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Research article

Alkyl carbamate ionic liquids for permeabilization of microalgae biomass to enhance lipid recovery for biodiesel production

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ABSTRACT

Microalgae are potential biomass source for biodiesel production. However, their strong cell walls make efficient lipid extraction problematic. Disrupting the cell wall is a key point in enhancing lipid yield from microalgae biomass. A new type of ionic liquid (IL) has been suggested in this work as a potentially viable solvent to permeabilize the strong microalgae cell structure for the efficient extraction of lipids. Morphological changes in microalgae cells were studied before and after ionic liquid permeabilization to understand the mechanism of ionic liquid treatment. Among the three selected CO_2-based alkyl carbamate ionic liquids, DIMCARP performed with the best extraction efficiency. The effects of extraction variables (temperature, time, ratio ionic liquid/Methanol, and solvent to biomass) on lipid extraction were examined via single-factor experiments coupled with response surface methodology (RSM) using a Box-Behnken design (BBD). The highest lipid yield (16.40%) was obtained after 45 min of extraction at 45 °C using a 9:1 ionic liquid to methanol and 7 mL of solvent to biomass ratio. Transesterification of lipids to make fatty acid methyl esters found that the most common fatty acids were C16:0, C18:2, and C18:3 (19.50%). The quality of the biodiesel made meets European and US standards.

1. Introduction

Sustainable energy research is needed due to concern about the rapid depletion of fossil fuels and climate change. Biodiesel fuel is regarded as a green energy resource due to its lower environmental impact, which reduce air pollution and the CO_2 footprint while ensuring sustainability [1]. Biodiesel is also seen as a promising way to reduce our reliance on petroleum-based fuels. Compared to conventional feedstocks, microalgae is a large potential feedstock for biodiesel because it offers many benefits, such as high photosynthetic efficiency, less land requirements, and little or no competition from the food supply [2].

Microalgae biomass is useful for the synthesis of biodiesel raw materials due to its high lipid content (up to 70% dry of the weight of biomass in some microalgal species). As a result, worldwide interest in microalgae research and commercialization is expanding [3]. The following procedures are commonly involved in the conversion of microalgae lipids into biodiesel: algal cultivation, cell harvest, lipid recovery, and lipid transesterification [4]. Lipid extraction is an important step in the production of microalgae biodiesel and has been identified as a major barrier to large-scale production because it uses high energy consumption, cause global pollution, and unsatisfactory extraction efficiency [5].

Disadvantages like the high toxicity and flammability of organic solvents have prompted researchers to look for new technologies

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that pose far fewer risks to both people and the environment. The process of cell disruption, which accounts for a significant amount of the entire process cost, is one of the barriers to the use of microalgae for commercial purposes. Physical, chemical, and biological techniques are commonly used for lipid extraction of microalgae to decrease or totally eliminate the use of harmful solvents and to shorten the duration and temperature of the process [6]. Mechanical (ultrasonication, bead-beating, milling, spray-drying, and high-pressure homogenization), chemical (acid, alkali, ozonation, organic solvents, and ionic liquid), and thermal (heating and cooling) treatments are the most popular methods to break up cells. To produce biodiesel, intensive energy is needed for mechanical and thermal processes, as well as chemical processes that could result in biotoxin production, device corrosion, and lipid degradation [7]. Then, it is essential to create effective techniques for microalgae cell disruption, that are both safe and non-toxic for the environment.

Ionic liquids (ILs) are organic salts that melt at temperatures below 100 °C. The term "ILs" refers to their potential use as "green solvents." Their nonvolatile nature and thermal stability make them viable alternatives to volatile organic solvents. Due to the low vapor pressure, fast reaction time, ability to recover and reuse of the ionic liquid, and good performance yield of ionic liquid treatment, an autoclave reactor is not necessary. Because the ions in ionic liquids alter the hydrogen bonds of microalgae cell walls; the modification of cell walls is expected to improve lipid extraction. While traditional ILs in combination with imidazolium- and pyridinium-based cations are poisonous, poorly biodegradable, and non-recyclable [8]. Nowadays, the goal is to boost extraction yield while reducing extraction steps, energy needs, and process costs [9].

In order to overcome these concerns, CO_2-based alkyl carbamate ILs were developed as an alternative solvent for cell disruption and lipid extraction. The distillation points of these ILs are low, allowing the ILs to be easily evaporated from the extracted compounds without significantly affecting the labile molecules. Using carbon dioxide to prepare these ILs also helps reduce the influence of this greenhouse gas on climate modification. CO_2-based alkyl carbamate ILs have demonstrated their ability to optimize extraction of bioproducts such as astaxanthin [10], lutein [11] and curcumin [12] from microalgae species. In this study, it was demonstrated that the cell wall could be permeabilized and lipids extracted from *Chlorella pyrenoidosa* using these ILs. According to our research area, no investigations using CO_2-based alkyl carbamate ILs have been done for lipid extraction. Through single-factor studies, it was possible to determine an ideal range for the ILs extraction parameters, which included extraction time, extraction temperature, ILs to methanol ratio, and solvent-to-biomass volume. Furthermore, the potential interference factors of this process were evaluated by response surface methodology (RSM), including ratio of ILs to methanol, reaction time, and temperature effect.

2. Experimental section

2.1. Materials

A powder of the *C. pyrenoidosa* strain was collected from Tianjin Norland Biotech Co., Ltd., China. The strain was kept fresh at 4 °C after being vacuum dehydrated for 24 h at 60 °C. According to the traditional modified Floch's approach [13], this strain had 17.21% of the total lipid. Additional compounds supplied by Shanghai Aladdin Biotechnological Co., Ltd. included *n*-hexane (97%), methanol (99.9%), sulfuric acid (98%), methyl pentadecanoate, and dipropylamine (99%) (Bidepharm, Beijing Standard Spectrum Testing Technology Co., Ltd). Diallyl amine was purchased from Shanghai Macklin Biomedical Co., Ltd. N, N′ Dimethylamine from Energy Chemical, and solid dry ice carbon dioxide (CO_2).

