1 Biomethane and biocrude oil production from protein extracted
2 residual *Spirulina platensis*

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19 **Abbreviations**

20 AD - Anaerobic digestion

21 HTL - Hydrothermal liquefaction

22 PERB - Protein extracted residual biomass

23 ORIB - Original biomass

24 DISB - High pressure homogenizer disrupted biomass

25 BMP - Biochemical methane potential

26 ACP - Aqueous co-product

27 SR - Solid residue

28 DCM - Dichloromethane

29 HHV - Higher heating value

30 ER – Energy recovery

31 E_{output} – Energy output

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Abstract

The performance of the residual biomass obtained after protein extraction from the cyanobacterium *Spirulina platensis* as a feedstock for biomethane production via anaerobic digestion (AD), and biocrude oil production via hydrothermal liquefaction (HTL) pathways was investigated. The experimental methane yield and kinetic rate of methane production from protein extracted residual biomass (PERB) were higher by 30.4 % and 161 % respectively, compared to original *S.platensis* biomass (ORIB). The rate of methane production for PERB was also higher (by 38.9 %) than high pressure homogenizer disrupted biomass (DISB), although the yield was slightly lower (by 7.8 %). The lag phase time for methane production was the least for PERB among all the three substrates. On the other hand, HTL of PERB resulted in biocrude oil with slightly lower nitrogen content than ORIB (6.2 % and 7 % respectively), although at a reduced yield. A composition analysis using GC-MS revealed that the biocrude from PERB had a higher number of hydrocarbons and fatty acids and lower number of nitrogenous compounds compared to that from ORIB. A comparison of energy output and energy recovery in the AD and HTL processes suggested a better performance of PERB in the former process.

Keywords: *Spirulina platensis*; protein extraction; protein extracted residual biomass; anaerobic digestion; hydrothermal liquefaction

1. Introduction

Algae (and cyanobacteria) are rich sources of protein for human and animal nutrition. However, extraction of algal proteins may not be an economically sustainable process by itself if the rest of the biomass is not used for any applications [1]. Integration of the protein extraction process with biofuel production from the by-product of the process, the protein-extracted residual biomass (PERB), may improve the economics and sustainability of the overall process. PERB is expected to have a lower nitrogen and protein content, and a higher content of non-protein components such as carbohydrates and lipids compared to the original biomass (ORIB). Such a composition is favorable for biofuel production. However, since protein extraction is usually carried out on wet algae biomass, PERB is expected to have low solids content.

Anaerobic digestion (AD) and hydrothermal liquefaction (HTL) are wet processing technologies capable of converting biomass at low solids content to biofuels, thereby reducing the costs involved in extensive dewatering and drying. AD is a biochemical process which converts wet biomass into biogas using bacteria under anaerobic conditions. The biogas, mainly composed of methane and carbon dioxide, may be used directly as a fuel or purified and upgraded to biomethane which can be used in place of natural gas for several applications such as domestic fuel, electricity generation and transportation fuel [2]. The AD process comprises of four stages namely hydrolysis, acidogenesis, acetogenesis and methanogenesis. The first stage (hydrolysis) is rate limiting for AD of insoluble organic matter. Pretreatments such as mechanical cell disruption, thermal and chemical treatments can enhance AD by improving the accessibility of intracellular components by the hydrolytic enzymes produced by the anaerobic bacteria, thus improving the hydrolysis kinetics [2-5]. Co-product extraction as a pretreatment has also been reported to enhance algal AD, primarily due to the cell disruptive effect and higher

solubilisation of organic matter. Ramos-Suárez and Carreras, 2014 investigated the anaerobic digestibility of residual *Scenedesmus* biomass obtained after enzymatic extraction of amino acids [6]. The amino acid extracted residues improved the yield and kinetic rate constant for methane production compared to the original *Scenedesmus* biomass. Astals et al., 2015 investigated anaerobic digestibility of *Scenedesmus sp.* after protein extraction using free nitrous acid pretreatment and found a 36% enhancement of methane production [7]. Protein extraction to decrease the nitrogen content of algal/ cyanobacterial biomass may also help in reducing the formation of ammonia, a major inhibitory factor [8]. McCarty, 1964 noted that ammonia inhibition in AD occurs at TAN (total ammonia nitrogen) concentrations of 1.5-3 g L⁻¹ at pH above 7.4 and a higher concentration of TAN becomes toxic irrespective of pH, leading to process failure [9]. A higher inhibitory range (1.7-14 g L⁻¹) was reported by several authors and was said to depend on the nature of substrates and inocula, operating conditions and acclimatization periods [8]. Free ammonia concentration of 1.1 g-N L⁻¹ was found to cause inhibition of batch AD of swine manure at pH 8 [10]. It was reported that AD of protein rich cyanobacterium *Spirulina maxima* containing upto 60 % protein content releases upto 7000 mg L⁻¹ of ammonia, a value in the inhibitory range [11]. The presence of high amounts of ammonia in the biogas generated from algae also prevents its direct use as a fuel due to the possibility of harmful NO_x emissions, and adds to the costs involved in its purification to produce biomethane [12].

