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Screening of biomethane production potential from dominant microalgae

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Abstract

The use of microalgae for biomethane production is considerably increasing during the recent years. In this study, four dominant species belonging to the genera *Scenedesmus, Chlorella, Dunaliella* and *Nostoc* were selected. The influence of different genera with several morphological, structural and physic-chemical characteristics on methane production was assessed in biochemical methane potential (BMP) tests. The ultimate methane yield values were 332±24, 211±2, 63±17 and 28±10 mL CH_4/g VS_{added} for *Scenedesmus obliquus, Chlorella sorokiniana, Dunaliella salina* and *Nostoc* sp., respectively. The highest methane production was achieved by microalga species that had no complex cell wall or wall basically composed by proteins and simple sugars such as in *S. obliquus*, while lower methane yields were found for *D. salina* and *Nostoc* sp., due to the salinity effects and cell wall composition in terms of complex polysaccharide and glycolipid layers, respectively. Kinetic constant values obtained in the BMP tests ranged between 1.00 ± 0.08 $days^{-1}$ and 0.097 ± 0.005 $days^{-1}$ for *D. salina* and *S. obliquus*, respectively.

Keywords: Microalga; specific strains; biomethane; anaerobic digestion.

Introduction

The energy demand keeps rising at a worrisome speed and the accessibility of easy fossil fuel reserves rapidly decrease which leads to increasing energy prices. The availability and affordability of energy is a critical element of economic wellbeing and, in many countries, also of industrial competitiveness. [1,2] Renewable energy sources – including biomass, geothermal, ocean, solar, and wind energy, as well as hydropower –have a huge potential to provide energy services for the world. Sustainable chemical products from second generation feedstocks can potentially provide environmental benefits as well. [3-5] Sunlight is by far the largest source of energy received by the Earth and the biological production in the water from the phytoplankton play a primary role in regulating the quality of the water resource. Some second generation feedstocks, such as algae, can be grown with saline or wastewater rather than utilizing freshwater resources. [6]

Anaerobic digestion can be applied to convert microalgae biomass to biogas [7-9] either using the total produced biomass or the residual fraction remaining after extraction of valuable products. [10] Anaerobic process not only recovers the energy stored in the biomass, but also leads to nitrogen and phosphorous release, which can in turn be source of nutrients for the microalgae culture. [7]

Anaerobic digestion of microalgae has also shown several constrains. Firstly, some microalgae have shown low biodegradability compared to other feedstocks. This is due to the cell walls of some microalgae species which are composed of complex carbohydrates hardly biodegradable. [11, 12] These cell walls act as a defence of the intracellular organic macromolecules from bacterial attack. Another obstacle to the anaerobic digestion of microalgae is its relatively high N content and low C/N ratio, due to its high protein fraction. Substrates of low C/N ratio are likely to produce excessive ammonia, which inhibits the growth of anaerobic microorganisms and consequently hindering or even stopping the digestion process. [13] In addition, high salinity levels, which can be usually found in

82 microalgae medium, are likely to be inhibitory as it can cause dehydration to bacteria cells
83 due to increased osmotic pressure. Salinity includes multiple elements such as sodium,
84 magnesium, calcium and aluminium and depends on the water source and its associated
85 environment. Specifically, the sodium ion is the most inhibitory of these metal cat-ions to
86 anaerobic digestion, being the light metal ion with the largest percentage found in seawater.
87 [13] Frigon et al. [14] found that the highest methane yield (410 mL CH_4/g VS) from a trail of
88 20 microalgae species was obtained with *Scenedesmus* sp.-AMDD, despite previous reports in
89 which *Scenedesmus* was supposed to be highly recalcitrant to digestion due to a tough
90 polysaccharide-based cell wall. [15] Frigon et al. [14] have also hypothesized that most probably
91 the specific inoculum used in their BMP assays had a stronger cytolytic activity than inocula
92 from other studies, allowing a higher methane production.
93 The aim of the present study was to evaluate the methane production and the kinetics of
94 methane generation for four dominant different microalgae species. These four species have
95 completely different structure and characteristics. The microalgae species selected for this
96 study are all dominant strains from natural habitats and show fast growth rates in nature and
97 lab conditions, or have specific characteristics and abilities, certainly among extremophiles.
98 Based on the foregoing, three eukaryotic microalgae belonging to green algae (*Scenedesmus*
99 *obliquus*, *Chlorella sorokiniana* and *Dunaliella salina*) were chosen for this research.
100 Chlorophyta division is considered the evolutionary line leading to the land plant and, like the
101 land plants, is able to store starch in their plastids and contains chlorophyll a and b. *D. salina*
102 is a halophilic species and its ability to grow at very high salt concentrations has made this
103 microalga an attractive candidate for the study. Finally, the prokaryotic cyanobacterium
104 *Nostoc* sp. (class Cyanophyceae) was also selected. Cyanobacteria are photosynthetic
105 prokaryotes with enormous environmental relevance, being responsible for a great percentage
106 of global N_2 and CO_2 fixation.

