Microalgae based biorefinery: evaluation of oil extraction methods in terms of efficiency, costs, toxicity and energy in lab-scale

Biorefinería basada en microalgas: evaluación de métodos de extracción de aceite en términos de eficiencia, costos, toxicidad y energía a escala laboratorio

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Abstract

Several alternatives of microalgal metabolites extraction and transformation are being studied for achieving the total utilization of this energy crop of great interest worldwide. Microalgae oil extraction is a key stage in microalgal biodiesel production chains and their efficiency affects significantly the global process efficiency. In this study, a comparison of five oil extraction methods in lab-scale was made taking as additional parameters, besides extraction efficiency, the costs of method performing, energy requirements, and toxicity of solvents used, in order to elucidate the convenience of their incorporation to a microalgae-based topology of biorefinery.
Methods analyzed were Solvent extraction assisted with high speed homogenization (SHE), Continuous reflux solvent extraction (CSE), Hexane based extraction (HBE), Cyclohexane based extraction (CBE) and Ethanol-hexane extraction (EHE), for this evaluation were used the microalgae strains *Nannochloropsis* sp., *Guinardia* sp., *Closterium* sp., *Amphiprora* sp. and N*avicula* sp., obtained from a Colombian microalgae bioprospecting. In addition, morphological response of strains to oil extraction methods was also evaluated by optic microscopy. Results shows that although there is not a unique oil extraction method which excels in all parameters evaluated, CSE, SHE and HBE appears as promising alternatives, while HBE method is shown as the more convenient for using in lab-scale and potentially scalable for implementation in a microalgae based biorefinery.

Keywords: *biorefinery, microalgae, oil extraction, sustainability.*

Resumen

Distintas alternativas de extracción y transformación de metabolitos de biomasa de microalgas están siendo estudiadas para lograr el aprovechamiento total de este cultivo energético. La extracción del aceite de microalgas es una etapa clave en cadenas de producción de biodiesel a partir de ellas y su eficiencia afecta significativamente la eficiencia global del proceso. En este estudio se realiza la comparación de cinco métodos de extracción de aceite de microalgas a escala laboratorio, tomando como criterios adicionales a la eficiencia de extracción, los costos de ejecución de cada método, requerimientos energéticos y toxicidad de los solventes utilizados, con el fin de definir la conveniencia de su incorporación en una topología de biorefinería a partir de microalgas.
Los métodos analizados fueron extracción con solvente asistida con homogenización (SHE), extracción con reflujo continuo de solvente (CSE), extracción con hexano (HBE) y ciclohexano (CBE), y extracción de aceite utilizando la mezcla etanol-hexano (EHE). Se emplearon las microalgas de bioprospección nacional

Nannochloropsis sp., *Guinardia* sp., *Closterium* sp., *Amphiprora* sp. y N*avicula* sp. Adicionalmente, se estudió la respuesta morfológica de las cepas mencionadas a los distintos métodos de extracción por medio de microscopía óptica. Los resultados muestran que aunque no hay un método que sobresalga en todos los parámetros evaluados, los métodos CSE, SHE y HBE se perfilan como promisorios, mientras que el método HBE se muestra como el más conveniente para utilizar a escala laboratorio en términos generales y potencialmente escalable para su implementación en una biorefinería basada en microalgas.

Palabras clave: *biorefinería, microalgas, extracción de aceite, sostenibilidad.*

Introduction

The progressive replacement of oil with biofuels will require certain changes in the current production of goods and services. For this reason, research about sustainability of biofuels production from renewable resources is increasing [1]. According to Chisti Y [2], energy production, goods and services are necessary, but they must be socially, economically and environmentally sustainable. Microalgae is an energy source that offers considerable amounts of fuel from small crop areas and lower production costs, which further helps in the mitigation of global warming; its culturing tolerates high concentrations of CO_2 and decreases the amount of nitrogen oxides released into the atmosphere. The most conventional biodiesel-from-microalgae production chain until now is composed by the stages of cultivation, harvesting of biomass, drying, lipid extraction and oil transesterification [3].

Despite of continuous and positive advances in algal research, biodiesel-from-microalgae production chain is not sustainable yet, in energy terms, comparison of energy demands for microalgal biodiesel production shows that energy required in all stages of production process is more than energy produced by third generation biodiesel [4], In this sense, results of studies related to bioprospecting, exploration and production of microalgae biomass made by research centers as the NREL In United States, the CISOT and CIEMAT in Spain [5], the CIDES and ICP in Colombia [6], among others, concludes that production of biodiesel from microalgae can be economically viable if total biomass components are used for obtaining biofuels and high value products and the concept of biorefinery is incorporated.

Biorefining is processing biomass in a sustainable way within a spectrum of marketable products and energy, this concept can be extended, according to Cherubini [7], to a laboratory or a set of laboratories that integrates biomass transformation processes and equipment for the production of fuels for transportation, energy and chemicals. The biorefinery concept has been identified as the most promising for the creation of an industry based on biomass. However, this concept has not been applied so far to the biomass of microalgae define a path-oriented technology for the production of biofuels and high added value products based on the physicochemical characterization of a promising species, a microalgae based biorefinery must take into account several issues for its sustainability as water requirements, production costs, environmental impacts and process efficiency [8].

The extraction of carbohydrates, lipids, pigments, proteins and special substances from microalgae biomass is under research for obtaining several bioproducts [9] focusing on the use of multifunctional processes for simultaneous extraction separation and transformation of two or more desired products [10], or in optimization of operating conditions and routes for obtaining a desired specific metabolite, pigments extraction can be made by cell breaking, solvent extraction and centrifugation, and purification is made using microfiltration, drying or lyophilization [11], reducing sugars can be obtained by hydrolysis reaction with simultaneous cell wall disruption for oil extraction [12], proteins are extracted for use as fertilizer [13], animal feed supplement [14] and substrate for fermentation [15].

