**Biodiesel and biogas production from *Isochrysis galbana* using dry and wet lipid extraction: A biorefinery approach.**

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**Abstract**

Wet lipid extraction combined with residual biomass anaerobic digestion are alternatives to reduce the overall energy consumption of biodiesel production from microalgae. Solvents with different polarities have been studied to assess dry and wet lipid extraction process from *Isochrysis galbana* microalga. Ethyl acetate (EA) and a chloroform:methanol (CM) mixture yielded the best lipid extraction results in the dry and wet route with suitable lipid compositions. Fatty acid methyl esters (FAMEs) conversion of dry and wet extracted lipids with these solvents was performed by using both homogeneous (H2SO4) and heterogeneous (resin CT 269) catalysts. FAME production from wet extracted lipids with the EA solvent using the CT-269 resin constitutes an advantageous process because it avoids the water elimination step, and the CT-269 is a heterogeneous commercial catalyst, readily to separate after reaction. Lipid-spent microalga was anaerobically digested, obtaining that waste biomass from the wet extraction with EA had the highest methane yield (310 mL CH4 / g volatile solids (VS). Energy balance analysis for FAMEs production with EA solvent (wet route) and heterogeneous catalyst yielded an energy recovery of about 80 % in terms of biodiesel and biogas. Therefore, this process constitutes a promising route under an energy-driven microalga biorefinery.

Keywords: lipid extraction; microalgae; biodiesel; FAME; anaerobic digestion; biorefinery.

1. **INTRODUCTION**

The production of biodiesel as fatty acid methyl esters (FAMEs) from microalgae has been widely studied as a promising alternative to satisfy the fuel demand for transport [1]. It is well known that microalgae can be grown on non-arable land and it does not compete with food production [2,3]. Furthermore, microalga cultivation can be used to capture CO2 emissions from human activities and mitigate their greenhouse gases emissions [4,5].

One of the most important drawbacks in attempting to increase FAME yield from microalgae is that an intensive drying process of the cultivated biomass must be carried out. According to Sander and Murthy [2], the biomass drying process represents about 90 % of the energy cost of the whole process. In order to overcome this bottleneck [6], it is mandatory to reduce the overall energy consumption [2,7]. In this context, the use of wet biomass along with the additional production of other energy products such as biogas in an energy-driven biorefinery scheme can improve the energy balance of the process.

The selection of an appropriate solvent has been identified as the key factor in wet lipid extraction, which follows a step-by-step process [8,9]. When a microalga cell is exposed to a non-polar organic solvent such as hexane, the solvent penetrates through the cell membrane into the cytoplasm (step 1) and interacts with neutral lipids through van der Waals forces (step 2). Then, a solvent-lipid complex is formed (step 3) and finally, this complex diffuses through the cell membrane (step 4). However, some neutral lipids are found in the cytoplasm forming complexes with polar lipids and therefore they cannot be extracted using non-polar solvents. These complexes are strongly linked through hydrogen bonds to proteins in the cell membrane. Therefore, polar organic solvents, such as methanol and isopropanol, are used to disrupt the lipid-protein associations by forming hydrogen bonds with the polar lipids in the complex. Once the solvent-lipid complexes have been released, a biphasic separation is induced. In this biphasic separation, neutral and polar lipids are mainly dissolved in the organic phase (a mixture of non-polar and polar organic solvents), while the aqueous phase (a mixture of water and polar organic solvent) mainly contain non-lipid contaminants such as proteins and carbohydrates [10].

Different solvents have been reported in the literature for wet lipid extraction from microalgae [11], the main ones being hexane [12,13] and a mixture of chloroform:methanol [14–16]. Nevertheless, other authors have tested different solvents or mixtures, such as ethyl acetate [17], hexane/ethanol [18] or hexane/isopropanol [19]. These solvents are more polar than hexane, which can improve the lipid extraction yield, and also less toxic than the chloroform:methanol mixture.