2.2. Synthesis of alkyl carbonate ionic liquids

In this study, three types of alkyl carbamate ILs were used, including diallylammonium diallylcarbamate (DACARB), dimethylammonium dimethylcarbamate (DIMCARB), and dipropylammonium dipropylcarbamate (DPCARB). The synthesis of selected CO_2-based alkyl carbamate ILs followed the procedure reported by N·C Song et al., [14]. Briefly stated, a 250 mL round-bottom flask was initially filled with the appropriate amount of dialkylamine solution (respectively, 2 mol for dipropyl amine, and diallyl amine; 1.8 mol for dimethyl amine) and placed in an ice bath (4 °C) for incubation. A mol of solid carbon dioxide was gradually introduced into the flask while it was stirred to prevent building up pressure. The synthesis reaction takes around 30 min for the dry ice to be dissolved and turned into gas. As illustrated in Eqs. (1) and (2), the first mol of alkyl amine's single pair of nitrogen reacted with CO_2 (Eq. (1)), converting the non-polar R_2NH molecule into a polar R_2NH–COOH. Next, as shown in Eq. (2), a powerful intermolecular interaction between the dialkylammonium cation and the dialkylcarbamate anion is created through proton transfer, the result of carbamic acid reacting with a second mol of alkylamine. At room temperature, the final product (IL) was obtained in a liquid state as the result of the H- transfer from dialkylcarbamate acid. Before conducting subsequent experiments, all alkyl carbonate ILs synthesized were sealed and kept dry in a desiccator. The FTIR and ^{1}H'NMR analyses supported the synthesis of different ILs.

$$R_2NH + CO_2 \leftrightarrow R_2\,N\text{–}COOH \quad \text{eq1}$$

$$R_2N\text{–}COOH + R_2NH \leftrightarrow [R_2N\text{–}COO^-]\left[R_2NH_2^+\right] \quad \text{eq2}$$

2.3. Analytical characterization

Scanning Electron Microscopy (SEM) from SU8010, Hitachi, Tokyo, Japan, was used at 3000x, 10000x, and 20000x magnification to monitor changes in the ultrastructure of untreated and pretreated microalgal cells that were placed on a 25 mm aluminum pin stub

with a conductive double-sided adhesive coated with gold.

To investigate the structural changes during cells permeabilization by ILs, the FTIR spectra of *Chlorella pyrenoidosa* after and before permeabilization, were obtained. As well, high-resolution spectral analysis of the synthesized alkyl carbamate ionic liquids in terms of their properties of functional groups was recorded. In addition, the synthesized ILs were analyzed in terms of purity, chemical properties, and functional groups by infrared spectrometry (FTIR) using the software (WQF-520 FTIR spectrometer with DTGS and MCT detectors, FTIR software (Beijing Rayleigh Analytical, China)) for the analysis within the range of 4000–500 cm^{-1}.

To determine their purity, $^{1}H'$ NMR measurements of the alkyl carbamate ILs were carried out at room temperature using ADVANCE II 400 MHz Bruker spectrometer. The NMR sample tube was filled with 200 L of ILs samples uniformly mixed with 500 μL of 6′-succinylskimmin (MeOH-d6) solution. δ ppm values relative to TMS were used to report chemical changes. Details on ^{1}H NMR characterization are found in the Supplementary information (Fig SI-1 to SI-3).

2.4. Screening of ionic liquids

The screening of the different ILs, including DIMCARB, DPCARB, and DACARB, was analyzed to evaluate their performance on cell permeabilization and the extraction of lipids. In brief, 200 mg of dry microalgae were treated with 3 mL of 100% ILs (1 w/w) for 60 min at 50 °C under magnetic stirring. The mixture was centrifuged at 6000 rpm for 10 min to separate the liquid phase from the cells after being cooled to room temperature. 2 mL of *n*-hexane and 1 mL of distilled water were added to the mixture to disperse the lipid and segregate the organic from the inorganic fractions. Vibration and centrifugation separated the different phases. The lipid-containing top phase was put into a flask that had been previously weighed. Then, using a rotary vacuum evaporator (RE-501, Zhengzhou Haiqi Instrument Technology, China), the solvent was evaporated from the mixture. The dry weight of the microalgae lipid production was evaluated and calculated gravimetrically as follows in Eq. (3).

2.5. Total lipid content and lipid extraction by CO_2-based alkyl carbamate ILs

The total lipid content of the biomass was determined using the modified Floch method [13]. In summary, lipid extraction was performed using 0.2 g of powdered biomass, 5 mL of a 2:1 concentrate of chloroform and methanol, mixed continuously for 30 min at room atmosphere, and then added 8 mL of pure water. By centrifugation at 4000*g* for 10 min, the residue in the mixture was separated from the layer of lipids in chloroform, which was then collected and placed into a container that had already been weighed. The leftover biomass was subjected to the same process twice, and at the end, all the supernatants collected were combined. The weighed flask containing the supernatants was dried with a rotary evaporator before being gravimetrically weighed. Following Eq. (3)., the value of lipid content was estimated:

$$Yield\ of\ lipid\ extracted = \left(\frac{lipid\ weighted\ from\ dry\ biomass}{Weight\ of\ dry\ biomass}\right) \times 100\% \quad \text{eq3}$$

Lipid extraction was conducted by a single-factor experiment which included effects of different factors such as the ILs/Methanol ratio, time, temperature, and volume solvent to biomass. In accordance with the specifications in the experimental design, 0.2 g of pretreated biomass was combined with ILs/Methanol and put in a 20 mL glass vial, where it was mixed under magnetic agitation using IKA RH basic 2 at a desired temperature. The mixture was centrifuged at 4000 g for 10 min to separate the liquid phase from the cells after being cooled to room temperature. To allow phase segregation and dissolve the lipid, 2 mL of *n*-hexane and 1 mL of distilled water were added to the mixture. Vibration and centrifugation separated the different phases, lipid-containing top phase was put into a flask that had been previously weighed. Then, using a rotary vacuum evaporator (RE-501, Zhengzhou Haiqi Instrument Technology, China), the solvent was evaporated from the mixture. The dry weight of the microalgae biomass was used to evaluate lipid production; here it is calculated gravimetrically as follows in Eq. (3).

2.6. Optimization of lipid extraction by using response surface methodology

After single-factor experiments were conducted to evaluate the effect of various parameters on the yield of lipid extraction, the best value of each parameter was reported and used for response surface methodology (RSM). The Design Expert software (version 11.01) was applied to RSM to further optimize the reaction conditions, and achieve the recommended yield of lipid extraction. A factorial Box-Behnken Design (BBD), RSM stool were used and developed to optimize factor experiments. The mathematical formula from Box-Behnken to RSM is provided in Equation. 4.

$$X = \beta_0 + \sum_{i-1}^{3} \beta_i Y_I + \sum_{i-1}^{3} \beta_{ii} Y_I^{\,2} + \sum_{i-1}^{3} \beta_{ij} Y_i Y_j \quad \text{eq4}$$

where X is the expected outcome, β_0 is a constant, βi and βii are linear and quadratic coefficients, respectively, and βij is the interaction coefficient between *i* and *j*. *Yi* and *Yj* are independent variables.

RSM prediction equation was then used to find the best extraction conditions. These conditions were used in lipid extraction test and Fatty Acid Methyl Ester analysis.

2.7. Fatty acid methyl ester analysis

In order to produce the FAME, the isolated lipid was methylated in accordance with the procedure described by Lu et al. [15]. Step by step, 16 mg of lipids produced under optimal conditions were transferred to the glass tube and transesterified using 2% H_2SO_4 as a catalyst for 150 min at 80 °C with magnetic stirring. The solution was cooled for 20 min at room temperature. Then, to extract the FAME, a combination of 10:1 lipid-hexane was injected, and together were thoroughly mixed for 30 s before centrifugation for 5 min in order to separate into two phases. The concentrated upper phase was collected and moved into a different column tube, and then left to dry under oven gas emissions.

