Extraction of chlorophyll from wild and farmed *Ulva* spp. using aqueous solutions of ionic liquids

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Abstract

Products extracted from natural resources are an increasing trend in several fields promoted by consumer demand. Allied to the importance attached to the concept of "natural product" should be the way the "natural product" is obtained. In this work, chlorophyll was extracted from batches of wild-harvested and farm-raised green macroalgae *Ulva* spp. from two different European locations, Portugal and France. The performance of different aqueous solutions of tensioactive compounds such as ionic liquids and common surfactants in the yield of extraction of chlorophyll was studied and the operational conditions of extraction were optimized. The effect of drying the biomass on the yield of extraction of chlorophyll was evaluated as well as the effect of both locations (and the specific conditions of each location in terms of nutrients, water temperature and light intensity) in chlorophyll production. After optimization of all operational conditions, a maximum yield of extraction of 5.96 $mg_{chl}.g_{dry\ algae}^{-1}$ was obtained using 250 mM of tributyltetradecylphosphonium chloride ($[P_{4,4,4,14}]Cl$). The use of this solvent has allowed the development of a cost-effective (conclusion obtained after the economic analysis) and efficient process capable of maintaining the stability of the final product for more than one month.

Keywords: Green macroalgae, *Ulva*, geographic location, seaweed, bioeconomy, blue biotechnology, chlorophyll, surfactants, tensioactive ionic liquids, economic analysis.

1. Introduction

Blue biotechnology is emerging as a solution to reduce the world's need of synthetic compounds from non-renewable raw materials. In this sense, the development of sustainable and integrated biorefineries based on abundant marine materials scarcely used is essential (1). Macroalgae are an example of such a biomass, which did not have, up to now, a multi-application approach of the biomass, being macroalgae used mainly for polysaccharide extraction for human food, or other lower volume sectors like cosmetics, feed and pharma. However, this type of biomass can benefit from its integration in processes answering the biorefinery challenges, by combining the extraction of added-value molecules with high-volume/low-cost applications as feed, plant biostimulants or even energy and bioplastics (2).

Due to their market value and usually lower contents in the biomass, added-value molecules should be the first molecules to be considered in a biorefinery chain (2). The added-value molecules present in macroalgae represent a large plethora of chemicals, with a wide range of properties, from antioxidant, to anti-inflammatory and anti-tumoral, with potential in biomedical, pharmaceutical, and cosmetic industries (3). In macroalgae, lipids, carbohydrates, proteins, minerals, vitamins and pigments are included in the most valuable bioactive compounds, which can supply consumer current demands for natural products. At the same time, these have been reported for the environmental aspects of sustainability allowing consequently to boost new economies and industrial sectors (4). Pigments are extremely important for macroalgae since they ensure the light capture required for photosynthesis (5). Besides, they have a significant number of applications attributed, currently mainly cosmetics but potentially including pharmaceutical products as well as food and even textile dyes (6,7).

The role of chlorophyll in the harvesting of light and in conversion of energy of absorbed photons to chemical energy (8) is already well-established. Moreover, their benefits to human health have been reported considering its antioxidant (9), anti-inflammatory (10), and anti-tumoral activities (11). Although chlorophyll extraction from living matrices is not new (12), the reported methodologies are in their vast majority based on the use of hazardous and volatile organic solvents or mechanical treatments that lead to the increase of temperature and partial thermo-degradation (13,14).

The use of water-based solvents at room temperature appears as a more sustainable and biocompatible approach. To use water as solvent to recover hydrophilic compounds is easy; the challenge is to use water to extract hydrophobic molecules like chlorophyll (15). Some articles dealing with extraction of hydrophobic pigments already suggest the use of aqueous solutions of tensioactive compounds as extracting solvents (16). Tensioactive compounds tend to form micelles above the critical micelle concentration, creating a friendly environment for solvation of hydrophobic molecules in water. Besides common surfactants, some ionic liquids (ILs) also have this tensioactive feature (17). ILs are salts with special interest due to their tunable nature. This results from the correlation between the IL structure-properties-application, allowing them to be recognized as “designer solvents” with affinity to a large set of biomolecules (15,18).

The main objective of this work is aligned with the objectives defined on GENIALG (GENetic diversity exploitation for Innovative macro-ALGal biorefinery), an European project with several academic and industrial participants from all around Europe. This project focuses specifically two macroalgae species, the *Saccharina latissima* (common names being sugar kelp or Kombu royal) and *Ulva* spp. (or sea lettuce), two of the species with high biomass production yield and with a validated farming expansion potential in

Europe. Under the scope of this European project, the intention is "to boost the Blue Biotechnology Economy by designing high-yielding seaweed cultivation systems and more sustainable downstream processes". In this sense, the objective of this work encloses the optimization of more sustainable extraction methodologies by replacing the conventional volatile organic solvents usually used to recover the pigments by more selective solvents, mainly composed of water, that provide higher yields of extraction and higher stability to the pigments, and that simultaneously lead to more sustainable and profitable processes with industrial potential. More specifically, aqueous solutions of common surfactants and tensioactive ILs were used in the extraction of dry and fresh samples of *Ulva* spp. harvested in different locations, namely in Portugal and France.

