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Valorisation of biodiesel production wastes: Anaerobic digestion of residual *Tetraselmis suecica* biomass and co-digestion with glycerol

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Abstract

One of the principal opportunity areas in the development of the microalgal biodiesel industry is the energy recovery from the solid microalgal biomass residues to optimise the fuel production. This work reports the cumulative methane yields reached from the anaerobic digestion of the solid microalgal biomass residues using different types of inocula, reporting also the improvement of biogas production using the co-digestion of microalgal biomass with glycerol. Results demonstrate that the solid microalgal biomass residues showed better biogas production using a mesophilic inoculum, reaching almost two-fold higher methane production than under thermophilic conditions. Furthermore, the solid microalgal biomass residues methane production rate showed an increase from 173.78 ± 9.57 to 438.46 ± 40.50 mL of methane per gram of volatile solids, when the co-digestion with glycerol was performed. These results are crucial to improve the energy balance of the biodiesel production from *Tetraselmis suecica*, as well as proposing an alternative way to treat the wastes derived from the microalgae biodiesel production.

Keywords

Anaerobic digestion, biodiesel, biogas production, microalgae, solid microalgal biomass residues, *Tetraselmis suecica*

Introduction

Biofuels, which are new, renewable and from biological origin alternative fuels, have been receiving much attention all over the world owing to energy needs and environmental consciousness (Rawat et al., 2013). These energy sources are referred to liquid, gas and solid fuels produced mainly from organic matter, and they are considered one of the most strategically important sustainable energy sources nowadays. A variety of fuels, such as ethanol, biodiesel, hydrogen and methane, can be produced from different biomass (Gouveia and Oliveira, 2009).

Among this range of possibilities, the biodiesel produced from microalgae is considered one of the more interesting alternatives (Fuentes-Grünewald et al., 2009). Considering the low land requirements, this biodiesel is a renewable resource of energy that could be supplied instead of classical chemical energy and can be used in existing engines and transport infrastructures. Moreover, it is better than diesel fuel in terms of flash point and biodegradability. The biofuel from microalgae (the so-called third generation biodiesel) avoids land crop competition for food, it is more efficient when the lipid/dry weight ratio is compared and, in the case of marine organisms, it also avoids the use of freshwater (Gouveia and Oliveira, 2009; Rawat et al., 2013). This is why the researchers are seeking lipid-rich biological materials to produce biodiesel effectively, especially from microalgae during the last two decades (Fuentes-Grünewald et al., 2012; Graham et al., 2012). Furthermore, the use of microalgae as a renewable biological resource also contributes to the removal of wastewater pollutants and generating value-added products in the form of proteins, pigments, biopolymers, carbohydrates and carotenoids (Kaštánek et al., 2010).

Autotrophic microalgae use carbon dioxide as a carbon source and sun as energy for oil accumulation under specific conditions (Graham et al., 2012). The lipid concentration may vary from 15%–80% of the dry weight depending on several factors

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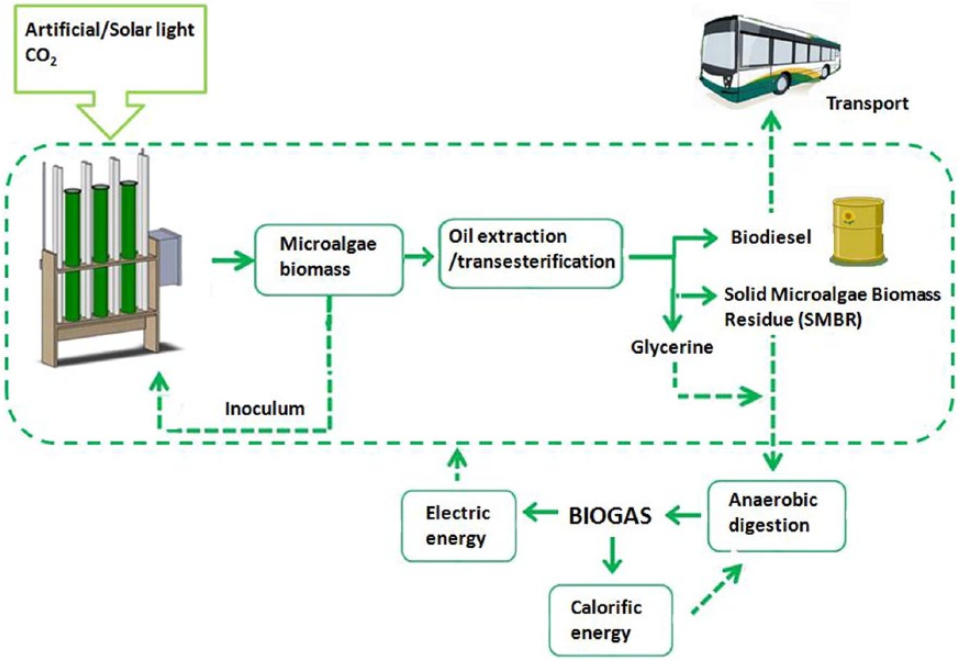

Figure 1. Schematic process of biodiesel production from microalgae biomass.

