Diseño y ajuste de métodos acoplados de extracción de aceite de microalgas para el desarrollo de una topología de biorefinería

Design and adjustment of coupled microalgae oil extraction methods for the development of a topology of biorefinery

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Recibido 29/11/11, Aceptado 22/05/2012

RESUMEN

La biomasa de microalgas tiene el potencial de producir una gran cantidad de biodiesel por unidad de área debido a su alto contenido lipídico. Para logar una producción sostenible de biodiesel a partir de microalgas, es necesario tener en cuenta el concepto de biorefinería, este concepto puede ser aplicado a la biomasa de microalgas para la obtención de biocombustibles y otros productos de alto valor agregado. La extracción del aceite de microalgas es un paso importante para el desarrollo de una topología de biorefinería. En este trabajo, tres métodos acoplados de extracción de aceite de algas fueron diseñados y ajustados a condiciones locales. Para los experimentos de disrupción celular, el efecto del autoclave, hidrólisis y pretratamiento organosolv sobre la eficiencia de extracción fueron evaluados, así como tres metodologías de extracción basadas en solventes orgánicos fueron diseñadas y ajustadas, extracción con la mezcla etanol/hexano, extracción con solvente de reflujo continuo y extracción con solventes asistida con homogenización a alta velocidad.

Las cepas utilizadas fueron *Closterium* sp., *Navicula* sp., *Amphiprora* sp., *Guinardia* sp., *Botryococcus* sp., *Desmodesmus* sp. y *Tetraselmis* sp., los resultados muestran que la hidrólisis y el pretratamiento organosolv como etapas de disrupción celular son las que mas incrementan la eficiencia de la extracción, mientras que para la extracción con solvente de reflujo continuo los mejores resultados son obtenidos al utilizar hexano durante 8 horas y para la extracción con solventes asistida con homogenización a alta velocidad las mayores eficiencias se obtienen a bajos tiempos y frecuencias se 5000 rpm.

Palabras clave: Microalgas, Biocombustibles, Biorefinería, Disrupción celular, Extracción de aceite

ABSTRACT

Microalgae have the potential to produce a big amount of biodiesel per area unit owing to its high lipid content. However, for a sustainable biodiesel production from microalgae, the concept of biorefinery must be taken into account; this concept can be applied to microalgae biomass for the production of biofuels and high added value products owing to different components present in microalgae strains. Microalgae oil extraction is an important step to take into account for the development of a topology of biorefinery. The main objective of this work is to establish different coupled methods for cell disruption and oil extraction of bioprospected microalgae. Three methods were designed and adjusted to local conditions. For cell disruption experiments, effect of autoclave, hydrolysis and organosolv pretreatment on the increase of extraction efficiency was evaluated. For oil extraction, three solvent based methodologies were developed, ethanol/hexane mixture, continuous reflux solvent extraction and solvent extraction with high speed homogenization.

Microalgae used in experimental development were *Closterium* sp., *Navicula* sp., *Amphiprora* sp., *Guinardia* sp., *Botryococcus* sp., *Desmodesmus* sp. and *Tetraselmis* sp. microalgae strains. Cell disruption procedures that presents better results were acid hydrolysis and organosolv pretreatment, best results for continuous reflux solvent extraction were reached when extraction time was 8 hours and hexane is used as extraction solvent, while solvent extraction with high speed homogenization shows higher oil yields when are used times around 14 minutes and frequencies of 5000 rpm.

Keywords: microalgae, biofuels, biorefinery, cell disruption, oil extraction

1. INTRODUCTION

Microalgae has the potential to produce a big amount of biodiesel per area unit owing to its high lipid content which exceeds lipid content of all biodiesel sources used currently [1], in addition, microalgae are cultivated in photobioreactors and open ponds which only needs water, some nutrients and sunlight to stimulate growing, these culture conditions makes feasible the using of non-crop lands for photobioreactor or open pond assembly. The use of microalgae for biodiesel production is an advantageous alternative because of the high lipid content and fatty acid profiles that suitable offers.

The biorefinery concept has been identified as the most promising way for the creation of an industry based on biomass. This concept can be applied microalgae biomass for the production of biofuels and high added value products based on the composition of 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 [2].

Studies about microalgae oil extraction for biodiesel production are taking significance because the efficiency of biodiesel production chain from microalgae depends in a great way of the oil extraction efficiency. In previous works, authors explain several physical, chemical and enzymatic microalgae oil extraction methods [3]. Solvent-based lipid extraction methods as Folch, and Bligh and Dyer's method [4], has been used for obtaining lipids from microalgae. Using a mixture hexane-ethanol, can be extracted around of 80% of fatty acids presents into biomass [5], hexane is frequently used for soxhlet extraction using microalgae biomass as a raw material [6], hexane is cheap, easy to recovery after extraction and is selective to neutral lipids, Ethanol with acid has been used for simultaneous cell disruption and lipid extraction using microalgae strains *Amphiprora* sp. and *Navicula* sp. [7].

The main objective of this work is to establish different solvent based high detailed methodologies for the cell wall disruption and lipid extraction of microalgae for the development of a topology of biorefinery through the evaluation of operating conditions for each step in each method, after that, best operating conditions as cell disruption as solvent extraction are assembled and adjusted to a coupled extraction method for future integration in a microalgae based biorefinery concept.

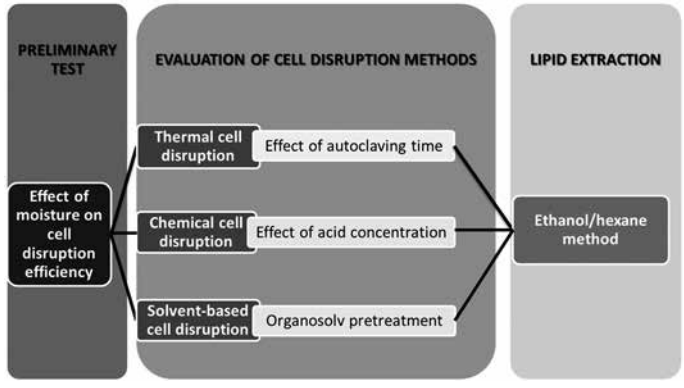
2. METHODOLOGY

Microalgae biomass was provided by Corporación Instituto de Morrosquillo (Punta Bolivar, Colombia), algae was cultured in F/2 medium, grown in open ponds and harvested by flocculation (150 ppm $FeCl_3$), biomass was sun-dried and frozen until using.