Hydrothermal liquefaction is a thermochemical process which converts wet biomass into biocrude oil in hot compressed water. This biocrude can be upgraded to generate liquid fuels for transportation and other applications [13]. A major limitation of microalgal/cyanobacterial HTL is the presence of high protein content in the biomass, resulting in high nitrogen content in the

final biocrude product [14] . This is highly undesirable as the combustion of this product would result in the formation of toxic oxides of nitrogen (NO_x) which significantly impact the environment [15]. Moreover, the proteins which are hydrolyzed to amino acids at the hydrothermal conditions undergo further deamination, decarboxylation and cross reactions with carbohydrates and lipids to form complex nitrogenous compounds [16, 17]. This poses a difficulty in further upgrading the biocrude to fuel oil as it can lead to possible poisoning and deactivation of the catalysts and increased hydrogen demand during hydrodeoxygenation [18]. Extraction of proteins from the microalgal/cyanobacterial biomass and the use of the residual biomass for HTL may result in the formation of biocrude oil with lower nitrogen content. Further, component extraction may help in reducing the formation of undesirable nitrogenous compounds by avoiding cross reactions. Cheng et.al., 2014 reported that the use of the residual biomass after lipid extraction from *Nannochloropsis oceanica* as a feedstock for HTL process prevented the formation of low grade, long chain amides and other nitrogenous compounds formed as a result of reaction between lipids and proteins [19]. Similar effect could be achieved by the use of protein extracted residual biomass as a HTL feedstock.

The current study investigated the performance of protein extracted residual *Spirulina platensis* biomass (PERB) as a feedstock for biomethane production via AD, in comparison to non-protein extracted biomass. Although AD studies on protein/ amino acid extracted microalgal biomass were reported previously [6, 7], the type of biomass (organism) and the method of component extraction reported in the current study are significantly different. Green algae such as *Scenedesmus* differ from cyanobacteria such as *S.platensis* in cell wall structure and biochemical composition [1]. Hence the impact of component extraction on the biodegradability and methane production is expected to be different among the two phyla. In addition, the method

of component extraction may also impact the digestibility because mechanical, chemical or thermal pretreatments are usually applied during the extraction process. Thus the results presented in this work uniquely describe the impact of protein extraction (using the specific method) on AD for cyanobacteria.

The current study also investigated the performance of PERB as a feedstock for HTL process in comparison to original *S.platensis* biomass (ORIB). Such a study, describing the impact of protein extraction (using the specific method) on HTL process has not been reported for any microalgal/ cyanobacterial biomass previously to the best of our knowledge.

2. Materials and Methods

2.1 Feedstock

Dry powder of *S.platensis* biomass was procured from Earthrise Nutritionals LLC (Calipatria, CA). Original biomass (ORIB) slurry at the desired total solids (TS) concentration was prepared by resuspending the dry *S.platensis* powder in deionized water. Disrupted biomass (DISB) was obtained by subjecting the ORIB slurry to cell disruption by high pressure homogenization (2 passes through a high pressure homogenizer (Constant systems LTD, UK) at 103.4 MPa (15 kpsi)). Protein extracted residual biomass (PERB) was generated by subjecting *S.platensis* biomass at 3.6 % solids content to cell disruption by high pressure homogenization and a subsequent protein isolation process involving two stages. The first stage involved protein solubilisation at pH 11.38 (adjusted using 1 M NaOH) for 35 min and subsequent centrifugation (8670 g, 35 min) in order to separate the pellet and the supernatant. The second stage involved protein precipitation from the supernatant (obtained from the first stage) at pH 4.01 (adjusted

using 1 M HCOOH) for 25 min and subsequent centrifugation (8670 g, 35 min) to separate the pellet (protein isolate) and the supernatant. The pellet obtained from first stage was combined with the supernatant from the second stage to obtain PERB. The protein extraction experiments were carried out in duplicate. A part of the PERB obtained after protein extraction was dried in an oven at 50 °C and resuspended in deionized water to obtain biomass slurry at 15 % solids. This biomass slurry was used as feed to the HTL process.

