R-phycoerythrin extraction and purification from fresh Gracilaria sp. using thermo-responsive systems†

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R-phycoerythrin is a high added-value protein found in red macroalgae with several interesting properties. Despite the promising results found when R-phycoerythrin is used as an optically active center in luminescent solar concentrators (LSCs), it still has some problems that can be attributed to the low stability of the R-phycoerythrin in the presence of the specific contaminant proteins found in the crude extract. The development of downstream strategies able to reduce the use of environmentally hazardous solvents, while improving the purification without compromising the R-phycoerythrin structural integrity is still the biggest challenge to overcome. Aqueous micellar two-phase systems (AMTPS) appear as an appealing fractionation approach since they allow the processing of systems with larger water contents, while displaying great selectivity and biocompatibility with several biomolecules. Moreover, AMTPS that mix surfactants and surface-active ionic liquids are shown to significantly enhance protein purification. In this work, mixed AMTPS were applied to the R-phycoerythrin purification from red macroalgae. After the process optimization, this work proposes the application of two consecutive steps of purification as the final process to isolate R-phycoerythrin from the remaining proteins composing the crude extract, while maintaining the structural integrity of R-phycoerythrin, as requested to be used in the LSC. Besides a good performance, the two-step approach developed was also shown to have a lower environmental impact with a carbon footprint decrease of 16%, when compared with the conventional AMTPS.

Introduction

Red macroalgae (Rhodophyta) are marine natural raw materials rich in chemicals of economic/industrial interest. The algal coloration comes from the presence of three specific proteins, namely R-phycoerythrin, R-phycocyanin and allophycocyanin. Phycobiliproteins act as photosynthetic pigments in Rhodophyta, with good stability from pH 4 to 10 and in temperatures up to 40 °C. These bioactive fluorescent compounds display a wide range of applications as probes, colorants, and nutraceuticals, due to their properties and biological activities, which explains their high commercial value. Nowadays, their use addresses mainly the food, pharmaceutical, biomedical, and cosmetic fields.

Recent works are addressing the development of innovative applications for R-phycoerythrin, e.g., in fluorescent-based detection systems and as optically active centers in luminescent solar concentrators (LSCs). To meet the different purity demands (cosmetic, energy, pharmaceutical, just to mention a few), the development of a sustainable purification process to obtain R-phycoerythrin of enough purity, without compromising its structural integrity and chromophores, is of utmost importance.

The most common industrial methodologies describe the R-phycoerythrin purification by water leaching, staged precipitation with ammonium sulphate and ionic exchange chromatography (Patent CN1587275A), or by ammonium sulphate precipitation and ionic exchange chromatography (Patents CN1271085C and CN101240009A). However, there is a worldwide demand to develop more cost-effective downstream processes for the most efficient purification of these fluorescent proteins from the aqueous crude extract.
Liquid–liquid extraction techniques appear as an attractive alternative, mainly those based in aqueous two-phase systems (ATPS). ATPS consist of the formation of two immiscible aqueous-rich phases formed by the mixture of two distinct phase formers, namely polymers, salts and/or ionic liquids (ILs). These are known for their high water content, sustainable nature and easy scale-up. In this sense, ATPS have been used to purify distinct compounds, from proteins and antibiotics to antibodies and dyes. However, in spite of the good results reported, their use on the purification of some proteins still lacks the possibility to reach the multi-product scenario envisaged under the scope of biorefineries.

Aqueous micellar two-phase systems (AMTPS) are a specific type of ATPS, being explored in the past few years. The poor interaction between the solvents of the AMTPS and proteins, in general was the criteria behind their selection in this work. These systems, composed essentially of water and a surfactant, have the particularity of being thermo-responsive systems, i.e., it is the temperature change that induces the phase separation. For systems with a lower critical solution temperature (LCST) e.g. non-ionic surfactants, when the temperature increases, the homogeneous system reaches the cloud point forming two aqueous phases. Indeed, the cloud point corresponds to the specific temperature at which the solution becomes turbid and the system coacervation occurs. The micelle coacervation induces a phase separation, creating two distinct environments: a hydrophobic surfactant-rich phase and a hydrophilic surfactant-poor phase. The determination of different cloud points as the surfactant concentration increases, allows the determination of the system binodal curve, which represents the boundary between the monophasic and biphasic regions. This means that, above the curve, the system displays two phases; below the curve the system is homogeneous. Although the development of systems with liquid–liquid immiscibility with an UCST or a LCST as thermo-responsive systems creating two phases after temperature increase is a ‘normal phenomenon’, this phase change can be used with advantage on separation processes. As previously shown by us and others, the cloud points can be modified by the addition of inorganic salts, ionic surfactants, and surface-active ionic liquids (SAILs). Several phase diagrams have recently been reported and characterized for a few nonionic surfactants belonging to the Tergitol family in the presence and absence of SAILs. Although being a ‘normal phenomenon,’ this does not make them less simple to obtain. If on the one hand, there isn’t an extensive database reporting tensioactive compounds presenting this behaviour in aqueous solution, on the other hand, there are still a limited number of compounds presenting critical points (UCST or LCST) included in appropriate temperature ranges usable for biomolecule purification. Thus, this makes the development of processes using AMTPS very challenging. Indeed, in addition to the SAIL influence on the cloud points, their incorporation into mixed micelles has also been proved to be helpful to enhance the AMTPS extractive performance and selectivity.