Several methodologies are under study in lab-scale for extracting and separating lipids from microalgae biomass, most methods are composed by the stages of cell wall disruption and lipid separation from biomass. For cell wall disruption, various thermal, chemical and physical methods have been evaluated. Gonzalez-Delgado AD & Kafarov V [16], evaluated cell disruption using autoclave, organosolv pretreatment and acid hydrolysis, while McMillan, Watson, Ali & Jaafar [17], evaluated microwave, water bath, blender, ultrasonic and laser treatment, Vanthoor-Koopmans, Wijffels, Barbosa & Eppink [18] also exposes in their review

other novel techniques of cell disruption. After this stage is necessary a further step of solvent addition for lipid recovery, several polar, non-polar and combination of solvents are being evaluated in microalgae oil extraction, methodologies and results of adjustment of solvent based methods can be seen in detail in the works of González A, Kafarov V & Guzman A [19], Fajardo AR, Cerdan LE, Medina AR, Fernandez FGA, Moreno PAG & Grima EM [20] and Halim R, Danquah M & Webley P [21]. More advanced methods are also been evaluated as enzymatic extraction [22], supercritical fluid extraction [23], wet extraction [24], Osmotic shock [25] and in-situ transesterification [26].

One of the goals pursued by researchers in this area, is to find a method for microalgae oil extraction which can be at the same time efficient, cheap, selective to lipids desired, reproducible and scalable, for achieve this goal, several studies must be developed in order to find the process that allows an effective oil extraction in terms of efficiency, purity of product desired, energy requirements, costs and environmental impacts. The main objective of this study, is the evaluation and comparison of five solvent-based microalgae oil extraction methods in lab-scale previously developed, incorporating additional criteria commonly used in literature (oil yield/extraction efficiency), these criteria are energy consumption during method performing, costs extraction in terms of materials, energy and equipment usage and toxicity of solvents selected for lipid extraction. Although is well known by the authors the availability of robust methodologies for evaluation of each one of parameters discussed in this study as energy, exergy, and emergy analysis from the energetic point of view [27], techno-economic analysis with scenarios comparison and sensitivity analysis for evaluation of technologies from the economic point of view [28], and optimization of biorefineries taking into account economic and safety objectives [29], the scope of this research is to provide a big picture of the behavior of several oil extraction methods used on several microalgae strains in lab-scale under several criteria in order to provide some lights for further deeper study of techniques. As secondary contribution, morphological response of bioprospected strains used for evaluation of oil extraction methods is also discussed such as some issues to consider for integration of technologies developed with other methods for extraction and separation of additional microalgae metabolites according to biorefinery concept.

Methodology

Microalgae Strains

Bioprospected microalgae strains were provided by Morrosquillo Corporation (Punta Bolivar, Colombia); biomass was cultivated in f/2 medium, harvested by flocculation, dried and refrigerated until use. Characterization of different strains was developed by the Colombian Petroleum Institute ICP-ECOPETROL. As is mentioned in abstract, microalgae strains used for this study were *Nannochloropsis* sp., *Guinardia* sp., *Closterium* sp., *Amphiprora* sp. and *Navicula* sp.

Oil extraction in lab-scale

Solvent-based oil extraction methods evaluated (hexane and cyclohexane based methods, solvent extraction with high speed homogenization, continuous reflux solvent extraction and ethanol-hexane method) were designed and adjusted by authors in previous works [16], finding the best operating conditions as the first stage of cell wall disruption as second stage of solvent oil extraction and lipid purification, for all methods cell disruption is intended to destroy the microalgae cell wall to facilitate the recovery of intracellular products and obtain greater amounts of lipids, all oil extraction experiments were made by triplicate, methods were performed as follows:

Improved Solvent extraction assisted with high speed homogenization (SHE). This is a rapid and effective method, which mainly includes the stages of strong homogenization, centrifugation and filtration, for its performance, methanol, chloroform and biomass are mixed in a mass ratio of 6:12:1 under environmental conditions, methanol is a polar solvent that dissolves polar lipids, on the other hand, chloroform is a non-polar solvent which dissolves the neutral lipids present in the extraction and water is a polar solvent allows separate methanol/polar lipids phase of the chloroform/ neutral lipids, the mixture is stirred and separated by filtration, obtaining a liquid phase with high percentage of lipids and a solid stream of biomass, liquid fluid is mixed with water in 4:1 ratio for phase separation, after that, hydrophilic/hydrophobic phases are separated using centrifugation for 15min at 3400rpm the upper phase methanol/water from the centrifuge tube was removed while lower phase biomass/lipids Chloroform, was filtered by gravity. Solvents are recovered by evaporation and condensation using a roto-evaporator. Finally, the

lipid extract was allowed to volatilize to constant weight for its measurement, cell disruption in this method is achieved by mechanical action in homogenization stage [16].

Improved Extraction with the mixture Ethanol/ Hexane (EHE). This method is based in a lipid extraction method developed by Fajardo AR, Cerdan LE, Medina AR, Fernandez FGA, Moreno PAG & Grima EM [20], this procedure uses two solvents for extraction and subsequent purification of the extract. Ethanol is used in the first stage to recover the lipid content of microalgae; the crude oil obtained with ethanol contains unsaponifiable lipids, such as pigments, proteins, amino acids and other lipid and non-lipid contaminants. As a second step, the addition of water and hexane to the crude extract, obtained above, generates the formation of a biphasic system, in which lipids are transferred to the hexane phase, and the impurities are retained in the hydroalcoholic phase. This phase separation occurs due to the difference in solubility between solvents. It is performed by decanting and is repeated five times by adding more water and hexane to the hydroalcoholic phase. The proportion water content has been optimized to displace the equilibrium distributions of lipids to the hexane phase, for cell disruption a solution with 5g of biomass and 0.5mol L^{-1} of hydrochloric acid was prepared and subjected to a stirring speed of 500rpm for 120 minutes at room temperature, subsequently, vacuum filtration was performed where the pH was raised about 6 or 7 with the addition of distilled water, thereby obtaining hydrolysed biomass and water-soluble phase. Hydrolyzed biomass was dried to 105ºC for 4h [16].

