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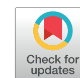
Research article

Effect of biomass pre-treatment on supercritical CO_2 extraction of lipids from marine diatom *Amphora* sp. and its biomass evaluation as bioethanol feedstock



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ABSTRACT

Marine diatoms are a potential source for lipids and carbohydrates, which can have several applications ranging from biofuels to nutraceuticals. Due to their siliceous cellular structure and the complex nature of different lipid classes, it is important to understand the effect of biomass pre-treatment on the extractability of marine diatom lipids. In the present study, we tested the effect of four biomass pre-treatments (acid, base, anionic detergent, and non-ionic detergent) conditions on the extractability of lipids from *Amphora* sp. biomass. Lipids were extracted under identical supercritical fluid extraction (SFE) conditions from each of the above mentioned pre-treated biomass of *Amphora* sp. grown with or without silica. The fatty acids profile of saponified lipids was analysed by LC-MS. Results obtained in this study suggest each pre-treatment has a specific effect on the fatty acids profile. Therefore, depending on the downstream application of lipids (biodiesel or nutritional), both types of biomass and their pre-treatment conditions need to be considered. From the fermentation study for biomass evaluation as bioethanol feedstock, it was found that the complex carbohydrates of *Amphora* sp. biomass were easily convertible by autoclaving to monomer sugars, which were suitable for bioethanol production by yeast fermentation.

1. Introduction

Amphora sp. is a type of pennate diatom microalga belonging to the family Catenulaceae of the class Bacillariophyceae. Most of the Bacillariophyceae microalgae are highly productive and environmentally flexible eukaryotic organisms with a high content of lipids and fatty acids (10–39% lipids and 4–28% fatty acids of dry weight biomass) [1]. Diatoms possess triglycerides (neutral lipids) as carbon storage, which is the best substrate to produce biodiesel [2,3]. However, the fatty acid profile would be crucial in determining the suitability of a specific diatom as biodiesel feedstock. Diatoms can also be an alternative source for essential fatty acids, such as omega-3 unsaturated fatty acids [α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 20:6n-3)] and omega-6 unsaturated fatty acids [linoleic acid (LA, C18:2n-6 *cis*), γ-linolenic acid (GLA, C18:3n-6), arachidonic acid (ARA, C20:4n-6)] are important for human health [4,5]. Therefore, diatoms received commercial interest for biofuel, essential lipid sources, and other applications [6,7].

The methods for extraction of lipids from microalgae are sub-optimum [8] and are traditionally based on toxic organic solvents such as chloroform, methanol, and hexane for the laboratory-scale extractions. Supercritical fluid extraction (SFE) using CO_2 is now an established choice in industrial-scale extractions for essential omega fatty acids as well as biodiesel lipids [9,10,11,12]. This green technology is replacing existing organic solvents based extraction of lipids from microalgae, because SFE is more efficient than harmful solvents like chloroform, methanol, hexane, etc. and offers a negligible environmental impact, and is highly recommended for food applications. The cell wall and cell membranes of various microalgae and especially the siliceous cell walls (frustules) of *Amphora* sp. (diatom) may interfere with the efficiency of lipid extractions due to high mechanical strength and chemical resistance [13]. Hence, biomass pre-treatment has been considered for the optimum extraction of biomolecules from microalgae as well as other organisms [9,10,14,15,16,17,18].

The microalgal feedstock containing carbohydrates can serve as an alternative for both first- and second-generation bioethanol production.

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The spent biomass obtained after supercritical CO_2 extraction of lipids from microalga *Chlorococcum* sp. was tested for suitability for bioethanol conversion [19]. The data suggest that residual algal biomass is a valuable feedstock for bioethanol production in addition to primary biomass use. The utilisation of spent diatom biomass for ethanol production deserves special consideration. However, diatoms differ from other microalgae in terms of storage carbohydrate type, which is a complex carbohydrate called chrysolaminarin within the chrysolaminarin vacuole. Chrysolaminarin is a soluble carbohydrate containing β(1→3) and β(1→6) linked glucose units in a ratio of 11:1 [20]. Hence, ethanol fermentation capability of diatom carbohydrates needs to be evaluated within the biorefinery concept, which is considered as the sustainable solution for biomass energy [21,22].

In the present manuscript, we wanted to identify if there are any effects of biomass pre-treatment conditions on the lipid quality extracted by supercritical CO_2 extraction. We also wanted to see if there are any effects of pre-treatment conditions on the lipid quality of differently-grown biomass extracted by supercritical CO_2. We used two types of biomass (grown with and without silica) which were pre-treated with four chemical conditions (acid, base, an anionic detergent, and non-ionic detergent) before SFE extraction of lipids. Additionally, we have tested the suitability of biomass carbohydrates, which were quantified as total carbohydrates in terms of glucose, and were supposed for the production of bioethanol using yeast fermentation.

2. Materials and methods

2.1. Amphora sp. maintenance and cultivation

The diatom *Amphora* sp. obtained from Shannon ABC biobank was originally isolated from Galway, Ireland, and was cultured in 250 mL Erlenmeyer flasks containing 100 mL of ASN-III medium [23] for actively growing biomass. Briefly, 200 μL of recently sub-cultured seed culture maintained in tubes was inoculated. The flasks were incubated in an environmental growth chamber at 20 °C, under the PAR (photosynthetically active radiation; 400–700 nm) illumination of 80 μmol photons m^{-2} s^{-1} with 16/8h light/dark cycle. Flasks were hand-shaken every day.