While improving the selectivity and yields, AMTPS also allow the reduction of solvents, minimizing or precluding the use of environmentally hazardous solvents, thus improving the processes for a successful biorefinery approach.

This work aims at the application of AMTPS using ILs as co-surfactants to promote the purification of both R-phycoerythrin and R-phycoerythrin from the red macroalga Gracilaria sp. It started by the optimization of the process variables, namely the surfactant concentration, the equilibration time, the concentration of the phycobiliprotein crude extract added to the AMTPS and pH. Moreover, the presence and absence of a pre-purification step using the ammonium sulphate precipitation was also evaluated. After selecting the highest performing AMTPS and the best process conditions to separate (i) fluorescent from non-fluorescent proteins and (ii) R-phycoerythrin from R-phycoerythrin, a complete process was investigated considering also the reuse of the solvents employed for the process. To evaluate the sustainability of this process, an environmental evaluation was carried out considering the carbon footprint as the final output. In this analysis two scenarios were evaluated, (i) the conventional AMTPS (i.e., without the SAIL addition as co-surfactant) and (ii) the mixed AMTPS (i.e., in the presence of the most efficient SAIL).

Results and discussion

Solid–liquid extraction

Characterization of phycobiliprotein crude extract. A solid–liquid extraction to remove the phycobiliproteins from the fresh biomass was firstly performed. Afterwards, the crude extract rich in phycobiliproteins that was obtained was characterized by a proteomic analysis. Through this analysis, the different proteins present in the phycobiliprotein crude extract and their relative abundance were identified, the results being depicted in Fig. 1 (and Table S3 of ESI†). A representative nanoHPLC chromatogram of the injection of the tryptic digest of a SDS-PAGE spot and a representative mass spectrum acquired during the run are also displayed in Fig. S1 of ESI†. The proteins present in the crude extract (Fig. 1) can be divided in two groups comprising (i) the fluorescent (phycobiliproteins) and (ii) the non-fluorescent proteins. As fluorescent proteins, Gracilaria sp. has in its composition R-phycoerythrin, which is the most abundant, and R-phycoerythrin and allophycocyanin. Regarding the non-fluorescent proteins, the crude extract contains mostly ribulose-1,5-bisphosphate carboxylase and the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit being the remaining proteins, detailed in Table S3 of ESI† and included in the fraction Others of Fig. 1.

Separation of phycobiliproteins and R-phycoerythrin. Taking into account the previous characterization of the crude extract, the presence of contaminants demands the development of a purification process, in which contaminants and fluorescent proteins are separated. In this work, thermo-responsive AMTPS were used. Proteins are thermolabile biomolecules, and as such, their purification must consider this limitation,
especially when thermo-responsive systems are used. The non-ionic surfactant Tergitol 15-S-7 was selected due to its low cloud point, ranging between 34 and 40 °C (cf. Fig. S2†), and which can be further controlled by the proper choice of a SAIL acting as a co-surfactant,20 being thus appropriate to be used in the purification of R-phycoerythrin, which is stable until 40 °C.3 Besides, these mixed AMTPS have shown enhanced selectivity when compared with the AMTPS without any SAIL.14

For the Tergitol 15-S-7/McIlvaine buffer-based AMTPS, the purification conditions were optimized considering the highest recovery of fluorescent and non-fluorescent proteins in the surfactant-poor and -rich phases, respectively. It represents the system with the highest selectivity of purification and lowest R-phycoerythrin contamination with R-phycocyanin (results shown in Fig. 2 and S3–S9 of ESI†). Herein, the McIlvaine buffer (pH 7.0) was used to assure the highest extraction of phycobiliproteins from the biomass, while maintaining R-phycoerythrin structural integrity.7 After the selection of the highest performing AMTPS, the surfactant concentration was the first parameter optimized (Fig. 2).

The bars of Fig. 2 represent the recovery of phycobiliproteins (pink bars) and total proteins (grey bars) in both phases while the line corresponds to the selectivity parameter. These results suggest that the phycobiliproteins and, particularly R-phycoerythrin, partitioned preferably towards the (most hydrophilic) surfactant-poor phase as shown by the recoveries (>60%) and the pink colour of the system’s bottom phase (Fig. 2), characteristic of the R-phycoerythrin presence. In contrast, the total proteins recovery in the surfactant-rich phase increases with the surfactant concentration, also contributing to enhance the selectivity. In addition, R-phycoerythrin contamination with R-phycocyanin also diminishes with surfactant concentration augment (cf. Fig. S3 of ESI†). Taking these data into account, 10 wt% of surfactant was selected as the optimum concentration. The extraction time was also evaluated, revealing that 3 h were enough to guarantee the phycobiliproteins’ complete partition between the two phases (Fig. S4†); however, the contaminant proteins need an extra
hour (4 h) to reach the partition equilibrium (Fig. S5†), thus justifying 4 hours as the time selected. After selecting the surfactant concentration and equilibration time, the effects of the extract concentration and system pH were tested. The results showed that, by increasing the concentration of the crude extract rich in phycobiliproteins (Fig. S6 and S7†), the system complexity is increased, resulting in a lower extraction of total proteins into the surfactant-rich phase. Thereby, it is preferable to use less extract if it means increasing the R-phycoerythrin purity in the lowest number of steps. Regarding the pH effect on the purification of the fluorescent proteins, it was evidenced that the system selectivity was the highest at neutral pH (Fig. S8 and S9†). Moreover, it was proved that an additional step of ammonium precipitation is not needed, since the results in its presence/absence were very similar (Fig. S8 and S9†).

