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Sustainable biorefining of *Chlorella vulgaris* into protein, lipid, bioethanol, and biogas with substantial socioeconomic benefits

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ABSTRACT

An integrated biorefinery was developed that utilizes microalgal biomass, *Chlorella vulgaris*, to sustainably produce proteins, fatty acids, bioethanol, and biogas. The microalgal soluble proteins and fatty acids were initially extracted through a cascading extraction process, including bead milling and solvent extraction. Subsequently, the investigation focused on utilizing the biomass residues for bioethanol and biogas production, ultimately improving energy recovery. Implementing the cascading process resulted in a 25 % enhancement in bioethanol yield and a 22.4 % increase in biomethane yield compared to untreated biomass. This approach resulted in 78.0 g of protein, 50.9 g of lipid, 20.8 ml of ethanol, and 136.5 L of methane from one kilogram of dry *C. vulgaris* biomass. Considering the potential of 8,640 k tons of annual microalgae production in Iran, an estimated 4.1 million tons of CO2 emissions could be averted. This reduction could result in saving approximately 1394.8 million USD in associated social costs of carbon. These improvements in fully valorizing biomass through practical cascading methods significantly advance microalgal biorefinery.

1. Introduction

The remaining CO_2 emission 'budget' to limit temperature increase to 1.5 °C stands at approximately 250 billion tonnes. With the current emission rate at about 54 billion tonnes per year, this restricted budget will likely be depleted before the decade concludes, necessitating urgent action to curb emissions and mitigate the severe impacts of climate change [1]. One of these crucial measures involves reshaping the utilization framework toward bio-based products, capitalizing on their innate attributes, carbon sequestration capabilities, and reduced carbon footprint. Bio-based products are derived from various biological sources classified into different generations: first (i.e., food crops, energy crops, edible oilseeds, and animal fats), second (i.e., lignocellulosic biomass), and third or fourth (i.e., algae and other microbes). While the first and second generations of feedstocks rely on food crops and non-food biomass, respectively, they present challenges such as competition with food production and limited availability. Conversely, the third and fourth generations offer more sustainable alternatives, including increased productivity, high carbon dioxide absorption, and reduced land usage [2].

In recent years, microalgae have garnered attention as a renewable source for producing bio-based products. These microorganisms possess remarkable capabilities for thriving in diverse environmental conditions such as lakes, ponds, seas, rivers, and even wastewater. They use water, CO_2, and sunlight to generate biomass used in the production of biofuels, food, feed, and valuable commodities [3]. Carbohydrates, proteins, and fatty acids constitute the main components of microalgae, accumulating at high concentrations within their cells. Carbohydrates find specific applications in pharmaceuticals, cosmetics, and biofuels like ethanol and hydrogen. Fatty acids serve as a suitable raw material for a wide range of products, from inexpensive biodiesel to high-value nutritional supplements and functional foods. While crude microalgal protein is not yet widely available in global nutrition markets, ongoing research demonstrates the remarkable potential of microalgal protein as a substantial source of human dietary protein [4,5].

Several studies concentrating on single-product extraction from microalgae reveal that this approach is not economically viable, especially when compared to inexpensive substitutes from fossil sources (e.g., biofuel at 0.2–0.5 $/kg) [6]. To fully exploit the potential of

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Nomenclature

Symbol	Description
FAME	Fatty acid methyl ester
F_M	Microalgal biomass, ton
W	Water, L
L_B	Liquid phase of bead milling, L
S_B	Solid phase of bead milling, kg
E	Ethanol, L
L_E	Liquid phase of solvent extraction, L
S_E	Solid phase of solvent extraction, kg
SSF	Simultaneous saccharification and fermentation
S_F	Amount of residual biomass input in high-solid fermentation, kg
S_{AD}	Amount of residual biomass input to anaerobic digestion, kg
$F_{biofuel}$	Annual production of biofuel, L
$Y_{biofuel}$	Yield of biofuel, L per kg dry biomass
F_{saving}	Saved fossil fuel, L
R	Energy ratio parameter, L fossil fuel per L biofuel
$C_{biofuel}$	well-to-wheel GHG emissions of biofuel, kg CO_2 eq/ L fuel
C_{fossil}	well-to-wheel GHG emissions of fossil fuel, kg CO_2 eq/ L fuel
GHG	Greenhouse gas emission, kg CO_2
SCC	Social cost of carbon dioxide, $
Δ_{GHG}	Reduction in greenhouse gas emission, kg CO_2 eq
Δ_{SCC}	Reduction in SCC, ($ per t emitted CO_2

microalgae, a shift to a biorefinery approach is crucial. This method involves recovering and separating the various biomass components while minimizing waste generation [7]. For this purpose, a biorefinery must contain suitable extraction methods to fractionate the various components while maintaining their yield and quality [8]. Currently, a cascade extraction method involving physical and chemical fractionation steps has been developed to recover feedstock components [9]. In this approach, the highest-value product is extracted first, with the remaining by-products valorized using different methodologies [7]. For instance, Francavila et al. [10] used a cascade approach to refine red macroalgae *Gracilaria gracilis* into phycobiliproteins, bio-oil, and biochar. They initially separated phycobiliproteins using a Potter homogenizer, followed by fast pyrolysis at 500 °C to transform cell residues into bio-oil and biochar. Similarly, Malik et al. [11] transformed *Chlamydomonas* sp. biomass into carotenoids, biodiesel, industrial enzymes, and mycoproteins through a cascading biorefinery, including solvent extraction and fungal fermentation. Efforts continue to seek processes capable of valorizing biomass entirely in a zero-waste and circular bioeconomy, focusing on the energy-efficient development of a sustainable algal-based biorefinery. Despite these efforts, developing efficient and scalable methods for separating and extracting different biomass components without extensive energy use and negative impacts on the properties and yield of the final product remains a challenge. Additionally, there is a lack of comprehensive socioeconomic analyses evaluating the practicality and sustainability of biorefinery approaches. To address these challenges, this study implemented practical methods (bead milling + solvent extraction) for initially separating proteins and fatty acids while preserving their quality. Subsequently, fermentation and anaerobic digestion were employed on the residual biomass to enhance overall efficiency and sustainability. Ultimately, a socioeconomic analysis was undertaken to evaluate the sustainability and greenhouse gas (GHG) emission reduction potential of biofuels derived from *Chlorella vulgaris* biomass cultivated in Iran, filling the gap in evaluating the real-world impact and economic feasibility of microalgal biorefineries.

2. Materials and methods

The proposed biorefinery was developed through microalgal cultivation, cascading extraction (bead milling and solvent extraction), fermentation, and anaerobic digestion (Fig. 1). Each step is elaborated in detail in the following section.