(Fuentes-Grünewald et al., 2009; Gouveia and Oliveira, 2009). Certain microalgae species show high theoretical productivities and oil accumulation under cellular stress conditions (Fuentes-Grünewald et al., 2011), this being the main reason that makes biodiesel production from algal oil transesterification a logical choice for research and development (Gouveia and Oliveira, 2009). The produced lipids are mostly neutral lipids and have a high level of saturation (Rawat et al., 2013). Another potential advantage is that microalgae possess a very short harvesting cycle, allowing multiple or continuous harvesting even in outdoor conditions (Fuentes-Grünewald et al., 2012; Kaštánek et al., 2010). Also, the lipid high productivity of microalgae imparts the potential for a high theoretical production with the proximately yield of 58,700 to 136,900 L ha^{-1}, reaching a potential biodiesel production of 15 to 300 times more than traditional crops on an area basis (Gouveia and Oliveira, 2009; Rawat et al. 2013).

It is important to highlight, however, that microalgal biodiesel has not yet reached a clear-cut economic feasibility; the biggest challenge being the relatively high costs of production of microalgal biomass and extraction/separation of lipids for biodiesel, besides the transformation of microalgae oil to biodiesel is considered an energy-consuming process (Rawat et al., 2013). In order to make biodiesel an economically suitable fuel and increase its marketability, its costs must be lowered (Sevigné-Itoiz et al., 2012). Regarding this, one of the principal concerns of the commercial production of biodiesel from microalgae is the fate of microalgae residues obtained after the extraction and transesterification process. The overall economic analysis indicates that the feasibility of microalgal biofuel development depends on the possibility of obtaining co-products with a high market value, as proposed in the so-called biorefineries, which implies the simultaneous production of biodiesel, animal feed, biogas and electrical power (Rawat et al., 2013; Schneider et al., 2014).

The microalgae biodiesel production process (Figure 1) results in the generation of large amounts of solid microalgae biomass residues (SMBRs) produced after the lipid extraction and/or fuel conversion processes, which accounts for approximately 65% of the original harvested biomass. This SMBR consists mainly of protein and carbohydrate, which makes possible its anaerobic digestion to obtain energy in the form of biogas, mainly methane (Park and Li, 2012). Glycerol is the main by-product formed during the transesterification process (Ehimen et al., 2011) representing up to 10% of the total weight (Kolesárová et al., 2011). Glycerol is one of the main economic problems of biodiesel production from microalgae (as other SMBRs) if is not adequately recovered (Ehimen et al., 2009). The use of this by-product to generate biogas is a clear target in the most recent studies on this topic (Ehimen et al., 2009; Kolesárová et al., 2011). In fact, the protein–carbohydrate waste product, together with the glycerol as a co-substrate for anaerobic digestion, could serve as an alternative source of energy for the production and processing of the SMBR (Sialve et al., 2009).

The technology for anaerobic digestion is well developed. The parameters to optimise the fermentation conditions, such as bioreactor materials, biophysical parameters and the inoculum and substrate concentrations, have been largely studied (Chen et al., 2008; Ferrer et al., 2008). Although the use of microalgal biomass directly to produce methane by anaerobic digestion has been studied during the last decades and it is technically feasible (González-Fernández et al., 2012; Ras et al., 2011; Ward et al., 2014; Zamalloa

et al., 2012), the biogas production from them cannot compete with other low-cost organic substrates that are widely available for anaerobic digestion.

The anaerobic digestion of the SMBR has been theoretically examined to improve the energy balance of the microalgae biodiesel production process; it is well known that the results are not always similar among microalgae species due to their variable lipid, carbohydrate and protein composition (Sialve et al., 2009); Zhu (2014) calculated the theoretical methane and energy production using the SMBR biochemical composition for some species of marine and freshwater microalgae obtaining values between 30–360 mL CH4 g^{-1} volatile solids added, representing calorific values of 1.4–8.3 MJ kg^{-1} volatile solids added. The author recommends that cost-effectiveness and the energy life cycle analysis should be determined for the species with the best potential to obtain the best option for the production of methane from microalgae.

Otherwise, there are few experimental investigations on methane production using SMBRs. Ehimen et al. (2009) investigated the batch anaerobic digestion of *Chlorella* residues, with average methane yields within 222–267.5 mL g^{-1} volatile solids of the microalgae residue digested. Co-digesting the SMBR with the glycerol as co-product was observed to increase the methane yields when compared with those of digestion of the residues alone, and using semi-continuous cultures of SMBR (*Chlorella* sp.), developing an optimisation of hydraulic loading rate, retention time and C:N ratio (co-digesting the SMBR with glycerol), biogas production increments higher than 50% were obtained (Ehimen et al., 2011). Zhao et al. (2014) analysed the biogas and methane production from whole microalgae and lipid-extracted biomass for *Chlorella vulgaris, Phaeodactylum tricornutum, Nannochloropsis* sp., *Nannochloropsis salina* and *Nanofrustulum* sp. obtaining values between 3.04–3.99 L CH_4 L liquid^{-1} and CH_4 content ranged from 60%–75%; besides they observed that the effluent total ammonia nitrogen and volatile fatty acids (VFA) levels were low, indicating effective digestion despite the C:N ratios well below that ideally recommended. Some authors (Alzate et al., 2014; Chandra et al., 2014) observed an improvement in the CH_4 production for the SMBR compared with the whole microalgae biomass, which suggested that lipid-extraction constituted itself a pretreatment to increase the biochemical CH_4 potential for some species as *Nannochloropsis* sp. and *Scenedesmus dimorphus*.

