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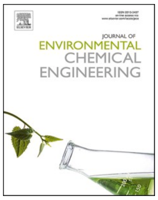

Process intensification of seaweed biorefinery using reusable natural deep eutectic solvents

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ABSTRACT

Conventional alginate extraction methods from brown seaweed typically rely on harsh chemicals that are not reused, and valuable pigments are lost during this process. This study applied a novel approach utilising reusable natural deep eutectic solvents (DES) in three-phase partitioning (TPP) to simultaneously extract alginate and pigments from *Saccharina latissima*. The hydrophobic DES effectively released alginate from the algal cell wall in the aqueous phase and served as a solvent for pigment extraction. Computational screening confirmed that all selected DES had an affinity for pigments chlorophyll *a* and fucoxanthin, while alginate extraction confirmed their role in disrupting the algal cell wall. Extraction conditions were optimised, resulting in an alginate yield of 101.8 ± 3.1 mg/g DW compared to 55.3 ± 14.1 mg/g DW for conventional alkaline extraction. According to physicochemical characterisation through FT-IR and M/G ratio (mannuronic to guluronic) analysis, the extracted alginate was comparable to that obtained via alkaline extraction, exhibiting similar functional groups and M/G ratios. The DES was reused successfully, showing that it could be reused for up to seven extraction cycles, during which pigments accumulated. After the seventh cycle, alginate yield declined, likely due to partial transfer into the DES phase, possibly driven by reverse micelle formation in the system. This study highlights a novel, mild multiproduct approach of a DES-based TPP system, enhancing economic feasibility by employing gentler and quicker extraction conditions. It facilitates the concurrent recovery of alginate and pigments while allowing for the repeated reuse of the DES.

1. Introduction

The need for alternative, greener extraction solvents is becoming increasingly urgent due to the environmental and health risks associated with conventional solvents [1]. Traditional solvents can be toxic, flammable, and volatile, contributing to pollution, human health issues, and hazardous waste. Therefore, the development of sustainable alternative solvents is essential. Deep eutectic solvents are an emerging class of novel solvents that could potentially replace these organic extraction solvents [2,3]. DES are solvents composed of one or more hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), that are formed upon a large depression in melting point when mixed at a certain ratio. They are favourable solvents due to their low volatility, ease of preparation, low costs, and potential recyclability [4–6]. Over the past decades, DES have gained attention in a wide range of fields, including pharmaceuticals [7,8], separation processes [7], and renewable energy [9]. Thanks to their tuneable properties and environmental compatibility, these solvents are being explored for applications such as drug solubilization, metal recovery, and used as electrolytes in energy systems [10,11]. Specifically, DES have been successfully used to extract various bioactive compounds, including polysaccharides [12,13], lipids [14], proteins [15], and polyphenols [16] from diverse biomass sources, including plants, microalgae, and seaweed. However, many studies do not report on recovery and reuse, which compromises the environmental sustainability of the process. The overall impact is also strongly influenced by the preparation step, as the choice of constituents used to form a DES largely determines it environmental footprint [17,18]. Furthermore, Life Cycle Assessment (LCA) studies have demonstrated

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that DES can be more sustainable than conventional organic solvents, provided they are efficiently recovered and reused [19]. Yet, their low vapour pressure poses challenges for product isolation and solvent recovery, meaning that research on reuse strategies remains limited and warrants further investigation [20].

Seaweed emerges as an attractive, sustainable, renewable biomass for DES extraction due to its high abundance, rapid growth rate, and diverse biochemical composition. Brown seaweeds, in particular, are rich in compounds such as alginate, fucoidans, and valuable pigments [21]. Among these components, alginate stands out for its versatility and wide range of applications (Figure S1). This polysaccharide is widely used within a variety of sectors, including food processing as a thickening and gelling agent and medicine for drug delivery and tissue engineering [22]. In addition to its polysaccharide content, brown seaweed is a rich source of high-value pigments, including chlorophylls and fucoxanthin (Figure S2). Chlorophyll *a* is a bioactive compound with health-related benefits, including antimutagenic and antioxidant activities [23]. Fucoxanthin is a xanthophyll that exhibits antioxidant, anti-inflammatory, and anti-cancer properties [24,25]. Due to their market value and diverse biological activities, both pigments present a wide range of potential applications. Conventional alginate extraction methods typically rely on hazardous chemicals and involve multiple acidic and alkaline processing steps, resulting in excessive water consumption and high energy demand [26,27]. Additionally, pigments are lost during the process and cannot be recovered due to the harsh chemical conditions employed.

Our previous study demonstrated that alginate can be efficiently extracted using DES [28]. However, DES reuse was not addressed, as alginate recovery relied on precipitation. Direct extraction with hydrophilic DES complicates recovery due to strong hydrogen bonding. Moreover, the study focused solely on alginate, without considering the recovery of other valuable compounds such as pigments, limiting full biomass utilisation. To overcome these challenges and maximise biomass utilisation, hydrophobic DES offer a promising alternative. They could be applied as a novel approach for indirect alginate extraction while facilitating easy DES recovery and subsequent reuse. As an extraction agent, hydrophobic DES would allow alginate to migrate to a separate, hydrophilic phase. Simultaneously, hydrophobic DES could also be employed for pigment extraction from brown seaweed. Several studies have already demonstrated the effectiveness of DES in extracting pigments from various biomasses, including brown seaweed [29], microalgae [30] and plants [31]. Building on this concept, three-phase partitioning (TPP) with DES presents an innovative approach to further enhance the extraction process. TPP is a liquid-liquid extraction technique that enables the simultaneous concentration and purification of biomolecules. It offers a mild and efficient approach for separating alginate and pigments from brown seaweed. In traditional TPP systems, an organic solvent and an aqueous salt solution are usually applied to the biomass [32], resulting in three distinct phases: an organic top phase containing pigments or lipids, a middle phase containing the residual biomass, and an aqueous bottom phase containing polysaccharides or soluble proteins. Replacing the widely used toxic solvent *t*-butanol with DES [33–35] could enhance the environmental sustainability of the system while preserving its extraction efficiency. DES have already been successfully integrated into TPP systems for the extraction and purification of polysaccharides from grape seeds [36] and tomato peroxidase [37]. However, they have not yet been applied in a multiproduct approach, utilising both the salt and DES phases. The dual application of DES as both a biomass pretreatment agent for alginate release and an extraction solvent for pigments represents a novel and innovative approach to integrated biorefinery processes. This strategy enhances resource efficiency and has the potential to maximise the economic viability of the process through the simultaneous recovery of both high- and low-value products, while enabling DES reuse.

In line with this, this study developed a three-phase partitioning system that uses natural deep eutectic solvents for a multi-product extraction of alginate and pigments from *Saccharina latissima*. Nine DES were computationally evaluated with the software COSMO-RS (COnductor-like Screening MOdel for Real Solvents) to evaluate their pigment extraction capabilities. This was followed by laboratory testing to determine their alginate extraction yield. Under optimised conditions, the DES's reuse potential within a three-phase partitioning (TPP) system was assessed while enabling pigment accumulation in the DES phase. This study introduces an innovative and sustainable multi-product approach to extract various compounds from brown seaweed efficiently.

