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Microalgae Based Biorefinery: Evaluation of Several Routes for Joint Production of Biodiesel, Chlorophylls, Phycobiliproteins, Crude Oil and Reducing Sugars

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In this work, several routes for obtaining high value pigments, fermentable sugars, crude algae oil and biodiesel were evaluated using the microalgae strain *Amphiprora* sp. in order to develop a topology of microalgae-based biorefinery. For chlorophylls and phycobiliproteins extraction, ethanol and methanol were evaluated as solvents, for reducing sugars production, Organosolv pretreatment (OSE), acid hydrolysis (HSE), and multifunctional systems using ethanol (MSE) and methanol (MSM) were compared. For lipid extraction, five solvent-based methods were compared with multifunctional process and for biodiesel production, transesterification, MSE and MSM were used. Results shows that several bioproducts can be obtained from *Amphiprora* sp. microalgae and can be a promissory strain for the development of a biorefinery, two routes were defined: one route without the use of multifunctional processes with includes pigments extraction using ethanol, reducing sugars production by organosolv pretreatment, lipid extraction by HBE method and oil transesterification by methanol/NaOH; and a second route composed by pigments extraction using ethanol, multifunctional process by MSE route and biodiesel production from algae oil by methanol/NaOH transesterification.

1. Introduction

Microalgae have recently been rediscovered as promising candidates for biotechnological applications and efficient energy production systems, biodiesel from microalgae production chain is being studied (Trabucco et al. 2011). However, for a sustainable biofuels production from microalgae, the concept of biorefinery must be applied. As in an oil refinery, a biorefinery uses all biomass components for obtaining several biofuels and high value products (Khan et al. 2009). In this work, several routes for obtaining high value pigments and phycobiliproteins, fermentable sugars, crude algae oil and biodiesel were evaluated using the microalgae strain *Amphiprora* sp. in order to develop a topology of microalgae-based biorefinery.

2. Materials and Methods

Microalgae biomass of *Amphiprora* sp. was provided by Morrosquillo Corporation (Punta Bolívar, Colombia). Figure1 shows stages of routes evaluated and different methods for obtaining several microalgae bio-products which will be explained in further sections.

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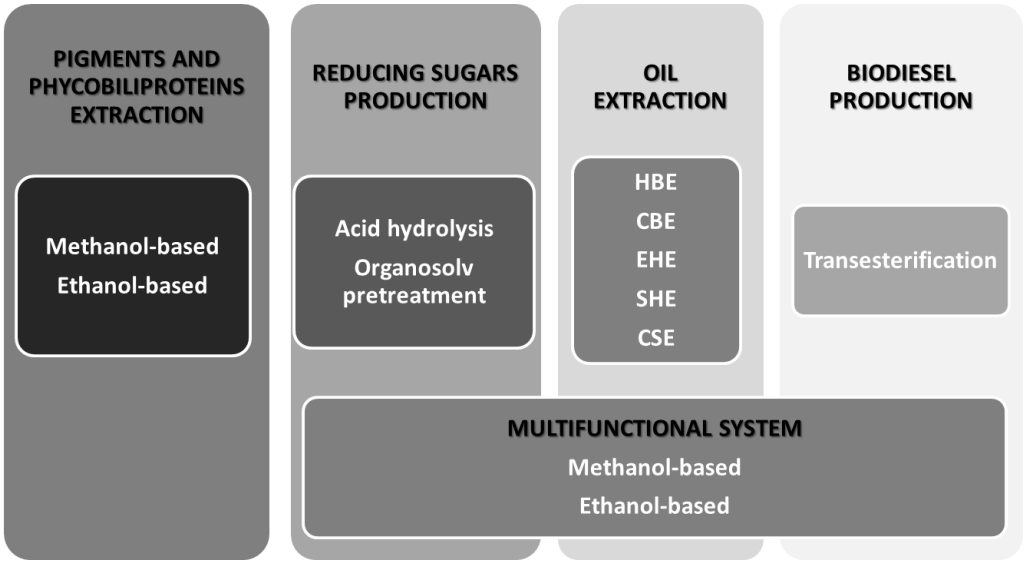

Figure 1: Methods evaluated for obtaining several bioproducts from Amphiprora sp.