Material and methods

Microalgae growth

The microalgae species selected for this study were three eukaryotic microalgae belonging to green algae (*S. obliquus, C. sorokiniana* and *D. salina*) from the class Chlorophyceae and one as the prokaryotic cyanobacteria *Nostoc* sp. (class Cyanophyceae).

S. obliquus, C. sorokiniana and *D. salina* were provided as a lyophilised by Huelva University, Huelva (Spain). *Nostoc* sp. was grown in an AGP-700-ESP incubator chamber (Radiber S.A., Barcelona, Spain) with illumination provided by 6 fluorescent tubes delivering 36 W photosynthetically active radiation (PAR, 400-700 nm), on a photoperiod of 16:8 hr (light:dark). Ambient air and CO_2 were delivered by aquarium air pumps to provide the CO_2 for each flask as well as the required agitation to keep the microalgae in suspension. The temperature was 25°C. *Nostoc* sp. was cultivated using 50% of BG-11 medium, [16] and 50% of F/2 medium. [17] Then, the biomass was collected by centrifugation for 3,500 rpm during 5 min (Avanti J25, Beckman).

Biochemical methane potential (BMP) tests

BMP tests were performed in a multi-batch vessel system, which provides continuous agitation by magnetic bars, set at 300 rpm for this study. The effective volume of reactors was 150 mL. A thermostatic water bath kept the tests at mesophilic temperature (35±2 ºC). The inoculum used in the BMP test was taken from a full-scale anaerobic reactor treating waste activated sludge from a municipal wastewater treatment plant operating at mesophilic (35 ºC) conditions. The main characteristics of the anaerobic biomass used as inoculum were: pH: 7.5; total solids (TS): 20 g/L and volatile solids (VS): 10 g/L.

The substrate to inoculum ratio was 0.5 (VS basis). For each reactor containing 128 mL of inoculum, the substrate needed to give the required substrate to inoculum ratio was added. In order to avoid micronutrient deficiency, 130 μL of micronutrients solution was supplemented. The composition of the micronutrients solution was: $FeCl_2 \cdot 4H_2O$ 2000 mg/L, $CoCl_2 \cdot 6H_2O$ 2000 mg/L, EDTA 1000 mg/L, $NiCl_2 \cdot 6H_2O$ 50 mg/L, $MnCl_2 \cdot 4H_2O$ 500 mg/L, $Na_2SeO_3 \cdot 5H_2O$ 194 mg/L, $AlCl_3 \cdot 6H_2O$ 90 mg/L, H_3BO_3 50 mg/L, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 50 mg/L, $ZnCl_2$ 50 mg/L and $CuCl_2 \cdot 2H_2O$ 38 mg/L. Two control reactors were used with inoculum and micronutrients solution but without substrate addition.

The reactors were sealed and headspace flushed with N_2 at the beginning of the test. pH around 7.5 was measured prior and after each BMP test. The produced biogas was measured by liquid displacement after going through 2N NaOH solution to capture the produced CO_2; the remaining gas was expected to be only methane. The BMP tests lasted until the accumulated methane production was essentially unaffected, i.e. lower than 5% of the accumulated methane produced and c.a 30 days. Each experiment was carried out in duplicate.

Analyses

Standard methods 2540B and 2540E were followed in order to determined TS and VS, respectively; [18] COD was determined as described by Raposo et al. [19] pH was measured with a pH-meter model Crison 20 Basic. C and N were determined through an Elemental Analyser LECO CHNS-932.