2.2 Biochemical methane potential

The biochemical methane potential (BMP) assay was used to evaluate the anaerobic digestibility of the substrates. BMP tests were conducted as batch experiments using the AMPTS II setup (Bioprocess Control, Sweden). Anaerobic sludge from a digester treating food waste was used as inoculum. ORIB, DISB and PERB were used as substrates. Each of the substrates and inoculum were premixed and loaded into the 500 mL bottle reactors after flushing with N₂ gas for 2-3 min to remove O₂ from the headspace. The experiments were carried out at a working volume of 150 mL, substrate loading of 1 g volatile solids (VS) and substrate to inoculum ratio of 9:1 by volume. The inoculum to each BMP reactors had a COD of 2.41 g L⁻¹, TS of 0.61 % and VS of 30 %. Each experiment was conducted in triplicate. Deionized water was used instead of substrate for control runs (carried out in duplicate) in order to determine the methane productivity of the inoculum alone. The total organic load of the substrate/inoculum mixture in each reactor was 1.42 g COD except for control reactors (0.04 g COD). The temperature was maintained at 38 °C during the 30 day runs. The daily biomethane accumulation was recorded online. The actual daily cumulative methane production by each substrate was calculated by subtracting the recorded cumulative methane accumulation for the control from the corresponding value for the substrate-inoculum mixture on that particular day. The kinetic

parameters for the BMP experiments were determined by fitting the cumulative methane production (mL g⁻¹VS) to the modified Gompertz equation shown below [20]:

$$M = P \times \exp \left\{ -\exp \left[\frac{R_m}{P} e (\lambda - t) + 1 \right] \right\} \quad (1)$$

where M is the cumulative methane production (mL g⁻¹VS), P is the methane production potential (mL g⁻¹VS), λ is the lag phase time (d) and R_m is the rate of methane production (mL g⁻¹VS d⁻¹).

2.3 Hydrothermal liquefaction and product separation

Hydrothermal liquefaction experiments were carried out in duplicate using PERB and ORIB at 15 % solids content as feedstock. The experiments were carried out in 100 mL batch reactors (Parr Instruments Co., Moline, PA) at a temperature of 350 °C and holding time of 30 min [21]. In a typical HTL run, the feed biomass slurry was transferred to the reactor which was then sealed and pressurized to about 3.45 MPa (500 psi) using helium gas. It was heated to 350 °C at a rate of 14 °C min⁻¹ using an electrical jacket and held at that temperature for 30 min. At the end of the run, the jacket was removed and the reactor was allowed to cool to ambient temperature. The ambient temperature and pressure were recorded, a fraction of the gas was sampled using Tedlar[®] sampling bags and the remaining gas was vented out. The biocrude oil, aqueous co-product (ACP) and the solid residue (SR) in the product mixture were separated from each other using a combination of solvent (dichloromethane (DCM)) extraction, vacuum filtration, gravity separation and rotary evaporation (at 38 °C using a rotary evaporator) steps (Fig. 1) [22]. The biocrude oil obtained from PERB is henceforth referred to as PERB biocrude and that obtained from ORIB is referred to as ORIB biocrude. Similar notation is followed for the other HTL products.

Fig. 1 Hydrothermal liquefaction and product separation process. SR – Solid residue; ACP – Aqueous co-product.

2.4 Analytical methods

C, H, N, S elemental analysis was carried out using a LECO brand analyzer (Model CHNS-932) [21]. Protein content was obtained by multiplying the N content with a factor of 6.25 [23-25]. Total solids (TS) were determined by drying the samples in crucibles at 105 °C for 4 h in a conventional oven. After measuring the total solids, the ash content and volatile solids (VS) were determined by incinerating the crucibles in a furnace at 575 °C for 3 h using a slightly modified version of the NREL procedure [26]. Elemental O content was obtained by the difference (based on C, H, N, S and ash contents). Chemical oxygen demand (COD) was

analyzed by the HACH Reactor Digestion method (Method 8000) using HACH DRB 200 spectrophotometer (HACH Corporation, Loveland, CO) and digestion kit [21].