The aim of the present study was to evaluate the methane production from SMBRs resulting from biodiesel production in the marine microalgae *Tetraselmis suecica*, a marine green flagellate that has been widely used in industrial aquaculture as feed for shrimps larvae, molluscs and rotifers (Zittelli et al., 2006). According to Zittelli et al. (2006), it is one of the most suitable green microalgae candidates for biodiesel production: although its lipid content per unit biomass is around 7%–23% and is not inside the highest values reported for microalgae (Fuentes-Grünewald et al., 2009), this strain is very resistant and shows a very high specific growth rate (0.23–0.69 d^{-1}), even in outdoor conditions, resulting in a fast, robust and suitable oil source (Zittelli et al., 2006). Different inocula in batch anaerobic digestion experiments were tested. The co-digestion of the microalgae residues with glycerol is also reported.

Materials and methods

Microalgae biomass production

Local strains of the microalgae species *T. suecica* obtained from the algal collection of the Facultad de Ciencias del Mar of the Universidad Autónoma de Sinaloa (Mexico) were used. The microalgae was cultivated using the medium reported by Guillard and Ryther (1962), consisting of natural seawater enriched with a solution that has a final composition per litre of: 75 mg KNO_3, 5.65 mg $NaH_2PO_4 \cdot 2H_2O$, 4360 mg EDTA, 3150 mg $FeCl_3 \cdot 6H_2O$, 0.010 mg $CuSO_4 \cdot 5H_2O$, 0.022 mg $ZnSO_4 \cdot 7H_2O$, 0.010 mg $CoCl_2 \cdot 6H_2O$, 0.180 mg $MnCl_2 \cdot 4H_2O$, 0.006 mg $Na_2MoO_4 \cdot 2H_2O$, 2 g crystalline cyanocobalamin (B_{12}), 0.100 mg thiamine hydrochloride (B_1) and 0.001 mg crystalline biotin. The cultures were grown at 25 °C (±1 °C) in 19 L plastic containers using an experimental capacity volume of 16 L. Microalgae cultures were subjected to a 12:12 h light/dark (L/D) conditions. Illumination was provided by four lamps with an intensity of 6000–6500 lux, generating an active radiation of 120–130 µmol photons m^{-2} s^{-1}. Filtered air was supplied continuously through a 1 µm cut-off cartridge and using a blower (2.5 hp). The aeration gas exchange facilitated and enabled the cells to keep in suspension, which also favoured the exposure of all the cells to the same amount of light.

Biomass samples were harvested by flocculation with chitosan and dried to a constant weight in a forced draft oven at 80 °C. Dried microalgae biomass was ground in a mortar and its oil content was obtained using the extraction method described by Folch et al. (1957). The final SMBRs were separated from the lipid solvent solution by filtration and then it was dried at 80 °C during 12 h, for elemental characterisation.

Anaerobic potential biogas production tests

Anaerobic batch tests for methane production evaluation and measurement as described by Ferrer et al. (2008) were adapted and used in this study as commented here.

Inoculum and substrate characteristics

The biomass samples obtained after the extraction and transesterification (biodiesel production) processes were collected and used in the anaerobic biogas test as a substrate. The inoculum was composed of digested sludge from three different anaerobic reactors (Table 1). One inoculum was collected from the mesophilic anaerobic digester of a municipal wastewater treatment plant, located in Sabadell, Barcelona (SAB). The second inoculum was obtained from a mesophilic anaerobic digester that processes the organic fraction of municipal solid wastes located in Montcada i Reixach, Barcelona (ECO). The third inoculum came

Table 1. Characteristics of the substrate and inocula.

Parameter	SMBR	SAB	ECO	TER
TS (%)	83.37 ± 0.07	2.44 ± 0.01	11.88 ± 0.06	18.4 ± 0.2
VS (% of TS)	57.4 ± 0.7	67.4 ± 0.2	58.0 ± 0.4	52 ± 1
pH	N/A	7.47	8.26	8.54

Data are the means of three measurements with standard deviations.
SMBR: solid microalgae biomass residues; SAB: Sabadell; ECO: Ecoparc; TER: Terrasa; TS: total solids; VS: volatile solids; N/A: no analysis.

Table 2. Characterisation of the SMBR.

