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Can mild alkaline pretreatment simultaneously enhance the antioxidant capacity of Beta-carotene extracts and biomethane yields in a sustainable *Dunaliella salina* biorefinery?

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ABSTRACT

This research aims to assess the effect of alkaline pretreatments on the antioxidant potential of β-carotene-rich extracts from the microalga *Dunaliella salina* and the cumulative biomethane production from its spent biomass, within the framework of a circular economy approach using four biorefineries. A solvent screening was performed, with ethyl acetate achieving the maximum β-carotene extraction yield (5.3% ± 0.03%). Alkaline pretreatments were applied to the initial biomass (direct) and extracts after a extraction with ethyl acetate (indirect), using two matrices: water (W) and a mixture water:ethanol (WE). Direct alkaline pretreatments (D) offered extracts with higher potential than indirect pretreatments (I) in terms of: i) antioxidant capacity, as measured by $ABTS^{\bullet+}$ assay (0.69±0.1 and 0.61±0.1 mmolTE/gDW for W-D and WE-D, respectively, and 0.55 ±0.1 and 0.53±0.1 mmolTE/gDW for W-I and WE-I, respectively) and •OH scavenging activity (1.89±0.2 and 2.05±0.5 mmolTE/gDW for W-D and WE-D, respectively, and 0.48±0 and 1.2±0.3 mmolTE/gDW for W-I and WE-I, respectively), ii) biomethane production from their spent biomass (301±14 $mLCH_4$/gVS and 289±9.0 $mLCH_4$/gVS for W-D and WE-D, respectively, compared to 235±57 $mLCH_4$/gVS without alkaline pretreatment), and iii) sustainability analysis, which includes the assessment of the biomass exploitation for β-carotene extraction and biomethane production. The most sustainable biorefinery was W-D as it achieved the highest biomass exploitation (33.8%), compared to WE-D (29.1%), W-I (33.1%) or WE-I (32.8%). This underscores the novelty and effectiveness of direct alkaline pretreatments for enhancing both antioxidant potential and energy recovery from *D. salina* biomass in a biorefinery context.

1. Introduction

Reactive oxygen species (ROS) are molecules that form in the human body in response to natural processes such as UV radiation, aging, or genetic conditions. Still, they can also be caused by other factors, such as an unhealthy diet or bad sleeping habits. Enzymatic processes such as phagocytosis or mitochondrial respiration, as well as non-enzymatic processes, result in the production of ROS. They include oxygen intermediates like superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) [1]. NADPH oxidase is one of the enzymes that generates $O_2^{\bullet-}$, which subsequently reacts to form •OH radicals. Oxidase enzymes produce H_2O_2, which then reacts with $O_2^{\bullet-}$ and Fe^{2+} or Cu^{+} (Fenton reaction), producing •OH radicals, the most reactive of all the free radical species [1]. When the accumulation of ROS is too high, oxidative stress is produced, damaging the skin tissues. The accumulation of ROS can lead to further adverse situations, particularly when enzymes such as matrix metalloproteinases (MMPs) are overexpressed, leading to cellular damage and degradation of biomolecules such as proteins [2]. In particular, •OH radicals promote lipid peroxidation, damaging structural lipids, the main biomolecules of the epidermis. Besides, if the damage is excessive, DNA can be affected, leading to acute severe effects such as cell apoptosis or necrosis [3].

Natural molecules like D-galactose-rich polysaccharides can

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Abbreviations

ROS:	Reactive oxygen species
AFEX:	Ammonia fiber expansion
W-D:	Direct alkaline pretreatment of the initial biomass in an aqueous medium
WE-D:	Direct alkaline pretreatment of the initial biomass in an aqueous and ethanolic medium
W-I:	Indirect alkaline pretreatment of the extract from initial biomass in an aqueous medium
WE-I:	Indirect alkaline pretreatment of the extract from initial biomass in an aqueous and ethanolic medium
BE :	Biomass Exploitation

contribute to eliminating ROS [4] and increasing the antioxidant defenses. Others, such as anthocyanins, are photoprotective [5], avoiding ROS formation and acting as natural filters. Those natural molecules can replace synthetic ones obtained through chemical processes that cause the formation of harmful waste with an impact on human health. In this context, microalgae constitute a natural source of pigments such as β-carotene, a precursor of vitamin A, that humans cannot synthesize. Its use is recommended due to its numerous beneficial properties, which include antioxidant effects [6]. Thus, β-carotene has been shown to scavenge $ABTS^{\bullet+}$ radicals, commonly used to estimate antioxidant capacity [7]. It is also an effective antioxidant against certain types of ROS, such as singlet oxygen ($^1O_2$) or superoxide radicals ($O_2^{\bullet-}$) [8]. A previous study demonstrated that carotenoids from the microalgae *D. salina* inhibited the enzyme lipid peroxidase, which promoted lipid peroxidation [9], showing the probable implication of those carotenoids in the scavenging of •HO radicals and thus protecting structural lipids. However, available research about the capacity of β-carotene to neutralize •OH radicals, one of the most reactive and damaging radicals produced in the human body, is limited [10]. These properties make β-carotene a key player in the body's antioxidant defense system. For instance, it protects the skin against sunlight damage, stimulating collagen and elastin production to enhance skin density, elasticity, and firmness. However, it is important to complement its action with other antioxidants that can neutralize the radicals for which β-carotene is less effective.

Carotenes, primarily β-carotene, accumulate within lipid globules in the chloroplasts and are abundant in *Dunaliella salina*, constituting 10–14 % of its dry weight, making it the primary natural source of this pigment. This green unicellular eukaryotic microalga lacks a rigid polysaccharide cell wall, instead having a thin elastic plasma membrane, which facilitates the release of β-carotene. Consequently, β-carotene-rich extracts obtained from this microalga are of interest to various industries, including cosmetics and food. *D. salina* is considered safe by various countries, including the USA, China, and Australia [11]. The global market of β-carotene is worth $339.12 billion in 2024 and is expected to reach $410.61 billion by the end of 2029 [12].

The traditional downstream processing of microalgae for extracting carotenoids includes harvesting, cell disruption (not necessary with the microalgae *D. salina*), and extraction with an organic solvent that can extract saponifiable lipids and carotenoids simultaneously. In this context, treating the harvested microalgae with a base like sodium or potassium hydroxide allows the separation of saponifiable lipids and carotenoids. This is because the hydrogen ions of the carboxyl group of fatty acids from the saponifiable lipids can be neutralized and replaced by metal ions to form fatty acid salts, which are soluble in water and ethanol [13]. Carotenoids do not participate in this reaction due to the absence of carboxyl groups, allowing them to be separated from fatty acid salts using an appropriate solvent.

Once separated from carotenoids, the biomass from this microalga offers several opportunities for utilization as by-products or bioenergy within a biorefinery framework. Anaerobic digestion is one of the most suitable and commercially proven routes in algal biorefinery design [14]. The residual algal biomass has undergone extensive testing as feedstock for anaerobic digestion, showing promising biogas yields [15,16], supporting thus the UN's sustainable development goal (SDG) 7 (affordable and clean energy). Anaerobic digestion of residual algal biomass not only enhances the biorefinery's energy balance, enabling the generation of bioenergy, but it also involves the mineralization of organic nitrogen and phosphorus present in the residual biomass, with the resulting digestate serving as a valuable soil amendment and fertilizer [14].

Pretreatments of microalgal substrates are commonly used to increase biogas production yields [17]. Alkaline pretreatment presents advantages over other methods, such as diluted acid or ammonia fiber expansion (AFEX). Acid treatments are more corrosive than alkaline ones, and the AFEX cannot be performed at atmospheric pressure. As a result, sophisticated and expensive equipment to counteract corrosion or high-pressure working conditions is necessary for microalgal pretreatment [18].

Previous research has utilized the microalgae *D. salina* in a biorefinery, optimizing the extraction of β-carotene through saponification and recovering polar lipids and glycerol [19]. In our work, *D. salina* was evaluated as a sustainable feedstock within various biorefinery approaches to produce: i) β-carotene-rich extracts with outstanding antioxidant properties for the cosmetic industry and ii) biogas with both exceptional yield and quality for bioenergy applications. Alkaline pretreatments were studied to enhance the effectiveness of the biorefinery, aiming to produce antioxidant β-carotene-rich extracts and improve the biogas yield. Our approach, which has not been previously explored in the literature, emphasizes the novelty of integrating alkaline pretreatments and their potential impact on the overall process efficiency, considering that while the production of β-carotene and biogas separately has been studied before, their combined enhancement through this method is unprecedented.

2. Materials and methods

2.1. Materials

D. salina powder was supplied by Monzon Biotech (Barcelona, Spain). The inoculum for the anaerobic digestion was obtained from the anaerobic digester of a wastewater treatment plant (Population Equivalent: ~600,000) in the province of Madrid, Spain. Chloroform, methanol, acetone, ethyl acetate, diethyl ether, heptane, hexane (analytical grade), iron (II) sulfate heptahydrate (>98% purity), hydrogen peroxide (30% w/w), sodium salicylate (≥99%), and NH_4^+, N, P and COD Spectroquant® kits were supplied by Merck (Darmstadt, Germany). β-carotene (99% purity) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (98% purity), were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Trolox (99.8% purity) was acquired from TargetMol, Boston, MA, USA, and potassium persulfate (≥98% purity) from Glentham Life Sciences, UK.

