Co-digestion of *Sargassum* sp. with glycerol and waste frying oil following a design of experiments

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ABSTRACT

A response surface methodology 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_4kg^{-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_4kg^{-1} $CODd^{-1}$) was obtained in the assay with 0.5% TS and 3.0 $g_{Gly}L^{-1}$. Co-digestion with Gly or WFO is a promising process to enhance the BMP from the macroalgae*Sargassum* sp..

KEYWORDS

Anaerobic co-digestion; Design of experiments; Glycerol; *Sargassum* sp.; Waste frying oil

INTRODUCTION

Algae potential was rediscovery recently. Seaweed (or macroalgae) can be used to produce bioenergy, namely bioethanol and biogas. This 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 large yields make them even more attractive(Borjensson and Mattisson, 2008).

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 CH_4kg^{-1} 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 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 (Costa et al., 2013b).

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., 2012; Costa et al., 2013a; Oliveira et al., 2014). For instance, the addition of 2% of crude glycerol increased the biogas production from the macroalgae*Gracilariavermiculophylla* by 18%. However, its addition may be inhibitory at higher concentrations(Oliveira et al., 2014).

The addition offat, oil, and grease (FOG) to a municipal sludge anaerobic digester increased the BMP 257%, reaching 418±14 L CH_4 kg^{-1} VS(Li et al., 2011). Neves et al. (2009) 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} $L^{-1}_{reactor}$ 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 theC: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 response surface methodology was adopted to determine in a systematic way the statistical significance of each parameter and to evaluate the possible interactions.

METHODS

Anaerobic granular sludge from a brewery industry was used as inoculum in the biodegradability assays. The sludge samples contained 0.081±0.001 g volatile solids (VS) g^{-1} inoculum. The specific methanogenic activity (SMA) in the presence of acetate (30 mM) was 136±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±65 mL $CH_{4@STP}$ g^{-1} VS d^{-1}. SMA was determined according to described in Costa et al. (2012b).

Sargassum sp. was collected in the Portuguese coast (Póvoa de Varzim), dried at 37 ºC and milled to less than 1 mm. Crude glycerol, from vegetable oils, and WFO, from a kitchen restaurant in Braga (Portugal), were used as co-substrates in the 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.

A factorial DOE 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. Five level (-α; -1; 0; +1 and +α) of X_1 (0.5; 1.31; 3.25; 5.19 and 6 % TS, respectively) and X_2 (0; 0.88; 3; 5.12 and 6 g_{waste} L^{-1}, respectively) were selected. The experimental design consist in a full factorial experimental design with 18 runs (Eq. 1):

$$n[N_f + N_\alpha + N_c] \quad (1)$$

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*≤ 0.05 indicated significant model terms.

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

Where Y_i indicates the predicted response variable; β_0 is the constant coefficient; β_i is the coefficient of the X_i; β_{ij} and $\beta_{ijk}$are the interaction coefficients; and X_i, X_j, X_k are the independent variables.

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 (0ºC and 1 atm). BMP was defined by the volume of methane produced per unit of COD of substrate added to the assay (Eq. 3):

$$BMP = kg\ COD - CH_4 \times 350\ L\ CH_4 kg^{-1}\ COD\ /kg\ COD_{added} \quad (3)$$

Where, $kg\ COD - CH_4$ is the cumulative methane produced during the anaerobic biodegradability assay and $kg\ COD_{added}$is the total COD added from the substrate in each vial.

Ammonium ($N\text{-}NH_4^+$), Total Kjeldahl nitrogen (TKN), TS, VS and pH were measured according to Standard Methods (APHA et al., 1998). Free ammonia ($N\text{-}NH_3$) was calculated based on total ammonium concentration and pH (Eq. 4):

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

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. Protein content was determined based on the TKN measurement using the correction factor 6.25 (Lourenço et al., 2008). Lignin, glucanandxylan quantifications were done as described in Sluiter et al. (2011). Volatile Fatty Acids (VFA), long chain fatty acids (LCFA) and methane content of biogas was analyzedaccording to dercribed in Oliveira et al. (2014).

