CHEMICAL ENGINEERING TRANSACTIONS
VOL. 57, 2017
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Sauro Pierucci, Jiří Jaromír Klemeš, Laura Piazza, Serafim Bakalis

ISBN 978-88-95608- 48-8; **ISSN** 2283-9216

Potential of Choline Chloride – Based Natural Deep Eutectic Solvents (NaDES) in the Extraction of Microalgal Metabolites

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In a typical chemical process, the solvents are widely used for the dissolution of the reagents, to favor the kinetics and the thermodynamics of a chemical reaction, for the extraction of products, for the separation of mixtures. However most of the currently used organic solvents are characterized by different properties harmful to human health and the environment. Among the principles of Green Chemistry are that solvents should be innocuous to Man and to the Environment (safer solvents) and that the substances used in a chemical process should be chosen to minimize the potential for chemical accidents (intrinsically safe processes).
Biorefining, the biomass Era counterpart of oil refining is most likely going to be extraction-based, and thus heavily solvent-dependent, much as the Oil Era was based on distillation and hence heat-dependent. Ionic Liquids (ILS) and eutectic mixtures exploited as solvents (DES) are two major classes of solvents that are making their way in Green Chemistry and, in particular, in biomass processing research.
NaDES ('Natural Deep Eutectic Solvents'), i.e. mixtures formed by natural primary metabolites present in all organisms, such as sugars, polyols, amino acids, organic acids, derivatives of choline, form intermolecular hydrogen bonds and, when mixed in a certain ratio, change their state from solid to liquid forming a eutectic system. The most interesting NaDESs are those in which water is one of a ternary system since the degree of dilution with water modifies such physical properties of the NaDES as the density, the viscosity, and the polarity. By modulating the water content the solvation power can be adjusted to specific needs.
In this work, the PCH (1,2-propanediol, choline chloride, water 1:1:1) NaDES was used to treat microalgal biomass and carry out the extraction of cellular components, such as lipids, proteins, carbohydrates and photosynthetic pigments (chlorophylls and carotenoids) from the biomass itself.
Three sets of experiments were carried out based on different contact time between biomass and PCH: 24 and 72 hours, with and without pre-treatment with ultrasound. Biomass was shaken together with the PCH solvent in the presence of glass beads to promote the extraction efficiency. The analysis of the extract composition was carried out spectrophotometrically for pigments (chlorophylls and carotenoids), with biochemical assays for proteins and carbohydrates and gravimetrically for the determination of lipids. The results showed the ability of PCH, coupled with the mechanical destruction of cell walls, to solubilize a wide range of polar biomolecules at room temperature.

1. Introduction

The overall goal of green chemistry combined with a biorefinery approach is the production of genuinely green and sustainable chemical products (Di Paola et al. 2015). Any new chemical process being developed should aim at using sustainable feedstocks. In the field of sustainable energy, microalgae can warrant renewable biofuels such as third-generation biodiesel obtained by the non-polar lipids, but the maximum utilization of the feedstock mass in final products is required. Lipids are not the only important fraction from the biomass, microalgae and cyanobacteria are important sources of carbohydrates, proteins, polyunsaturated fatty acid (PUFA) with high nutritional values, carotenoids such as β-carotene and astaxanthin, phycobiliproteins, which are used as natural dyes and antioxidant compounds for pharmaceutical and cosmetic applications. Biorefineries of the future will incorporate the production of fuels, energy and value-added chemicals, via the processing of biomass, into a single site (Kamm et al., 2006). The most popular extraction method for