2.2. Methods

2.2.1. Microalga and extracts characterization

Carbohydrates (29.8±1.2, wt%) were measured using the phenol-sulfuric method [20], and proteins (22.5±0.9, wt%) were determined following Lowry's protocol [21]. Lipids (37.5±0.1, wt%) were analyzed with the Bligh & Dyer modified protocol [22], in which a mixture of water, chloroform, and methanol (2:2:1, v/v) was used. Ash (7.4±0.2, wt%) was determined by a developed optimized method for microalgae [23] submitting the sample at 600°C overnight in a muffle furnace (Nabertherm. Lilienthal, Germany). The elemental analysis was conducted in a Flash 2000 equipment (Thermo Fisher Scientific. Waltham,

MA, USA) equipped with a thermal conductivity detector (TCD). The carbon, hydrogen, nitrogen, and sulfur content were determined by an oxidation/reduction reactor at 900°C, while the oxygen content was determined independently from a specific pyrolysis reactor at 1,060°C. The quantification of the analysis was performed using 2,5-bis (5-*tert*-butyl-benzoxazol-2-yl) thiophene as the calibration standard. HPLC was used to quantify the lipophilic pigments of the microalga *D.salina*. Calibration was performed using the following pigment standards: fucoxanthin, lutein, zeaxanthin, 19′hexanoiloxyfucoxanthin, peridinin, violaxanthin, alloxanthin and β-carotene. Molar extinction coefficients from Jeffrey et al., 1997 [24] were used for the quantification of pigments that were not calibrated with commercial standards.

Fig. S1 of the supplementary material shows the lipophilic pigment profile of the microalga *D.salina* analyzed at the Basque Microalgae Culture Collection Service (University of the Basque Country, Spain). For this purpose, 2 mg of the sample were dissolved in 20 ml of 90% acetone, filtered (0.22 μm) and analyzed by HPLC. Pigments were separated and quantified according to the protocol described by Zapata et al. [25], with modifications and equipment described in Seoane et al. [26].

2.2.2. *β-carotene extraction*

Five solvents with different polarities were used to extract β-carotene: acetone, ethyl acetate, diethyl ether, heptane, and hexane. 1 g of biomass was stirred for 5 min in a Vortex device (IKA-Werke GmbH. Staufen, Germany) with 40 mL of the solvent. The mixture was centrifuged (5,000 rpm, 10 min). The supernatant was collected and filtered (0.45 μm), and β-carotene extraction yield and purity were analyzed.

Four distinct alkaline pretreatments were conducted. Fixed conditions in all the pretreatments were a temperature of 55°C (higher temperatures lead to the degradation of the pigment), 35 min, and 35 wt% of potassium hydroxide regarding the dry biomass. The set variables were the type of alkaline pretreatment and the reaction matrix. Types of alkaline pretreatment were: i) direct (D) when the pretreatment was performed with the whole initial *D. salina* microalga; or ii) indirect (I), when the pretreatment was run using the extract after a simple extraction using the best solvent chosen in the screening of solvents. The matrix of the reaction was: i) water (W-D and W-I in direct and indirect reactions, respectively) or ii) water:ethanol in a ratio of 1:13 (v/v) (WE-D and WE-I, in direct and indirect reactions, respectively). Details of the alkaline pretreatment experiments are summarized in Table 1.

Direct alkaline pretreatment was performed with 1 g of the microalga and 20 mL of water (W-D) or 20 mL of a 1:13 v/v water:ethanol mixture (WE-D) in a flask. The mixture was stirred under the conditions mentioned above. When the alkaline treatment was completed, it was left at room temperature for 30 min. Then, 40 mL of the chosen solvent was added, stirring the mixture for 5 min in a vortex. The mixture was centrifuged (5,000 rpm, 10 min) in an 5910 Eppendorf centrifuge (Hamburg, Germany), and the supernatant was collected, filtered (0.45 μm), washed with MiliQ water, and analyzed. Solvent residues were eliminated from the spent biomass by drying it at 40°C for 24 h in an oven. Spent biomass was then analyzed and used to produce biogas through anaerobic digestion reactions. Direct alkaline pretreatment extraction experiments are depicted in Fig. 1A.

Table 1
Alkaline pretreatment: experiments description.

Experiment	Raw material	Alkaline pretreatment	Matrix
W-D	Initial biomass	Direct	Water
WE-D	Initial biomass	Direct	Water: Ethanol
W-I	Extract from initial biomass	Indirect	Water
WE-I	Extract from initial biomass	Indirect	Water: Ethanol

Indirect alkaline reactions were performed following the same protocols as direct experiments, with a slight difference: 35 mL of solvent-filtered extract (obtained from a simple extraction) was used in the alkaline treatments instead of 1 g of *D. salina* biomass. Supernatants were analyzed, and spent biomass was dried, analyzed, and used as a substrate for anaerobic digestion reactions to produce biogas. Indirect alkaline pretreatment extraction experiments are depicted in Fig. 1B.

2.2.3. *Analysis of β-carotene extracts*

Extracts were analyzed regarding β-carotene extraction yield and purity, composition (including β-carotene content and biochemical composition), and antioxidant capacity.

2.2.3.1. β-carotene extraction yields and composition. To quantify the β-carotene extraction yields, a calibration curve was established using stock solutions of β-carotene and dissolved in ethyl acetate. The concentrations of the pigment used in the standard curve preparation were 1, 2, 4, 6, and 8 μg/mL. The maximum peak was detected at 454 nm. Calibration was performed in triplicate obtaining a first-order equation (R^2 =0.99) used to quantify the concentration of β-carotene by spectrophotometric absorption in a Cary 500 UV–Vis–NIR spectrophotometer (Varian, Inc. Palo Alto, CA, USA):

$$\beta\ carotene\ extracted\ (\mu g\,/\,mL) = (4.1947 A_{454}) + 0.041 \quad (1)$$

where A_{454} is the absorbance measured at 454 nm.

β-carotene extraction yields are expressed in percentage as the amount of β-carotene extracted regarding the initial biomass (dry weight basis), using the following equation:

$$\beta carotene\, extraction\, yield\,(\%) = \beta carotene\,(\mu g\,/\,mL)\, V(mL)\,/\,W(\mu g)\, 100 \quad (2)$$

where β-carotene is the concentration of β-carotene determined with equation (1), V is the volume of solvent used in the extraction, and W corresponds to the weight of the initial dry biomass.

The purity of β-carotene extracts was determined with the following equation:

$$\beta carotene\, extract\, purity\,(\%) = \beta carotene\,(\mu g\,/\,mL)\, Vd(mL)\,/\,PW(\mu g) 100 \quad (3)$$

where β-carotene is the concentration of β-carotene determined with equation (1), Vd is the volume of solvent used in the gravimetrical determination, and PW corresponds to the weight of the phase (supernatant).

In addition, the biochemical composition of β-carotene extracts was analyzed according to the protocols described above.

2.2.3.2. Antioxidant capacity of β-carotene extracts. Two mechanisms of radical scavenging were assessed in vitro to test the antioxidant power: 1) $ABTS^{\bullet+}$ radical scavenging [27], and 2) hydroxyl radical (•OH) scavenging [28]. The samples analyzed included β-carotene-rich extracts obtained from the different alkaline pretreatment protocols (W-D, WE-D, W-I, and WE-I), as well as a standard of pure Trolox.

In the $ABTS^{\bullet+}$ scavenging assay, 7 mM solution of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and 2.45 mM of potassium persulfate solution were prepared in MiliQ water, mixed in a ratio 1:1 (v/v) and kept in darkness for 16 h. Then, the stock solution was diluted with methanol until its absorbance was set at 0.73±0.03 (734 nm). 600 μL of the stock solution was mixed with 30 μL of the sample for 10 s and kept at room temperature for 7 min in darkness. A Trolox standard curve ranging from 0 to 400 μM was used to calculate the antioxidant capacity of the extracts in terms of $ABTS^{\bullet+}$ scavenging.