Figure 1. Set-up showing cultivation of *Amphora* sp. inside the environmental growth chamber at 20 °C, under 45–80 μmol photons m^{-2} sec^{-1} illumination with 16/8h light/dark cycle, and micro-filtered aeration (0.8 L/min) through aquarium pump.

Actively grown cultures (100 mL) after about 8–10 days of growth were inoculated in 10L of ASN-III medium and 10L of ASN-III + silica (5 ppm) medium in carboys (Nalgene, USA) (Figure 1). The commercially available aqueous solution of sodium metasilicate (Na_2SiO_3) (VWR, catalog no. 28079.320) with 25–27% silica content was used to obtain a 5 ppm concentration of silica in ASN-III medium. The initial cell density (*in vivo* absorbance) was ~0.1 at 680 nm. Both the carboys were incubated in an environmental growth chamber at 20 °C, under the PAR illumination of 45–80 μmol photons m^{-2} sec^{-1} with 16/8h light/dark cycle. The cultures were aerated with filtered air (700 cc/min) using an aquarium pump (Marina-100) and the cells were homogenously suspended through a magnetic stirrer from the bottom of the carboy. After 12 days of growth, the cells were flocculated using NaOH at pH 10.5, and the biomass was harvested by centrifugation at 4,800 rpm for 8 min at room temperature. The biomass pellets were then washed twice with a saltwater solution (25 g NaCl/L water) to remove excess NaOH. The biomass was then freeze-dried (Thermo Electron Heto Power Dry LL3000) for 24 h so that biomass contained no moisture before use for pre-treatment and extraction of lipids as well as to test as bioethanol feedstock.

2.2. Reagent chemicals and standards

The chemicals required for growth medium were obtained from VWR, Ireland, Thermo Fisher Scientific, Ireland, and Sigma Chemicals, Arklow, Ireland. HCl, NaOH, and sodium sulfate crystals were purchased from VWR, Ireland Acetonitrile, ammonium acetate, dichloromethane, chloroform, methanol, spectrophotometric grade 95% ethanol, sodium dodecyl sulphate, Tween-20, potassium dichromate, sulphuric acid, formic acid, phenol were also purchased from Sigma Chemicals. Fatty acid standards such as lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9 cis), linoleic acid (LA, C18:2n-6 cis), γ-linolenic acid (GLA, C18:3n-6), arachidonic acid (ARA, C20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were obtained from Sigma Chemicals (Arklow, Ireland). All solvents used in this study were HPLC or analytical grade, and the water was ultrapure (TKA High Purity Water Systems, Niederelbert, Germany).

2.3. Biomass pre-treatment

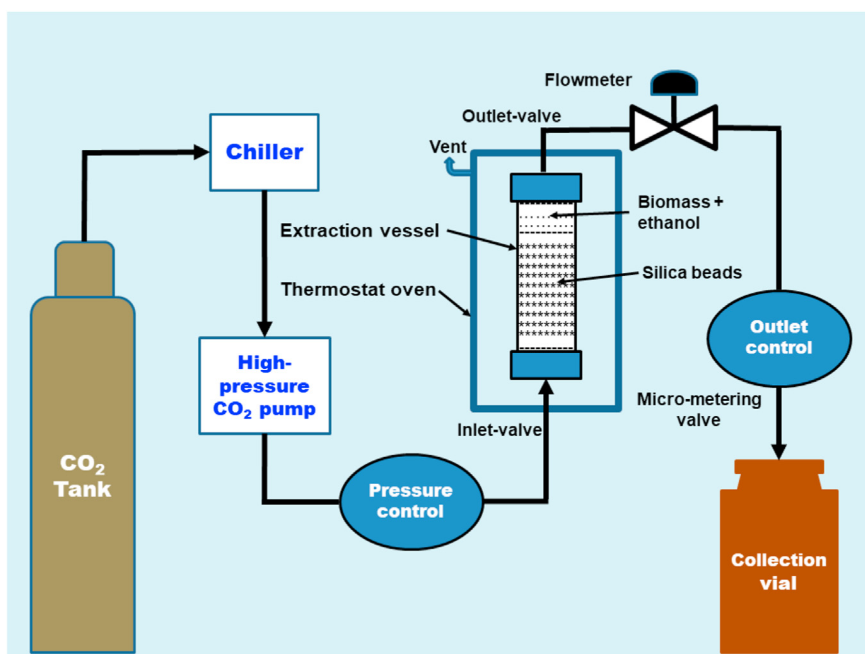
A known amount of freeze-dried biomass (500 mg) was taken for each of the four pre-treatment conditions: 1) acid (0.5 M HCl), 2) base (0.5 M NaOH), 3) anionic detergent (0.5% sodium dodecyl sulphate [SDS]) and 4) non-ionic detergent (0.5% Tween-20). Each biomass was homogenously mixed with 10 mL of appropriate treatment solutions and was agitated in a shaker (New Brunswick Excella E24) at 120 rpm for 4 h at 20 °C. After that, the biomass samples were centrifuged (Heraeus Biofuge Stratos, Fisher Scientific Ltd., Dublin, Ireland) at 5,000 rpm for 8 min and the supernatants were discarded. The resultant biomass pellets were washed thoroughly with 10 mL of distilled water and centrifuged again at 5,000 rpm for 8 min. The pellets were then dried overnight at 55 °C oven and considered as pre-treated biomass for supercritical fluid extraction (SFE) of lipids.