Results and discussion

157 ***Microalgae structure and composition***

158 The green alga *Scenedesmus* contains a multi-layered cell wall that forms 4-celled colonies.
159 The sugar constituents of the rigid cell wall are glucose (major), galactose and mannose. [20]
160 The trilaminar structure of the outer wall layers is resistant to enzymes like cellulases,
161 hemicellulases, lysozyme and other hydrolases. Furthermore, the inner wall layers contribute
162 to the cell walls rigidity due to the glucosamine-containing biopolymers and glicoproteins
163 content. [20] *Scenedesmus* contains high proportion of proteins and lipids in its inner
164 composition. [21]

165 *Chlorella* is a genus of single-cell green algae with a diameter of 4-10 μm, belonging to the
166 phylum Chlorophyta. *Chlorella* appears to have a stable cell wall with a high hemicellulose
167 content. [22] *Chlorella* is morphologically very simple but is diverse in physic-chemical
168 characteristics. Its hemicellulotic cell wall accounts for the rigidity of the cells. [23] The cell
169 wall of *Chlorella* species could be divided into two groups: a glucose-mannose type and a
170 glucosamine type. *C. sorokiniana* can be classified into the second group. [6]

171 *Dunaliella* is a green unicellular microalga highly adaptable to a wide range of salt
172 concentrations, from 0.02% to almost salt saturation. [24] *Dunaliella* is a single cell organism
173 without a protective cell wall which has extremely effective mechanisms for tolerating
174 osmotic stress such as changes in phospholipid metabolism. [25] The chloroplast pigments in
175 *Dunaliella* have been reported to include xanthophylls, zeaxanthin, cryptoxanthins, β-carotene
176 and other carotenoids. [24]

177 *Nostoc*, a genus of cyanobacteria, is a group of photosynthetic prokaryotes that exists in
178 extensive diversity and distribution in the world. *Nostoc* is a genus of blue-green
179 cyanobacteria with cells organised in beadlike chains that are congregated in a gelatinous
180 mass. Their photosynthetic system is similar to eukaryotes because both have chlorophyll a
181 and photosystem II, and carry out oxygenic photosynthesis. [26] *Nostoc* sp. forms filamentous

colonies and is especially relevant their capacity of N_2 fixation. Nitrogen-fixation happens in the heterocyst where the enzyme nitrogenase transforms N_2 to NH_4^+ which is protected by a thick cell wall from oxygen-inactivation. This thick cell wall consists of distinct glycolipid and polysaccharide layers that limit gas diffusion into the cell. [27, 28] Heterocystous cyanobacteria are commonly observed in both aquatic and terrestrial habitats. Species of the genus *Nostoc* are among the most widespread of all nitrogen-fixing cyanobacteria and contain two pigments, phycocyanin and phycoerythrin.

In order to give an overview on the major constituents in microalga species, data of various micro-algal species are shown in Table 1. Proteins are usually in the range of 40-60%, lipids around 2-20% and carbohydrates around 10-35% (Table 1). This distribution shows that microalgae is usually a protein base substrate for anaerobic digestion.

Methane production

Figure 1 shows the evolution of the methane yield obtained (mL CH_4/g VS_{added}) against time (days) for the BMP assays carried out with the tested algal biomasses. As can be seen, the ultimate methane yield values were found to be 332±24, 211±2, 63±17 and 28±10 mL CH_4/g VS_{added} for *S. obliquus*, *C. sorokiniana*, *D. salina* and *Nostoc* sp., respectively. The highest values were found for *Scenedesmus* and *Chlorella*, whose percentages of VS in relation to the TS were much higher (93% and 95%, respectively) than those observed for *Dunaliella* and *Nostoc* (48% and 78%, respectively) (Table 2). It is worth to notice that the highest C/N ratio, found in *D. salina* (Table 2), resulted in a low methane yield. Apparently, no correlation between C/N ratio (Table 2) and methane yield was observed.

Table 3 shows values of biochemical methane potential of different species of microalgae belonging to different genera reported in the literature. All reported values corresponded to BMP experiments carried out without pretreatments and without lipid extraction.