The recovered FAME was degassed and filtered before being quantitatively analyzed using gas chromatography (Agilent, USA 7890) with a 5975C Inert MSDGS system. Equipped with a 30 m 0.25 mm i.d. and capillary column with 0.5 m film thickness (HP-5MS), was used to determine the qualitative analysis (GC–MS). Agilent Mass Hunter Software was used to process the data, and it was compared to the NIST mass spectral library.

Previously, the software "Biodiesel Analyzer V 1.1" (http://www.brteam.ir/biodieselanalyzer) was used to predict the characteristics of biodiesel generated from yeast and microalgae biomass. This program was used to examine the fundamental biodiesel parameters (fatty acid distribution).

2.8. Test of reusing of DIMCARB

The recyclability of DIMCARB was evaluated by employing the recovered DIMCARB in three lipid extraction tests. After being filtered and mixed with the right amount of methanol, the recovered IL is still being used.

We used a modified method reported in other studies [10,11,16] the recyclability of IL was carried out as follows: IL DIMCARB was recovered from the lipid extract and reused in the new extraction batch. In order to get enough ionic liquid for distillation, the scale of extraction was multiplied by 6. A total of 42 mL of IL DIMCARB was applied to 1.2 g of biomass according to the optimum conditions of the experiment, supported by cell disruption and centrifugation. The supernatant was treated with 6 mL of *n*-hexane volume, the recovery phase was washed three times with distilled water; then, using the rotary evaporator, the crude lipid was obtained. The gravimetric yield was calculated by weighing the residue.

2.9. Statistical analysis

At least two copies of each sample were used for more precision in the data obtained. Origin Pro 8.5.1 (Origin Lab, USA) and Excel 2010 (Microsoft Office Enterprise) were used for data analysis, and analysis of variance (ANOVA) when appropriate. Results from experiments were presented as a mean value ± SD, and p 0.05 was the threshold for statistical significance.

3. Results and discussions

3.1. Confirming the formation of ionic liquid

The synthesis of DIMCARB, DPCARB, and DACARB was confirmed using the FTIR spectroscopic by detection of functional groups in carbon dioxide-based alkyl carbamate ILs. Fig. 1 displays the FTIR spectra of the CO_2-based alkyl carbamate ILs used in this research. The symmetric carbamate and carbamate C–O stretching peaks were present in the FTIR spectra of the synthesized ILs at, respectively, 1408 cm^{-1} and 1622 cm^{-1}.

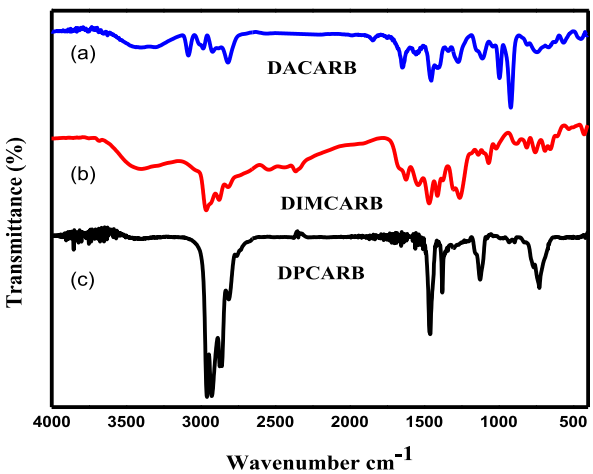

Fig. 1. FTIR spectrum data for the synthetized ILs (a) DACARB, (b) DIMCARB, and (c) DPCARB, respectively.

3.2. Screening of different ILs on lipid extraction

The capacity of the alkyl carbamate ILs (i.e., DACARB, DIMCARB, and DPCARB) to permeabilize cells and extract lipids from the *Chlorella* microalgae was tested. Fig. 2 revealed that DIMCARB had a higher lipid yield (9.32%), followed by DACARB (8.97%), and lastly, DPCARB (7.5%). This shows that the permeability of CO_2-based alkyl carbamate ILs in the cell wall of microalgae was affected by the chemical properties of the cation and anions. The excellent performance of DIMCARB in cell permeabilization and lipid extraction was hypothesized to be due to the long anion chain as well as the hydrophilic nature of DIMCARB. DIMCARB results in a less hydrophobic and more miscible solvent, which makes it easier to permeabilize through the amorphous, multilayered extracellular matrix and extract the lipid from the inside of microalgae. The results were consistent with those of Desai et al. (2016), where ILs with a long anion chain were used [17], It was discovered that imidazolium-based ILs were crucial for removing various substances from the *Chlorella* microalgae's cell wall. According to screening data, DIMCARB has a bright future as a cell-permeabilizing solvent for lipid extraction. Numerous studies have determined that the main mechanism for cell permeabilization is the development of a hydrogen bond between anions and the hydroxyl group of cellulose [18]. The cellulose's hydrogen bond network could be broken by the hydrophilic ILs, which also caused the cellulose to disintegrate inside the cell. This claim was supported by our findings, which demonstrated that the hydrophobic DPCARB produced the lowest lipid extraction yield when compared to other, more hydrophilic ILs (i.e., DACARB and DIMCARB) [19].

The DIMCARB was chosen for the next step of the experiment (Fig. 3). We included methanol in the subsequent experiment because it has been suggested that methanol may influence the extraction parameter. Additionally, it is suggested that the main effects of the polar molecule (methanol) are to disturb the cytomembrane and increase the effectiveness of lipid extraction from biomass [20]. Methanol makes it easier for lipids to precipitate out of cells, which opens up the cell wall.

The suitability of the lipid extracted for biodiesel production was confirmed by FTIR spectroscopy of the lipid extracted as shown in Fig. 3.

FTIR spectra with groups of distinctive peaks for lipids in the ranges of 30,002,900 cm^{-1}, 2900–2850 cm^{-1}, 2850–2750 cm^{-1}, 1750–1700 cm^{-1}, and 1500–1400 cm^{-1}, for proteins in the range of 1700–1500 cm^{-1}, for carbohydrates in the range of 1200–800 cm^{-1}, and for nucleic acids, polyphosphates, proteins, and phospholipids in the range of 1300–1200 cm^{-1}. The following principal distinctive peaks are shown in the biological lipid FTIR profiles (Fig. 3) of the investigated biomass: 2955 cm^{-1} and 2915 cm^{-1} which represent the stretching of the CH2 bond in triacylglycerol (TAG) fatty acids; and 2870 cm^{-1}, which represent the stretching of the CH_3 bond in TAG fatty acids. The entire amount of lipid in the cell is represented by 1741 cm^{-1}, which is the result of C═O stretching in ethyl esters, and then, 1461 cm^{-1}, which represents the stretching of the CH_2 and CH_3 bonds in TAG fatty acids [21].