Another way to improve the overall energy balance of biodiesel production from microalgae is the valorisation of the residual biomass resulting from lipid extraction [7,8,20]. Several studies reported in the literature have shown the possibility of producing biogas from the above mentioned waste biomass [21–23]. Lipids have a higher theoretical methane potential than proteins and carbohydrates [23]. Therefore, the lipid extraction previous to anaerobic digestion would reduce the biogas production. However, a high concentrations of lipids could cause inhibition due to the formation of intermediate products such as long chain fatty acids and volatile fatty acids during anaerobic digestion [24,25]. This effect was also observed in the anaerobic digestion of untreated *Nannochloropsis gaditana*, *Isochrysis galbana* and *Arthrospira platensis* biomasses in a previous study [26]. In addition, some authors [27,28] have reported that it is necessary to disrupt the cell walls of microalgae to obtain high methane production yield and therefore, the waste biomass from lipid extraction could be suitable as a substrate for anaerobic digestion [29,30].

Lipid extraction for biofuels production is a well-known field and several studies reported biorefinery approaches to optimize energy production, driven by biodiesel [7,10,31–33] or biogas production [22,24,30,34,35]. However, a biorefinery can extract the most of the potential energy from microalgae biomass by combining the production of both biofuels [36]. This approach is novel and requires optimization, as well as a dedicated analysis of the energy balance in order to set-up potentials for further scale-up of the biorefinery to an industrial process.

In the present work, solvents with different polarities have been studied to evaluate the wet lipid extraction process from the microalgae *I. galbana*. For comparison purposes, the dry extraction was also studied with the same solvents. Based on the extraction results, the best solvents were selected to produce FAMEs from the extracted lipids with two different acid catalysts: H2SO4 as homogeneous catalyst [37] and CT-269 ion exchange resin as heterogeneous catalyst. Those catalysts have been previously tested in these reactions with *N. gaditana* [33,38] and the results showed high levels for the FAME yield and purity. The waste biomass obtained from the lipid extraction was used to produce biogas through anaerobic digestion. Finally, the energy balance of biodiesel and biogas production was calculated.

1. **MATERIALS AND METHODS**
	1. **Microalga biomass**

The strain of microalga used for this work was *I. galbana*, supplied by AlgaEnergy S.A. (Madrid, Spain). The inoculum for the anaerobic digestion experiments was obtained from a full-scale anaerobic digester located in a sewage treatment plant in Móstoles (Spain).

* 1. **Lipid extraction and characterisation**

Both dry and wet extraction processes were carried out by triplicate in 12 mL glass tubes with a dry microalga:solvent ratio of 1:20. The selected solvents purchased from Sigma-Aldrich (Spain) were an alkane - hexane (H, 99 wt % purity), a cycloalkane - methylcyclohexane (MCH, 99 wt % purity) and an ester compound - ethyl acetate (EA, 99.8 wt % purity). In addition, the results for the lipid extraction yield were compared with a control consisting of a standard method proposed by Folch et al [39], where a 2:1 chloroform (>99.5 wt % purity):methanol (99.8 wt % purity) mixture (C:M) was used (both provided by Sigma-Aldrich). The solvent properties are shown in Table 1.

Table 1. Solvent properties. Hexane (H), Methylcyclohexane (MCH), ethyl acetate (EA), chloroform (C) and methanol (M).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Solvent | H | MCH | EA | C | M |
| Molecular formula | C6H14 | C7H14 | C4H6O3 | CHCl3 | CH4O |
| Purity | > 95 | > 99 | > 99.8 | > 99 | > 99.9 |
| Density (20 ºC) (kg L-1) | 0.655 | 0.77 | 0.90 | 1.48 | 0.79 |
| Viscosity (20 ºC) (cP) | 0.294 | 0.732 | 0.45 | 0.57 | 0.59 |
| Tbp (ºC) | 68 | 101 | 77.1 | 61 | 65 |
| Solubility in H2O (g L-1) | Insoluble | Insoluble | 83 | 8.22 | Miscible |
| Dielectric constant (25 ºC) | 1.89 | 2.02 | 6.02 | 4.81 | 32.7 |
| Class | Alkane | Cycloalkane | Ester | Halogenated | Alcohol |