RESULTS AND DISCUSSION

Substrates characterisation. The wastes characterisation is shown in Table 1. 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 biodegradability. Bird and co-workers (1990) refer to *Sargassum* sp. as a poor feedstock to methane production. The co-digestion with Gly 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.

Table 1

The sample of crude glycerol and WFO had 25 and 6% VS of LCFA, respectively. 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.

Anaerobic biodegradability assay. The experimental design matrix and the results obtained are presented in Table 2. 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 withGlyas 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. Addition of Gly and WFO showed similar results between them, although the yields are slightly higherusing Gly as co-substrate. An increase in the concentration of *Sargassum* sp. leads to a decrease on the BMP, except the assays 17 and 18 (Table 2) that showed a considerable increase. For lower concentrations of *Sargassum* sp., the BMP decreased with increasing concentrations of co-substrates. However, for

concentration of *Sargassum* sp. >4% TS, the addition of different amounts of Gly did not influence significantly the BMP. On other hand, the addition of WFO slightly increased the BMP (assays 17 and 18).

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. The buffering capacity was capable of prevent the pH drop levels prejudicial to the methanogenesis (>6.8) (Table 2).
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 and co-workers (2004) concluded that a specific content higher than 1g COD-LCFA g^{-1} VS was a limiar of toxicity for the anaerobic 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.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).

Table 2

Regarding the *k*, it was observed a variation between 26.4 to 65.9L CH_4 kg^{-1} COD d^{-1} for Gly and 28.7 to 56.7L CH_4 kg^{-1} COD d^{-1} for WFO (Table 3). The biodegradability of *Sargassum* sp. without co-substrate was47.7±2.5L CH_4 kg^{-1} COD d^{-1}. As in the BMP, the concentration of *Sargassum* sp. and co-substrate had significant influence in the *k*. BMP and *k*showed a similar behaviour (Table 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 selected for the response variables Y_1 (BMP) andY_2(*k*). The model with lower standard error for regression was selected. To significantly represent the BMP prediction aquadratic 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*<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*<0.0001. The quadratic effects were not considered significant in this case.

The ALOVA analysis gave the significance of the selected models for each response variable, as well as for all independent variables and their interactions. In the quadratic model for Y_1, only the variable X_2(*p-value*=0.6167), the interactions X_2X_3(*p-value*=0.7123)(co-substrate concentration and type) and ${X_2}^2$(*p-value*=0.8521) (quadratic effect of the co-substrate concentration) had no significant effect in the BMP. 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 thechosen interaction (X_1X_2, $X_1X_3$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_2X_3(*p-value*=0.0919).
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 Figure 1, while the contour plot in Figure 2 shows the response surface of *k* from the co-digestion of *Sargassum* sp. with Gly and WFO.
The surfaces are described by equations 5 and 6 (*p-values*<0.0001).

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

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

Equations5 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% $TS_{Sargassum\ sp.}$ with Gly as co-substrate at 0.88 gL^{-1}. These results can be explained by the characteristics of the substrates. *Sargassum* sp. is difficult to biodegrade in large amounts, due to some recalcitrant material present in the samples (Bird et al., 1990). The addition of Gly should be very careful because high concentrations can inhibit the methanogenesis(Oliveira et al., 2014).

Figure 1

Figure 2

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 CH4 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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Table 1: Characterisation of *Sargassum* sp., glycerol and 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	$g\ g^{-1}_{substrate}$	0.60 ± 0.06	1.60 ± 0.01	2.55 ± 0.29
sCOD	$g\ g^{-1}_{substrate}$	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)

Table 2: Design matrix of the factorial experimental design and the observed response variables (BMP and k).