In the present work, the highest methane yield (332 mL CH_4/g VS) was obtained for *S. obliquus*, value higher than that obtained by Mussgnug et al., [15] who reported a value of 278 mL CH_4/g VS. Mussgnug et al. [15] highlighted the role of the cell wall in the digestion process. Their results indicated that high gas production is connected to microalgae species that had either cell wall made from proteins and other simple compounds or no cell wall at all. *Scenedesmus* contain high proportions of proteins and simple sugars such as glucose, galactose and manose. [20] The composition of the biomass of *Scenedesmus* could explain the high methane production achieved in the present study. High methane yield (410 mL CH_4/g VS) was also reported by Frigon et al. [14] in the batch anaerobic digestion of *Scenedesmus* sp.-*AMDD.* Frigon et al. [14] pointed out that the strain of *Scenedesmus* sp.-*AMDD* is a promising model strain for continuous anaerobic digesters.

Gas production decreased for microalgal species that presented a carbohydrate-based cell wall containing hemicellulose. This is the case of *C. sorokiniana*, which has a stable cell wall with a high hemicellulose content providing a high rigidity to the cells, [22] for which a lower ultimate methane yield (211 ± 2 mL CH_4/g VS) was obtained. This methane yield is similar to that reported by Polakovicová et al. [32] (212 mL CH_4/g V_{added}) and somewhat lower than that reported by Frigon et al. [14] (283 mL CH_4/g VS_{added}).

It has been reported that some species of green microalgae such as *D. salina* are able to accumulate high quantities of lipids. [41] Lipids are attractive for anaerobic digestion due to a higher theoretical methane potential compared to proteins and carbohydrates. However, low methane production (63 ± 17 mL CH_4/g VS) was obtained for *D. salina* in this study, which can be attributed to the effects of salinity. [14] Salinity and more specifically sodium monovalent cations do pose a problem to bacteria associated with anaerobic digestion. [13] Rinzema et al. [42] demonstrated that acetoclastic methanogens were inhibited by 10%, 50% and 100% with sodium concentration of 5, 10 and 14 g/L, respectively. In the present work,

mineral fraction of *D. salina* slightly higher than 13 g/L were measured at the beginning of the BMP test, which, in addition to the low ratio g COD/g VS found for this microalgae (0.68±0.01 g COD/g VS) could have caused its low methane yield.

Finally, *Nostoc* sp. gave the lowest methane yield, which can be attributed to its rigid and complex cell wall. This cell wall has been reported to include several polysaccharide and glycolipid layers. [43] *Nostoc* contains two pigments, red phycoerythrin and blue phycocyanin. These compounds usually combined to form clusters that stick to the cell membrane, [44] which may be an additional cause of the low methane yield obtained for *Nostoc* sp.

Kinetics of methane production

First-order exponential model (equation (1)) is commonly used to correlate methane production from biodegradable substrates in batch anaerobic digestion processes with time: [45]

$$B = B_{max} \cdot [1 - \exp(-k \cdot t)] \qquad (1)$$

where: B (mL CH_4/g VS_{added}) is the cumulative specific methane production, B_{max} (mL CH_4/g VS_{added}) is the ultimate methane production, k is the specific rate constant ($days^{-1}$) and t (days) is the time.

This first-order model was applied for all the microalgae tested. Sigmaplot software (version 11.0) was used to calculate parameters k and B_{max} for these BMP assays (Table 4) by non-linear regression adjustment of the pairs of experimental data (B, t). The low values of the standard error of estimate (S.E.E.) and high values of the R^2 demonstrate the goodness of the fit of experimental data to the first-order exponential model. Table 4 shows the specific rate constants (k) and ultimate methane yield obtained for the four microalgae tested.