The test of SEM was examined before and after permeabilization to evaluate the DIMCARB permeabilization of the wall of microalgae *pyrenoidosa*. Fig. 4(X) shows that *C. pyrenoidosa* cells that had not been exposed to IL had smooth surfaces, clear cell shape, and were completely intact. There were no indications of pitting or cell wall damage. After the permeabilization of the microalgae, cell morphology is illustrated in Fig. 4(Y). The algal cells that were present had significant cellular debris and were rough. It's probable that DIMCARB's permeabilization of the cell wall caused the microalgae's cell wall polysaccharides to change into short-chain oligosaccharides and/or monosaccharides, giving the algal cells their fragmented appearance. DIMCARB ionic liquid can dissolve cell wall polysaccharides (such as cellulose and hemicellulose analogs), making it possible for lipids to more easily move from cells, thus convenient for lipid extraction. A similar phenomenon was observed in other reports, with significant damage to microalgae cells when using ILs [7].

The structural changes in microalgae biomass after and before DIMCARB permeabilization were investigated using the FTIR spectroscopy technique (Fig. 5). The wavelength was in the 4000-500 cm^{-1} region. Hydrogen bond (O–H), alkane (C–H), and amide (N–H) stretching frequencies were found to be responsible for the wavenumber band at 3500-2700 cm^{-1}. The amide I band corresponds to (C═O) stretching vibrations and amide II band to (N–H) deformation vibrations were attributed to the peak observed at 1662 cm^{-1} and 1533 cm^{-1}, respectively. In addition, the wavenumber at 1455 cm^{-1} was attributed to proteins' methylene groups

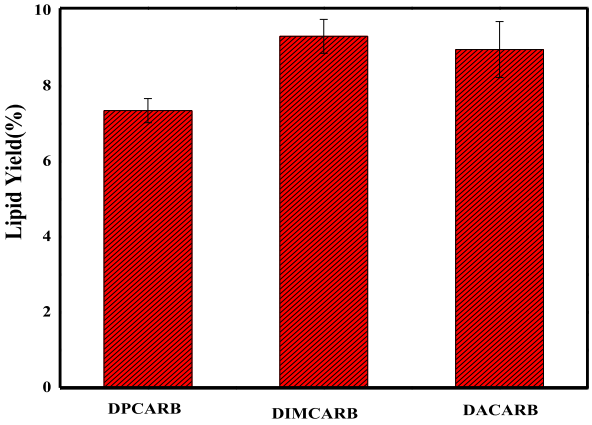

Fig. 2. Lipid extraction by the selected CO_2-based alkyl carbamate ILs.

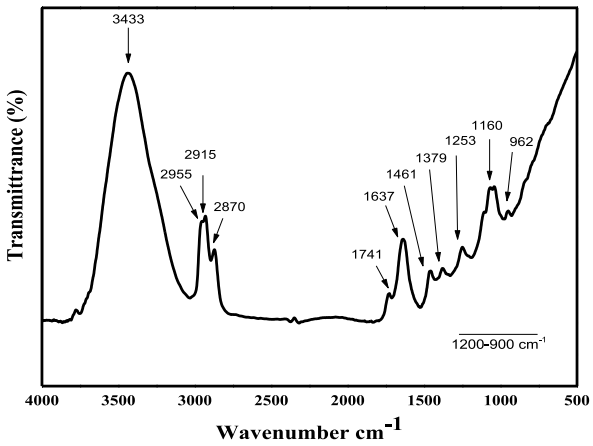

Fig. 3. FTIR of lipid extracted from biomass after permeabilization by IL.

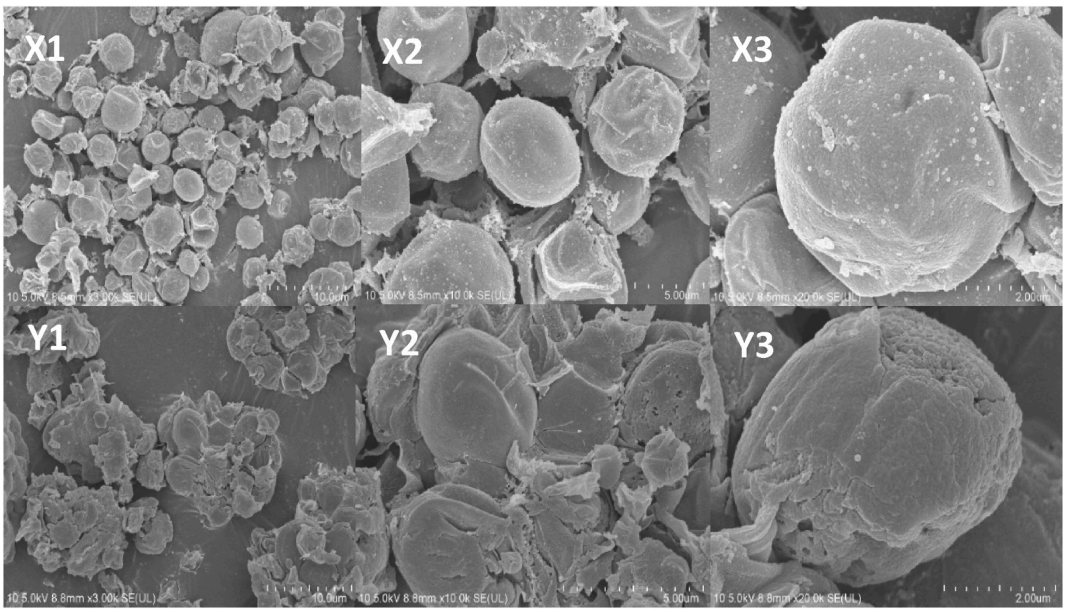

Fig. 4. *Chlorella* sp. cell surface morphology (X) before and (Y) after DIMCARB permeabilization. The magnification levels at 3000×, 10,000x, and 20,000x, respectively, are indicated by the numbers 1–3.

being bent by methyl (CH_3). After permeabilization of the cell wall by DIMCARB, the intensity of bands amide I, amide II, and methyl increased significantly; which can be explained by the diffusion of IL in cellulose and polysaccharide analogs to the cell wall [22]. Functional groups related to carbohydrates, like the band at 1240 cm^{-1} (phosphate groups, P═O stretch), the peak at 1157 cm^{-1} (glycosidic ether, C–O–C stretch), and the strong band at 1020 cm^{-1} (hydroxyl/ether, C–O stretch), dominated the spectral region between 1241 and 1047 cm^{-1}. These changes can be attributed to the breakdown of the cell wall structure because the polysaccharide in algal cell walls contains glucose, mannose, galactose, rhamnose, and xylose. Monosaccharides present in hemicelluloses, cellulose, pectins, fucans, chitins, and alginates were primarily linked together by glycosidic bonds [23].

To evaluate the interaction occurring during the treatment, investigations on the mechanism of cell permeabilization by ILs are crucial. Cellulose or hemicellulose has a highly crystalline structure and is insoluble in water and many organic solvents due to the presence of branched H-bonding networks [24]. According to some research, changes in the ultrastructure may be attributed to the bonding interaction between the hydroxyl group of cellulose and the charged molecules of IL, which form electron-donor acceptor (EDA) complexes. Literature says that hydrogen bonding between the hydroxyl group (-OH) of cellulose and the cell wall of biomass forms EDA complexes with the charged species of ILs.