2.1 Cell disruption experiments

General methodology for cell disruption experiments is shown in Figure 1, all raw materials supplied were dried in a convection digital oven (make WTC binder) at 105 °C for 4 hours, based on the standard NREL/TP-510-42621, and then, the sample was homogenized. A 25 L Autoclave was used for thermal cell disruption experiments, microalgae biomass was exposed at autoclaving conditions of 394.15 K and 103410 Pa. by 1 and 3 hours.

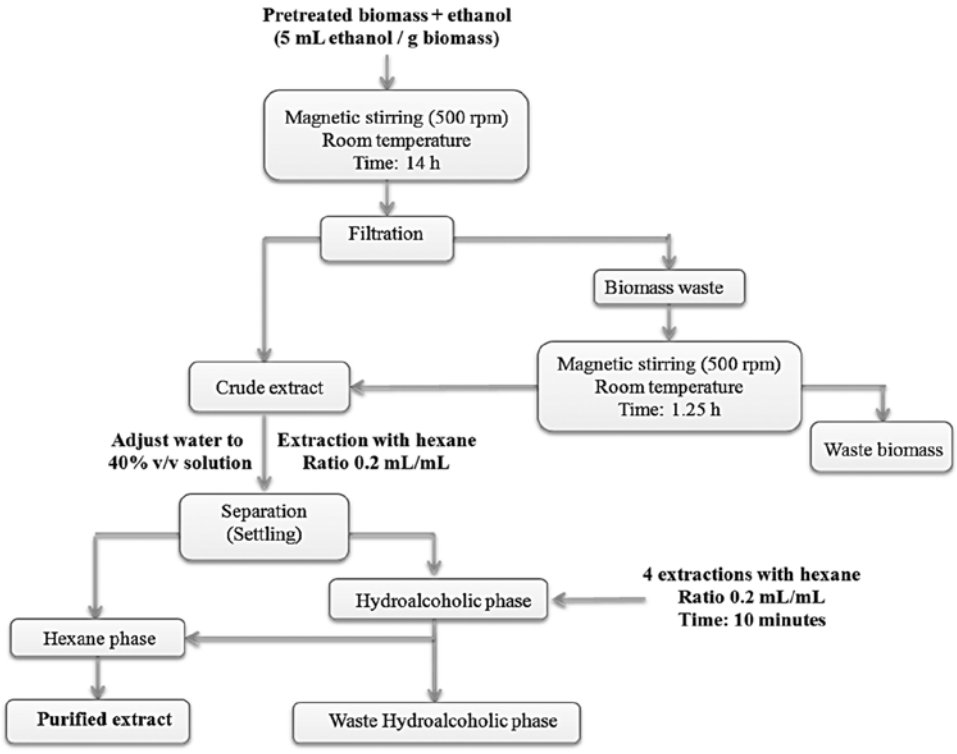
Figure 1. Methodology for evaluation of cell disruption methods using microalgae biomass of *Amphiprora* sp.
Figura 1. Metodología para la evaluación de métodos de disrupción celular utilizando biomasa de la microalga *Amphiprora* sp.



For solvent-based cell disruption, an organosolv pretreatment previously developed by authors was performed using a mixture of water, an organic solvent and an acid at high temperatures [8]. For cell disruption using acid hydrolysis, microalgal biomass were dried in an oven at 378 K for 4 hours, after that, 3g of biomass were mixed with different HCl solutions at concentrations of 0.1 mol L^{-1}, 0.5 mol L^{-1}, 1 mol L^{-1} and 3 mol L^{-1} with an exposure time of 0.5 hours with magnetic stirring at room temperature. Solid and liquid phases were separated by filtration and biomass is washed with distilled water, biomass was dried and submitted to lipid extraction, all cell disruption experiments were made by triplicate.

After pretreatment, biomass was separated from the liquor by vacuum filtration. Separate biomass was washed with distilled water and dried in oven at 378.15 K for 4 hours. For measurements of the effect of pretreatments on oil yield, lipid extraction using the mixture ethanol/hexane method (EHE) described in Figure 2 was used, biomass was mixed with ethanol using a ratio of 1:5, mixture was stirred by 14 hours at 500 rpm. After that, the mixture was

Figure 2. Lipid extraction methodology for cell disruption experiments on microalgae biomass (EHE method).
Figura 2. Metodología para la extracción de lípidos en la evaluación de métodos de disrupción celular (método EHE).



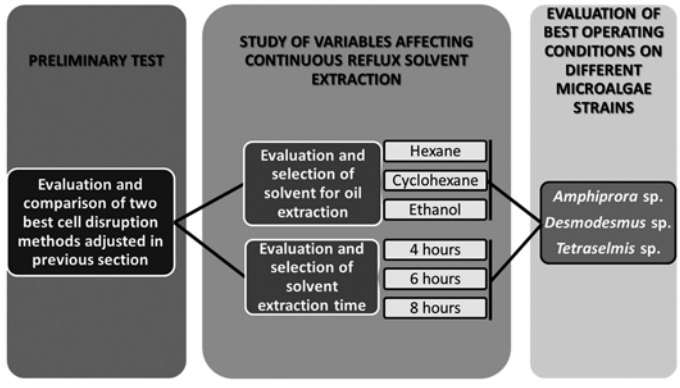
filtered and solid phase was stirred again with fresh ethanol, two liquid phases were combined, hexane and water were added for two liquid phases formation, the phases were separated and fresh hexane is added again to hydroalcoholic phase, this process is repeated two times, the four hexane phases were mixed and lipid extract is separates of hexane by distillation, The quantification of lipid extract was determined with the aim of evaluating the performance of the process and obtains an indirect measure of the effect caused by pretreatment of cells. Deeper explanation of development and results of this method can be seen in dissertation presented by Sarmiento y Amaya in which is based this subsection [9].