2.1. Cultivation and harvesting of microalgae

The green microalgae *C. vulgaris* UTEX 259 (the Culture Collection of algae, the University of Austin, USA) was cultivated in a 1300 L tubular photobioreactor (*LGem*, GemTube™ MK2-750, The Netherlands). Cultivation conditions, growth medium composition, and the harvesting procedure were reported in our previous work [12]. Briefly, M8a medium was used as a feedstock, and the pH of the culture medium was maintained at 6.7 by injecting CO_2 whenever deviations occurred from this optimal value. The pH was continuously monitored, with CO_2 injections adjusted to restore the optimal pH of 6.7. Natural light during February 2019 in Wageningen, The Netherlands, provided illumination, supplemented by high-pressure mercury greenhouse lamps (18 h on, 8 h off). The temperature was maintained within the range of 25 °C and 30 °C. Once the microalgal biomass concentration reached 2.4 g/L, it was collected and subsequently concentrated to achieve a concentration of 20 % w/w. This process was implemented using an Evodos type 25 spiral plate centrifuge at 0.75 m^3/h, ~3000 × g, and 80 Hz. The obtained biomass was divided into 100-gram portions in small bags and kept at −20 °C until further use. All experimental trials were performed with biomass that defrosted slowly at 4 °C.

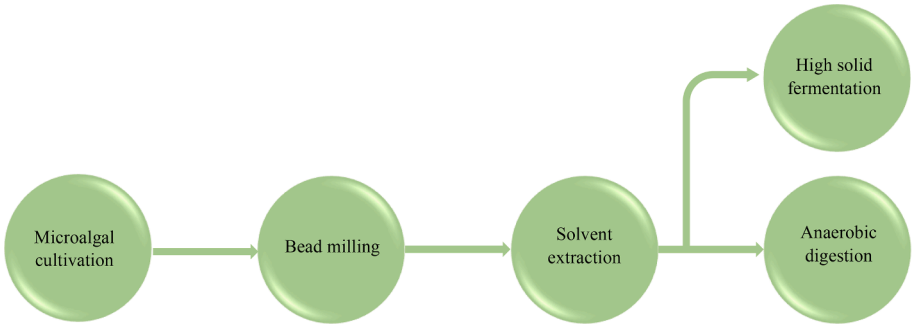

Fig. 1. The methodology employed for developing the biorefinery in this study.

2.2. Biomass composition characterization

The composition of *C. vulgaris* biomass, encompassing total carbohydrate, protein, lipid, and ash content, was determined using the following methods.

The Lowry method was employed to assess protein content. Microalgal cells were initially digested in 0.4 M sodium hydroxide and incubated at 100 °C with a 15 % w/w solid loading for 30 min. Protein content was determined using modified Lowry protein assessment kits provided by BioRad, utilizing bovine serum albumin as a reference standard and measuring absorbance at 750 nm.

Carbohydrate content was determined using the modified National Renewable Energy Laboratory (NREL) method for algal cell carbohydrates analysis [13]. This method involved treating 25.0 mg of microalgal biomass with 250 μL H_2SO_4 (72 % w/w) at 30 °C for 1 h. After the addition of 7 mL Milli-Q water and autoclaving at 121 °C for 1 h, the cooled samples were analyzed by high-performance liquid chromatography (HPLC) to measure monomeric sugars such as glucose and galactose.

The determination of lipids, fatty acids, and their characterization into polar and non-polar types followed the method outlined by Breuer et al. [14]. Briefly, 10 mg of microalgal biomass was processed with a 4 mL solution containing 2.22 mL methanol and 1.78 mL chloroform, and the resulting lipid was extracted by beating the bead to disrupt cell walls. The extracted lipid was collected after phase separation. Polar and non-polar fatty acids were separated using a SEP column. Additionally, the amount of total lipid released into the aqueous phase was ascertained using the procedure presented by Liang et al. [15]. The released lipids were transformed into fatty acid methyl ester (FAME) and identified by gas chromatography (GC) using internal standard, i.e., glyceryl tripentadecanoate (C15:0 TAG). Additionally, the FAME content (mg/mg biomass) is calculated using the following equation [14]:

$$FAME = \frac{M_{IS} \times A}{A_{IS} \times RRF} \times \frac{1}{M_{biomass}} \quad (1)$$

where M_{IS}, A_{IS}, $M_{biomass}$, and A refer to the weight of internal standard per sample (mg), the peak area of internal standard, the weight of analyzed biomass (mg), and the peak area of each FAME, respectively. Also, *RRF* (relative response factor) represents the ratio of the detector response of an analyte ($A_{analyte}$) to the detector response of an internal standard compound (A_{IS}). It is calculated for each fatty acid according to the following equation [14]:

$$RRF = \frac{A_{analyte} \times C_{IS}}{A_{IS} \times C_{analyte}} \quad (2)$$

which $C_{analyte}$ and C_{IS} refer to the concentration of analyte and internal standard, respectively.

For ash content determination, the biomass was firstly dehydrated at 100 °C in an oven for 24 h and then burned at 575 °C in a furnace. The ash content was calculated according to equation (3) [13], utilizing the difference between biomass weight before and after burning in the furnace:

$$\text{Ash content} = \frac{W_1 - W_2}{W_1} \quad (3)$$

with W_1 and W_2 referring to the weight of biomass (g) before and after burning in the furnace, respectively.

2.3. Cascading extraction

A cascading extraction method, utilizing bead milling and solvent extraction, was employed to extract proteins and fatty acids from *C. vulgaris* biomass. In this method, a horizontal bead mill with capacity of 75 mL (Dyno-Mill Research Lab, Willy A. Bachofen AF Maschinenfabrik, Switzerland) was used to disrupt the cell walls. The bead mill was filled with 0.4 mm Y_2O_3 stabilized ZrO_2 beads up to 65 % capacity. A suspension of microalgal biomass (25 g/L) in distilled water was then introduced into the bead mill, which operated in cyclic batch mode at a consistent stirring rate (2039 rpm) at 25 °C for 8 min. After operation, the suspension was collected and centrifuged (4000 × g, 4 °C, 45 min) to separate the liquid phase, primarily containing released intracellular components, such as proteins, from cell debris.

The subsequent step involved the selective fractionation of lipids (triglycerides, phospholipids, and cholesteryl esters) from the harvested pellets. The pellets were mixed with ethanol at a concentration of 37.5 mg/mL and kept at 30 °C for 30 min, following the method described by Figueiredo et al. [16]. The resulting mixture was centrifuged (2000 rpm, 10 min) to separate the liquid and solid phases. The liquid phase was collected in a pre-weighed glass tube. The solid phase was subjected to the same process eight times, with the collected liquid phase, containing ethanol and dissolved fatty acids, dried under a nitrogen gas stream to recover the fatty acids. The dried solid phase was preserved for further use.