The $ABTS^{\bullet+}$ scavenging capacity was determined according to the following equation:

$$ABTS^{\bullet+}\, scavenging\ capacity\ (\%) = [(A_{734B} - A_{734S})/A_{734B}]\, 100 \quad (4)$$

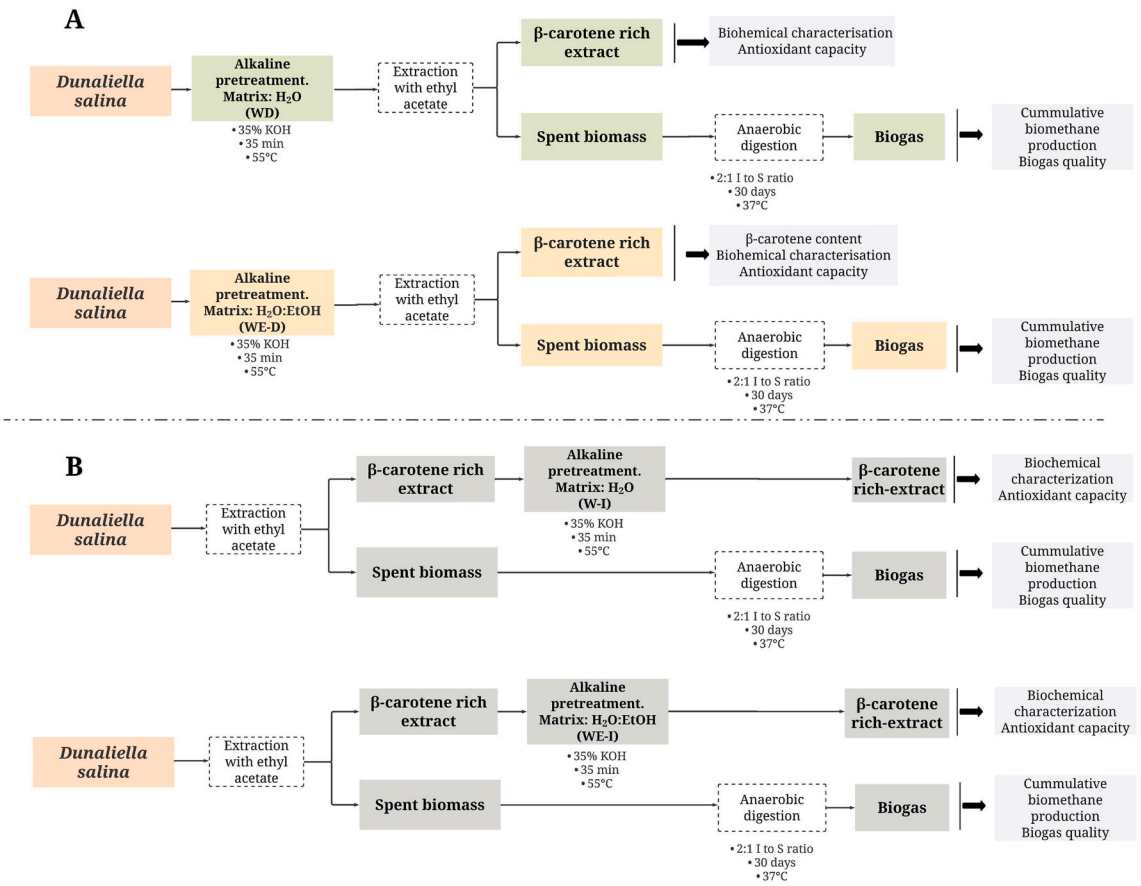

Fig. 1. Alkaline-based treatments of *D. salina* to obtain β-carotene extracts and biogas using direct (A) and indirect (B) procedures.

where A_{734B} and A_{734S} are the absorbances measured at 734 nm, for the negative control (MiliQ water) and the sample, respectively. $ABTS^{\bullet+}$-scavenging potential was also expressed in millimoles of Trolox Equivalents (mmolTE) per gram of dry weight (gDW) of the extract according to the first-order equations obtained from the Trolox standard curve. High values of mmolTE/gDW point out high antioxidant capacities (greater ability to scavenge $ABTS^{\bullet+}$ radicals). IC_{50} was calculated for Trolox standard and is the sample dosage needed to reach 50% scavenging capacity of $ABTS^{\bullet+}$ radicals. In this case, higher values of IC_{50} mean less antioxidant capacity.

In the •OH scavenging assays, 1 mL of 1.5 mM $FeSO_4{\cdot}7H_2O$ was mixed with 1 mL of sample and 0.7 mL of H_2O_2 (30 % w/w), both prepared in methanol. Then, 0.3 mL 20 mM of sodium salicylate or 0.3 mL of methanol (blank) was added. The mixtures were then incubated for 1 h at 37°C. Absorbances were measured at 562 nm. A Trolox standard curve ranging from 0 to 12,000 μM was used to calculate the antioxidant capacity of the extracts in terms of •OH scavenging.

•OH scavenging capacity was calculated according to the following equation:

$$\bullet OH\ \text{scavenging capacity}\ (\%) = [1 - (A_1 - A_2)/A_0\,]\,100 \qquad (5)$$

where A_1, A_2, and A_0 correspond to the absorbances at 562 nm of the sample with sodium salicylate, the sample without sodium salicylate (replacing with 0.3 mL methanol), and the negative control of the assay (1 mL of methanol and sodium salicylate added), respectively.

Again, high mmolTE/gDW indicate high antioxidant capacities (greater ability to scavenge •OH radicals, in this case). IC_{50} was calculated for Trolox standard and it is the sample dosage needed to reach 50 % scavenging capacity of •OH radicals. In this case, higher values of IC_{50} mean less antioxidant capacity.

2.2.4. Biogas production

Spent biomass were used in the biochemical methane potential (BMP) experiments to assess their capability to produce biogas. The initial biomass of *D. salina* was also used for comparison purposes. Biomethane production from control experiments, containing only the inoculum, was subtracted from the rest of the experiments. The BMP experiments carried out in this work are summarized in Table 2. BMP tests were performed in triplicate.

Spent biomass after β-carotene extraction was dried at 40°C for 24 h and an aliquot was frozen at −20°C for further analyses. The C/N ratio of the spent biomass was analyzed. All the substrates were treated with a bead-beater (Biospect Products, Inc. Bartlesville, OK, USA) with 0.5 mm glass beads for 3 min to enhance their solubility in the medium of reaction. The inoculum, which had a volatile solids (VS) content of 71.6 ±0.8%, was mixed with 23 mL of MiliQ water, and the substrate was added at an inoculum to substrate ratio of 2:1 ratio.

BMP tests conditions, moisture, total solids, and volatile solids calculation and measurements of biomethane production and quality were performed following the specifications of a previous work [15].

Table 2
BMP experiments description.

Content	Substrate origin
Only sludge	Control
Sludge + initial biomass	Initial biomass
Sludge + spent biomass	Extraction with ethyl acetate. Process: Indirect
Sludge + spent biomass	Alkaline pretreatment + extraction. Matrix: Water: Process: Direct
Sludge + spent biomass	Alkaline pretreatment + extraction. Matrix: Water:Ethanol. Process: Direct

When BMP tests were completed, the digestate left in the bottles was centrifuged (10,000 rpm, 10 min) and filtered (0.45 μm Nylon filter), obtaining the soluble fraction, which was analyzed in terms of NH_4^+, N, P, and Chemical Oxygen Demand (COD).

2.2.5. Statistical analysis

All the experiments were carried out in triplicate, and statistical analyses were performed using the R software [29]. The Shapiro-Wilk test (p-value >0.05) was employed to check the normal distribution of the data followed by Levenne's test (p-value>0.05) to assess the homogeneity of the variance. One-way ANOVA tests were carried out when examining the influence of a single independent variable or factor. The solvent was the only factor in the solvent screening experiments, while the dependent variables were the β-carotene yield and purity.

For the alkaline pretreatments, a one-way ANOVA test was performed to check the impact of alkaline pretreatment types on various dependent variables, including β-carotene yield, content of β-carotene (purity), lipids, proteins, carbohydrates, and antioxidant capacity of the produced extracts in terms of $ABTS^{\bullet+}$ and •OH radical scavenging. This analysis was repeated by using the factor matrix of alkaline pretreatment. Furthermore, two-way ANOVA was performed to check the combined influence of these factors on the dependent variables. Moreover, Pearson correlation coefficients (r) were calculated to determine the correlation between each pair of variables. We analyzed the correlation between i) the polarity of solvents and the purity of the extracts and ii) the antioxidant capacity of the extracts according to the β-carotene yields and purities, protein, carbohydrate, and lipid contents. One-way ANOVA was performed in the BMP, COD, and NH_4^+ tests. Factors were the type of substrate, and dependent variables were cumulative methane production, COD reduction, and NH_4^+ increase. Tukey's HSD (honestly significant difference) tests were carried out to identify significant differences among data pairs.

2.2.6. Sustainability assessment

The biorefineries analyzed in this work have been subjected to sustainability assessment using the calculation of Biomass Exploitation (BE). This metric was calculated according to the next equation:

$$BE(\%) = mass\ of\ products/mass\ of\ biomass\ \ 100 \quad (6)$$

The mass of the products includes the mass of the β-carotene-rich extract and the valuable portion of biogas (biomethane), while the mass of biomass corresponds to the initial *D. salina* dry biomass.

3. Results and discussion

3.1. Initial screening of solvents for extraction of β-carotene

A preliminary screening was conducted using five organic solvents of varying polarity to assess their ability to extract β-carotene from the cells of *D. salina*. Solvents compliant with European regulation framework [30] for cosmetic ingredient production (acetone, diethyl ether, and ethyl acetate) were selected. Heptane and hexane, deemed unsafe solvents for this purpose, were also compared due to their low polarity, similar to β-carotene. Fig. 2 shows the β-carotene yield and purity for all the extracts.

