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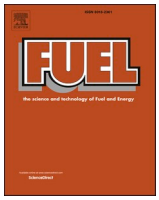

Full Length Article

# Effect of ulvan extraction and process temperature on dark fermentation of the marine macroalgae *Ulva*

James Lawrence [a,b,*], Armando Oliva [a,b], Jerry D. Murphy [c,d], Piet N.L. Lens [a,d]

[a] University of Galway, University Road, H91 TK33 Galway, Ireland
[b] Department of Civil, Architectural and Environmental Engineering, University of Naples Federico II, Via Claudio 21, 80125 Naples, Italy
[c] Civil, Structural and Environmental Engineering, School of Engineering and Architecture, University College Cork, Ireland
[d] Science Foundation Ireland MaREI Centre for Energy, Climate and Marine, Ireland

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ABSTRACT

Investigation of circular bioeconomy renewable fuel systems is currently a key area of research due to climate change. Biohydrogen production through dark fermentation of biomass wastes offers a pathway to renewable fuel, with numerous pretreatment techniques enabling the enhancement of this biological process. The macroalgal species *Ulva* offers significant potential as it contains the polysaccharide ulvan within the cell wall, which has uses in the medical industry due to its antiviral, antibacterial and anticancer activities. This study aims to investigate the impact of ulvan extraction and lyophilisation on the biohydrogen potential of *Ulva* biomass at different process temperatures (25, 37 and 55 °C). Among the investigated conditions, ulvan-extracted *Ulva* was the most promising biomass in terms of $H_2$ production, achieving 12.8 ± 0.2, 15.0 ± 1.2, and 15.3 ± 1.5 mL $H_2$/g VS at 25, 37, and 55 °C, respectively. The return of $H_2$ at thermophilic conditions was negligibly higher than 25 °C and 37 °C. Thus, it is more energy-beneficial to operate at lower temperatures as the investment of energy to elevate the temperature does not equate to the return of $H_2$ obtained. In terms of volatile fatty acids, a peak concentration of 783.7 ± 31.8 mg $HAc_{eq}$/L was observed at the end of the incubation of ulvan-extracted *Ulva* at 55 °C. On the other hand, the highest concentration of carbohydrates was obtained with lyophilised *Ulva* at 25 °C 1381.5 ± 141.1 mg/L. This data indicates that the removal of the polysaccharide ulvan did not hinder the biohydrogen potential of *Ulva* due to the effect of the pretreatment, both physical and chemical, intrinsic to the ulvan extraction process. Furthermore, this study highlights the biorefinery potential of *Ulva* biomass, aiming at maximising the bioproduct recovery from this macroalgae species, such as carbohydrates and volatile fatty acids.

## 1. Introduction

The use of alternative feedstocks to produce advanced biofuels in circular economy systems has gained interest in the research world over recent years. The heavy energy demand required for biofuel conversion processes suggests that first-generation and second-generation biofuels have an uncertain future [1]. This led to third-generation biofuels obtained from algae offering a solution to finite arable land [2] while also not contributing to excessive water usage or the need for pesticides. Macro and microalgae are viable substrates in producing various biofuels [3,4] with fast algal growth leading to high rates of renewable fuel production [5]. In particular, gaseous biofuels such as hydrogen gas ($H_2$) can be produced from algae more effectively than other biomass sources by dark fermentation [6]. The macroalgae species *Ulva* contains low lignin levels (5 %) [7], meaning the energy conversion process does not demand as many chemicals and energy for pretreatment as compared to other lignocellulosic feedstocks such as straw and wood residues [8]. Thus, more cost-intensive pretreatment methods such as thermochemical, catalytic, and enzymatic processes were not considered [9]. This low level of energy demand is very beneficial for the $H_2$ production pathway, especially as compared to more energy-intensive high-carbon methods such as steam reforming of methane ($CH_4$) [10].

Fermentative $H_2$ production methods require mild conditions for the bioreactions, which is not the case for conventional processes like water electrolysis or $CH_4$ reforming. Dark fermentation is a favourable method for biohydrogen production due to its high production rate [11]. It is carried out by obligate and facultative anaerobes without the requirement of light [12]. Following the hydrolysis of complex polymers, $H_2$ is

* Corresponding author at: Department of Microbiology and Ryan Institute, University of Galway, University Road, H91 TK33 Galway, Ireland.
*E-mail address:* jameslawrence172@gmail.com (J. Lawrence).

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produced through the acetate or butyrate pathway, or both [13]. Despite the limited lignin content, macroalgae contain other recalcitrant organic compounds such as polyphenols, the concentration of which varies depending on several factors such as species and environmental growth conditions [14]. Due to microbes being unable to readily digest such compounds, their presence in the fermentation stage can result in decreased biomass degradability and ultimately limit the $H_2$ yield [15].

Macroalgae are typically classified by their colour, i.e., red, brown, and green. The algae cell wall (specifically in brown algae) has a rigid structure composed of cellulose and proteins, that functions as a barrier and separates microorganisms from the biodegradable cell content [16]. Similarly, green algae have a firm cell wall made up of cellulose and other polysaccharides. For this, macroalgae are often considered a more difficult feedstock to work with compared to microalgae due to their complex cell walls [17]. Green algae are the most diverse algae group, accounting for approximately 7000 species [18]. Their wide availability makes them a favourable substrate for biofuel production [19]. Macroalgae have potential in terms of biohydrogen production due to their high carbohydrate content, approximately 50 % of the algae biomass [20]. Furthermore, algae have a reduced hemicellulose content compared to other biomass types, allowing for a more efficient fermentation process [21]. Macroalgae have a short harvesting cycle, making the turnover rate for growth much quicker than other biomasses [22].

One such species that has shown potential for biorefinery applications is the green seaweed *Ulva* sp. [23], also commonly referred to as sea lettuce. *Ulva* sp. can tolerate a variety of environmental conditions and climates. This stability has made them perfect candidates for biotechnological processes such as biofuel production [24]. This macroalgae species can thrive in different water environments, including saltwater, wastewater, and some cases even freshwater [25]. This wide range of habitats makes them easier to cultivate and harvest [26]. Further interest in terms of the valorisation of *Ulva* sp. is the presence of the polysaccharide ulvan [27]. Ulvan has a variety of uses in sectors such as the pharmaceutical industry, and it is considered a strategic alternative to the use of fully synthetic materials, particularly in the design of tissue engineering procedures [28]. Such advantages offer a clear incentive for the extraction and utilisation of this value-added product prior to processing the *Ulva* biomass.