In this work, the extraction of chlorophyll from *Ulva* spp. from two different geographic locations was investigated. Several aqueous solutions of common surfactants and tensioactive ILs were studied and the results obtained compared with the data obtained for a conventional organic solvent, in this work, ethanol. Moreover, the process conditions of solid-liquid ratio, time of extraction, concentration of tensioactive, type (dry or fresh) and geographic location (farm-raised @ Portugal, *Ulva rigida* and wild-harvested @ France, *Ulva armoricana*) of the biomass were optimized. Then, the stability of the chlorophyll content extracted was also studied. Finally, the economic evaluation of the traditional *versus* the alternative extraction process was performed, where different scenarios were evaluated in such costs.

2. Experimental section

2.1. Material

2.1.1. Macroalgae

The biomass used in this work was kindly provided by two different companies, ALGAplus (Ílhavo, Portugal) and Olmix (Bréhan, France). ALGAplus farms *Ulva rigida*. at Ria de Aveiro lagoon (40°36'44.7" N, 8°40'27.0" W) in coastal Portugal under the EU organic aquaculture standards (EC710/2009). This aquaculture is performed in a land-based/on-shore within the concept of integrated multi-trophic aquaculture system (meaning that the dissolved inorganic nitrogen (DIN) input to the seaweed farm is higher due to the use of the effluent water from fish production). Olmix harvests *Ulva* sp. in the north Brittany coast near Plestin-les-Grèves (48°40'49.9" N, 3°35'40.1" W), France. Dry and fresh biomass samples from the Portuguese company were harvested in September 2018 and June 2018, respectively, and fresh biomass from the French company was harvested in July 2017, being these three samples studied. Fresh biomass was washed and kept frozen until needed.

2.1.2. Chemicals

Absolute ethanol (HPLC- grade) was purchased from Fisher Scientific being used as a standard organic solvent. Tensioactive compounds in aqueous solution were used on the extraction of chlorophyll from the green macroalgae. The series of 1-alkyl-3-methylimidazolium chloride as 1-hexyl-3-methylimidazolium, $[C_6C_1im]Cl$ (98 wt%), 1-methyl-3-octylimidazolium chloride, $[C_8C_1im]Cl$ (99 wt%), 1-decyl-3-methylimidazolium chloride, $[C_{10}C_1im]Cl$ (98 wt%), 1-dodecyl-3-methylimidazolium chloride, $[C_{12}C_1im]Cl$ (> 98 wt%), 1-methyl-3-tetradecylimidazolium chloride, $[C_{14}C_1im]Cl$ (98 wt%), 1-hexadecyl-3-methylimidazolium chloride, $[C_{16}C_1im]Cl$ (> 98 wt%) were all acquired from Iolitec. The tributyltetradecylphosphonium chloride, $[P_{4,4,4,14}]Cl$ (95 wt%) and the decyltrimethylammonium chloride, $[N_{1,1,1,10}]Cl$ (98 wt%) were purchased from Iolitec

and Tokyo Chemical Industry, respectively. The dodecyltrimethylammonium bromide, $[N_{1,1,1,12}]Br$ (99 wt%), and tetradecyltrimethylammonium bromide, $[N_{1,1,1,14}]Br$ (98 wt%), were acquired from Alfa Aesar, while the hexadecyltrimethylammonium bromide, $[N_{1,1,1,16}]Br$ (99 wt%) was purchased from Merck. The surfactants sodium dodecyl sulfate, SDS (99 wt%) and polyethylene glycol tert-octylphenyl ether, Triton X-114 were purchased from Acros Organics. The chemical structures of the tensioactive compounds used are depicted in Fig. S1 from ESI.

2.2. Chlorophyll extraction

The fresh samples of macroalgae were washed at least three times with distilled water and stored at -20ºC. Before the extraction, the samples were frozen with liquid nitrogen and ground in a coffee grinder until powder (< 0.5 mm). The drying procedure of the dry samples of macroalgae was carried out by ALGAplus, in which the algae were washed with seawater, centrifuged to remove excess water and then dried in a forced air-tunnel at a set temperature of 25ºC until reaching a moisture content of 10%-11%. The dried samples were milled and sieved to obtain powder (< 1 mm).

The extractions were performed at room temperature (20-25ºC) under a constant agitation of 80 rpm. Ethanol was used in parallel as a control solvent. Initially, solutions of 250 mM of the tensioactive compound in water (common surfactants and tensioactive ionic liquids) were used (19) at an incubation time of 30 minutes and a solid-liquid ratio (SLR) of 0.01 $g_{biomass}.mL_{solvent}^{-1}$. The type of solvent, SLR, solvent concentration, and time of extraction were systematically changed as they were optimized. All assays were performed at least in triplicate. In order to remove the cell

debris, a centrifugation step was added in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700 xg for 30 minutes at 4ºC.

2.3. Chlorophyll quantification

The absorption spectra were measured between 200 and 700 nm using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek) within one hour after the extraction process. The chlorophyll content was quantified at 667 nm being the interference of the solvents considered and the chlorophyll concentration calculated according to a calibration curve previously prepared. The results are expressed in terms of yield of extraction ($mg_{chl}.g_{dry\ algae}^{-1}$).