Among all the tested solvents for the β-carotene extraction, ethyl acetate released the highest extraction efficiency (5.3% ± 0.03%), showing significant differences with the nonpolar solvents such as heptane (3.8% ± 0.2%, p = 0.047) and hexane (3.7% ± 0.1%, p = 0.029). Given that β-carotene is a non-polar carotenoid and both hexane and heptane are non-polar solvents, it was initially expected that these solvents would provide better extraction outcomes. However, ethyl acetate, being a medium-polar solvent, exhibited better results than anticipated. This discrepancy suggests that additional factors are influencing the extraction results. The microalga *D. salina*, while predominantly rich in β-carotene, also contains smaller amounts of chlorophyll *a*

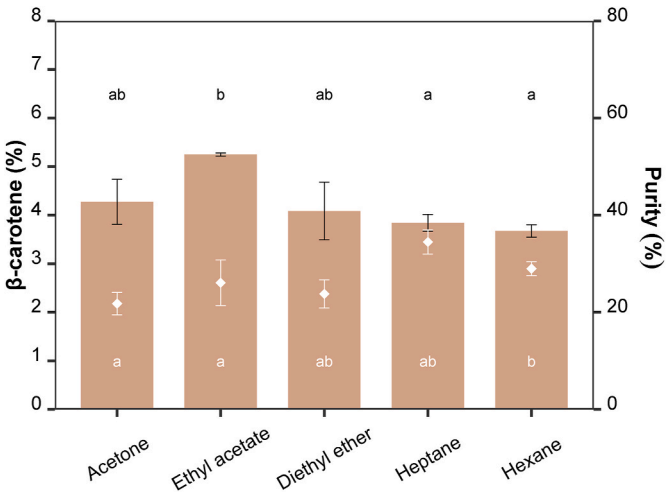

Fig. 2. β-carotene yield dry basis (brown bars) and purity (white dots) of the extracts from *D. salina* using different organic solvents. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and b, and very small quantities of xanthophylls such as lutein and zeaxanthin (Fig. S1, supplementary material) which can be extracted in varying quantities depending on the polarity of the solvent. Complete extraction of chlorophylls and xanthophylls typically requires the use of more polar solvents such as acetone, due to the strong bonds between chlorophylls and other components in the chloroplast and the presence of oxygen in the chemical composition of the xanthophylls. Thus, ethyl acetate, with its intermediate polarity, is capable of extracting β-carotene and the above-mentioned pigments of more polarity. This co-extraction is evidenced by spectrophotometric analysis, where the maximum absorption peak of β-carotene at 454 nm overlaps with absorption peaks of lutein, zeaxanthin and chlorophyll *a*. Thus, the presence of those pigments in the ethyl acetate extracts may contribute to the overall calculated extraction yield of β-carotene.

The extraction with diethyl ether, which has a polarity between the non-polar solvents (hexane and heptane) and ethyl acetate, yielded a lower β-carotene extraction efficiency (4.1% ± 0.6%) compared to ethyl acetate. However, there were no significant differences in β-carotene extraction efficiency between ethyl ether and either of the non-polar solvents.

Besides, no significant differences were observed when comparing the extraction yield using ethyl acetate to that of the more polar solvent acetone (4.3% ± 0.5%), although, given the non-polar nature of β-carotene, the results might have been expected to be somewhat lower. The solubility of this pigment in hexane is higher (600 mg/mL) than the corresponding one in ethyl acetate (500 mg/mL) [31]. In this case, the results are attributed to a slight decrease in β-carotene extraction yield and a minor increase in chlorophyll and xanthophyll extraction, which correlates with the polarity of acetone. Another factor could be the influence of solvents on the cell membrane, which is crucial for extracting intracellular compounds. Polar solvents are more effective at reaching the concentrations needed to disrupt and break down the membrane [32]. However, the relatively thin cell membrane of *D. salina* facilitates β-carotene extraction, thereby reducing the need for highly polar solvents. These results have been corroborated by HPLC analysis of the β-carotene-rich extract obtained from acetone extraction (Fig. S1, supplementary material), which primarily detected β-carotene (85%) and smaller amounts of α-carotene (6.5%), chlorophyll *a and b* (5%), lutein (2.5%), and zeaxanthin (1.5%). Previous studies on organic solvent extraction of β-carotene from microalgae released similar results, with ethyl acetate being one of the best solvents to extract β-carotene [33,34].

The purity of the extracts (the amount of β-carotene in the extract)

was also assessed. Purities between 21% and 35% were obtained in all cases due to the significant amount of lipid co-extracted with β-carotene using these solvents. The Pearson correlation coefficient (−0.83) revealed a tendency: a decrease in the polarity of the solvent led to somewhat purer extracts. For instance, hexane and heptane yielded extracts with purities of 29.0% ± 1.5% and 34.5% ± 2.5%, respectively, while solvents with higher polarity indexes, such as acetone, resulted in less pure extracts (21.8% ± 2.3%). The purity obtained using ethyl acetate was 26.1% ± 4.7%, but this value is not significantly different from the highest obtained with n-heptane. The only significant differences in purity were found between heptane extracts and acetone and diethyl ether extracts (21.8% ± 2.3% and 23.8% ± 2.9%, respectively). Therefore, the low to medium-polar solvents used in this work exhibited higher purities of non-polar β-carotene in *D. salina* compared to the high-polar solvents, corroborating that the extraction efficiency depends on the polarities of both the carotenoid and the solvent.

Ethyl acetate was selected as the best solvent to extract β-carotene due to i) its highest β-carotene yield, ii) the closest purity to non-polar solvents, iii) the possibility of commercialization in the cosmetic sector according to the EU legal framework.

3.2. Alkaline pretreatments for β-carotene extraction

3.2.1. β-Carotene yield and extract composition

After completing the solvent screening process and selecting ethyl acetate as the best solvent for β-carotene extraction, alkaline pretreatment experiments were conducted. Two different alkaline pretreatments were performed: direct treatment of the biomass of *D. salina* before the extraction stage and indirect treatment of the extract obtained using ethyl acetate. Additionally, two media were employed in the alkaline treatments: water and a water:ethanol mixture. Extracts were analyzed based on their β-carotene yield and purity, lipid, protein, and carbohydrate contents, and antioxidant capacity (Table 3).

These experiments aimed to achieve two main objectives: i) obtain β-carotene-rich natural extracts to assess their bioactivity in terms of antioxidant capacity, and ii) serve as pretreatments of the microalgal spent biomass after the extraction in the direct processes to increase the biogas yields.

β-carotene extraction yields were mainly influenced by the type of pretreatment performed as indirect pretreatments in water and in the mixture water:ethanol offered higher β-carotene yields (5.4% ± 0.2% and 5.5% ± 0.4%, respectively) than direct pretreatments with water (4.3% ± 0.3%) and water:ethanol (4.6% ± 0.1%). However, the reaction medium was found to be insignificant in each pretreatament considered separately (p =0.38). The lower recovery of β-carotene pigment in the alkaline direct experiment with water may be due to the low miscibility of ethyl acetate in water, which hindered the solubility of this pigment in the ethyl acetate phase. However, the same direct pretreatment using a mixture water:ethanol allowed a higher retrieval of β-carotene, because of the miscibility of ethyl acetate in ethanol, which implies an increase in the solubility of β-carotene in the ethyl acetate phase.

Table 3
Characterization of extracts produced from *D. salina*. W-D: direct alkaline pretreatment/water, WE-D: direct alkaline pretreatment/water:ethanol, W-I: indirect alkaline pretreatment/water, WE-I: indirect alkaline pretreatment/water:ethanol.

Variable	W-D	WE-D	W-I	WE-I	*Pearson's r*	
					$ABTS^{\bullet+}$	•OH
β-carotene yield (%, dry basis)	4.3 ± 0.3^{a}	4.6 ± 0.1^{ab}	5.4 ± 0.2^{b}	5.5 ± 0.4^{b}	−0.85	−0.77
β-carotene purity (%, dry basis)	26.0 ± 2.8^{a}	16.9 ± 0.2^{b}	26.5 ± 0.7^{a}	23.5 ± 2.1^{ab}	−0.10	−0.53
Lipids (%, dry basis)	51.1 ± 3.2^{a}	58.4 ± 1.3^{a}	54.4 ± 2.5^{a}	59.4 ± 3.3^{a}	−0.22	−0.04
Proteins (%, dry basis)	15.2 ± 1.4^{a}	16.1 ± 0.8^{a}	10.3 ± 0.8^{b}	8.6 ± 0.3^{b}	**0.51**	**0.78**
Carbohydrates (%, dry basis)	7.8 ± 1.0^{a}	8.6 ± 0.4^{a}	8.9 ± 0.6^{a}	8.6 ± 0.9^{a}	−0.36	−0.25
$ABTS^{\bullet+}$ radical scavenging (mmolTE/gDW)	0.69 ± 0.1^{a}	0.61 ± 0.1^{a}	0.55 ± 0.1^{a}	0.53 ± 0.1^{a}	–	–
•OH radical scavenging (mmolTE/gDW)	1.89 ± 0.2^{a}	2.05 ± 0.5^{a}	0.48 ± 0.0^{b}	1.2 ± 0.3^{ab}	–	–

The alkaline direct experiment in water:ethanol medium did not show significant differences compared to the corresponding indirect experiment using the same medium. The miscibility of ethanol in ethyl acetate helps to better mix the phases, facilitating the solubility of compounds like β-carotene, which is less polar. Therefore, ethanol decreases the polarity of the medium compared to pure water, which favors the solubility of β-carotene in the ethyl acetate phase. Furthermore, alkaline pretreatments did not improve β-carotene extraction yields compared to a simple extraction with ethyl acetate (5.3% ± 0.03%, Fig. 2), as β-carotene is in a free form and does not require alkaline pretreatment for its release. By contrast, other kinds of carotenoids, such as lutein, do not exist in free form or as stable fatty acid esters and require saponification. Besides, saponification is typically used to separate carotenoids from lipids [13].