This study aims to unlock the full potential of *Ulva* utilisation by exploring different approaches to maximise high-value product recovery from this substrate. In particular, the impact of extracting the cell wall polysaccharide ulvan on the macroalgae biomass in terms of value-added product yield was examined. The experimental activities investigated the effects of process temperature, ulvan extraction and lyophilisation, i.e. freeze drying, a low-temperature dehydration process which involves freezing the seaweed and lowering pressure without destroying its physical structure, on the biohydrogen yield obtained from dark fermentation of the pretreated *Ulva* sp. In addition, the changes in carbohydrate availability and volatile fatty acids (VFAs) concentration during the fermentative process were studied. The experimental batch studies were conducted at 25, 37, and 55 °C as a further operating aspect that can maximise the valorisation of this substrate. The *Ulva* biomass was subject to a variety of pretreatment techniques featuring lyophilisation, milling and acid-thermal treatment as part of the ulvan extraction process.

## 2. Materials and methods

### 2.1. Source of biomass and inoculum

The substrate used was composed of variations of *Ulva* seaweed which was harvested in August 2021 on the Spiddal coast (53° 24′ 00.6″ N−9° 31′ 20.2″ W) in Co. Galway (Ireland). The samples were composed of the species *Ulva lacinulata* and *Ulva rigida*, identified through the Cleaved Amplified Polymorphic Sequences (CAPS) assay [29]. Both *Ulva lacinulata* and *Ulva rigida* are foliose species showing subtle differences morphologically hence molecular identification was utilised for identification. After collection, the biomass was rinsed with tap water to remove sand and salt attached to the biomass. The total solid (TS) and volatile solids (VS) content of the *Ulva* was 13.6 % and 9.3 % respectively, with a VS/TS ratio of 68 % [30].

The steps involved in the preparation of the substrate are summarised in Table 1. Any excess water was removed by gently squeezing the *Ulva* biomass before storage at −40 °C. Following storage at −40 °C, the *Ulva* biomass which was to undergo lyophilisation was stored at −80 °C for 24 h. Freeze drying took place for 5 days to ensure complete moisture content removal. Following this, the macroalgae was broken down to a size of 4–5 mm using a 2-speed Laboratory Blender (Waring, USA). The ulvan-extracted biomass was obtained following the protocol proposed by Manikandan and Lens [27]. This process involved mixing the lyophilised *Ulva* biomass with a 0.1 M hydrochloric acid solution before submersion in a water bath at 90 °C for two hours. Following this, the solid fraction was utilised in the biohydrogen production tests.

The inoculum used was an anaerobic dairy granular sludge from an up-flow anaerobic sludge bed (UASB) reactor at a wastewater treatment plant at Arrabawn dairy farms (Killconnell, Co. Galway, Ireland). The sludge was collected from a 200 $m^3$ UASB reactor treating dairy wastewater at 20 °C with a hydraulic retention time (HRT) of 9–12 h. The TS and VS content in the inoculum after the heat shock were 6.9 % and 5.9 %, respectively, corresponding to a VS/TS ratio of 86 % [30]. Pretreatment of the inoculum was carried out at 100 °C for 1 h to inhibit methanogenic bacteria present in the dairy sludge [31]. The procedure was carried out on a heat plate to allow for agitation and the inoculum to homogenise.

### 2.2. Activity tests

The dark fermentation process was carried out using 250 mL serum bottles. The activity tests were performed to measure the volumes of biohydrogen produced through dark fermentation of each substrate at different temperatures, i.e., 25, 37 and 55 °C. Each bottle was loaded with 2.0 g volatile solids (VS) of *Ulva* feedstock and 1.0 g VS anaerobic granular sludge, thus keeping a substrate-to-inoculum ratio of 2:1 in terms of grams VS [32]. Distilled water was added to reach a working volume of 150 mL and a headspace volume of 100 mL. The initial pH of each bottle was brought to 7.5 using a 1 M HCl or NaOH solution. Tests were carried out in triplicate for each condition, with the varying substrates being raw *Ulva*, lyophilized *Ulva*, and ulvan-extracted *Ulva* (Table 1). Control tests containing solely the inoculum and demineralised water were also carried out. The bottles were sealed using a rubber bung and an aluminium steel cap. Each bottle was fitted with a 5 cm needle and a pressure valve for the withdrawal of liquid and gas samples. The bottles were then attached to a gas exchanger and purged with nitrogen gas ($N_2$) for 10 min to ensure anaerobic conditions in the bottles.

The activity tests were monitored for up to 4 days with gaseous samples taken at multiple points throughout the day, whereas the liquid phase was sampled daily. The volume sampled was taken into consideration in the calculation for gas production in relation to an increasing headspace following the sampling of the liquid fraction. The final gaseous sampling took place at hour 88 for the tests at 25 °C and 55 °C and at hour 72 for the tests conducted at 37 °C. Experiments were stopped on the 3rd/4th day of dark fermentation due to issues regarding $H_2$ consumption which can result in the microbes consuming $H_2$ and ultimately leading to an unstable $H_2$ production rate [33]. Gas and liquid samples were taken by attaching a syringe to the open-air valves of each bottle. The pressure in the bottles was released through the air valve every time the sampling was completed to ensure each bottle was returned to atmospheric pressure. Interval gas release can be beneficial for $H_2$ production while also leading to high acetic and butyric acid accumulation, showing similar results to constant gas release [34]. The

**Table 1**
Pretreatment and storage steps (marked with **x**) of the *Ulva* biomass. Arrows (→) indicate the order of each of the steps in the pretreatment process.

| Substrate | Rinsing and squeezing | → | Storage at −40 °C | → | Storage at −80 °C | → | Freeze drying | → | Grinding | → | Ulvan extraction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw *Ulva* | x | | x | | | | | | | | |
| Lyophilised *Ulva* | x | | x | | x | | x | | x | | |
| Ulvan-extracted *Ulva* | x | | x | | x | | x | | x | | x |

pressure reading before sample withdrawal and after headspace release was performed using a Leo 1 pressure gauge (Keller, Winterthur, Switzerland) and recorded to be used in the calculation of total gas production as described previously by Oliva et al. [35].