2.4. Chemical stability of the extracts over time

Extracts obtained with the most promising solvents at the optimized conditions were analyzed in terms of their stability over time at 25ºC and 4ºC, protected from light, for the wild-harvested and farmed-raised algae. The assay was done during 33 days by analysing the percentage of chlorophyll loss, being the NMR spectra (H^1 and C^{13}) of the samples analysed at least once a week and compared with the respective extract just after the extraction.

2.5. Statistical analysis

Analysis of variance (ANOVA) was performed using the BIOESTAT 5.3 to compare the significance of the obtained extraction yields for each operational condition and solvent at a time, using a degree of significance of 95% ($p < 0.05$, n=3). This analysis was always

performed considering a comparison of significance in the yield of extraction of chlorophyll for the same algae, solvent and parameter tested.

2.6 Economic evaluation

The economic evaluation performed focused mainly on the material consumption between the IL and ethanol process options. Production costs were calculated *per* milligram of chlorophyll produced (Cost of goods *per* milligram – CoG/mg). To calculate the production costs, the following equation (**Equation 1**) was employed:

$$\frac{CoG}{mg} = \frac{\sum_{i=1}^{n} \frac{Use\ of\ material_i}{Batch} * \frac{Price\ of\ material_i}{Unit\ of\ material_i}}{\frac{Amount\ of\ chlorophyll}{Unit\ of\ dry\ biomass} * Mass\ used\ of\ dry\ biomass} \quad \text{(Eq. 1)}$$

This evaluation consisted of two analyses. Firstly, a deterministic analysis where the CoG.mg^{-1} is calculated using the best conditions selected after the experimental work was performed. Then, a sensitivity analysis was performed to determine the impact of the material costs (higher or lower than the base cost) and the concentration of IL applied. These variables were defined in the equation presented. It can be seen from **Equation 1** that the only cost related variables are the price of the materials. For this analysis, the price of the IL considered was of 409.3 €.kg^{-1} (Ionic Liquid Technologies, Heilbronn, Germany) and for Ethanol 75 €.L^{-1} (Fischer Scientific, Portugal).

3. Results and discussion

3.1. Screening of alternative solvents and operational conditions optimization: comparison of fresh and dry algae

A comparison among fresh and dry samples of farm-raised *Ulva* sp. (*Ulva rigida* in this case) from the same location was done, being the screening of aqueous solutions of

different alternative solvents and the optimization of the process operational conditions performed. Ethanol was studied simultaneously as an example of a conventional solvent reported for the chlorophyll extraction (16).

In the screening of the alternative solvents (Fig. 1) common surfactants and tensioactive ILs, namely imidazolium-, phosphonium-, and ammonium-based ILs were studied in a concentration of 250 mM, SLR of 0.01 $g_{biomass}.mL_{solvent}^{-1}$ for 30 minutes. The effect of the alkyl side chain length was studied for imidazolium- and ammonium-based ILs. However, the aqueous solutions of SDS and $[P_{4,4,4,14}]Cl$ stand out as the most efficient solvents with similar or even higher results than the ones reported for ethanol. For fresh biomass, the results obtained follow the trends previously described for other biomolecules (19). For the dry algae, aqueous solutions of SDS showed a colour change of the extract, probably due to chlorophyll degradation.

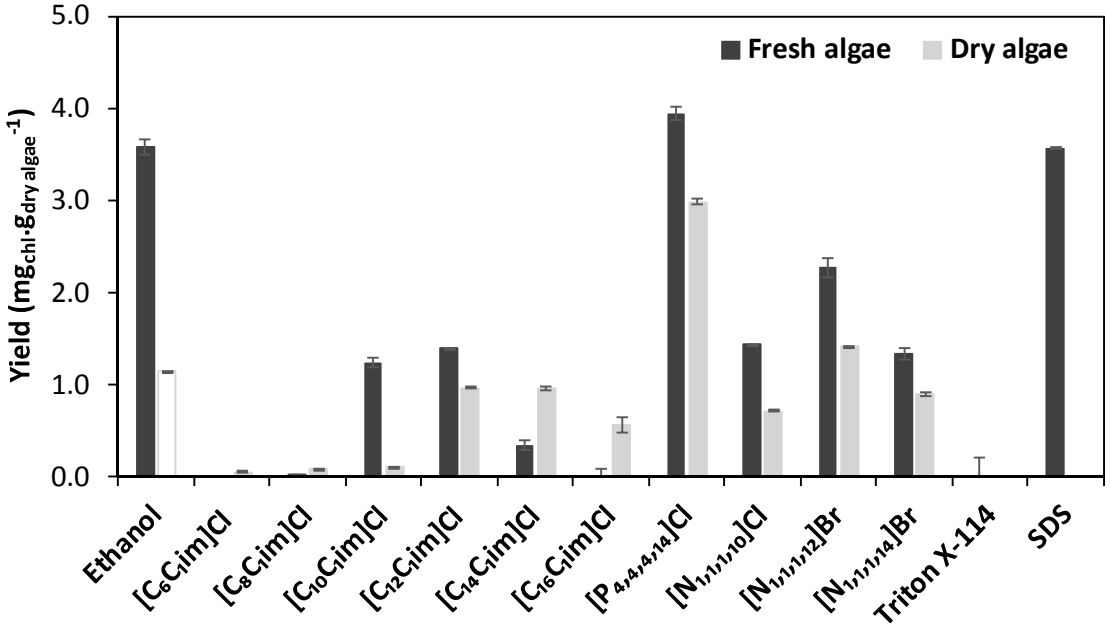

Fig. 1: Yield of extraction of chlorophyll using fresh and dry farm-raised *Ulva* sp. regarding the screening of aqueous solvents of different tensioactive solvents.