The appearance on the cell surface, as shown in Fig. 6, is caused by an interaction between oxygen and hydrogen atoms in the cellulose and hemicellulose chains of microalgae *Chlorella* sp. cell walls first and the ionic DIMCARB [$(CH_3)2NH_2$] + [$(CH_3)2NH$–COO] on the other hand, resulting in the alteration of hydrogen bonds in hemicellulose and cellulose in the cell walls of microalgae.

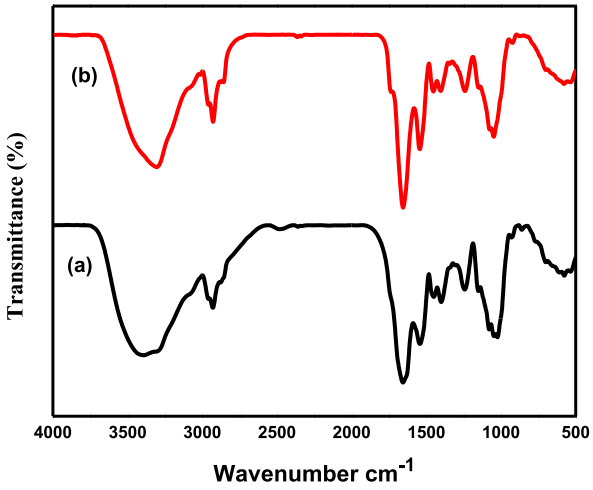

Fig. 5. FTIR spectrums of microalgae (a) before and (b) after permeabilization by DIMCARB.

3.3. Single factor experiment

3.3.1. Ratio IL/methanol

Lipid extraction was significantly influenced by the ratio of ionic liquid-to-methanol. Methanol (the diluent) was used to modify the ratio of DIMCARB because it was reported that methanol molecules were segregated from one another by reacting with the anion of IL via H-bonding; ionic liquid/methanol (IL/M) system created a hydrophobic environment to enable lipid transfer [20]. A number of combination ratios of DIMCARB to methanol were selected to evaluate the potential extraction in a range from 5:5 to 9:1. Fig. 7(a) depicts the change in DIMCARB and methanol volume ratios from 5:5 to 9:1. For comparison, DIMCARB and methanol were utilized as distinct extraction solvents.

Experimental results from Fig. 7(a) show that the ILs ratios of 9:1 and 8:2 had higher extraction efficiencies. It was suggested that, lipid extraction increases as the ratio increases, and a low ratio of DIMCARB to methanol is not favorable for cell permeabilization [10]. A study by Miazek et al. reported that ionic liquid might easily release lipids from inside the microalgae cells by disorganizing the structure of the fiber bundle in the cell wall [9]. The fact that carbamate IL changes from an "ionic" state to a "molecular liquid" base [25] can explain why the ratios 5:5 and 6:4 have low yields. This can cause the polarity of IL to drop, thereby reducing the extraction efficiency. A similar phenomenon was observed in the report [12].

3.3.2. Temperature

The operating temperature is a crucial factor to consider while extracting bioactive chemicals. One of the key variables that can influence and damage the hydrogen bond network of cellulose is the reaction temperature. Regarding the effectiveness of complete lipids [26]. The lipid yield decreased from 12.65% to 10.7% as the temperature increased from 25 to 55 °C. The maximum lipid yield was obtained at a temperature of 25 °C, as shown in Fig. 7(b); this can be explained by the fact that ILs carbamate are a single endothermic transition that appears in the range between 30 and 70 °C. Their application for the extraction of compounds requires a low temperature due to their low distillation point. The high temperature could cause ILs to evaporate away and also change the optimal concentration of solvent. This would leave the biomass alone in the extraction process, which would reduce the amount of lipids that can be extracted.

3.3.3. Effect time

The lipid yield increased from 11.5% to 12.03% by extending the duration of time from 30 min to 75 min (Fig. 7(c)). It was thought that a longer contact period would allow greater cellular permeabilization, so DIMCARB was used to infiltrate and disintegrate the cellular structure of *Chlorella pyrenoidosa.* Incubation for 90 min did not increase the lipid yield. *Chlorella* sp cell wall. is composed of cellulose, xylans and mannan compounds, with numerous chains of -linked D-glucose units formed between oxygen atoms and hydrogen networks [27]. It was hypothesized that DIMCARB, a polar and hydrophilic IL, would make it easier for hydrogen bonds to form and deprotonate the cellulose chain in the *C. pyrenoidosa* cell wall. This makes it easier for the lipid to dissolve into the solvent by weakening the cellulose of the encysted *Chlorella* sp cell. Furthermore, the amount of lipid that can diffuse from the microalgal cells to the solvent may be limited by the maximal mass transfer of lipids between the inner and outer walls of *Chlorella* sp*'s* cellular structure. For these reasons, 75 min was determined to be the best time.

3.3.4. vol solvent to biomass

Fig. 7(d) illustrates how the volume solvent to biomass affected the yield of lipid extraction. The range of volume solvent-to-biomass used in the extraction experiments was 3–12 mL at 45 °C temperature, ratio IL/M 9/1 (v/v), and time for 60 min,