The composition of the biocrudes and the ACPs was analyzed using a Hewlett-Packard (Model HP-6890) gas chromatograph in conjunction with a Hewlett-Packard (Model HP-5973) mass spectrometer with a mass selective detector. The dimensions of the GC column were: 30 m length, 0.25 mm internal diameter and 0.25 μm film thickness. The method comprised of the following parameters: inlet temperature of 250 $^{\circ}\text{C}$, detector temperature of 280 $^{\circ}\text{C}$, flow at 1 mL min^{-1} Helium, oven temperature at 40 $^{\circ}\text{C}$ held for 4 min followed by ramp up at the rate of 5 $^{\circ}\text{C min}^{-1}$ to 275 $^{\circ}\text{C}$ and held for 5 min. Sample size for injection was 1 μL . A split ratio of 50:1 was used. The compounds were identified using Agilent Technologies Software (MSD Chemstation D.03.00.611) which carries out a probability-based matching of the unknown spectra to mass spectral library using National Institute of Standards and Technology's 2008 version. The ACPs were analyzed using high performance liquid chromatography (LC-20 AT, Shimadzu Corp., USA) equipped with a RID-10A refractive index detector and a 7.8 $\times$ 300 mm Corajel 64-H transgenomic analytical column for simple sugars and organic acids according to the method described by Jena et al., 2011 [21]. The gaseous products were analyzed by a gas chromatograph equipped with a thermal conductivity detector (Agilent 3000A micro-GC) to determine the concentrations of the gases (H_2 , N_2 , CO , CO_2 , CH_4 and other C_2 - C_5). The columns used on the GC-TCD analyzer were: MS 5A PLOT (10 m length $\times$ 32 mm diameter), PLOT U (8 m $\times$ 0.32 mm), alumina PLOT (10 m $\times$ 0.32 mm), and OV-1 (10 m $\times$ 0.15 mm $\times$ 2.0 μm). The method was operated at the following parameters: sample size of 1 μL , oven temperature at 35 $^{\circ}\text{C}$ for 5 min, temperature ramp at the rate of 20 $^{\circ}\text{C min}^{-1}$ to 200 $^{\circ}\text{C}$ and hold for 5.75 min.

2.5 Theoretical calculations

225 All recoveries and yields are reported on a percentage dry weight basis. Higher heating values
 226 (MJ kg⁻¹) for the biocrudes and the feeds were calculated based on the elemental composition as
 227 described by Biller & Ross, 2011 [27]. Energy output (MJ kg⁻¹) and energy recovery (%) in the
 228 biocrudes and biomethane were calculated as follows [28]:

$$E_{output} \text{ (MJ kg}^{-1}\text{)} = HHV_{product} \times Y_{product} \quad (2)$$

$$ER \text{ (\%)} = \frac{HHV_{product}}{HHV_{feed}} \times Y_{product} \times 100 \quad (3)$$

229 where $Y_{product}$ is the product yield expressed as dry weight fraction (w/w).

230 Theoretical maximum yield of methane was calculated based on the elemental
 231 composition of the substrates according to the Buswell and Mueller, 1952 stoichiometric
 232 equation as modified by Richards et al., 1991 [29-31]:

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234 3. Results and Discussion

235 3.1 Feedstock composition

236 Table 1 shows the elemental composition, protein, ash and VS contents of ORIB and
 237 PERB. It was experimentally determined that there was not much variation in the composition
 238 of ORIB and DISB. Thus the theoretical parameters such as theoretical methane and HHV which
 239 were calculated based on these values were assumed to be the same for both these biomass. C, H,

N, protein and VS content were lower, and ash content and C/N ratio were higher in PERB than ORIB.

Table 1 Composition of the original *S.platensis* biomass (ORIB) and the protein extracted residual biomass (PERB). All values are reported as weight percentages on a dry basis^a.

Parameter	ORIB	PERB
C (%)	50.1(2.0)	35.3 (2.5)
H (%)	6.4 (0.3)	5.5 (0.2)
N (%)	11.0 (0.1)	7.6 (0.1)
O (%)	25.9 (1.4)	31.3 (3.2)
Ash (%)	6.7 (0.5)	20.3 (0.1)
VS (%)	93.3 (0.5)	79.7 (0.1)
C/N ratio	4.58 (0.1)	4.64 (0.3)
Protein content (%)	68.8 (1.9)	47.5 (0.8)

^a Values in parentheses are standard deviations of means

3.2 Anaerobic digestibility and methane production

The cumulative methane production by the three substrates is shown in Fig. 2 (a). The experimental total methane production (yield) was the least for ORIB (181.1 mL g⁻¹VS) owing to the presence of intact cell wall which limits hydrolysis and access to the intracellular organic matter, resulting in low digestibility [32]. This value is lower than the methane yields reported in literature for *S.platensis* (290-330 mL g⁻¹VS) [2, 33], possibly due to the low inoculum input to the reactors in our study and inhibition of AD. Higher methane yields were obtained from DISB (254.5 mL g⁻¹VS) and PERB (236.1 mL g⁻¹VS). In both of these substrates, the *S.platensis* cells were subjected to cell disruption using high pressure homogenization (and additional protein extraction process in the case of PERB) which improved the access to intra cellular components and thus the digestibility. Enhancement of microalgal methane production by high pressure homogenization based cell disruption and protein/ amino acid extraction by various methods was reported previously [3, 6, 7]. The total methane production (averaged for the two replicates) by