The lowest kinetic constant was found for the species *S. obliquus*, 0.097±0.005 $days^{-1}$, which is practically coincident with that obtained by Ramos-Suarez and Carreras [11] (0.0902 ±

257 0.0025 days^{-1}) in BMP tests of *Scenedesmus* biomass. Although the kinetic constant of the
258 BMP of *S. obliquus* was very low, it gave the highest methane yield in the present study.
259 After the intracellular material become available to the anaerobic microorganisms the
260 methane production resulted in high values, even with the low degradation kinetics measured
261 in the BMP test. It has been reported that the kinetics of anaerobic degradation of
262 *Scenedesmus* can be improved after extraction of amino acids and lipids. [11] Kinetic
263 enhancements were attributed to the disruption of microalgae cell walls and increase in
264 organic matter solubilisation. [11] Specifically, the amino acid extraction process improved the
265 digestion in a higher extent compared to the lipid extraction because of its higher hydrolytic
266 effect. [11]
267 The ultimate methane yield of *C. sorokiniana* was achieved after 10 days of digestion with a
268 kinetic constant of 0.48±0.03 days^{-1}. Some authors have pointed out the slow degradation of
269 *Chlorella* genre which has cell wall composed of some complex carbohydrates that partially
270 impede the action of the microorganisms responsible for the anaerobic degradation. [15] In any
271 case, the kinetic constant obtained in the present work was higher than those reported by
272 Mendez et al. [46] in batch anaerobic digestion tests of thermochemically pretreated *Chlorella*
273 *vulgaris* (0.08-0.14 days^{-1}).
274 Although *D. salina* showed a low ultimate methane yield, the kinetic constant obtained was
275 quite high (1.00 ± 0.08 days^{-1}). As it is well known the use of salt-containing organic residues
276 entails the presence of salts which inhibits methanogenesis [13, 47] as shown by the low
277 methane yield obtained. However, it has not severely affected the degradation rate.
278 Although the lowest value of the methane yield was obtained for *Nostoc*, the quick
279 degradation of the most biodegradable compounds present in this cyanobacteria is the main
280 responsible for the high kinetic constant (0.94±0.23 days^{-1}) obtained in its BMP test.
281

Conclusions

A screening of the biomethane production potential from different microalgae genera was performed in this study in order to compare the most promising strains with different characteristics to produce maximum methane yields. Maximum methane yields were obtained with the strains *S. obliquus* and *C. sorokiniana*, which do not contain a very complex cell wall. In contrast, *D. salina* and *Nostoc* sp. gave the lowest methane yield values, although the degradation kinetics were faster in these cases due to the fast degradation of the most biodegradable compounds present in this microalgae and cyanobacteria, respectively.

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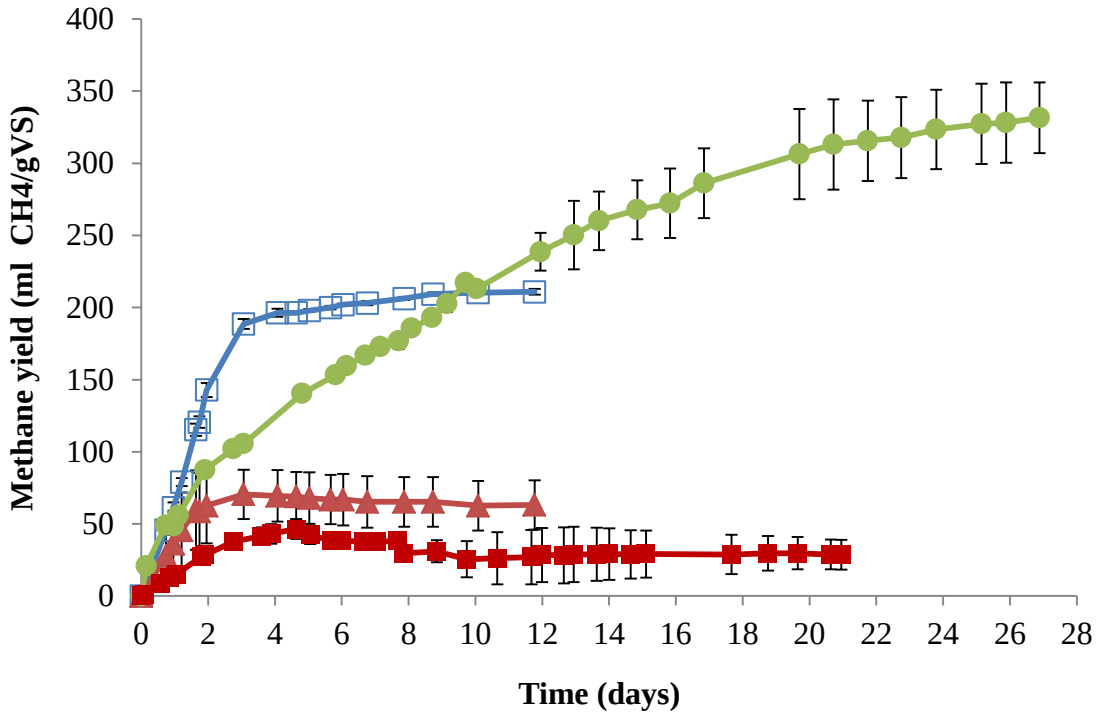
FIGURE CAPTIONS