2. Materials & methods

2.1. Materials

Brown seaweed *Saccharina latissima* was harvested in May 2023 from Kamperland (Zeeland, The Netherlands). The collected biomass was stored at −80 °C until it was utilised. Prior to extraction, the biomass was ground into a fine powder using a GRINDOMIX GM 200 (Retsch) grinder. The ground seaweed was then freeze dried until it reached a dry weight content of at least 93 % After freeze drying, the seaweed was sieved using 710 μm and 90 μm mesh sizes. The fraction between 90 and 710 μm was collected and used for subsequent extraction. To extract and isolate bioactive compounds from the seaweed using TPP, nine different DES were employed. Fatty acid-based and linalool-based HBAs and HBDs were selected (Table 1). Dodecanoic acid (purity 98 %), octanoic acid (purity > 99 %), nonanoic acid (purity > 97 %), decanoic acid (purity > 98 %), hexanoic acid (purity > 98 %), linalool (purity > 97 %), ammonium sulphate (purity > 99.5 %), Jeffamine D-230, hydrochloric acid (purity 37 %), menthol (purity 99 %) and thymol (purity >98.5 %) were purchased from Sigma Aldrich (Merck).

2.2. DES preparation

The DES were prepared according to the heating method [6]. The HBA and HBD were mixed in the appropriate molar ratio provided from literature and experimental screening (Table 1). The mixture was then heated at 50 °C for approximately 15 min until a translucent solution was formed. These DES solutions were subsequently used for the extraction of alginate and pigments with TPP.

2.3. Computational DES screening for pigment affinity

The software COnductor-like Screening MOdel for Real Solvents (COSMO-RS) was applied for the initial affinity screening of the pigments chlorophyll *a* and fucoxanthin, and for determining the octanol-water partitioning coefficient (logP) of the DES and pigments. The octanol-water partitioning coefficient (logP) was estimated using COSMO-RS to assess the hydrophobicity of both the DES and pigments. Less polar compounds have higher logP values than polar compounds. COSMO-RS is a quantum chemical software that can predict the thermodynamic properties of fluid mixtures [40]. With COSMO-RS (Software for Chemistry and Materials BV (SCM), Amsterdam, The Netherlands), the DES were screened by generating the activity

Table 1
Composition of DES used in this study.

	HBA	HBD	Mole ratio	Reference
DES1	Dodecanoic acid	Octanoic acid	1:1	[36]
DES2	Dodecanoic acid	Nonanoic acid	1:1	[36]
DES3	Dodecanoic acid	Decanoic acid	1:1	[36]
DES4	Dodecanoic acid	Hexanoic acid	1:1	
DES5	Octanoic acid	Decanoic acid	2:1	[38]
DES6	Linalool	Dodecanoic acid	1:1	
DES7	Linalool	Decanoic acid	1:1	[39]
DES8	Linalool	Nonanoic acid	1:1	[39]
DES9	Linalool	Octanoic acid	1:1	[39]

coefficient of the solvents, followed by calculating the capacity of each DES. The activity coefficient (γ) is defined as the ratio of a species' activity to its mole fraction, and it reflects the deviation from ideal solution behaviour. It represents the interaction between the pigments and each DES at infinite dilution. COSMO-RS within the ADF (Amsterdam Density Functional) was used for screening. The settings used were selected according to Xu *et al.* [30]. The capacity of each solvent, indicating the maximum solubility in the DES, was calculated using the inverse of the activity coefficient as shown in Eq. 1:

$$Capacity = 1/\gamma \tag{1}$$

where γ represents the activity coefficient of chlorophyll *a* or fucoxanthin at infinite dilution in the solution.

2.4. Alginate extraction, characterisation and quantification

Alginate was extracted by combining dried seaweed and an ammonium sulphate solution (30 wt%) with a DES in a solid to liquid ratio of 1:40. Ammonium sulphate was chosen as salt in the aqueous phase due to its widespread use in three-phase partitioning [35,37,41,42]. The DES and ammonium sulphate were added in a 0.5:1 vol ratio. As a control, alginate was extracted with only the ammonium sulphate solution. A second control involved alkaline extraction to allow comparison with conventional alginate recovery methods. For alkaline extraction, acid water containing H_2SO_4 (pH 1.9) was added to the seaweed in a 1:1 (w/w) ratio and left to rest at room temperature for one hour. Next, Na_2SO_4 was added in a ratio of 0.0325:1 (w/w) to the stream for a one-hour extraction at 55 °C [27]. The extractions were performed at 55 °C for 30 min, and the solutions were vortexed every 5 min. After extraction, solutions were centrifuged, resulting in a three-phase separation. The DES phase and precipitation phase were separated from the bottom salt solution phase. The salt phase was dialysed and thereafter analysed for alginate content. Subsequently, extraction time, extraction temperature, solid to liquid ratio, mass fraction of ammonium sulphate, type of salt, and DES to salt ratio were altered to assess the influence of these factors on the extraction yield. For alginate quantification, a colorimetric assay adapted from Cesaretti *et al.* [43] was used. The alginate extraction yield was determined following Eq. 2:

$$Yield\left(\frac{mg}{gDW}\right) = \frac{C_{extract} * V_{total}}{m_{biomass} * x_{DW}} \tag{2}$$

where $C_{extract}$ is the concentration of the extract, $m_{biomass}$ is the weight of the initial biomass, x_{DW} is the weight fraction of the dry biomass and V_{total} is the total volume of the extract.

The alginate fraction obtained through TPP was dried and subsequently characterised using Fourier transform Infrared Spectroscopy (FT-IR), and its spectral profile was compared to those of alginate extracted via alkaline and acid treatment. Additionally, the DES fraction before and after extraction was characterised using FT-IR. Spectral analysis was performed with a Nicolet Summit X attenuated total reflection (ATR) FT-IR Spectrometer (ThermoFisher Scientific), covering a range from 400 to 4000 cm^{-1}.

The mannuronic to guluronic (M/G) ratio of the extracted alginate was determined through methanolysis, followed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as described by Bojorges *et al.* [44]. The analysis was conducted using a Dionex ICS-6000 system (Thermo Fisher) equipped with a CarboPac 1 mm column. Alginate extracted using DES was compared to that obtained through conventional alkaline extraction. The extracted alginate was precipitated using 80 % ethanol with 1 % NaCl, followed by two washing steps with 80 % ethanol. The samples were subsequently re-solubilised in Milli-Q water, and 50 μL aliquots were used for methanolysis.

2.5. Pigment extraction and quantification

Pigments chlorophyll *a* and fucoxanthin were extracted in the DES phase under the previously mentioned conditions (Section 2.4). Pigment quantities were estimated using a UV-Vis microplate reader (TECAN Infinite M200) as this provides a rapid and non-destructive means for assessing pigment contents in solution [45] and has been applied for quantification [46]. The absorption spectra of the DES were measured from 300 to 800 nm. Chlorophyll and fucoxanthin contents were estimated using calibration curves established in DES1, based on absorbance at 667 nm for chlorophyll (R^2 = 0.9985) and 457 nm for fucoxanthin (R^2 = 0.9967), as shown in Figure S3 [46].

2.6. DES reusing

To reuse the DES fraction, the optimal DES was selected, and optimal extraction conditions were applied. The multiproduct extraction of alginate and pigments was performed 12 times to assess the impact on the yield by reusing the DES fraction. Alginate content and pigment extraction yields were assessed per extraction cycle.

Fig. 1 gives a schematic overview of the integrated multi-product extraction, phase separation and DES regeneration process. Initially, seaweed is added to the DES and aqueous salt solution to facilitate the simultaneous extraction of alginate and pigments. Following extraction, phase separation results in three distinct fractions: (1) a hydrophobic DES-rich upper phase containing lipophilic pigments, (2) an aqueous salt-rich lower phase containing solubilised alginate, and (3) an intermediate solid fraction composed of undissolved residual biomass. The DES phase containing pigments is recovered and reused for subsequent extraction cycles, while the alginate fraction is dialysed. The residual biomass fraction is discarded.