Improved Continuous reflux solvent extraction (CSE). This is a multiple-extraction procedure that consists in a first cell disruption stage in which 5g of biomass are mixed with water, methanol and sulphuric acid in a 1:5:0.8:0.32 ratio, mixture is placed in a 25L Autoclave by 4h, water-soluble compounds in the cell were dissolved by the acid and formed a compound called solubilised mass, which is separated from the non-polar phase by vacuum filtering, followed by a neutralization of the biomass to stop cell degradation and drying at 105ºC during 4h, for solvent extraction, a typical Soxhlet extractor with 45/50 outer/upper and 24/40 lower/inner joint for 250mL capacity was used, pre-treated dry biomass was put in a cartridge and solvent was heated to boiling point, then condensing it on the cartridge of biomass, giving way to the solid-liquid extraction of present lipids, the process described is repeated for 16h, during solvent extraction, the amount of biomass and the ratio biomass/solvent were kept constant, solvent used for this method was hexane. After extraction, extract-solvent mixture was filtered, distilled and the remnant solvent was evaporated. Total lipids were also quantified by gravimetric methods [16].

Improved Hexane and Cyclohexane based extraction (HBE and CBE respectively). In the first stage of cell disruption, 5g of microalgae biomass are mixed with hydrochloric acid 0.5molL^{-1}. Mixture was stirred for 120min at room temperature, after that, vacuum filtration was performed where the pH was raised about 6 or 7 with the addition of distilled water, finally, hydrolyzed biomass was dried to 105ºC for 4h, for solvent extraction, biomass was mixed with fresh hexane or cyclohexane in a 1:20 ratio and stirred at 500rpm for 24h in order to promote the solvent-biomass contact, finally, solvent-extract solution is separated from biomass by vacuum filtration and solvent is recovered by distillation [30].

Parameters for comparison of oil extraction methods

Lipid yield and lipid extraction efficiency. It was estimated the yields and efficiencies for each of the methods based on the gravimetric analysis done to each, oil yield in every test was calculated using the Equation 1, from amount of biomass used and oil obtained. To calculate lipid extraction effectiveness, the term Relative Extraction Ratio is introduced; this ratio is defined as the lipid yield reached using any extraction method evaluated respect to lipid yield reached performing SHE method, which is used for total lipid determination, Equation 2 was used for calculation of Relative Extraction Ratio.

$$Lipid\ Yield = \frac{lipid\ extract\ weight}{total\ biomass\ weight} \cdot 100\% \qquad (1)$$

$$Relative\ Extraction\ Ratio\ (RER) = \frac{Oil\ Yield}{total\ lipid\ determined} \cdot 100\ \% \qquad (2)$$

Statistical comparison of lipid yield. Results of oil extraction for methods evaluated were compared in order to determine significant differences between methodologies performed, comparison was made for the five methods in one strain, and process was repeated for rest of strains evaluated, statistical procedure used was the One-way Anova, which test differences among three or more sets of data, for the special case where two extraction methods are compared t-test is used and relation between Anova and t-test was made using the expression $F=t^2$. Confidence interval was set on 95%, in addition, values of variance and standard error were calculated for each method in each strain evaluated, consideration of equal variances was not assumed for statistical comparison, for statistical analysis was used the online application SISA (Simple Interactive Statistical Analysis) in options Oneway Anova and T-test [31].

Cost of extraction. An estimate of the value of application of each method in lab-scale was calculated using an economic gross evaluation taking into account the cost of solvents and volume used in each extraction method, cost of microalgae was not taking into account in order to provide an estimated non-dependent of biomass production costs, costs of utilities which includes electric energy, water, heating and cooling services were also calculated according to their prices in local conditions, a percentage of 10% corresponding to equipment depreciation and consumption of minor materials was assumed according to heuristic rules. Excepting the CSE method, cost decrease by solvent reutilization was not taken into account.

$$C_{met} = \left[\sum_{1}^{m} C_{solv} \cdot V_{solv} + \sum_{1}^{m} \left[C_{utilities} \cdot U_{utilities} \right] \cdot 1.1 \right] \quad (3)$$

Toxicity. As all microalgae oil extraction methods evaluated in this study are solvent-based, toxicity is considered as a very important aspect due to the implications of the use of these substances; toxicity was used as safety gross evaluation criteria. $LD_{50\%}$ is a measure of inherent toxicity of a solvent that is defined as the lethal concentration that would kill the half of the affected population. $LD_{50\%}$ was chosen as toxicity criteria because values are available in literature for solvents evaluated. Exists other toxicity values as IDLH, AEGL and ERPG, however IDLH and AEGL were not used due to inconsistencies in their values reported in literature, ERPG was also discarded because in comparison to $LD_{50\%}$, is less applicable for solvents. In methods with solvent mixtures for extraction, the solvent with lower $LD_{50\%}$ was taken as reference. The method whit higher $LD_{50\%}$ was considered more tolerable in comparison to other lower values. In order to obtain a better data analysis, values were normalized to the same biomass amount (1g of dry biomass) and extraction time (1h).

Energy requirements. Energy requirements were calculated for each extraction method taking into account electric and/or heating services required for performing. Values were estimated according to the electric power of the equipment used in each stage (homogenization, drying, vacuum separation, solvent recovery etc.) and time spent in extraction procedure which depends of each oil extraction method, power values were taken from equipment handbooks, internal power loses were not taken into account calculations were made using Equation 4, for detailed explanation of terms used in Equations 1-4, please see nomenclature section.

$$E_{met} = \sum_{1}^{n} P_{eq} \cdot t_{et} \quad (4)$$

Morphological response. Observation in optical microscope is performed to the biomass of the five strains at objective 100x before and after every procedure in order to see its influence in the cell and its damage on the morphology of the same.

Results and Discussion

Characterization of microalgae strains

According to the characterization of studied microalgae strains shown in Table 1, *Amphiprora* sp. presents the highest lipid percentage (wt%), followed by *Navicula* sp., *Nannochloropsis* sp. presents the highest composition of proteins and can be potentially used for food and feed, while *Guinardia* sp. is mostly composed by carbohydrates, cellulose and hemicelluloses, and could be used for reducing sugars production and transformation to third generation bioethanol. Profile more suitable for the development of a topology of biorefinery corresponds to *Amphiprora* sp. owing to their balanced composition of lipid and non-lipid components.

Table 1. Microalgae strains composition (modified from UIS-ICP-Morrosquillo [5]).