2.2 Continuous reflux solvent extraction (CRSE)

For continuous reflux solvent extraction evaluation, a typical Soxhlet extractor with 45/50 outer/upper and 24/40 lower/inner joint, for 250 mL capacity was used, each experiment was performed with 5 grams of dry treated biomass, three commonly used extraction solvents were evaluated; hexane, cyclohexane and ethanol. These solvents were chosen taking into account their low boiling point, costs, safety factors and toxicity. In the next phase, after selecting the cell disruption method and the solvent for lipid extraction, the extraction time was evaluated, using values of 4, 6 and 8 hours (based on literature review [6]).

During solvent extraction, the amount of biomass and the ratio biomass/solvent were kept constant. After extraction, extract-solvent mixture was filtered, distilled and the remnant solvent was evaporated. Total lipids were also quantified gravimetrically, in the final phase, the best experimental conditions for the oil yield were applied to the three genera studied, figure 3 shows the methodology proposed. Deeper explanation of development and results of this method can be seen in dissertation presented by Cordoba y Lopez in which is based this subsection [10].

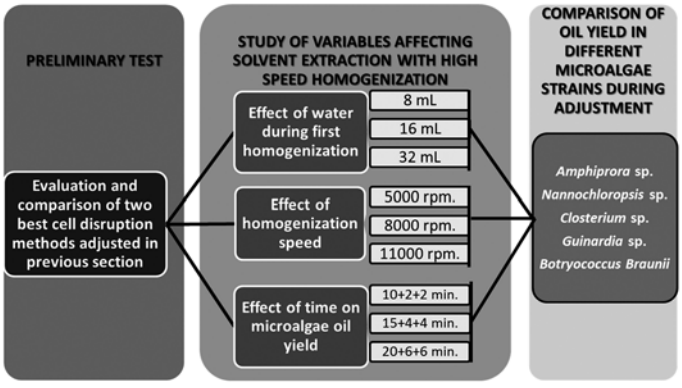
Figure 3. Methodology for continuous reflux solvent extraction adjustment (CRSE).
Figura 3. Metodología para el ajuste del método de extracción con solventes de reflujo continuo (CRSE).



2.3 Solvent extraction with high speed homogenization (SHE)

The solvent extraction method combined with high speed homogenization is based Folch and Bligh & Dyer's method, solvents chosen were methanol and chloroform, the methodology to adjust is shown in Figure 4, and includes the steps of stirring, centrifugation, separation and volatilization. In the stirring phase, two rates of biomass/ solvent were evaluated 1:10 and 1:20 based on preliminary test results, the effect of adding water in the first part of homogenization and the effect of time and frequency of homogenization, accord to an experimental design. Centrifugation was carried out for 15 minutes and it was assessed a frequencies of 2500 and 3400 rpm. The phase separation was performed by removing the upper phase methanol/water from the centrifuge tube while lower biomass/lipids Chloroform, was filtered by gravity. Finally, the lipid extract was allowed to volatilize to constant weight for its measurement.

Figure 4. Methodology for adjustment of Solvent extraction with high speed homogenization (SHE).
Figura 4. Metodología para el ajuste de la extracción con solventes asistida por homogenización a alta velocidad (SHE).



For each experiment, 5 g. of disrupted biomass were mixed with methanol and chloroform in a ratio 2:1 three homogenization frequencies were evaluated (5000, 8000 and 11000 rpm.) using a Heidolph® SilentCrusher homogenizer. variables were evaluated following a 2^2 central composite experimental design, phases were separated by centrifugation and filtration and lipids were recovered from chloroform phase by evaporation, statistical analysis of main effects was made using STATISTICA 7.0 software taking as a response variable the lipid extract yield concentration. Oil yield for all experiments was measured by gravimetric method. Each experiment was performed by triplicate in order to give reproducible results. Deeper explanation of development and results of this method can be seen in dissertation presented by González y Galindo in which is based this subsection [11].

3. RESULTS AND DISCUSSION

3.1 Design and adjustment of cell disruption methods

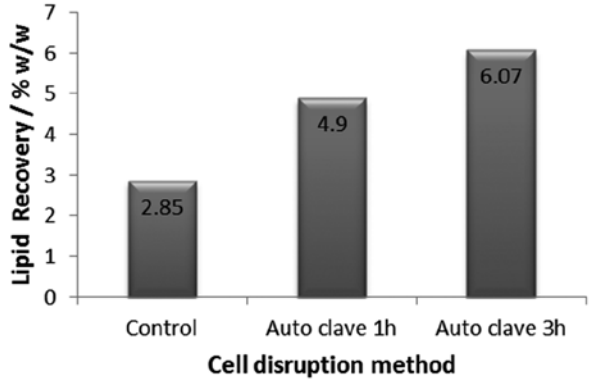
3.1.1 Effect of moisture on cell disruption efficiency. Table 1 shows the yields obtained from the lipid extraction process using wet and dry biomass. These tests were performed with a mixture of *Navicula* sp. and *Amphiprora* sp., it is shown that the water content in the sample is not favorable for the extraction of lipids due to two reasons; Presence of water in the sample decreases the concentration of ethanol in the biomass / solvent mixture during the first stage of the process, reducing the efficiency of solvent extraction of crude oil.