Multi-product strategy development

A multi-product strategy requires the separation of the contaminant proteins previously identified in the crude extract. After optimizing the processual conditions considering AMTPS use, a complete purification process able to separate (i) the fluorescent and non-fluorescent proteins and to isolate (ii) R-phycoerythrin from R-phycocyanin was developed. For that purpose, thermo-responsive AMTPS based on Tergitol 15-S-7 with SAILs as co-surfactants were used, and the purification of phycobiliproteins, more precisely R-phycoerythrin, was evaluated by the analysis of two parameters, namely the system selectivity and R-phycoerythrin contamination index. A screening was performed using SAILs from distinct families, namely imidazolium, phosphonium, quaternary ammonium, pyridinium, cholinium and alkyl sulfonates, as presented in Fig. 3. These were selected not only because the mechanisms of formation of the two-phases were already understood, but also because their cloud points were in the appropriate range of temperatures regarding the phycobiliproteins’ thermostability.

The Recovery data for R-phycoerythrin (pink bars) and total proteins (grey bars) in both phases, as well as the Selectivity (table) were the parameters determined. The results suggest that, apart from the [C\textsubscript{14}mim]Cl-based AMTPS, all the mixed AMTPS maximized the R-phycoerythrin migration for the opposite phase of the contaminant proteins, thus increasing the system selectivity. By analysing the Selectivity results (Fig. 3II), it seems that only the mixed AMTPS composed of SDS showed a lower selectivity ($S = 2.51 ± 0.02$) than the system without SAILs, while the [P\textsubscript{4,4,4,14}]Cl-based AMTPS displayed a similar selectivity ($S = 3.1 ± 0.3$) to this non-additivated AMTPS ($S = 3.28 ± 0.08$). The remaining mixed AMTPS improved the R-phycoerythrin purification as follows: SDS $< \text{[P\textsubscript{4,4,4,14}]Cl} \approx$ without SAIL $< \text{[C\textsubscript{14}mim]Cl} < \text{[C\textsubscript{16}mim]Cl} < \text{[C\textsubscript{14}im-6-C\textsubscript{14}im]Br\textsubscript{2}} < \text{[Ch\textsubscript{[Tetradec]}} \approx \text{SDBS} < \text{[N\textsubscript{1,1,12,8,C\textsubscript{7}H\textsubscript{7}]}Br} \approx \text{[C\textsubscript{16}py]Br}$. This trend suggests that the R-phycoerythrin purification is independent of the IL family under study since very distinct results were observed within the quaternary ammonium family, for instance. Here, [Ch][Tetradec] and [N\textsubscript{1,1,12,8,C\textsubscript{7}H\textsubscript{7}]}Br showed outstanding results compared with the average results attained with [Ch][Tetradec]. Therefore, the different characteristics and properties of the mixed micelles being formed, and/or the establishment of more specific interactions should dictate the protein partitioning. Regarding the R-phycoerythrin contamination with R-phycocyanin (Fig. 4), there is only one system that does not display any contamination, the AMTPS based in [N\textsubscript{1,1,12,8,C\textsubscript{7}H\textsubscript{7}]}Br. The best results displayed by the latest system may be justified by the different sizes of both phycobiliproteins or even by some specific interactions taking place between the micelles formed and the contaminant fluorescent protein. Indeed, the contamination with R-phycocyanin follows a different trend than the one obtained

![Fig. 3](image-url) Extractive performance of distinct AMTPS in the absence and presence of SAILs using the mixture point composed of 10 wt% of Tergitol 15-S-7 + 0 or 0.3 wt% of SAIL + 10 wt% of phycobiliprotein extract + 80 or 79.7 wt% of McIlvaine buffer at pH 7.0: (i) and (ii), R-phycoerythrin Recovery (%) in the surfactant-poor and surfactant-rich phases, respectively; (iii) and (iv), total proteins Recovery (%) in the surfactant-poor and surfactant-rich phases, respectively. Table in inset (ii) shows the values of Selectivity obtained for each AMTPS under study.
for selectivity: $[N_{1,1,12,(C7H7)}]Br < [C14im]Cl < [Ch][Dec] \approx [C16py]Br \approx SDS < without SAIL < SDBS < [C14im-6-C14im]Br_2 < [P_{4,4,4,14}]Cl < [Ch][Tetradec]$. In these systems, the purification is normally dependent upon the hydrophobicity/hydrophilicity of the biomolecules and the micelle size.\(^{21}\)

It would be thus assumed that the systems displaying micelles with larger diameters may induce a higher purification since they extract a higher amount of contaminants. However, it is not the case here. As we recently showed,\(^{20}\) SDBS and [Ch][Tetradec] are, from the SAILs studied in this work, the ones inducing the formation of the largest micelles (between 40 and 50 nm) when compared with the 7–8 nm of the remaining mixed AMTPS and the \(\sim 14\) nm of the micelles obtained for the non-additivated AMTPS. The results here obtained show a higher purification performance for the AMTPS with lower micelle diameter, again suggesting the strong influence of interactions between the proteins and the micelles, rather than the micelles size.