2.4. Preparation of ethanol-producing microorganisms

Saccharomyces cerevisiae CCUG 53310 and the zygomycete fungus, *Mucor indicus* CCUG 22424 (the Culture Collection of the University of Gothenburg, Sweden) were utilized in the fermentation processes to produce ethanol from microalgal biomass residues. The biomass of *S. cerevisiae* was weighed under sterile conditions and added to the fermentation medium (sec. 2.5) until it reached a concentration of 5 g/L (dry weight). Regarding *M. indicus*, to promote spore formation, it was initially incubated on an agar slant medium consisting of 10 g/L peptone, 40 g/L D-glucose, and 20 g/L agar, at a pH of 5.5, and kept at a temperature of 32 °C for 5 days. Subsequently, to achieve a cell density of 5 g dry weight/L, the spores (at a concentration of approximately $6 \pm 3 \times 10^6$ spores/ mL) were isolated, suspended in sterilized distilled water, and cultivated in a solution containing 50 g/L glucose, 3.5 g/L KH_2PO_4, 7.5 g/L $(NH_4)_2SO_4$, 1.0 g/L $CaCl_2$, 0.75 g/L $MgSO_4{\cdot}7H_2O$, and 5 g/L yeast extract. The cultivation was carried out at 150 rpm and 32° C for 24 h. The obtained fungal biomass was then harvested by centrifugation (4000 rpm, 10 min), rinsed twice with distilled water, and used to inoculate the fermentation medium.

2.5. Ethanol production

The potential for ethanol production from microalgal biomass residues was investigated using *S. cerevisiae* and *M. indicus*. The fermentation process involved whole cells of *C. vulgaris* to evaluate the impact of the cascading extraction on ethanol yield. The SSF medium was prepared by mixing sodium citrate buffer (50 mM) with yeast extract (5 g/L), $CaCl_2{\cdot}2H_2O$ (1 g/L), $(NH_4)_2SO_4$ (7.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.75 g/L), K_2HPO_4 (3.5 g/L), with pH adjusted to 5.5 using 2 M NaOH. Following this, the medium was added to microalgal residues (200 g/L) in 2.5 mL glass bottles and autoclaved at 121 °C for 20 min. After cooling, cellulase enzyme (Cellic® CTec2, 125 FPU mL^{-1}) and the fungal inoculum (*S. cerevisiae* or *M. indicus*) was added at concentrations of 15 FPU/g substrate and 5 g/L, respectively. The bottles were sealed tightly and the remaining air within the bottles was removed by purging nitrogen to create an oxygen-free environment. The samples were incubated at 37 °C, withdrawn at 12-, 24-, and 72-hour intervals, and analyzed using HPLC to assess the resulting ethanol and sugars (sec. 2.7). The solid phase was dried and stored for further analysis. All experiments were performed in duplicate.

The ethanol production yield (%) was calculated using equation (4) [17]:

$$Ethanolproductionyield(\%) = \frac{Producedethanol(g/L)}{Glucancontent(g/L) \times 1.111 \times 0.51} \times 100 \quad (4)$$

where 0.51 represents the theoretical ethanol yield and 1.11 is the glucan-to-glucose conversion factor.

2.6. Anaerobic digestion

After lipid extraction, the microalgal biomass residue underwent mesophilic anaerobic digestion to assess its potential for biogas production following the procedure outlined by Hansen et al. [18]. Additionally, the whole biomass of *C. vulgaris* was used to explore the effect of the extraction process on biogas production. The defatted microalgal biomass (0.25 g) was introduced to 20 mL of inoculum obtained from a wastewater treatment digester (Municipal Wastewater Treatment, Isfahan, Iran) inside a 118 mL dark bottle. Then, deionized water (5 mL) was added to the mixture, the bottles were sealed with rubber stoppers and aluminum lids, and filled with nitrogen to ensure an oxygen-free environment. The incubator, set at 37 °C, housed the bottles for 40 days, with manual shaking once a day. Biogas composition from the resulting samples, collected every 3 days, was analyzed using gas chromatography (GC). All experiments were carried out in duplicate. The biogas production yield (mL/g dry biomass) was determined according to equation (5) [19]:

$$Biogasproductionyield\left(\frac{ml}{gdrybiomass}\right) = biogasyield\left(\frac{ml}{gVS}\right) \times VS\left(\frac{g}{gVS}\right) \quad (5)$$

where VS (volatile solid) determination was performed following the standard method reported by Sluiter et al. [20].

2.7. Analytical analysis

A HPLC (Shimadzu, Nexera X2, Japan) was used to quantify sugars (including glucose and galactose) with a Phenomenex organic acid H + column (Rezex, CA, USA) and a refractive index detector. The eluent used was 8 mM sulfuric acid with a flow rate of 0.6 mL/min at 60 °C. The same HPLC supplied with an ion exchange Aminex column (HPX-87H, Bio-Rad, CA, USA) was used for ethanol quantification. The eluent used was 5 mM sulfuric acid with a flow rate of 0.6 mL/min at 60 °C.

A gas chromatograph (GC, Agilent 7890, CA, USA) supplied with a column 30 m × 530 μm × 1 μm (Supelco Nucol 25357) was used for quantification of fatty acid methyl esters (FAMEs). The carrier gas utilized was helium at a flow rate of 20 mL/min. The injector and flame ionization detector (FID) temperature were maintained at 250 °C and 270 °C, respectively, with a split flow adjusted to 1.6 mL/min and a split ratio of 0.1:1.

The composition of the resulting biogas was determined using the GC equipped with a Porapak Q column (Chrompack, Germany) and thermal conductivity detector (TCD, SP-3420A). The detector and injector temperature were set at 150 °C and 100 °C, while the column temperature was at 40 °C. Nitrogen was used as the carrier gas at a flow rate of 20 mL/min. All biogas yields are expressed under standard conditions.

2.8. Mass balance analysis

The mass balance calculations were performed using the resulting data obtained through bead milling, solvent extraction, SSF, and anaerobic digestion. Yields for proteins, fatty acids, ethanol, and biogas were calculated based on 1 kg of *C. vulgaris* biomass used as biorefinery feedstock. Equation (6) [19] was applied to calculate the mass yield (X_i):

$$X_i = Y_i \times m_i \quad (6)$$

where Y_i represents the yield of each process within the biorefinery, including bead milling, solvent extraction, SSF, and anaerobic digestion, while m_i denotes the weight of inputs to each process.