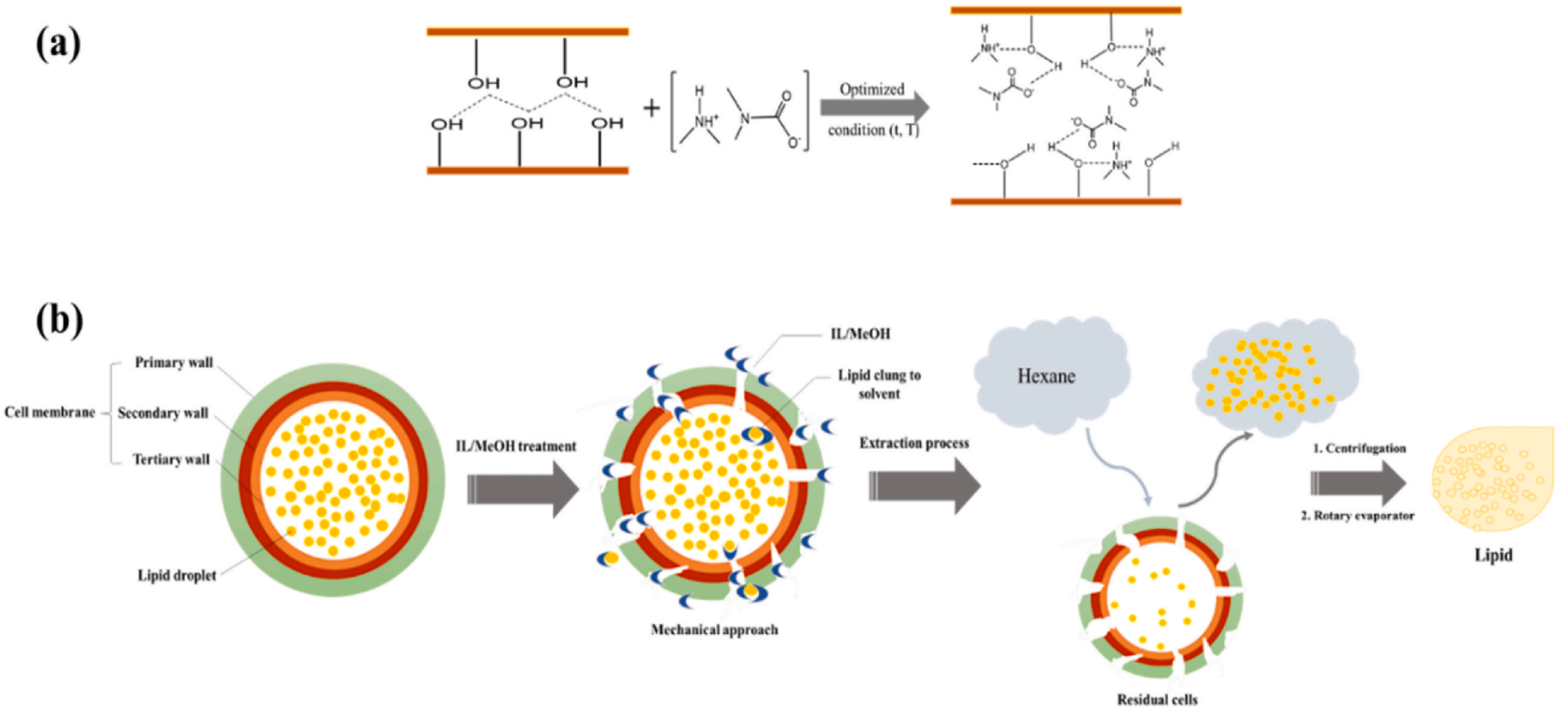

Fig. 6. Schematic illustration of microalgae cell wall permeabilization by DIMCARB.

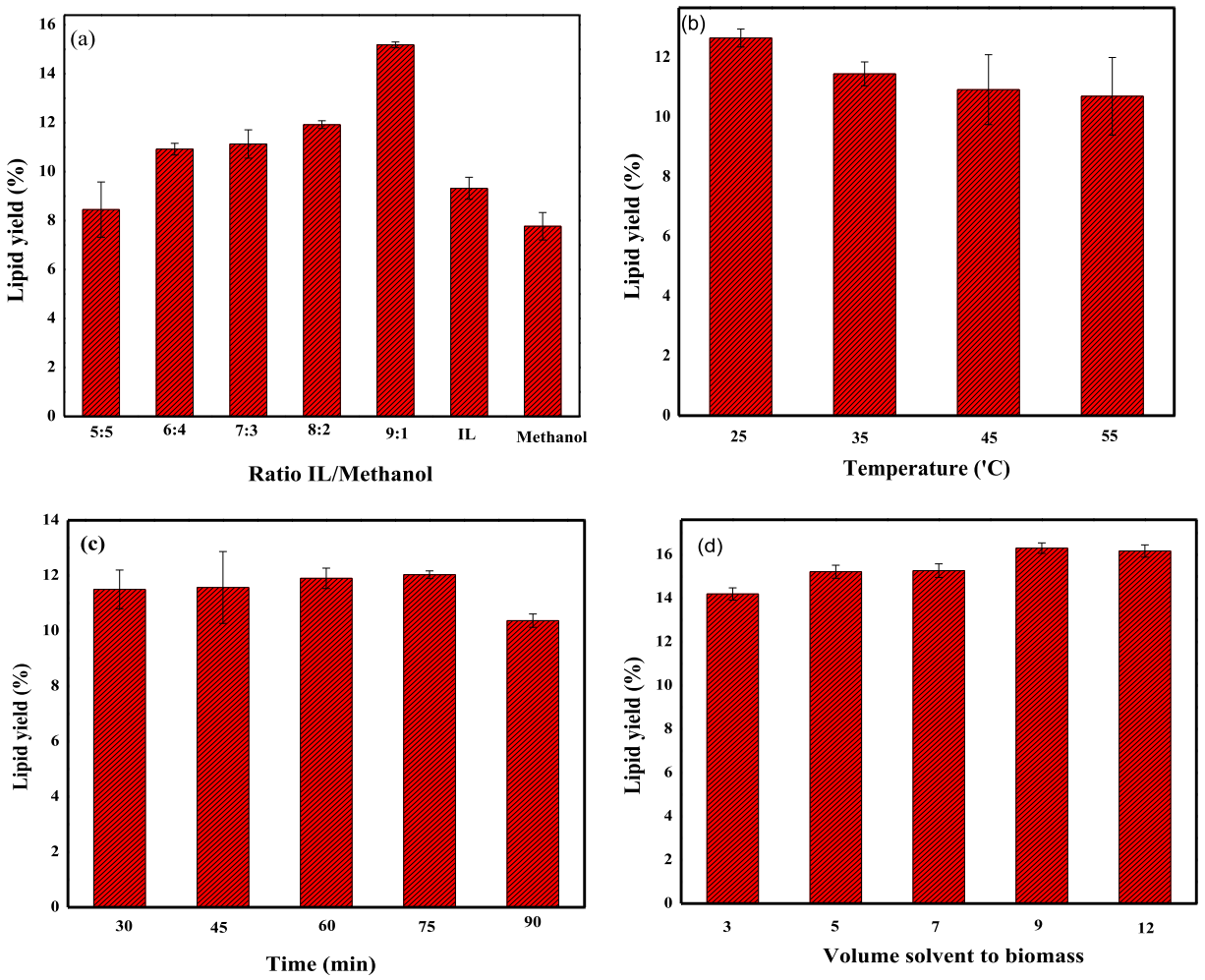

Fig. 7. Lipid extraction conditions are optimized using IL/M. Variable parameter's reaction (a) Ratio solvent, (b)Temperature, (c) Time, and (d) Volume solvent.

respectively. Fig. 7(d) shows that, depending on the amount of solvent used, the yield of lipid increased from 14.10% to 16.30%. The lowest lipid yield (14.2%) was noted at a low amount of solvent (3 mL), and the highest lipid (16.30%) was noted at a high amount of solvent (12 mL). This is probably due to a rise in the concentration gradient between the biomass and the solvent, which leads to a rise in mass transfer efficiency. Even though the yield of lipid extraction increased along with the volume solvent-to-biomass, using too much solvent is not economically feasible because the process of separating lipid and solvent uses a lot of energy. We have chosen 7 mL volume for future experiments to reduce operation costs while keeping high lipid yield.

Table 1
FTIR spectra analysis of lipid.