### Complete process using sequential AMTPS steps

For some applications of R-phycoerythrin, a high purity degree is required. To achieve higher purity levels, the use of sequential steps of purification was evaluated. To optimize this process, the Recovery of R-phycoerythrin and total proteins, the Selectivity, and the R-phycocyanin Contamination Index were determined (Table 1). The most promising systems selected from the SAILs screening were tested, namely the mixed AMTPS composed of $[C16py]Br$, [Ch][Dec], SDBS and $[N_{1,1,12,(C7H7)}]Br$. The non-additivated AMTPS was used once again as reference. It should be noticed that due to its high selectivity and ability to isolate R-phycoerythrin from R-phycocyanin, $[N_{1,1,12,(C7H7)}]Br$ was always used as co-surfactant in the second purification step.

<table>
<thead>
<tr>
<th>Cycle of purification</th>
<th>System</th>
<th>R-phycoerythrin</th>
<th>Total proteins</th>
<th>Selectivity</th>
<th>R-phycocyanin contamination index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st step</td>
<td>Without SAIL</td>
<td>17.4 ± 0.4</td>
<td>55.0 ± 0.8</td>
<td>5.9 ± 0.3</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>2nd step</td>
<td>$[N_{1,1,12,(C7H7)}]Br$</td>
<td>15.3 ± 0.9</td>
<td>49 ± 1</td>
<td>5.86 ± 0.04</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>1st step</td>
<td>$[C16py]Br$</td>
<td>15.4 ± 0.9</td>
<td>58.6 ± 0.5</td>
<td>5.4 ± 0.3</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>2nd step</td>
<td>$[N_{1,1,12,(C7H7)}]Br$</td>
<td>15.4 ± 0.9</td>
<td>59 ± 1</td>
<td>5.4 ± 0.3</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>1st step</td>
<td>[Ch][Dec]</td>
<td>15.4 ± 0.9</td>
<td>59 ± 1</td>
<td>6.1 ± 0.3</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>2nd step</td>
<td>SDBS</td>
<td>15.4 ± 0.9</td>
<td>59 ± 1</td>
<td>7.1 ± 0.6</td>
<td>0.147 ± 0.004</td>
</tr>
<tr>
<td>1st step</td>
<td>$[N_{1,1,12,(C7H7)}]Br$</td>
<td>22 ± 1</td>
<td>64 ± 6</td>
<td>7 ± 1</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>2nd step</td>
<td>$[N_{1,1,12,(C7H7)}]Br$</td>
<td>25 ± 3</td>
<td>71.4 ± 0.9</td>
<td>8.00 ± 0.02</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>1st step</td>
<td>$[N_{1,1,12,(C7H7)}]Br$</td>
<td>25.2 ± 0.8</td>
<td>82.1 ± 0.5</td>
<td>13.60 ± 0.09</td>
<td>0.047 ± 0.004</td>
</tr>
</tbody>
</table>
The goal behind the development of this process is to assess and optimize a two-step approach to firstly separate phycobiliproteins from the non-fluorescent proteins, and a second step used to separate R-phycoerythrin from R-phycocyanin. Table 1 shows very similar results regarding the Recovery of R-phycoerythrin towards the surfactant-poor phase in both steps. On the other hand, the Recovery of total proteins on the surfactant-poor phase tends to decrease in all systems with the second step: [C16py]Br-based AMTPS being the exception. In this system, the replacement of the SAIL in the second step seems to induce a higher partition of both fluorescent and non-fluorescent proteins towards the bottom phase, thus reducing the process purification performance. In the end, a purification trend of these systems can be established as follows: [C16py]Br/[N1,1,12,(C7H7)]Br < without SAIL/[N1,1,12,(C7H7)]Br ≈ [Ch][Dec]/[N1,1,12,(C7H7)]Br < SDBS/[N1,1,12,(C7H7)]Br < [N1,1,12,(C7H7)]Br/[N1,1,12,(C7H7)]Br. These results clearly show an enhanced performance of the mixed systems compared to the traditional one (without any SAIL). Moreover, the proper manipulation of the AMTPS is also driving the Selectivity of the systems, which was more than doubled (maximum $S = 13.6 \pm 0.1$) when compared with the traditional AMTPS ($S = 5.86 \pm 0.04$). Concerning the Contamination Index data (Table 1), it is evidenced that the presence of [N1,1,12,(C7H7)]Br considerably reduces the contamination of R-phycoerythrin with R-phycocyanin. Unexpectedly, the first step of the [N1,1,12,(C7H7)]Br-based AMTPS presented some contamination with R-phycocyanin and lower Selectivity, which is thus overcome in the second step of the process. This is probably a result of algal biochemical heterogeneity, promoted by changes in the weather and daylight length and intensity during cultivation. The biochemical heterogeneity of the cells results in higher amounts of R-phycocyanin produced by the macroalgae and consequently being extracted from the cells together with phycobiliproteins, thus influencing the process performance.

Summing up, a two-step process using sequential AMTPS steps was developed and efficiently implemented as an effective downstream process to isolate R-phycoerythrin, R-phycocyanin and non-fluorescent proteins, under a multi-product strategy. However, and foreseeing its industrial implementation, the recycling and reuse of the main solvents are essential steps to implement.