Thus, for dry experiments, 10 mL of the solvent were added to 0.5 g of dry microalga, whereas wet extractions were performed with a microalga concentration of 50 g L-1 (5 % in dry weight) adding 10 mL of the solvents. The samples were vigorously stirred with a vortex (FINECORP, Korea) during 5 min at room temperature. Then, the mixture was centrifuged for 5 min at 11000 rpm (Centrifuge 5810, Eppendorf, Germany to separate the lipid organic fraction from the aqueous phase and the solid residue. The organic phase was extracted and the solvent was removed by vacuum distillation at 60 ºC during 24 hours until reaching constant weight. The extraction yield was determined by the weight difference between the initial biomass and the extracted lipids.

The lipid composition was analysed by Thin Layer Chromatography (TLC) analysis using the method developed by Vicente et al [40]. Chromatographic separation was carried out in 20 cm x 20 cm silica-coated aluminium plates (Alugram Sil G/UV. Macherey-Nagel GmbH, Düren, Germany) using a solvent mixture of 88.5 vol. % n-hexane and 11.5 vol. % diethyl ether. Then, 1 mL of glacial acetic acid was added to the mixture. Visualization was carried out by staining with iodine. Digital image was analysed with Un-Scan-It Gel 6.1 software (Silk Scientific Inc. Orem, UT, USA), and lipid composition was quantified with the corresponding calibration curves. Non-saponifiable products (non-saponifiable lipids and non-lipidic compounds) present in the extracted oil were determined by a gravimetric procedure [41].

The oil samples were transformed into their corresponding FAMEs using the boron trifluoride method described in ISO 5509. The fatty acid profile was then determined by gas chromatography (GC) in a CP-3800 gas chromatograph (Bruker, Germany) fitted with a flame-ionization detector (FID) and a capillary column of fused silica Teknokroma Zebron ZB-WAX (0.32 mm x 30 m, 0.25 µm standard film, from Phenomenex, USA). Identification of chromatographic peaks was performed by comparison with a FAME standard mixture (Sigma-Aldrich). Quantification of different FAMEs was carried out using external standards and their corresponding calibration curve [42]. Finally, the iodine value of the extracted oils was calculated with EN 14111 [43].

* 1. **FAME production**

FAME production tests were performed by triplicate in 100 mL glass flasks using H2SO4 (Scharlab, Spain) and the CT-269 resin (Sigma–Aldrich, USA) as catalysts. For this purpose, 0.5 grams of extracted oil were weighed and the necessary quantities of solvent and catalyst were added [33,37]. A catalyst/solvent/oil ratio of 1.74/15/1 was used for the reaction using H2SO4 as catalyst, whereas this ratio was 1.1/70/1 for the reactions with the CT-269 resin. In each experiment, the mixture was stirred during 4 hours at 900 rpm and 65 ºC. Then, the sample was cooled down and the upper phase containing FAMEs was carefully removed and washed with 50 mL of water to remove impurities and traces of catalyst and methanol. Finally, the sample was dried at 60 °C and the product yield was calculated by the difference in weight. The molar conversion of the saponifiable lipids in the microalga oil to FAMEs was calculated through 1H-NMR analyses performed in a Varian Mercury Plus 400 unit, following the procedure of Gelbard et al. [44].

* 1. **Biomethanization of raw and waste biomass**

Biochemical methane potential tests (BMP) were performed by triplicate in 150 mL glass serum bottles, leaving 1/3 of the bottle empty for gas accumulation, with a 2/1 inoculum/substrate ratio (I/S) [41,45,46]. 0.33 g of biomass was weighed from both the residual biomass and the entire microalgae, 66 mL of inoculum with a volatile solids content of 1 % was added to the mixture and mili-Q water was added to a final volume of 100 mL. Once all the bottles were filled, they were closed with rubber septa and aluminium seals and purged with argon to displace the oxygen from the inside that could interfere with the process and were incubated in static mode at 38 ºC. A blank test containing the inoculum and no substrate was used to correct for background methane potential of the added inoculum. The BMP tests were conducted during a minimum of 30 d and were performed in triplicate. All error bars indicate 95 % confidence limit on the average of the triplicates.