The study proceeded with the optimization of the most relevant operational conditions, namely the SLR, solvent concentration in water, and time of extraction (Fig. 2A, 2B, and 2C, respectively). For the dry algae, aqueous solutions of SDS were not considered for the reasons discussed above. In any case, the extraction yield obtained using the fresh biomass was always the highest. Moreover, even in the case of dry algae, the yield of extraction of chlorophyll is more than the double using the aqueous solution of $[P_{4,4,4,14}]Cl$ (250 mM) instead of ethanol (Fig. 1), which is a consequence of the poor capacity of ethanol to penetrate the dry biomass.

Regarding the effect of the SLR (Fig. 2A), the choice falls on the condition that provides the highest yield of extraction of chlorophyll, meaning the least amount of solvent for the highest concentration of chlorophyll possible. When fresh biomass is used, the yield of extraction is maximum for SLR of 0.04 and 0.02 $g_{biomass}.mL_{solvent}^{-1}$ for $[P_{4,4,4,14}]Cl$ and SDS, respectively. Meanwhile, when using dry algae, the maximum of chlorophyll extracted was observed for a SLR of 0.013 $g_{biomass}.mL_{solvent}^{-1}$ for $[P_{4,4,4,14}]Cl$. This means that, when the dry biomass is used, to achieve the highest yield of extraction, more volume of solvent is needed. This could be justified by the impact that the drying process may have on the structures of chloroplasts or thylakoidal membranes, but it may also be explained by the negative impact towards the chlorophyll structure.

After selecting the most efficient SLR as being 0.04 and 0.013 $g_{biomass}.mL_{solvent}^{-1}$, for fresh and dry biomass, respectively, the effect of $[P_{4,4,4,14}]Cl$ concentration was tested and for that, aqueous solutions of the IL in concentrations between 50 and 500 mM were tested (Fig. 2B). The main results suggest that the yield of extraction increases with the tensioactive concentration up to 250 mM, a profile that is independent of the biomass being fresh or dry, for both solvents. Interestingly, this same trend was previously

observed found for the extraction of green fluorescence protein from recombinant *Escherichia coli* cells (19). As a third condition, it was studied the time of extraction as depicted in Fig. 2C. From the experimental data, it is possible to observe an increase in the yield of extraction up to 60 min, for both fresh and dry biomass.

In general, even after the optimization of extraction parameters, a lower performance regarding chlorophyll extraction from dry biomass when compared with fresh biomass is evident and is in agreement with data already reported in literature for carotenoids (20,21). As mentioned before, this can be due to the structural changes in membranes, hindering the extraction of chlorophyll, but also due to the photosystem degradation that many times is irreversible even after rehydration, making this biomass less useful for photosynthetic pigments extraction (22).

3.2. Comparison of fresh algae from different geographic locations

Fresh wild-harvested *Ulva* sp. from the north of France and farm-raised *Ulva rigida* from Portugal were compared (Fig. 3). From the results obtained, it seems that the chlorophyll content in the wild-harvested algae is higher than the farm-raised algae, by *circa* 2 $mg_{chl}.g_{dry\ algae}^{-1}$. As already discussed by Powley and collaborators (23), these differences may be attributed to the different habitat conditions, mainly in terms of light intensity, but also temperature and nutrients supply at the time of harvest (e.g. phosphorus (P) and nitrogen (N)) on both locations that will interfere with biomass composition, namely the chlorophyll production (24–26). Moreover, in the case of farm-raised *Ulva*, we are sure of dealing with only one species (*Ulva rigida*), while in wild-harvested biomass it is possible to have a mixture of different *Ulva* species as well as a small percentage of other contaminant species.