### 2.3. Carbon recovery

A carbon (C) balance among the products generated during the dark fermentation process was performed considering $CO_2$, carbohydrate, and VFAs as the main C-rich products. All products were expressed as mg per litre of working volume. $CO_2$ (mL/g VS) production was converted to mg/L by considering the g VS from *Ulva* fed into the bottles and the density of $CO_2$ at 20 °C (i.e., 1.8393 kg/m$^3$) [36]. The carbon aliquot from carbohydrates was calculated assuming all the carbohydrates as glucose ($C_6H_{12}O_6$). The carbon share from VFAs was calculated considering the VFA speciation.

### 2.4. Analytical methods

A pH reading was taken on the liquid samples on each day of the activity tests. The pH was measured using a pH controller (pH/ORP 300, Cole-Parmer, Vernon Hills, USA) which was connected to a Hamilton SlimTrode electrode (Darmstadt, Germany).

Total carbohydrate measurements were carried out on the liquid samples to evaluate the ability of the inoculum to ferment the carbohydrates hydrolysed from the substrates. The liquid samples from days 0, 1, 2, 3, and 4 from each bottle were analysed after centrifugation (Eppendorf, Hamburg, Germany) at 12000 rpm to remove the solid fraction. Each sample was then passed through a 0.45 µm filter to further remove all solids. The samples were mixed with a 5 % phenol solution and sulfuric acid, and subsequently incubated at 100 °C following the Dubois method (Dubois, 1956). The carbohydrate content was assessed by measuring the absorbance at 492 nm using a UV–VIS spectrophotometer UV-1900 (Shimadzu, Kyoto, Japan) with the analysis carried out in triplicate.

VFAs analysis was carried out on liquid samples taken from the bottles as described by Oliva et al. [35] using a 1260 Infinity II high-performance liquid chromatograph (HPLC) (Agilent, Santa Clara, USA) equipped with a Hi-Plex H (300 × 7.7 mm) column, heated at 60 °C and a refractive index detector (RID) set at 55 °C. A 0.005 M $H_2SO_4$ solution was used as the mobile phase at a flow rate of 0.7 mL/min. Before analysis, the liquid samples were centrifuged for 12 min at 14500 rpm and filtered with 0.22 µm polyethersulfone membranes (Filtropur S 0.2, Sarstedt, Germany).

The gas composition was evaluated using a 7890B gas chromatograph (Agilent, Santa Clara, USA) equipped with a thermal conductivity detector heated at 250 °C, able to detect $CH_4$, $CO_2$, $N_2$, $O_2$, and $H_2$ as described by Braga and Lens [30]. Helium was used as the carrier gas with a flow rate of 10 mL/min. Specific gas production was calculated by subtracting the aliquot produced by the controls and then dividing the net production by the input grams VS from the substrate.

### 2.5. Statistical comparison

Statistical comparison of the cumulative hydrogen production from raw and pretreated *Ulva* for each of the process temperatures was performed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. All analyses were performed with Minitab 17 Statistical Software (Minitab LCC, Chicago, IL, USA), where a difference marked with a p-value lower than 0.05 was considered statistically significant. Statistical analysis is reported in the supplementary materials accompanying the manuscript (Table S1).

## 3. Results

### 3.1. Carbohydrate availability

Fig. 1 shows the carbohydrate accumulation throughout the dark fermentation process carried out at 25 (Fig. 1A), 37 (Fig. 1B), and 55

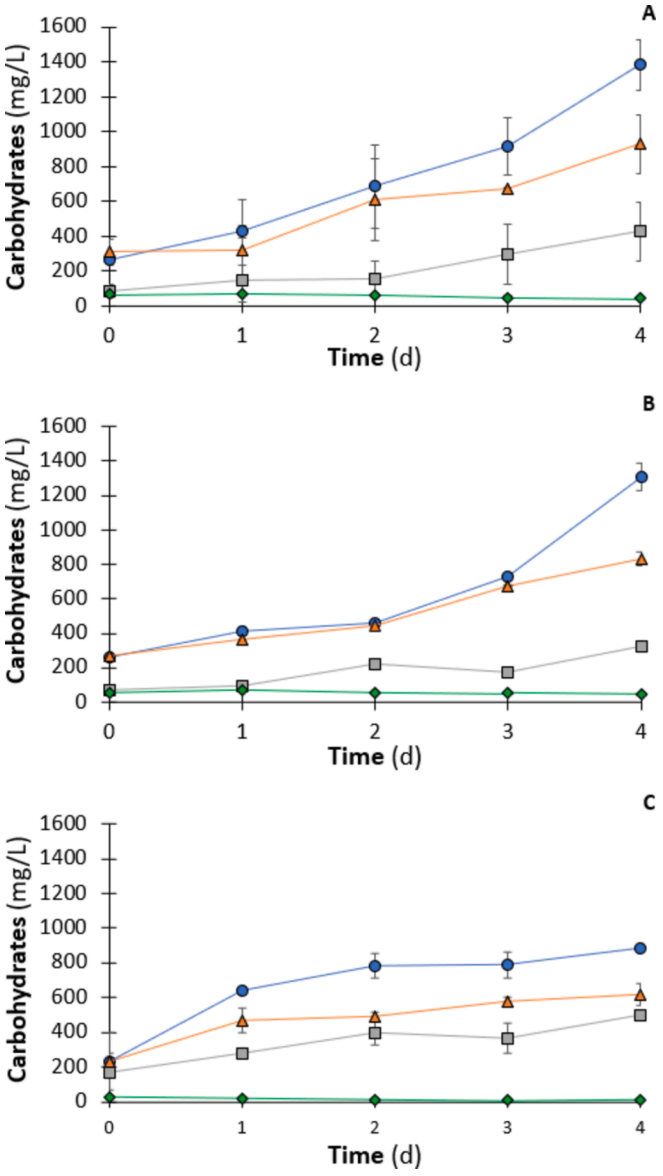


**Fig. 1.** Carbohydrates present in the fermentation broth along the dark fermentation process at 25 °C (A), 37 °C (B), and 55 °C (C). Lyophilised *Ulva* (●), ulvan-extracted *Ulva* (▲), raw *Ulva* (■), and control test (◆).