2.7. Alginate determination in DES

To determine the potential alginate content in the hydrophobic DES, the DES was switched to a hydrophilic solution to release the alginate content. This was achieved by adding water to the DES in a 5:1 ratio, along with Jeffamine D-230 at a 1:10 ratio relative to water, to increase the pH and induce the formation of a hydrophilic fraction (Figure S4). The aqueous fraction was precipitated and subsequently analysed with Dionex through methanolysis (Section 2.4).

3. Results and discussion

3.1. DES screening for alginate and pigment extraction

Before assessing the ability of each DES to release alginate from the cell wall within the TPP, the affinity of these DES towards pigments was determined computationally. This assessment ensured that the DES selected for subsequent alginate extraction with TPP could also effectively extract pigments, thereby enabling a multi-product biorefinery. A multiproduct biorefinery approach maximises biomass valorisation by allowing the extraction of not only alginate but also high-value pigments, thereby enhancing both economic viability and sustainability. This integrated strategy aligns with circular bioeconomy principles by reducing waste, diversifying product streams, and meeting the growing demand for natural bioactives in food, cosmetic, and pharmaceutical sectors [47].

3.1.1. Pigment affinity screening

The capacity of each DES and benchmark solvent ethanol was calculated as the inverse of the activity coefficient at infinite dilution (Section 2.3). The solvent capacity indicates the maximum amount of pigment that can be dissolved in DES, making it an important parameter to consider for extraction solvents. Screening results from COSMO-RS showed that all DES have affinity with the pigments chlorophyll *a* and

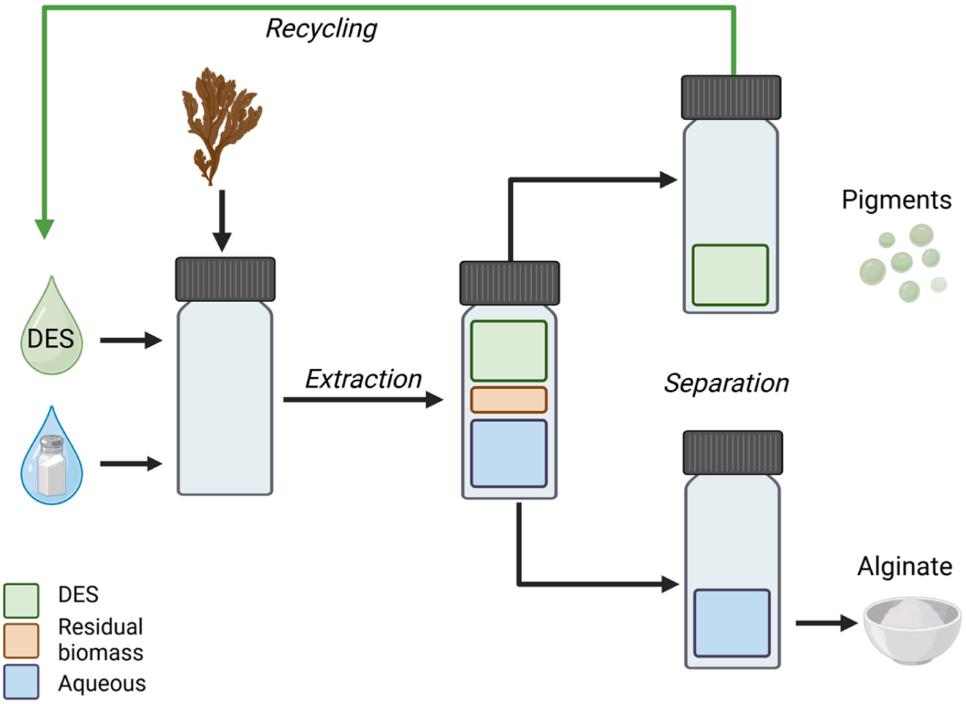

Fig. 1. General overview of multi-product brown seaweed biorefinery using three-phase partitioning with a hydrophobic DES and an ammonium sulphate aqueous solution.

fucoxanthin, with capacities ranging from 2.14×10^4–2.99 (Table 2). When comparing the linalool-based DES (DES6-DES9) with the fatty acid-based DES, we observed that the fatty acid-based DES (DES1-DES5) had higher capacities for both pigments. This difference could be due to the lower logP of linalool compared to the C9, C10, and C12 fatty acids (Table S1). Capacities could reach a factor 10^4 for chlorophyll *a*, while for fucoxanthin, this could reach a factor of 10^3 (Table 1). For fucoxanthin, the capacity decreased with increasing chain length of the carboxylic acid HBD in dodecanoic acid-based DES. Conversely, an increased capacity was observed for chlorophyll a as the carbon chain length of the HBD increased when using dodecanoic acid as HBA. The difference in capacities could be due to the higher logP chlorophyll exhibits (18.4) compared to fucoxanthin (13.6) (Table S1), indicating its greater hydrophobicity. This suggests that chlorophyll has a stronger affinity toward more non-polar solvents. Fatty acid-based DES, composed of long hydrophobic chains, exhibit a lower polarity than DES based on monoterpenes like linalool (Table S1). As a result, these fatty acid-based solvents provide a more compatible environment for solubilising highly non-polar compounds such as chlorophyll, which may explain their higher extraction capacity in this case compared to fucoxanthin. Moreover, each DES displayed a higher capacity compared to the benchmark extraction solvent ethanol. highlighting their superior solubilising ability for algal pigments. This supports the potential of DES as promising green alternatives for efficient and selective pigment extraction.

Table 2
Calculated capacities of DES and ethanol for chlorophyll a and fucoxanthin.

	Chlorophyll *a*	Fucoxanthin
DES1	2.04×10^4	2.10×10^3
DES2	2.10×10^4	2.02×10^3
DES3	2.14×10^4	1.94×10^3
DES4	1.88×10^4	2.23×10^3
DES5	1.88×10^4	2.31×10^3
DES6	1.12×10^1	2.87
DES7	1.08×10^1	3.15
DES8	1.36×10^1	3.89
DES9	7.98	2.99
Ethanol	8.86×10^{-3}	3.01×10^{-1}

By plotting the σ-potential of the pigments and DES, we could assess the affinity of the pigments towards the DES [30]. σ-Potential plots provide insight into the interaction tendencies between the pigments and the DES. A more negative σ-potential indicates a stronger attractive interaction, whereas less negative or positive values suggest weaker affinity or repulsion between the species [30,48]. As observed from the σ-potential, chlorophyll *a* and fucoxanthin display high negative values in their HBD regions and positive values in both the neutral and HBA regions (Figure S5). This indicates that these pigments can be solvated best in DES with strong HBD groups, meaning DES with more negative σ-potential values in the HBD region ($\sigma \leq -0.085$ e/A^2) (Figure S5). Additionally, to enhance the extraction efficiency of DESs for both pigments, the DES should exhibit negative σ-potential values in the HBA region to enable strong complementary interactions with the pigments, together with increased capacity values (Table 2). The fatty acid-based DES (DES1–5) have stronger negative values in the HBA region ($>+0.085$ e/A^2) compared to the linalool-based DES (DES6–9) (Figure S5). This indicates that the fatty acid-based DES have a stronger affinity with HBA molecules compared to the linalool-based DES. Additionally, the fatty acid-based DES display less negative values in the HBD region (<-0.085 e/A^2) compared to the linalool-based DES, thereby having less affinity to HBD molecules.

As a result, the fatty acid-based DES should solvate the pigments more effectively than the linalool-based DES due to complementary hydrogen bonding interactions that can occur, thereby reducing the self-association between the HBA and HBD of the DES. Due to the high capacity all DES exhibited for chlorophyll *a* and fucoxanthin (Table 2), all DES were considered in the TPP for alginate extraction.

