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# Protic ionic liquid-assisted cell disruption and lipid extraction from fresh water *Chlorella* and *Chlorococcum* microalgae

Mukund Shankar[a], Pratap K. Chhotaray[b], Ayushi Agrawal[c], Ramesh L. Gardas[d], Krishnamurthi Tamilarasan[c], Mathur Rajesh[c,*]

[a] Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur 603203, India
[b] Department of Chemistry, Louisiana State University, Baton Rouge 70803, LA, USA
[c] Department of Chemical Engineering, School of Bioengineering, SRM University, Kattankulathur 03203, India
[d] Department of Chemistry, Indian Institute of Technology Madras, Chennai 600036, India

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ABSTRACT

Protic ionic liquids (PILs) with their ability to host labile protons and form hydrogen bonds are likely to be efficient catalyst for bioprocessing. This is one of the first reports investigating PIL-assisted microalgal cell disruption and lipid extraction. The applicability of several PILs with butyrolactam, caprolactam, propylammonium and hydroxypropylammonium cations in combination with formate, acetate and hexanoate anions for cell disruption and lipid extraction from fresh water *Chlorella* and *Chlorococcum* sp. have been evaluated in this study. The investigations were performed with de-watered wet microalgae under ambient temperatures and hence do not require any energy intensive drying and/or processing steps. The PILs with formate and hexanoate anions when compared with conventional pre-treatment techniques: sonication, microwave and cellulase provided better microalgal cell disruption efficiencies. The FESEM analysis for the Butyrolactam hexanoate (BTH) treated cells show extensive pattern of microalgal cell disruption. The fluorescence imaging analysis coupled with culturing experiments indicates a near-complete disruption and loss in viability of the PIL-treated microalgal cells. The lipid yield from the formate and hexanoate anions-PILs was statistically comparable and/or higher to the Bligh & dyer control method. Among the PILs, BTH recorded 1.86 and 1.72 folds higher lipid yields than the control method with *Chlorella* and *Chlorococcum* sp. respectively. The lipids show predominance of $C_{16}$ and $C_{18}$ fatty acids similar to lipid profile obtained with the control method. Furthermore, the PILs were also found to have a beneficial role in reducing the chlorophyll pigments in the final lipid product. Overall, the results show PILs with formate and hexanoate anions can be utilized for a relatively energy efficient, one-step cell disruption and lipid extraction process from wet microalgae.

## 1. Introduction

Microalgae in comparison to edible oil sources, animal fat and spent cooking oil is a sustainable lipid feedstock for production of liquid transportation fuels such as biodiesel [1,2]. The advantages of microalgae over traditional oil crops have led researchers to isolate various microalgal species from aquatic environments. Some of the high lipid yielding candidates reported include *Chlorella*, *Chlorococcum*, *Dunaliella*, *Scenedesmus, Botryococcus*, *Schizochytrium* species, *etc.* [1,3,4]. The development of energy efficient, economically viable, sustainable downstream processing and production strategy is crucial for biodiesel production from these high lipid yielding microalgae.

Conventional microalgae based biodiesel production methods are labour intensive and require large infrastructural investment [5,6]. These production methods involve cell harvesting followed by two significant pre-processing steps: (i) cell lysis and (ii) lipid extraction. Cell lysis may involve physical and/or chemical methods. Physical treatment methods though effective, are energy intensive. Chemical treatment methods are effective, consume relatively less energy, but need effluent disposal. Moreover, microalgae with their plant-like complex cell walls are rigid enough to resist these cell lysis methods [7–9]. The lipid extraction step may be conducted with organic solvents

*Abbreviations:* (ILs), ionic liquids; PILs, protic ionic liquids; HCDS, high cell density suspension; BTF, butyrolactam formate; BTA, butyrolactam acetate; BTH, butyrolactam hexanoate; CPF, caprolactam formate; CPA, caprolactam acetate; CPH, caprolactam hexanoate; PAF, propylammonium formate; PAA, propylammonium acetate; 3HPAF, 3-hydroxypropylammonium formate; 3HPAA, 3-hydroxypropylammonium acetate; FAMEs, fatty acid methyl esters; FESEM, field emission scanning electron microscope

* Corresponding author.
*E-mail addresses:* mprajesh@gmail.com, rajesh.m@ktr.srmuniv.ac.in (M. Rajesh).

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and are reported to co-extract pigmented lipophilic components such as chlorophyll [10,11]. Hence, additional steps are required to remove these contaminants in the final biodiesel product. The organic solvents also possess high volatility and are toxic to the environment [12,13].

The need for an alternative approach devoid of these problems, which are also energy efficient, environmental friendly and relatively inexpensive has led researchers to focus on green solvents such as ionic liquids (ILs). ILs are molten salts, liquid at room temperature, non-volatile, thermally stable and relatively less toxic to the environment [12–15]. The applications of ILs in enzyme stability, lignocellulosic biomass hydrolysis, lipid extraction and lipid transesterification have been explored [14,16,17]. However, IL-assisted microalgal lipid extraction is a relatively new area of interest to researchers and most reports focus on extraction of *Chlorella vulgaris* lipids with imidazolium ILs [17–19]. Protic ionic liquids (PILs) unlike aprotic ILs are relatively inexpensive and less toxic alternative [20]. PILs are synthesized through proton transfer from a Brønsted acid to a base (or a Brønsted base) without any by-product formation. PILs have already been reported for dissolution of lignocellulosic biomass for bioethanol production and microalgal lipid transesterification to biodiesel [16,21]. Herein, PIL-assisted one-step microalgal cell lysis and lipid extraction has been reported. The effectiveness of various PILs: caprolactam, butyrolactam, propylammonium and hydroxypropylammonium for microalgal (fresh water *Chlorella* and *Chlorococcum* sp.) cell disruption and lipid extraction have been investigated. The PIL-treatments have been conducted with dewatered wet microalgae under ambient temperatures. The PILs treatment is able to simultaneously lyse microalgal cells, extract lipids and significantly reduce the co-extraction of green pigments from the biomass.