	Lipids (% TS)	TOC (% TS)	NTK (% TS)	C:N
SMBR	1.05 ± 0.03	26.6 ± 0.9	8.6 ± 0.5	3.1

Data are the means of three measurements with standard deviations.
SMBR: solid microalgae biomass residues; TS: total solids; TOC: total organic carbon; NTK: total Kjeldahl nitrogen; C:N: carbon:nitrogen ratio.

from a dry thermophilic anaerobic digester which treats the organic fraction of municipal solid wastes located in Terrassa, Barcelona (TER) at 55 °C. Prior to the experiments, the mesophilic and thermophilic inocula were starved for 14 days in 37 °C and 55 °C incubators, respectively, until the biogas production was not detectable to start the biochemical methane potential assays (Ferrer et al., 2008).

Digester configuration and experimental conditions

The biogas production experiments were carried out in triplicate using sealed aluminium bottles (1 L) equipped with gas valves for biogas sampling and gas volume measurement. As the total solids (TS) content of the three inocula clearly differed (Table 1), distilled water was added to the TER and ECO inocula to homogenise the TS content of the mixtures to similar levels. Blank assays (containing only inoculum) were performed for monitoring the biogas production of the inocula from endogenous activity (Ferrer et al., 2008). The results of the blank probes were used to correct for the residual methane production. Control assays were run for each inoculum, using 1.7 g of cellulose substrate and 600 mL inoculum in each case. SMBRs were weighed (1.7 ± 0.2 g) and placed into the reactor bottles. The prepared inoculum was then introduced to reach a target test volume of 600 mL. Residual oxygen was then removed from the headspace of the vessels by flushing with pure nitrogen gas. The filled bottles were incubated for 30 days at 37 ± 1 °C (55 ± 1 °C for the thermophilic experiments) (Table 2). Reaction vessels were homogenised by shaking the bottles once a day to ensure the complete resuspension of the sediments and scum layers (Ehimen et al., 2009). Biogas volume and concentration measurements were performed on a daily basis.

Glycerol co-digestion

Glycerol solution (86%–89% purity, Sigma Aldrich) was used for the experiments of co-digestion. Crude glycerol is the major by-product of the biodiesel industry. In general, an average of about 10–20 L crude glycerol is produced for every 80 L of biodiesel (Kolesárová et al., 2011). Crude glycerol generated by homogeneous base-catalysed transesterification contains approximately 81% to 85% of glycerol, and the rest is formed by alkalis, methyl esters, methanol and water, so the used glycerol can be considered acceptable in its composition for the purposes of the experiment (Ehimen et al., 2009, 2011).

The anaerobic digestion was performed simulating the co-digestion of proportional amounts obtained of SMBRs and residual glycerol from the extraction and transesterification processes of oil from the *T. suecica* biomass. The contribution of endogenous methane production by the inoculum, SMBR or glycerol, was deducted from the entire cumulative yield of methane. Equation (1) was used to calculate the quantity of glycerol for co-digestion:

$$\begin{matrix}\text{Equivalent Glycerol Production} \\ \text{(g / g dry biomass transesterified)}\end{matrix} = \frac{\alpha M_{Glycerol}}{M_{Oil}} \quad (1)$$

where α is the oil fraction of microalgae biomass, $M_{glycerol}$ is the molecular weight of glycerol and M_{oil} is the molecular weight of the oil from microalgae. For *T. suecica* the average lipid content is 23% and the average molecular weight of the obtained oil is 896 g mol^{-1} (Fuentes-Grünewald et al., 2009), obtaining a relation of 0.024 g glycerol produced per 0.77 g of dry SMBR. The anaerobic experimental set-up was carried out using these theoretical proportions in the reactor bottles.

Analytical methods

For its use for the anaerobic digestion trials, the inoculum was characterised on the basis of the TS and volatile solids (VS) content. These parameters were determined according to Standard methods (APHA, 1999). The pH was measured manually using a model Delta 320 pH Meter (Mettler-Toledo, Germany). pH, TS and volatile solids were determined directly from sludge samples and the SMBRs. The protein content was determined based on the total Kjeldahl nitrogen (TKN) (Ferrer et al., 2008), meanwhile total

Table 3. Operative conditions and summary of the result obtained in the anaerobic batch digestion of SMBR.

Parameter	SAB[a]	ECO[a]	TER[b]
Operating temperature (°C)	37 ± 1	37 ± 1	55 ± 1
pH	7.47	8.26	8.54
Hydraulic retention time (HRT) (days)	30	30	30
Biogas production yield (mL biogas gVS^{-1})	310 ± 21	173 ± 9	133 ± 17
Content of methane in biogas (%)	59.6	73.2	68.1
Methane production yield (mL CH_4 gVS^{-1})	174 ± 9	127 ± 6	91 ± 12

SAB: Sabadell; ECO: Ecoparc; TER: Terrasa; VS: volatile solids.
[a]Mesophilic conditions.
[b]Thermophilic conditions.