(Fig. 1C) °C. Carbohydrates followed a similar trend across substrates for each of the temperatures. The highest carbohydrate concentrations for lyophilised *Ulva* were measured on day four at each temperature, i.e., 1381.5 ± 141.1 mg/L at 25 °C, 1311.1 ± 79.1 mg/L at 37 °C, and 886.7 ± 9.7 mg/L at 55 °C. The second highest carbohydrate concentration was obtained with the ulvan-extracted samples following the same trend at each temperature, i.e., 928.8 ± 170.5 mg/L at 25 °C, 832.5 ± 36.5 mg/L at 37 °C, and 618.3 ± 59.2 mg/L at 55 °C. The raw *Ulva* fermentative tests gave the lowest carbohydrate concentration across the studied temperatures, i.e., 426.1 ± 168.5 mg/L at 25 °C, 329.4 ± 18.9 mg/L at 37 °C, and 497.9 ± 12.1 mg/L at 55 °C. Carbohydrates in each of the control samples (inoculum only) were negligible.

### 3.2. Gas analysis

Gas analysis was carried out to determine both gas composition and overall gas volume produced during the dark fermentation process (Fig. 2). The gas composition was made up of $H_2$ and $CO_2$, whereas $CH_4$ was not observed in any of the incubations. The three sets of batch experiments followed a similar trend in terms of cumulative biohydrogen production for the ulvan-extracted biomass, each reaching its highest volume at the final sampling point, i.e., 12.8 ± 0.2 mL $H_2$/g VS at 25 °C, 15.0 ± 1.2 mL $H_2$/g VS at 37 °C, and 15.3 ± 1.5 mL $H_2$/g VS at 55 °C. The cumulative biohydrogen production from the lyophilised *Ulva* was significantly lower than ulvan-extracted *Ulva* ($p < 0.05$), peaking at 1.7 ± 0.1 mL $H_2$/g VS at 25 °C, 5.9 ± 1.2 mL $H_2$/g VS at 37 °C, and 0.9 ± 0.1 mL $H_2$/g VS at 55 °C. Minimal $H_2$ production was observed in the raw *Ulva* samples fermenting at 37 °C, whereas the $H_2$ production from raw *Ulva* was higher ($p < 0.05$) at 25 and 55 °C (Fig. 2A-C). When examining the biogas composition produced from ulvan-extracted *Ulva,* the $H_2$ content recorded was 44 % at 25 °C, 56 % at 37 °C, and 52 % at 55 °C (Table 2). Following statistical analysis, no significant difference ($p > 0.05$) was found among process temperatures for the ulvan-extracted *Ulva* in terms of $H_2$ production, despite 37 and 55 °C being beneficial to achieve $H_2$-rich biogas production. On the other hand, 37 °C was the most effective ($p < 0.05$) temperature to produce biohydrogen from lyophilised *Ulva* (Table 2). Overall, the ulvan-extracted *Ulva* performed optimally across each process temperature in terms of $H_2$ production.

The gas composition analysis showed that $CO_2$ was produced in a larger quantity in comparison to $H_2$ (Table 2). In relation to $CO_2$ production, peaks were observed at 16.6 ± 0.8 mL $CO_2$/g VS at 25 °C (Fig. 2D) in the ulvan-extracted bottles and 17.5 ± 0.5 mL $CO_2$/g VS at 55 °C from the raw *Ulva* (Fig. 2F). At 37 °C, $CO_2$ production peaked at 11.8 ± 0.6 mL $CO_2$/g VS in the ulvan-extracted bottles (Fig. 2E). The untreated substrate followed the same trend when dark fermentation was operated at 25 and 55 °C, recording the highest overall $CO_2$ yield at 55 °C, i.e., 17.5 ± 0.5 mL $CO_2$/g VS. This trend in $CO_2$ production was not observed at 37 °C with the cumulative production in the raw samples being 3 ± 1.1 mL $CO_2$/g VS.

**Table 2**
Biogas yield and percentage of $H_2$ and $CO_2$ present in the biogas at the final sampling point, i.e., hour 88 at 25 and 55 °C and hour 72 at 37 °C.

| Substrate | Incubation temperature(°C) | Biogas yield ($H_2$ + $CO_2$) (mL/g VS) | Biogas composition | |
|---|---|---|---|---|
| | | | $H_2$ (%) | $CO_2$ (%) |
| Ulvan-extracted *Ulva* | 25 | 29.4 ± 0.2 | 44 | 56 |
| | 37 | 27.1 ± 1.0 | 56 | 44 |
| | 55 | 29.4 ± 1.6 | 52 | 48 |
| Lyophilised *Ulva* | 25 | 5.8 ± 0.4 | 29 | 71 |
| | 37 | 16.3 ± 2.1 | 37 | 63 |
| | 55 | 3.7 ± 1.0 | 23 | 77 |
| Raw *Ulva* | 25 | 17.6 ± 0.9 | 24 | 76 |
| | 37 | 3.5 ± 1.2 | 15 | 85 |
| | 55 | 19.1 ± 0.9 | 8 | 92 |

### 3.3. Volatile fatty acids profile

The dominant VFAs produced during the dark fermentation process were acetic, propionic, and butyric acid (Fig. 3). Fig. 3 shows the total VFAs produced for each of the pretreatment conditions at 25 (Fig. 3 A-D), 37 (Fig. 3 E-H), and 55 °C (Fig. 3 I-L). The control for each experiment produced minimal VFAs at each condition due to the lack of substrate (*Ulva* biomass). At each temperature, the ulvan-extracted biomass gave the highest yield of total VFAs with the peak concentrations observed at the end of the incubation, being 666.6 ± 76.0 mg $HAc_{eq}$/L at 25 °C, 610.7 ± 66.9 mg $HAc_{eq}$/L at 37 °C, and 783.7 ± 31.8 mg $HAc_{eq}$/L at 55 °C. The lyophilised *Ulva* sp. exhibited the lowest VFAs concentration, i.e., 151.9 ± 31.96, 188.9 ± 41.7, and 295.4 ± 27.1 mg $HAc_{eq}$/L, at 25, 37, and 55 °C, respectively. The raw *Ulva* sp. showed maximum concentrations of 328.1 ± 52.7, 295.9 ± 37.8, and 312.5 ± 52.6 mg $HAc_{eq}$/L, respectively, at 25, 37, and 55 °C during the incubation.