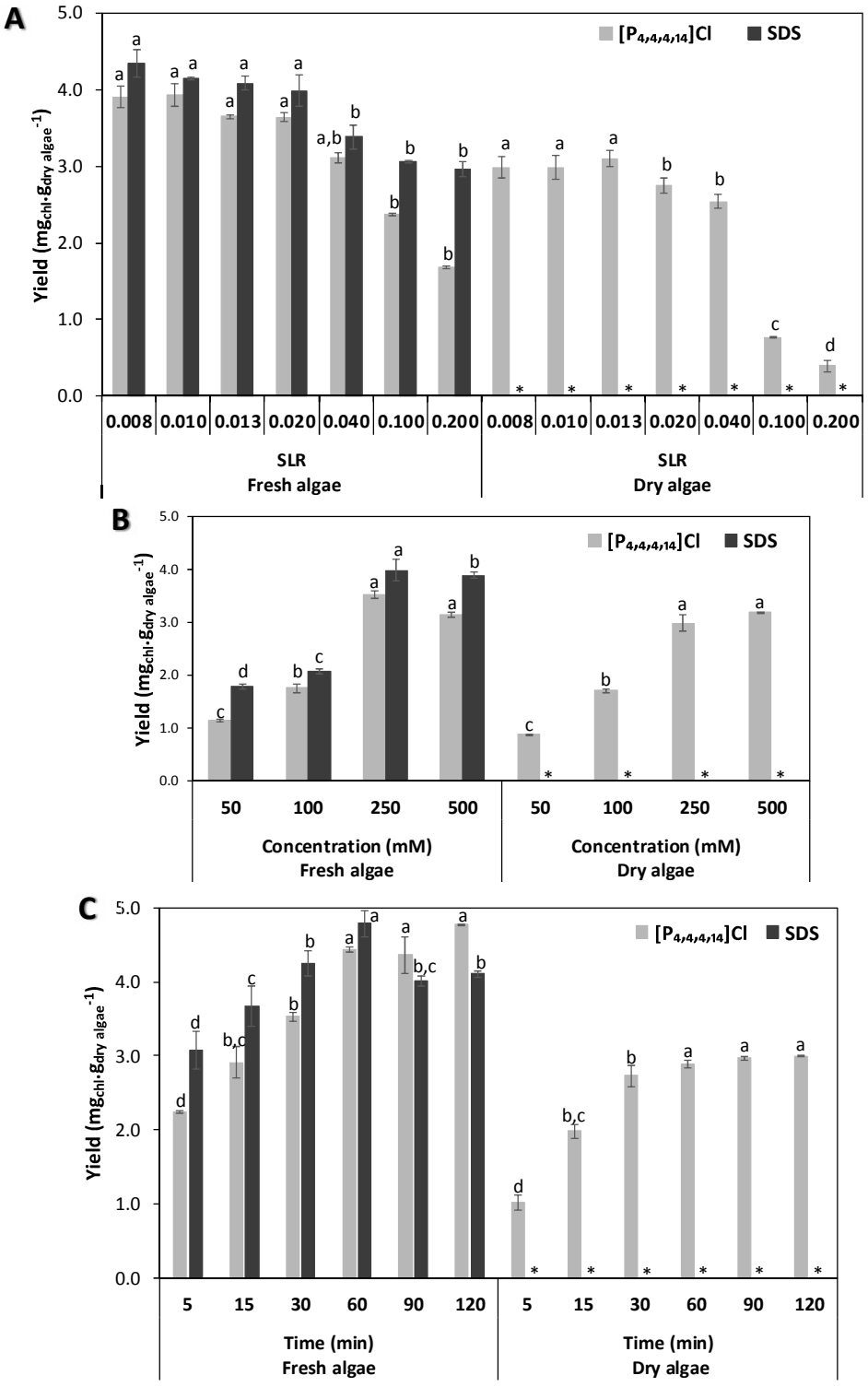

Fig. 2. Yield of extraction of chlorophyll using fresh and dry farm-raised *Ulva* sp. regarding the effect of operational conditions: (A) SLR, (B) solvent concentration, and (C) time of extraction. *SDS was not considered for dry algae. Equal letters in the same column represent statistically equivalent values.

Despite this difference, the same trends were identified for the different alternative solvents and operational conditions under study (Fig. 3). As previously seen for the fresh farm-raised algae, the $[P_{4,4,4,14}]Cl$ and the SDS stand out as the best solvents in the wild-harvested algae. The SLR study revealed a different maximum, being 0.01 and 0.013 $g_{biomass}.mL_{solvent}^{-1}$ for $[P_{4,4,4,14}]Cl$ and the SDS, respectively (Fig. 3B), which may be related with the different chlorophyll contents found in the two samples. The optimum solvent concentration of the alternative solvent in water using fresh and dry algae is still the same, 250 mM. Finally, a decrease in the time of extraction was observed for the wild-harvested algae to 30 minutes for $[P_{4,4,4,14}]Cl$, in comparison with the 60 minutes obtained for the SDS and as well as for both solvents when the farm-raised algae is used.

3.3. Chlorophyll stability over time

Given that small differences in terms of yield of extraction were seen using both aqueous solutions of $[P_{4,4,4,14}]Cl$ and SDS, the chlorophyll stability was studied in both solvents. In this case, the stability of chlorophyll extracted with ethanol (standard solvent), and aqueous solutions of both $[P_{4,4,4,14}]Cl$ and SDS, was studied for 33 days, at 25ºC and 4ºC and in the absence of light. The results are displayed in terms of chlorophyll content loss being the chlorophyll content periodically measured (Fig. 4).

