# Optimization of biogas production from *Sargassum* sp. using a design of experiments to assess the co-digestion with glycerol and waste frying oil

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J.V. Oliveira, M.M. Alves *, J.C. Costa

*CEB-Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal*

## HIGHLIGHTS

- *Sargassum* sp. was co-digested with glycerol (Gly) and waste frying oil (WFO).
- Co-digestion was optimized through a design of experiments methodology.
- Gly and WFO increased 56% and 46% the BMP of *Sargassum* sp. (188 L $CH_4$ $kg^{-1}$ COD).
- The methane production rate increased 38% and 19% with Gly and WFO, respectively.
- 1.31% TS of *Sargassum* sp. with 0.88 $g_{Gly}$ $L^{-1}$ gave the best co-digestion performance.

## ARTICLE INFO

## ABSTRACT

A design of experiments was adopted to assess the optimal conditions for methane production from the macroalgae *Sargassum* sp. co-digested with glycerol (Gly) and waste frying oil (WFO). Three variables were tested: % total solids of algae (%$TS_{Sargassum\ sp.}$), co-substrate concentration ($g_{Gly/WFO}$ $L^{-1}$), and co-substrate type (Gly or WFO). The biochemical methane potential (BMP) of *Sargassum* sp. was 181 ± 1 L $CH_4$ $kg^{-1}$ COD. The co-digestion with Gly and WFO increased the BMP by 56% and 46%, respectively. The methane production rate ($k$), showed similar behaviour as the BMP, increasing 38% and 19% with Gly and WFO, respectively. The higher BMP (283 ± 18 L $CH_4$ $kg^{-1}$ COD) and $k$ (65.9 ± 2.1 L $CH_4$ $kg^{-1}$ COD $d^{-1}$) was obtained in the assay with 0.5% TS and 3.0 $g_{Gly}$ $L^{-1}$. Co-digestion with glycerol or WFO is a promising process to enhance the BMP from the macroalgae *Sargassum* sp.

## 1. Introduction

In the present scenario of limited fossil reserves, the increasing demand for food, animal feed, chemicals, materials and energy is closely associated to the use of arable land. Alternative renewable energy sources, alternative sources of biomass, sustainable processes and products that optimize the arable land area use, valorize the by-products and wastes, and minimize the corresponding environmental impact are of huge importance for the world society in the coming years. After the sudden interest in the cultivation of highly productive algae due to the oil crisis in the 1970's (van Hal et al., 2014), we are currently witnessing a rediscovery of algae potential. Algae can be used to produce bioenergy, namely biodie-sel, bioethanol and biogas. This source of biomass has several advantages over terrestrial crops since it does not compete with land use and water consumption necessary for food crops production. Their fast growth rates and high yields make them even more attractive (Borjesson and Mattiasson, 2008). Macroalgae have also great potential as energy crops for anaerobic digestion because they contain easily hydrolysable sugars and low lignin content.

*Sargassum* sp. is a brown macroalgae widely distributed in tropical and subtropical seas, and one of the most abundant seaweed in the Portuguese coast. The biochemical methane potential (BMP) of *Sargassum* sp. ranged between 119 and 380 L of methane ($CH_4$) per kg of volatile solids (VS) (Bird et al., 1990; Chynoweth, 2005; Gunaseelan, 1997). Using *Sargassum* sp. as substrate in anaerobic digestion processes not only gives a solution for their disposal, but also provides a renewable source of energy. However, some problems have been reported in anaerobic digestion of seaweeds. Recalcitrant materials, like polyphenols, cellulosic fibers and lignin

* Corresponding author at: Universidade do Minho, Departamento de Engenharia Biológica, Campus de Gualtar, 4710-057 Braga, Portugal. Tel.: +351 253 604 417; fax: +351 253 604 429.

*E-mail address:* madalena.alves@deb.uminho.pt (M.M. Alves).

type components, difficult their biodegradability (Ward et al., 2014).

Co-digestion can enhance the anaerobic biodegradability of two or more complementary substrates due to synergetic effects. *Sargassum* sp. has high content in protein, therefore its co-digestion with substrates with high C/N content, may be a promising alternative to increase the methane yield of *Sargassum* sp.