2.4. Lipids extraction by SFE and Folch method

In this manuscript, SFE was applied only for the extraction of lipids from both untreated and pre-treated *Amphora* sp. biomass. Each pre-treated and untreated biomass was gently homogenised on a mortar and pestle. Then the biomass (500 mg) was mixed with a double amount (1 g) of silica beads material (*Spe-ed* Matrix, Product no. 7950, Applied Separations, USA) and transferred to the top-end of the stainless steel 5 mL extraction vessel. Then the large void space of the vessel was filled with the same silica beads material so that the sample bed was firmly packed for uniform diffusion of supercritical fluids through the sample matrix. As per the standard instructions of the SFE instrument (Spe-ed

SFE-4, Applied Separations, USA) extraction vessels were packed in such a way that both ends of the biomass matrix contained polypropylene wool and a frit. Finally, 1 mL of ethanol was added as co-solvent through the careful opening and quick closing of the top-end of the vessel and was connected to the instrument (Figure 2). Then, the vessel was pressurised with supercritical CO_2 (SC–CO_2) at 50 MPa while maintaining the extraction vessel temperature at 70 °C for 45 min prior to extraction for 30 min. This state of SFE is called 'static period' in which the system is prepared for the setup conditions and no extracts are collected in the 'static period'. The above conditions were found optimum for two other microalgal biomass tested recently in our lab (unpublished data). Then, the extracts along with the co-solvent were collected for 30 min (extraction period) using a continuous flow (2–2.5 L CO_2/min) of supercritical CO_2 pressurised at 50 MPa and by regulating precisely the outlet control valve. This state of SFE is called the 'dynamic period', when the supercritical CO_2 flows continuously throughout the vessel, and the extracts are collected. The extracts were collected in glass vials (Figure 2), dried-off any eluted co-solvent with a nitrogen flush. The dried lipids were then measured gravimetrically and stored at -20 °C until used for LC-MS analysis.

A solvent extraction method of Folch et al. [24] was used for the extraction of lipids, which served as a control for comparison with SFE extracted lipids. Briefly, 500 mg of freeze-dried untreated biomass was soaked overnight at 4 °C in 5 mL of extraction solvent (2:1 chloroform: methanol). Then, the biomass was ground by adding washed sand powder in a mortar and pestle. For the complete extraction of lipids, further grinding was performed by adding 10 mL of the above extraction solvent. All extracts were pooled in a tube and 5 mL of ultrapure water was added, and vortexed gently to remove water-soluble impurities. Then the tubes were centrifuged (Heraeus Biofuge Stratos, Fisher Scientific Ltd., Dublin, Ireland) at 5000 rpm for 5 min. After the separation of two layers, the lower lipid layer was transferred carefully to a new



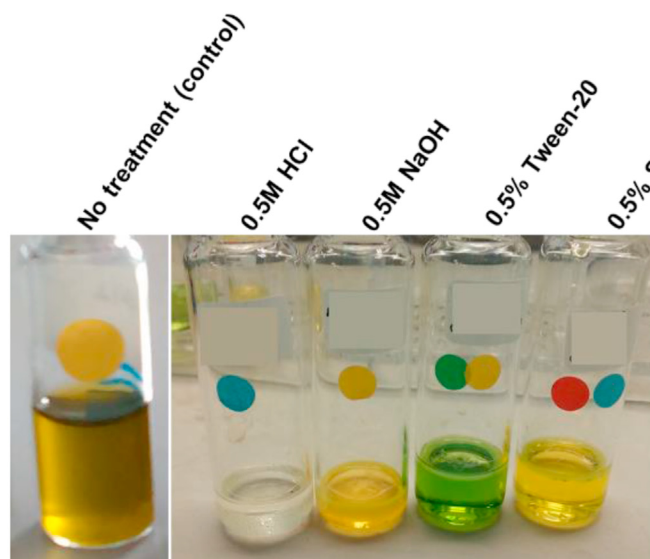


Figure 2. Upper panel showing the experimental work-flow diagram of supercritical CO_2 extraction of lipids from *Amphora* sp. biomass. Lower panel showing the effect of pre-treatment of *Amphora* sp. biomass on the colour of extracts before drying.

tube. Then, approximately 1 g of sodium sulfate crystals was added to remove the moisture content of lipids. Finally, the clear supernatants were transferred to a pre-weighed tube, and lipids were dried in a fume hood for gravimetric estimation. The dried lipids were then stored at -20 °C until used for LC-MS analysis.