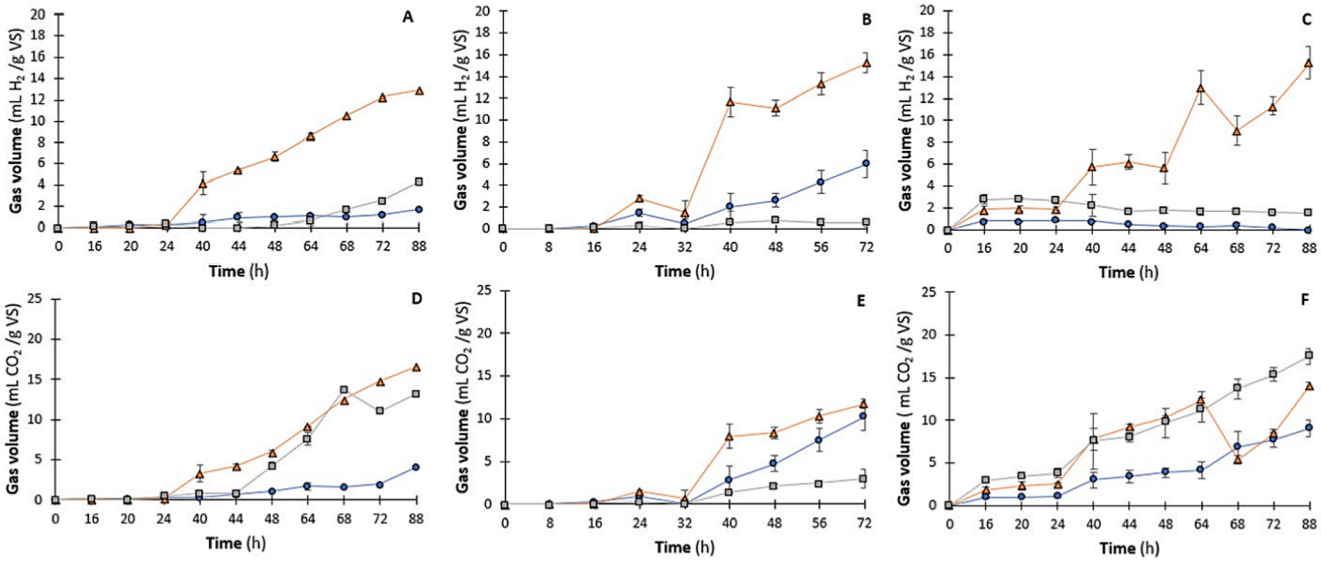


**Fig. 2.** Cumulative hydrogen (A, B, C) and carbon dioxide (D, E, F) production at 25 °C (left column), 37 °C (middle column), and 55 °C (right column). Lyophilised *Ulva* (●), ulvan-extracted *Ulva* (▲), and raw *Ulva* (■).

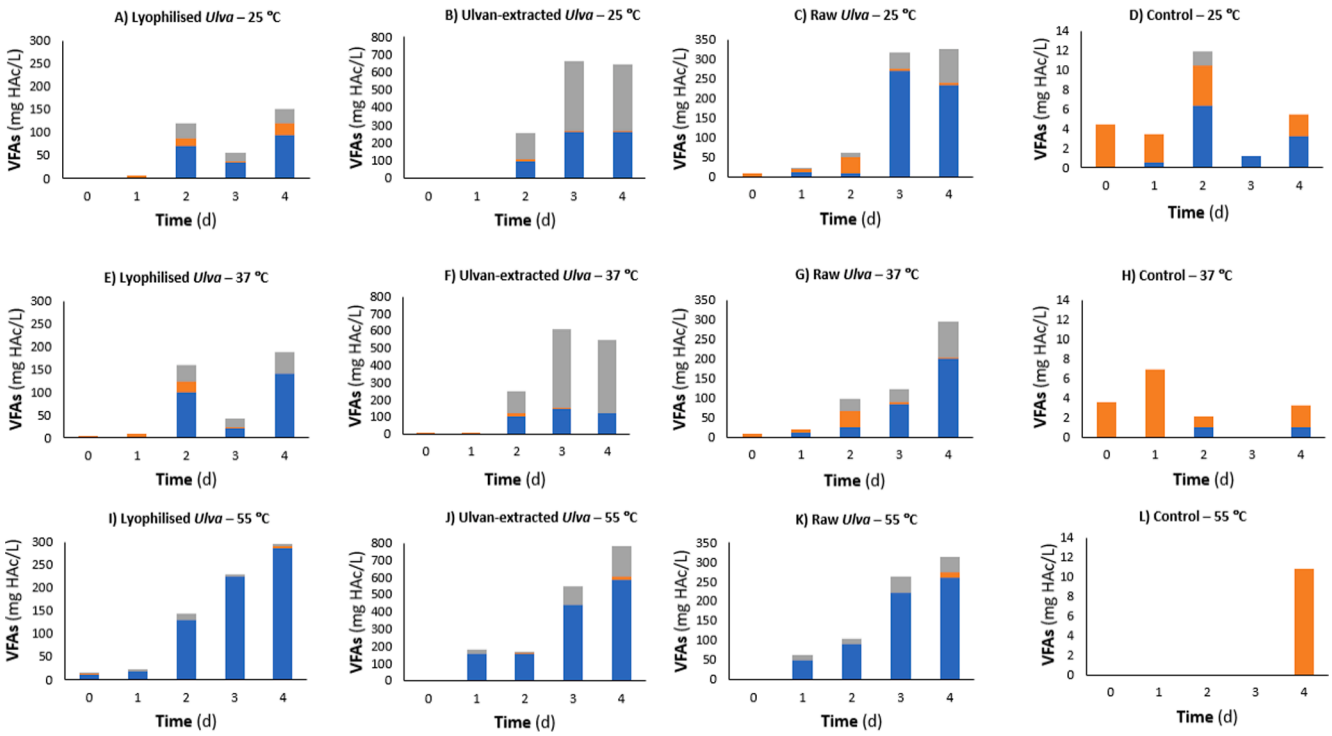


**Fig. 3.** Volatile fatty acids (VFAs) profile observed at 25 °C (top row), 37 °C (middle row), and 55 °C (bottom row) while fermenting lyophilised *Ulva* (A, E, and I), ulvan-extracted *Ulva* (B, F, and J), raw *Ulva* (C, G, and K) and in control tests (D, H, and L). Acetic (◆), propionic (■), and butyric (■) acids.