β-carotene purity was only influenced by the matrix of treatment. Alkaline treatment utilizing the mixture of water and ethanol resulted in less pure extracts than treatments in water medium. This difference was particulary notable for the direct alkaline treatment. The β-carotene purities were 26.0% ± 2.8% and 16.9% ± 0.2% for the direct treatment using water and a mixture of water and ethanol, respectively. This may be attributed to the higher solubility of lipids in ethanol, leading to increased lipid content (the primary component of *D. salina* biomass) in the β-carotene extracts. Thus, the lipid content of these extracts was 58.4% ± 1.3% and 59.4% ± 3.3% for the direct and indirect treatments with KOH, respectively, using an alcoholic medium. However, when the experiments were conducted only with water, lipid amounts in the carotenoid-rich fraction were lower (51.1% ± 3.2% and 54.4% ± 2.5% for the direct and indirect treatments, respectively).

Proteins constituted the second most abundant group of biomolecules found in the β-carotene extracts. Alkaline pretreatments are frequently used to disrupt microalgal cell walls. Yet, it has been demonstrated that they can also enhance protein solubility due to the increased solubility of proteins under basic conditions [35,36]. Results showed that the type of pretreatment (direct or indirect) greatly influenced the protein content in the β-carotene extracts ($p < 0.001$): direct treatments yielded higher protein content (15.2% ± 1.4% and 16.1% ± 0.8%) than indirect ones (10.3% ± 0.8% and 8.6% ± 0.3%) for the water and water:ethanol media of reaction, respectively. Indirect experiments were less effective in recovering proteins due to the initial extraction using ethyl acetate, which primarily recovers lipids with a smaller amount of proteins (and carbohydrates). Consequently, fewer proteins were available in the ethyl acetate extract subjected to the alkaline treatment. Furthermore, direct biomass treatments with KOH led to protein solubilization in the basic reaction medium, which was then recovered in the β-carotene ethyl acetate extracts.

Carbohydrates constitute the minority group of biomolecules quantified in the produced extracts, and neither the matrix nor the type of alkaline pretreatment influenced their yields, as ethyl acetate is less efficient in extracting carbohydrates compared to lipids or proteins [37,38].

3.2.2. Antioxidant capacity of the extracts: •OH and $ABTS^{\bullet+}$ scavenging

β-carotene extracts of *D. salina* were also assessed in terms of antioxidant capacity to scavenge $ABTS^{\bullet+}$ and hydroxyl radicals (•OH). For this purpose, standard of pure Trolox (vitamin E analog), a well-known antioxidant, was used as a reference to express the antioxidant capacity

of natural extracts. This standard was assessed in the ABTS$^{\bullet+}$ (Fig. 3A) and •OH (Fig. 3B) radical scavenging assays.

Trolox offered higher antioxidant power in scavenging ABTS $^{\bullet+}$ than •OH radicals, as their IC_{50} were 244.3 μM and 7,619 μM, respectively.

First-order equations (R^2 =0.99) were obtained and used to calculate the ABTS $^{\bullet+}$ and •OH radical scavenging potential (antioxidant capacity) of the β-carotene extracts produced in this work and the results are represented in Table 3:

$$ABTS^{\bullet+}\ scavenging\ (\mu M\ TE) = -670.38\ (A_{734}) + 478.35 \quad (7)$$

$$\bullet OH\ scavenging\ (\mu M\ TE) = -23851\ (A_{562}) + 15759 \quad (8)$$

Where A_{734} and A_{562} are the absorbances measured at the wavelengths 734 nm and 562 nm, respectively.

Extracts obtained through direct pretreatments with KOH of *D. salina* biomass exhibited higher scavenging activity than those obtained through indirect pretreatments. Direct experiments offered an ABTS$^{\bullet+}$ radical scavenging potential of 0.69 ± 0.1 and 0.61 ± 0.1 mmolTE/gDW for the water and water:ethanol media, respectively, while those obtained through the indirect pretreatments offered an ABTS$^{\bullet+}$ radical scavenging potential of 0.55 ± 0.1 mmolTE/gDW and 0.53 ± 0.1 mmolTE/gDW using water or water/ethanol media, respectively.

Results of antioxidant capacity in terms of hydroxyl radical scavenging of the produced *D. salina* extracts in this work showed the same pattern than for the ABTS$^{\bullet+}$ radicals: direct pretreatments released the highest antioxidant capacities. Their •OH scavenging power were 1.89 ± 0.2 mmolTE/gDW and 2.05 ± 0.5 mmolTE/gDW for the water and water:ethanol mixtures, respectively, whereas indirect experiments offered lower antioxidant capacities (0.48 ± 0.0 mmolTE/gDW and 1.2 ± 0.3 mmolTE/gDW for the water and water:ethanol pretreatments, respectively).

Our results of antioxidant capacity in terms of ABTS$^{\bullet+}$radical scavenging were similar to the ones reported by other authors who used different methods or solvents, obtaining antioxidant capacities in the range of 0.4–0.76 mmolTE/gDW [39–42]. For two of those studies [39,40], an alkaline treatment was performed with the biomass of *D. salina*, and the assessed extracts released similar ABTS$^{\bullet+}$radical scavenging activity to that reported in our study. However, the antioxidant capacity results in works that did not include an alkaline treatment step [41,42] were lower, indicating that alkaline treatments as those carried out in our work are suitable in terms of enhancing the antioxidant capacity. Additionally, other factors, such as the solvent, affect the composition of the extracts and, therefore, their antioxidant capacity.

To identify the type of antioxidant molecule potentially responsible for capturing the ABTS$^{\bullet+}$and •OH radicals, Pearson's r coefficients were calculated for the extract composition (Table 3): β-carotene, lipids, proteins, and carbohydrates. Although carotenoids have been reported as ABTS$^{\bullet+}$ and •OH radical scavengers [43,44], the β-carotene purity of the extracts of *D. salina* were not positively correlated with their antioxidant capacity. This suggests that the antioxidant capacity of the β-carotene extracts may have originated from a synergy of β-carotene with other antioxidants present in the extracts [8].

Antioxidant capacity did not increase with higher lipid and carbohydrate content. However, a relationship was observed between antioxidant capacity and protein content in the extracts. Pearson's r coefficients revealed a positive correlation between protein content and scavenging activity (r =0.51 and r =0.78, for the ABTS$^{\bullet+}$and •OH radicals, respectively), indicating that the antioxidant power increased with the protein content. For that reason, extracts obtained through direct alkaline pretreatments, containing protein amounts of 15.2% ± 1.4% (water medium) and 16.1% ± 0.8% (water:ethanol medium), offered higher antioxidant capacities than indirect alkaline pretreatment extracts, which contained 10.3% ± 0.8% (water medium) and 8.6% ± 0.3% (water:ethanol medium) of proteins. These results can be explained by the fact that chemical hydrolysis with KOH during the direct pretreatment in both media may have released protein fractions (peptides or even amino acids) with high antioxidant capacity. Thus, chemical hydrolysis has been performed previously to recover peptides from microalgae and bacteria [36], and peptides extracted from the microalga *D. salina* have been proven to be a potent antioxidant [45].

In the case of indirect treatment, the protein concentration was lower in both media used: water (10.3% ± 0.8%) and water-ethanol mixture (8.6% ± 0.3%). For this reason, the extracts offered lower antioxidant capacities. In this case, ethyl acetate is also present during the pretreatment, as extraction with this solvent is the first step in the indirect process. Therefore, the lower protein values are due to the immiscibility of water and ethyl acetate during the indirect pretreatment. Thus, KOH remains in the water phase, hindering protein hydrolysis and protein fractions dissolution in the ethyl acetate phase.

3.3. Biogas production

The spent biomass after β-carotene extraction was used in anaerobic digestion reactions to assess its potential to produce biogas within a circular economy framework, aligning with the UN's SDG 7 on affordable and clean energy. Four different substrates derived from *D. salina* were used: the initial biomass, the spent biomass after a straightforward extraction with ethyl acetate (indirect process), and the spent biomass

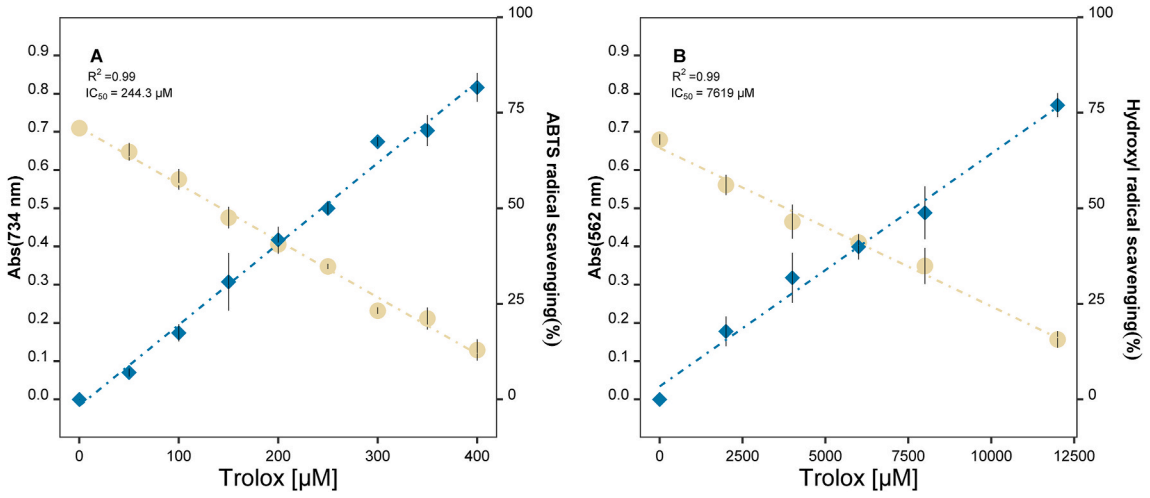

Fig. 3. ABTS $^{\bullet+}$(A) and •OH (B) radical scavenging assays using Trolox standard. Light brown color depicts absorbances. Blue color represents % of radical scavenging. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

after an alkaline treatment with either water or a water-ethanol media followed by the extraction of β-carotene with ethyl acetate.