258 the control after 30 days was only 14.4 mL. This was probably due to the low VS input (0.03 g)
 259 to the control reactors. However, on a VS basis the control exhibited a total methane productivity
 260 of 519 mL g⁻¹VS. Several authors reported methane productivities from AD of various food
 261 wastes in the range of 298-482 mL g⁻¹VS [34-36]. Thus, the inoculum used in this study had a
 262 good methanogenic activity.

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266 **Fig. 2** AD of original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual
 267 biomass (PERB). (a) Methane production per gram VS (input). (b) VS and COD consumed.

268 The calculated theoretical maximum and experimental methane yields for the three
 269 substrates are presented in Table 2. The theoretical yield of CH_4 for original *S.platensis* reported
 270 in our work was comparable to the maximum theoretical yield reported for this species by
 271 Heaven et al., 2011, although our calculations were based on reaction stoichiometry and
 272 elemental composition (according to eq. 4), in contrast to Heaven et al., 2011 whose calculations
 273 were based on the empirical formulae and biochemical composition of proteins, lipids and
 274 carbohydrates [37]. The experimental methane yield represented as percentage of theoretical is a
 275 clear indication of the extent of digestibility of a given substrate. Thus, the extent of digestibility

was the highest for PERB and least for ORIB. The COD and VS consumed at the end of the BMP experimental runs were also the highest for PERB, supporting the inference that this substrate had a better digestibility than the other two (Fig. 2 (b)). The alkali treatment and acid hydrolysis steps involved in the generation of PERB must have resulted in the degradation of complex cellular organic compounds/ polymers into simpler, easily digestible compounds (for instance, complex carbohydrates into simple sugars) during the protein isolation process, resulting in higher digestibility and methane yield.

Table 2 Theoretical and experimental CH₄ yields for the original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB)^a.

Substrate	Theoretical CH ₄ yield (mL g ⁻¹ VS)	Experimental CH ₄ yield (mL g ⁻¹ VS)	CH ₄ yield as % of theoretical
ORIB	558.4	181.1 (11.8)	32.4
DISB	558.4	254.5 (16.0)	45.6
PERB	374.0	236.1 (13.1)	63.1

^aValues in parentheses are standard deviations of means

It can also be inferred from Fig. 2 (a) that the rate of methane production was much higher for PERB compared to the other two substrates. Table 3 shows the kinetic parameters for methane production obtained by curve fitting using the modified Gompertz equation for all three substrates. All the predicted parameter values were highly significant ($p < 0.001$). The regression coefficients for curve fitting between the experimental and calculated methane production values for all the substrates was greater than 0.99 indicating a very good fit. Both the PERB and DISB substrates had a higher predicted methane production potential (BMP) and rate of methane production, and lower lag phase time than ORIB. Although the predicted BMP of PERB was lower than DISB (by 12.3 %), the rate of methane production was higher (by 38.9 %) and the lag phase much lower (by 6.3 days). This clearly indicated that the anaerobic bacteria carrying out

the biochemical conversion could adapt to and digest the PERB substrate far more readily and at a faster rate compared to the other two substrates. This may be attributed to the presence of simpler organic compounds in PERB (as discussed above) which could easily be metabolized and converted by the anaerobic bacteria, and the possibility of lower ammonia inhibition during AD of PERB. It has been reported that ammonia inhibition results in increased lag phase time and decrease in methane production rate [38, 39]. Substrates with better kinetics and low lag phase times for methane production are highly favorable for scale up in industrial processes which operate in continuous mode as they can reduce the energy required for maintenance of the digesters, decrease the substrate residence times and improve the efficiency of the AD processes. Thus, PERB could potentially prove to be a much better substrate compared to the other two for commercial AD applications.