	Nannochloropsis sp.	*Guinardia sp.*	*Closterium sp.*	*Amphiprora sp.*	*Navicula sp.*
Carbohydrates (wt%)	3	13	14	12	9
Lipids (wt%)	23	13	19	33	32
Proteins (wt%)	46	29	40	25	37
Cellulosic Material (wt%)	18	35	17	20	12
Ash (wt%)	10	10	10	10	10
Total	100	100	100	100	100

Multicriteria comparison of oil extraction methods in lab-scale

Extraction Efficiency. As is shown in Table 2, extraction efficiency depends as extraction method performed as microalgae strain used, according to extraction results is clear that microalgae strain *Amphiprora* sp. presents the highest oil yield for all five methods evaluated, followed by *Navicula* sp. except when EHE method is performed, this behavior can be explained from the biologic point of view, owing to these two strains belong to the *Naviculales* order, which presents seams in their valvs, while the strain *Nannochloropsis sp.* whose cell wall is composed by several xylan layers making difficult chemical disruption and decreasing extraction efficiency. *Guinardia* sp. microalgae strain presents the highest reproducibility of third generation energy crops studied, this can be owed to a very low percentage of polar lipids and chlorophylls, which increases the standard deviations when selective and non-selective methods are compared, however, relative extraction ratio is lower than values obtained for *Amphiprora* sp., *Navicula* sp. and *Closterium* sp.

Table 2. Extraction efficiency results.

Microalgae strain	SHE		EHE		CSE		HBE		CBE	
	RER (%)	Stdev	RER (%)	Stdev	RER (%)	Stdev	RER (%)	Stdev	RER (%)	Stdev
Nannochloropsis sp.	100.0	1.71	4,87	0.13	10.65	0.37	16.75	7.87	15.15	1.72
Guinardia sp.	100.0	1.70	9.28	1.70	13.15	1.00	9.55	3.60	12.83	0.40
Closterium sp.	100.0	1.10	22.62	4.90	50.57	10.50	36.15	0.40	29.04	4.00
Amphiprora sp.	100.0	1.90	43.66	2.10	92.04	2.60	74.52	2.40	72.49	3.90
Navicula sp.	100.0	1.65	22.01	2.48	73.06	7.35	64.05	3.66	68.39	2.39

By comparing RER of methods evaluated in five strains can be seen that extraction method used as reference for calculations (SHE method), presents the highest average extraction efficiency, derived by the combination of polar/non-polar solvents and high speed homogenization, which contributes to increase the amount of final product obtained. However, as is reported by Archanaa S, Moise S, & Suraishkumar G. [32], methods which uses methanol-chloroform as solvents can over-estimate the amount of biofuel-related lipids, because these methods also extracts other products as chlorophylls, in Figure 1 can be seen that SHE extract presents darker tone in comparison to other extracts, which shows the presence of non-lipid components, purity of extracted oil affects quality of final product desired from this microalgae metabolite (High value fatty acids or biodiesel).

After solvent extraction with high speed homogenization (SHE method), Continuous reflux solvent extraction method (CSE) presents the highest average relative extraction ratio, being potentially used for effective lipid extraction in lab scale, however, the scaling-up of this method can represent a process design challenge, owing to equipment, energy and solvent requirements. Batch methods as hexane and cyclohexane based extraction (HBE and CBE respectively) presents good extraction ratios in comparison to CSE method, with the advantage of an easier scaling-up, and lower solvent requirements, HBE extraction can be more attractive for a large scale microalgae processing owing to solvent cost, oil extraction using the ethanol-hexane mixture

presents the lowest average standard deviation of methods evaluated which could be positive for ensure reproducibility of the oil extraction, however relative extraction ratio of this method does not overcome relative extraction ratio of any other method evaluated for the same strain.

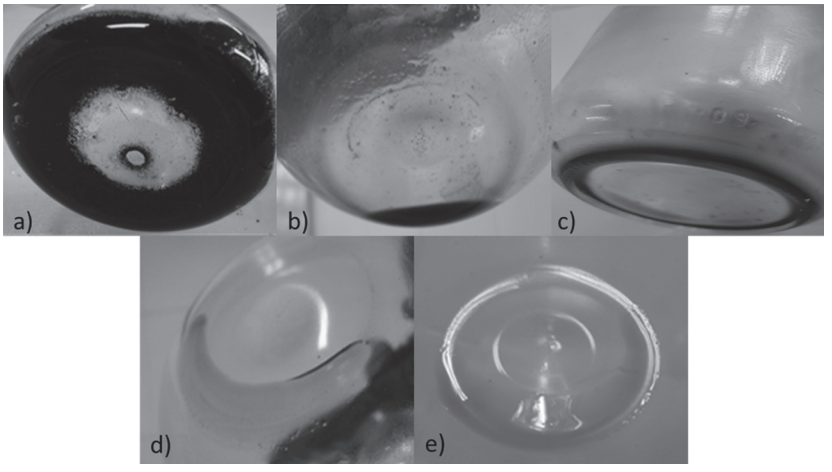

Figure 1. Lipid extracts from microalgae obtained in lab-scale, a) SHE, b) EHE, c) CSE, d) HBE and e) CBE.

Costs of extraction. If extraction costs in lab-scale are compared, lowest value belongs to EHE method and followed by EHE method, these values are due to low solvents amount needed to perform these methods and low cost of ethanol and hexane in comparison to other organic solvents, while higher extraction costs belongs to CBE method, which is drastically increased by the costs of cyclohexane which is near to 13 times more expensive than hexane in local market.

Table 3. Statistical comparison results.