The methane production for the manometric method was calculated from the accumulated headspace pressure increase and the biogas composition. Methane yields are reported at standard conditions (i.e. 0 °C and 1 bar). A Boyle-Mariotte oil manometric apparatus was used for measuring the biogas pressure in the headspace (U30046 Deluxe Boyle's Law Mariotte Apparatus, 3B Scientific, Hamburg, Germany). Gas composition was determined from 8 mL gas samples by using a 7820 Gas Chromatography – Thermal Conductivity Detector (GC-TCD) apparatus (Agilent Technologies, Santa Clara, CA, USA).

Analysis of total and volatile solids (TS, VS), total and soluble chemical oxygen demand (TCOD, SCOD) and Kjeldahl’s nitrogen were determined according to APHA Standard Methods [47]. Phosphates and ammonium nitrogen were analysed by using Merck kits (Merck, Darmstad, Germany).

* 1. **Model implementation and data analysis**

Kinetic parameters of methane production in BMP tests were obtained by fitting first order models to the data as per Segura et al. [48], thereby calculating *kH* (in d−1) and BMP (*B0*, in mL CH4/g volatile solids (VS)). Parameter uncertainty was determined using two-tailed t-tests calculated from the standard error in the parameter value, obtained from the Fisher information matrix. Where parameter optimization problems involve multiple parameters (*kH*, *B0*), parameter uncertainty surface (J = Jcrit, 5% significance threshold) has also been assessed as described in Batstone et al., 2003 [49]. Confidence intervals (at 95%) were also calculated based on two-tailed t-tests from the parameter standard error, as above, and used for statistical representative comparisons. All the statistical analyses of BMP assays were performed by using Aquasim 2.1d (Eawag, Dübendorf, Switzerland) [50].

* 1. **Energy balance of the proposed biorefinery**

The energy balance of the best process was checked to calculate the energy recovery in the form of biodiesel and biogas from *I. galbana*. For this purpose, elemental composition (H, C, N, S, O) from *I. galbana*, FAMEs and the residual biomasses were measured using a Vario EL III Element Analyzer (Elementar Analysensysteme GmbH, Germany). From these values, elemental composition of liquid subproducts (liquid waste) were determined through mass balances of the biorefinery scheme.

Based on the elemental compositions, the high heating value (HHV) was determined using the Boie equation (eq. 1) [51], as follows:

HHV (MJ kg-1) = 0.3516·C + 1.16225·H + 0.1109·O + 0.0628·N (1)

1. **RESULTS AND DISCUSSION**
	1. **Lipid extractions**

Figure 1 shows the lipid extraction yield (wt %) with the selected solvents obtained by dry and wet extractions. The extraction process was conditioned by the polarity of the solvent (Table 1, Figure 1). Thus, H and MCH, solvents with lower dielectric constant values, registered lower values of extracted lipids due to the low affinity of these solvents to the polar lipids such as phospholipids and glycolipids. These results are in agreement with those reported by Angles et al. [11] showing that extraction of polar lipids is promoted by solvents with higher polarity. Therefore, only non-polar lipids (free fatty acid and neutral lipids such as glycerides) can be dissolved in the non-polar solvents such as MCH and H, which has been also observed previously [52]. This can explain the low lipid extraction yield obtained when using these solvents [51].

This effect of solvent polarity was clearly evidenced during the dry extraction, where lipid yield increased from 7.5 wt % for H to 38 wt % for the 2:1 C:M mixture (dielectric constant of 1.89 and 10.6, respectively [53]). In this case, the extraction using non-polar solvents such H also involved a kinetic limitation often found when dry material needs to be wetted and impregnated with this type of solvents [11].