In this study, biomethane production from alkaline pretreated biomass was aimed at not disrupting the cell walls, as *D. salina* lacks a rigid polysaccharide cell wall. Instead, the goal was to hydrolyze complex organic molecules into simpler ones, facilitating their solubilization in the sludge and enhancing access for methanogenic bacteria. The kinetics of biomethane production and biogas quality are represented in Fig. 4.

The substrates derived from the initial biomass and the spent biomass after the ethyl acetate extraction stage of the indirect process exhibited comparable patterns in terms of biomethane production yield. Both substrates achieved similar final values, with 218 ± 47 $mLCH_4$/gVS and 235 ± 57 $mLCH_4$/gVS, respectively. In contrast, biomethane production using alkaline-pretreated biomass via the direct processes yielded significantly higher values. Specifically, biomethane production reached 301 ± 14 $mLCH_4$/gVS and 289 ± 9.0 $mLCH_4$/gVS for the spent biomass subjected to aqueous and alcoholic alkaline pretreatments, respectively. Notably, no significant differences were observed between these two alkaline pretreatment methods in terms of biomethane production yield. Alkaline pretreatment of microbial biomass significantly enhances biogas production through anaerobic digestion. This enhancement is primarily due to the increased solubilization of organic matter, which improves substrate availability for microbial digestion, leading to higher biogas yields [46]. Additionally, alkali can induce the ionization of hydroxyl groups in some complex molecules. This ionization promotes the swelling and bioavailability of molecules by causing anion repulsion and disrupting the hydrogen bonds of the molecule granules [47]. It is observed that the pretreatment also reduces the lag phase in biogas production (Fig. 4), allowing for a quicker onset of methane generation due to the improved biodegradability of the pretreated biomass, which was also reported for the anaerobic digestion of alkaline pretreated pulp and paper biomass [48].

Consistent with our findings, previous studies have reported substantial increases in methane yield with alkali pretreatment compared to untreated biomass. For instance, Solé-Bundó et al. (2017) [49] observed increased biogas production when using lime to pretreat a mixed culture of microalgae and bacteria, highlighting the effectiveness of alkaline pretreatment in enhancing biogas yields.

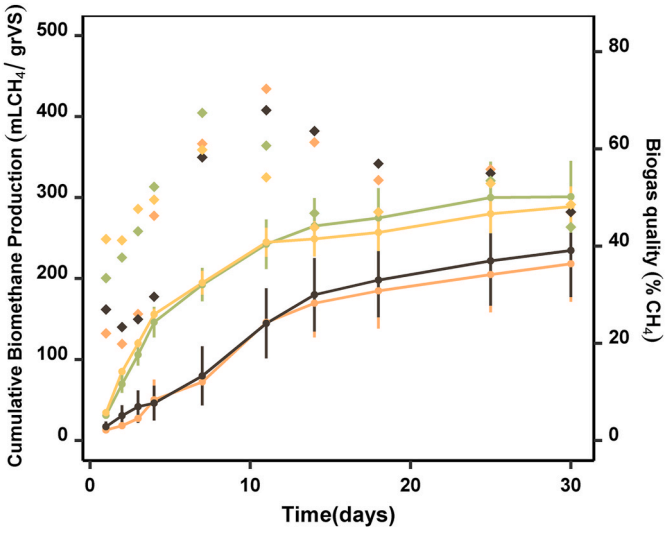

Fig. 4. Cumulative biomethane production of *D. salina* substrates. Initial biomass (orange). Spent biomass from ethyl acetate extraction in the indirect process (brown). Spent biomass from alkaline direct process, water medium (green). Spent biomass from alkaline direct process, water:ethanol medium (yellow). Rhombuses depict biogas quality. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

These results align with findings from other studies [17] and underscore the potential of alkaline pretreatment as a viable strategy for optimizing biogas production processes and advancing sustainable bioenergy systems.

Increased COD reduction during anaerobic digestion highlighted an efficient methanogenesis and higher biomethane yields (Fig. 5).

Consequently, a more significant decrease in COD was observed in the alkaline-pretreated spent biomass, regardless of the medium used (81.5% ± 0.1% and 82.8% ± 1.1% using water and a mixture of water and ethanol, respectively), compared to both the untreated substrates: the initial biomass of *D. salina* (58.9% ± 1.2%) and the spent biomass obtained in the indirect process using ethyl acetate for carotenoid extraction (41.4% ± 3.8%, i.e., spent biomass after extraction with ethyl acetate). The discrepancy in COD reduction values between the initial biomass and the spent biomass obtained through ethyl acetate extraction also corresponds to the observed differences in biomethane yields (218 ± 47 $mLCH_4$/gVS and 235 ± 57 $mLCH_4$/gVS, respectively).

The initial and final values of NH_4^+ in the soluble fraction of each experiment and its increase are represented in Table 4.

In all the experiments, there has been an increase in the percentage of ammonium due to the conversion of organic nitrogen to ammonium during anaerobic digestion. However, this increase was more noticeable in the alkaline-pretreated biomass using the mixture water:ethanol (3.02 fold increase). This could be explained by the initial ammonium concentration in this experiment (194 ± 14 $mgNH_4^+$/L). Values below 200 mg/L are reported to be beneficial to microorganisms during anaerobic digestion, as nitrogen is an essential nutrient [50]. This result is also consistent with one of the highest value of biomethane production (289 ± 9 $mLCH_4$/gVS) obtained in this experiment. By contrast, the lowest increase of ammonium was achieved using the initial biomass as substrate (2.02 fold increase), which aligns with the lowest results of biomethane obtained during its anaerobic digestion (218 ± 14 $mLCH_4$/gVS).

The C/N ratios of microalgae substrates are depicted in Table 5. The C/N ratio of the initial biomass was the highest because the other substrates resulted from previous extraction steps that released some carbon compounds, especially lipids (Table 3). These extractions caused a slight decrease of the C/N in all spent biomass substrates (13.7 ± 0.4, 15.2 ± 0.7 and 14.7 ± 0.4) compared to the initial biomass (17.3 ± 0.1). Typically, an increase in the C/N ratio implies higher yields of biomethane production. However, this is not the case with the initial biomass in this study, despite having the highest C/N ratio, as it yielded low biogas production compared to the other substrates of this study. Therefore, the C/N values were not the determining factor for anaerobic digestion of the substrates in this study; instead, the alkaline pretreatment was the controlling issue for achieving higher biogas production, as observed in the results of biomethane production using alkaline-treated spent biomass (Fig. 4).

The speed of anaerobic digestion can be assessed using the hydrolysis constant outlined in Table S1 (supplementary material). Initial biomass and spent biomass obtained in the indirect process with ethyl acetate exhibited low hydrolysis constants ($38{\cdot}10^{-4} \pm 2{\cdot}10^{-4}$ d^{-1} and $5.7{\cdot}10^{-4} \pm 1{\cdot}10^{-4}$ d^{-1}, R), indicating a slower conversion of biomolecules into biogas. In contrast, the values for spent biomass obtained through alkaline treatment were significantly higher: $0.01 \pm 8.5{\cdot}10^{-3}$ d^{-1} and $0.03 \pm 4.3{\cdot}10^{-3}$ d^{-1} when aqueous KOH and a combination of ethanolic and aqueous KOH were used, respectively, implying a faster conversion from organic compounds to biogas. This suggests that alkaline pretreatment leads to an increase in the rate of microalgal biomolecules conversion to biogas, regardless of the KOH solution. Consequently, as mentioned above, the solubilization of biomass biomolecules caused by the basic treatment of *D. salina* biomass also enhances the hydrolysis rate during anaerobic digestion. Thus, this study demonstrates that the alkaline treatments increased biomethane yields and improve the hydrolysis rate during anaerobic digestion.

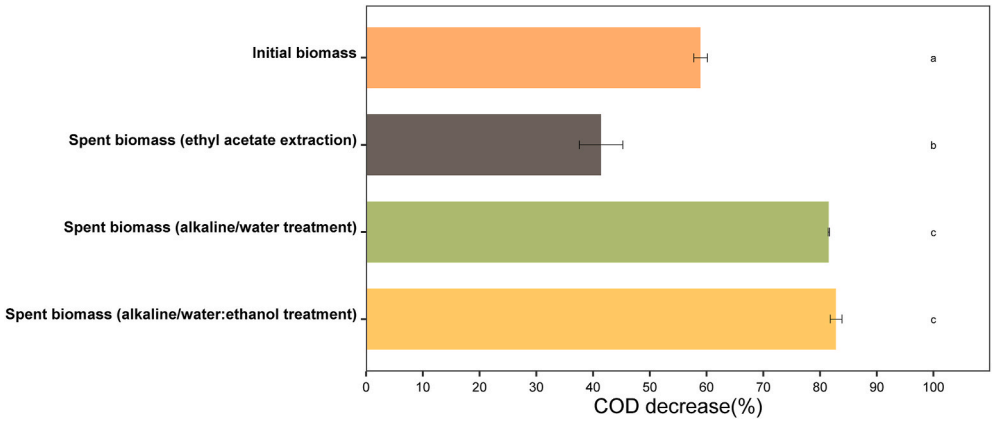

Fig. 5. Chemical oxygen demand (COD) reduction (%) during anaerobic digestion of *D. salina* substrates.