Strain	Method	Variance	Standard Error	95wt% of C.I.		Non-Significant differences
Nannochloropsis sp.	SHE	2.92	0.99	95.75	104.25	-
	EHE	0.02	0.08	4.55	5.19	3
	CSE	0.14	0.21	9.73	11.57	2
	HBE	61.94	4.54	2.80	36.30	1,2,3
	CBE	2.96	0.99	10.88	19.42	1
Guinardia sp.	SHE	2.89	0.98	95.78	104.22	-
	EHE	2.89	0.98	5.06	13.50	1,2
	CSE	1.00	0.57	10.67	15.63	1,3,4
	HBE	12.96	2.08	0.61	18.49	2,3,5
	CBE	0.16	0.23	11.84	13.82	4,5
Closterium sp.	SHE	1.21	0.64	97.27	102.73	-
	EHE	24.01	2.83	10.45	34.79	1
	CSE	110.25	6.06	24.49	76.65	2
	HBE	0.16	0.23	35.16	37.14	2
	CBE	16.00	2.31	19.10	38.98	1
Amphiprora sp.	SHE	3.61	1.10	95.28	104.72	-
	EHE	4.41	1.21	38.44	48.88	-
	CSE	6.76	1.50	85.58	98.50	-
	HBE	5.76	1.39	68.56	80.48	1
	CBE	15.21	2.25	62.80	82.18	1
Navicula sp.	SHE	2.72	0.95	95.90	104.10	-
	EHE	6.15	1.43	15.85	28.17	-
	CSE	54.02	4.24	54.80	91.32	1,2
	HBE	13.40	2.11	54.96	73.14	1,3
	CBE	5.71	1.38	62.45	74.33	2,3

Statistical comparison of methods. Table 3 shows the results of statistical comparison of oil extraction methods taking into account the extraction efficiency, results shows that although behaviour of oil extraction methods is affected by the strain evaluated which is coherent with the analysis made in previous section, however, it can be seen that in most of cases (strains) there is no significant differences between performing HBE and CBE methods, showing that not worth it to continue using both methods in lab-scale for future work, nevertheless, is also clear that selection criteria between HBE and CBE cannot be efficiency, for selecting the more convenient method, must be compared using additional criteria discussed in further sections of this work. It also can be seen that there is no significant differences between CSE and HBE for most of strains evaluated, so, other criteria must be taken into account for a more robust comparison of these two methods. On the other hand, EHE method presents significant differences in comparison to other C6-based extraction methods in all cases.

Toxicity. Values of solvents used shows that SHE method is the most harmful of methods evaluated, owing to the use of highly toxic solvents as methanol and chloroform which is disadvantageous for a large-scale processing without appropriate safety-based process design, extraction methods which uses hexane as solvent (CSE and HBE) presents the lowest toxicity. If is analyzed the toxicity parameter together with solvent recovery for studied methods, can be seen a disadvantage of performing this method frequently in lab-scale, by the release of high amounts of highly toxic solvents, requiring adequate facilities and protection, can be convenient to use SHE method once for an estimation of total lipid content of feedstock and used as reference. However, using an adequate large-scale process design which takes into account all safety aspects or appropriate assumptions, can be interesting the evaluation of this method. CSE presents higher solvent loses in comparison to HBE, however, in SCE case solvent is lost by continuous evaporation and condensation and for HBE, bulk of the solvent non-recovered is in mixture with algae meal after extraction, for this reason is recommendable a further drying of algae meal and condensation of vapours released for a more effective hexane recovery.

Energy Requirements. it can be seen that lower energy requirements corresponds to SHE method followed by HBE/CBE and highest energy requirements are presented by CSE method (Table 4.), this difference can be explained by the heating and cooling requirements that Soxhlet extraction system needs, extraction methods with high energy requirements must be discarded for a large scale microalgae processing if the final use of microalgae components is energetic, EHE method presents high energy requirements and low efficiency as is shown in previous section. When solvent recovery is considered for evaluation of oil extraction methods, is understandable that energy requirements increases, because an additional energy input is necessary for condensing the solvent separated from the lipid extract, and for separating solvent mixtures in methods where is required, in this scenario, method with higher energy requirements is EHE, for efficient first-step extraction with ethanol, recovered solvent must be separated from water added for phase separation, and hexane must be condensed after lipid extraction and separation.

Table 4. Comparison of oil extraction methods in lab-scale.

Extraction method	Cost of extraction [USD/g of biomass]		$LD_{50\%}$ [mg/kg]		Energy requirements [kWh]	Solvent recovered [%]	
	Basic	Solvent recovery	Basic	Solvent recovery	Basic	Solvent recovery	
SHE	0.28	0.18	**1194**	**1194**	0.72	1.59	**55**
EHE	0.11	0.04	10600	10600	1.75	**2.62**	85
CSE	1.90	1.90	28710	28710	**2.37**	2.37	80
HBE	0.18	0.05	28710	28710	1.51	2.26	85
CBE	**2.39**	**1.36**	6200	6200	1.51	2.26	85

Taking into account results obtained in Table 4, can be established that for a lab-scale microalgae oil extraction, method most convenient to perform is HBE, because its low energy consumption compared to other methods, low extraction costs and relatively low toxicity of solvent used, on the other hand, CBE method becomes non-convenient for oil extraction from microalgae due to its high cost of cyclohexane and high toxicity, in addition, lipid yield obtained with this method is similar to yields of HBE method.

Influence of solvent recovery on parameters evaluated

Solvent recovery plays an important role on selection of oil extraction methods for a large-scale processing and can change results obtained in lab-scale, is important to take into account that depending on the extraction method, bulk of the solvent must be recovered from the algae meal and/or from the lipid extract, and there is an amount of solvent which cannot be recovered, this affects negatively the impacts of method performing from the safety point of view, and the cost of extraction by including the costs of solvent recovery and input of fresh solvent for replacement of the non-recovered solvent, from the energy point of view, must be taken into account the energy consumption of solvent evaporation and condensation for re-use.

In SHE method, chloroform must be separated as from lipid extract as from algae meal, owing to low boiling point of this solvent and the high speed of homogenization which produces an increase of temperature of the extraction system, chloroform loses are significant (around of 50%vol), and after extraction, algal meal also contains a significant amount of solvent which is not recovered affecting safety of process and economics by fresh solvent requirements and commercialization potential of algal meal or utilization of algal meal for obtaining other bioproducts under biorefinery concept.

For EHE method, algal meal contains only ethanol, because there is no contact between hexane and biomass, which allows higher possibilities of further processing of algae meal without significant co-product purification, if is desired to convert meal carbohydrates into reducing sugars, can be used a organosolv pretreatment which includes ethanol with an acid for hydrolysis reaction, in this sense, is more convenient the EHE method in comparison to SHE method, hexane is also easily recovered from hydrophobic phase and can be used again for extraction decreasing processing costs.