2.5. Lipids saponification and fatty acids analysis by LC-MS

The dried lipids were saponified by adding 1.5 mL of saponification reagent [2.5 M KOH: methanol (1:4, v/v)] [8]. Lipids with the saponification reagent were then vortexed gently and the vials were incubated at 72 °C for 15 min just before the addition of 225 μL of formic acid. Then, equal volume (i.e., 1725 μL) of chloroform followed by 375 μL of ultrapure water was added and vortexed. The vials let stand for the separation of two layers and the lower chloroform layer containing saponified lipids was transferred carefully to amber vials. The solvent was evaporated to obtain saponified dry lipids and stored at -20 °C.

For the analysis of fatty acids by LC-MS [25], saponified lipids were dissolved in 500 μL of methanol: dichloromethane (2:1 v/v) and filtered using 0.45 μm Ultrafree-MC centrifugal filters (Millipore, USA). Then, 10 μL of the filtrate was injected to obtain free fatty acids profile in HPLC (Agilent 1260 series) system equipped with Q-TOF mass spectrometer (Agilent 6520). Fatty acids were resolved by an Agilent C-18 Poroshell 120 column (2.7 μm, 3.0 × 150 mm) with gradient elution. Mobile phase A consisted of 2 mM ammonium acetate in water and mobile phase B consisted of 2 mM ammonium acetate in 95% acetonitrile. The mass spectrometer was operated in negative ionisation mode, scanning from 50 - 1100 m/z. Drying gas flow rate, temperature, and nebuliser pressure was at 5 L min^{-1}, 325 °C, and 0.21 MPa, respectively. Fragmentor and skimmer voltages were maintained respectively at 175 V and 65 V.

Standard fatty acids such as lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9 *cis*), linoleic acid (LA, C18:2n-6 *cis*), γ-linolenic acid (GLA, C18:3n-6), arachidonic acid (ARA, C20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were used to validate the LC-MS method for fatty acids analysis by comparing their retention time (RT) and specific accurate mass (Sigma, Ireland). All fatty acids in the samples were then identified based on their known accurate mass and their relative content was recorded as per their peak area. The peak area of each fatty acid was the average of triplicate samples.

2.6. Substrate preparation and carbohydrate determination

The biomass of *Amphora* sp. grown in complete ASN-III medium was freeze-dried and used as the substrate for fermentation for bioethanol production. Five hundred milligram biomass was weighed out in triplicates and 3.5 mL of distilled water was added. Then the biomass was agitated with a glass rod for homogenous suspension before autoclave (Priorclave, Ireland) at 121 °C at 0.1 MPa pressure for 15 min. Once autoclaved, each sample was then transferred to a new sterile tube aseptically and centrifuged (Heraeus Biofuge Stratos, Fisher Scientific Ltd., Dublin, Ireland) at 5,000 rpm for 15 min at room temperature. The clear supernatant thus obtained was transferred to another sterile tube and stored at 4 °C until used within a week as a fermentation substrate.

The total carbohydrate content of the above substrate was determined by using the method developed by Dubois et al. [26]. Briefly, an aliquot of 50 μL of the supernatant was diluted with 950 μL of distilled water in triplicates. Then, 1 mL of 5% aqueous phenol was added followed by 5 mL of concentrated H_2SO_4. The assay samples were left at room temperature for 10 min and then incubated at 30 °C for 20 min. Then the absorbance was read at 490 nm by inserting 100 μL of each reaction mix in the appropriate well of a 96-well plate reader (BioTek Synergy 4). The total carbohydrate content of each sample was determined based on the standard curve obtained by using a range of D-glucose (Sigma Chemicals, Arklow, Ireland) concentrations (0, 50, 100, 250, 500, and 750 μg/mL) with R^2 value of 0.9991.

2.7. Fermentation and ethanol estimation

For the fermentation assay, 1 g of "Young's U Brew" lager yeast (*Saccharomyces pastorianus*, viable cell count >5.0 × 10 CFU/g) was added to 5 mL of 3% aqueous D-glucose and activated for 10 min before inoculation. Thereafter, *Amphora* biomass extract (3 mL containing 1.11–1.31 g/L glucose) in the stoppered glass tube was inoculated with 50 μL of the above-activated yeast. *Amphora* biomass (untreated) extract served as a source for carbohydrate (concentration estimated by the Dubois method mentioned above) that was used as the test sample. The positive control contained 3 mL of 10 g/L D-glucose and 50 μL of activated yeast. The negative control contained 3 mL of sterile distilled H_2O and 50 μL of activated yeast. All the assay tubes in triplicates were then incubated at 25 °C for 48 h. During this incubation period, fermentation tubes were carefully hand-shaken twice. After 48 h of fermentation, 500 μL of representative fermentation solutions were taken for ethanol estimation.

The concentration of ethanol produced by known amounts of *Amphora* sp. biomass was estimated by using a modified method described earlier [27]. Briefly, 500 μL of clear fermentation solution was added to a volumetric flask. Then, 2.5 mL of acetate buffer (pH 4.3) and 2.5 mL of 12% potassium dichromate solution were added, and gently mixed. Later, 12.5 mL of 1N sulphuric acid was added to the assay mixture and stirred gently. The assay solution was then allowed to stand at room temperature for 2 h. Finally, 100 μL of each reaction mix was inserted in each well of a 96-well plate reader and read the absorbance at 578 nm (BioTek Synergy 4). A standard curve was constructed using 95% ethanol (at concentrations of 0, 1.5, 3.0, 4.5, 6, 9, and 12 μL/mL) to estimate the concentration of ethanol produced by tested samples, and was expressed as μL/mL as ethanol equivalent.