organic carbon (TOC) was determined by standard methods (EPA, 1999). Total lipid content was extracted with n-hexane (95% purity, Sigma Aldrich) in a Soxhlet E-812/816 extraction unit (Büchi, Germany) (EPA, 1996). TKN, TOC and total lipid content were determined from SMBR samples. The volume of the produced biogas was determined by pressure measurements using an ISE30A type pressure gauge (SMC Corporation, Japan). The cumulative volumetric biogas production, in millilitres, at normal conditions was calculated from the pressure increased in the headspace volume. Biogas samples were taken periodically for the analysis of methane content by gas chromatography. After each pressure determination, the reactor was returned to atmospheric pressure by releasing the formed biogas. At the end of the experiment the average biogas produced by the SMBR was calculated subtracting the volumes of the blank samples. The generated biogas and methane volumes where then reported at normal conditions (1 atm, 273 K). Biogas composition was determined using a gas chromatograph (Hewlett Packard 5890A) equipped with thermal conductivity detector. The column type was Porapack Q 3 mts × 1/8" × 2.1 mm D.I. (Teknokroma, Barcelona, Spain). Helium was used as a carrier gas in splitless mode with a back pressure of 338 kPa. The oven was maintained at a constant temperature of 70 °C for 3 min. The injector and detector temperatures were at 150 °C and 180 °C, respectively. The system was calibrated with analytical methane and CO_2.

Results and discussion

Characterisation of the SMBRs and anaerobic inocula

The results of the characterisation of different inocula are summarised in Table 1. The TS content of the three inocula were clearly different, according to their origin. The highest value was registered on the TER inoculum, with 18.43% TS, and the lowest was the SAB inoculum with only 2.44% TS. The VS percentage also differs, from 51.72% (TER) to 67.36% (SAB). In general, the three inocula presented a relatively high organic content, typical from fresh non-stabilised materials. Values above neutral pH were registered for each inoculum; the highest pH was observed in the TER inoculum, probably because of the high ammonia content, typical of wastes digested under thermophilic conditions (Chen et al., 2008).

The characteristics of the substrate (Table 2) are comparable with values reported on biomass and SMBRs of other species of microalgae. For the C:N ratio, the SMBR had a low ratio of 3.1, which was lower than the obtained from *Taihu* blue algae (5.9) and *Chlorella* (8.60) biomass, and even lower than SMBRs obtained from *Chlorella* (5.4) (Ehimen et al., 2009; Zhong et al., 2012). The C:N ratio of the sample was found below the range of 15–30, which was recommended to be the most suitable for the optimal operation of the anaerobic digestion and the highest methane production (Yen and Brune, 2007). Although this low C:N ratio could form ammonia and provoke an increase of pH, thus generating a toxic inhibition of the bacterial population in the digester (Chen et al., 2008), this was clearly not present in this experiment.

Lipid content was also determined in SMBRs (Table 2). Results showed that *T. suecica* SMBR had a lipid composition of 1.05% TS, which could indicate a successfully lipid extraction from the biomass, corroborating the efficiency of the extraction method used. Moreover, this value is comparable with those reported from residual biomass obtained in the oil extraction from other species (lipids contents within 0.19%–1.96%) (Gonzáles-Fernández et al., 2012).

Biogas production rates, methane content and the operative conditions for the digestion of *T. suecica* SMBRs using different mesophilic and thermophilic inocula are shown in Table 3. The cumulative biogas production after 30 days of incubation was 309.77 ± 20.59 mL biogas gVS^{-1} for SAB, 173.43 ± 8.76 mL biogas gVS^{-1} for ECO and 133.00 ± 17.53 mL biogas gVS^{-1} for TER. Cumulative values were higher for the mesophilic inocula than the thermophilic inoculums. On the other hand, methane concentrations of the produced biogas were registered between 59.64% and 73.18%, being the SAB and ECO, the lowest and the highest, respectively. Methane content is one of the main issues in anaerobic digestions, where high levels of CH_4 in biogas indicate an efficient digestion process, leading to minimum purification steps. Moreover, high methane content in an anaerobic digester implies a steady balance of methane and carbon dioxide formation. On the contrary, low methane content implies some form of inhibition that diminishes the methanogenic activity within the microbial consortium (Chen et al., 2008; Kolesárová et al., 2011).

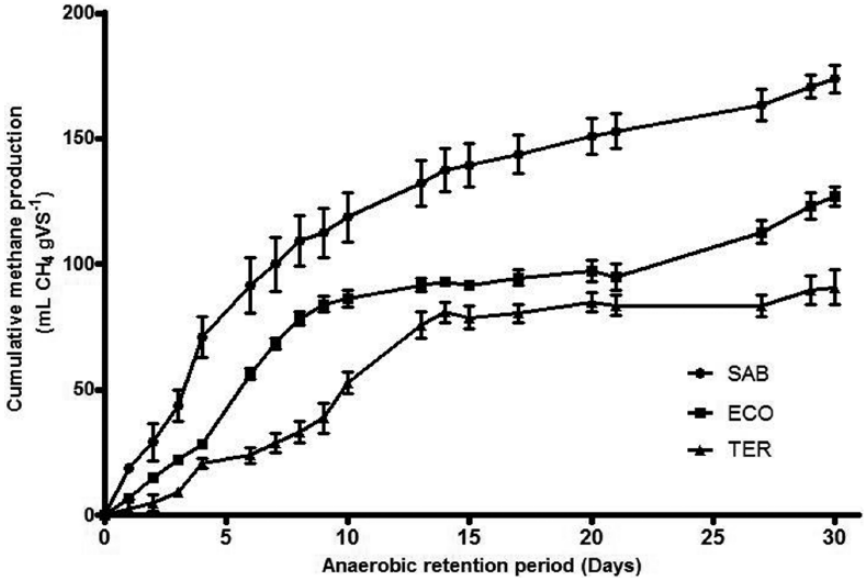

Figure 2. Average cumulative methane production during batch anaerobic digestion of *T. suecica* SMBR using mesophilic (SAB and ECO) and thermophilic (TER) inocula.
Data represent the mean ± standard deviation ($N = 3$).