## 2. Materials and methods

### 2.1. Chemicals

Algae culture media M342 (HiMedia Laboratories Pvt. Ltd., India), Acid Cellulase (SynkroMax Biotech Pvt. Ltd., India.), Calcofluor white M2R (Sigma-Aldrich, USA), ε-Caprolactam (Alfa Aesar, USA), 2-Pyrrolidone and 3-Amino-1-Propanol (Spectrochem Pvt. Ltd., India) were procured from respective sources. All the other chemicals were obtained from Sisco Research Laboratories Pvt. Ltd., India.

### 2.2. Microalgal culturing

Fresh water strains of *Chlorella* and *Chlorococcum* species were gifted by Shri. A.M.M. Murugappa Chettiar Research Centre, Chennai, India. Algae culture media M342 (pH 7.5 ± 0.1), supplemented with filter sterilized vitamin solution (Vitamin B1 and B12, 10 μg/L each) was used for all culturing experiments. The seed cultures were grown in a controlled environment at 25 ± 1 °C, 200–250 RPM, 2600 ± 100 lx with an automatic adjustment of light and dark cycle of 12 h each. 10% of the seed (15–17 days) were aseptically transferred to a 2.5 L media in 5 L Erlenmeyer flasks. The cultures were then incubated at room temperature (28–32 °C) with natural day light and continuously sparged with 0.2 μm filtered air for 25–27 days before harvest.

### 2.3. Synthesis of protic ionic liquids

The PILs were synthesized with the methods mentioned elsewhere [22,23]. In brief, respective bases were placed in a two-necked round bottom flask equipped with a reflux condenser and a dropping funnel. The respective acid was added drop-wise to the contents of the flask maintained below 10 °C and incubated with stirring at room temperature for 24 h. At the end of the incubation period, the liquid obtained was subjected to high vacuum for 48 h at room temperature with continuous stirring followed by storage under nitrogen atmosphere. The PILs synthesized are listed in Table 1.

### 2.4. Treatment methods

#### 2.4.1. Conventional microalgae treatment methods

Microalgal cells were harvested with centrifugation (5000 g, 15 min). The cell pellet was resuspended in distilled water to produce a high cell density suspension (HCDS) of 15 g/L dewatered cells and subjected to three different conventional treatment methods as follows: (1) Sonication experiments were carried out at 20 kHz, 100 W for 5, 10, 20 and 40 min with a probe (QSonica XL-2000 Microson) placed in the HCDS; (2) Microwave experiments were performed in a non-thermostated microwave system at 700 W for 30, 60, 90 and 180 s in a beaker containing HCDS, and (3) Cellulase treatment was carried out by preparing the HCDS in citric acid buffer (pH 4.5) prior to addition of acid cellulase (activity: 250,000 U/g). The final enzyme solution were maintained at 10% v/v and 20% v/v followed by incubation for 24 h at 50 °C.

#### 2.4.2. PIL treatment of wet microalgae

The cell disruption efficiency by PILs on wet microalgae was investigated by dewatering the cells with centrifugation (5000 g, 15 min). The cell pellet was resuspended in PILs (PILs: cells, 10:1 w/w) and incubated for 24 h at room temperature with constant low speed stirring to avoid settling of the cells. The control samples had the PILs replaced with distilled water.

### 2.5. Estimation of extent of microalgal cell disruption

The reduction in the total number of microalgal cells post treatment was considered as the extent of cell disruption and expressed as a percentage. The cell counts were performed with a Neubauer haemocytometer and observed at 40 × magnification under Leica DM LS2 microscope with phase contrast. The control and the treated samples were diluted and the cell densities were determined.

$$\%\text{Cell disruption} = 100\left[1 - \left(\frac{T}{C}\right)\right]$$

where $C$ and $T$ are the number of cells/mL in control and treated samples respectively.

$$\text{cells} \Big/ \text{mL} = \left(\sum_{i=1}^{5} N \times df \times 10^6\right) \Big/ 20$$

$N$ is the number of cells counted in 5 haemocytometer chambers of 4 nL each and $df$ is the dilution factor.

### 2.6. Assessment of PIL treated microalgal cell viability and structural integrity

To assess the microalgal cell viability, the PILs treated and control cells were washed twice with sterile distilled water, centrifuged and the pellet was aseptically inoculated into 5 mL sterile nutrient media and incubated under controlled conditions as stated in Section 2.2. Growth was monitored by periodically aspirating samples into micro-titre plates. The change in absorbance was measured at 610 nm.

Fluorescence analysis was performed on BTH treated and control microalgal cells to assess cell structural integrity. The samples were pelleted and rinsed thrice with distilled water. The cells were resuspended in 0.2 mL calcofluor white M2R solution (0.2% in distilled water) and incubated in the dark for 10 min. The stained cell pellets were washed with distilled water and observed at 40 × magnification under Zeiss LSM 700 confocal fluorescence microscope operated with laser excitation at 405 nm and emission between 440 and 490 nm. The scanned confocal microscopy images (brightfield and fluorescence) were superimposed with the Zen 2010 software from Zeiss International, Germany [24].