2.8. Statistical analysis

All experiments were carried out with three replicates. The results of carbohydrate estimation as well as ethanol production are expressed as averages ±standard error (SE) of triplicate samples. The data presented in Table 1 and Table 2 are based on averages of three chromatographic peak areas of each fatty acid obtained by LC-MS analysis and the variations of peak area of each fatty acid were less than 5% within three individual injections in LC-MS.

3. Results and discussion

The end-point biomass yield of *Amphora* sp. grown in two media conditions was similar with a respective yield of 4.014 g dry weight biomass/10L of complete ASN-III and 4.24 g dry weight biomass/10L of ASN-III + silica medium. The total extracted lipid amounts by Folch method of ASN-III and ASN-III + silica grown biomass were respectively 12.6% and 13.71%. The lipids content of *Amphora* sp. studied earlier varied relatively such as 6.9% in *Amphora coffeaformis* [28], 11.14% in *Amphora* sp. of the Sfax Solar [29], and 23.4% in *Amphora* sp. isolated from Banting, Malaysia [30]. Therefore, the lipid yield of the present studied *Amphora* sp. remained in the middle range as per available literature on *Amphora* sp. lipid content. It is to be noted that the addition of ethanol might have modified the polarity of supercritical CO_2 and thus might have impacted the extractability of non-polar lipids. The relative comparison of SFE extracted lipids with that of the Folch method extracted lipids needs caution. Interestingly, the addition of silica to the growth medium aided in an 8% increase in lipid yield compared to the silica-free ASN-III medium. On the contrary, the addition of silica to the growth medium negatively impacted cell dry weight and total fatty acids including EPA content in diatom *Phaeodactylum tricornutum* [31]. Likewise, in another diatom *Cyclotella cryptica*, slightly increased total lipid

Table 1. Relative percentage of extractable fatty acids of untreated and pre-treated biomass of *Amphora* sp.

Fatty acid name	Control (Folch method extraction)	Untreated biomass	0.5M HCl_ ASN-III Biomass	0.5M HCl_ ASN-III + Silica Biomass	0.5M NaOH_ ASN-III Biomass	0.5M NaOH_ ASN-III + Silica Biomass	0.5% SDS_ASN-III Biomass	0.5% SDS_ASN-III + Silica Biomass	0.5% Tween-20_ ASN-III Biomass	0.5% Tween-20_ ASN-III + Silica Biomass
Caprylic (C8:00)	ND	ND	0.5	0.5	1.2	0.1	0.1	ND	0.1	0.1
Pelargonic (C9:00)	0.7	1.6	2.9	2.9	1.6	1.1	1.0	0.2	1.2	1.2
Capric (C10:00)	0.1	ND	0.6	0.6	0.2	0.2	ND	ND	0.1	0.1
Undecylic (C11:00)	ND	ND	0.1	0.1	0.1	ND	ND	ND	ND	ND
Lauric (C12:00)	0.1	0.1	0.2	0.2	0.1	0.2	0.3	0.1	21.2	21.2
Tridecylic (C13:00)	ND	0.1	0.2	0.2	0.1	0.1	ND	0.1	ND	ND
Myristic (C14:00)	8.6	9.4	1.7	1.7	4.3	17.9	6.3	3.4	8.2	8.2
Pentadecylic (C15:00)	ND	ND	0.3	0.3	0.1	0.1	ND	0.1	0.1	0.1
Palmitic (C16:00)	0.4	0.5	0.6	0.6	0.6	1.7	0.7	0.4	0.4	0.4
Palmitoleic (C16:01)	33.2	32.0	17.0	17.0	20.2	39.3	21.7	16.8	0.8	0.8
Margaric (C17:00)	ND	0.2	ND	ND	ND	ND	ND	ND	ND	ND
Stearic (C18:00)	ND	ND	16.9	16.9	7.3	7.8	5.0	8.2	7.3	7.3
Oleic/Elaidic (C18:01)	0.1	0.1	6.7	6.7	8.9	16.9	9.8	5.5	7.5	7.5
Linoleic (C18:02)	ND	ND	3.2	3.2	3.1	3.3	3.9	3.3	1.7	1.7
Linolenic (α + γ) (C18:03)	0.1	0.4	0.3	0.3	0.7	0.2	1.0	0.8	0.3	0.3
Nonadecylic (C19:00)	ND	ND	3.7	3.7	1.1	0.8	0.3	0.8	4.3	4.3
Arachidic (C20:00)	0.1	0.1	11.4	11.4	4.5	2.5	1.0	3.0	13.2	13.2
Gadoleic (C20:01)	0.1	0.1	1.0	1.0	0.3	0.6	0.1	0.3	0.3	0.3
Dihomolinoleic (C20:02)	ND	0.1	0.2	0.2	0.3	0.2	0.4	0.3	0.1	0.1
Dihomolinolenic (C20:03)	0.2	0.1	0.5	0.5	1.0	0.6	1.4	1.0	0.4	0.4
Arachidonic (C20:04)	1.4	1.8	0.6	0.6	1.4	0.1	2.0	0.9	0.4	0.4
EPA (C20:05)	53.9	53.1	14.8	14.8	35.5	0.8	41.7	48.4	16.1	16.1
Heneicosylic (C21:00)	ND	0.1	13.0	13.0	4.6	2.9	1.1	3.7	13.8	13.8
Behenic (C22:00)	ND	ND	3.0	3.0	1.3	2.2	0.3	0.9	2.0	2.0
Erucic (C22:01)	ND	ND	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1
Docosadienoic (C22:02)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Eranthic (C22:03)	ND	ND	ND	ND	0.1	0.1	ND	0.1	ND	ND
Ardenic (C22:04)	0.1	ND	0.2	0.2	0.6	ND	0.6	0.8	0.2	0.2
DPA (C22:05)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DHA (C22:06)	0.6	0.3	0.2	0.2	0.7	ND	1.1	0.7	0.2	0.2