recovering oil and high value products from microalgae is solvent extraction because it guarantees reproducible results and it is a relatively inexpensive technique; its drawbacks are the high flammability and/or toxicity of the organic solvents, the high cost of solvent recovery and the request of large volume of solvent. Organic solvents can lead to contamination in the form of solvent residues present in the final product. These are the reasons why most organic solvents may have limited application in food processing. Downstream processing for the potential commercial production of microalgae products not only must consider economic costs, but should also consider minimizing environmental impacts, in order to attain sustainable production processes. (Mercer and Armenta, 2011). The green technology facilitates the minimum use of non-hazardous media, new environmentally acceptable solubilization techniques by controlling physical properties of media such as temperature and pressure, and developing new green solvents. Among the diverse ways of green technology, developing new green solvents may be the most important subjects. Therefore, several groups have reported the use of supercritical CO_2, less toxic solvent mixtures, and ionic liquids to replace toxic organic solvents (Demirbas and Demirbas, 2011; Young et al., 2010). Abbott et al. (2004), developed DES mixing choline chloride with urea, which results in a liquid with a freezing point of 12 °C. This liquid was found to have interesting solvent properties that are comparable to ambient temperature ionic liquids and a wide variety of solutes were found to exhibit high solubility. DES are obtained by mixing solid compounds forming a eutectic mixture with a melting point much lower than either of the individual components due to the formation of hydrogen bonds. Natural products are a plentiful and ideal source of ILs and DES due to their enormous chemical diversity, biodegradablility and pharmaceutically acceptable toxicity profile. Different mixtures of various abundant cellular constituents (primary metabolites) such as sugars, sugar alcohols, amino acids, organic acids, and choline derivatives were tested and many combinations of these compounds were found to be liquids. The exploration of different combinations of these common metabolites abundantly present in all types of cells and organisms provided over 100 combinations of NaDESs. These eutectic solvents present densities higher than that of the water, the viscosity is determined by water content and temperature. The polarity range varies from 44.81 kcal mol^{-1} (higher than water) to 51.89 kcal mol^{-1} (comparable to methanol), but the polarity depends on the amount of water present in the solvent. (Dai et al., 2013).
In this work the potential of the 1,2-propanediol choline chloride water (PCH) in the extraction of metabolites was investigated. The treatment of the biomass includes the coupling of the NaDES extraction with the ball mill disruption technique in order to maximize the recovery of microalgal compounds. Carbohydrates, proteins, chlorophylls, carotenoids and polar lipids were then quantified and the extraction efficiency was compared with that of hexane.

2. Materials and Methods

1,2-propanediol-choline chloride-water (PCH) was prepared with the molar ratio of 1:1:1. Components were mixed in a pyrex bottle with a magnetic bar, capped and heated in a water bath at 50 °C under agitation for 45 minutes, until the solution became clear and transparent. Choline chloride (>99 %), 1,2-propanediol (≥ 95 %), dichloromethane (>99.8 %) and methanol (>99.9 %) were purchased from Sigma-Aldrich. *Scenedesmus dimorphus* (UTEX 1237) was obtained from the Culture Collection of Algae at the University of Texas at Austin, USA. The strain on agar was inoculated into 3NB culture medium that in a liter contains: $CaCl_2$ 0.17 mmol, $NaNO_3$ 8.82 mmol, $MgSO_4 \bullet 7H_2O$ 0.3 mmol, K_2HPO_4 0.43 mmol, KH_2PO_4 1.29 mmol, NaCl 0.43 mmol, $Na_2EDTA \bullet 2H_2O$ 2 mmol, $FeCl_3 \bullet 6H_2O$ 0.36 mmol, $MnCl_2 \bullet 4H_2O$ 0.21 mmol, $ZnCl_2$ 0.037 mmol, $CoCl_2 \bullet 6H_2O$ 0.0084 mmol and $Na_2MoO_4 \bullet 2H_2O$ 0.017 mmol. Cultures were grown in 400 mL cylindrical glass tubes, with a diameter of 6 cm, fed with filtered and humidified air; flow rate was $130*10^3 Nm^3/h$. A 16 h photoperiod of light provided by cold white fluorescent lamps (400-700 nm, 865 K, 32 W, 80 mmol photons m^2 s^{-1}) was followed by a period of darkness equal to 8 h. The temperature was maintained constant at 28 ±1 °C. The biomass was harvested every 8-10 days when it reached the stationary phase of growth. Physical properties of the PCH were investigated. Viscosity was measured with the rotational viscometer Haake instruments Rotovisco RV 12, conductivity with the Delta Ohm probe HD 8706-R1 and pH with pH-meter of Hanna Instruments HI 8418. Carbohydrates and proteins were quantified colorimetrically with an UV-Vis Mapada spectrophotometer; total carbohydrates were quantified through the Dubois assay (Dubois et al. 1956) and total proteins were quantified with Lowry assay (Lowry et al. 1951). Chlorophylls and carotenoids were determined spectrophotometrically adopting Lichtenthaler equations (Lichtenthaler, 1987). Cell destruction was obtained in the presence of PCH with ball mill method with glass beads of 2.5 mm diameter and it lasted for 24 h. It was compared with the Ultrasound Assisted Extraction (UAE) coupled with the solvent. Ultrasound pulsed cycle was effectuated in a refrigerated vessel and lasted for 40 minutes with a frequency of 20 kHz and a range of 70%. The amount of extracted chlorophylls and carotenoids was quantified spectrophotometrically in the solvent, extracted lipids were evaluated gravimetrically, proteins and carbohydrates were quantified as the difference in content between biomass collected and residual biomass after treatment.