By-products and wastes from the biodiesel production, namely crude glycerol, biodiesel processing wastewaters and crop waste after oil extraction (cake), still contain high energy potential (van Hal et al., 2014). The use of crude glycerol as co-substrate, has proven to increase the methane yields and rates of several substrates (Costa et al., 2012b; Costa et al., 2013; Oliveira et al., 2014). For instance, the addition of 2% of crude glycerol increased the biogas production from the macroalgae *Gracilaria vermiculophylla* by 18%. However, its addition may be inhibitory at higher concentrations (Oliveira et al., 2014).

The addition of fat, oil, and grease (FOG) to a municipal sludge anaerobic digester increased the BMP 257%, reaching $418 \pm 14$ L $CH_4$ $kg^{-1}$ VS (Li et al., 2011).Neves et al. (2009a) used oily waste, from a canned fish processing, to apply pulses of oil in an anaerobic reactor fed with cow manure and food waste. A 9 g $COD_{oil}$ $L_{reactor}^{-1}$ pulse reached almost 100% of biomethanation.

The efficiency of a co-digestion process depends on several variables, improving the balance of the mixture of co-substrates, including the C:N ratio, pH, macro and micronutrients, inhibitory or toxic compounds and dry matter content (Mata-Alvarez et al., 2000). Usually the effects are studied independently and possible interactions are not properly considered. A statistical analysis, using a design of experiments (DOE), is an efficient way to optimize the factors that are interrelated, for instance optimizing the mixture ratio of two or more substrates.

This work aimed at study the anaerobic co-digestion of *Sargassum* sp. with crude glycerol (Gly) and waste frying oil (WFO). The effects on the BMP and methane production rate ($k$) of three operating conditions (*Sargassum* sp. and co-substrate concentrations, and type of co-substrate) were investigated. A DOE was adopted to determine in a systematic way the statistical significance of each parameter and to evaluate the possible interactions.

## 2. Methods

### 2.1. Inoculum and substrate

Anaerobic granular sludge from a brewery industry was used as inoculum in the biodegradability assays. The sludge samples contained $0.081 \pm 0.001$ g VS $g^{-1}$ inoculum. The specific methanogenic activity (SMA) in the presence of acetate (30 mM) was $136 \pm 17$ mL $CH_{4@STP}$ $g^{-1}$ VS $d^{-1}$, and in the presence of $H_2/CO_2$ (80/20 v/v, 1 atm) was $592 \pm 65$ mL $CH_{4@STP}$ $g^{-1}$ VS $d^{-1}$. SMA was determined according to described inCosta et al. (2012b).

*Sargassum* sp. was collected in the Portuguese coast (Póvoa de Varzim), dried at 37 °C and milled to less than 1 mm. Crude glycerol was obtained from a biodiesel producing industry (from vegetable oils) located near Lisbon. Prior use it was stored at 4 °C. WFO was collected from a kitchen restaurant located in Braga (Portugal). Gly and WFO were used as co-substrates in the anaerobic biodegradability assays.

### 2.2. Anaerobic biodegradability assays

Anaerobic biodegradability batch assays were used to determine the BMP and $k$ from *Sargassum* sp. co-digested with Gly or WFO, following a response surface methodology DOE.

### 2.2.1. Factorial experimental design

A factorial experimental design was used to define the experiments matrix. The effect of two numeric factors, concentration of *Sargassum* sp. ($X_1$) and concentration of co-substrate ($X_2$), and one categorical factor, co-substrate type ($X_3$) (100% of Gly or 100% of WFO), were studied on two response variables, BMP ($Y_1$) and $k$ ($Y_2$), using a response surface methodology. The levels used in the anaerobic biodegradability assays are shown in Table 1. The factorial design consist in a full factorial experimental design with 18 runs (Eq. (1)):

$$\text{Runs number} = n[N_f + N_\alpha + N_c] \tag{1}$$

where $N_f = 2p$ is the number of factorial points, $N_\alpha = 2^p$ is the number of axial points, $N_c$ is the central point, $p$ is the number of numerical factors, and $n$ is the number of levels of the categorical factor.

The experiments were randomly performed. The software package Design-Expert® (Stat-Ease, Inc., Minneapolis, USA) was used to determine the experiments design matrix and its statistical analysis. BMP and $k$ data were processed for Eq. (2), including the analysis of variance to obtain the interaction between the process variables and the responses. The $p$-values of the parameters estimation were used to validate the model, where $p$-value $\leqslant 0.05$ indicated significant model terms.

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \beta_{ijk} X_i X_j X_k \tag{2}$$

where $Y_i$ indicates the predicted response variable; $\beta_0$ is the constant coefficient; $\beta_i$ is the coefficient of the $X_i$; $\beta_{ij}$ and $\beta_{ijk}$ are the interaction coefficients; and $X_i, X_j, X_k$ are the independent variables.