The complete process proposed is shown in Fig. 5, in which three main steps were included. The process starts with the (conventional solid–liquid) extraction of phycobiliproteins from the biomass using water. Then, the aqueous crude extract rich in phycobiliproteins proceeds to the purification step, in which two mixed AMTPS were sequentially applied. When the first AMTPS step was applied, it was possible to recover around 77%....
of R-phycoerythrin into the surfactant-poor phase and around 71% of total proteins, including most of contaminant non-fluorescent proteins, into the opposite phase. The surfactant-poor phase was then applied in the second AMTPS step, where ~79% of the R-phycocyanin was removed from the R-phycoerythrin. In addition, from the remaining ~29% of total proteins remaining in the surfactant-poor phase of the first step, it was possible to remove ~82% of contaminants, improving the R-phycoerythrin purification even more. After the separation of the different proteins, an isolation step was experimentally tested to separate the surfactant-rich phase from protein, this step envisioning the reuse of the major part of the surfactant. Cold acetone was added to the surfactant-rich phase, containing most of the system phase formers (surfactant and SAIL), to precipitate the contaminant proteins. To prove the efficiency of the precipitation with cold acetone in isolating the proteins and recycling the phase formers, both resuspended pellet and supernatant were analysed by FTIR. The resuspended pellet contains the precipitated proteins, whereas the acetone supernatant contains the system phase formers. The results indicate that all the tensioactive compounds were completely removed from the contaminant proteins (cf. Fig. S10 of ESI†). Afterwards, an evaporation can be introduced to separate acetone from the surfactant, allowing their consequent reuse. Meanwhile, the use of ultrafiltration21–25 is pointed out as a strategy to separate the surfactant-poor phase from R-phycoerythrin. In this work, this step was only envisaged theoretically, since this task will be only needed for some specific final applications of R-phycoerythrin, a scenario not further explored in this work.

**Structural integrity of R-phycoerythrin after purification**

The stability and structural integrity of R-phycoerythrin were analysed. Circular dichroism (CD) is one of the techniques most used to infer the structural integrity of proteins.

Through the CD evaluation, the secondary structure of proteins is analysed, in what concerns their content in α-helix, β-sheet, β-turn and random coils. This is possible by analysing the spectrum in the range of 240 nm and below, to identify the peptide bonds.26 The macroalgal crude extract was analysed and compared to the spectra of the most selective AMTPS ([N1,1,12,(C7H7)]Br) and the conventional AMTPS, after both steps of purification, to conclude about the stability of R-phycoerythrin. This was possible through the direct comparison between our data and the R-phycoerythrin analysis found in the literature,2 where it was shown that the secondary structure of pure R-phycoerythrin presents more than 70% α-helix. In this context, all samples were diluted until a good spectrum was obtained, and the spectra were normalized (Fig. 6). This data shows that the crude extract is mostly composed of α-helix due to the negative bands found at 210 and 220 nm and the positive band below 200 nm, just like pure R-phycoerythrin. The spectra show that, during the complete purification process, the proteins can maintain their integrity, since these bands are retained in all AMTPS spectra after both purification steps.

![Circular dichroism analysis of R-phycoerythrin stability after two consecutive steps of purification from macroalga crude extract](image)

**Fig. 6** Circular dichroism analysis of R-phycoerythrin stability after two consecutive steps of purification from macroalga crude extract: –, crude extract; –, without SAIL (1st step); –, without SAIL/[N1,1,12,(C7H7)]Br. –, [N1,1,12,(C7H7)]Br (1st step); –, [N1,1,12,(C7H7)]Br/[N1,1,12,(C7H7)]Br.

![Carbon footprint for the two scenarios considered](image)

**Fig. 7** Carbon footprint for the two scenarios considered: (i) conventional AMTPS in the first liquid–liquid extraction and with the addition of [N1,1,12,(C7H7)]Br in the second liquid–liquid extraction, (ii) AMTPS with [N1,1,12,(C7H7)]Br in both extractions. Results are expressed in kg CO2eq. mg−1 R-phycoerythrin purified: , algae preparation, , solid–liquid extraction, , 1st liquid–liquid extraction, and , 2nd liquid–liquid extraction.
To evaluate the sustainability of the process here proposed, which included the steps of extraction of R-phycoerythrin, followed by the separation of R-phycoerythrin, R-phycocyanin and non-fluorescent proteins, an environmental evaluation was carried considering the carbon footprint as the main output. Both scenarios proposed, namely the application of a conventional AMTPS and the best mixed AMTPS (using $[\text{N}_{1,1,12}(\text{C}_{7}\text{H}_{7})]\text{Br}$ as co-surfactant) for R-phycoerythrin purification were addressed, the main results being depicted in Fig. 7. The carbon footprint is 81.30 kg CO$_2$e q.m $g^{-1}$ R-phycoerythrin when the conventional AMTPS (1st step) + mixed AMTPS (2nd step) is used and the GHG emissions are decreased by 16% when the mixed AMTPS is used in both steps (carbon footprint = 68.14 kg CO$_2$ eq. $g^{-1}$ R-phycoerythrin). The steps of polishing and reuse of phase formers were not taken into account in this analysis and, therefore, the carbon footprint of the production of acetone and energy consumed in these steps was not considered. Besides, possible emissions of acetone to air would not be accounted for in the carbon footprint, since acetone is not a greenhouse gas.

Briefly, the main contribution to the carbon footprint in both scenarios comes mainly from the energy consumption (first and second AMTPS steps, 46% and 45%, respectively, of the total). In fact, the energy consumption to promote the phase separation in these two stages represents almost 90% of the total carbon footprint. A comparison of the results achieved in this work is not possible, considering not only the lack of published data on the carbon footprint evaluation but also due to the lack of similar processes. Summing up, if on the one hand, the extraction efficiency of the process increases, on the other hand, the GHG emissions associated with the application of mixed AMTPS to purify R-phycoerythrin are lowered.