2.9. Greenhouse gas emissions and socioeconomic analysis

The substitution of fossil fuels with biofuels produced in this biorefinery would result in the reduction of GHG emissions and subsequently reduced connected social cost, known as the social cost of carbon dioxide (SCC). These positive effects were quantified using equations 7–10. For this purpose, it was estimated the capacity production of microalgal biomass (F_M, ton) per year according to the available information in the literature. Then, equation (7) was employed to calculate the annual production of each biofuel ($F_{biofuel}$, L) achievable from harvested microalgal biomass using the proposed biorefinery.

$$F_{biofuel} = Y_{biofuel} \times F_M \quad (7)$$

where $Y_{biofuel}$ refers to the yield of each biofuel (L kg^{-1} dry microalgal biomass). Equation (8) was utilized to calculate the amount of fossil fuel that could be saved (F_{saved}, L) as a result of replacing biofuels. For this purpose, the energy ratio parameter (R) was used, which was obtained from the division of the energy density of biofuels by that of fossil fuels.

$$F_{saving} = R \times F_{biofuel} \quad (8)$$

The decrease in GHG emissions (Δ_{GHG}, kg CO_2 eq) was estimated using equation (9). According to this equation, the annual greenhouse gas emissions resulting from fossil fuel consumption would be reduced by an equivalent amount to the fossil fuel saved by substituting it with biofuels. Additionally, the equation takes into account the emissions associated with the own biofuel production.

$$\Delta_{GHG} = C_{biofuel} \times F_{biofuel} - C_{fossil} \times F_{saving} \quad (9)$$

where C_{fossil} and $C_{biofuel}$ refer to the well-to-wheel GHG emissions of fossil fuel and biofuel (kg CO_2 eq/ L fuel), respectively.

The reduction in SCC (Δ_{SCC}, $) was calculated by multiplying Δ_{GHG} by the SCC ($/ t emitted CO_2), as can be seen in equation (10).

$$\Delta_{SCC} = \Delta_{GHG} \times SCC \quad (10)$$

According to the estimates provided by the United States Environmental Protection Agency (EPA) in 2020, the *SCC* of 340 USD per ton of emitted carbon dioxide was used.

3. Results and discussion

The following sections present and discuss the results obtained at different stages of the biorefinery, which uses microalgal biomass as a primary feedstock for producing valuable products and biofuels. These stages include cascading extraction, ethanol production, biogas production, mass balance analysis, and socioeconomic assessment.

3.1. Cascading extraction

The approach used in this study for separating protein and fatty acids is illustrated in Fig. 2. Additionally, Table 1 reveals the detailed composition of each inlet and outlet shown in Fig. 2. As can be seen in Table 1, cultivated *C. vulgaris* comprised 19 % protein, 37 % carbohydrate, and 27 % lipid, respectively. In this study, water-soluble components, predominantly proteins and sugars, were initially extracted through bead milling, as a scalable mechanical disruption technique, followed by centrifugation and protein concentration for the food market. The findings of our previous study [12] revealed that the protein released is Ribulose-1,5-biphosphate carboxylase oxygenase (Rubisco) which is a promising potential sustainable protein source for plant-based food products [21]. The optimum time for releasing the maximum amount of proteins was determined, as indicated in Table 1, which illustrates the amount of intracellular components before and after bead milling. Approximately 29.7 % of carbohydrates and 41.0 % of soluble proteins dissolved in the aqueous phase after bead milling.

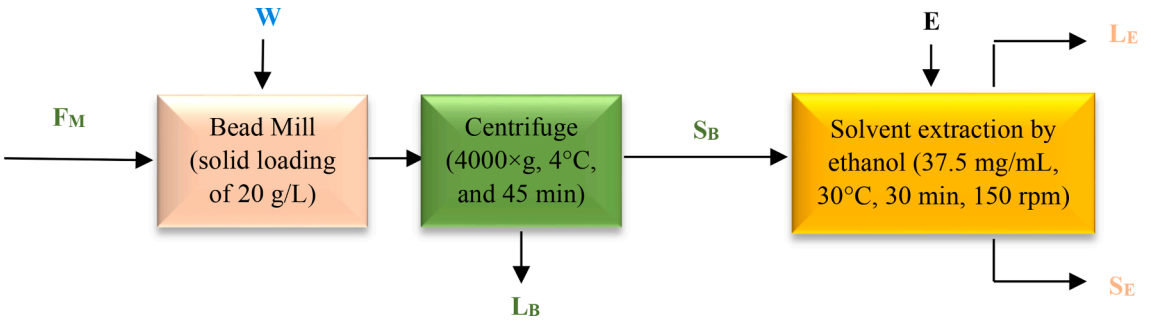

Fig. 2. Cascading extraction of *C. vulgaris* for separating protein and fatty acids. FM: microalgal biomass, W: water, LB: liquid phase of bead milling, SB: solid phase of bead milling, E: ethanol, LE: liquid phase of solvent extraction, and SE: solid phase of solvent extraction.

Table 1
Composition of inlets and outlets in the cascading extraction process.

Flow	Amount	Composition (g) Carbohydrate	Protein	Lipid
F_M (Microalgal biomass)	100.0 g (dry weigth)	37.0 ± 0.2	19.0 ± 0.5	27.0 ± 0.1
W (Water)	4.0 L	–	–	–
L_B (Liquid phase of bead milling)	3.7 L	11.0 ± 0.3	7.8 ± 0.4	0.4 ± 0.3
S_B (Solid phase of bead milling)	85.0 g (dry weigth)	24.6 ± 0.1	15.3 ± 0.5	23.8 ± 0.2
E (Ethanol)	2.3 L	–	–	–
L_E (Liquid phase of solvent extraction)	2.1 L	–	–	5.0 ± 0.1
S_E (Solid phase of solvent extraction)	66.4 (dry weight)	27.6 ± 0.1	12.0 ± 0.3	19.8 ± 0.2

Subsequently, to extract food-grade microalgal polar lipids, an ethanol treatment was applied to the bead-milled biomass. Ethanol, considered a food-grade, inexpensive, easy-to-handle, and environmentally friendly solvent, has recently been used for lipid extraction from various microalgal species [16]. The results (Table 1) showed that this method enabled to extract around 20 % of lipids from the bead-milled biomass. However, lipid extraction by ethanol resulted in lower purity and recovery compared to organic solvents like chloroform or methanol, which are not permitted in the food and feed industries due to associated toxicity. For example, Couto et al. [22] reported a lipid yield from *C. vulgaris* biomass of 10.6 % for ethanol compared to 90.6 % for dichloromethane. The fatty acid profiles of untreated microalgal biomass, bead-milled biomass, and the extracted lipid were identified and depicted in Fig. 3. The fatty acid profiles of lipids extracted by ethanol were similar to those of lipids contained in the microalgal biomass, suggesting that ethanol could serve as an extractor of all polar lipids and triglycerides.

Numerous efforts have been made to separate valuable macromolecules like proteins and fatty acids at the initial stage of biorefinery, often reporting higher yields through harsh chemical pretreatment. For example, Phusunti et al. [23] developed an integrated biorefinery that extracted nearly 80 % of proteins using hydrolysis and sonication under alkaline conditions. Despite the higher yields of these methods, they often alter protein properties and generate waste. In contrast, combining bead milling with ethanol solvent extraction, as presented in this study, resulted in the separation of nearly 40 % of soluble proteins and 20 % of fatty acids while preserving their quality. Additionally, bead milling and ethanol solvent extraction are both cost-effective, waste-free, and suitable for large-scale operations, making this cascading approach more practical for industrial applications.