### 2.2.2. Experimental procedure

The anaerobic biodegradability assays were performed according to the guidelines defined in Angelidaki et al. (2009), with a work volume of 50 mL and 50% (v/v) of inoculum, at 37 °C. All the assays were performed in duplicate, except the central point of factorial design and the blanks (without substrate), which were performed in triplicate. The blank was used to discount for the residual substrate present in the inoculum.

The methane accumulated in the headspace of the closed vessel was measured by gas chromatography (GC) using a gas tight syringe to sample 500 μL. Methane production was corrected for standard temperature and pressure (STP) conditions (0 °C and 1 atm). BMP was defined by the cumulative volume of methane produced per unit of COD of substrate added to the assay (Eq. (3)):

$$\text{BMP} = \frac{\text{kg COD} - CH_4 \times 350 \text{ L } CH_4 \text{ kg}^{-1} \text{ COD}}{\text{kg COD}_{added}} \tag{3}$$

where kg COD – $CH_4$ is the cumulative methane produced during the anaerobic biodegradability assay, 350 L $CH_4$ $kg^{-1}$ COD is the theoretical methane production per mass of COD and kg $COD_{added}$ is the total COD added from the substrate in each vial. The maximum initial methane production rate ($k$) is obtained by the highest slope of the linear initial region of the cumulative methane production curve divided by the initial substrate COD, being expressed as L $CH_4$ $kg^{-1}$ COD $d^{-1}$.

**Table 1**
Levels of factors selected for the response surface methodology.

| Factors | Substrate | Units | Real values of coded levels | | | | |
|---------|-----------|-------|:---:|:---:|:---:|:---:|:---:|
| | | | $-\alpha$ | $-1$ | $0$ | $+1$ | $+\alpha$ |
| $X_1$ | *Sargassum* sp. | %TS (m/v) | 0.50 | 1.31 | 3.25 | 5.19 | 6.00 |
| $X_2$ | Gly or WFO | g $L^{-1}$ | 0.00 | 0.88 | 3.00 | 5.12 | 6.00 |

The biodegradability assays lasted approximately 42 days. At the end, pH, sCOD, ammonium (N-NH$_4^+$), volatile fatty acids (VFA) and long chain fatty acids (LCFA) were measured for each bottle.

## 2.3. Analytical methods

Ammonium (N-NH$_4^+$) was determined using the Nessler method, and total Kjeldahl nitrogen (TKN), total solids (TS), VS and pH were measured according to standard methods (APHA, 1998). Free ammonia (N-NH$_3$) was calculated based on total ammonium concentration and pH (Eq. (4)):

$$[N - NH_3] = \frac{[N - NH_4^+] \times 10^{pH}}{\exp\left(\frac{6344}{273+37}\right) + 10^{pH}} \tag{4}$$

The concentration of ammonia [N-NH$_3$] and ammonium [N-NH$_4^+$] are expressed in mg L$^{-1}$ (Handbook of Chemistry and Physics, 1989-1990). Total and soluble COD (tCOD and sCOD, respectively) were determined using standard kits (Hach Lange, Düsseldorf, Germany). Lipid content was extracted with chloroform and methanol, based in Bligh and Dyer (1959) method. The samples (0.4 g) were mixed with water (1.6 mL), chloroform (6 mL) and methanol (2 mL), and then filtrated (0.45 μm) and transferred to a separatory funnel. The lower phase (chloroform and lipids) was dropped into a beaker, previously weighted, allowed to evaporate and weighted. The amount of lipids was calculated with the difference between weights. Protein content was determined based on the TKN measurement using the correction factor 6.25 (Lourenço et al., 2002). Lignin, glucan, xylan and acetate quantifications were done as described in Sluiter et al. (2011). VFA were determined by a Jasco HPLC (Tokyo, Japan), using an Agilent Hi-Plex H column (300 × 7.7 mm), maintained at 80 °C and with UV/VIS detection at 210 nm. The mobile phase was sulfuric acid (5 mM) fed at a rate of 0.9 mL min$^{-1}$. Crotonic acid was used as internal standard. LCFA (lauric C12:0, myristic C14:0, palmitic C16:0, palmitoleic C16:1, stearic C18:0, oleic C18:1 and linoleic C18:2 acids) analysis was done as described in Neves et al. (2009b). The LCFA were analyzed in the liquid and solid matrix since they were adsorbed/accumulated onto the solid matrix. Free fatty acids present in the samples were esterified with HCl:1-propanol and extracted with dichloromethane. Quantification was done with a GC equipped with a FID. LCFA were separated using a Teknokroma TRB-WAX column (30 m × 0.25 mm × 0.25 μm) with helium as the carrier gas, fed at a rate of 1 mL min$^{-1}$. Temperatures of the injector and detector were 220 and 250 °C, respectively. The initial oven temperature was 50 °C, maintained for 2 min, followed by a 10 °C min$^{-1}$ ramp up to 225 °C and finally isothermal conditions were maintained for 10 min. The methane content of biogas was analyzed with GC (Chrompack 9000), as described in Costa et al. (2012a). The GC was equipped with a FID and Carbowax 20 M (80–120 mesh) (2 m × 2 mm) column. Nitrogen was used as carrier gas (30 mL min$^{-1}$). The detector, injector, and oven temperatures were 35, 110, and 220 °C, respectively.