Table 1. Effect of moisture on cell disruption: L_R: (%) lipid recovery in the total biomass; Y: (%) losses of biomass by handling (strains: mixture of *Navicula* sp. and *Amphiprora* sp.).
Tabla 1. Efecto de la humedad sobre la disrupción celular de microalgas: L_R: (%) porcentaje de lípidos recuperados; Y: (%) perdidas de biomasa por manipulación (cepas utilizadas: mezcla de *Navicula* sp. y *Amphiprora* sp.).

Moisture (%)	Extracts Weight (g)	Standard deviation	L_R (%)	Y (%)
80	0.0337	0.0028	1.5	30.8
>5	0.0629	0.0033	2.8	9.4

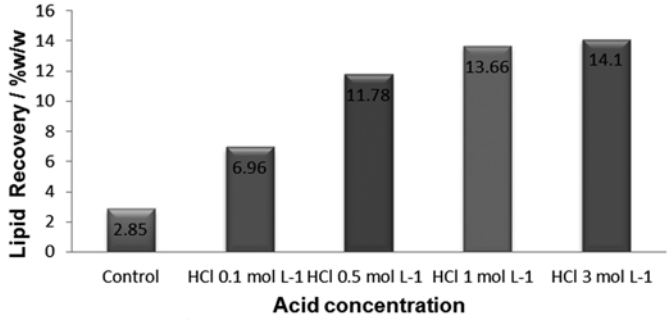
3.1.2 Effect of autoclaving time. Thermal pretreatment results for microalgae strain *Amphiprora* sp. are shown in Figure 5. Although cell disruption process shows a significant increase in the recovery rate of lipids for an autoclaving time of 3 hours, failed to overcome any of the results obtained with the other pretreatments. The recovery percentages for autoclave times evaluated do not differs more than 1.2 % w/w despite increased exposure time to 2 hours. This allows inferring that long times represents large and unnecessary energy expenditure.

Figure 5. Effect of autoclave time on the recovery rate of lipids for microalgae strain *Amphiprora* sp. **Figura 5.** Efecto del tiempo de autoclave sobre la separación de lipidos utilizando la cepa *Amphiprora* sp.



3.1.3 Effect of HCl concentration. Effects of hydrochloric acid concentration on the extraction yield were also evaluated, using concentrations of 0.1 mol L^{-1}, 0.5 mol L^{-1}, 1 mol L^{-1} and 3 mol L^{-1}. Figure 6 shows that the extraction yield increases when acid concentration is also increased within the range set but at concentrations higher than 0.5 mol L^{-1}, this effect is less pronounced with a tendency to stabilize.

Figure 6. Effect of acid concentration on the recovery rate of lipids using microalgae biomass of *Amphiprora* sp.
Figura 6. Efecto de la concentración de acido sobre la recuperación de lipidos utilizando la biomasa de la microalga *Amphiprora* sp.



Cell disruption with HCl 3 mol L^{-1} presents the highest percentage of lipids recovery, however, this concentration involves the increase of acid amount several times for very little yield increase in comparison with the oil yield obtained with an acid concentration of 0.5 mol L^{-1}, corresponding to 11.78 % w/w. In addition, higher concentrations of hydrochloric acid might increase the levels of corrosion in the equipment involved throughout the process. Therefore, a solution of 0.5 mol L^{-1} hydrochloric acid was the most suitable for pretreatment of biomass, 250 % decline in spending on chemical agent worked to the maximum concentration, without affecting performance deeply.

3.1.4 Organosolv Pretreatment. Results obtained of applying organosolv pretreatment to microalgae biomass of *Amphiprora* sp. are shown in table 2, although organosolv pretreatment increased the recovery rate of lipids in more than 3 % w/w over 3 hours of autoclave treatment, did not surpass the results obtained with HCl 0.5 mol L^{-1}. In addition, this pretreatment involves high energy costs, a longer exposure time and use of more chemicals making it inconvenient to use as pretreatment method prior to cell disruption with ethanol-hexane if the only one product desired is microalgae crude oil.

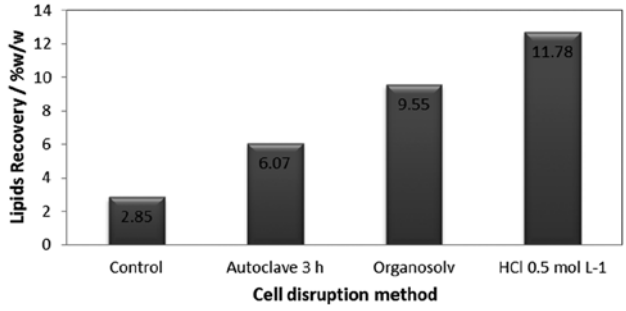
3.2 Comparison of cell disruption methods

Values obtained in the response variable (L_R) shows clearly that the chemical treatment with HCl 0.5 mol L^{-1} and organosolv pretreatment gives the highest oil yield when ethanol/hexane method is used for microalgae oil extraction (Figure 7).

Table 2. Organosolv pretreatment results for *Amphiprora* sp. strain: L_R: % w/w lipid recovery in the total biomass; L_R: % w/w % w/w Biomass unrecovered.
Tabla 2. Resultados del pretratamiento organosolv aplicado a la microalga *Amphiprora* sp. L_R: % p/p de recuperación de lípidos, L_R: % p/p de biomasa no recuperada.

Pretreatment	L_R (%)	B_N (%)
Organosolv	9.55	71.6

Figure 7. Comparison of microalgae biomass cell disruption methods for *Amphiprora* sp. biomass.
Figura 7. Comparación de los métodos ajustados de disrupción celular utilizando la microalga *Amphiprora* sp.



Method selected for oil extraction in this section presents lower yields compared to traditional procedures for the recovery of lipids, but the product obtained is mainly composed of neutral lipids due to the selectivity of hexane, this being the most suitable fraction for later processes of esterification, transesterification or hydro-treatment, taking into account this results, acid hydrolysis and organosolv pretreatment were taken into account as cell disruption methods in further sections of this work.