Wavenumber (cm^{-1})	Assignments
3500–3000	O–H stretching of water, N–H stretching of amide C–O stretching of carbohydrate
2955, 2870	CH2 symmetric and CH3 symmetric of fatty acids
2915,	CH2 asymmetric of fatty acid
1741	C=O stretching of ester functional groups from lipids and fatty acids
1637	C=O stretching of amides associated with protein (Amide I)
1461	CH2 and CH3 asymmetric deformation, stretching of lipids and proteins
1379	CH2 symmetric and CH2 asymmetric of proteins, C–O of carboxylic groups
1253	P=O asymmetric, stretching of nucleic acids, phosphoryl groups, and phosphorylated proteins
1200–900	C–O–C stretching of polysaccharides
1160	C–O stretching of carbohydrates

3.4. Box-Behnken

After single factor experiment, based on the impact different parameters on lipid extraction, we tried to establish a model relationship between the variables and determined the responses. The RSM optimization approach was used to calculate the best reaction conditions. (Supplementary informationSI-Table 1), The average of three independent runs constitutes each value obtained experimentally. To achieve optimum results, 17 different sets of tests were conducted as shown in SI-Table 1. According to the RSM design run order, the highest yield was obtained at 9, followed by 8, 1, 6, 14, 16, 12, and others. The results showed that 45-min extraction period at a temperature of 45 °C and IL/M ratio of 9:1 produced the highest lipid extraction yield (16.40%) (Run 9).

The R2 determination coefficient is a measure of model accuracy in the prediction of the response that should tend to unity for well-fitted models. The adjusted coefficient is another important factor in determining the type of model. Adequate precision is also an important coefficient for the evaluated type of model. It can be measured to determine the signal–to–noise ratio. For fitted models, this ratio should be greater than 4. As a result, a cubic model was chosen since it produced the highest R2 and adjusted coefficient values, which are reported in the next step, which involved performing an ANOVA analysis to determine whether the factors and their interactions had any discernible effects on lipid yield, F and P values were used to examine the effects of the factors. The calculated F value was 54.29, which confirms the model's statistical relevance and importance. Additionally, the model's P value was found to be less than 0.0001, which suggests that the probability of obtaining such an F value due to noise or randomness is less than 0.01%. The significance of the model and its variables is clear given the high value of F and the low value of P. As seen in Table 2, the extraction of lipid is significantly impacted by certain factors.

The linear coefficient, as shown in Table 2 suggested that the ratio IL/M (9/1) was the independent variable having the greatest influence on lipid extraction, with a p-value less than 0.0001. Lipid yield increased as the ratio of IL/M increased. Additionally, the temperature had a favorable individual impact on the extraction yield, followed by the time. Then, 3D response surface graphs from the model were shown in Fig. 8, which showed how independent variables interacted on the microalgae lipid extraction yield. The combined effects of extraction time and temperature on extraction yield are shown in Fig. 8(c). It demonstrates that the extraction yield is not significantly affected by the interaction between the two factors, with regard to the correlation between extraction temperature and IL/M, it is clear from Fig. 8(b) that when the ratio of IL/M increases, lipid extraction efficiency improves considerably. The lipid yield decreased as the extraction temperature was raised to around 45 °C, but increased as the IL/M ratio was increased. The interaction between extraction time and the ratio IL/M, on the other hand, had a considerable effect on extraction yield, as seen in Fig. 8(a). In the contour diagram, the smallest ellipse is the limit for the value that the surface shows to be the highest.

3.5. Recyclability of DIMCARB IL

The reusability of IL is crucial, they could hypothetically reduce the financial and ecological aspects of using IL for industrial applications. DIMCARB recyclability studies were performed based on optimized parameters for the extraction of lipids by DIMCARB for three extraction cycles. The DIMCARB was evaporated by a rotary evaporator and collected for the subsequent extraction of the microalgae biomass. According to the result of the lipid yield obtained from Table 3, three successive extraction cycles were not satisfactory for an industrial application process. The dilution of distilled DIMCARB produced by water droplets during the distillation process, which complicates the lipid separation process may be the probable cause. Nevertheless, in-depth studies remain to be carried out for a satisfactory industrial lipid yield from the recovery and recyclability of DIMCARB.

3.6. Fatty acid methyl esters (FAMES) profile of optimum condition from lipid extraction

The FAMES of an algal lipid can be used to determine its suitability for use as a feedstock for biodiesel [28]. It is crucial to note that the fatty acid profile of lipids can be affected by different extraction solvents, resulting in varied lipid yields. It is important to note that various extraction solvents may not only result in varying lipid yields but may also alter the fatty acid profile of lipids along with their

Table 2
ANOVA analysis for response surface methodology.

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	13.13	9	1.46	11.77	0.0019	Significant
A-ratio	6.62	1	6.62	53.45	0.0002	
B-time	0.0800	1	0.0800	0.6455	0.4482	
C-temperature	2.08	1	2.08	16.79	0.0046	
AB	0.0625	1	0.0625	0.5043	0.5006	
AC	0.0256	1	0.0256	0.2065	0.6632	
BC	0.1225	1	0.1225	0.9884	0.3533	
A^2	2.12	1	2.12	17.13	0.0044	
B^2	0.1146	1	0.1146	0.9249	0.3682	
C^2	1.57	1	1.57	12.64	0.0093	
Residual	0.8676	7	0 .1239			
Lack of Fit	0.6376	3	0.2125	3.70	0.1195	Not significant
Pure error	0.2300	4	0.0575			
Cor Total	14.00	16				