Specific CH_4 yield of batch experiments with different inocula

The cumulative methane yield of *T. suecica* SMBR, inoculated with different mesophilic (SAB, ECO) and thermophilic (TER) inocula, was assessed by means of anaerobic batch tests by triplicate. Figure 2 shows the evolution of the net cumulative yields during 30 days of anaerobic digestion. During the first 12 days of digestion, methane production rates were higher for all the inocula used for digestion of SMBR and afterwards, methane production rates started to decrease for all samples. Specifically, the mesophilic inocula exhibited higher methane production during the first days of digestion than that of thermophilic inoculum, and this behaviour was maintained during the whole experiment. The highest methane yield was achieved by anaerobic digestion of SMBRs with the SAB inoculum, with a value of 173.78 ± 9.57 mL CH_4 gVS^{-1}, while the lowest yield was for the TER inoculums with 90.69 ± 11.95 mL CH_4 gVS^{-1}.

The results of the methane yield in this study were similar to previous reports: the cumulative CH_4 production was higher than the 100–140 mL CH_4 gVS^{-1} obtained for SMBRs from a mixture of *Scenedesmus* and *Chlorella* (Yen and Brune, 2007), the 101 mL CH_4 gVS^{-1} produced by *S. dimorphus* SMBR (Chandra et al., 2014) and the value reported by Park and Li (2012) for *N. salina* SMBR (133 mL CH_4 gVS^{-1}). However, the cumulative production obtained in the present work was lower than that of *Chlorella* SMBR, which registered 267.5 mL CH_4 gVS^{-1} (Ehimen et al., 2009); also for *Nannochloropsis* sp. SMBR, which produced 271–284 mL CH_4 gVS^{-1} (Zhao et al., 2014), and for SMBR from *Scenedesmus*, which shows 290.3 mL CH_4 gVS^{-1} (Yang et al., 2011).

Is worthwhile mentioning that the methane yield for an anaerobic process depends on different parameters, such as type of digestion, temperature, anaerobic retention time and the characteristics of the inoculum and substrate; thus, the results reported here can be considered close, although it is clear that the source of SMBR, the efficiency of oil extraction and the operational conditions can be also very important in the variation observed for the different works (Chen et al., 2008, Zhu, 2014).

Otherwise, thermophilic experiments produced nearly half of the methane with respect to the other inocula. The reasons for this difference in gas productivity are not yet clear, but this behaviour has been previously reported for this kind of substrates (Gonzáles-Fernández et al., 2012); these authors obtained double methane production when comparing mesophilic and thermophilic anaerobic digestion of *Scenedesmus* and *Chlorella* biomass. This could be explained because microbial growth rates and VFA concentration are affected by temperature change; usually an increase in the process temperature has a positive effect on the metabolic rate of the micro-organisms, but it also results in a higher concentration of VFA (Chen et al. 2008). Some authors have found that the anaerobic fermentation of compounds with a high concentration of nitrogen was more easily inhibited and less stable at thermophilic temperatures than at mesophilic temperatures (Zamalloa et al., 2012).

SMBRs and glycerol co-digestion performance

The results of cumulative methane yield for the co-digestion of *T. suecica* SMBR and glycerol, using the inoculum SAB (which shows the best performance in the first experiment), are shown in Figure 3. The co-digestion of the theoretical amounts of residual material obtained from oil extraction and transformation to biodiesel, starting from *T. suecica* biomass, shows a significant

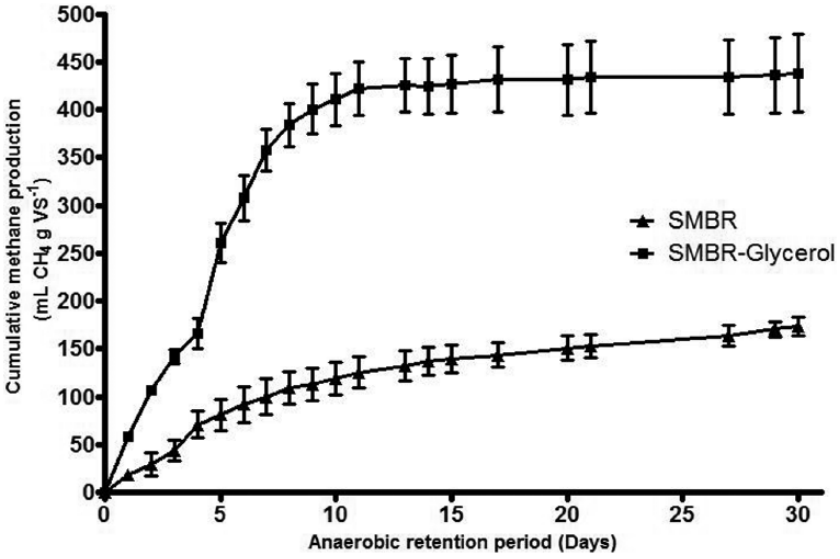

Figure 3. Average cumulative methane production during batch anaerobic digestion of SMBR and co-digestion with glycerol, using the inocula SAB.
Data represent the mean ± standard deviation ($N = 3$).
SMBR: solid microalgae biomass residues.