Note: ND, mostly these fatty acids could not be detected under the detection limit of the equipment used or detected only at a negligible amount that the relative percentage could not be presented within one decimal point value in the table.

content was recorded during silica depleted cultivation, however with suppressed polyunsaturated fatty acids biosynthesis [32].

In the present study, the fatty acids profile and their relative content in untreated biomass were essentially similar in both Folch et al. method extracted lipids and in SFE extracted lipids (Table 1). A total of 25 fatty acids were identified in the lipid extracts, while SFE extracted lipids additionally contained margaric acid (C17:0) in untreated biomass. EPA, palmitoleic, and myristic acids are the prominent fatty acids followed by the next major fatty acids (pelargonic, arachidonic, DHA, and palmitic) in the above two lipids from untreated biomass. The fatty acids profile and their relative content of SFE extracted lipids of each pre-treated biomass were essentially different. A total of 27 fatty acids were identified in the lipids of each pre-treated biomass with varied relative content. Interestingly, caprylic, stearic, linoleic, nonadecylic, and behenic fatty acids were only detected in lipids of pre-treated biomass, while margaric, docosadienoic, docosapentaenoic (DPA), and tricosylic fatty acids were not detected in lipids of any pre-treated biomass. Fatty acids profile and their relative content of pre-treated biomass grown in complete ASN-III medium and ASN-III supplemented with silica remained essentially similar. However, NaOH treated biomass grown in ASN-III supplemented with silica medium showed drastically reduced relative content of EPA but with a relatively high content of myristic, palmitoleic, and oleic/elaidic fatty acids. EPA is a nutritionally essential fatty acid, while myristic, palmitoleic and oleic/elaidic fatty acids are relevant to biodiesel because of their short carbon length and low unsaturation levels. The relative content of EPA in untreated biomass remained the same in both Folch et al. method (control) and SFE extractions, while all pre-treatments negatively impacted its relative content (Table 1). The relative content of DHA, another nutritionally essential fatty acid, was slightly increased in NaOH and SDS pre-treated biomass compared to untreated biomass extractions (Tables 1 and 2). It is to be noted that SDS can solubilize membrane proteins and thus release membrane lipids during aqueous treatment, although this may be difficult due to the silicified nature of cell walls of diatoms. However, because of the lack of data on lipid loss during aqueous SDS treatment, the original released content of DHA might even be higher. So, it appears that depending on the availability of nutritionally important fatty acids (EPA or DHA or both) in a specific diatom, the method of pre-treatment needs to be chosen for optimum SFE extraction yield. In general, all pre-treatments had a positive effect on releasing certain fatty acids (caprylic, heneicosylic, arachidic, stearic, linoleic, nonadecyclic, and behenic) at different levels from the biomass as reflected by an increase in their relative content compared to untreated biomass extracted either by Folch method or SFE method (Table 2). For example, caprylic, stearic, linoleic, nonadecyclic, and behenic fatty acids were not detected in untreated biomass extracted either by the Folch method or by supercritical CO_2 extraction method. Therefore, the peak abundance values of these fatty acids detected in any of the pre-treated biomass represent their relative percentage compared to the Folch method values. In the case of arachidic and heneicosylic fatty acids, which were detected in untreated biomass extracted either by Folch method or by supercritical CO_2 extraction method, however, their relative percentage yield was increased in all pre-

Table 2. The percentage yield of each specific fatty acid obtained by supercritical CO_2 extraction of untreated and pre-treated biomass of *Amphora* sp. relative to their extraction yield by Folch et al. method of extraction.