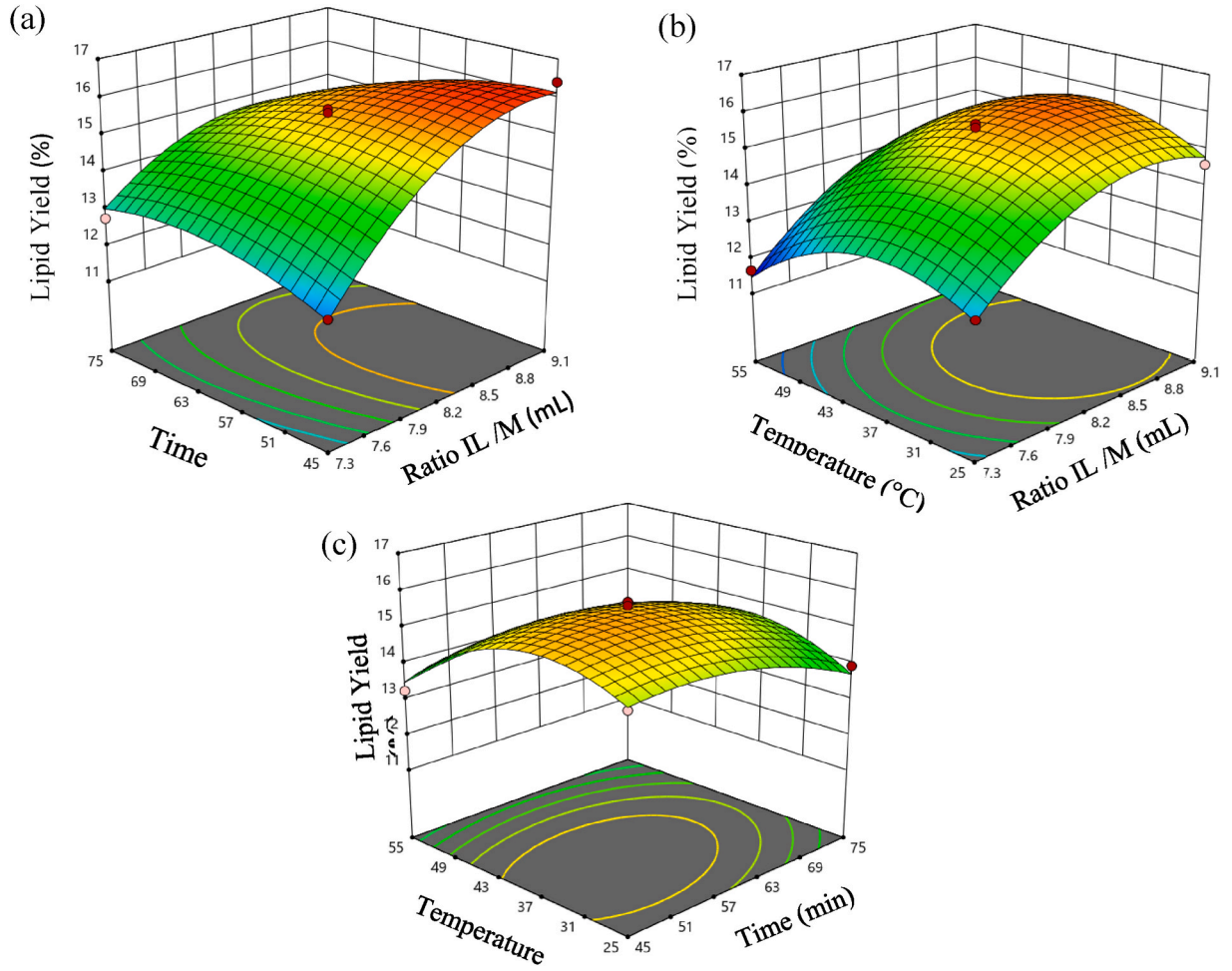

Fig. 8. Response surface graphs for the yield of lipid extracted for the following conditions: (a) time and IL/M ratio; (b) temperature and IL/M ratio; and (c) temperature and time.

Table 3
Recyclability test performance of DIMCARB for lipid extraction.

Reuse of DIMCARB	Lipid Yield (%)
Initial extraction	16.40
1st cycle	11.40
2nd cycle	8.35
3rd cycle	3.55

chemical properties like carbon number and degree of saturation. Fig. 9 shows the fatty acid profiles of the lipid recovered from *Chlorella* sp. under ideal IL/M conditions.

In this study, the FAME mixture is mostly made up of C16–C18 methyl esters. As show in Table 4, the primary fatty acids found were palmitic acid (C16:0), (C16:2), 7, 10, and 13 hexadecatrienoic acids (C16:3), octadecanoic acid, methyl ester (C18:0), linoleic acid (C18:2), and linolenic acid (C18:3). Those components account for 96.95% of the total fatty acids, defining the best quality biofuel. Other fatty acids were found in trace amounts. It is well known that a methyl ester of the C18 group signifies fuel property. Qualitative analysis, shown the distribution of fatty acids as follows: 73.33% polyunsaturated fatty acids (PUFA), 1.73% monosaturated fatty acids (MUFA), and 24.30% saturated fatty acids (SFA). FAME composition affects biodiesel characteristics.

In this study, IV and SV are favorably compared, IV provides information on the degree of unsaturation, which affects the viscosity of the fuel. The IV value for good quality biodiesel should be less than 120 g I2 100 g^{-1}, according to EN 14214. The overall mass of the FAMEs present in the biodiesel is determined by SV; when evaluating the quality of biodiesel in cold areas, freezing point and cold-filter plugging point are crucial parameters. The predicted CFPP and CP values of −9.06 and 7.42, respectively, are in accordance with

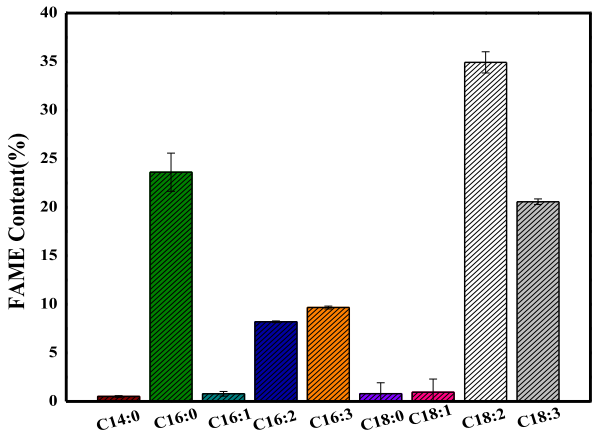

Fig. 9. Fatty acid profile of the lipid recovered from *Chlorella* sp. under ideal IL/M conditions.

Table 4
Fatty acyl methyl ester property.

Fuel property	International standards		Obtained biodiesel
	EN 14,214	ASTM D6751	
Density (kg. cm^{-3})	860–900	820–860	880
Saponification value (SV)	–	–	200.22
IV (g$I_2$100g−1 oil)	<120		195.93
Cetan number		–	
Oxidation stability (OS)	>6		5.049
Long chain saturated factor (LCSF)			–
Cloud point (CP)	>4	–	7.42
Cold filter plugging point (CFPP) ‘C	−20 to 5	–	−9.06

international norms. This suggests improved fuel performance at low-temperature settings. These results revealed that the microalgae lipids from *C. pyrenoidosa* were successfully isolated.

4. Conclusion

Challenges associated with the mechanical disruption approach were overcome by using a novel CO_2-based alkyl carbamate ionic liquid as an efficient solvent to permeabilize the robust microalgae cell wall and extract lipids. It has been clearly demonstrated that pretreatment with CO_2-based alkyl carbamate IL could permeabilize the cell wall of microalgae *pyrenoidosa* and improve the extraction of lipid. SEM images and FTIR measurements showed thatpermeabilization of the microalgae cell wall is the cause of the increase in lipid extraction efficiency. The effects of extraction parameters (temperature, time, ratio of ionic liquid/Methanol and volume solvent to biomass) on lipid extraction were investigated via single-factor experiments. DIMCARB IL produced the highest lipid yield (9.32%) of any CO_2-based alkyl carbamate IL tested. However, a low yield of lipid was obtained when using Methanol only (7.77%). Following optimization of best value of each parameter via response surface methodology (RSM), the highest lipid yield (16.40%) was obtained after 45 min of extraction at 45 °C using ratio of 9.1 and 7 mL volume solvent-to-biomass. The extraction efficiency of lipid was 95.23% based on the lipid yield according to the conventional Floch’s method (17.21%). There are nine distinct fatty acids found in the *C. pyrenoidosa* lipid composition. By examining the fatty acid profile of the lipid mixture and comparing its properties to a global industry standard, it was shown that *C. pyrenoidosa* biomass could be used as a feedstock to generate biodiesel. However, further research on the reusing procedure or the recyclability of these ILs should be investigated for industrial applications in lipid extraction.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2022.e12754.

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