## 3. Results and discussion

### 3.1. Substrates characterisation

The wastes characterisation is shown in Table 2. Seaweeds were collected in their natural environment, where they were drying at ambient temperature. They contain several impurities, which could influence the anaerobic digestion process. The low value of VS and high concentration of nitrogen, like those found in literature 0.9–2.0% (dry basis) (Bird et al., 1990), may limit their

**Table 2**
Characterisation of *Sargassum* sp., glycerol and waste frying oil (WFO) used in the anaerobic biodegradability assays.

| Parameter | | *Sargassum* sp. | Glycerol | WFO |
|---|---|---|---|---|
| TS | % | 89.5 ± 0.3 | 67.9 ± 1.0 | 100 |
| VS | % TS | 53.8 ± 0.8 | 93.8 ± 0.1 | 100 |
| tCOD | g g$_{substrate}^{-1}$ | 0.60 ± 0.06 | 1.60 ± 0.01 | 2.55 ± 0.29 |
| sCOD | g g$_{substrate}^{-1}$ | 0.015 ± 0.001 | 1.60 ± 0.01[a] | 2.55 ± 0.29[a] |
| TKN | % VS | 3.87 ± 0.08 | nd | nd |
| Protein | % VS | 23.6 ± 0.5 | nd | nd |
| Lipid | % VS | 2.73 ± 0.05 | 49.3 ± 15.0 | 98.2 ± 0.7 |
| Lignin | % VS | 4.6 ± 0.9 | nd | nd |
| Xylan | % VS | 11.7 ± 1.3 | nd | nd |
| Glucan | % VS | 32.9 ± 2.6 | nd | nd |
| LCFA | % VS | nd | 24.5 ± 1.2 | 6.19 ± 1.38 |

nd – not detected.
[a] The sCOD was similar to tCOD, so it was considered the average of all values determined (tCOD and sCOD).

biodegradability. Bird et al. (1990) refer to *Sargassum* sp. as a poor feedstock to methane production. The co-digestion with glycerol and WFO can be a good alternative to bring the C:N ratio near to the optimum ratio for anaerobic digestion (around 20–30:1), since both co-substrates have high concentration of soluble COD and negligible content in nitrogen. However, the low lignin content and the high carbohydrates concentration makes it a good candidate to anaerobic valorisation through the production of biogas.

The sample of crude glycerol had 25% VS of LCFA. The total amount of LCFA is composed by linoleic acid (54% w/w), oleic acid (31% w/w), palmitic acid (11% w/w), and stearic acid (4% w/w). On the other hand, the sample of WFO only had 6% VS of LCFA, consisting in linoleic acid (62% w/w), oleic acid (27% w/w), palmitic acid (8% w/w), and stearic acid (4% w/w). Theoretically, 1 g of oleic acid (C18:1) can produced 1.01 L of methane at standard temperature and pressure (STP), whereas 1 g of glucose only produced 0.37 L. The high content in lipids (49% and 98% VS for Gly and WFO, respectively) make the co-substrates optimal for methane production, regarding the theoretical biogas potential of lipids, compared with carbohydrates and proteins.