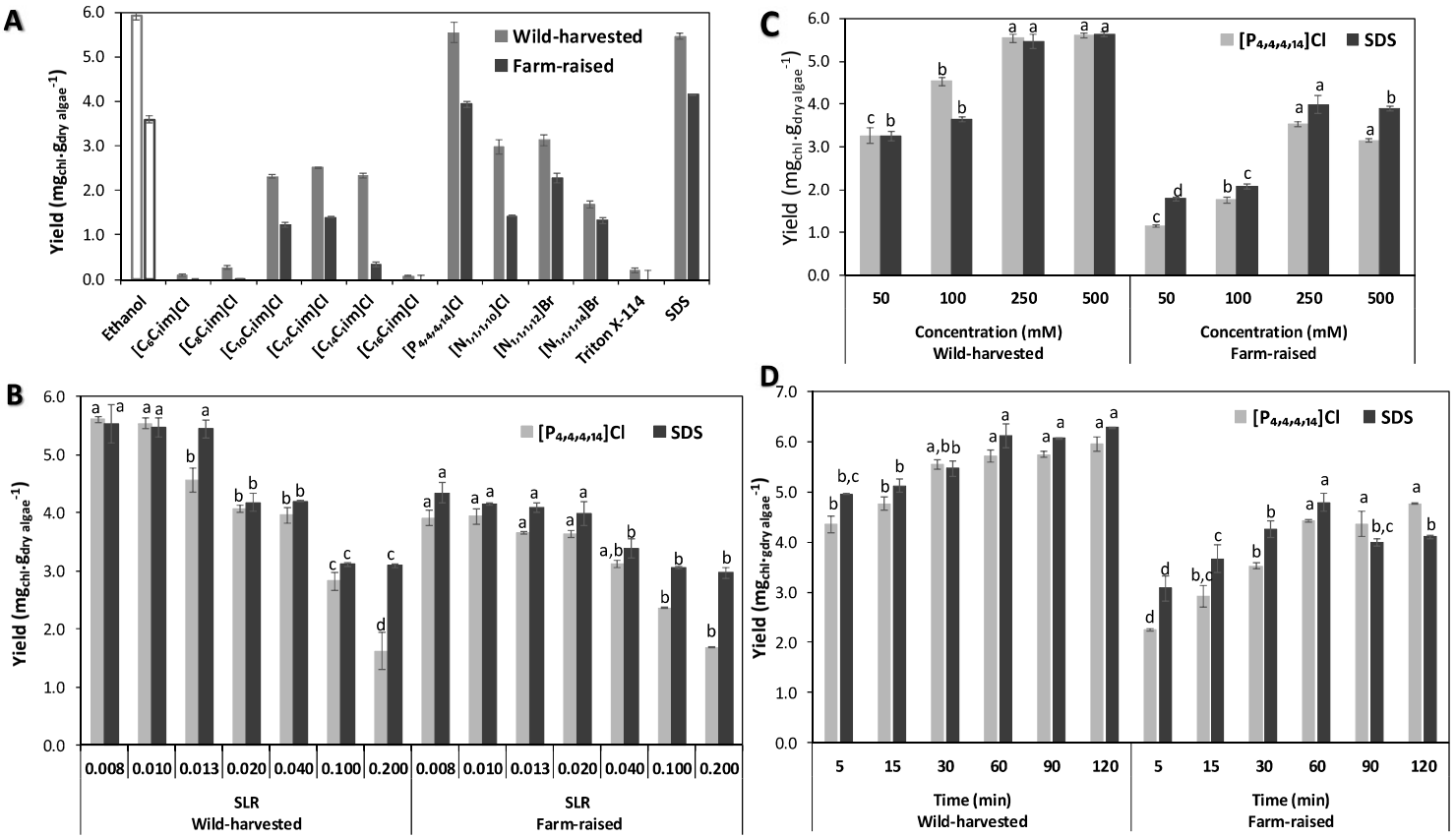

Fig. 3. Yield of extraction of chlorophyll using fresh wild-harvested and farm-raised *Ulva* sp. regarding the (A) screening of alternative aqueous solvents; and the effect of operational conditions such as the (B) SLR, (C) solvent concentration of alternative solvents in water, and (D) time of extraction. Equal letters in the same column represent statistically equivalent values. Results obtained for the farm-raised biomass are also here displayed to facilitate the comparison.