3. Results and discussion

3.1 Physical-chemical properties of PCH in function of its water content

The effect of the water contained in the solvent on properties of conductivity, viscosity and polarity, was studied. Conductivity increases with increasing of water amount, reaching a maximum when water is 60 weight% (Figure 1.).

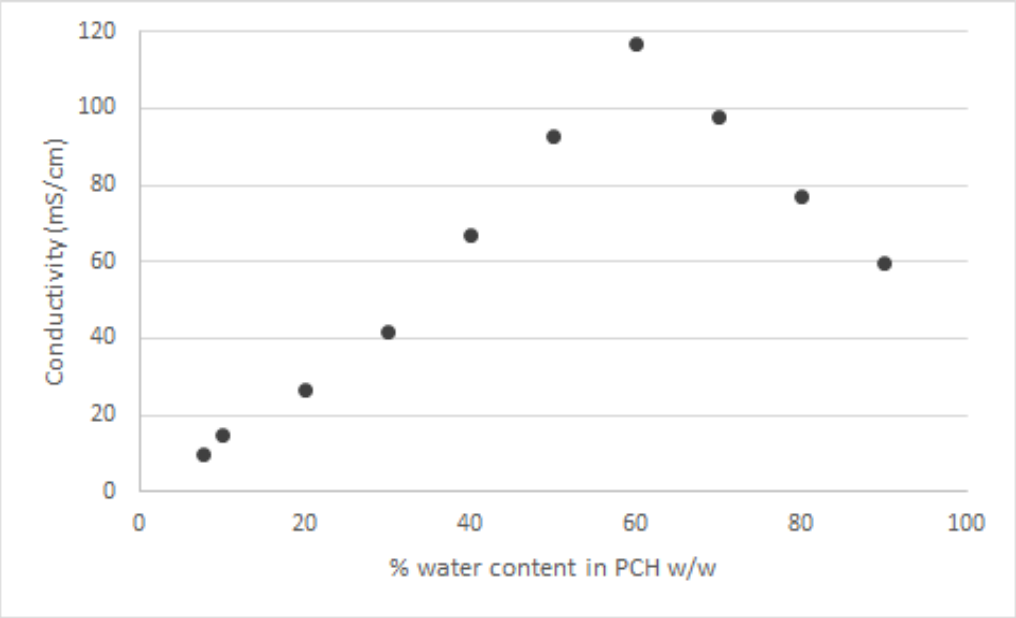

Figure 1. Variation of conductivity with the water amount in PCH.

The huge viscosity of NaDES, due to the presence of a large net of hydrogen bonds established among its components, is a big obstacle in extraction protocols. When PCH is diluted with water, the interactions among the components are weakened and so viscosity decreases as shown in Figure 2. When the degree of dilution reaches 50%, the viscosity value is close to the one of water and the solvent still maintains its intermolecular structure.