### 3.2. Anaerobic biodegradability assay

The experimental design matrix and the results obtained are presented in Table 3. The BMP of *Sargassum* sp. (without co-substrate) was 181 ± 1 L CH$_4$ kg$^{-1}$ COD, corresponding to around 52% of the theoretical maximum methane production. In the co-digestion assays, the BMP varied significantly from 157 to 283 L CH$_4$ kg$^{-1}$ COD with glycerol as co-substrate and from 172 to 265 L CH$_4$ kg$^{-1}$ COD with WFO. These results suggest that the two parameters (concentrations of *Sargassum* sp. and co-substrate) had significant effects on the efficiency of the anaerobic digestion process.

An inhibitory effect was observed with higher concentration of *Sargassum* sp. with Gly (assays 8 and 9, complementary to 17 and18, respectively), possibly due to accumulation of VFA, although the pH was in the range 7.0–7.4 (Table 3).

The concentration of ammonia did not reach inhibitory values, i.e. >0.1 g NH$_3$-N L$^{-1}$ (Oliveira et al., 2014). Regarding the LCFA analysis, no significant accumulation was observed. Pereira et al. (2004) suggested that a specific content higher than 1 g COD-LCFA g$^{-1}$ VS should not be overcome in order to guarantee a well-balanced microbial activity. The assay 15 (Table 3) had the highest concentration of LCFA (210 ± 99 mg LCFA L$^{-1}$) in the end of the biodegradability test, corresponding to a specific content of 15 mg COD-LCFA g$^{-1}$ VS. Palmitic acid (C16:0) was the main constituent (>50%) of the LCFA detected at the end of the assays.

**Table 3**
Design matrix of the factorial experimental design and the observed response variables (BMP and $k$).

| Assay | $X_1$ [S] %TS | $X_2$ [CS] g L$^{-1}$ | $X_3$ CS type | $Y_1$ BMP L CH$_4$ kg$^{-1}$ COD | $Y_2$ $k$ L CH$_4$ kg$^{-1}$ COD d$^{-1}$ | pH | sCOD g L$^{-1}$ | NH$_3$-N mg L$^{-1}$ | VFA g L$^{-1}$ | LCFA mg L$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | $-\alpha$ | 0 | Gly | 283 ± 18 | 65.9 ± 2.1 | 7.24 ± 0.03 | 0.56 ± 0.08 | 25 ± 6 | 0.19 ± 0.03 | nd |
| 2 | $-1$ | $-1$ | Gly | 216 ± 27 | 58.2 ± 2.3 | 7.29 ± 0.05 | 0.85 ± 0.14 | 30 ± 1 | 0.25 ± 0.06 | nd |
| 3 | $-1$ | $+1$ | Gly | 235 ± 3 | 47.9 ± 1.9 | 7.22 ± 0.01 | 1.20 ± 0.03 | 29 ± 0 | nd | 17 ± 17 |
| 4 | 0 | $-\alpha$ | Gly | 181 ± 1 | 47.7 ± 2.5 | 7.26 ± 0.06 | 1.78 ± 0.07 | 32 ± 5 | 0.23 ± 0.01 | nd |
| 5 | 0 | 0 | Gly | 188 ± 3 | 38.9 ± 1.2 | 7.30 ± 0.01 | 3.92 ± 0.11 | 54 ± 10 | nd | 62 ± 39 |
| 6 | 0 | $-\alpha$ | Gly | 172 ± 2 | 31.4 ± 0.2 | 7.24 ± 0.00 | 4.70 ± 0.17 | 43 ± 1 | 0.15 ± 0.15 | 115 ± 49 |
| 7 | $+1$ | $-1$ | Gly | 157 ± 2 | 35.3 ± 1.1 | 7.29 ± 0.01 | 6.25 ± 0.05 | 57 ± 4 | 0.58 ± 0.01 | 114 ± 42 |
| 8 | $+1$ | $+1$ | Gly | 170 ± 11 | 31.7 ± 2.9 | 7.24 ± 0.03 | 9.73 ± 0.74 | 46 ± 1 | 2.43 ± 1.38 | 171 ± 13 |
| 9 | $+\alpha$ | 0 | Gly | 172 ± 3 | 26.4 ± 3.9 | 7.29 ± 0.01 | 9.21 ± 0.28 | 60 ± 1 | 2.02 ± 0.07 | 168 ± 16 |
| 10 | $-\alpha$ | 0 | WFO | 213 ± 0 | 33.0 ± 0.2 | 7.15 ± 0.01 | 0.66 ± 0.29 | 16 ± 1 | 0.24 ± 0.00 | nd |
| 11 | $-1$ | $-1$ | WFO | 265 ± 25 | 56.7 ± 2.0 | 7.13 ± 0.02 | 0.81 ± 0.15 | 18 ± 0 | 0.28 ± 0.03 | nd |
| 12 | $-1$ | $+1$ | WFO | 196 ± 5 | 29.5 ± 1.6 | 7.05 ± 0.01 | 0.73 ± 0.00 | 14 ± 0 | 0.19 ± 0.01 | nd |
| 13 | 0 | $-\alpha$ | WFO | 181 ± 1 | 47.7 ± 2.5 | 7.26 ± 0.04 | 1.78 ± 0.07 | 32 ± 5 | 0.23 ± 0.02 | nd |
| 14 | 0 | 0 | WFO | 172 ± 14 | 35.5 ± 3.4 | 7.28 ± 0.08 | 2.05 ± 0.06 | 38 ± 7 | 0.17 ± 0.06 | 45 ± 4 |
| 15 | 0 | $+\alpha$ | WFO | 180 ± 3 | 28.7 ± 2.1 | 7.15 ± 0.02 | 1.98 ± 0.15 | 27 ± 3 | 0.14 ± 0.04 | 210 ± 99 |
| 16 | $+1$ | $-1$ | WFO | 173 ± 0 | 36.0 ± 1.0 | 7.27 ± 0.01 | 5.94 ± 0.05 | 56 ± 3 | 0.14 ± 0.01 | 32 ± 1 |
| 17 | $+1$ | $+1$ | WFO | 204 ± 1 | 30.0 ± 0.4 | 7.28 ± 0.01 | 6.19 ± 0.21 | 52 ± 5 | 0.28 ± 0.06 | 173 ± 68 |
| 18 | $\alpha$ | 0 | WFO | 189 ± 3 | 30.6 ± 3.0 | 7.32 ± 0.02 | 8.69 ± 0.93 | 71 ± 1 | 0.89 ± 0.54 | 104 ± 35 |