3.1.2. *Alginate extraction and isolation*

Alginate was successfully extracted with TPP using the nine studied DES (Fig. 2). During the extraction process, the hydrophobic DES interact with seaweed biomass primarily through hydrogen bonding and hydrophobic interactions. These DES may disrupt the polysaccharide-rich cell wall structure, aiding the release of alginate. Due to

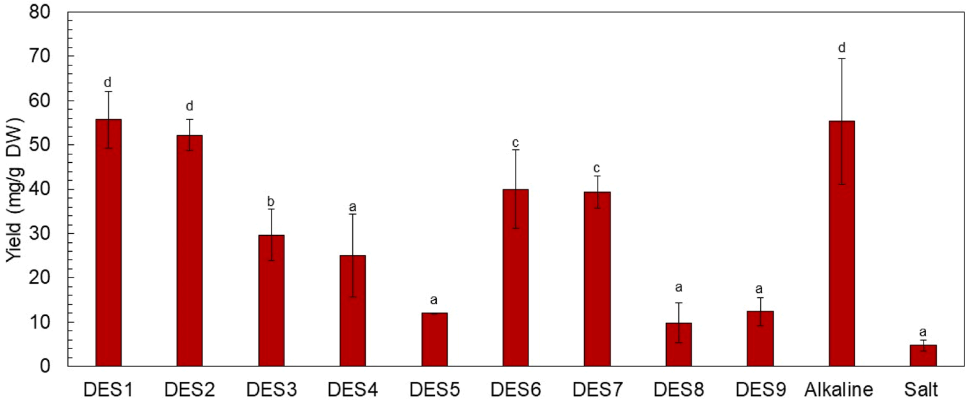

Fig. 2. Alginate extraction yield for TPP systems with DES1-DES9. Alkaline extraction and extraction with only salt (ammonium sulphate) were used as controls.

alginate's hydrophilic and anionic nature, it has limited solubility in the hydrophobic DES phase. Therefore, the aqueous phase permits the selective migration of alginate into the aqueous phase. In the aqueous phase, alginate can strongly interact with ammonium sulphate through electrostatic interactions between the negatively charged carboxylate groups on the alginate backbone and the positively charged ammonium ions [49], thereby influencing the solubility of alginate [50].

Among the DES types, DES1 (dodecanoic acid: octanoic acid) resulted in the highest alginate release, with an extraction yield of 55.7 ± 6.4 mg alginate per g DW. The control using the conventional alkaline extraction resulted in similar amounts of alginate with a yield of 55.3 ± 14.1 mg/g DW (Fig. 2). DES1 and DES2 performed comparably to the alkaline extraction regarding alginate yield (Fig. 2), while the other DES had a lower performance compared to the alkaline extraction. Although the alkaline treatment provides an extraction yield similar to those of DES1 and DES2, this method can be objectionable for safety, environmental and engineering (corrosion) reasons, as it requires strong alkaline and acidic conditions. Furthermore, solvent regeneration cannot be applied with this treatment.

When only the aqueous salt solution was applied without adding a DES, the extraction yields were considerably lower (4.8 ± 1.2 mg/g DW) compared to extracting with salt and DES (Fig. 2). This indicates that, even though the hydrophobic DES is not directly involved in extracting alginate, the DES can act as a synergistic extracting agent together with the aqueous phase, facilitating enhanced alginate extraction into the aqueous salt phase. Previous studies have demonstrated that certain DES can affect the permeability and integrity of the cell walls of plants [51–53] and microalgae [54]. Therefore, in the TPP system, alginate could have been released in the aqueous phase through the disruption of the cell wall with DES. However, scanning electron microscopy would be required to confirm this. Hydrophobic DES can disrupt cell walls primarily by reconfiguring the hydrogen-bond network within structural polysaccharides of the biomass [55]. The hydrophobic DES constituents interact with non-polar cell wall regions through van der Waals forces [56], enhancing membrane permeability, thereby facilitating the release of compounds, including alginate. It must be noted that the effect of a single component (HBA or HBD separately) could also influence permeability rather than the DES. However, comparing DES and single components is difficult because part of the used fatty acids are in a solid state at room temperature as single components, resulting in less exposure to cellular material [57].

For the dodecanoic acid-based DES (DES 1–4), it was observed that increasing the chain length of the HBD resulted in a decrease in alginate extraction yield. (Fig. 2). Conversely, for the linalool-based DES (DES 6–9), using dodecanoic and decanoic acid as HBD resulted in a higher alginate extraction yield compared to using nonanoic and octanoic acid as HBDs (Fig. 2). Differences in alginate yield may be due to the differences in polarity of these DES. The DES with the lowest alginate extraction yields (DES5, DES8 and DES9) displayed a logP below 4 (3.9, 3.8 and 3.6, respectively) (Table S1), while all the other DES had logP values larger than 4. However, DES3 displayed a logP of 5.1 and had a decreased yield compared to DES1 and DES2. This suggests that a certain degree of non-polarity (logP between 4 and 5) of the DES is required to act on the algal cell wall and to destabilise it. Furthermore, greater hydrophobicity of DES could cause more alginate to migrate into the aqueous phase because of repulsion between alginate molecules and the hydrophobic solvent, enabling alginate to partition into the more hydrophilic phase.

For the dodecanoic acid-based DES, an increased chain length of HBD decreased the alginate extraction yield (Fig. 2). These observations are in line with Chen *et al.* [36], where grape seed polysaccharides were extracted in a TPP system using fatty acid-based DES. Here, the highest extraction yield of polysaccharides was also obtained with dodecanoic acid: octanoic acid. The viscosity of the DES may be a factor influencing the extraction yield. Out of the dodecanoic acid-based DES, DES1 displayed the lowest viscosity.

The higher extraction yields observed with dodecanoic acid-based DES compared to linalool-based DES may be due to the ability of fatty acids to interact with the seaweed's cell wall. Cao *et al.* [58] stated that hydrophobic DES containing fatty acids can interact with the outer cell membrane of recombinant *E.coli* cells, causing it to destabilise. When fatty acids are deprotonated, they can function as surfactants known for their cell-disrupting properties [57]. As the permeability of the outer membrane increases, the hydrophobic DES can penetrate the inner phospholipid layer, thereby disrupting the phospholipid bilayer. This phenomenon could have led to the release of more alginate with the dodecanoic acid-based DES compared to the linalool-based DES.

In a separate study on the pretreatment of lignocellulosic biomass with hydrophilic DES [59], it was demonstrated that choline chloride-based DES disrupts the cell wall structure by dissolving lignin from the lignocellulosic microfibrils. This disorganisation led to an increased cellulose availability. In another study performed by Huang and coworkers [60], a choline chloride-based DES (choline chloride: formic acid 1:2) was used as a pretreatment to extract lipids from the microalga *Nannochloropsis oceanica*. Density Functional Theory (DFT) calculations revealed that DES could disorganise the intermolecular interactions between microalgal molecules through the reconstruction of the hydrogen bond network, therefore increasing lipid availability for extraction. In our study, the hydrophobic DES may have disrupted the cell membrane, while also disorganising the algal cell wall. This could have facilitated the penetration of the aqueous salt phase, enhancing the release of alginate. As a result, the application of DES likely led to a significant increase in alginate extraction yield compared to using the aqueous solution alone.

Given its high alginate extraction efficiency and strong capacity for chlorophyll *a* and fucoxanthin, DES1 was chosen for further single-factor experiments.

3.2. Extraction conditions

TPP is a technique that involves both extraction and separation within a one-pot system. Operational parameters can affect the system's extraction yield. Therefore, the effect of extraction time, extraction temperature, the concentration of ammonium sulphate, the liquid to solid ratio, DES to ammonium sulphate ratio and the type of salt on the alginate yield were studied (Fig. 3 a-e).