3.3 Adjustment of continuous reflux solvent extraction coupled with cell disruption (CRSE)

Best cell disruption methods obtained in previous section were applied to microalgae biomass and a continuous reflux solvent extraction was applied for lipids recovery. Highest oil yield was obtained with organosolv pretreatment (6.8%) in comparison with HCl 0.5 mol L^{-1}. In addition, when organosolv pretreatment was used, the oil yield was increased three times in comparison with the control. This difference can be attributed by the degree of hydrolysis of the cellulosic cell wall components of microalgae according to each disruptor agent and operation conditions of treatment. Then, the lipids are exposed to higher or lower proportion to the solvent extraction and the oil yield is affected. Efficiencies of the extraction process using cell disruption methods are shown in Table 3, it can be seen also that all extraction efficiencies using continuous reflux solvent extraction are higher than efficiencies obtained using ethanol/hexane method.

Table 3. Comparison of best adjusted cell disruption methods using continuous reflux solvent extraction (CRSE) for microalgae biomass of *Amphiprora* sp.
Tabla 3. Comparación de los mejores métodos ajustados de disrupción celular utilizando extracción con solventes de reflujo continuo (CRSE) en biomasa de la microalga *Amphiprora* sp.

Cell disruption method	Extraction efficiency (%)	Standard deviations
Control	18.0	2.4947
Organosolv	56.5	2.5495
Hydrochloric acid 0.5 mol L^{-1}	37.9	1.7802

3.3.1 Solvent Selection. By using hexane, cyclohexane and ethanol as solvents in extraction process, it was shown that the hexane presents higher loss of solvent. However, as evidenced in Figure 8, this solvent produced the greatest oil yield (6.8%) relative to cyclohexane (3.2%) and ethanol (2.3%). It is also the cheapest solvent of the three tested, also is selective to neutral lipids and commonly used in solvent extraction processes chemicals. Besides, when performing the extraction with cyclohexane was obtained the second highest oil yield (3.2%), but this is the solvent most expensive of the three solvents studied.

Figure 8. Oil yield with different solvents for *Amphiprora* sp. microalgae biomass.
Figura 8. Extracción de lípidos utilizando diferentes solventes para biomasa de la microalga *Amphiprora* sp.

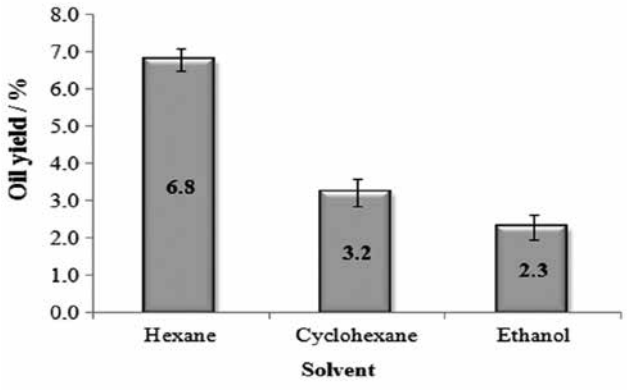


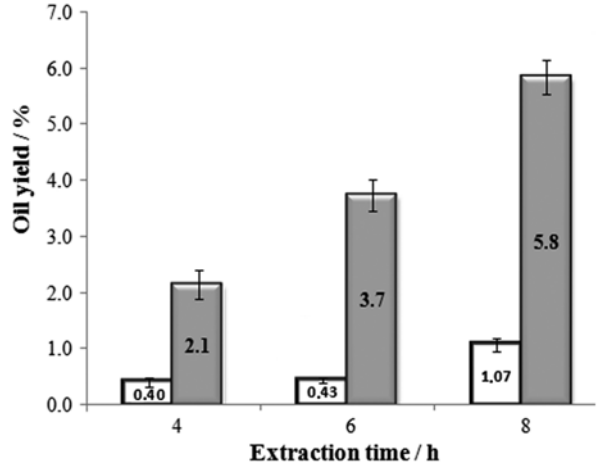
Table 4. Effect of solvent on extraction efficiency using microalgae strain *Amphiprora* sp.
Tabla 4. Efecto del solvente sobre la eficiencia de extracción utilizando la microalga *Amphiprora* sp.

Solvent	Extraction efficiency (%)	Standard deviations
Hexane	56.5	2.5495
Cyclohexane	26.9	3.0353
Ethanol	19.3	2.7426

Ethanol is known to be a good solvent for extraction, but its selectivity towards the lipids is relatively low compared with hexane and cyclohexane, and it is necessary to perform a purification process (e.g. treating the crude extract with non-polar solvents) to obtain the extracts. Ethanol had the lowest oil yield (2.3%). Also, as shown in Table 4 with the use of hexane was achieved, the highest extraction efficiency (56.5%) is reached, and solvent hexane shown higher reproducibility of the data according to the standard deviation calculated.

3.3.2 Effect of extraction time. Effect of extraction time is observed clearly in Figure 9, when the contact time between solvent and biomass was increased, there was a significant impact on the oil yield, because it promotes the mass transfer of lipid components into the solvent, reaching a higher oil yield (5.8%) when the sample was extracted for eight hours and with an increasing trend for higher times. In the same way when compared the results with (dark bars) and without cell disruption method (white bars), there was an increase of more than five times in the oil yield, for all operation times evaluated.

Figure 9. Effect of extraction time on *Amphiprora* sp. microalgae oil yield.
Figura 9. Efecto del tiempo sobre la extracción de lípidos en la microalga *Amphiprora* sp.