Fatty acid name	Control (Folch method extraction)	Untreated biomass	0.5M HCl_ ASN-III Biomass	0.5M HCl_ ASN-III + Silica Biomass	0.5M NaOH_ASN-III Biomass	0.5M NaOH_ ASN-III + Silica Biomass	0.5% SDS_ ASN-III Biomass	0.5% SDS_ ASN-III + Silica Biomass	0.5% Tween-20_ ASN-III Biomass	0.5% Tween-20_ ASN-III + Silica Biomass
	(100%)	(Percentage yield relative to control Folch. method extraction)								
Caprylic (C8:00)	ND	ND	2.7×10^7	1.3×10^7	2.6×10^8	1.2×10^7	2.0×10^7	1.3×10^6	1.5×10^7	7.8×10^6
Pelargonic (C9:00)	1682161.2	79.3	95.2	47.6	208.4	149.5	181.0	14.5	168.9	84.5
Capric (C10:00)	190772.9	7.1	167.8	83.9	277.7	186.3	72.6	23.6	79.9	40.0
Undecylic (C11:00)	15732.3	14.7	338.6	169.3	852.3	66.9	708.6	341.0	721.2	360.6
Lauric (C12:00)	305956.2	27.2	40.3	20.1	88.0	178.1	269.0	44.9	15887.3	7943.6
Tridecylic (C13:00)	16259.4	338.9	668.5	334.3	1000.2	1204.2	916.4	684.5	79.3	39.6
Myristic (C14:00)	19894997.3	40.3	4.7	2.4	48.7	197.8	94.8	18.3	94.5	47.2
Pentadecylic (C15:00)	84349.6	28.5	185.4	92.7	256.2	174.5	97.6	134.4	371.5	185.8
Palmitic (C16:00)	982895.5	39.2	34.8	17.4	141.5	383.4	216.6	46.3	82.7	41.3
Palmitoleic (C16:01)	76802092.6	35.7	12.1	6.1	58.4	112.5	84.7	23.7	2.4	1.2
Margaric (C17:00)	ND	1.4×10^7	ND	ND	ND	ND	ND	ND	ND	ND
Stearic (C18:00)	ND	ND	9.2×10^8	4.6×10^8	1.6×10^9	1.7×10^9	1.5×10^9	8.8×10^8	1.6×10^9	8.3×10^8
Oleic/Elaidic (C18:01)	196347.9	44.3	1879.7	939.8	10062.0	18964.7	14962.0	3049.2	8749.0	4374.5
Linoleic (C18:02)	ND	ND	1.7×10^8	8.7×10^7	6.8×10^8	7.2×10^8	1.1×10^9	3.5×10^8	3.9×10^8	1.9×10^8
Linolenic (α + γ) (C18:03)	230304.7	161.3	69.1	34.6	705.7	172.9	1331.8	373.0	289.3	144.6
Nonadecylic (C19:00)	ND	ND	2.0×10^8	1.0×10^8	2.4×10^8	1.7×10^8	1.0×10^8	8.2×10^7	9.9×10^8	4.9×10^8
Arachidic (C20:00)	193598.5	32.0	3216.5	1608.3	5130.3	2814.1	1598.6	1674.8	15612.0	7806.0
Gadoleic (C20:01)	119276.0	38.2	470.4	235.2	599.4	1194.6	242.0	253.0	562.9	281.5
Dihomolinoleic (C20:02)	99618.7	59.6	121.2	60.6	728.4	530.0	1239.0	285.0	332.1	166.1
Dihomolinolenic (C20:03)	426380.4	27.6	58.9	29.5	537.7	295.9	959.1	252.1	203.6	101.8
Arachidonic (C20:04)	3321157.8	45.5	9.3	4.6	91.8	9.5	177.6	30.9	30.3	15.1
EPA (C20:05)	124651392.9	36.5	6.5	3.3	63.5	1.4	100.5	42.0	29.6	14.8
Heneicosylic (C21:00)	33466.0	139.8	21222.4	10611.2	30830.3	19136.1	10133.0	11934.6	94644.3	47322.2
Behenic (C22:00)	ND	ND	1.6×10^8	8.3×10^7	3.0×10^8	4.8×10^8	8.8×10^7	9.3×10^7	4.4×10^8	2.2×10^8
Erucic (C22:01)	109751.1	28.8	73.3	36.7	246.2	311.9	299.2	139.0	209.5	104.8
Docosadienoic (C22:02)	94441.5	12.1	ND	ND	ND	ND	ND	ND	ND	ND
Eranthic (C22:03)	82498.0	12.9	22.9	11.5	140.4	160.3	170.6	129.8	82.5	41.2
Ardenic (C22:04)	219470.3	14.8	48.4	24.2	580.6	28.5	842.9	396.3	254.5	127.2
DPA (C22:05)	12118.7	29.6	ND	ND	ND	ND	ND	ND	ND	ND
DHA (C22:06)	1275034.8	22.8	8.1	4.1	118.8	2.3	252.7	57.8	32.2	16.1
Tricosylic (C23:00)	42732.8	14.3	ND	ND	ND	ND	ND	ND	ND	ND

Note: 2nd column represents the peak abundance of each specific fatty acids extracted by the Folch method, and these peak values were considered as 100% to estimate the relative yield (columns 3–11) by comparing the peak abundance values of corresponding fatty acids obtained by supercritical CO_2 extraction. ND, these fatty acids could not be detected under the detection limit of the equipment used.

treatment conditions with highest respectively in 0.5% Tween-20_ASN-III + Silica Biomass (7806%) and 0.5% Tween-20_ASN-III Biomass (94644%) (Table 2).The relative content of lauric acid was increased in non-ionic detergent Tween-20 treated biomass grown in both complete ASN-III and ASN-III supplemented with silica medium (Table 2). It appears that most of the tested pre-treatments are effective in extractability of the above biodiesel relevant fatty acids, and hence a suitable pre-treatment method needs to be considered depending on the target biodiesel relevant fatty acids from *Amphora* sp. biomass. There were reports on free nitrous acid (FNA) pre-treatment on microalga *Tetraselmis striata* M8 biomass and the enzyme alginase pre-treatment on macroalgae *Undaria pinnatifida* biomass, which aided in enhanced lipid extraction by organic solvents [14,33]. There was essentially no influence of pre-treatments (drying under air-flow and freeze-drying) of *Nannochloropsis oculata* biomass on supercritical CO_2 extraction of lipids and fatty acids, however, drying under airflow was the most adequate pre-treatment for rapid extraction kinetics [9]. This appears as the first report on the effect of acid, alkali, and detergents pre-treatment of *Amphora* sp. biomass on the supercritical CO_2 extraction of specific fatty acids.