3.2. Ethanol production

After protein and lipid extraction, the residual biomass was divided into two equal parts for biofuel production. One part underwent high solid SSF to produce bioethanol, while the other was utilized in anaerobic digestion for biomethane production. In recent years, high-solid processing (>15 % w/w) has gained more attention for industrial bioethanol production compared to low- and moderate-content processing.

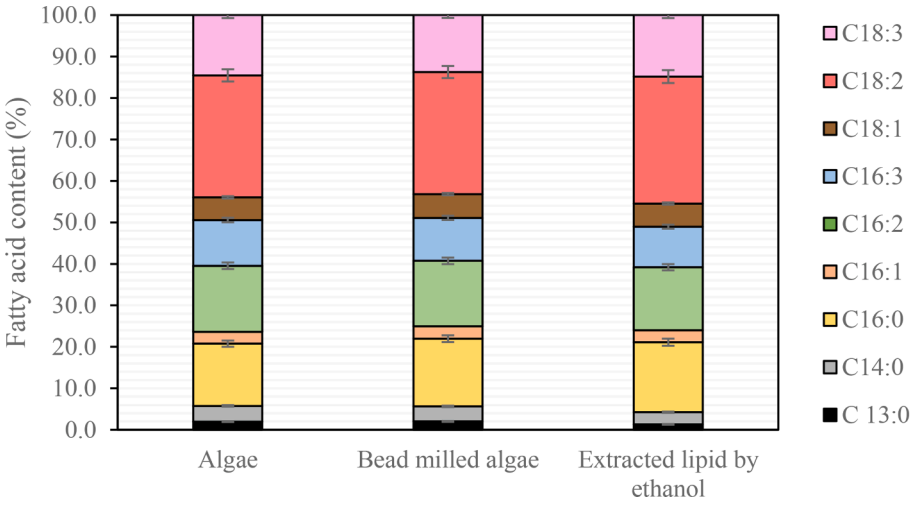

Fig. 3. Comparison of fatty acid content (%) for algae, bead-milled algae, and extracted lipid by ethanol.

High-solid processes usually follow two approaches: simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). SSF offers advantages over SHF, such as lower energy consumption and shorter process time [24].

Selecting a suitable microorganism that can utilize solid materials and grows in reduced moisture conditions is critical in achieving high ethanol yields. In this study, *M. indicus*, known for its robustness and improved performance in SSF processes, was chosen and compared with *S. cerevisiae* (baker's yeast) for ethanol production from microalgal biomass. According to the obtained results (Fig. 4), *S. cerevisiae* exhibited a higher ethanol production capacity compared to *M. indicus*. The impact of initial fungal and yeast cell concentration on ethanol yield in high-solid SSF was also examined to explore whether increasing the initial inoculum dosage led to a higher rate of sugar utilization and ethanol conversion. Fig. 4 illustrates the essential results of ethanol concentration during high solid SSF of microalgal biomass for different inoculum dosages.

The results in Fig. 4 show that the maximum ethanol yield was obtained after 12 h. With increasing fermentation time, ethanol yield decreased due to increased evaporation or depletion of monomeric sugars in the environment leading to utilizing ethanol as a carbon source for survival by fungi/yeast [25]. Lower initial concentrations of fungal and yeast as inoculum resulted in higher ethanol yield. For instance, 1 g/L *M. indicus* achieved a 51.8 % ethanol yield, whereas 10 g/L of fungal biomass attained 33.9 % yield. This pattern was observed for *S. cerevisiae*, which exhibited a 12 % lower ethanol yield at 10 g/L yeast concentration. These findings contrast with those of Molaverdi et al. [26], who reported that a higher initial fungal concentration led to higher ethanol yield in high solid SSF of sweet sorghum stalks. The accumulation of heat and the formation of hot spots may decrease fungal/yeast activity at high cell concentrations, leading to the over-utilization of fermentable sugars for growth and maintenance, rather than ethanol production.

Moreover, the effect of cascading extraction on bioethanol production was analyzed. Bead milling, as a pretreatment, not only extracted soluble proteins but also increased ethanol yield by 25 %. This finding aligns with the results presented by Juarez et al. [27], where bead milling significantly increased enzymatic hydrolysis yield and solubilized components like proteins and lipids. Previous studies [28–31] reported higher ethanol yields for defatted algal biomass compared to the results in this study. However, most of those studies used chemical pretreatments (acidic, alkali, and physicochemical) before the saccharification and fermentation process.

To the best of the authors' knowledge, there are few studies employing microalgal biomass at high solid loading for ethanol production. In studies using high solid loading for microalgal biomass fermentation, Condor et al. [32] fermented *C. vulgaris* biomass treated with dilute acid hydrolysate at a solid loading of 200 g/L, obtaining ethanol concentrations of 8.9 g/L and 10.1 g/L using *Zymomonas mobilis* and *S. cerevisiae* as fermenting microorganisms, respectively. In another study, Kim et al. [33] used a hydrothermal process at a solid loading of 25 % w/w for fractionating valuable components and pretreating *Schizocytrium* sp. biomass. They reported an ethanol yield of 11.8 g/L using *E. coli* KO11 through an SSF process, but fractionated products such as proteins were obtained in a non-native form due to treatment at high temperatures. In the present research, a similar ethanol productivity range (7.5–10.5 g/L) was achieved by high-solid SSF, as a practical and scalable method for ethanol production. Notably, *S. cerevisiae*, as a commercial microorganism with well-established fermentation operation conditions, was utilized which further supports the feasibility and efficiency of our approach. Additionally, pretreatments before fermentation effectively separated valuable components like proteins and fatty acids with their inherent qualities.

3.3. Biogas production

After protein and lipid extraction, the potential of pretreated microalgal biomass was evaluated to assess its suitability for biogas production. Additionally, the impact of lipid and protein extraction on biogas production was investigated by applying untreated microalgal biomass to anaerobic digestion. In Fig. 5, the concentration of methane and carbon dioxide (mL/g VS) is displayed for untreated *C. vulgaris* biomass (Fig. 5-a) and the biomass after lipid and protein extraction (Fig. 5-b). It is evident from Fig. 5 that the maximum biogas yields from untreated and pretreated microalgal biomass were obtained at 523.8 mL/g VS and 608 mL/g VS, respectively, after 25 days. Moreover, methane accounted for approximately 65–69 % of the total biogas production, with carbon dioxide contributing to 31–35 % of the biogas composition. Comparing the results of untreated and pretreated microalgal biomass (Fig. 5) revealed that bead milling and lipid extraction by ethanol improved the methane yield and total biogas yield by 22.4 % and 16 %, respectively.