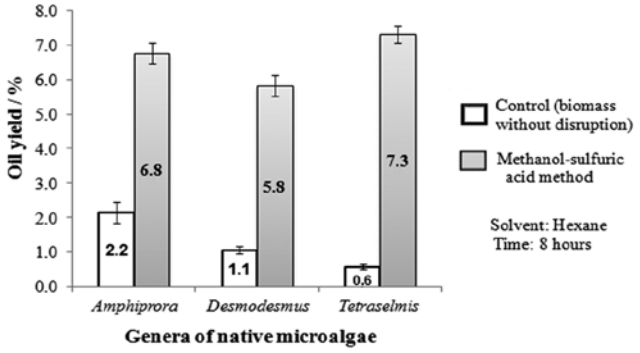
Furthermore, the extraction time of eight hours produced the best extraction efficiency of 53.6% as is reported in Table 5.

3.3.3 Best experimental conditions obtained. When the best experimental conditions according to the higher oil yield (organosolv pretreatment as cell disruption method, hexane as solvent and 8 hours of operation time) were applied, results obtained were the shown in Figure 10 for the three genera studied.

Table 5. Lipid extraction efficiency in relation to operation time for *Amphiprora* sp. strain.
Tabla 5. Eficiencia de extracción de lípidos en relación al tiempo para la microalga *Amphiprora* sp.

Cell disruption method	Extraction time (h)	Extraction efficiency (%)	Standard deviations
Biomass without disruption (control)	4	3.7	0.8325
	6	3.9	0.3648
	8	9.8	0.9874
Biomass with organo-solv pretreatment	4	19.6	2.3862
	6	34.3	2.5732
	8	53.6	2.7439

Figure 10. Oil yield with the best (CRSE) conditions for the three genera of microalgae (*Amphiprora, Desmodesmus* and *Tetraselmis)*.
Figura 10. Aplicación del método de extracción (CRSE) ajustado a tres géneros de microalgas (*Amphiprora, Desmodesmus* y *Tetraselmis)*.



These results confirm the advantage of applying a cell disruption method before extraction process, it can achieve significant increases in the oil yield for biomass without disruption, and increments of three, five and twelve times oil yield for genera *Amphiprora, Desmodesmus* and *Tetraselmis* respectively. Also as shown in Table 6, for all genera of microalgae was obtained a superior process efficiency to 50% using the best conditions of the variables analyzed, getting the highest value 57.6% for the genus *Tetraselmis*.

Table 6. Oil extraction efficiency with adjusted (CRSE) method for several microalgae strains.
Tabla 6. Eficiencia de extracción de lípidos con el método (CRSE) ajustado para varias cepas de microalgas.

Strain	Extraction efficiency (%)	Standard deviation
Amphiprora sp.	56.5	2.5495
Desmodesmus sp.	53.6	2.7439
Tetraselmis sp.	57.6	1.8656

3.4 Adjustment of solvent extraction with high speed homogenization (SHE)

In the stirring stage, when the biomass/solvent ratio 1:10 was initially evaluated, there was no lipid extract obtained because the rate of volatilization of chloroform was higher than the rate of filtration of the mixture, leaving all biomass retained in the filtration stage. While performing the extraction at a biomass/solvent ratio of 1:20 this problem was overcome and it was decided to maintain this ratio for further experiments. On the other hand, in the stage of centrifugation, when the frequency was adjusted according with literature in 2500 *rpm*, there was no a complete separation of the solvents mixture, for this reason, centrifugation frequency was increased to 3400 *rpm*, in this case, it was identified the biphasic system composed by a methanol and water in the upper phase and lower chloroform-lipids -biomass. Therefore, for the development of the extraction method, centrifugation stage was tuned in 3400 *rpm* during a time of 15 minutes.

3.4.1 Effect of cell disruption. Given that cell wall of microalgae is destroyed by the degradation of the polysaccharides present in biomass, and these and other components of the solid matrix are soluble in liquor of hydrolysis, a large percentage of the biomass subjected to the cell disruption process becomes part of the liquor, reducing the biomass used for extraction.

Figure 11. Effect of cell disruption on the percentage of extraction for. a) *Guinardia* sp. b) *Amphiprora* sp.
Figura 11. Efecto de la disrupción celular sobre el porcentaje de extracción para a) *Guinardia* sp. b) *Amphiprora* sp.

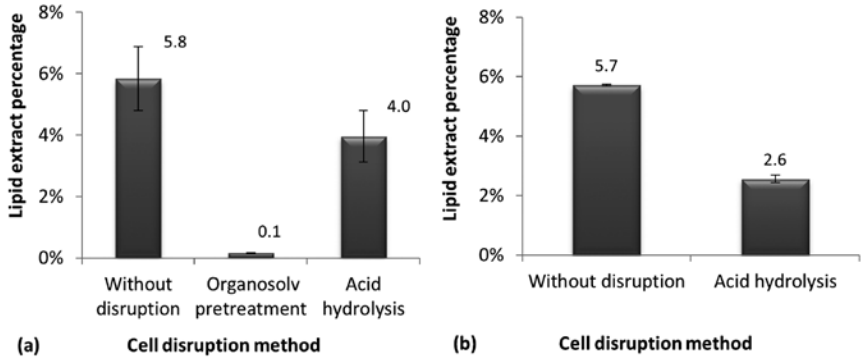


Figure 11a shows that acid hydrolysis and organosolv pretreatment did not increase the percentage of lipid extract in this extraction method. Based on these results, the organosolv pretreatment was discarded for further testing due to low percentages of lipid extracts obtained an the difficulty in the development of extraction. While the cell disruption with acid hydrolysis even when it reported a 32% decrease in performance continued to be the subject of study because it made easier the steps of centrifugation and filtration when extracting. After that, new tests were performed using acid hydrolysis in *Amphiprora* specie (Figure 11b) to verify that the negative effect of this method to other specie was still getting a 55% reduction in the yield of extraction.

The low extraction yields using biomass with cell disruption respect to biomass without cell disruption are due to the microalgae solvent extraction with high speed homogenization in particular, in addition to lipids, it also extracts significant amounts of non-lipid components. By previously applying cell disruption method this lipid components become part of hydrolysis liquor thus obtaining a purer lipid extract compared with the extraction using biomass without cell disruption. That is, the application of a cell disruption method allows obtaining purer extracts after lipid extraction performed with the solvent extraction with high speed homogenization, but decreases the percentages of extraction. For third method can be concluded that use of acid hydrolysis or organosolv pretreatment is not necessary because cell disruption is performed by the high speed homogenization process.