### 3.4. Carbon recovery

Carbon recovery calculations were carried out to quantify both the carbon recovery percentage (Fig. 4A) and the absolute amount (mg/L) of carbon recovered (Fig. 4B). Ulvan-extracted *Ulva* performed similarly at each temperature returning the highest amount of carbon recovery from VFAs at each temperature (410.7 mg/L at 25 °C, 387.0 mg/L at 37 °C, and 387.4 mg/L at 55 °C). In contrast, carbon recovery from VFAs was minimal when fermenting lyophilised *Ulva* (93.2 mg/L at 25 °C, 94.5 mg/L at 37 °C, and 120.6 mg/L at 55 °C). Although the percentage recovery of VFAs of the raw *Ulva* at 37 °C was the highest (50.9 %), its overall return in terms of mg/L was low (156.1 mg/L) compared to the ulvan-extracted *Ulva*. In terms of carbon recovery from carbohydrates, the lyophilised *Ulva* returned the highest percentage across each temperature (81.8 % at 25 °C, 75.0 % at 37 °C, and 64.3 % at 55 °C). Recovery from $CO_2$ returned the lowest value in terms of mg/L across each substrate, following the same trends for each of the examined temperatures.

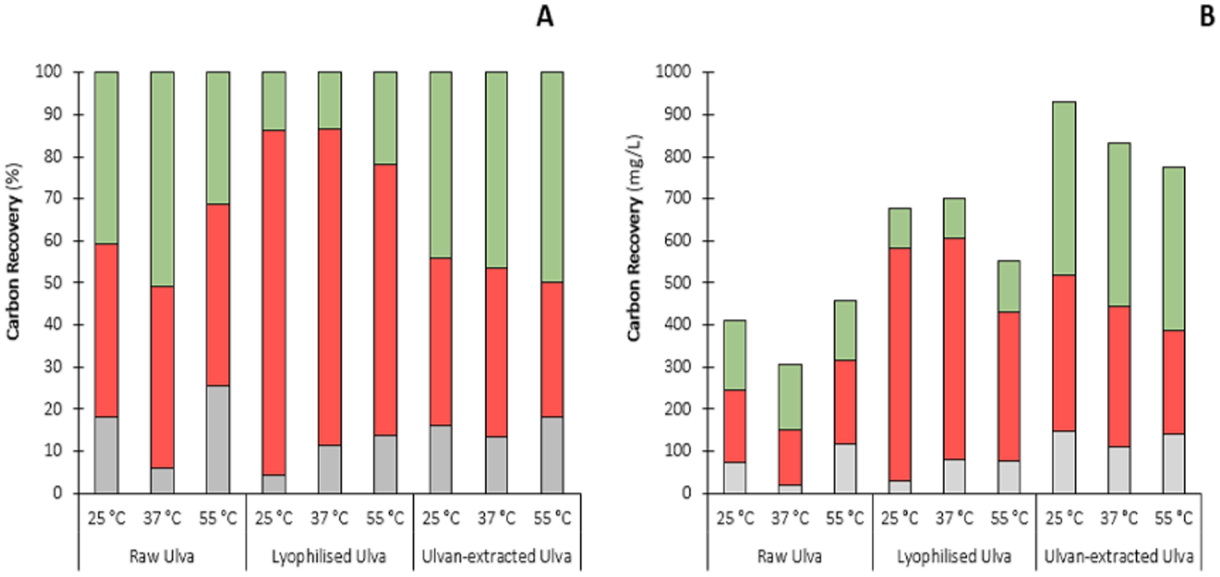


**Fig. 4.** Carbon recovery from raw, lyophilised, and ulvan-extracted *Ulva* expressed as a percentage of the total carbon (A) and as concentration (mg carbon/L) observed in the fermentation broth (B). $CO_2$ (■), carbohydrates (■), and volatile fatty acids (■).

## 4. Discussion

### 4.1. Hydrogen production from Ulva sp.

This study is, to the best of our knowledge, the first to report the impact ulvan extraction has on the dark fermentation of *Ulva* sp., while also looking at the biorefinery benefits of extracting the polysaccharide ulvan and the possibilities for obtaining value-added products alongside $H_2$ production. Fig. 2 and Table 2 showed that ulvan extraction had no negative impact on biohydrogen potential. On the contrary, the highest $H_2$ yield produced at each temperature was achieved by the ulvan-extracted biomass, as this extraction procedure involves multiple steps that can also be considered a pretreatment. In addition, the ulvan-extracted *Ulva* also performed better as seen by the lower $CO_2$ percentage (Table 2). Kidgell et al. [37] recommended acid extraction of ulvan which in turn had a positive effect on the residual biomass. Thus, this method was chosen for the current study as a means of enhancing biomass biodegradation during the dark fermentation process. The polysaccharide ulvan provides numerous biological activities that ensure benefits to its extraction. Immunomodulating [38], antioxidant [39], cytotoxicity [40], and anticoagulant [41] activities all provide benefits of ulvan, thus its extraction before the use of the leftover biomass in a dark fermentation process provides economic incentives. Braga and Lens [42] showed that the sulfate present in ulvan can impede the dark fermentation process because the microbial community's sulfate-reducing bacteria can reduce the sulfate both heterotrophically—using soluble metabolites through the incomplete sulfate reduction pathway—and autotrophically—using $H_2$ as the electron donor thus reiterating positives for its extraction. The economic and environmental benefits of utilising *Ulva* sp. [43,44] highlight further the importance of coupling the ulvan extraction to dark fermentation of residual biomass as reported in this study.