Except for EA, extraction yields for the dry process were higher than the values obtained by the wet route. The water of the wet biomass may inhibit the penetration of solvents, avoiding lipid release [54]. However, it is remarkable that in the case of EA the lipid extraction yield was similar in both dry and wet routes (17.9 and 17.6 wt %, respectively). In the wet extraction, the increase in lipid extraction yield with solvent polarity was less significant in the extractions using MCH, H and EA. However, the lipid extraction yield was very similar for the solvents with higher dielectric constant like EA (17.6 wt %) and the C:M mixture (16.2 wt %) in the wet route. Therefore, the use of the C:M mixture is less unfavourable in wet conditions since methanol is water-miscible, so water inhibits the lipid extraction process.



Figure 1. Dry and wet extraction yields (wt %) from *I. galbana* (bars) and dielectric constant of solvents (black symbol).

In conclusion, EA and C:M exhibit the higher extraction yield values in the wet extractions and therefore, they were selected for further study of biodiesel and biogas production.

Saponifiable lipids, free fatty acids, non-saponifiable lipids and non-lipidic compounds extracted with the selected solvents (EA and C:M) were analysed by TLC (Table 2).

Table 2. Composition (wt %) of the lipids extracted from *I. galbana*

|  |  |
| --- | --- |
| Compound | Solvent |
|  | EAdry | EAwet | C:Mdry | C:Mwet |
| Free Fatty Acids and saponifiable lipids (wt %) | Free Fatty Acids | 20.9 | 16.9 | 17.9 | 19.5 |
| Triglycerides | 3.7 | 5.4 | 4.7 | 4.8 |
| Sterol esters | 0.2 | 0.5 | 0.2 | 0.4 |
| Polar lipids | 63.2 | 76.7 | 50.2 | 74.7 |
| **Saponifiable lipids (wt %)** | 88.0 | 99.5 | 73 | 99.4 |
| Non-saponifiable lipids (wt %) | Carotenoids | n.d | n.d | n.d | n.d |
| Sterols  | n.d | n.d | 0.2 | 0.1  |
| **Non-saponifiable compounds (wt %)** | n.d | n.d | 0.2 | 0.1 |
| **Non-lipidic compounds (wt %)** | 12.0 | 0.5 | 26.8 | 0.5 |

(n.d: non-detectable).

The high content of saponifiable compounds is remarkable in all the extraction processes. This denotes the large amount of saponifiable lipids that could be transformed into FAMEs using the *I. galbana* microalga. On the other hand, the saponifiable lipid content was significantly higher in the wet route than in the dry extraction one (99.5 and 99.4 wt % vs 88 and 73 wt % for EA and C:M, respectively). These results show that EA and C:M extracted more polar compounds in the wet route. Thus, the content of polar lipids was 63.2 and 50.2 wt % using EA and C:M in the dry process, respectively. However, the polar lipid concentration increased to 76.7 and 74.7 wt % for the wet extraction with EA and C:M, respectively. These results could be a consequence of the solid-liquid-liquid equilibrium that would promote the extraction of these polar lipids in the wet process.

The non-saponifiable lipid fraction consisted mainly of carotenoids and sterols and it represented less than 0.2 wt % in all cases (Table 2). This low content is desirable as these compounds cannot be converted into FAMEs. The content of non-lipidic compounds, which are not suitable for obtaining FAMEs, was higher in the dry extraction than in the wet one. Non-lipidic compounds are mainly polar molecules that dissolved in the water layer during the wet process, reducing the amount of these undesirable compounds in the extracted lipid. These results show that the wet extraction route is more suitable since higher saponifiable lipid concentrations and lower contents of non-saponifiable compounds were extracted. However, EA is more suitable solvent for lipid extraction because it is less toxic than the C:M mixture.

Table 3 shows the fatty acid profile together with the iodine values for the saponifiable lipids extracted from *I. galbana*. Similar fatty acid profiles and consequently iodine values were obtained in all cases. Therefore, they do not depend on EA and C:M polarity and the presence of water.