3.2.1. Extraction time

The extraction time was assessed, ranging from 5 to 60 min. The alginate extraction yield initially increased between 5 and 10 min, then gradually decreased from 20 to 60 min (Fig. 3a). The highest alginate extraction yield of 86.6 ± 13.0 mg/g DW was achieved after just 10 min of extraction. In contrast to traditional alkaline extraction, which generally involves one hour for the acid treatment followed by an additional hour of alkaline extraction (Section 2.4), TPP extraction is more time-efficient. The decrease in yield from 10 to 60 min (Fig. 3a) could be attributed to the increased dissolution of other components [36] and an increase in viscosity of the DES over time [55]. However, a notable reduction in alginate yield was observed at 60 min, suggesting that alginate might be partially transferred into the DES phase due to the saturation of the DES. This could occur through the formation of reverse micelles, created by the algal phospholipids or proteins, as they can act as surfactants [61], similar to methods used for encapsulating polysaccharides, proteins and enzymes [62].

3.2.2. Extraction temperature

The effect of the extraction temperature was investigated across a range of 20–70 °C. The alginate extraction yield increased with increasing temperature, reaching its highest value of 100.2 ± 2.4 mg alginate/g DW at 70 °C (Fig. 3**b**). The increase in yield with higher temperatures (Fig. 3**b**) may be due to the reduced viscosity of the DES at elevated temperatures, which enhances mass transfer [33,63]. In addition, higher temperatures within the TPP can lead to increased thermal molecular movement [36], facilitating the formation of more hydrogen bonds, as more hydroxyl groups of alginate are exposed. This results in increased solubility of alginate in the aqueous phase, yielding a higher extraction [35]. Additionally, higher temperatures can help in rupturing the cell wall [64], thereby improving the alginate accessibility. Even though the alginate yield increased with increased temperatures, fucoxanthin is susceptible to thermal degradation above 36 °C [30]. For energy efficiency and pigment protection, 35 °C was chosen as the optimal extraction temperature.

3.2.3. $(NH_4)_2SO_4$ concentration

The mass fraction of $(NH_4)_2SO_4$ was tested from 0 to 40 wt%. The alginate extraction yield increased as the amount of $(NH_4)_2SO_4$ rose to

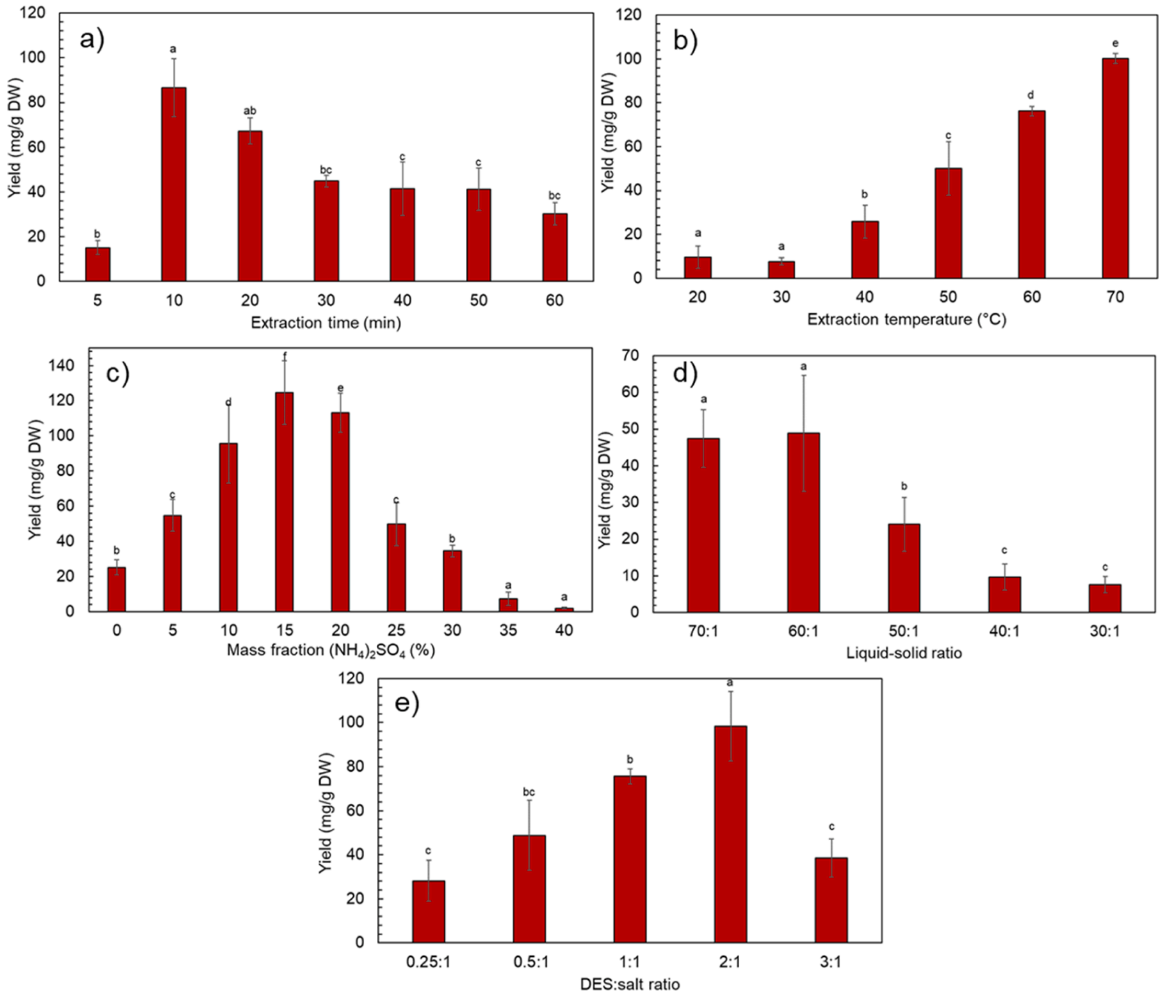

Fig. 3. Effect of (a) the extraction time, (b) extraction temperature, (c) mass fraction of $(NH_4)_2SO_4$, (d) the liquid-solid ratio and (e) the DES to salt ratio on the extraction yield of alginate. Conditions were standardised at 30 wt% $(NH_4)_2SO_4$, 55 °C, liquid-solid ratio of 60:1, 30 min extraction time and a DES:salt ratio of 0.5:1 when one of the extraction parameters was altered.

15 wt%, above which the alginate yield gradually decreased (Fig. 3**c**). A clear optimum was observed at 15 wt%, with an extraction yield of 124.6 ± 18.1 mg/g DW. The increased presence of $(NH_4)_2SO_4$ in the solution enhanced the alginate extraction yield, as NH_4^+ and SO_4^- ions help stabilise macromolecular interactions [35]. Specifically, NH_4^+ ions can interact with the negatively charged carboxylate groups on alginate, reducing intermolecular aggregation and thereby enhancing alginate solubilisation [50]. However, further increases in the mass fraction of $(NH_4)_2SO_4$ led to a decrease in yield, likely due to reduced dissolution as the aqueous layer became increasingly saturated [34,65]. This saturation reduced the amount of water available for dissolving alginate, lowering the extraction yield. Additionally, higher salt concentrations can interfere with hydrogen bonding between alginate and water, further decreasing the yield [66]. Therefore, a 15 wt% mass fraction of $(NH_4)_2SO_4$ was selected as optimal.