In CSE method, as the solvent is continuously evaporated and condensed during extraction for effective lipid recovery, this continuous reflux increases solvent loses during extraction process, and is more significant at long extraction times, issue that is characteristic of this method. On the other hand, if the extraction process is stopped when the amount of solvent in contact with biomass is minimum, cost of processing will decrease by more solvent recovery and further processing of algal meal for obtaining other products will be chapter. By the nature of the process, solvent separation from lipid extract can be performed in the same extraction system, which is a benefit in lab-scale, but difficult to achieve in large scale without additional equipment.

For the cases of HBE and CBE methods, separation of solvent from biomass is difficult with loses of biomass/solvent mixture during the process, however, this disadvantage can be avoided in large-scale with appropriate equipment, for CBE extraction, there is a higher impact derived of solvent loses from the safety point of view, despite amount of solvent recovered is similar to HBE extraction, lower $LD_{50\%}$ makes more dangerous the exposition to solvent vapours. Solvent loses in CBE also impacts strongly in operating costs of extraction owing to high cost of cyclohexane, in lipids-solvent separation for both methods, no significant hexane/cyclohexane loses are presented.

Morphological response by strain to oil extraction methods

Guinardia sp. Morphological comparison of a microalgae strain to all oil extraction methods performed was made using the strain *Guinardia* sp. (Figure 2), when this microalgae is submitted to SHE extraction the cell shape is strongly affected and broken, can be seen pieces of frustules, free chloroplasts and other fragments of totally destroyed cells (Figure 2b), cells after EHE method keeps still their frustules, the only significant change observed by optic microscopy is related to the shape of the strain, all cells individually observed keeps their two chloroplasts within the cell wall (Figure 2c), with performing of CBE extraction can be observed cell disruption in several cells and absence of lipid drops which were extracted by cyclohexane in higher percentage than other methods (Figure 2d), microalgae exposed to HBE method showed a change in cell shape and cell disruption in high percentage evidenced by the

presence of free chloroplasts, in come cells there was not disruption but inner metabolites looks disordered dislocated (Figure 2d), finally, when microalgae strain is submitted to CSE method there is a higher percentage of non-broken cells, however, this method presented the higher relative efficiency, this behaviour can be explained because CSE method does not use mechanical or magnetic stirring, for this reason the possibility of cell rupture by mechanical action is lower, but solvent can remove lipid components going across the damaged cell wall (Figure 2f).

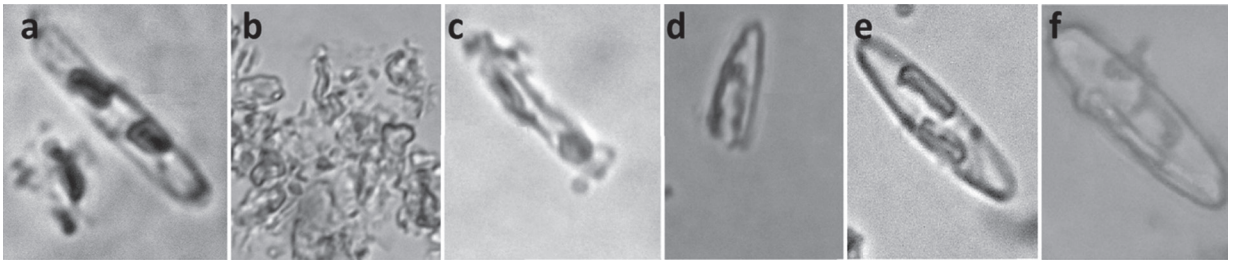

Figure 2. Morphological response of *Guinardia* sp. strain to SHE oil extraction method (b) EHE method (c), CBE method (d), HBE method (e) and CSE method (f) in lab-scale. Left-side image correspond to cells before oil extraction (a).

***Amphiprora* sp**. After observation of cells before extraction process can be seen that *Amphiprora* sp. strain presents an irregular shape which is not common in diatoms (Figure 3. a), this phenomenon can be derived of previous stages of microalgae biomass production chain as drying, in which some cell wall components can be degraded because of high temperature used for this step.

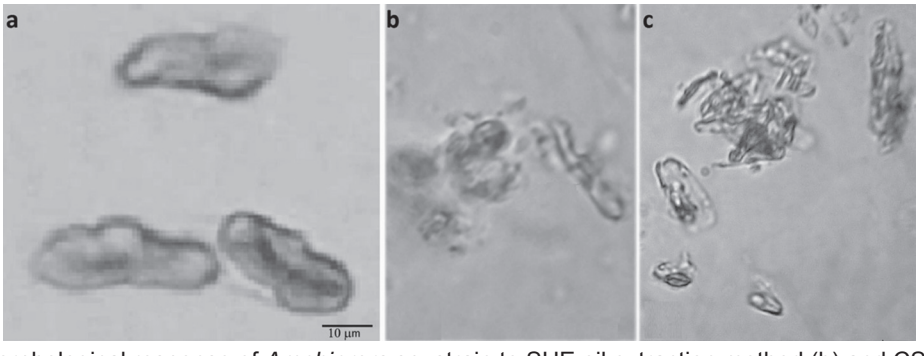

Figure 3. Morphological response of *Amphiprora* sp. strain to SHE oil extraction method (b) and CSE (c) in lab-scale. Left image correspond to cells before oil extraction (a).

After performing SHE extraction using this biomass (Figure 3b), can be observed significant changes in the morphology of the cell as the presence of chloroplast outside of the cell and changes in shape and colour of the cell, this changes are promoted by two main factors, mechanical destruction by high speed homogenization and effectiveness of solvents mixture used for microalgae compounds removal, however, degree of cell destruction confirms the low selectivity of SHE method for extraction of lipids usable in biodiesel production. When biomass is submitted to CSE method can be seen that microalgae cell wall is still present although is drastically deformed and damaged, is also shown that most of intracellular content including lipids was released, hexane could break through the degraded cell wall dissolving neutral lipids and other non-polar components (Figure 3c).