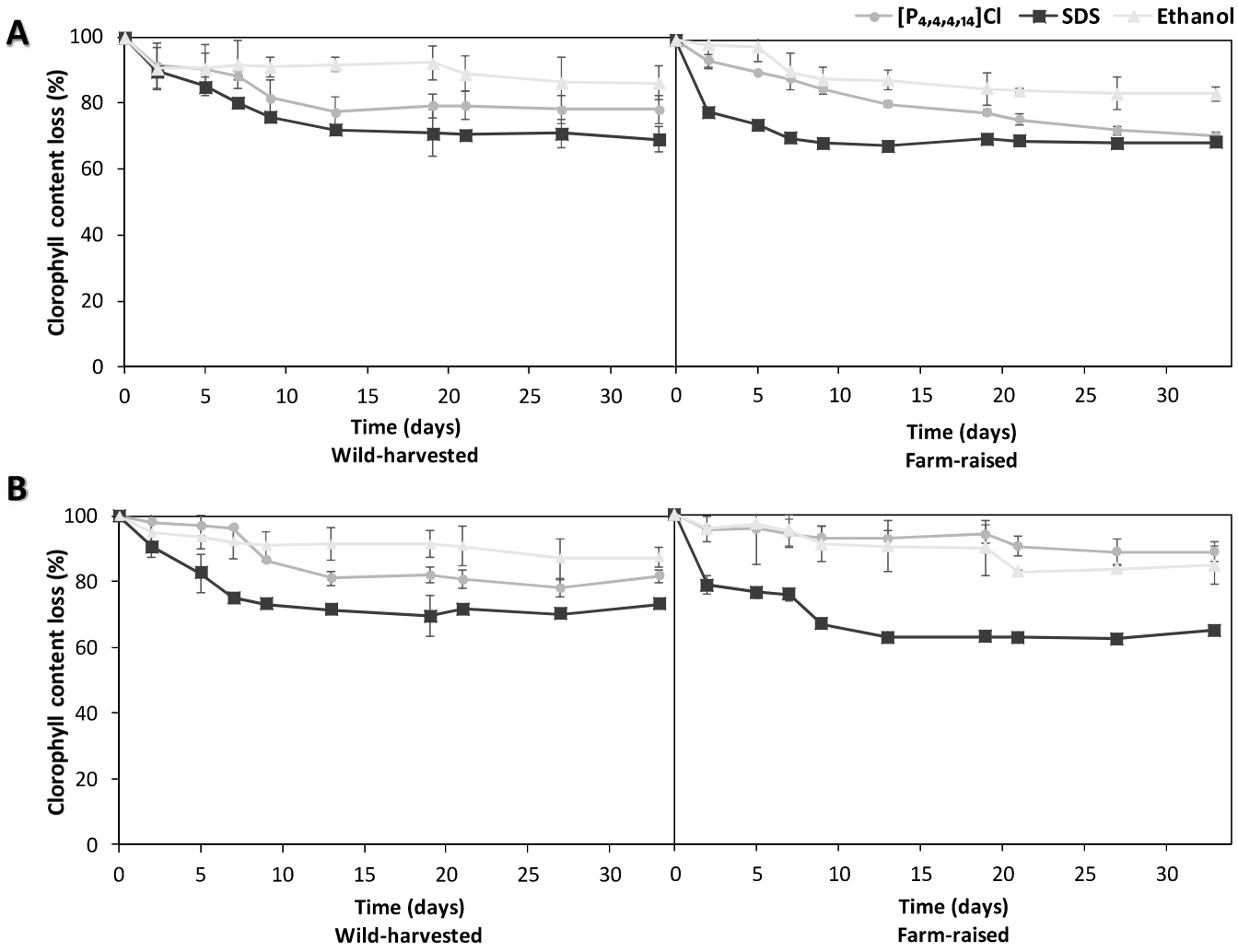

Fig. 4. Chlorophyll stability represented by the chlorophyll content loss in the extract over time and in the dark, at (A) 25°C and (B) 4°C.

Despite the conclusions previously reported for the effect of temperature (27), in this case the results are not so different. In all cases, the stability seems to be affected by the solvent. In general, the aqueous solutions of SDS provide the lowest stability, with losses in the chlorophyll content up to 40%, which may justify the lower contents of chlorophyll described during the optimization of the extraction process. On the other hand, the ethanol seems to have a slightly better performance maintaining the stability of the chlorophylls over time, which may be contradicted by the maintenance of the pigments at low temperature (4ºC), for which the results representing the IL as solvent are better (case of farm-raised) or similar (wild-harvested) when compared with the traditional solvent.

3.4 Economic evaluation

In addition to the yields of extraction and stability of the products, to define the most efficient downstream process and industrially more appropriate, an economic evaluation is required. In this work, the processes with the best results in terms of yield of extraction and chlorophyll stability were selected (i.e. those based in ethanol and $[P_{4,4,4,14}]Cl$). Results for both analyses are summarized in Fig. 6. The deterministic analysis comprises the calculation of the production costs using the optimum conditions previously determined during the optimization step. For both, it was at 30 min, SLR of 0.01 $g_{biomass}mL_{solvent}^{-1}$, with 250 mM for $[P_{4,4,4,14}]Cl$ and ethanol 100%. In general, the results suggest that the $[P_{4,4,4,14}]Cl$ has a lower production cost when applied on the extraction step (1.7 times lower) (Fig. 6A). This makes the use of $[P_{4,4,4,14}]Cl$ as a more attractive approach. Indeed, despite the higher cost of the Il when compared with the ethanol, in the alternative process using IL much less material is used, which decrease

the cost of the alternative downstream process. This specifically contradicts the general assumptions normally found in literature, and shows that the cost of the IL is not the only condition to be considered in the analysis of a process but also the amount of solvent employed, the operational conditions, the yields of extraction and the stability of the products obtained.