Table 4
Initial and final values of NH_4^+ concentration in the soluble fraction.

D. salina substrates		[NH_4^+] day 1 (mg/L)	[NH_4^+] day 30 (mg/L)	Fold-change
Initial Biomass		249 ± 9	503 ± 8	+2.02
Spent Biomass	Ethyl acetate extraction (indirect process)	233 ± 48	544 ± 13	+2.33
	Alkaline/water treatment (direct process)	200 ± 41	518 ± 5	+2.59
	Alkaline/water: ethanol treatment (direct process)	194 ± 14	586 ± 21	+3.02

Table 5
C/N ratio of the initial substrates of anaerobic digestion.

D.salina substrates		C/N
Initial Biomass		17.3 ± 0.1
Spent Biomass	Ethyl acetate extraction (indirect process)	13.7 ± 0.4
	Alkaline/water treatment (direct process)	15.2 ± 0.7
	Alkaline/water: ethanol treatment (direct process)	14.7 ± 0.4

The results of this study were compared with those available in the literature for anaerobic digestion of *D. salina* (Table 6). All the substrates of the present work were subjected to bead-beating to standardize all the samples and asses the influence of alkaline treatments.

Our result of biomethane production for initial *D. salina* biomass (218 ± 47 $mLCH_4$/gVS) was slightly lower than the one reported by Roberts et al. [53] (248 $mLCH_4$/gVS). Still, it exceeds the results reported by other research [51,52] on biomethane production with *D. salina*. This may be caused by bead-beating use for the standardization of the samples in this work.

Jeon et al. (2012) [52] explored various pretreatment methods for *D. salina* biomass, including thermal treatment, ultrasound, or combinations of those treatments. However, those methods did not result in increased biomethane yields (197 ± 9, 197 ± 4 and 184 ± 12 $mLCH_4$/gVS, respectively) compared to the biomethane values obtained with the initial *D. salina* in the present work (218 ± 47 $mLCH_4$/gVS), treated with bead-beating. In addition, those methods have been compared to the values obtained in our study using indirect pretreatment of *D. salina* and biomethane values observed in our study (235 ± 57 $mLCH_4$/gVS) were higher than those reported in the literature for pretreated *D. salina* biomass. As observed in Table 6, the best results for biomethane production were obtained with the spent biomass of the present study using the direct treatment with an alkali, both in aqueous and ethanolic solutions (301 ± 14 and 289 ± 9.0 $mLCH_4$/gVS, respectively). Jeon et al. (2012) [52] also studied alkaline pretreatment coupled with ultrasounds on the microalga *D. salina*, obtaining a value quite inferior (196 ± 3 $mLCH_4.gVS^{-1}$) to those reported in the present study with both types of media.

Biogas quality was also assessed, revealing two distinct patterns (Fig. 4). Biogas generated from both the initial and spent biomass after ethyl acetate extraction exhibited low-quality values on the first day (22% ± 2 % and 27 ± 3%, respectively). However, biogas quality experienced a remarkable increase until day 11 (reaching 72% ± 6% and 68% ± 6%, respectively). Beyond this point, biogas quality declined, remaining higher than the initial values, with biomethane percentages reaching 47% ± 0.2% and 47% ± 2%, respectively. In contrast, biogas produced from spent biomass after alkaline aqueous pretreatment or alkaline aqueous/ethanolic pretreatment exhibited higher initial biogas quality than the substrates above (33% ± 4% and 41% ± 2%, respectively). Within seven days, biogas quality increased until reaching concentrations of 67% ± 2% for spent biomass after alkaline aqueous treatment and 60% ± 7% for spent biomass after alkaline water:ethanol treatment. Subsequently, biogas quality declined, culminating in final biomethane concentrations of 44% ± 0.2% and 49% ± 0.8% for spent biomass after aqueous and aqueous/ethanolic alkaline treatment.

Table 6
Comparison of cumulative biomethane production (BMP) for *D. salina* substrates.

Biomass pretreatment	Time (day)	BMP $mLCH_4$/gVS	Reference
Bead-beating	30	218 ± 47	This study
Untreated	30	63 ± 17	[51]
Untreated	90	206 ± 3	[52]
Untreated	87	248	[53]
Bead-beating after ethyl acetate extraction	30	235 ± 57	This study (indirect process)
Thermal + Ultrasounds	90	184 ± 12	[52]
Thermal	90	197 ± 9	[52]
Ultrasounds	90	197 ± 4	[52]
Alkaline (water) + bead-beating	30	301 ± 14	This study (direct process)
Alkaline (water:ethanol) + bead-beating	30	289 ± 9.0	This study (direct process)
Alkaline + Ultrasounds	90	196 ± 3	[52]

3.4. Sustainability assessment

Biomass exploitation (BE) was used as a metric to evaluate the sustainability assessment for the biorefineries developed in the present study. For comparison purposes, BE was also calculated for similar biorefineries with other microalgae species such as *Haematococcus pluvialis, Phaeodactylum tricornutum*, and *Chromochloris zofingiensis*, as

assessed in other studies (Table 7).

Scenario 1, which involved the direct alkaline treatment using an aqueous medium, was the most sustainable among the four biorefineries presented in this work (BE =33.8%) due to its higher biomethane yield production (0.140 kg/kg DW) and mass recovery in the extract (0.198 kg/kg DW). Scenario 3 implied the indirect treatment with aqueous KOH of the β-carotene-rich extract, being the second most suitable biorefinery in terms of sustainability (BE =33.1%) due to the high mass recovery found in the extract (0.230 kg/kg DW), which was similar in the scenario 4 (0.227 kg/kg DW), the indirect one in a medium with water and ethanol. Both scenarios involved the use of the same *D. sallina* biomass, as the indirect alkaline treatment was carried out on the extract. Therefore, their biomethane production values are identical (0.101 kg/kg DW). Thus, this scenario also had a similar BE value of 32.8%. The scheme that included a direct alkaline treatment in alcoholic and aqueous medium (Scenario 2) obtained the lower mass recovery in the extract (0.166 kg/kg DW), being the least sustainable scheme studied (BE =29.1%) in terms of biomass harness, despite the high yield of biogas obtained during anaerobic digestion (0.125 kg/kg DW).

Scenario 1 was selected as the most sustainable biorefinery in this work because it achieved the highest harness of the initial biomass of *D. salina*. In future works, the aqueous phase after the separation of the ethyl acetate phase should be used to obtain valuable fatty acids, and the digestate needs to be valorized as soil fertilizer to improve BE.

Other biorefinery-based microalgae research has studied the extraction of carotenoids and subsequent biomethane production employing anaerobic digestion. We calculated the BE values of those works and compared them with the BE values of our study. Hosseini et al. (2020) [54] developed a biorefinery based on the microalgae *H. pluvialis* (Scenario 5) in which they produced an astaxanthin extract and biomethane from its spent biomass. The BE value calculated for this biorefinery was 16.5%. Scenario 6, involves the production of a fucoxanthin extract from the diatom *P. tricornutum* and the valorization of the residual biomass to produce biogas [55], releasing a value of 29.7%, similar to our Scenario 2, but lower than the BE values offered by our biorefineries of *D.salina* in Scenarios 1, 3 and 4. Scenarios 7 and 8, based on the microalgae *C. zofingiensis* [56], offered values of 14% and 16.9%, respectively. The biorefineries of our work are then proposed as biorefineries schemes with enhanced biomass exploitation percentages compared to similar microalgal biorefineries reported in the literature. This increased biomass harness is due to different variables such as the strain selection or the type of pretreatment employed that influences the extraction yields. *D. salina* lacks rigid cell walls and extraction yields are higher than for other strains. Moreover, alkaline pre-treatments are presented as an efficient solution to enhance BE compared to other pretreatments such as ultrasounds.

3.5. Industrial application and scale up-design

Direct alkaline pretreatment in an aqueous medium was the most successful of the experiments assessed in this study. Whereas the efficacy of this pretreatment has been proven at laboratory scale, it is important to address the final use of the obtained products in a real application in the industry, together with the analysis of possible constraints and improvements of the process in the scale up process. A scaling up process proposal is depicted in Fig. 6.

We propose a scenario of a company that produces natural extracts and preserves them, producing the final ingredient that will be sold to a final cream or serum manufacturer for the cosmetic industry. We assume that the company will produce a total amount of 3 tons per year of the β-carotene rich extract according to the procedure described in this work.