nd – not detected.

Therefore, no inhibitory thresholds were achieved. The accumulation of sCOD suggests inhibition of the methanogenesis step. One possible justification for the inhibition of methane production in the assays with higher concentration of *Sargassum* sp. was described by Bird et al. (1990). The authors identified a high percentage (>30%) of an acid and alkaline insoluble component, considered herein as fibre, in the VS of this macroalgae. Although a low content of lignin was determined, there are several types of recalcitrant material present in the macroalgae composition which reduce their biodegradability potential (Bird et al., 1990).

Regarding the methane production rate ($k$), it was observed a variation between 26.4–65.9 L CH$_4$ kg$^{-1}$ COD d$^{-1}$ for glycerol and 28.7–56.7 L CH$_4$ kg$^{-1}$ COD d$^{-1}$ for WFO (Table 3). The biodegradability rate of *Sargassum* sp. without co-substrate was 47.7 ± 2.5 L CH$_4$ kg$^{-1}$ COD d$^{-1}$. As in the BMP, the concentration of *Sargassum* sp. and co-substrate had significant influence in the methane production rate. BMP and $k$ showed a similar behaviour (Table 3).

### 3.3. Statistical analysis

The effect of independent variables i.e., concentration of *Sargassum* sp. ($X_1$) and co-substrate ($X_2$), and co-substrate type ($X_3$) on methane production, in terms of BMP ($Y_1$) and methane production rate ($Y_2$), were investigated by a statistical analysis, based on a factorial experimental design. Response surface methodology is a collection of mathematical and statistical techniques useful for designing experiments, building models, evaluating relative significance between the independent and response variables and their combinations, accessing the optimum conditions for desirable methane production (Gilmour, 2006).

Two different models were suggested for the response variables $Y_1$ (BMP) and $Y_2$ ($k$). The model with lower standard error for regression was selected. To significantly represent the BMP prediction a quadratic response surface model was suggested and used. A $p$-value <0.05 indicates that the model is significant. The quadratic model shows a $p$-value of <0.0001, with a determination coefficient ($R^2$) of 0.98. For the prediction of methane production rate was recommended a response surface 2FI (2-factor interaction) model, with a $p$-value of <0.0001. The quadratic effects were not considered significant in this case.