***Navicula* sp.** For *Navicula* sp. microalgae biomass can be seen that morphology of the cell is not affected by previous drying step (Figure 4a), this is due to the thickness of the microalgae frustule, which protects the cell from external damage factors. After oil extraction using EHE method (Figure 4b), can be still found cells without damage and other with most of metabolites present within the cell, this morphological response helps to explain the low efficiency of EHE method in

comparison to other microalgae oil extraction methods evaluated, Figure 4c shows microalgae biomass after performing HBE method where can be seen a higher percentage of broken cell walls in comparison to EHE method, can be observed several chloroplast outside of the cell which means that metabolites were released, but were not dragged by the solvent, behaviour of microalgae biomass after CBE method performing was very similar (Figure 4d), this observation confirms the selectivity of non-polar solvent based extraction methods to microalgae lipids.

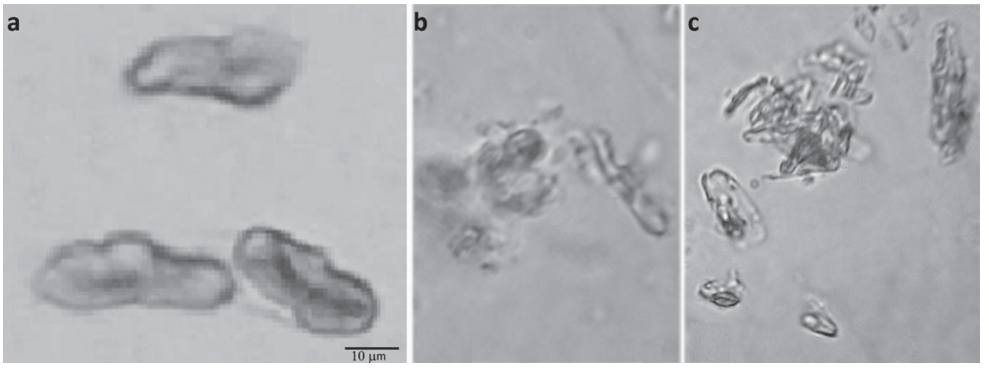

Figure 4. Morphological response of *Navicula* sp. strain to EHE oil extraction method (b) HBE method (c) and CBE method (d) in lab-scale. Left image correspond to cells before oil extraction (a).

Conclusions

Extraction method showed different yields depending on microalgae strain evaluated, for all cases, variation of oil yield and oil extraction efficiency as function of microalgae strain used for evaluation is an important issue to consider, because a large scale extraction method must show high yields for several strains, this can depend on nature of microalgae strain and/or cultivation, harvesting and drying conditions, *Amphiprora* sp. presented the highest oil yield of strains evaluated for all five extraction methods, followed by *Navicula* sp., this can be explained because both strains belongs to the same order (Naviculales), with similar cell walls and compositions as is shown in Table 1. On the other hand, *Nannochloropsis* sp. presented the lowest oil yield for all methods studied, which is not consistent with literature, inferring that a previous biomass processing stage could decrease and/or degrade neutral lipid percentage of strain. Taking into account biomass composition, morphologic response and oil yield, microalgae genera *Amphiprora* sp. emerges as a potential strain for the development of a topology of biorefinery.

SHE method shows the highest yield as result of combination of polar and non-polar solvents, as disadvantage presents the extraction of non-desirable lipids for biodiesel production, as sterols, pigments and other non-lipid metabolites, taking into account that, in lab-scale is convenient the utilization of this method for total lipid determination in non-characterized strains, however, overestimation of lipid percentage derived of extraction of other microalgae metabolites must be taken into account, in addition, SHE method presents the highest toxicity and lowest percentage of solvent recovery of methods evaluated, which makes expensive and risky the continuous utilization of this method even with solvent recovery strategies.

Statistical comparison showed that there is no significant differences between C6-based extraction methods (CSE, HBE and CBE) for most of strains studied, taking into account lipid extraction efficiency criteria, then, is convenient to choose only one of these methods for application in lab scale and evaluation as emerging technology in large scale and for further synthesis of a microalgae-based biorefinery topology. CSE method shows good results in terms of efficiency, low toxicity and higher yields than other methods evaluated, besides, selectivity of hexane to neutral lipids usable for biodiesel production promotes its inclusion in a microalgae based biorefinery. however, scaling-up of CSE could be not feasible in terms of energy requirements owing to energy input necessary for continue evaporation and condensation of solvent, HBE method also uses hexane and presents lower energy requirements than CSE for both scenarios evaluated, also presents lower costs of extraction and energy requirements in solvent recovery scenario than CSE, derived of lower biomass/solvent ratio, and higher amount of solvent recovered. For CBE

method in terms of technology implementation, the purchase of an expensive and more toxic solvent with similar yields and recovery percentage to hexane is not attractive in any scale. Taking into account all issues mentioned, HBE method is the most convenient for utilization in lab-scales under the criteria evaluated, also becomes as a promising alternative for scaling-up and further evaluation in a biorefinery superstructure.

Solvent recovery must be a mandatory parameter for performing solvent-based oil extraction methods in lab-scale, with benefits in all aspects evaluated in this work, in addition is a fixed stage in large-scale sustainable production processes. Deeper evaluation of methods evaluated in this work can be made from several points of view, authors are making parallel work in evaluation of promising oil extraction alternatives using process simulation and taking into account energy and environmental aspects by the use of methodologies of Life Cycle Assessment and Exergy Analysis, additional evaluations can be made from safety point of view such as methodologies of process optimization, mass and energy integration and experimental tests in pilot-plant scale can be also developed. Comparison can also be extended to wet-based supercritical and enzymatic oil extraction methods.

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Nomenclature

$C.I.$: Short name for Confidence Interval
C_{met}: Cost of application of certain method
C_{solv}: Cost of a specific solvent per volume units
E_{met}: Energy requirements of a given method
$LD_{50\%}$: Median Lethal Dose of a substance used as indicator of its acute toxicity
m: Number of solvents used performing a given method
m_e: Amount of extract obtained after carrying certain method
m_0: Initial amount of biomass subjected to extraction of certain specie
m_p: Amount of biomass obtained after pre-treatment
n: Number of equipment used to perform a given method
P_{eq}: Nominal electric power of equipment
RER: Short name for Relative Extraction Ratio
$Stdev$: Short name for Standard deviation
t_{eq}: Time of use of equipment
V_{solv}: Volume of solvent used in a given method
w_i: Variable weighting value assigned to particular criteria.