Table 3 Methane production kinetic parameters for the original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB)^a

Substrate	P (mL g ⁻¹ VS)	R _m (mL g ⁻¹ VS d ⁻¹)	λ (d)	R ²
ORIB	213.4 (8.9)	15.6 (1.4)	14.9 (0.2)	0.9938
DISB	262.4 (2.4)	29.3 (1.1)	10.4 (0.1)	0.9979
PERB	230.0 (1.7)	40.7 (2.3)	4.2 (0.2)	0.9942

^aValues in parentheses are standard errors

3.3 Hydrothermal liquefaction products

The biocrude oils obtained from PERB and ORIB were both dark brown in color and had a similar, strong odor. Fig. 3(a) compares the yields of biocrude, aqueous soluble, solid and gaseous products obtained from PERB and ORIB. The yield of biocrude from ORIB was 26.9 % and from PERB was 13.7%, which was about half of the former. The lower yield from PERB was a result of its lower protein content because proteins contribute about 11-18 % of the total biocrude yield in HTL process [27]. The decrease in biocrude yield was clearly reflected in the

increase in gas and solid products yields from PERB. The increase in gas yield by about 21% may be attributed partly to gasification being better favored by PERB compared to ORIB, and partly to the breakdown of formic acid (added during the protein isolation process) present in PERB into CO and H₂ gases [18]. The presence of a higher proportion of simpler, low molecular weight compounds in PERB formed as a result of disruption of cell structure and degradation of some of the cell components such as carbohydrates, proteins or lipids into simpler compounds during the protein isolation process may have favored gasification to liquefaction under the operated HTL conditions, also resulting in a reduction of biocrude yield. On the other hand, ORIB was not subjected to any treatments prior to the HTL process and thus the slower hydrolysis and conversion kinetics may have favored liquefaction resulting in higher biocrude yields [14]. Several authors reported that algal HTL is influenced by the operating conditions [14, 21, 22]. Thus, optimization of the HTL parameters for PERB may improve the biocrude yield by enhancing liquefaction. GC-TCD analysis of the gaseous products revealed that the relative composition (%) of CO and H₂ were higher in the PERB gas compared to the ORIB gas (2.2 % CO and 5 % H₂ in the former, and 0.3 % CO and negligible H₂ in the latter) confirming formate decomposition. CO₂ was slightly lower in the former compared to the latter (15.1 % and 16.1 % respectively). The higher SR yield from PERB compared to ORIB may be attributed to the higher ash content of PERB.

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Fig. 3 HTL of original biomass (ORIB) and protein extracted residual biomass (PERB).
(a) Products yield (b) Nitrogen content of products and feedstock. SR – Solid residue; ACP –
Aqueous co-product.

Fig. 3 (b) shows the nitrogen contents in ORIB and PERB, and the biocrudes, ACP and SR products obtained from them. ORIB biocrude had a nitrogen content of 7.0 % while PERB biocrude had 6.2 %. Thus, the nitrogen content in the HTL biocrude decreased by 0.8 % as a result of protein isolation. This decrease was not as high as desired and may be attributed to the undesirably high nitrogen content (7.6%) still remaining in PERB, which was the feed to the HTL process. The review article by Barreiro et al., 2013 highlighted that higher nitrogen content in the feed is reflected as higher nitrogen content in the biocrude and vice versa [14]. The nitrogen content of the SR also showed a similar trend. Thus, additional unit operations or alternative methods to further decrease the nitrogen content in PERB may result in obtaining biocrude with lower nitrogen content.

Fig. 4 shows the cumulative chromatographic peak areas of different classes of organic compounds present in ORIB biocrude and PERB biocrude (further information on the compounds present in the biocrudes may be found in Table S1 and Table S2). PERB biocrude comprised of a higher number of long chain aliphatic hydrocarbons (C10-C20) such as heptadecane and pentadecane and fatty acids such as n-hexadecanoic acid compared to ORIB biocrude. ORIB biocrude comprised of a number of long chain aliphatic amides such as hexadecanamide, N-methyl dodecanamide etc., which were not present in PERB biocrude. Such compounds have been identified in the biocrude obtained from *Spirulina* biomass previously and are formed by reactions between protein and lipid hydrolysates [19, 27]. In addition, both the biocrudes contained a number of heterocyclic nitrogenous compounds such as derivatives of pyrrolidine and piperidine. The presence of such heterocyclic nitrogenous compounds in algal

biocrude was also reported previously [27]. These compounds are formed as a result of repolymerization of the decarboxylated and deaminated amino acid hydrolysates or their recombination with carbohydrate hydrolysates through Maillard reactions [14, 40]. It may be inferred from the cumulative peak areas that PERB biocrude had a higher amount of long chain aliphatic hydrocarbons and fatty acids compared to ORIB biocrude. Although the cumulative peak area for nitrogenated heterocyclic compounds was higher for PERB biocrude, the cumulative peak area of all nitrogenous compounds (nitrogenated heterocyclic compounds, long chain aliphatic amides and other nitrogenous compounds) was higher for ORIB biocrude.