While dark fermentation is a successful method for converting natural biomass and organic waste into biofuels with particular attention to $H_2$ [45], it is clear that the choice of feedstock and the inoculum used are highly important. Table 3 summarises the results achieved in terms of mesophilic and thermophilic biohydrogen production from *Ulva* biomass. Acid-treated *Ulva* sp. has been used for the first time as a substrate for $H_2$ production by Margareta et al. [6]. They highlighted the difficulties related to *Clostridia* sp.'s ability to utilise the reducing sugar xylose as the substrate, thus producing only low amounts of $H_2$. In contrast, many studies have reported successful $H_2$ production from both brown and red macroalgae [46,47]. These difficulties were also apparent in the current study as seen by the low specific yield of $H_2$ production of raw *Ulva*. Considering this, a change in pretreatment technique could be beneficial to allow for further acclimatisation of the inoculum and towards enhanced microbial diversity for $H_2$ production without methanogenesis thus allowing for a shorter incubation period. Acid treatment [48], aeration [49] and ultrasonic treatment [50] are alternative methods of pretreatment that both suppress methanogens and enhance $H_2$ production. Further studies have demonstrated the need for a combination of substrate pretreatment methods to enhance biohydrogen production [51]. A recent study showed that particle size reduction alone is insufficient for the enhancement of biohydrogen production from *Ulva* sp. Dung et al. [52] showed that a combination of particle size reduction followed by acid treatment produced the most $H_2$ (i.e., 60 mL $H_2$/g COD), an 81 % increase in comparison to untreated *Ulva reticulata*. This aspect is confirmed in the present study since the ulvan-extracted biomass showed a higher $H_2$ yield across each of the temperatures investigated (Fig. 2 and Table 2).

In the analysis of the $H_2$ yield in this study, thermophilic conditions were successful in terms of $H_2$ production. Nevertheless, compositional analysis of the biogas showed that 37 °C gave the highest $H_2$ percentage, i.e. 56 % (Table 2). Although it is reported in the literature that the optimum temperature for dark fermentation is 37 °C [53], higher-temperature reactors can enhance the fermentation performance by promoting hydrolysis and decreasing $H_2$ solubility in the fermentation broth due to the higher temperature inhibiting non-hydrogen-producing microorganisms [54]. This is highlighted in the present study with the overall $H_2$ production peaking at 55 °C with 15.3 ± 1.5 mL $H_2$/g VS (Fig. 2C) and a cumulative $CO_2$ yield of 14.1 ± 0.5 mL $CO_2$/g VS. A further confirmation of these findings is reported by Valdez-Vazquez [55], who showed that thermophilic conditions perform more efficiently in terms of $H_2$ production. Further research at thermophilic temperatures found that two times more extracellular enzymes can form in thermophilic bacteria than in their mesophilic counterparts. These extra enzymes led to a greatly improved hydrolysis rate that can speed up the entire process [56]. The present study reports *Ulva* sp. to possess low biohydrogen potential but has ample possibilities from a biorefinery viewpoint. Given the feedstock demands and expensive pretreatment methods [57], it is pivotal to focus on multiple product recovery streams to realise the full value of *Ulva* sp. as a feedstock. In addition, the return of $H_2$ at thermophilic conditions was negligibly higher than 25 °C and 37 °C. Thus, it is more energy-beneficial to operate at lower temperatures as the investment of energy to elevate the temperature does not equate to the return of $H_2$ obtained.

### 4.2. Carbohydrate release during Ulva sp. pretreatment

Lyophilisation and ulvan extraction had a clear impact on

**Table 3**
Comparison of hydrogen yields obtained from different *Ulva* sp. under different pretreatment conditions.

| Seaweed | Inoculum | Pretreatment | Biohydrogen yield (incubation temperature) | Reference |
|---|---|---|---|---|
| *Ulva fasciata* | Rumen fluid | Sonication | 40.5 mL/g COD (37 °C) | [58] |
| *Ulva fasciata* | Rumen fluid | Surfactant coupled sonication | 91.7 mL/g COD (37 °C) | [58] |
| *Ulva reticulata* | Seed slurry | Surfactant + disperser | 63 mL/g COD (37 °C) | [59] |
| *Ulva reticulata* | Digested sludge | Microwave + $H_2O_2$ + acidic | 92.5 mL/g COD (37 °C) | [60] |
| *Ulva reticulata* | Digested sludge | Surfactant-induced microwave disintegration | 54.9 mL/g COD (37 °C) | [61] |
| *Ulva* sp. | Granular sludge | Acid thermal | 66.8 mL/g glucose (55 °C) | [30] |
| *Ulva reticulata* | Methanogenic bacteria archaea | Physical and chemical | 60.0 mL/g COD (37 °C) | [52] |
| *Ulva* sp. | Granular sludge | Ulvan extraction (i.e. physical and chemical) | 27.83 mL/g COD [a] (55 °C) | This study |

[a] The specific methane potential (mL $H_2$/g COD) was calculated from the theoretical COD [62], obtained considering the elemental analysis of Ulva reported by Kumar et al. [63].

polysaccharide release as compared to the untreated bottles (Fig. 1). This trend was visible across each temperature with both the ulvan-extracted and lyophilised samples maintaining a higher carbohydrate concentration throughout the experimental run as opposed to the raw *Ulva* incubations. In the dark fermentation process, an increased availability of carbohydrates to be metabolised is favourable in terms of the production of both gases and value-added products [64]. During the first two phases of anaerobic digestion, namely hydrolysis and acidogenesis, polymeric carbohydrates are broken down by hydrolytic enzymes to yield sugar monomers [65]. In the acidogenic phase, facultative and obligate anaerobes use up the carbohydrate monomers to produce VFAs [66]. Fig. 1 shows the abilities of the bacterial species present in the pretreated microbial inoculum to ferment and utilise the carbohydrates present, which determined the microorganism's ability to produce gaseous and acidic end-products. The gradual increase of carbohydrates presented in Fig. 1A and 1B shows that the microorganisms had not fully metabolised all the available carbohydrates, likely due to difficulties in breaking down the substrate. This is further represented by the significantly low $H_2$ yield observed across each of the substrates at all temperatures investigated (Fig. 2) in comparison to the $H_2$ yields reported in Table 3. Notably, both the growing and harvesting conditions can impact the $H_2$ potential of *Ulva* [67]. Furthermore, the cellulose content of *Ulva* (up to 19 %) [68] can also result in a lack of readily available carbohydrates following pretreatment, hence the lower concentration of carbohydrates recorded on day 0 in comparison to day 4. Cellulose is often resistant to both chemical and physical pretreatment due to its compact structure which includes hydrogen bonds between glucose monomers [69].