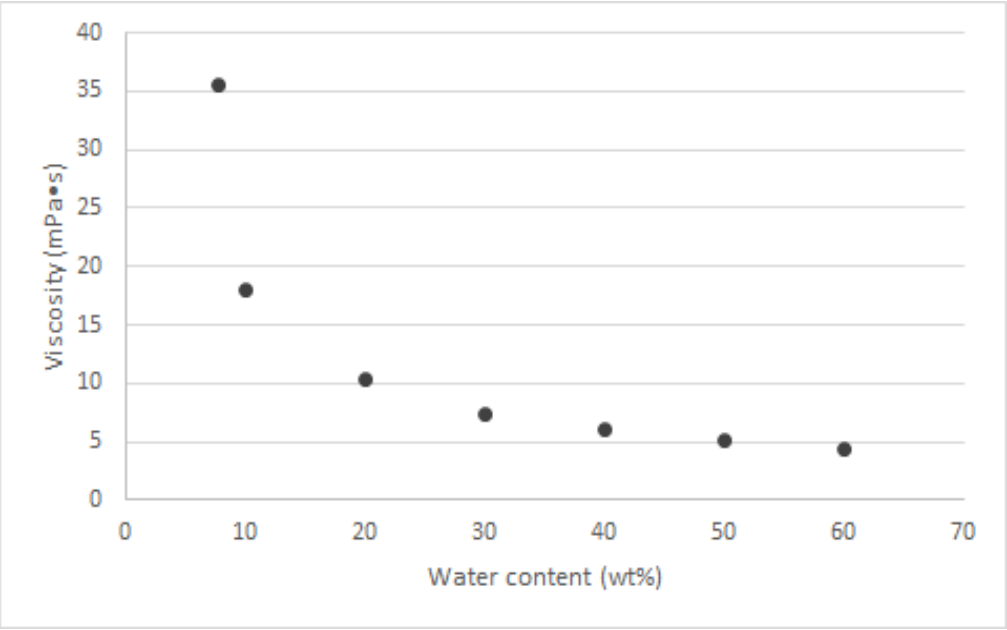

Figure 2. Variation of viscosity in accordance with the water amount in PCH

To understand polarity variation under the influence of water amount in PCH, miscibility tests were performed with an organic solvent. Since PCH has a polarity close to that of methanol it is expected to have the same miscibility behaviour. Moreover, Butyl alcohol is a solvent miscible with methanol and with PCH, they form an only phase, but it is immiscible with water (and in theory with PCH with high water content). When the water content in the NaDES is 25 wt% the system PCH – butyl alcohol forms two separated liquid phases, because PCH reaches a polarity value close to the one of water. The investigated properties of PCH are summarized in Table 1.

Table 1. Physical-chemical properties of PCH prepared with the molar ratio of 1:1:1.

	PCH
pH	5
Conductivity at 25 °C (mS/cm)	10.1
Viscosity at 40 °C (mPa•s)	35.6
Density at 40 °C (g/cm^3)	1.1

3.2 Extraction of biomolecules with PCH from wet samples of *S. dimorphus* biomass

Firstly, the action of PCH on microalgae cell walls, without any intervening mechanical rupturing method, was investigated. A volume of 30 mL of PCH containing 0.6 g of wet biomass was kept at room temperature and was shaken at 250 rpm for 24 hours in an orbital shaker. After the sample centrifugation, the PCH recovered was completely transparent and no peaks were observed by UV-vis spectrophotometric analysis. Presuming a solvent diffusional problem, due to its huge viscosity, a new extraction lasting 72 hours was performed with the same results. Hence, PCH alone is not able to destroy and penetrate microalgae cell walls. For this reason, mechanical destruction was applied on the biomass and two protocols were set up.
Ball mill extraction with PCH is a simple and economic way to destroy cell walls and can be easily accomplished by means of glass beads. Before the incubation in the orbital shaker, the solution containing 0.6 g of microalgae, 2.5 g of glass beads and 50 mL of NaDES, was vortexed for 15 minutes at 1500 rpm. Then it was put in a 250-mL flask with 50 mL of PCH. The beads-assisted extraction lasted for 24 hours at room temperature in an orbital shaker set to 250 rpm. The solution was recovered and ultracentrifuged at 17000 rpm at 20 °C for 30 minutes, 2.5 mL of solvent with the accompanying extract were spectrophotometrically analyzed to quantify the extracted photosynthetic pigments, while the residual was put in a (previously weighed) flask with 100 mL of hexane for 24 hours. In this way, extracted lipids were quantified gravimetrically. The cell pellet was washed twice to remove salts and its content of proteins and carbohydrates was quantified, the extracted were calculated through the difference with the starting content. A sample from the cell pellet was observed by optical microscopy, showing that a very small quantity of cells was effectively lysed (Figure 3a). To improve the efficiency in the cell walls breaking, the next step was UAE in presence of PCH. A solution with a volume of 100 mL of NaDES, containing 0.6 g of wet biomass, was treated in a jacketed vessel, refrigerated with water and ethylene glycol at 0 °C, for 40 minutes. Every ten minutes a sample of 1 mL was seen at microscope to supervise the effective break of cells. Fig. 3b shows the lysed biomass after 40 minutes of UAE.