3.2.4. *Liquid to solid ratio*

Total liquid (DES and ammonium sulphate solution) to solid ratios ranging from 30:1–70:1 were evaluated. The extraction yield improved as the liquid to solid ratio increased, with a maximum extraction yield at a ratio of 60:1 (48.8 ± 15.8 mg/g DW) (Fig. 3d). Further increasing the ratio to 70:1 did not result in a higher extraction yield. Higher liquid to solid ratios provide a larger extraction volume, allowing more alginate to dissolve in the aqueous phase. A 60:1 ratio was found to be optimal for alginate extraction, as increasing the ratio to 70:1 did not result in a further improvement in yield. When applying low liquid to solid ratios, the extraction yield was low (Fig. 3d). This could be due to solubility-limited extraction, in which alginate reaches saturation in the aqueous phase [67]. On the other hand, mass transfer constraints could have caused no extraction improvement from 60:1–70:1. An insufficient amount of extractant results in incomplete extraction, while excessive amounts lead to waste and negatively impact the extraction process [36,66]. Therefore, a 60:1 ratio was selected as the ideal for extraction.

3.2.5. *DES to salt ratio*

Various DES to salt volume ratios, ranging from 0.25:1–3:1, were tested. It was found that increasing the amount of DES relative to salt resulted in higher alginate extraction yields (Fig. 3e). The highest extraction yield, 98.3 ± 15.8 mg/g DW of alginate, was achieved with a 2:1 DES to salt ratio. Increasing the concentration of DES could enhance van der Waals interactions with hydrophobic regions of the cell structure [56], thereby improving membrane permeability and promoting the release of alginate. However, increasing the volume ratio to 3:1 resulted in a reduced extraction yield (38.6 ± 8.6 mg/ g DW). The optimal DES to salt ratio was determined to be 2:1 (Fig. 3e), aligning with findings from previous studies [63,68]. At lower DES to salt ratios, the DES and $(NH_4)_2SO_4$ cannot effectively synergise, compromising the system's stability [36]. These findings suggest that DES enhances the buoyancy of macromolecules, including alginate, facilitating its separation into the aqueous phase while solubilising the lipid fraction into the DES [33]. Further increasing the amount of DES may raise the system's overall viscosity, which could impede mass transfer of alginate to the salt phase [63]. Thus, a 2:1 DES to salt volume ratio was selected as the optimal condition.

3.3. *Extraction optimisation and characterisation*

Subsequently, the optimal extraction conditions were selected for DES reusing, except for the extraction temperature. For temperature, 35 °C was selected. The following conditions were applied: 10 min, 35 °C, 15 wt% $(NH_4)_2SO_4$, liquid to solid ratio of 60:1 and DES to salt of 2:1. For temperature, the suboptimal extraction condition at 35 °C was selected as higher temperatures lead to increased energy consumption. Under optimised conditions, 101.8 ± 3.1 mg/g DW of alginate was extracted, while 0.144 mg/g DW and 0.026 mg/g DW of chlorophyll *a* and fucoxanthin were obtained, respectively. Taking into account that *S. latissima* contains approximately 19 % alginate and 90 % of the biomass is dry weight, this indicates that approximately 55 % of the total alginate content was extracted.

Although a temperature of 35°C was chosen, which was not the optimal temperature, high alginate extraction yields were still achieved under the optimised conditions. This temperature, combined with optimal extraction parameters, could have facilitated a synergistic effect, resulting in a high yield with a relatively low extraction temperature. Additionally, lower extraction temperatures are advantageous for pigment extraction [30]. Compared to the initial extraction conditions, the optimised process employed shorter extraction times, lower temperatures, and reduced salt concentrations, thereby enhancing its economic viability.

Compared to other novel extraction methods, similar yields were achieved. Direct alginate extraction using hydrophilic DES choline chloride: urea (1:2) resulted in 74 % alginate after optimisation [28]. However, the reuse of DES was not examined since alginate was recovered through precipitation. Additionally, the approach focused only on alginate extraction, without considering the recovery of other valuable bioactive compounds. In a study by Bojorges *et al.* [69], hydrostatic pressure-assisted extraction was used for *S. latissima*, using 0.1 M $NaHCO_3$ for 2 h at room temperature. An alginate yield of 13.9 % was obtained, which is significantly lower than the TPP extraction of alginate. Moreover, the treatment is relatively long (2 h compared to 10 min), and no other compounds were extracted. Saravana *et al.* [70] employed subcritical water extraction at 125 °C and 10 bar using a choline chloride: glycerol (1:2) DES containing 70 % water, achieving an alginate yield of 28 %. Despite its potential, this approach yielded lower extraction efficiencies and requires high-pressure equipment, which increases capital investment and raises safety and material corrosion concerns**.**

The FT-IR spectra of alginate extracted using TPP and alkaline treatment exhibited similar characteristic patterns (Fig. 4). Both extracts exhibited peaks at 1020 cm^{-1} and 1080 cm^{-1}, as well as at 1600 cm^{-1} and 1400 cm^{-1}. Additionally, a minor peak at 2900 cm^{-1} was detected for both extracts (Fig. 4). The sharp peaks observed at 1080 and 1020 cm^{-1} are attributed to mannuronic and guluronic acid units [71]. The peaks around 1600 and 1400 cm^{-1} correspond to the asymmetric and symmetric stretching modes of carboxylate groups, respectively [71–73]. Additionally, the low-intensity peak at 2900 cm^{-1} is associated with $-CH_2$ groups [72]. The presence of these characteristic bands indicates that the proposed TPP system is effective for extracting alginate from brown seaweed, producing a profile similar to that of the conventional alginate extraction method.

The M/G ratio is an important factor in alginate extraction, as it impacts the physical properties of the alginate [44]. Alginates with a higher M/G ratio generally form more permeable gel matrices, whereas those with a lower M/G ratio produce mechanically stronger structures. This difference is attributed to the higher affinity of guluronic acid blocks for calcium ions, which leads to enhanced cross-linking and gel strength compared to mannuronic acid blocks [74].

The M/G ratio of alginate extracted using the TPP system was 1.05 ± 0.00, whereas the conventional alkaline extraction yielded a higher ratio of 1.57 ± 0.02. These values are comparable to values reported in literature (ranging from 1.17 to 1.40) for *S. latissima* [44,75], indicating that the M/G ratios of TPP and alkaline extraction methods are similar. However, alginate extracted with TPP could probably produce stronger and less permeable gels, whereas

the conventional method could yield softer and more elastic gels. Both the FT-IR analysis and M/G ratio results suggest that alginate extracted and recovered using TPP is comparable to that obtained through conventional alkaline extraction, with similar functional groups and M/G ratios. Therefore, physicochemical characterisation indicates that TPP could be a viable method for alginate extraction.

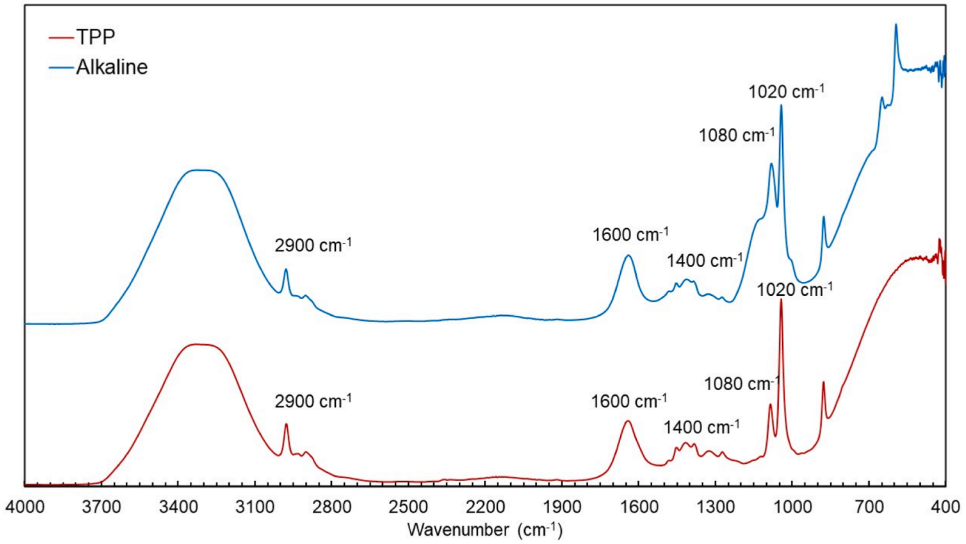

Fig. 4. FT-IR spectra of alginate extracted with DES-based three-phase partitioning and conventional alkaline extraction.