Kumari et al. [34] investigated the effects of ultrasonic, thermal, hydrothermal, and Feton pretreatments on biogas production from *C. pyrenoidosa*, reporting an increase in biogas production of up to 18 % due to these pretreatments. However, their study did not emphasize the valorization of valuable components before applying microalgal biomass in anaerobic digestion. In another study, Juarez et al. [35] investigated the effects of various pretreatments, including acidic or alkali treatment, steam explosion, and bead milling, on biogas

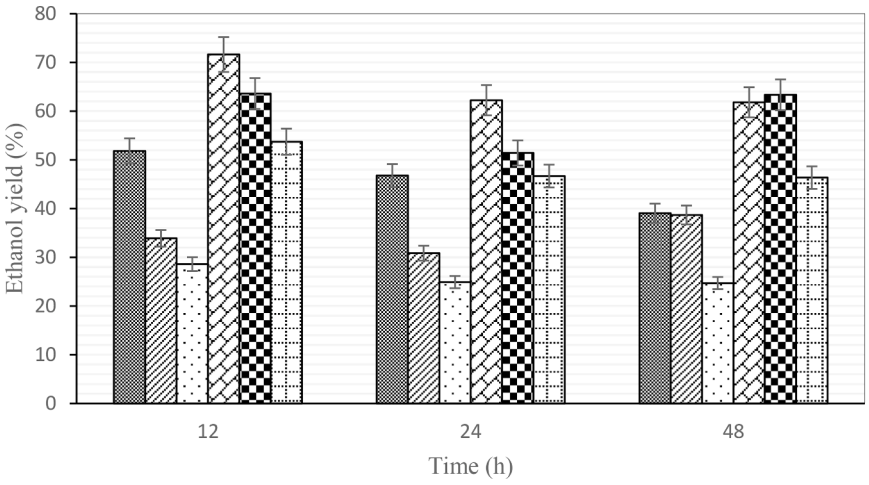

Fig. 4. Ethanol yield (%) during simultaneous hydrolysis and fermentation (SSF) of defatted *C. vulgaris* by: *M. indicus*, 1 g/L (); *M. indicus*, 10 g/L (); *S. cerevisiae*, 1 g/L (); *S. cerevisiae*, 10 g/L (); whole cell of algal biomass inoculated by *M. indicus*, 10 g/L (); and whole cell of algal biomass inoculated by *S. cerevisiae*, 10 g/L ().

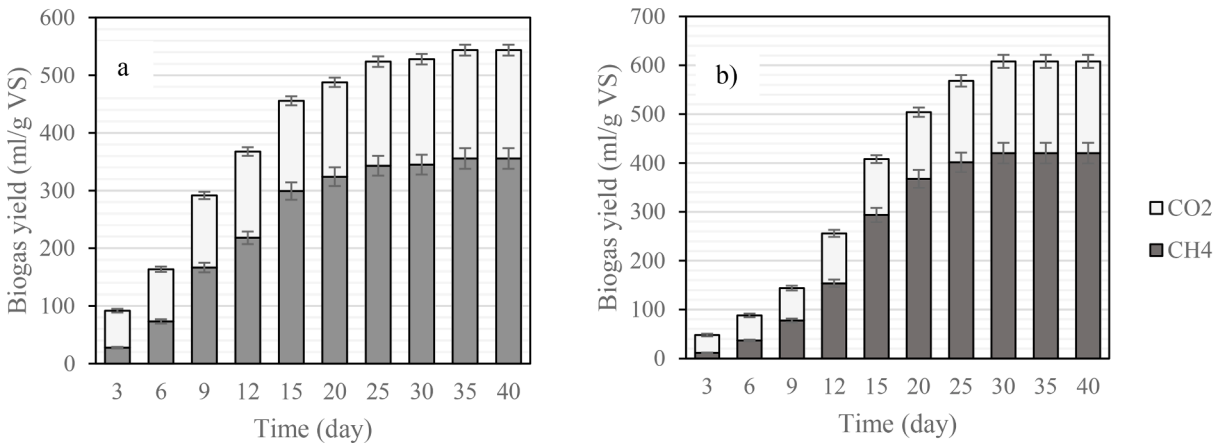

Fig. 5. Biogas yield (ml/g VS) of a) *C. vulgaris* biomass and b) *C. vulgaris* biomass after lipid and protein extraction.

production from mixed microalgae biomass grown in pig manure. They reported the highest yield of 377 mL CH_4/g VS for alkali-pretreated biomass. Ayala-Parra et al. [36] also evaluated the effects of lipid extraction by sonication pretreatment on biogas yield, finding that the energy content was not significantly reduced by lipid extraction, which accounted for 9 % of algal dry weight, while sonication improved the access of hydrolytic enzymes to algal biopolymers, compensating for the energy lost due to lipid extraction.

Considering theoretical methane production yields of 1014, 496, and 415 mL per g of VS from pure lipids, proteins, and carbohydrates, respectively, an expected 521.6 mL/g VS of methane from untreated *C. vulgaris* and 563.7 mL/g VS from pretreated *C. vulgaris* can be calculated based on their composition. The results in Fig. 5 demonstrated that 74.5 % of the theoretical yield of methane production was attained from pretreated microalgal biomass and 65.8 % from untreated biomass. Bohutskyi et al. [37] studied the potential of lipid-extracted *Auxenochlorella protothecoides* biomass for methane generation and nutrient recovery. They reported that methane production was limited to nearly 50 % of theoretical yield due to biomass recalcitrance and inhibition effects from residual solvents (acetone and hexane) in the defatted residual biomass.

The increased biomethane production observed in this study could be attributed to the advantageous impacts of extractive extraction using ethanol, which occurred simultaneously during lipid extraction. This pattern was also observed by Tajmirriahi et al. [38], where the extraction process positively impacted biogas production from lignocellulosic feedstocks by reducing the presence of extraneous materials in the feedstock. Another contributing factor could be the positive impact of protein extraction before anaerobic digestion, improving the C/N ratio, which is beneficial for the production of bioethanol, biohydrogen, and biomethane [39]. According to the benefits of protein and lipid extraction before anaerobic digestion on biomethane yield by implementing scalable methods, this process promises an effective and practical approach for industrial applications.

3.4. Mass balance

The obtained results for the conversion of *C. vulgaris* into proteins, lipids, bioethanol, and biomethane led to the illustrated mass balance in Fig. 6. Accordingly, from every 1 kg of dry microalgal biomass, it is feasible to obtain 78 g of proteins, 50.9 g of lipids, 20.8 mL of ethanol, and 136.5 L of biomethane. Fig. 6 illustrates that the initial step involves pretreating *C. vulgaris* using a bead mill to extract its most valuable and sensitive component, i.e., soluble proteins previously identified as Rubisco [12]. Post bead milling, the microalgal biomass undergoes further processing in a mixer to extract valuable fatty acids by combining them with ethanol. This ethanol-based extraction method yields food-grade lipids, which are deemed safer compared to commonly used solvents like methanol and chloroform. The necessary solvent for lipid extraction is retrieved through evaporation and distillation of the solvent phase obtained from the mixer.