Fig. 4 GC-MS characterization of the biocrude from original biomass (ORIB biocrude) and the biocrude from protein extracted residual biomass (PERB biocrude). LHC – Long chain hydrocarbons (C10 - C20); AHC – Aromatic hydrocarbons; NHCC – Nitrogenated heterocyclic

compounds; LAA – Long chain aliphatic amides; ANC – Other nitrogenous compounds; Ket – Ketones; Phe – Phenolics; FA – Fatty acids; Oxy – Oxygenates.

The yield of PERB ACP was lower than ORIB ACP. Both ACPs were light brown in color and had pH around 9. The COD was high, implying the presence of high amounts of organics. Both the ACPs were enriched in oxygen (about 90%) and had low nitrogen content (around 0.5 %). GC-MS analysis showed that PERB-ACP comprised primarily of nitrogenous compounds (several aliphatic straight chain, branched and heterocyclic amines) and ethanol. HPLC analysis of this aqueous co-product showed the presence of a few organic acids and sugars - formate (3 mg mL⁻¹), acetate (2 mg mL⁻¹), lactate (2 mg mL⁻¹), ethanol (0.8 mg mL⁻¹) and succinate (0.6 mg mL⁻¹). Analysis of the mineral composition revealed that the content of most of the mineral elements present in significant amounts was higher in PERB ACP than in ORIB ACP (Table S3). The PERB biomass generation process resulted in the concentration of the mineral elements, as was evident from the higher ash content (about 3 times) present in this biomass compared to ORIB. Most of these mineral elements may have originated from original *S.platensis* biomass, except sodium which was added during the protein extraction process. The growth limiting nutrients of phosphorus and nitrogen were present in excess in both the ACPs compared to standard algal growth media [41]. Thus, both the ACPs may potentially be used for algal cultivation. Such an application has previously been explored and validated using ACPs obtained from *S.platensis* and other microalgae [42, 43].

3.4 Comparison of AD and HTL

Table 4 presents the elemental composition and HHV (energy content) of the feedstocks and the biomethane and biocrude products obtained from them, and the energy output and energy recovery in the AD and HTL processes. The HHV of PERB was lower than ORIB and DISB by

34 %. Yet, the energy recovery in the biomethane produced from PERB was higher than that from ORIB and DISB by 68.4 % and 19.8 % respectively. In terms of energy output from AD, PERB performed better than ORIB (by 11.3 %) but worse than DISB (by 20.7 %). The lower energy output from PERB compared to DISB was a result of lower biomethane yield.

The HHV of PERB biocrude was slightly lower than ORIB biocrude (by 8.3 %). Both these HHV values were about 72-78 % of the HHV of petroleum crude oil (42 MJ kg⁻¹) [44]. The energy recovery in PERB biocrude was 29.9 % lower than ORIB biocrude. This was clearly a result of lower biocrude yield from PERB. A comparison of energy output and energy recovery in the biofuel products (biomethane and biocrude) obtained from PERB and ORIB showed that AD performed better than HTL for the former biomass and vice-versa for the latter.

Table 4 Elemental composition and HHV of the feedstocks and biofuel products, and the energy output (E_{output}) and energy recovery (ER) in AD and HTL processes.

Sample	Elemental analysis (% w/w) ^a				HHV (MJ kg ⁻¹)	Yield (% w/w)	E _{output} (MJ kg ⁻¹)	ER (%)
	N	C	H	O				
AD								
<i>Biomass</i>								
ORIB	11	50.1	6.4	25.9	21.5			
DISB	11	50.1	6.4	25.9	21.5			
PERB	7.6	35.3	5.5	31.3	14.2			
<i>Biomethane</i>								
ORIB					55.5	11.2	6.2	28.8
DISB					55.5	15.7	8.7	40.5
PERB					55.5	12.4	6.9	48.5

HTL								
<i>Biomass</i>								
ORIB	11	50.1	6.4	25.9	21.5			
PERB	7.6	35.3	5.5	31.3	14.2			

Biocrude

ORIB	7	68.6	8.7	15.8	32.7	26.9	8.8	41.1
PERB	6.2	64.3	8.5	21.1	30.0	13.7	4.1	28.8

^aS (% w/w) detected in all the samples was found to be very low

4. Conclusion

This study showed that biofuel production after protein extraction has some advantages over direct biofuel production from original *S.platensis* biomass. AD tests revealed that the protein extracted residual biomass was a far more biodegradable substrate with a higher kinetic rate of methane production and capable of producing higher amount of methane gas compared to the original biomass. HTL of PERB resulted in a biocrude with slightly lower nitrogen content and higher number of long chain aliphatic hydrocarbons and fatty acids compared to ORIB. Thus, the use of PERB proved to be beneficial to both the AD and HTL processes, although AD performed better in terms of product yield, energy output and energy recovery. Further optimization of the protein extraction and biofuel production processes may improve the yield of biomethane and biocrude oil that could be generated from PERB in order to better impact the economics and sustainability of the integrated process.