Assay	X_1 [S]	X_2 [CS]	X_3 CS type	Y_1 BMP	Y_2 *k*	pH	sCOD	NH_3–N	VFA	LCFA
	%TS	g L^{-1}		L CH_4 kg^{-1} COD	L CH_4 kg^{-1} COD d^{-1}		g L^{-1}	mg L^{-1}	g L^{-1}	mg L^{-1}
1	-α	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	0.00 ± 0.00	17 ± 17
4	0	-α	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	0.00 ± 0.00	62 ± 39
6	0	+α	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	+α	0	Gly	172 ± 3	26.4 ± 3.9	7.29 ± 0.00	9.21 ± 0.28	60 ± 1	2.02 ± 0.07	168 ± 16
10	-α	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	-α	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	+α	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	+α	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

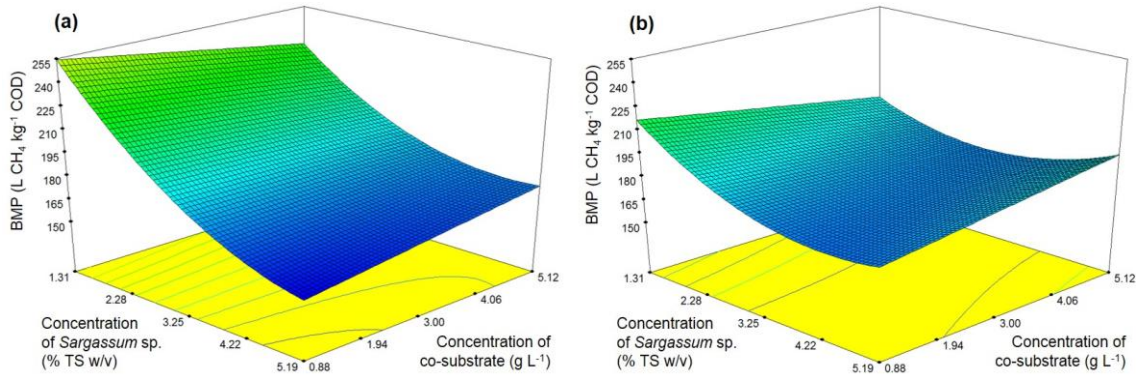

Figure 1: Response surface of the BMP of *Sargassum* sp., co-digested with glycerol (**a**) and WFO (**b**).

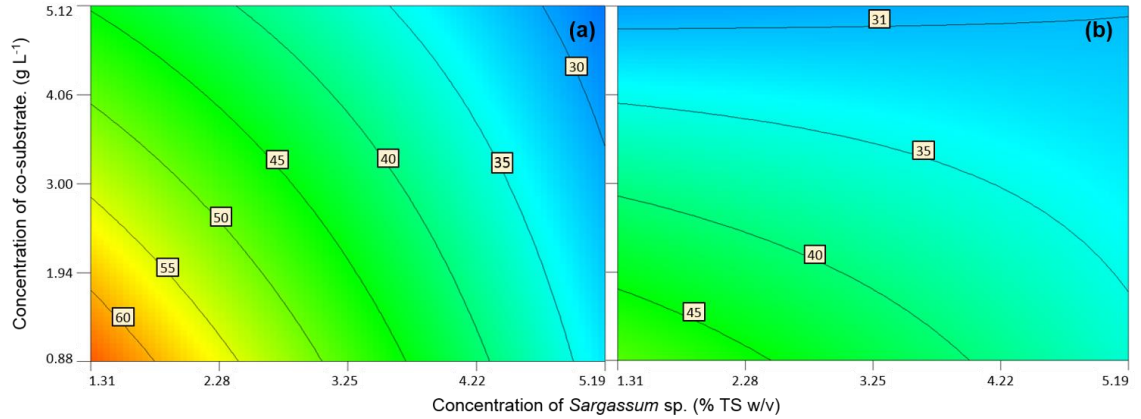

Figure 2: Contour plot of the methane production rate (L CH_4 kg^{-1} COD d^{-1}) from the anaerobic co-digestion of *Sargassum* sp. with glycerol (**a**) and WFO (**b**).