Conclusions

R-phycoerythrin purification was successfully developed in this work using thermo-responsive systems, namely mixed AMTPS. The mixed AMTPS have proved to be more selective than the traditional AMTPS (without co-surfactants), the best results being achieved with two consecutive steps of the $[\text{N}_{1,1,12}(\text{C}_{7}\text{H}_{7})]\text{Br}$-based AMTPS. This system was not only able to isolate the R-phycoerythrin from the contaminant proteins composing the initial crude aqueous extract, under a multiproduct strategy, but it was also able to maintain the R-phycoerythrin structural integrity. In addition, the process developed also included the recycle and reuse of the surfactant through an ultrafiltration process, which could be easily implemented at an industrial level. Aiming to evaluate the sustainability of the process, an environmental analysis was developed. As the final output, the carbon footprint of the complete process was evaluated (per mass of R-phycoerythrin) and found to decrease 16% when the mixed AMTPS was applied instead of using the conventional AMTPS.

Experimental

Materials

The non-ionic surfactant Tergitol 15-S-7 (purity ≥99%) was acquired from Sigma-Aldrich as well as hexadecylpyridinium bromide – $[\text{C}_{16}\text{py}]\text{Br}$ (purity = 97.0%), benzylidododecyldimethylammonium bromide – $[\text{N}_{1,1,12}(\text{C}_{7}\text{H}_{7})]\text{Br}$ (purity >99%) and sodium dodecylbenzenesulfonate, SDBS (technical grade). The imidazolium-based SAILs, 1-tetradecyl-3-methylimidazolium chloride, $[\text{C}_{14}\text{mim}]\text{Cl}$ (purity >98%) and 1-hexadecyl-3-methyl-
imidazolium chloride \([C_{16}\text{mim}]\)Cl (purity >98%), were purchased from Iolitec (Ionic Liquid Technologies, Heilbronn, Germany), while 3-(1-tetraethyl-3-hexylimidazolium)-1-tetradecyldimidazolium dibromide \([C_{14}\text{im}-6\cdot C_{14}\text{im}]\)Br2 was synthesized in-house using well-established procedures.\(^27\) The same protocol was used to synthesize cholinium decanoate, \([\text{Ch}\]Dec\), and cholinium tetradecanoate, \([\text{Ch}\]Tetradec\). The phosphonium-based SAIL tributyldodecylphosphonium chloride, \([P_{4,4,4,14}]\)Cl (purity = 97.1%), was kindly offered by Cytec. Sodium dodecylsulphate, SDS (purity = 99%), was supplied by Acros Organics. These structures are presented in Ref. 8. McIlvaine buffer (0.18 M) constituted by citric acid monohydrate \(\mathrm{C}_6\mathrm{H}_8\mathrm{O}_7\cdot\mathrm{H}_2\mathrm{O}\) (purity ≥99%) was used in all systems, and sodium phosphate dibasic heptahydrate \(\mathrm{Na}_2\mathrm{HPO}_4\cdot7\mathrm{H}_2\mathrm{O}\) (purity ≥99%), both acquired from Panreac AppliChem.

Methods

**Solid-liquid extraction.** Fresh *Gracilaria* sp. was cultivated by ALGPlus Ltda, a Portuguese company specializing in the Integrated Multi-Trophic Aquaculture (IMTA) production of marine macroalgae, located in Ilhavo, Portugal. Macroalgae samples were collected from aquaculture between April and November of 2016, and in January of 2017. After collecting the macroalgae, the samples were cleaned and washed with fresh and distilled water at least 3 times, weighed and stored in a freezer at −20 °C pending further use.

Algae samples were previously ground while frozen with liquid nitrogen, homogenized in distilled water (with a solid–liquid ratio of 0.7) at room temperature and placed in an incubator shaker (IKA KS 4000 ic control) for 20 min, at 250 rpm and room temperature. During the solid–liquid extraction step, all samples were protected from light exposure due to the high light sensitivity of phycobiliproteins. Then, the solution was filtered and, subsequently, the filtrate that originated was centrifuged in a Thermo Scientific Heraeus Megafuge 16 R Centrifuge at 3500 rpm for 30 min, at 4 °C. The resultant pellet was discarded, and the phycobiliprotein-rich supernatant was collected (phycobiliprotein crude extract) for further purification studies.

**SDS-PAGE procedure.** The phycobiliprotein crude extract was analysed through an electrophoresis that was prepared on polyacrylamide gels (stacking: 4% and resolving: 20%) with a running buffer consisting of 250 mM of Tris HCl, 1.92 M of glycine, and 1% of SDS. The proteins were stained with the usual staining procedure [Coomassie Brilliant Blue G-250 0.1% (v/v), methanol 50% (v/v), acetic 7% (v/v), and water 42.9% (v/v)] in an orbital shaker, at moderate speed, for 2–3 hours at room temperature. The gels were destained in a solution containing acetic acid 7% (v/v), methanol 20% (v/v), and water 73% (v/v) in an orbital shaker at a moderate speed (±60 rpm) for 3–4 hours at room temperature. SDS-PAGE Molecular Weight Standards and Marker molecular weight full-range (VWR), were used as protein standards. All gels were analysed using the Image Lab 3.0 (BIO-RAD) analysis tool.