Table 3. Free fatty acid composition and iodine values in the saponifiable lipids and fatty acids extracted from *I. galbana*.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Solvent | EAdry | EAwet | C:Mdry | C:Mwet |
| Fatty acid |  | Concentration (wt %) |
| Caprylic | C8:0 | n.d | n.d | 0.1 | 0.1 |
| Capric | C10:0 | n.d | n.d | 0.1 | 0.1 |
| Lauric | C12:0 | 0.1 | 0.1 | n.d | 0.1 |
| Myristic | C14:0 | 12.7 | 15.9 | 14.1 | 14.1 |
| Palmitic | C16:0 | 19.4 | 18.7 | 20.8 | 18.7 |
| Palmitoleic | C16:1 | 11.4 | 11.4 | 10.2 | 9.6 |
| Stearic | C18:0 | 0.6 | 0.4 | 0.5 | 0.5 |
| Oleic | C18:1 | 15.4 | 14.0 | 13.9 | 16.2 |
| Linoleic | C18:2 | 5.1 | 4.8 | 5.1 | 5.0 |
| Linolenic | C18:3 | 15.3 | 14.6 | 14.0 | 14.1 |
| Arachidic | C20:0 | 6.0 | 6.2 | 6.2 | 6.3 |
| Behenic | C22:0 | 2.8 | 3.2 | 3.9 | 2.8 |
| Erucic | C22:1 | 0.2 | 0.1 | 0.2 | 0.2 |
| Lignoceric | C24:0 | 5.5 | 5.6 | 5.5 | 6.4 |
| Others | 5.4 | 4.9 | 5.5 | 5.8 |
| Saturated | 49.9 | 52.7 | 54.1 | 52.1 |
| Monounsaturated | 28.5 | 26.8 | 25.7 | 27.6 |
| Polyunsaturated | 21.6 | 20.4 | 20.2 | 20.3 |
| Iodine value (g I2/100 g) | 73 | 69 | 67 | 69 |

The iodine value of the saponifiable lipids was determined according to the EN 14111 [43] standard and ranged 65-75 g I2/100 g, which is lower than the 120 g I2/100 g limit specified by the European Standard biodiesel test method [55]. A similar value was obtained for the lipid extracted for this microalga [56], but some authors have also reported somewhat lower iodine values [57,58]. This parameter provided suitable values because of the high concentration of saturated and monounsaturated fatty acids (mainly myristic, palmitic and oleic acids) that represents about 80 wt % of fatty acid as shown in Table 3. As previously reported [33], microalga oils contain higher proportion of polyunsaturated fatty acids than most vegetable oil, which provides lower oxidative stability but excellent fuel properties at low temperatures. However, the iodine values obtained in this work reveal a low content of polyunsaturated fatty acids (~20 wt %), which could improve the oxidative stability of the biofuel obtained. Likewise, it could imply poor cold properties as the total content of saturated compounds is about 50 wt %.

* 1. **FAME production**

Table 4 summarises the product weight yield (wt %) related to extracted lipids and the molar conversion of the saponifiable lipids in the microalga oil to FAMEs. Product weigh yield showed similar values, although slightly higher when the previous extraction stage was carried out with EA. Regarding molar conversion of saponifiable lipids, it can be observed how the values obtained after extractions with the EA solvent and the C:M mixture were very high (97.3–98.9 mol%) when using the homogeneous catalyst in both dry and wet processes. In fact, the values obtained with the homogeneous catalyst (H2SO4) met the ester content specification included in the European EN 14214 biodiesel Standard [55]. In this case, the extract composition did not significantly influence the molar conversion of free fatty acid and saponifiable lipids. Therefore, the results demonstrated that the esterification of free fatty acids and the transesterification of non-polar and polar saponifiable lipids can be catalysed by the homogeneous catalyst.