*Ulva* sp. is favourable due to its low lignin content, but it also possesses a complex microbial cell wall resulting in it being difficult for microbes to fully metabolise the available carbohydrates [70]. This indicates that an increased carbohydrate concentration would be favourable for high-rate dark fermentation as opposed to those obtained from the *Ulva* sp. during this experimental run. While increasing the incubation time would have allowed for further metabolisation of the carbohydrates, it could also have coincided with the onset of methanogenesis [71] and further $H_2$ consumption [33]. Moreover, $H_2$ in the headspace can be consumed by microorganisms to elongate VFAs [72]. Therefore, the incubation period was set at four days to optimise $H_2$ production as previously reported by Soares et al. [73]. Furthermore, the duration of this study was set to four days to focus on the biorefinery aspect of generating multiple products i.e. $H_2$, ulvan, VFAs, and $CO_2$, from *Ulva* biomass.

This study showed that ulvan extraction and lyophilisation had a favourable effect on the dark fermentative process, allowing an increased availability of carbohydrates compared to the raw *Ulva* (Fig. 1). Carbohydrates available in the ulvan-extracted tests were lower in comparison to lyophilisation due to the prior ulvan extraction process. On the other hand, the complex cell walls of raw *Ulva* made it difficult for the microbes present in the granular sludge to adequately access and metabolise these carbohydrates and thus successfully produce a higher $H_2$ gas yield with different types of *Ulva* sp. Due to the fact the initial carbohydrate release and conversion were quite low as opposed to alternative studies [30], possible solutions would be the addition of a nutrient medium or focusing on alternative pretreatment methods. Costa et al. [74] found that the addition of a phosphate-buffered medium enhanced biohythane production from *Sargassum* sp., achieving up to 91.3 ± 3.3 L $H_2$/kg VS of *Sargassum* sp., this $H_2$ yield being much higher than those reported in the literature (Table 3). The slow release of carbohydrates observed in the present study also reflects on the gas production which under all conditions began after only 24 h. This slow release of carbohydrates provides evidence that alternative pretreatment methods ought to be looked at. Yin and Wang [75] found that a combination of heat and acid pretreatment allowed for the highest polysaccharide release from *Saccharina japonica*, i.e., 7.9 g/L, in the liquid phase after pretreatment. This release had a further effect on the energy conversion which saw an energy conversion efficiency increase to 35.4 % due to the readily available polysaccharides. In the present study, it was observed that carbohydrate concentrations were higher at each of the lower temperatures (25 and 37 °C) in comparison to that of the thermophilic incubation (55 °C). This indicates that the fermentation process was less efficient at lower temperatures. This finding is further supported by both the $H_2$ and VFA results, thus concluding the microorganisms were not able to convert the carbohydrates to alternative metabolites before further conversion to $H_2$. Furthermore, carbohydrates can be seen to be utilised better at 55 °C illustrated by a plateau in concentration after 2 days (Fig. 1C).

Braga and Lens [30] optimised the diluted acid and thermal pretreatment approach to *Ulva* sp. A desirability function was employed to maximise the carbohydrate concentration and found that a condition of 35 % (*w/v*) wet *Ulva* sp. within a 0.3 N HCl solution for 2.5 h exposure time at 100 °C was optimal. Employing such alternative pretreatment methods in future studies can overcome the slow early release of polysaccharides in the process and allow for increased enzymatic activity to break down the more complex carbohydrates. Further confirmation of the success of the pretreatment approach in terms of releasing carbohydrates for $H_2$ production is given by Liu and Wang [76,] who examined heat, acid, ultrasonic, and alkaline treatments. The results of Liu and Wang [76] confirmed that heat, i.e., 66.7 ± 5.7 mL $H_2$/g wet weight, and acid, i.e., 43.7 ± 6.9 mL $H_2$/g wet weight, pretreatment yielded the most $H_2$.

### 4.3. VFA production from Ulva sp. during dark fermentation

During fermentation, microorganisms utilize biopolymers and in return produce VFAs. In the present study, production of acetic, butyric and propionic acid was observed at each of the examined temperatures. The results showed that the ulvan extracted *Ulva* performed optimally across each of the fermentation tests (Fig. 3) with VFA accumulation peaking on day four. In terms of speciation, acetic acid was the dominantly produced acid from each substrate other than the tests carried out on the ulvan extracted *Ulva* at 25 and 55 °C in which case a higher rate of butyric acid was produced. The maximum VFA yield of the ulvan extracted biomass amounted to 783.7 ± 31.8 mg $HAc_{eq}$/L produced at 55 °C. In comparison, Manikandan and Lens [77] obtained a maximum of 3.48 ± 0.14 g/L of volatile fatty acids produced following dark fermentation of *Ulva* biomass following ulvan extraction. This higher concentration indicated an increased presence of hydrolysed products present in the fermentation broth following the ulvan extraction process [78]. The VFA production observed in this study gives a clear indication as to the effectiveness of the pretreatment methods applied. Ultimately, the increased carbohydrate concentration made available following the lyophilisation and ulvan extraction process (Fig. 1) led to the fermentative microbe's present producing a higher amount of VFAs, accompanied by higher $H_2$ production, as opposed to the untreated *Ulva* (Fig. 2).

## 5. Conclusion

*Ulva* sp. has been shown to harbour potential from a biorefinery and no-waste approach by utilisation of the leftover biomass following extraction of ulvan. Extraction of the polysaccharide ulvan had no negative effect on the dark fermentation of the residual *Ulva* biomass. The optimal substrate to produce $H_2$ was the ulvan-extracted *Ulva* owing to the acid-heat pretreatment following lyophilisation the biomass underwent. This further indicated that the extraction of the polysaccharide ulvan did not limit the $H_2$ potential of the substrate. Following examination of the pretreated *Ulva* sp. biomass, the optimum temperature for biohydrogen production was found to be 55 °C, while the ulvan-extracted biomass recorded the highest biohydrogen yield across each temperature, i.e., 12.8 ± 0.2 mL $H_2$/g VS at 25 °C, 15.0 ± 2.2 mL $H_2$/g VS at 37 °C, and 15.3 ± 1.5 mL $H_2$/g VS at 55 °C. Carbohydrate and

VFAs production profiles showed fermentation of the ulvan-extracted *Ulva* biomass produced the highest concentration of VFAs, i.e., 666.6 (25 °C), 610.7 (37 °C) and 783.7 (55 °C) mg $HAc_{eq}$/L.

## CRediT authorship contribution statement

**James Lawrence:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Armando Oliva:** Writing – review & editing, Validation, Supervision. **Jerry D. Murphy:** Writing – review & editing, Supervision, Resources. **Piet N.L. Lens:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

## 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fuel.2024.133404.

## Data availability

Data will be made available on request.

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