**Extract analysis by proteomics.** Tryptic digestion was performed according to Ref. 28 with a few modifications. Protein spots were manually excised from the gel and transferred to Eppendorf tubes. The gel spots were washed with 25 mM ammonium bicarbonate/50% acetonitrile and then with acetonitrile. Gel pieces were dried in a SpeedVac (Thermo Savant) and rehydrated in digestion buffer containing 12.5 µg mL\(^{-1}\) sequence grade-modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate. 100 µL of 25 mM ammonium bicarbonate were then added and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by the addition of 10% formic acid/50% acetonitrile three times. Tryptic peptides were lyophilized in a SpeedVac (Thermo Savant) and resuspended in 5% acetonitrile/0.1% formic acid solution. The samples were analysed with a QExactive Orbitrap (Thermo Fisher Scientific, Bremen) system that was coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA) HPLC (high-pressure liquid chromatography) system. The trap (5 mm × 300 µm I.D.) and analytical (150 mm × 75 µm I.D.) columns used were C18 Pepmap100 (Dionex, LC Packings). Peptides were trapped at 30 µL min\(^{-1}\) in 95% solvent A (0.1% formic acid/5% acetonitrile \(v/v\)). Elution was achieved with solvent B (0.1% formic acid/100% acetonitrile \(v/v\)) at 300 nL min\(^{-1}\). The 50 min gradient was as follows: 0–3 min, 95% solvent A; 3–21 min, 5–45% solvent B; 21–35 min, 45–90% solvent B; 35–37 min, 90% solvent B; 37–40 min, 10–95% solvent A; 40–50 min, 95% solvent A. The mass spectrometer was operated in the data-dependent acquisition mode. An MS2 method was used with an FT scan from 400 to 1600 m/z (resolution 70 000; AGC target 1E6). The 10 most intense peaks were subjected to HCD fragmentation (resolution 17 500; AGC target 5E4, NCE 28%, max. injection time 60 ms, dynamic exclusion 35 s). General mass spectrometer parameters were: nano electrospray voltage, 1.8 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 275 °C; S-lens RF level 60.0. Spectra were processed and analysed using Proteome Discoverer (version 2.2, Thermo), with MS Amanda (version 2.0, University of Applied Sciences Upper Austria, Research Institute of Molecular Pathology) and the SequestHT search engines. The Uniprot Swiss-Prot protein sequence database (June 2018 version) was used for all searches under *Gracilaria* (genus). Database search parameters were as follows: carbamidomethylation and carboxymethyl of cysteine as a variable modification as well as oxidation of methionine, and allowance for up to two missed tryptic cleavages. Peptide mass tolerance was 10 ppm and fragment ion mass tolerance was 0.05 Da. To achieve a 1% false discovery rate, the Percolator (version 2.2, Thermo) node was implemented for a decoy database search strategy, peptides were filtered for high confidence and a minimum length of 6 amino acids, a minimum of two peptides and proteins were filtered for only rank 1 peptides. The average of intensities of the three most intense peptide ions was used to generate relative quantitative data.

**Purification of phycobiliproteins using AMTPS.** For phycobiliprotein purification using AMTPS, falcon tubes were weighed with specific amounts of each component: 10 wt% of phycobiliprotein crude extract, 10 wt% of surfactant and 0 or 0.3 wt% of SAIL, the system being completed with McIlvaine buffer.
(0.18 M) at pH 7.0 up to a final volume of 10 mL. The systems were homogenized for around 2 h using a tube rotator apparatus model 270 from Fanem, at 40 rpm. Then, the tubes were left in a temperature above the cloud point of the systems (40 °C) for 4 h, allowing thermodynamic equilibrium to be reached, resulting in the formation of surfactant-rich (top) and a surfactant-poor (bottom) phases. Both phases were carefully separated, and their volumes and weight composition measured. Then, the quantification of both phycobiliproteins and total proteins was assessed for each phase, using UV spectroscopy (Molecular Device Spectramax 384 Plus|UV-Vis Microplate Reader) at 565 nm and 280 nm, respectively. The analytical quantifications were performed in triplicate. Blank controls, i.e., identical systems without the phycobiliproteins extract, were performed for each system to eliminate any possible interference of the phase formers upon the quantification. The concentration of R-phycocyanin and total proteins in the extracts was assessed according to calibration curves previously determined in the same UV-Vis equipment.

In this work, the purification performance of each AMTPS was analysed through the Partition coefficient, Recovery, and Selectivity. The Contamination index was also determined to investigate the elimination of R-phycocyanin contamination from R-phycoerythrin. The partition coefficient ($K_{R\text{-phycoerythrin}}$) was calculated as the ratio between the amount of R-phycoerythrin present in the surfactant-poor (bottom) and the surfactant-rich (top) phases, as described by eqn (1). The partition coefficient of total proteins ($K_{\text{total proteins}}$) was determined identically (eqn (2)).

$$K_{R\text{-phycoerythrin}} = \frac{[\text{R-phycoerythrin}]_{\text{bot}}}{[\text{R-phycoerythrin}]_{\text{top}}} \quad (1)$$

$$K_{\text{total proteins}} = \frac{[\text{total proteins}]_{\text{bot}}}{[\text{total proteins}]_{\text{top}}} \quad (2)$$

where $[\text{R-phycoerythrin}]_{\text{bot}}$ and $[\text{R-phycoerythrin}]_{\text{top}}$ are, respectively, the concentration of R-phycoerythrin (in mg mL$^{-1}$) in the bottom and top phases; and $[\text{total proteins}]_{\text{bot}}$ and $[\text{total proteins}]_{\text{top}}$ are the total protein concentration (in mg mL$^{-1}$) in the bottom and top phases, respectively. The Recovery ($R$) parameter of R-phycoerythrin and the total protein content with respect to the bottom ($R_{\text{bot}}$) and top ($R_{\text{top}}$) phases were determined following eqn (3) and (4), respectively:

$$R_{\text{bot}} = \frac{100}{1 + \left(\frac{1}{K_{R\text{-phycoerythrin}}}\right)} \quad (3)$$

$$R_{\text{top}} = \frac{100}{1 + R_{\text{bot}} + K_{\text{total proteins}}} \quad (4)$$

where $R$ stands for the ratio between the volumes of the bottom and top phases. Finally, the selectivity ($S_{R\text{-phycoerythrin}/\text{total proteins}}$) of the AMTPS herein applied was described as indicated by eqn (5):

$$S_{R\text{-phycoerythrin}/\text{total proteins}} = \frac{K_{R\text{-phycoerythrin}}}{K_{\text{total proteins}}} \quad (5)$$

The phycocyanin contamination index (eqn (6)) was determined by the ratio between the absorbance at 620 nm and 565 nm, which belongs to the R-phycocyanin and R-phycoerythrin wavelengths, respectively. For ratios lower than 0.05, it is considered that no significant contamination by R-phycocyanin is found, as previously described in the literature.$^3$

$$\text{Phycocyanin contamination index} = \frac{\text{Abs}_{620}}{\text{Abs}_{565}} \quad (6)$$

**Purification of R-phycoerythrin using consecutive extractions.** To increase the R-phycoerythrin purification, the surfactant-poor phase was reused for a second step. The first step was carried out as previously described but this time for a total of 25 g. Here, only the SAILs that led to the highest selectivity for the elimination of the contaminant non-fluorescent proteins were used. The second step was carried out by adding 10 wt% of Tergitol 15-S-7 + 0.3 wt% of the selected SAIL + 89.7 wt% of the surfactant-poor phase of the first AMTPS, up to a total of 10 g.

**Study of the stability of R-phycoerythrin.** To guarantee that, at the end of the purification, R-phycoerythrin maintains its structural integrity, circular dichroism (CD) measurements were used to evaluate the protein secondary structure. The surfactant-poor phases of (i) the conventional AMTPS (i.e., without SAIL) and (ii) the best SAIL-based AMTPS were analysed using CD spectroscopy (Jasco-1500). The spectra were collected in a 1 mm path length quartz cuvette at a scan rate of 100 nm per minute, at 20 °C cell temperature. The response time and the bandwidth were 2 seconds and 0.2 nm, respectively. Three spectra were recorded consecutively to give single average data.

**Environmental evaluation: carbon footprint analysis.** Environmental evaluation was carried out taking into account two scenarios for R-phycoerythrin purification, namely using (i) the conventional system, where the AMTPS is used in the absence of SAIL as co-surfactant, and (ii) the mixed AMTPS, with the presence of the most promising SAIL selected.

The environmental assessment of the two systems chosen was performed by calculating the carbon footprint as output. This indicator is the sum of greenhouse gas (GHG) emissions expressed as carbon dioxide equivalent (CO2 eq.) from a life cycle perspective. The carbon footprint allows quantifying various GHG emissions associated with the two different systems tested and identifying their main causes.

This analysis includes the production of all reagents (Mcllvaine buffer, non-ionic surfactant Tergitol 15-S-7, and SAIL selected), and other consumables (tap and distilled water, and liquid nitrogen), besides the electricity consumed by the equipment used throughout the different processes.

Data on the amount of reagents, tap and distilled water, liquid nitrogen and electricity consumed were obtained during the experiment and from equipment catalogues (Table S1). Data on GHG emissions from the production of reagents, liquid nitrogen and electricity were sourced from Ecoinvent database version 3.4$^29$ and are present in Table S2.$^1$ The GHG emissions for the production of distilled water were calculated...
based on GHG emissions from tap water production\textsuperscript{30} and GHG emissions from electricity consumption during the distillation process. All data refer to 5 g of \textit{Gracilaria} sp.

**Conflicts of interest**

There are no conflicts to declare.

**Acknowledgements**

This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, FCT Ref. UID/CTM/50011/2019, financed by national funds through FCT/MCTES, CESAM (UID/AMB/50017 – POCI-01-0145-FEDER-007638), and QOPNA research unit (FCT UID/QUI/00062/2013) financed by national funds through FCT/MEC and when appropriate co-financed by the FEDER under the PT2020 Partnership Agreement. It was also supported by the Integrated Programme of SR&TD “SusPhotoSolutions – Soluções Fotovoltaicas Sustentáveis” (reference CENTRO-01-0145-FEDER-000005), co-funded by the Centro 2020 program, Portugal 2020, European Union, through the European Regional Development Fund. The authors are grateful for the national fund through the Portuguese Foundation for Science and Technology (FCT) for the doctoral grants SFRH/BD/101683/2014 of F. A. Vicente and SFRH/BD/122220/2016 of M. Martins. S. P. M. Ventura and A. C. R. Dias acknowledge FCT for the contracts IF/00402/2014 and IF/00587/2013, respectively. Thanks are also due to Fundação para a Ciência e a Tecnologia (FCT, Portugal), for the Macroalgae project, the Fundação para a Ciência e a Tecnologia (FCT, Portugal), for the contracts IF/00587/2013, and IF/00402/2014, respectively. Authors thank ALGAplus company for the macroalgae samples.

**Notes and references**

29. Ecoinvent, \url{http://www.ecoinvent.org}.