After selecting the most cost-efficient and sustainable process, the one based in $[P_{4,4,4,14}]Cl$, a sensitivity analysis (Fig. 6B) was performed. As previously indicated, a sensitivity analysis details the impact that changes in the process parameters have on the production costs. This analysis is done by the representation of different scenarios for the conditions selected as most important for each process. In this work, it was studied the effect of variations in the materials costs (50, 100 and 150%) and concentration of $[P_{4,4,4,14}]Cl$ (100, 250 and 500 mM). Considering the use of 250 mM and 100% of materials costs as the base scenario, the results indicate that the largest impact is provided by the $[P_{4,4,4,14}]Cl$ concentration employed [which is typically observed for other liquid-liquid or solid-liquid extractions (28)], closely followed by the cost variation of the IL. A critical aspect of the concentration effect is that as it changes, the yield of chlorophyll obtained *per* mass unit of biomass is also affected (Fig. 3C). This means that the solvent concentration has a combined effect from a change in the amount of IL being used and the amount of product generated as a result of the extraction efficiency. From these results, it can also be concluded that the use of less IL (100 mM), even with a reduced extraction yield, will assure lower production costs.

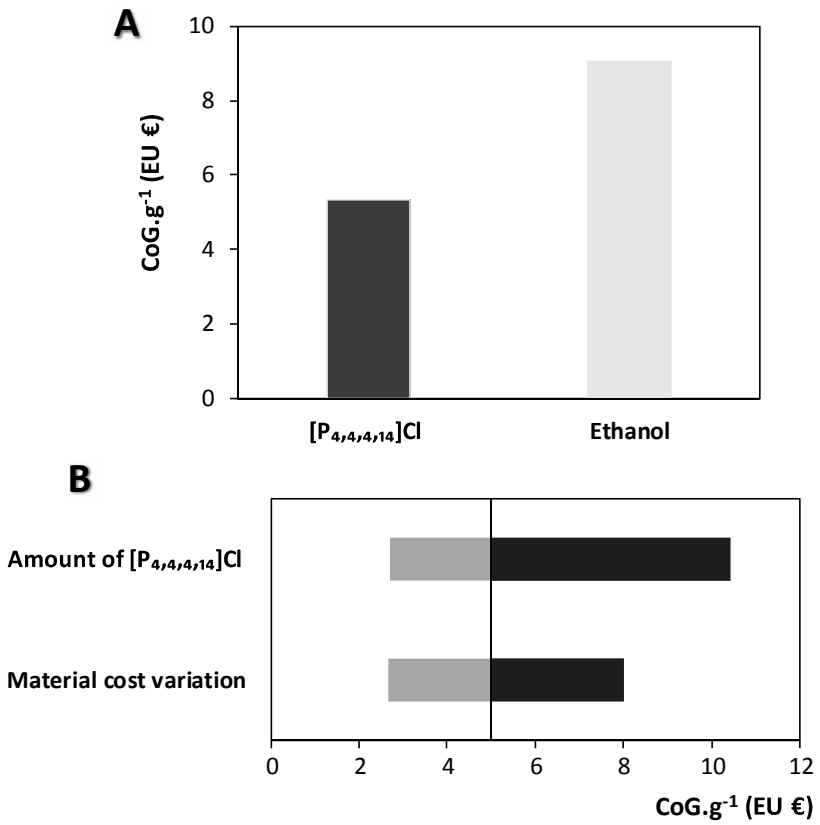

Fig. 6. Economic evaluation considering (A) the comparison of the extractions performed with pure ethanol and the aqueous solution of $[P_{4,4,4,14}]Cl$ for the wild-harvested algae, and (B) the amount of IL and material cost variation in the economic impact on the alternative process suggested in this work.

Considering its high extraction performance, good chlorophyll stability and lower cost of the IL-based process when compared with the ethanol-based process, the final process was defined. Industrially, a complete downstream process should be considered, including the optimized solid-liquid extraction of chlorophyll from *Ulva* sp., a recovery of chlorophyll from the aqueous solution of $[P_{4,4,4,14}]Cl$, and lastly the recycling of solvents. As an alternative, and considering the literature background, a back-extraction may be optimized and strategically applied on the recovery of chlorophyll from the aqueous solution of $[P_{4,4,4,14}]Cl$, allowing at the same time, the

aqueous solution of $[P_{4,4,4,14}]Cl$, now free of chlorophyll, to be reused in the solid-liquid extraction step (18).

4. Conclusion

In this work, aqueous solutions of tensioactive ILs and common surfactants were used, and compared with ethanol as a conventional solvent, to extract chlorophyll from different batches of *Ulva* sp. Operational conditions of extraction, such as SLR, solvent concentration in water, and time of extraction were also considered. Although the differences found between the dry and fresh samples from the same location and the wild-harvested and farm-raised *Ulva* sp. biomass on the chlorophyll content, the process of extraction optimization was successfully applied independently of the type of biomass. The best operational conditions were fixed at 250 mM of $[P_{4,4,4,14}]Cl$ in aqueous solution, for 30 minutes with a SLR of 0.01 $g_{biomass}.mL_{solvent}^{-1}$ for the fresh wild-harvested algae from the north of France, being a maximum yield of extraction of 5.96 $mg_{chl}.g_{dry\ algae}^{-1}$ obtained. In the end, the IL-based extraction process has proved to be the most efficient and less expensive (conclusion obtained after the economic analysis), while maintaining the stability of the final product for more than one month.

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