Table 4: Product yield and conversion to FAME with H2SO4 and CT-269 catalysts.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SOLVENT | EAdry | EAwet | C:Mdry | C:Mwet |
| Product yield by weight (wt %) with H2SO4 | 64.8 | 70 | 45.3 | 47.8 |
| Product yield by weight (wt %) with CT-269 | 74.1 | 75.9 | 53.3 | 52 |
| Molar conversion to FAMEs with H2SO4 | 98.9 | 97.6 | 98.3 | 97.3 |
| Molar conversion to FAMEs with CT-269 | 88.6 | 95.0 | 86.9 | 93.6 |

After the wet extraction, the conversions of saponifiable lipids with the heterogeneous (CT-269) catalyst were also very high (93.6 - 95.0 mol %), although slightly lower than the ones obtained with the homogeneous catalyst. These results are very interesting since the wet extraction process is a very promising alternative for lipid extraction from microalga biomass, avoiding the previous costly water elimination step. In addition, the use of the CT-269 resin in the FAME production is adequate as it is a heterogeneous commercial catalyst, simply to obtain and separate after reaction, and gives good results in the FAME production under mild conditions. However, conversions were lower in the case of the reaction with this catalyst after the dry process (86.9 – 88.6 mol%) because of the presence of non-polar compounds in the extracts obtained with the dry route, which is in agreement with the literature [33]. In this sense, the previous extraction route is not adequate when the resin is used as catalyst in the FAME production stage.

* 1. **Anaerobic digestion**

Initial biomass and residual biomass obtained after the lipid extraction (for wet and dry processes) with the selected solvents (EA and C:M) were digested anaerobically to obtain methane. Figure 2 shows the accumulated methane yield for the substrates under study. In addition, first-order models were used to fit experimental values [59].

As can be observed, anaerobic digestion of raw microalga showed a maximum methane yield of about 300 mL/g volatile solids (VS). These results are in agreement with other ones reported in literature [36,60–63].

As shown in Figure 2, the biochemical methane potential (BMP) is lower for the waste biomass after lipid extraction, except for the residual biomass obtained with EA in the wet process. As Heaven et al. [29] reported in literature, the BMP of lipid compounds is around 1.18 times greater than that of proteins and 2.4 times greater than for carbohydrates. Thus, the lipids allow a greater production of methane (1014 mL CH4/g VS) in comparison with proteins and carbohydrates (850 and 450 mL CH4/g VS, respectively) [64]. In this sense, if the lipid extraction is carried out, the BMP should decrease. Nevertheless, an inhibition effect can be observed by the production of long-chain fatty acids and volatile fatty acids from lipids during anaerobic digestion [24,25]. The results demonstrated that lipids of *I. galbana* did not produce inhibition of this raw biomass anaerobic digestion as very low concentration of substrate has been used in the BMP tests. In addition, the increase in BMPs obtained by wet lipid extraction with the EA solvent may be due to remaining traces of EA in the spent biomass than can be converted into ethanol and acetic acid, which act as substrates in the methanogenic phase [65,66]. This leads to higher methane yields, thus supporting the results with this solvent.

On the contrary, a low BMP value was obtained when using C:M as a solvent in the dry route (130 mL CH4/g VS), being negligible in the case of the wet route which means that the digestion process was completely inhibited. Chloroform present in the C:M is a toxic compound for the anaerobic digestion process. Despite the lipid-free biomass was carefully washed before anaerobic digestion, just chloroform traces can act as an inhibitor of the anaerobic process [67,68]. Yu and Smith [69] reported a chloroform concentration limit of 0.09 mg L-1, which can completely supress the methanogenic phase in the biogas production, leading to a low or non-existent methane concentration.



Figure 2. Biochemical methane potential assays experimental (symbols) and first order-modeled (lines) methane production obtained in the anaerobic digestion of *I. galbana* raw and residual biomasses.

The experimental data were used for obtaining the kinetic parameters of the simplified first order model. Figure 3 represented 95 % confidence regions for *kH* and *B0* values. As can be seen, the biodegradability significantly increases with the spent biomass obtained in the wet route using EA and this result cannot be observed for the other spent biomasses. This is in agreement with the positive behaviour of the EA in the biogas production. However, none of the solvent extraction procedures significantly improved the kinetics of the methane production, as the *kH* values remained in similar values irrespective of the BMP test. As explained before, the extraction of the lipids by EA apparently improved the biodegradability of the remaining substrate, but the rate remained invariable, probably due to a limitation of the hydrolysis step of complex proteins and/or polysaccharides [70,71].