References

[1] Singh PN, Singh A. Production of liquid biofuels from renewable resources. Progr Energ Combust Sci. 2011;37(1):52-68.
[2] Chisti Y. A bioeconomy vision of sustainability. Biofuels Bioprod Biorefin. 2010;4:359–61.
[3] Lam MK, Lee KT. Microalgae biofuels: A critical review of issues, problems and the way forward. Biotechnol Adv. 2012;30(3):673-90.
[4] Khoo HH, Sharratt PN, Das P, Balasubramanian RK, Naraharisetti PK, Shaik S. Life cycle energy and CO_2 analysis of microalgae-to-biodiesel: Preliminary results and comparisons Bioresour Technol. 2011;102:5800-7.
[5] Oltra C. Stakeholder perceptions of biofuels from microalgae. Energy Policy. 2011;39(3):1774-81.
[6] UIS-ICP-Morrosquillo. Bioprospecting of Colombian microalgae for biodiesel production. Final Report, Bucaramanga, Colombia. 2011.
[7] Cherubini F. The biorefinery concept: using biomass instead of oil for producing energy and chemicals. Energy Convers Manage. 2010;51:1412–21.
[8] González-Delgado AD, Kafarov V. Microalgae based biorefinery: Issues to consider. CT&F Cien Tecnol Futuro. 2011;4(4):5-21.
[9] Singh J, Gu S. Commercialization potential

of microalgae for biofuels production. Renew Sustain Energy Rev. 2010;14:2596–610.
[10] González A, Kafarov V. Design of a multifunctional reactor for third generation biofuels production. Chem Eng Transact. 2010;21:1297-302.
[11] Henriques M, Silva A, Rocha J. Extraction and quantification of pigments from a marine: A simple and reproducible method. Commun Curr Res Educ Top Trends Appl Microbiol. 2007;1:586-93.
[12] Peñaranda LA, Sepúlveda KJ, Álvarez, YE, González-Delgado AD, Kafarov V. Evaluation of lipid and monosaccharide obtaining routes of microalgae biomass under the biorefinery concept. rev.ion. 2011;24(2):13-22.
[13] Wilkie A, Mulbry W. Recovery of dairy manure nutrients by benthic freshwater algae. Bioresour Technol. 2002;84(1):81-91.
[14] Harun R, Singh M, Forde G, Danquah M. Bioprocess engineering of microalgae to produce a variety of consumer products. Renew Sust Energ Rev. 2010;14:1037-47.
[15] Mussgnug J, Klassen V, Schlüter A, Kruse O. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. J Biotechnol. 2010;150(1):51-6.
[16] González-Delgado AD, Kafarov V. Design and adjustment of coupled microalgae oil extraction methods for the development of a topology of biorefinery. Prospectiva. 2012;10(1):113-23.
[17] McMillan JR, Watson IA, Ali M, Jaafar W. Evaluation and comparison of algal cell disruption methods: Microwave, waterbath, blender, ultrasonic and laser treatment. Appl Energy. 2013;103:128-34.
[18] Vanthoor-Koopmans M, Wijffels RH, Barbosa MJ, Eppink MHM. Biorefinery of microalgae for food and fuel. Bioresour Tech. 2013;135:142-9.
[19] González AD, Kafarov V, Guzmán A. Development of a methodology of microalgae oil extraction in the biodiesel from microalgae production chain. Prospectiva. 2009;7:53–60.
[20] Fajardo AR, Cerdan LE, Medina AR, Fernandez FGA, Moreno PAG, Grima EM. Lipid extraction from the microalga Phaeodactylum tricornutum. Eur J Lipid Sci Technol. 2007;109:120–6.
[21] Halim R, Danquah MK, Webley PA. Extraction of oil from microalgae for biodiesel production: A review. Biot Adv. 2012;30(3):709-32.
[22] Sari YW, Bruins ME, Sanders JPM. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. Indust Crops Prod. 2013;43(1):78-83.
[23] Halim R, Gladman B, Danquah MK, Webley PA. Oil extraction from microalgae for biodiesel production. Bioresource Technology. 2011;102(1):178–85.
[24] Liu C, Zheng S, Xu L, Wang F, Guo C. Algal oil extraction from wet biomass of botryococcus braunii by 1,2-dimethoxyethane. Appl Energy. 2013;102:971-4.
[25] Yoo G, Park W, Kim CW, Choi Y, Yang J. Direct lipid extraction from wet chlamydomonas reinhardtii biomass using osmotic shock. Bioresour Technol. 2012;123:717-22.
[26] Ehimen EA, Sun Z, Carrington GC. Use of ultrasound and co-solvents to improve the in-situ transesterification of microalgae biomass, Procedia Environ Sci. 2012;15:47–55.
[27] Peralta-Ruiz Y, González-Delgado A, Kafarov V. Evaluation of alternatives for microalgae oil extraction based on exergy analysis. Appl Energy. 2013;101:226-36.
[28] Brandenberger M, Matzenberger J, Vogel F, Ludwig C. Producing synthetic natural gas from microalgae via supercritical water gasification: A techno-economic sensitivity analysis. Biomass Bioenergy. 2013;51:26-34.
[29] El-Halwagi AM, Rosas C, Ponce-Ortega JM, Jiménez-Gutiérrez A, Mannan MS, El-Halwagi MM. Multiobjective optimization of biorefineries with economic and safety objectives. AIChE J. 2013; In press.
[30] García J, Miranda J, González A D, Kafarov V. Comparison of microalgae oil extraction methods for biodiesel production. In: Barajas A & Gonzalez A. Editors. IV International Congress of Biofuels Science and Technology CIBSCOL. 2010 nov-dec 30-03. Bucaramanga, Colombia. ISBN: 978-958-44-8185-6; 2010. p. 81.
[31] Uitenbroek DG. SISA Binomial. (internet website). Southampton: D.G. Uitenbroek 1997. Available in: http://www.quantitativeskills.com/sisa/distributions/binomial.htm. Accessed on July 2th 2013.
[32] Archanaa S, Moise S, Suraishkumar GK. Chlorophyll interference in microalgal lipid quantification through the bligh and dyer method. Biomass Bioenergy. 2012;46:805-8.