and silver nanofibers. Therefore, the optimal method for lipid extraction should still be microwave assisted lipid extraction with 200–1000 $\mu g\, g^{-1}$ silver nanofibers at 70 °C for 5 min heating, which gave highest lipid extraction efficiency.

4. Conclusions

The silver nanofibers were able to rupture the cell walls of the microalgae and increase the lipid extraction efficiency for both Folch's method and microwave assisted lipid extraction. The addition of the silver nanofibers saved large amounts of solvent and energy for the lipid extraction process, as a reduction of solvent to 50% and time of 5 min resulted in equal or higher lipid yield compared with traditional the Folch's extraction method. The silver nanofibers had significant impact on the fatty acids profile of the lipids. The microwave extraction with nanofibers resulted in an increase in saturated shorter chain fatty acids. However, the lipid extraction with microwave and silver nanofibers should be the optimal extraction method for biodiesel production, considering the overall increase in lipid content. The oil extracted using microwave extraction and nanofibers and the one obtained with the Folch's extraction method without nanofibers had cetane numbers congruent with the ASTM standard D 6751.

Methods to reduce the cost of the nanofibers and recovery and reuse of the nanomaterials should be explored to make this method competitive with other extraction techniques. A balance should be made between the cost of the nanomaterials at larger scales and the savings on time, energy and solvents for the extraction process.

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