Table 4 shows the significance of the selected models for each response variable, as well as for all independent variables and their

**Table 4**
$p$- value of fitting model for BMP and $k$.

| Source | Prob > F | |
|---|---|---|
| | $Y_1$ | $Y_2$ |
| Model | <0.0001 | <0.0001 |
| $X_1$ | <0.0001 | <0.0001 |
| $X_2$ | 0.6167 | <0.0001 |
| $X_3$ | 0.0458 | 0.0004 |
| $X_1 X_2$ | 0.0014 | 0.0055 |
| $X_1 X_3$ | <0.0001 | <0.0001 |
| $X_2 X_3$ | 0.7123 | 0.0919 |
| $X_1^2$ | <0.0001 | – |
| $X_2^2$ | 0.8521 | – |

interactions, after ANOVA analysis. In the quadratic model for $Y_1$, only the variable $X_2$ and the interactions $X_2 X_3$ (co-substrate concentration and type) and $X_2^2$ (quadratic effect of the co-substrate concentration) had no significant effect in the BMP, i.e. they presented a $p$-value > 0.05. Nevertheless, the variable $X_2$ was considered in the statistical analysis to respect the hierarchy of the model, i.e. all the variables present in the chosen interaction ($X_1 X_2$, $X_1 X_3$ and $X_1^2$) need to be selected. In the 2FI model, for $Y_2$, all independent variables and interaction were considered significant, except the interactions $X_2 X_2$ ($p$-value = 0.0919) (Table 4).

Afterwards, new models were defined considering only the significant factors (and $X_2$ for $Y_1$). The response surface of the specific methane production from the co-digestion of *Sargassum* sp. with Gly and WFO, depending on the substrates concentration, is shown in a three dimensional graph in Fig. 1, while the contour plot in Fig. 2 shows the response surface of $k$ from the co-digestion of *Sargassum* sp. with glycerol and WFO. Addition of glycerol and WFO showed similar results between them, although the yields are slightly higher using glycerol as co-substrate. For lowest concentrations of *Sargassum* sp. tested (<2% TS), the addition of both co-substrates slightly decreased the BMP (Fig. 1). Increasing the *Sargassum* sp. concentration until 4% TS led to a decrease in the BMP, more significant with Gly. However, different concentration of co-substrate did not affect significantly the methane production (Fig. 1). For concentration of *Sargassum* sp. >4% TS, the addition of different amounts of co-substrate slightly increased the BMP, more significant with WFO (Fig. 1). This fact was explained by the production of methane achieved by the assays 17 and 18, compared with the same assays with Gly (Table 3).
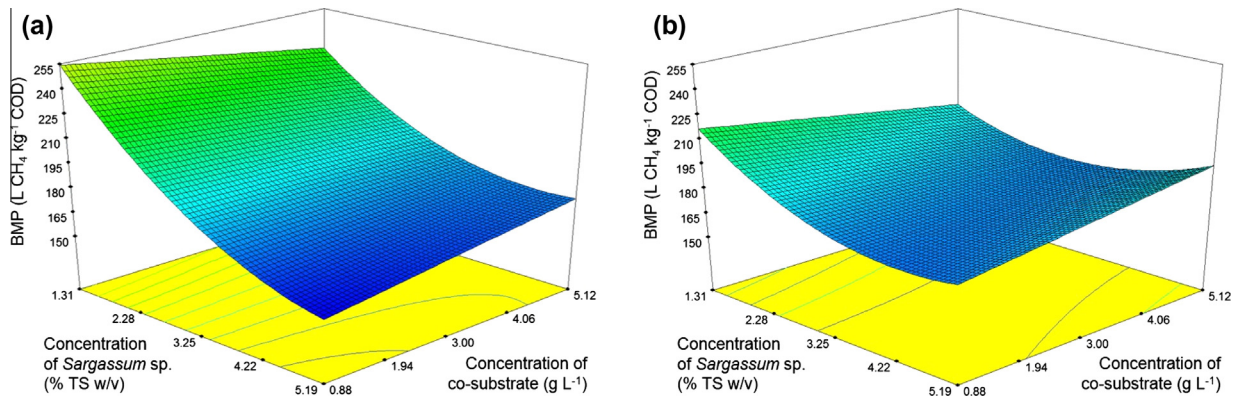
**Fig. 1.** Response surface of the BMP of *Sargassum* sp., co-digested with glycerol (a) and WFO (b).
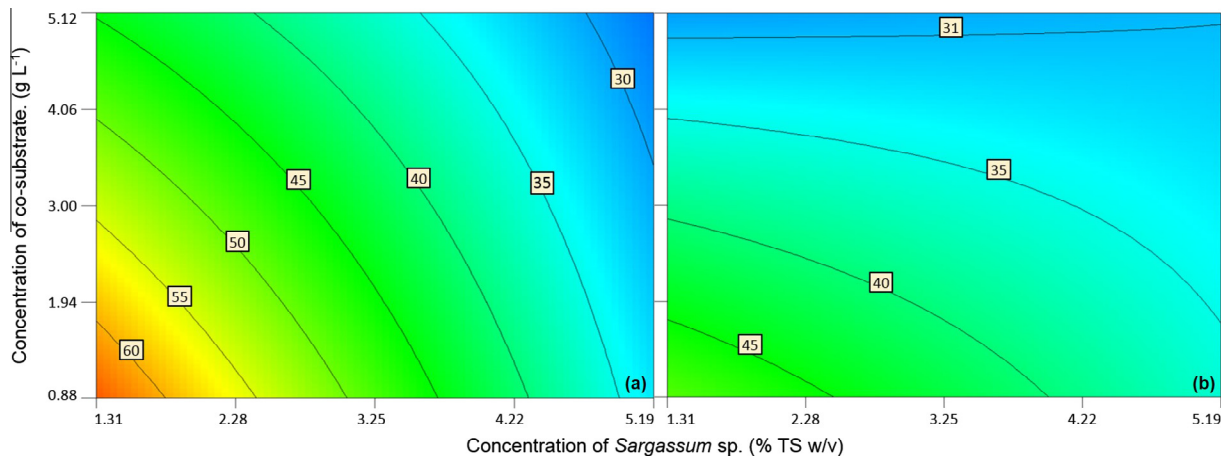