Figure 1. Biochemical methane potential (BMP) (mL CH_4/g VS_{added}) of biomasses of *Scenedesmus obliquus* (●), *Chlorella sorokiniana* (□), *Dunaliella salina* (▲) and *Nostoc sp.* (■). Vertical bars represent standard deviations.


Methane yield (ml CH4/gVS)
400
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
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Time (days)

Fig. 1

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477 **Table 1**. Composition of several microalgae genera. [29-31]

Genus	Proteins (%)	Lipids (%)	Carbohydrates (%)
Chlorella	51-58	2-22	12-26
Dunaliella	57	6	32
Scenedesmus	50-56	12-14	10-17
Nostoc	37-47	8-13	15-37

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Table 2. VS, VS/TN and C/N ratios of the tested algal biomasses.

Species	g VS/kg	% VS/TS	C/N
Scenedesmus obliquus	252±1	93	4.78±0.03
Chlorella sorokiniana	898±2	95	5.2 ±0.4
Dunaliella salina	435±4	48	12.6 ± 0.3
Nostoc sp.	5.4±0.8	78	4.79 ± 0.09

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504 **Table 3.** Values of biochemical methane potential of different strains of microalgae belonging
505 to different genera reported in the literature. All reported values corresponded to BMP
506 experiments carried out without pretreatments and without lipid extraction.

Phylum	**Genus**	**Species**	**BMP (ml CH_4/g VS)**	**Ref.**
Chlorophyta	*Chlorella*	*Chlorella kessleri*	218	[15]
		Chlorella sorokiniana	212	[32]
		Chlorella vulgaris	361	[14]
		Chlorella sorokiniana	283	[14]
		Chlorella sp. Island-R	302	[14]
		Chlorella vulgaris-FGP1	263	[14]
		Chlorella sorokiniana-RBD8	331	[14]
		Chlorella sp.-RB1a	309	[14]
		Chlorella vulgaris	286	[33]
		Chlorella vulgaris	240	[34]
	Chlamydomonas	*Chlamydomonas debaryana*-AMB1	302	[14]
		Chlamydomonas sp.-AMLS1b	333	[14]
		Chlamydomonas reinhardtii	387	[15]
	Scenedesmus	*Scenedesmus* sp.-PN2	258	[14]
		Scenedesmus obliquus	178	[15]
		Scenedesmus sp.-AMDD Nov-2010	306	[14]
		Scenedesmus dimorphus	397	[14]
	Botryococcus	*Botryococcus braunii*	343	[14]
		Botryococcus braunii	326	[35]
	Dunaliella	*Dunaliella salina*	323	[15]
		Dunaliella salina	63	[36]
		Dunaliella tertiolecta	24	[33]
Ochrophyta	*Nannochloropsis*	*Nannochloropsis oculata*	204	[37]
		Nannochloropsis gaditana	228	[14]
Cyanobacteria	*Spirulina*	*Spirulina maxima*	353	[38]
		Spirulina maxima	330	[39]
	Arthrospira	*Arthrospira maxima*	173	[40]
		Arthrospira platensis	293	[15]

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513 **Table 4.** Kinetic parameters obtained from the exponential model in the BMP tests of the different

514 microalgae assessed.

Substrate	B_{max} (mL CH_4/g VS_{added})	k ($days^{-1}$)	R^2	Standard Error of Estimate
Chlorella sorokiniana	215 ± 5	0.48 ± 0.03	0.977	10.597
Dunaliella salina	67 ± 1	1.00 ± 0.08	0.959	3.999
Scenedesmus obliquus	356 ± 8	0.097 ± 0.005	0.989	10.596
Nostoc sp.	33 ± 1	0.94 ± 0.23	0.705	5.960

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