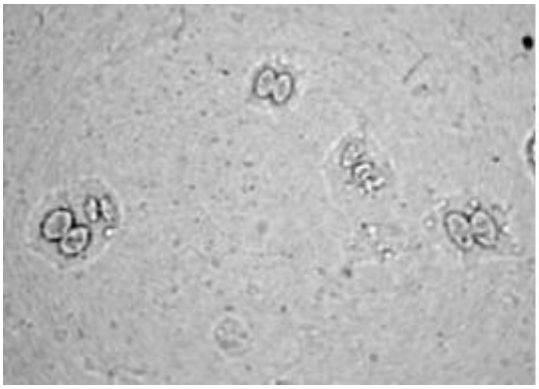

Figure 3a. Living cells after 24 PCH treatment.

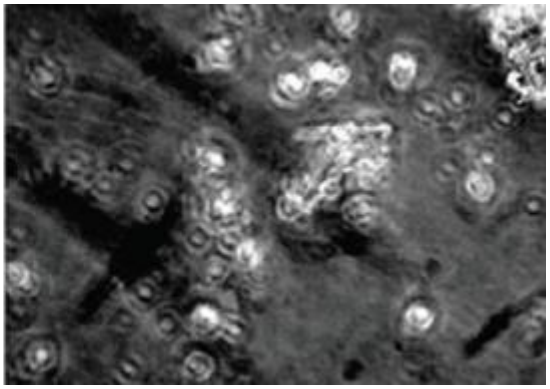

Figure 3b. Lysed cells after 40 minutes of UAE in PCH.

After the ultrasound cycle the suspension was transferred into a flask and shaken for 24 hours. Biomolecules extraction was evaluated as explained before. Table 2 reports the percent of extracted molecules, for the two methods of cell destruction. The comparison between the two protocols shows a better extraction efficiency for the UAE. This was predictable due to the strong lytic action of ultrasounds on cell walls, but it implicates a higher energy waste. In both cases, PCH showed low affinity for nonpolar compounds as lipids, a satisfactory extractive ability for photosynthetic pigments, carbohydrates and proteins.

Table 2. Profile of biomolecules extracted from biomass with PCH and mechanical disruption (ball mill or ultrasound assisted extraction - UAE)

Extraction efficiency %		
	Ball mill	UAE
Proteins	10	27
Carbohydrates	7	12
Lipids	0.7	1.8
Chlorophylls	2.8	3.4
Carotenoids	0.06	0.11

4. Conclusions

Mixed in a suitable ratio, primary metabolites can form Natural Deep Eutectic Solvents. Most attractive NaDES have water as a key constituent because, by varying the water content, some major physical-chemical properties of the solvent can be tuned. Here, the PCH NaDES properties and solubility toward microalgal biomass was investigated. The (water-free) PCH has shown to possess a high viscosity, that can be reduced significantly by tuning the PCH:Water composition. NaDES are liquid solvents and maintain their supermolecular structure at room temperature. PCH covers a fairly large range of polarity, that can be modulated, again, by changing the H_2O content in the DES. For this reason, NaDESs come close to the concept of “switchable solvents” generally attached to tertiary amines and, even though their change of polarity is not as large as that of the mentioned amines case, NaDESs fully implement the nature of “tunable” solvent that switchable solvents do not feature because they only possess two states. Thanks to these features, natural eutectic solvents are suitable to solubilize a wide selection of molecules, which are scarcely soluble, or completely insoluble, in water, such as DNA, pigments, proteins and polysaccharides, as it was shown here with microalgal biomass. One of its most attractive qualities is its sustainability and biodegradability, conferred by their natural and nontoxic ingredients. NaDES ingredients are cheap, the mixtures are easy to prepare with a low energetic waste and solvents have a high temperature of breakdown. These features encourage their utilization in the food, cosmetics, chemical and pharmaceutical industry. Combining PCH with a mechanical destruction method (UAE/Ball mill) a protocol with potential application in biorefinery for principal classes of biomolecules can be devised. To date, the limited change to nonpolar behaviour limits the PCH potential in lipids extraction. Although PCH investigation is still in its infancy a deeper study on PCH molecular structure could widen its polarity limits and lead to an improvement of its solubility toward nonpolar substances and enable a more complete extraction from the whole biomass.

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