For that purpose, one cycle will be operated to process 70 kg of *D. salina* biomass per day. Considering 220 working days per year and a β-carotene extract yield of 20 %, 3 tons of dry β-carotene extract will be produced annually, which is equivalent to 14 kg/day. Direct alkaline treatment (Step 1) will be performed in a large-scale reactor with a capacity of 5,000 L, to which 1,400 L/day of water and 24.5 kg/day of KOH will be added. When performed at larger scale, mixing time should be optimized (improvement 1,i1), and temperature will be set at 55°C as described in the methodology of this study. Step 2 consists of the extraction of β-carotene and associated molecules by adding 2,800 L/day of ethyl acetate to the same reactor. The mixing time should also be optimized (improvement 2, i2). The mixture will be centrifugated (step 3) in two large scale centrifuges with a capacity of 2,100 L/h each, instead of just one, to save time (improvement 3, i3). This will separate the mixture into three phases: i) ethyl acetate phase, ii) aqueous phase, and iii) residual biomass. Ethyl acetate phase processing (step 4) includes filtration trough a hollow fiber membrane filter of a minimum capacity of 2,800L/h, evaporation through a falling film evaporator and recovering of ethyl acetate (improvement 4, i4) to increase the rentability of the industrial process. Recovered ethyl acetate will be used for further extractions, and dry biomass after ethyl acetate elimination should be preserved to produce the final active ingredient which will be encapsulated to facilitate the skin penetration trough topical application [57]. According to the results of this study, 1 g of the β-carotene dry extract possesses the same antioxidant capacity in terms of •OH scavenging than 0.47 g of the vitamin E analogue, pure Trolox, which is used as an antioxidant ingredient in cosmetics. However, to make the production of this active ingredient more profitable, it should be assessed in terms of bioactivity, including not only antioxidant capacity but also other skin-related functions with molecules found in the extract, such as β-carotene (improvement 5, i5). An ideal application of this cosmetic active could be its inclusion in suncream formulations due to the reported activity of β -carotene as depigmenting [58], photoprotective [59], an antioxidant (this study).

Moreover, dry extract will be diluted to a maximum of 5% (w/w) in the final product So, 280 kg/day of final product with antioxidant activity will be produced, increasing the profitability of the product (improvement 6, i6).

Spent biomass is then used to produce biogas (step 5). The spent biomass will be processed in a anaerobic digester with a minimum capacity of 10,000 L. The quantity of spent biomass, anaerobic sludge and water added to the anaerobic digester depends on the percentage of recuperation at large scale (improvement 7, i7). For instance, if 40 kg/day of spent biomass (containing 31.2 kg of volatile solids) would be recovered from the large-scale centrifuge, then we will add 87.2 kg/day of sludge (containing 62.4 kg of volatile solids) and water until reaching

Table 7
Sustainability assessment through BE calculation considering dry extract biomass and biomethane.

Microalga	Scenario	Extract	CH_4 (Time, d)	Other Product	BE (%)	Reference
D.salina	1	β-carotene	30		**33.8**	This study. Direct. Water
D.salina	2	β-carotene	30		**29.1**	This study. Direct. Water:ethanol
D.salina	3	β-carotene	30		**33.1**	This study. Indirect. Water
D.salina	4	β-carotene	30		**32.8**	This study. Indirect. Water:ethanol
H. pluvialis	5	Astaxanthin	40	Ethanol	16.5	[54]
P. tricornutum	6	Fucoxanthin	4.2		29.7	[55]
C. zofigiensis	7	Carotenoids	35		14.0	[56]
C. zofigiensis	8	Carotenoids	35	Ethanol	16.9	[56]

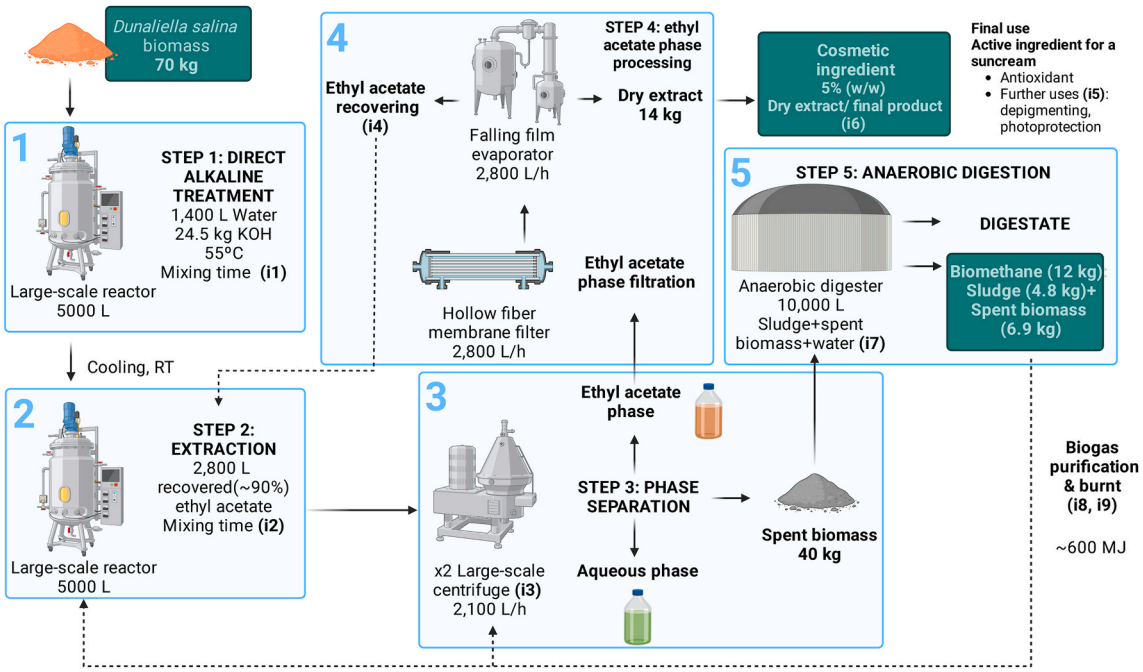

Fig. 6. Scheme of scaling-up the direct alkaline pretreatment process in aqueous medium of *D. salina* biomass for a calculation base of 1 day. Created in BioRender. Águila Carricondo, P. (2024) BioRender.com/e79e230.

a final volume of 6,961 L. Final product is biogas containing mainly biomethane and CO_2. Calorific value of the methane is withing the range of 50–55 MJ/kg, density at 37°C is 0.7 g/L, and the quantity of biomethane produced in this industrial scheme is 12 kg/day (considering 109 mL CH_4/gVS produced by sludge and 315 mL CH_4/gVS produced from the microalga spent biomass). Calorific value of biomethane is approximately 50 MJ/kg, so we will produce 600 MJ/day.

Biogas should be purified (improvement 8,i8) and then burnt using an internal combustion engine to feed the equipments used in the process. As an example, we will calculate the needed energy to feed the reactor used in steps 1 and 2 resulting that roughly 176 MJ/day are necessary to feed the reactor, leaving an excess of over 400 MJ from the produced biogas. This excess of energy can be used to power additional reactors or other equipments such as the large-scale centrifuge.

Moreover, there are two fractions of interest in the biorefinery scheme that have not been assessed in this study: the aqueous phase, that contains soaps which if acidulated can be converted into fatty acids, valuable as preservative of cosmetic ingredients, or to be assessed as a new ingredient with bioactivity, and the digestate that can be considered to be commercialized as biofertilizer. For that purpose, total nitrogen and phosphorous content were analyzed obtaining a nitrogen to phosphorous ratio of 1:0.94. This ratio may be beneficial for the growth of certain crops such as adzuki bean (*Vigna angularis*) [60].

Further studies should assess if the potential uses described for all the fractions are feasible and valuable at industrial scale.

4. Conclusions

The question of whether mild alkaline pretreatment can simultaneously enhance the antioxidant capacity of β-carotene extracts and biomethane yields in a sustainable *D. salina* biorefinery can be answered positively. The findings of this study demonstrate that mild alkaline pretreatment applied directly to *D. salina* biomass in an aqueous medium not only enhances the antioxidant power of β-carotene extracts by increasing the scavenging capacity against $ABTS^{\bullet+}$ and •OH radicals by 1.3 and 3.9-fold, respectively, compared to the lowest value obtained (indirect experiment in an mixture of ethanol and water, in the case of ABTS radicals, and indirect experiment in an aqueous medium for the hydroxyl radicals), but also significantly increases 1.4-fold the biomethane production from the spent biomass, relative to the lowest value observed, which correspond to biogas production from the initial biomass. The sustainability analysis highlights that this pretreatment is the most advantageous, as biomass exploitation was 1.2 times greater than that obtained from the less sustainable biorefinery evaluated in this study. (direct alkaline treatment in water:ethanol). These benefits support the development of more efficient and sustainable biorefineries with applications in both the cosmetic and bioenergy sectors. In this sense, a scale-up scheme for the alkali pretreatment in an aqueous medium has been described in this work, including industrial-scale improvements, to obtain 280 kg/day of a β-carotene rich active ingredient for the cosmetic industry and 12 kg/day of biomethane, using 70 kg/day of *D. salina* biomass.

CRediT authorship contribution statement

Pilar Águila-Carricondo: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Raquel García-García:** Investigation, Formal analysis. **Juan Pablo de la Roche Cadavid:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Pedro Luis Galán:** Writing – review & editing, Resources, Project administration. **Luis Fernando Bautista:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Gemma Vicente:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2024.107474.

Data availability

Data will be made available on request.

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