**Fig. 2.** Contour plot of the methane production rate (L $CH_4$ $kg^{-1}$ COD $d^{-1}$) from the anaerobic co-digestion of *Sargassum* sp. with glycerol (a) and WFO (b).

The *k* perdition (Fig. 2) showed a similar behaviour to BMP perdition. However, the addition of WFO only increased the *k* for the lowers concentrations of *Sargassum* sp. (<1.6% TS) and co-substrate (<1.1 g $L^{-1}$). Aiyuk et al. (2006) described a COD:N ratio of 60 for the adequate star-up of the process, nevertheless the higher rate was reached with a COD:N ratio of 40, due to possible recalcitrant materials present in *Sargassum* sp. and to toxic compounds in WFO.

The surfaces of Figs. 1 and 2 are described by Eqs. (5) and (6) (*p*-values < 0.0001), respectively.

$$Y_1 = 180.8 - 24.6X_1 - 1.08X_2 - 4.06X_3 + 11.3X_1X_2$$
$$+ 14.5X_1X_3 + 18.1X_1^2 \qquad (5)$$

$$Y_2 = 39.4 - 7.41X_1 - 6.06X_2 - 3.02X_3 + 3.48X_1X_2$$
$$+ 4.46X_1X_3 \qquad (6)$$

Eqs. (5) and (6) provide the optimum conditions for both response variables. According to the models, when the independent variables assume a coded level of −1, a BMP of 254 L $CH_4$ $kg^{-1}$ COD and a *k* of 63.8 L $CH_4$ $kg^{-1}$ COD $d^{-1}$ are achieved. Therefore, the best results would be obtained using 1.31% TS of *Sargassum* sp. with glycerol as co-substrate at 0.88 g $L^{-1}$. These results can be explained by the characteristics of the substrates. *Sargassum* sp. is difficult to biodegrade in large amounts, due to some recalci-trant material present in the samples (Bird et al., 1990). The addition of glycerol should be very careful because high concentrations can inhibit the methanogenesis (Oliveira et al., 2014).

## 4. Conclusions

A DOE was applied to study the co-digestion of *Sargassum* sp. with Gly and WFO. The BMP of *Sargassum* sp. without co-substrate was 181 ± 1 L $CH_4$ $kg^{-1}$ COD. The co-digestion caused an increase on the methane production up to 56% (with 0.5% $TS_{Sargassum\ sp.}$ and 3.0 $g_{Gly}$ $L^{-1}$), and 46% (with 1.31% $TS_{Sargassum\ sp.}$ and 0.88 $g_{WFO}$ $L^{-1}$). The methane production rate, increased 38% and 19% in the same assays with Gly and WFO, respectively. According to the model defined, the optimal conditions, maximizing the BMP and *k*, were 1.31% TS of *Sargassum* sp. and 0.88 $g_{Gly}$ $L^{-1}$.

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