3.4.2 Effect of water addition during first high speed homogenization. The percentage of lipid extract obtained for two different microalgae genera with and without addition of water in the first part of the stage of agitation is shown in Table 7, where it is observer that the addition of water decreased the rate of extraction for *Amphiprora* sp., *Botryococcus* sp. and *Nannocloropsis* sp., by 15% and 40% respectively. This is because water is soluble in methanol and insoluble in chloroform and lipids, which affects the solubility of chloroform - methanol and make it difficult to extract lipids. Based on these results it was decided that to adapting the solvent extraction with high speed homogenization methodology to the extraction of lipids from microalgae biomass is not convenient to add water in the first part of the stirring.

3.4.3 Effect of shaking rate. In order to study the effect of shaking rate on extraction yield, cell disruption was performed by organosolv pretreatment and the extraction was carried out homogenizing the biomass/solvent mixture for 14 minutes at frequencies of 5000, 8000 and 11000 *rpm.*

Table 7. Effect of water addition in the first stirring step during the solvent extraction with high speed homogenization (SHE) for three microalgae strains.
Tabla 7. Efecto de la adición de agua durante la primera homogenización utilizando el método (SHE) para tres cepas de microalgas.

Microalgae genera	Water (*mL*)	Oil yield (%)
Amphiprora sp.	_	8.83
	8	8.19
Botryococcus sp.	_	5.60
	16	4.74
Nannocloropsis sp.	_	1.45
	32	0.89

Figure 12. Effect of shaking frequency on oil yield using *Amphiprora* sp. microalgae biomass.
Figura 12. Efecto de la frecuencia de agitación sobre la extracción de aceite utilizando biomasa de la microalga *Amphiprora* sp.

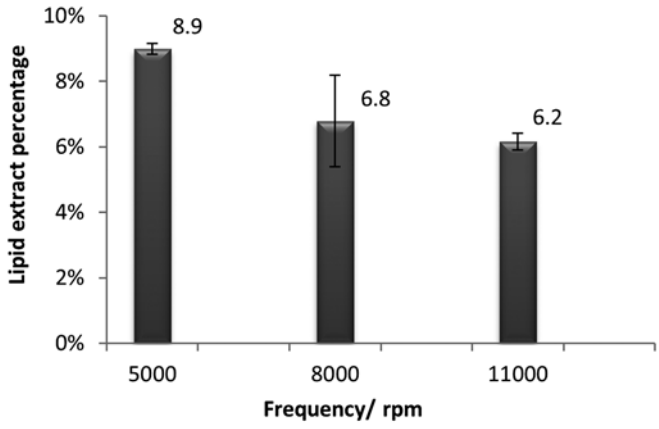


Figure 12 shows that an increase in the shaking frequency decreases the percentage of lipid extract, this result agrees with that reported by Cravotto et al [12], who evaluated the ultrasound-assisted extraction using frequencies between 19 and 300 *kHz* obtaining higher extraction yields at lower frequencies. For that reason it was proposed an experimental design in order to examine together the variables time and frequency of shaking.

The variables studied in the experimental design were: the total of homogenization time (*min*) and the frequency of shaking (*rpm*). Table 8 shows the values of the levels selected for each of the variables of experimental design.

The experimental design matrix and its respective percentages of lipid extract obtained are shown in Table 9. The best results are at the lowest level of each variable and are corresponding to experiments 1 and 6.

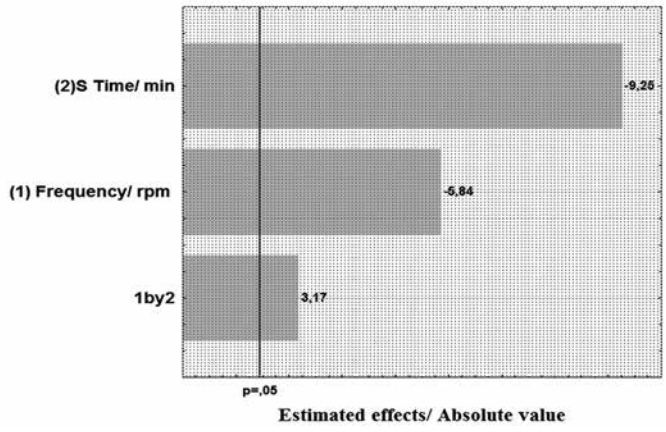
Table 8. Values and levels of the studied variables.
Tabla 8. Valores y niveles de las variables estudiadas.

Factor	Levels		
	-1	0	1
Time / *min*	14	23	32
Frequency / *rpm*	5000	8000	11000

Table 9. Experimental design matrix and oil yield obtained during extraction of microalgae oil from *Amphiprora* sp.
Tabla 9. Matriz del diseño experimental y porcentaje de aceite obtenido durante la extracción de aceite de la microalga *Amphiprora* sp.