As the separation of microalgal components through mechanical and non-mechanical methods involves high costs and energy requirements, multiple studies have explored the energy balance in biofuel production, encompassing methane, hydrogen, syngas, and bioethanol. Achieving optimal results in biofuel production yield and energy efficiency demands choosing specific processes based on the primary components remaining in the residue. For instance, anaerobic digestion and fermentation entail considerably lower energy requirements than hydrothermal gasification or pyrolysis [40].

Taking into account the remaining components in the exhausted microalgal biomass post protein and lipid extraction, the biomass was divided into two equal parts: one for ethanol production (S_F) and the other for biogas production (S_{AD}). According to the results inFig. 6, 41.5 % of the carbohydrates present in the exhausted biomass yielded 16.6 g (20.8 mL) of ethanol, while the same quantity of carbohydrates, along with 30 % lipids and 26 % non-soluble proteins, produced 136.5 L of biomethane and 61.3 L of carbon dioxide. A portion of the ethanol produced in this process could fulfill the initial ethanol requirement for lipid extraction. Moreover, since carbon dioxide is utilized in microalgal biomass production, it could be separated from methane using a membrane separator and stored in a CO_2 pod for use in microalgal cultivation.

Additionally, *C. vulgaris* has been extensively studied as a supplement or substitute for animal feed due to its high content of essential nutrients [41–43]. A literature review has shown that adding *C. vulgaris* to poultry and swine diets enhances animal health and immunity. However, the digestibility and accessibility of nutrients increase through pretreatments such as biological treatments [44]. Conversely, *M. indicus* is a generally recognized as safe (GRAS) source with substantial protein and lipid content, making it a promising option for animal feed production. Hence, it is anticipated that the residue obtained from the fermentation process in this study would have high-quality suitability as animal feed.

In comparing the outcomes of this study with existing literature, Table 2 summarizes the equivalent energy produced and the associated byproducts. According to the results in Table 2, several existing biorefineries that reported higher recovered energy have centered on using microalgal biomass for energy-based product production. However, the present study presents a more delicate and scalable cascading process (bead milling + solvent extraction) for the initial separation of the valuable components, i.e., proteins and lipids while preserving their intrinsic values. The integration of bead milling with ethanol solvent extraction also enhanced the overall efficiency of the energy-based products, i.e., bioethanol and biomethane. Additionally, the present

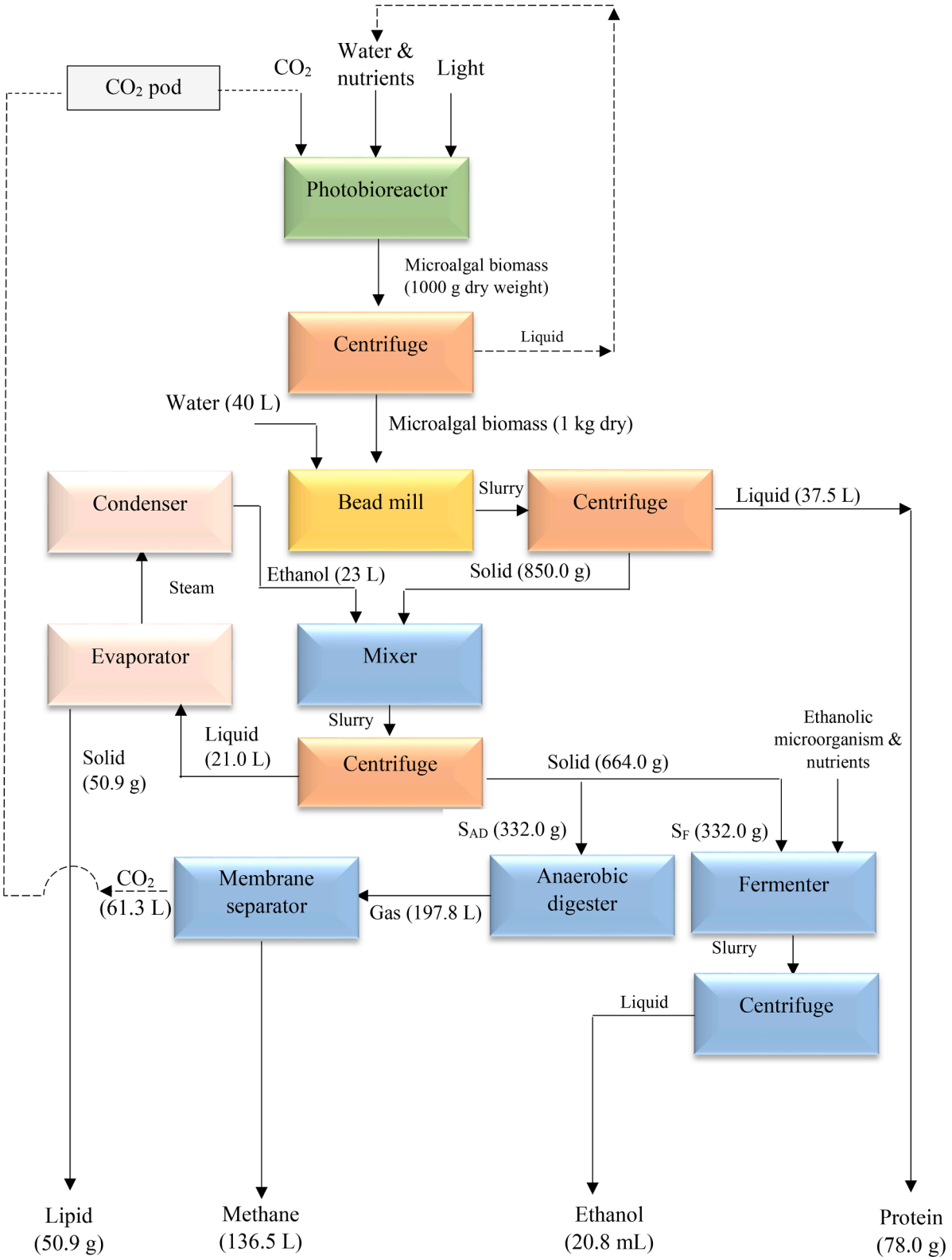

Fig. 6. Mass balance over biorefining of *C. vulgaris* (based on the best results obtained).

biorefinery benefits from high-solid SSF for producing ethanol which offers greater practicality and enhances environmental benefits by reducing water usage and wastewater generation. Therefore, it presents a comprehensive and scalable solution for industrial applications.