increase in the methane production rate (from 173.78 ± 9.57 to 438.46 ± 40.50 mL CH_4 gVS^{-1}); this value represents an increase of 152.30% over that observed in SMBR digestion alone (Figure 3). The content of methane for the biogas produced in the co-digestion was improved to 67.03%, compared with the 59.64% of methane obtained for the digestion of SMBRs with SAB inoculum. Although there are only a few reports that perform co-digestion of microalgae residues, the present results are comparable with other reports, where they reached improvements of between 18% and 204.54% using different co-substrates with a high C:N ratio such as waste paper, corn straw, lipid waste and glycerol (the latter being the best option for the integral use of the biodiesel by-products, and where the average increase varies from 100% to 200%) (Ehimen et al., 2009; Kolesárová et al., 2011; Park and Li, 2012; Yen and Brune, 2007; Zhong et al., 2012). Although the introduction of co-substrates may cause unstable digestion processes (Kolesárová et al., 2011), this was not the case in the present experimental set up.

The difference in biogas and methane yields after co-digestion may be mainly attributable to an increase in the organic matter available, added in the form of glycerol, which is a liquid phase easier to degrade (Ehimen et al., 2009). Moreover, the difference can also be caused by a synergistic effect, probably related to the C:N ratio. It is well known that balancing the C:N ratio is one of the main issues for the co-digestion process. In fact, ideal co-substrates for substrates with high nitrogen contents and high alkalinity are wastes that have a high C:N ratio, like crude glycerol (Ehimen et al., 2011; Kolesárová et al., 2011). This study involved the co-digestion of the proportional quantities produced for the production of biodiesel from *T. suecica*, searching the valorisation for the by-products of the process. Several authors propose the residual glycerol as the ideal co-substrate to improve the energy and economic efficiency for microalgae biodiesel production, providing an alternative route for glycerol use where an excess volume exists (Gouveia and Oliveira, 2009; Rawat et al., 2013). This step is essential mainly for three reasons: (1) glycerol is one of the main by-products of biodiesel production, and it is necessary to incorporate it into the same system of production of the fuel plant (Kolesárová et al., 2011); (2) it is necessary to understand how to use part of the by-products as the protein–carbohydrate waste coming from the biodiesel transesterification process, and the biogas production seems to be a good option to use it; and (3) the end cost of biodiesel depends, among other things, on the price of the feedstock (Alzate et al., 2014; Ward et al., 2014), and this kind of optimisations seems to be one of the ways to improve the productivity generating part of the energy needed for the biodiesel production.

Conclusions

The results of this study confirm that the inoculum type has a substantial impact on the methanogenic potential of the *T. suecica* SMBR. Differences as high as 190% in specific methane yields were observed for the different inocula used in the methane production assays. The highest specific methane yield was observed in the mesophilic inoculum obtained from an anaerobic digester of a municipal wastewater treatment plant, which shows values close to similar reports. Moreover, the co-digestion using the microalgae biodiesel by-products increases the production of biogas and the methane content of it, reaching a methane production rate near to two-fold higher than observed in the SMBR digestion alone. This demonstrates that biodiesel production can be improved, a basic step in the economical and energy balance of the microalgae biofuel generation in a near future.

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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References

Alzate ME, Muñoz R, Rogalla F, et al. (2014) Biochemical methane potential of microalgae biomass after lipid extraction. *Chemical Engineering Journal* 243: 405–410.

APHA (1999) AWWA, WPCF. Standard methods for the examination of water and wastewater. Washington DC, USA: American Public Health Association, American Water Works Association, Water Environment Federation.

Chandra TS, Suvidha G, Mukherji S, et al. (2014) Statistical optimization of thermal pretreatment conditions for enhanced biomethane production from defatted algal biomass. *Bioresource Technology* 162: 157–165.

Chen Y, Cheng JJ and Creamer KS (2008) Inhibition of anaerobic digestion process: A review. *Bioresource Technology* 99: 4044–4064.

Ehimen EA, Connaughton S, Sun Z, et al. (2009) Energy recovery from lipid extracted, transesterified and glycerol codigested microalgae biomass. *Global Change Biology Bioenergy* 1: 371–381.

Ehimen EA, Sun ZF, Carrington CG, et al. (2011) Anaerobic digestion of microalgae residues resulting from the biodiesel production process. *Applied Energy* 88: 3454–3463.

EPA (1996) Method 3540C. Soxhlet extraction. United States Environmental Protection Agency, SW-846 Manual. Washington, DC: Government Printing Office, , 1–8.