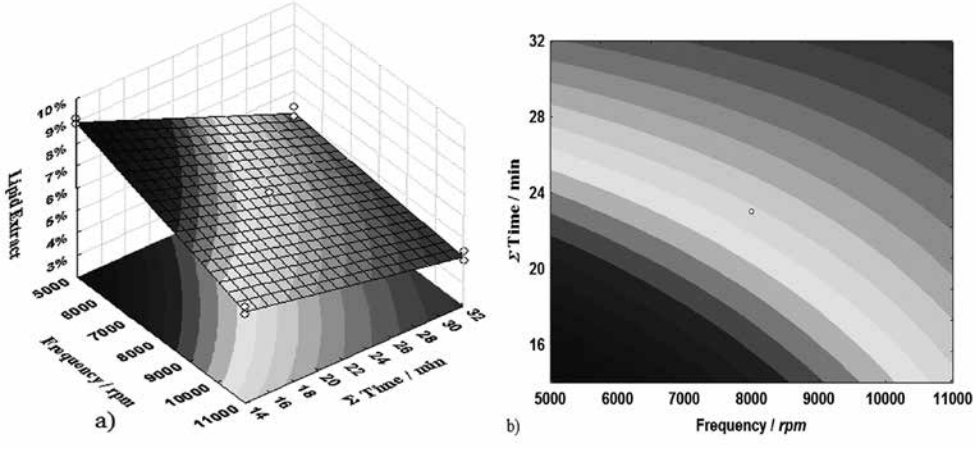
N° Experiment	Frequency	Time	Oil yield (%)
1	-1	-1	9.13
2	1	-1	6.34
3	-1	1	5.30
4	1	1	4.46
5	0	0	6.43
6	-1	-1	8.86
7	1	-1	5.98
8	-1	1	4.87
9	1	1	4.03
10	0	0	5.21

Figure 13. Effect of frequency and shaking time on the percentage of lipid extract from *Amphiprora* sp. strain.
Figura 13. Efecto de la frecuencia y el tiempo de agitación sobre el porcentaje de lipidos utilizando la microalga *Amphiprora* sp.



The Pareto's chart (Figure 13) shows that time, frequency and their interaction have significant effects on extraction, because all the blocks pass the threshold. In addition, can be inferred that the variables of time and frequency have negative effects on the performance of the extraction of lipids from microalgae, being the time the factor that mostly negatively affects the response variable. Finally, it should be noted that the combination of the independent variables has a positive effect on the response variable studied.

Figure 14. Effect of frequency and shaking time on the percentage of lipid extract for *Amphiprora* sp. microalgae. a) response surface plot b) contour diagram.
Figura 14. Efecto de la frecuencia y tiempo de agitación sobre el porcentaje de extracto lipídico para la microalga *Amphiprora* sp. a) diagrama de superficie de respuesta b) diagrama de contorno.



The interaction between time and frequency of homogenization can be seen in Figure 14 where the surface and the level curve show a region with the higher (bottom right side) and another with a lower percentage of lipid extract (upper left side). Finding then that the percentage of lipid extract is maximized when the variables of time and shaking are found on the lowest level within the experimented region, i.e. 14 minutes and 5000 *rpm.*

3.4.4 Extraction efficiency. The extraction efficiency is shown in Table 10. The *Nannocloropsis* genera presented the lowest yield, *Closterium*, and *Botryococcus* approached a yield of 50% and best results were obtained *Amphiprora* and *Guinardia* strains. This high performance was due to the rapid separation of the lipid extract and the solid in the filtration stage. Are also shown the differences in the efficiencies with the adjusted method and operating conditions reported in literature.

Table 10. Extraction efficiency for several species of microalgae using SHE method: [a, b,c] base extraction method, [d]method adjusted by authors.
Tabla 10. Comparación de la eficiencia de extracción para varias cepas de microalgas usando el método (SHE): [a, b,c] método de extracción base, [d]método ajustado por los autores

Species	Total lipids	Extracted Lipids	Efficiency
Nannocloropsis[a]	11	1.50	13%
Botryococcus braunii[b]	15	5.60	37%
Closterium[c]	22	9.10	41%
Amphiprora[d]	12	9.03	75%
Guinardia[d]	7	5.80	87%

4. CONCLUSIONS

- Three ways of microalgae oil extraction by combining cell disruption and solvent based lipid removal and recuperation were designed and adjusted, towards the development of a topology of biorefinery, different alternatives for microalgal biomass rupture were evaluated, with chemical cell disruption, the recovery rate of lipids was proportional to the concentration of hydrochloric acid within the range established for the pretreatment of biomass. However, an acid concentration of 0.5 mol L^{-1} was the most suitable for the cell disruption process, reducing by 250 % w/w reagent consumption compared to the maximum concentration worked, without significantly affecting the extraction yield, organosolv pretreatment also showed high efficiency on the increase of lipid yield for extraction methods without homogenization.
- Adjustment of continuous reflux solvent extraction also corroborate that the addition of a step of cell disruption before extraction process increases the efficiency of lipid extracted from microalgae, and the cell disruption using organosolv pretreatment (56.5%) was the most efficient. higher oil yield was reached using hexane as solvent and an operating time of eight hours, these conditions increased significantly the efficiency of the process (56.5% and 53.6% respectively). Furthermore, using the best experimental conditions, the extraction efficiency was over 50% for the algae strains *Amphiprora* sp., *Desmodesmus* sp. and *Tetraselmis* sp.
- For solvent extraction with high speed homogenization best operating conditions were: Biomass/ solvent ratio 1:20, homogenization frequency 5000 rpm, homogenization total time 14 minutes and centrifugation time of 3400 rpm by 15minutes. Moreover, the addition of water in the first part of stirring facilitated the filtration but decreased the percentage of oil extraction in a range between 15-40% . Use of acid hydrolysis or organosolv pretreatment is not necessary because cell disruption is performed by the high speed homogenization process.

ACKNOWLEDGMENTS

The authors thank to the Colombian Agriculture & Rural Development Ministry, project 2008D32006-6710 "Bioprospección de microalgas Colombianas para la producción de biodiesel" the Colombian Department of Science, Technology and Innovation COLCIENCIAS, project "creación y fortalecimiento de una red de transferencia de conocimiento y tecnología entre Estados Unidos y Colombia mediante el desarrollo de procesos de biorefinería para la obtención de biocombustibles y productos de alto valor agregado a partir de biomasa de microalgas", to Silvia González, Leislly Galindo, Luz Córdoba, Lina López, Ronald Sarmiento, Andrés Amaya y Javier Anaya for cooperation in experimental development and the Colombian Petroleum Institute ICP-ECOPETROL for supporting this work.

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