3.5. Socioeconomic analysis

According to Lababpour et al. [47], who studied site assessment and estimated the micro-algal production potential in Iran, they identified 57 suitable sites with a total area of 18,000 km^2 along the coasts of the Persian Gulf and the Oman Sea for the production of microalgal biomass.

Table 2
Products and total energy produced from different microalgal biorefineries (based on one kg of dry biomass).

Algal biomass	Processes	Products	Total energy produced (MJ)*	References
C. vulgaris	Bead milling, lipid extraction by ethanol, high solid SSF (200 g/L), and anaerobic digestion	• Protein (78.0 g) Lipid (50.9 g) Ethanol (20.8 mL) Methane (136.5 L)	5.3	Current study
C. vulgaris	Dilute acid hydrolysis (2 % H_2SO_4, 200 g/L substrate, 121 °C, and 20 min), and SHF	• Ethanol (50.6 g)	1.3	[32]
Scenedesmus. dimorphus	Blight and Dyer (24 h, 25 °C), SSF	• Ethanol (260.0 g) Lipid (not mentioned)	6.5	[45]
Scenedesmus sp.	Ultrasonic followed by dilute acid hydrolysis (0.3 N, 121 °C, and 20 min), SHF	• Ethanol (85.6 g) Biodiesel (349.0 g)	15.2	[46]
Chlorella sp. and *Scenedesmus* sp.	Hydrothermal pretreatment (121 °C and 15 min), SHF	• Ethanol (145.0 g) PHB** (43.0) Lipid (not mentioned)	3.6	[29]

* The energy content of ethanol, biodiesel, and biomethane was calculated based on 25.1 MJ per kg of produced ethanol, 37.8 MJ per kg of produced biodiesel, and 36.0 MJ per kg of produced methane, respectively.
** Polyhydroxy butyrate.

Considering the possibility of four cultivation periods, ranging from the second half of December to the first half of April, it is projected that approximately 2,160 kilotons of microalgal biomass can be cultivated in an open culture system. Given the higher productivity of 3–5 times that photobioreactors offer compared to open systems, it is estimated that up to 8,640 kilotons of microalgal biomass could be attained for processing in a biorefinery.

The potential biofuels that could be produced using the outlined biorefinery were determined based on the results presented in Fig. 6 and equation (7). Additionally, the fossil fuels saved by substituting them with the corresponding biofuels were estimated using equation (8). Reductions in greenhouse gas emissions and the associated social costs were calculated using equations (9) and (10). The findings are summarized in Table 2.

Upon subjecting the estimated 8,640 kilotons of cultivated *C. vulgaris* biomass to the proposed biorefinery, it is anticipated that 179.7×10^6 L of bioethanol and 1179.4×10^6 m^3 of methane could be produced. Substituting fossil fuels with the produced biofuels in the transportation sector of Iran would potentially save approximately 122.2×10^6 L of gasoline and 1179.4×10^6 m^3 of fossil methane, which would lead to a reduction in greenhouse gas emissions and social costs by approximately 4102.4×10^6 kg CO_2 eq and 1394.8×10^6 USD, respectively.

Table 3 displays the results obtained from a lignocellulosic biorefinery that utilized corn stover residue collected from arable land in Iran in 2020. The biorefinery focused on producing bioethanol, biodiesel, and biomethane [19]. Based on the results, microalgal biomass, as a biorefinery feedstock, can reduce greenhouse gas emissions and associated social costs similar to corn stover residue, a lignocellulosic waste. The ability of microalgal biomass to thrive in non-arable lands and diverse environmental conditions makes it a more favorable choice for developing biorefineries in Iran when compared to corn stover residues. However, further analyses such as techno-economic studies and life cycle assessments are necessary to determine which biorefinery is more efficient in terms of energy consumption, economic feasibility, and environmental impacts.

Table 3
The potential of produced biofuels, saved corresponding fossil fuels, and a reduction in GHG emissions as well as SCC from collectible amount of *C. vulgaris* biomass and corn stover in Iran.

Specification		Feedstock	
		C. vulgaris	**Corn stover**[a]
Capacity of production (k ton)		8.6×10^3	2.7×10^3
Available land for feedstock cultivation (ha)		1.8×10^6	0.2×10^6
Produced biofuels[b]	Bioethanol (L)	179.7×10^6	40.7×10^6
	Biodiesel (m^3)	–	2.8×10^6
	Biomethane (m^3)	1179.4×10^6	449.2×10^6
Saved fossil fuels[c]	Gasoline (L)	122.2×10^6	27.7×10^6
	Diesel (m^3)	–	2.6×10^6
	Methane (m^3)	1179.4×10^6	449.2×10^6
Total Δ_{GHG} (kg CO_2 eq)[d]		4102.4×10^6	4158.3×10^6
Total Δ_{SCC} (USD)		1394.8×10^6	1413.8×10^6

[a] According to the results presented by Alavijeh et al.[19].
[b] Estimated by equation (7).
[c] Estimated by equation (8); a value of R corresponded to 1.00 m^3 methane m^3 biomethane, 0.91 L diesel L^{-1} biodiesel, and 0.68 L gasoline L^{-1} cellulosic ethanol [48].
[d] Well to wheel CO_2 emission evaluated to be 2.99 kg CO_2/L ethanol, 0.6 kg CO_2/L bioethanol [44], 2.44 kg CO_2/m^3 biomethane, and 5.7 kg CO_2/m^3 methane [49].

4. Conclusion

This study introduces an integrated biorefinery designed to produce valuable products and biofuels from *C. vulgaris* biomass through a cascading process involving bead milling, solvent extraction, fermentation, and anaerobic digestion. A key advantage of this biorefinery is its use of mild and scalable processes for extracting high-value products, including soluble proteins and unsaturated fatty acids while serving as an effective pretreatment before fermentation and anaerobic digestion. The ability of biorefinery to supply the required CO_2 for microalgal cultivation and solvents for fatty acid extraction during biofuel production adds another layer of sustainability, bringing the system closer to a circular approach.

Implementing high solid SSF within this biorefinery offers several advantages, including reduced water usage, higher product concentration, and lower wastewater generation, thereby ensuring a more sustainable and practical approach to bioethanol production. Moreover, the proposed biorefinery shows significant potential in reducing GHG emissions and their associated social costs. However, achieving a fully circular zero-waste biorefinery requires improvements to minimize waste streams during fermentation and anaerobic digestion. Further research is necessary to assess the commercial viability of this biorefinery, considering both techno-economic and life cycle assessment perspectives.

CRediT authorship contribution statement

Razieh Shafiei-Alavijeh: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Michel Eppink:** Writing – review & editing, Resources, Investigation. **Joeri F.M. Denayer:** Writing – review & editing, Supervision, Project administration, Investigation. **Eveline Peeters:** Writing – review & editing, Supervision, Methodology. **Keikhosro Karimi:** Writing – review & editing, Validation, Supervision, Project administration, 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.

Data availability

No data was used for the research described in the article.

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