3.4. DES reuse

3.4.1. Pigment extraction

Reusing the DES was conducted to evaluate the alginate and pigment yield per extraction cycle. As the DES is reused without removal of pigments, it is anticipated that chlorophyll and fucoxanthin will progressively accumulate until the solvent reaches saturation. The extraction yield for both chlorophyll and fucoxanthin increased from cycles 1–10 (Fig. 5). For chlorophyll *a*, the extraction yield reached a plateau at cycle 7 (1.98 ± 0.04 mg/g DW) with no further increase up to cycle 12. The yield for fucoxanthin increased up to cycle 6 (0.18 ± 0.00 mg/g DW), followed by a decrease in cycle 7. Extraction yields increased again between cycles 7 and 12, reaching 2.08 ± 0.21 mg/g DW. The fluctuating fucoxanthin yields could be due to partial degradation of this pigment in the DES.

Previous research focused on fucoxanthin extraction from *S. latissima* [29] found an optimised extraction yield of 0.137 mg fucoxanthin/g DW and 0.08 mg/g DW chlorophyll using menthol: levulinic acid (1:1). These results are similar to the fucoxanthin yields obtained in our study after seven reuse cycles of the DES. In a study by Fernandes *et al.* [76], fucoxanthin yields ranging from 0.02 to 0.12 mg/g DW were obtained from *S. latissima* using acetone containing 0.05 % butylated hydroxytoluene, depending on the season. These values are comparable to those achieved with the TPP system. In contrast, Martins *et al.* [77] reported 1.96 mg/g DW and 4.93 mg/g DW of fucoxanthin and chlorophyll, respectively, when using a phosphonium-based ionic liquid combined with sunflower oil. Although high pigment yields were obtained, the isolation step relies on back-extraction using toluene, which limits the process's suitability for sustainable industrial applications. However, it must be noted that fucoxanthin content is subject to seasonal variation as well as differences related to the geographic location of seaweed collection [78]. Therefore, direct comparison of pigments extracted per dry weight between different studies is challenging due to variability.

To evaluate the DES extract, FT-IR analysis was conducted on both the pure DES and the DES extract. The pure DES exhibited characteristic peaks, including a strong stretching vibration of the carbonyl group (COOH) at 1703 cm^{-1} and a bending vibration of the hydroxyl group (-OH) in the carboxyl functional group at 917 cm^{-1} [36] (Figure S6). Upon extraction, the DES extract exhibited additional peaks that could be attributed to the presence of fucoxanthin and chlorophyll. Specifically, the low-intensity absorption at 3427 cm^{-1} corresponds to O–H stretching vibrations, present in fucoxanthin [45], while distinct carbonyl (C═O) groups were observed through a high-intensity peak at 1623 cm^{-1} [45,79], which is consistent with functional groups found in chlorophyll and fucoxanthin. These additional peaks may suggest the solubilisation of these compounds in the DES.

For product recovery, a biphasic system can be employed, as proposed by Kholany *et al.* [29]. By introducing a hydrophilic choline chloride-based DES, fucoxanthin and chlorophyll can be effectively separated, with fucoxanthin partitioning into the hydrophilic phase. Subsequently, fucoxanthin can be recovered through water addition to the hydrophilic DES. Chlorophyll remains in the hydrophobic DES and could be directly applied, for instance, for cosmetic applications [29].

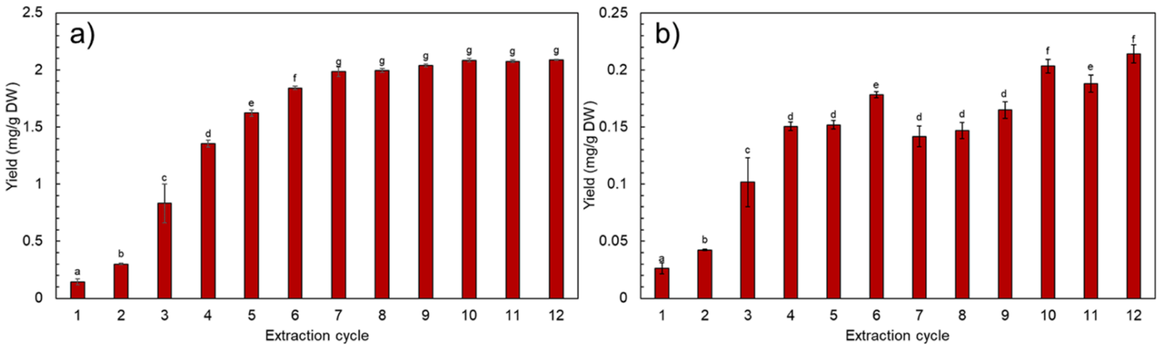

Fig. 5. Extraction yield of (a) chlorophyll a and (b) fucoxanthin when reusing the DES fraction.

3.4.2. Alginate extraction

DES reusing was performed for twelve cycles using the optimised extraction conditions (Fig. 6). Per extraction cycle, the alginate yield was determined in the aqueous phase. Interestingly, the alginate extraction yield decreased from cycle 1–3 (101.8 ± 3.1–54.1 ± 12.5 mg/g DW), and increased again from cycle 4 (95.4 ± 15.2 mg/g DW) up to cycle 7 (118.0 ± 22.4 mg/g DW). From cycle 8, the alginate yield decreased again. The decrease in extraction yield from cycle 1–3 and from cycle 7 could be due to increased dissolution of other components in the DES, therefore lowering the effectiveness of the DES [36]. However, this does not explain the increase in alginate yield from cycle 4–7. As discussed in Section 3.2, this trend could be due to the formation of reverse micelles by the DES, capturing alginate in the DES phase.

An overview of the extracted target analytes and approximate quantities are given in Table 3.

3.5. Proposed extraction mechanism

As previously mentioned (Section 3.2), the fluctuations in alginate yield could be attributed to the formation of reverse micelles, potentially caused by the release of phospholipids or proteins acting as emulsifiers when DES is applied to the biomass. However, the fatty acid-based DES could also play a role in reverse micelle formation, as studies have reported the use of fatty acids in reverse micellar systems [80,81]. Bukman *et al.* [80] utilised dodecanoic acid, hexadecenoic acid and octadecanoic acid to extract methylene blue from an aqueous solution via reverse micelle formation. Under optimised conditions, this method achieved a dye removal efficiency of 99 %.

The combination of phospholipids from the seaweed and fatty acids from DES is hypothesised to have promoted enhanced micellar formation. A study by Kittipongpittaya *et al.* [82] examined the influence of phospholipids and free fatty acids on reverse micelle formation. Their findings demonstrated that higher fatty acid concentrations significantly increased the concentration of micelles. This effect was attributed to the ability of fatty acids to alter the pH, which enhanced the positive charge on the phospholipid head groups. The resulting electrostatic repulsion between phospholipid molecules further facilitated the formation of reverse micelles. Penttila *et al.* [81] found that adding fatty acids to phospholipids improved the stability of reverse micelles against precipitation. Additionally, it was observed that the cores of the reverse micelles expanded, allowing them to accommodate more water.