Microalgal biomass after removal of lipids for fatty acids source holds great promise for bioethanol production from their biomass carbohydrate through the process of fermentation using fungi or bacteria. The storage carbohydrate in diatoms may range from 10% to 50% in ash-free dry weight [34]. In the present study, the carbohydrate content of *Amphora* sp. estimated was 68.6 ± 2.95% of dry weight biomass. Earlier, carbohydrate content in *Amphora* sp. reported was 18.4% of dry weight biomass [30]. However, there is a report in diatom *Skeletonema costatum*, where the cellular β-1,3-glucan level increased to 75–80% of cellular organic carbon after nitrogen limitation [35]. Starch and cellulose are the most common carbohydrates found in microalgae and were tested suitable for bioethanol production [36]. However, diatoms store a complex but soluble carbohydrate called chrysolaminarin [6]. The concentration of chrysolaminarin may reach up to 50% of cell dry weight depending on culture conditions in diatom *Odontella aurita* [37]. However, there may only be few studies focused on bioethanol production from chrysolaminarin of diatom biomass [38], which may be interesting from the biorefinery perspective.

Microalgal carbohydrates to be converted to bioethanol are first broken down into individual monomer sugars through a process called

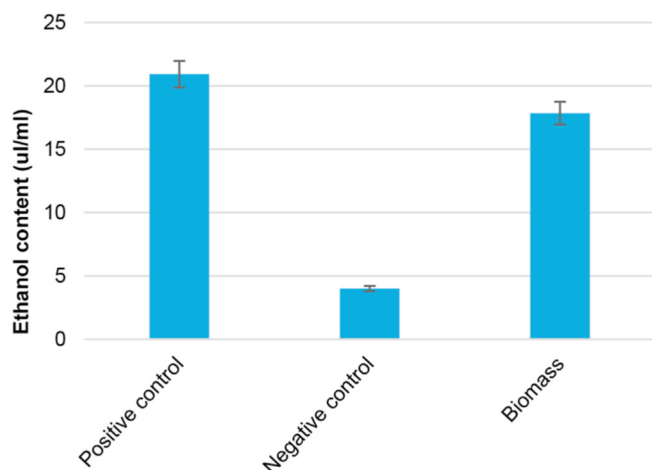


Figure 3. Production of bioethanol from the biomass of *Amphora* sp. after 48 h of fermentation using the yeast *Saccharomyces pastorianus*. Data presented are averages of triplicates ±standard error (SE).

saccharification or hydrolysis. This process of hydrolysis can be achieved by either chemical or enzymatic means. Chemical hydrolysis methods by acids or alkalis are rapid and are cheaper than enzymes, and generally acidic hydrolysis (by H_2SO_4, HCl, H_3PO_4, HNO_3, and TFA) is the most common degradation reaction of glycosidically linked glycosides used in the industry [38]. In the present study, the bioethanol produced from the autoclave hydrolysed *Amphora* sp. biomass was essentially comparable to the standard D-glucose based fermentation (Figure 3). The concentration of ethanol produced from the extracted carbohydrate of *Amphora* sp. biomass was 17.84 μL/mL of fermentation broth. The negative control (no additional carbohydrate except the inoculum) produced a very low quantity of ethanol of 4 μL/mL as expected. While the positive control containing 10 g L^{-1} of D-glucose produced the highest amount of ethanol of 20.92 μL/mL of fermentation broth. The results of this study suggest that biomass carbohydrates of the diatom *Amphora* sp. are easily convertible to monomer sugars by autoclave and are suitable for bioethanol production as good as standard D-glucose. The most common carbohydrate in green microalgal biomass is starch, which is composed of amylose and amylopectin. They are different in their molecular structures and their (amylose/amylopectin) ratio was found inversely related to ethanol production [39]. The advantages of the present bioethanol production process are avoiding the use of corrosive acids for biomass hydrolysis, and to ensure the complete use of spent-biomass after lipids extraction for other uses.

4. Conclusions

Marine diatom *Amphora* sp. biomass can be used for extraction of lipids containing EPA as a nutritionally important fatty acid source using SFE but no pre-treatment of biomass. While appropriate pre-treatment of biomass methods needs to be considered if *Amphora* sp. would be used for lipids extraction by SFE for biodiesel applications. The spent-biomass containing carbohydrates can be used for bioethanol production. Thus the maximum use of *Amphora* sp. biomass as feedstock for biodiesel or nutritional ingredients, and bioethanol may reduce the production cost altogether.

Declarations

Author contribution statement

Paul Hogan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Paz Otero: Performed the experiments; Analyzed and interpreted the data.

Patrick Murray: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sushanta Kumar Saha: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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