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Figure Captions

Fig. 1 Hydrothermal liquefaction and product separation process. SR – Solid residue; ACP – Aqueous co-product.

Fig. 2 AD of original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB). **(a)** Methane production per gram VS (input). **(b)** VS and COD consumed.

Fig. 3 HTL of original biomass (ORIB) and protein extracted residual biomass (PERB). **(a)** Products yield **(b)** Nitrogen content of products and feedstock. SR – Solid residue; ACP – Aqueous co-product.

Fig. 4 GC-MS characterization of the biocrude from original biomass (ORIB biocrude) and the biocrude from protein extracted residual biomass (PERB biocrude). LHC – Long chain hydrocarbons (C10 - C20); AHC – Aromatic hydrocarbons; NHCC – Nitrogenated heterocyclic compounds; LAA – Long chain aliphatic amides; ANC – Other nitrogenous compounds; Ket – Ketones; Phe – Phenolics; FA – Fatty acids; Oxy – Oxygenates.

Table captions

Table 1 Composition of the original *S.platensis* biomass (ORIB) and the protein extracted residual biomass (PERB). All values are reported as weight percentages on a dry basis^a.

Table 2 Theoretical and experimental CH₄ yields for the original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB)^a.

Table 3 Methane production kinetic parameters for the original biomass (ORIB), disrupted biomass (DISB) and protein extracted residual biomass (PERB)^a

Table 4 Elemental composition and HHV of the feedstocks and biofuel products, and the energy output (E_{output}) and energy recovery (ER) in AD and HTL processes.

figure 1

figure 2a

figure 2b

figure 3a

figure 3b

figure 4

Table 1

Parameter	ORIB	PERB
C (%)	50.1 (2.0)	35.3 (2.5)
H (%)	6.4 (0.3)	5.5 (0.2)
N (%)	11.0 (0.1)	7.6 (0.1)
O (%)	25.9 (1.4)	31.3 (3.2)
Ash (%)	6.7 (0.5)	20.3 (0.1)
VS (%)	93.3 (0.5)	79.7 (0.1)
C/N ratio	4.58 (0.1)	4.64 (0.3)
Protein content (%)	68.8 (1.9)	47.5 (0.8)

^a Values in parentheses are standard deviations of means

Table 2

Substrate	Theoritical CH ₄ yield (mL g ⁻¹ VS)	Experimental CH ₄ yield (mL g ⁻¹ VS)
ORIB	558.4	181.1 (11.8)
DISB	558.4	254.5 (16.0)
PERB	374.0	236.1 (13.1)

^aValues in parenthesis are standard deviations of means

Table 3

Substrate	P (mL g ⁻¹ VS)	R _m (mL g ⁻¹ VS d ⁻¹)	λ (d)	R ²
ORIB	213.4 (8.9)	15.6 (1.4)	14.9 (0.2)	0.9938
DISB	262.4 (2.4)	29.3 (1.1)	10.4 (0.1)	0.9979
PERB	230.0 (1.7)	40.7 (2.3)	4.2 (0.2)	0.9942

^aValues in parenthesis are standard errors

Table 4

Sample	Elemental analysis (% w/w) ^a				HHV (MJ kg ⁻¹)	Yield (% w/w)	E _{output} (MJ kg ⁻¹)	ER (%)
	N	C	H	O				
AD								
<i>Biomass</i>								
ORIB	11	50.1	6.4	25.9	21.5			
DISB	11	50.1	6.4	25.9	21.5			
PERB	7.6	35.3	5.5	31.3	14.2			
<i>Biomethane</i>								
ORIB					55.5	11.2	6.2	28.8
DISB					55.5	15.7	8.7	40.5
PERB					55.5	12.4	6.9	48.5

HTL								
<i>Biomass</i>								
ORIB	11	50.1	6.4	25.9	21.5			
PERB	7.6	35.3	5.5	31.3	14.2			
<i>Biocrude</i>								
ORIB	7	68.6	8.7	15.8	32.7	26.9	8.8	41.1
PERB	6.2	64.3	8.5	21.1	30.0	13.7	4.1	28.8