To confirm the presence of alginate in the DES phase, this phase was converted into an aqueous solution by adding water and an amine solution (Section 2.7). Notably, mannuronic and guluronic acid were detected in the aqueous DES phase (Figure S7), suggesting that alginate was indeed present in the DES. Therefore, alginate may have been encapsulated within reverse micelles formed by phospholipids released during DES treatment, in combination with fatty acids present in the DES. This process could have facilitated the transfer of alginate to the upper phase (Fig. 7). Initially, the fatty acid-based DES functioned as a pretreatment, disintegrating the algal cell wall and releasing the alginate. During this release, phospholipid fractions and fatty acids from the DES could have interacted, forming reverse micellar structures (Fig. 7). These micelles, present in the DES fraction, could encapsulate alginate, thereby reducing the alginate yield in the aqueous phase. The formation and release of reverse micelles may account for the fluctuations in extraction yield across the extraction cycles.

Table 3
Approximate concentrations of alginate, chlorophyll a, and fucoxanthin after seven extraction cycles with DES1.

Target analyte	Alginate	Chlorophyll *a*	Fucoxanthin
mg/g DW	118.0 ± 22.4	1.98 ± 0.04	$1.4*10^{-1}$

Given the low total lipid content (0.69–1.88 % per DW) [83] and protein content (5–13 % per DW) [15] in brown seaweed, reverse micelle formation is likely primarily driven by the addition of fatty acids. To evaluate the role of fatty acids in this system, a different hydrophobic DES without fatty acids was used in TPP. When TPP extraction was performed with menthol: thymol (1:1), a different trend emerged when the DES was reused five times (Figure S8). The alginate extraction yield decreased from cycles 1–2 (95.9 ± 4.8 mg/g to 87.4 ± 5.5 mg/g) and did not significantly change between cycles 3 and 5 (Figure S8). No fluctuations in alginate yield were observed, likely due to the absence of fatty acids. The decrease in yield between cycles 1 and 3 is probably attributed to the accumulation of impurities in the DES. The stable yield observed in cycles 3–5 suggests that alginate may have been encapsulated when using fatty acid-based DES, possibly enhanced by phospholipid release from the seaweed. To overcome this limitation, reverse micelle structures can be destructed [80]. Through basification, the fatty acids can form a homogeneous aqueous phase, thereby releasing the micelle structure [63]. This, in turn, could result in alginate release. The DES can be recovered through subsequent acidification and reused within the TPP extraction.

Biodegradable fatty acids and terpenes are relatively inexpensive compounds and display low solubility in water, making them suitable, bio-based hydrophobic extraction solvents [30,84]. Moreover, they can be easily reused and facilitate simultaneous pigment extraction in three-phase partitioning. Overall, natural hydrophobic DES based on fatty acids are suggested to be effective both as biomass pretreatment for alginate release from the cell wall and as an extraction solvent for pigments. For the first time, a DES-based TPP was employed to enable a

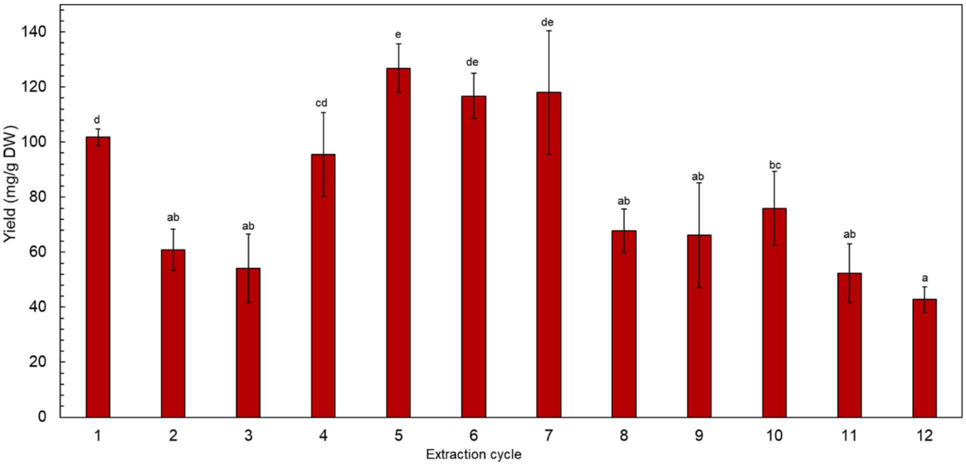

Fig. 6. Extraction yield of alginate when reusing the DES fraction twelve times.

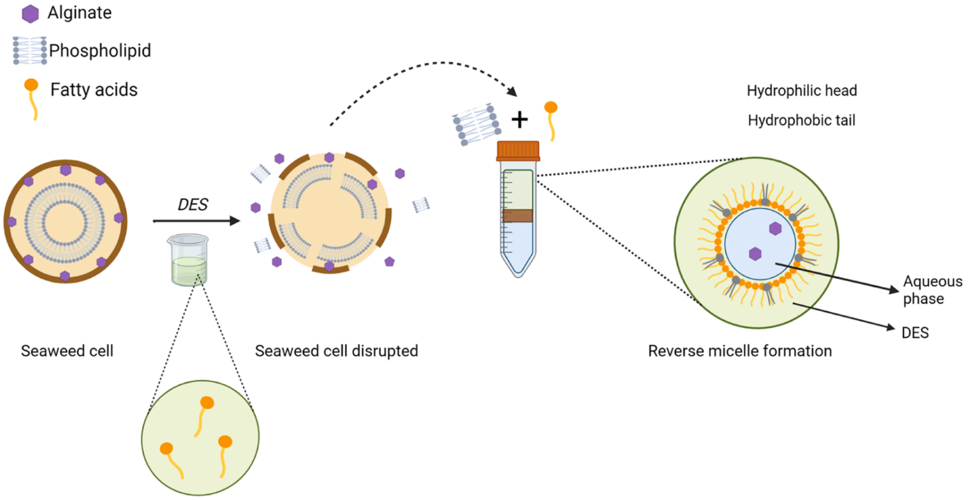

Fig. 7. Proposed TPP extraction mechanism with fatty acid-based DES.

multi-product biorefinery, wherein the DES served a dual function as both pretreatment agent and extractant. Reuse of the DES up to seven cycles resulted in a relatively stable alginate yield and pigment saturation, demonstrating its potential for sustainable and efficient resource utilisation.

4. Conclusion

This study investigated the use of natural deep eutectic solvents (DES) in three-phase partitioning (TPP) as a novel and mild approach for simultaneous extraction and purification of alginate and pigments from *Saccharina latissima* as an alternative to conventional alkaline processing. A multi-product DES-based TPP was employed, with DES functioning as both a pretreatment and extraction solvent. Alginate extraction showed that hydrophobic DES disrupted the algal cell wall, releasing alginate in the aqueous phase while extracting pigments with DES. DES1 (dodecanoic acid: octanoic acid) yielded the highest alginate output (55.7 ± 6.4 mg/g DW). A short extraction time (10 min) at a low temperature (35 °C) improved alginate yield to 101.8 ± 3.1 mg/g DW. The DES proved reusable for up to seven extraction cycles, although partial alginate transfer into the DES phase was observed due to reverse micelle formation. This work highlights the multi-product potential of the DES-based TPP system, improving economic viability through milder and shorter extraction conditions while facilitating the recovery of both alginate and pigments, along with DES reuse.

CRediT authorship contribution statement

Antoinette Kazbar: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Wijffels Rene H.:** Validation, Supervision. **Marco Bravi:** Validation, Investigation. **Michel H.M. Eppink:** Validation, Supervision. **Hiemstra Isa Sara Aimee:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.119184.

Data availability

Data will be made available on request.

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