Figure 3. 95 % confidence regions for BMP and kH values obtained in the anaerobic digestion of *I. galbana* residual biomasses.

* 1. **Biorefinery energy balance**

According to the results obtained, EA is a promising solvent for the lipid extraction from wet *I. galbana* that allows the joint production of FAMEs (biodiesel) from the extracted lipids and biogas from the spent biomass obtained. In addition, the use of an heterogenous commercial catalysts such as the CT-269 resin in the FAME production stage is adequate to obtain high yield and quality FAMEs to be used as biodiesel. In this context, the energy balance calculation of this proposed energy-driven biorefinery is crucial to evaluate the energy recovery in the form of biodiesel and biogas.

The results obtained for all the input and output fractions are depicted in Figure 4. Microalga biomass and methanol are inputs whereas the main energy outputs are related to biodiesel (FAMEs) and biogas (methane). In addition, glycerine and other compounds are obtained as by-products from the transesterification of triglycerides and polar lipids (evaluated as phosphoglycerides). The esterification of free fatty acids and transesterification of triglycerides and phosphoglycerides are provided in the Supplementary Material (Figure S1). The rest of the extracted fractions are considered as wastes, although they can be transformed to useful material such as fertiliser.

The input HHV is 17.5 MJ/kg and 22.2 MJ/kgfor *I. galbana* and methanol, respectively, whereas the output HHV mainly corresponds to FAMEs (39.8 MJ/kg) and methane (55.4 MJ/kg). If only biodiesel is considered when assessing the total energy associated to each compound, the energy recovery respect in comparison to input energy (microalga + methanol) is about 30 %. Nevertheless, this percentage increases up to approximately 80 % if biogas is produced along with biodiesel.

According to the results obtained, it can be concluded that the production of biogas from extracted lipid waste biomass clearly enhances the energy feasibility of biodiesel production from *I. galbana*. The results obtained in this study show that wet lipid extraction is a promising alternative to recover lipids from microalgae and that anaerobic digestion can largely increase the economic feasibility of a microalgal biorefineries



Figure 4. Mass and energy balance (wt, dry basis) for the biodiesel and biogas microalgal biorefinery.

1. **CONCLUSIONS**

Solvents with different polarities had been studied to evaluate the dry and wet lipid extraction process from microalga *I. galbana*. The best lipid extraction results were obtained through the dry and wet processes using the EA solvent and the C:M mixture (C:M) with satisfactory lipid compositions and appropriate iodine values. High conversion of saponifiable lipids (93.6 – 98.9 mol%) was obtained except for the reactions of dry extracted lipids with EA and C:M solvents using the resin CT-260. The results of the FAME production of the wet extracted lipids with the EA solvent using the CT-269 resin are very promising because the wet method constitute a very suitable alternative for lipid extraction from microalga biomass, avoiding the previous costly water elimination step, and the CT-269 is a heterogeneous commercial catalyst, simply to obtain and separate after reaction. Anaerobic digestion of residual biomass from lipid extraction with EA and C:M was performed to evaluate biogas production. Waste biomass obtained with the wet extraction with EA solvent exhibited the best behaviour as substrate to obtain biogas. Energy balance of the whole process from wet *I. galbana* biomass extracted with EA show that is possible to recover up to approximately 80 % of the input energy when biogas is produced along with biodiesel and the latter is obtained using a heterogeneous catalyst. Therefore, this is a promising process to produce biodiesel and biogas under an energy-driven biorefinery scheme which can maximise the economic profitability of the full wet microalga-to-biofuels chain.

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**Figure captions**

Figure 1. Dry and wet extraction yields (wt %) from *I. galbana* (bars) and dielectric constant of solvents (black symbol).

Figure 2. Biochemical methane potential assays experimental (symbols) and first order-modeled (lines) methane production obtained in the anaerobic digestion of *I. galbana* residual biomasses.

Figure 3. 95 % confidence regions for BMP and kH values obtained in the anaerobic digestion of *I. galbana* residual biomasses.

Figure 4. Mass and atomic balance (wt, dry basis) for the biodiesel and biogas biorefinery.