EPA (1999) Total organic carbon in water. Method 415.1. United States Environmental Protection Agency, SW-846 Manual. Washington, DC: Government Printing Office, 1–3.

Ferrer I, Ponsá S, Vázquez F, et al. (2008) Increasing biogas production by thermal (70ºC) sludge pre-treatment prior to thermophilic anaerobic digestion. *Biochemical Engineering Journal* 42: 186–192.

Folch J, Lees M and Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* 226: 497–509.

Fuentes-Grünewald C, Alacid E, Garcés E, et al. (2012). Biomass and lipid production of dinoflagellates and raphidophytes in indoor and outdoor photobioreactors. *Marine Biotechnology* 15: 37–47.

Fuentes-Grünewald C, Garcés E, Alacid E, et al. (2011). Improvement of lipid production in the marine strains *Alexandrium minutum* and *Heterosigma akashiwo* by utilizing abiotic parameters. *Journal of Industrial Microbiology and Biotechnology* 39: 207–216.

Fuentes-Grünewald C, Garcés E, Rossi S, et al. (2009). Use of the dinoflagellate *Karlodinium veneficum* as a sustainable source of biodiesel production. *Journal of Industrial Microbiology and Biotechnology* 36: 1215–1224.

Gonzáles-Fernández C, Sialve B, Bernet N, et al. (2012) Impact of microalgae characteristics on their conversión to biofuel. Part II: Focus on biomethane production. *Biofuels, Bioproducts and Biorefining* 6: 205–218.

Gouveia L and Oliveira AC (2009) Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology and Biotechnology* 36: 269–274.

Graham JM, Graham LE, Zulkifly SB, et al. (2012) Freshwater diatoms as a source of lipids for biofuels. *Journal of Industrial Microbiology and Biotechnology* 39: 419–428.

Guillard RL and Ryther JH (1962) Studies on marine planktonic diatoms I. *Cyclotella nana* Husted and *Detonula confervacea* (Cleve) Gran. *Canadian Journal of Microbiology* 8: 229–239.

Kaštánek F, Šabata S, Šolcová O, et al. (2010) In-field experimental verification of cultivation of microalgae *Chlorella* sp. using the flue gas from cogeneration unit as a source of carbón dioxide. *Waste Management & Research* 28: 961–966.

Kolesárová N, Hutnan M, Bodík I, et al. (2011) Utilization of biodiesel by-products for biogas production. *Journal of Biomedicine and Biotechnology.* Epub ahead of print 23 February 2011. DOI: 10.1155/2011/126798.

Park S and Li Y (2012) Evaluation of methane production and macronutrient degradation in the anaerobic co-digestion of algae biomass residue and lipid waste. *Bioresource Technology* 111: 42–48.

Ras M, Lardon L, Bruno S, et al. (2011) Experimental study on a coupled process of production and anaerobic digestion of *Chlorella vulgaris. Bioresource Technology* 102: 200–206.

Rawat I, Ranjith-Kumar R, Mutanda T, et al. (2013) Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. *Applied Energy* 103: 444–467.

Schneider M, Zimmer GF, Cremonese EB, et al. (2014) By-products from the biodiesel chain as a substrate to citric acid production by solid-state fermentation. *Waste Management & Research* 32: 653–660.

Sevigné-Itoiz E, Fuentes-Grünewald C, Gasol CM, et al. (2012) Energy balance and environmental impact analysis of marine microalgal biomass production for biodiesel generation in a photobioreactor pilot plant. *Biomass & Bioenergy* 39: 324–335.

Sialve B, Bernet N and Bernard O (2009) Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. *Biotechnology Advances* 27: 409–416.

Ward AJ, Lewis DM and Green FB (2014) Anaerobic digestion of algae biomass: A review. *Algal Research* 5: 204–214.

Yang Z, Guo R, Xu X, et al. (2011) Hydrogen and methane production from lipid extracted microalgal biomass residues. *International Journal of Hydrogen Energy* 36: 3465–3470.

Yen HW and Brune DE (2007) Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresource Technology* 98: 130–134.

Zamalloa C, Boon N and Verstraete W (2012) Anaerobic digestibility of *Scenedesmus obliquus* and *Phaeodactylum tricornotum* under mesophilic and thermophilic conditions. *Applied Energy* 92: 733–738.

Zhao B, Ma J, Zhao Q, et al. (2014) Efficient anaerobic digestion of whole microalgae and lipid-extracted microalgae residues for methane energy production. *Bioresource Technology* 161: 423–430.

Zhong W, Zhang Z, Lou Y, et al. (2012) Biogas productivity by codigesting *Taihu blue* algae with corn straw as an external carbon source. *Bioresource Technology* 114: 281–286.

Zhu L (2014) The combined production of ethanol and biogas from microalgal residuals to sustain microalgal biodiesel: A theoretical evaluation. *Biofuels, Bioproducts and Biorefining* 8: 7–15.

Zittelli GC, Rodolfi L, Biondi N, et al. (2006) Productivity and photosynthetic efficiency of outdoor cultures of Tetraselmis suecica in annular columns. *Aquaculture* 261(3): 932–943.