2.1 Pigments and phycobiliproteins extraction

For pigments and phycobiliproteins extraction was used a methodology developed by Garcia and Amaya (2012) in which 10 g. of microalgae biomass is mixed with solvent in a 1:30 ratio at 328 K during 2 h, using a stirring speed of 350 rpm., solvents evaluated were methanol and ethanol, products concentration was measured using a spectrophotometer MERCK Spectroquant® Pharo 300. Pigments and phycobiliproteins quantified were chlorophyll a, chlorophyll b, phycocyanin and allophycocyanin, using Eqs. 1-4, where *A* corresponds to absorbance at a specific wavelength.

$$Chlorophyll\ a = (16.5*A665) - (8.3*A650) \quad (1)$$

$$Chlorophyll\ b = (33.8*A650) - (12.5*A665) \quad (2)$$

$$Phycocyanin = (A650 - 0.7*A650) / 7.38 \quad (3)$$

$$Allophycocyanin = (A650 - 0.19*A620) / 5.65 \quad (4)$$

2.2 Reducing sugars production

For this stage, four methods were performed and yields were compared. Organosolv pretreatment (OSE) was implemented using operation conditions found by González and Kafarov (2010) for reducing sugars production from microalgae biomass. For acid hydrolysis method (HSE), biomass was mixed with HCl 0.5M during 2 h; liquid phase was separated and used for reducing sugars determination, for all methods evaluated, total reducing sugars were quantified using the DNS method.

2.3 Multifunctional System

This route includes the implementation of joint actions of reducing sugars production, oil extraction and in situ transesterification. Pathway previously proposed and evaluated by Peñaranda et al. (2011), conducted two simultaneous systems, in which ethanol (MSE) and methanol (MSM) were evaluated as solvents/reagents. Biomass-alcohol ratio was fixed in 1:6; sulphuric acid was used as catalyst for transesterification in oil-acid ratio of 1:1. Reaction systems were continuously stirred at 500 rpm for 4 h. after that, hexane and distilled water were added in order to obtain a three-phase system consisting of hexane phase, residual biomass and hydro-alcoholic layer. Reducing sugars were measured using DNS method, biodiesel production was monitored by Infrared Spectroscopy using a Shimadzu FTIR-

8400S (Fourier Transform Infrared Spectrophotometer) in the wavelength range of 400-4000 cm^{-1}, and lipid yield was determined by gravimetric techniques and a mass balance after solvents recovery and residual biomass drying.

2.4 Oil extraction

Five solvent-based oil extraction methods previously developed by authors were evaluated and compared as follows: for hexane based extraction, (HBE) and cyclohexane (CBE) based methods, biomass was mixed with fresh hexane or cyclohexane in a 1:20 ratio and stirred at 500 rpm for 24 h 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. For solvent extraction with high speed homogenization (SHE), methanol, chloroform and biomass are mixed in a mass ratio of 6:12:1 under environmental conditions, 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 15 min at 3400 rpm, the upper phase methanol/water was removed while lower phase biomass/lipids Chloroform, was filtered by gravity. For continuous reflux solvent extraction (CSE), a typical Soxhlet extractor with 45/50 outer/upper and 24/40 lower/inner joint for 250 mL capacity was used, dry biomass was put in a cartridge and solvent was heated to boiling point, then condensing it on the cartridge of biomass, process is repeated for 16 hours, 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. Finally, for ethanol-hexane method (EHE), 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 other lipid and non-lipid contaminants. As a second step, the addition of water and hexane to the crude extract, generates a biphasic system in which lipids are transferred to the hexane phase, process is repeated five times by adding more water and hexane to the hydroalcoholic phase.

2.5 Biodiesel production

For microalgae oil transesterification, previous study of variables was performed by authors (Plata et al. 2010) in which best operating conditions were found (methanol/oil molar ratio of 14:1, temperature of 316 K and catalyst concentration of 0.42 %. information published in mentioned work was used for comparison with MSE and MSM routes in this study.

3. Results

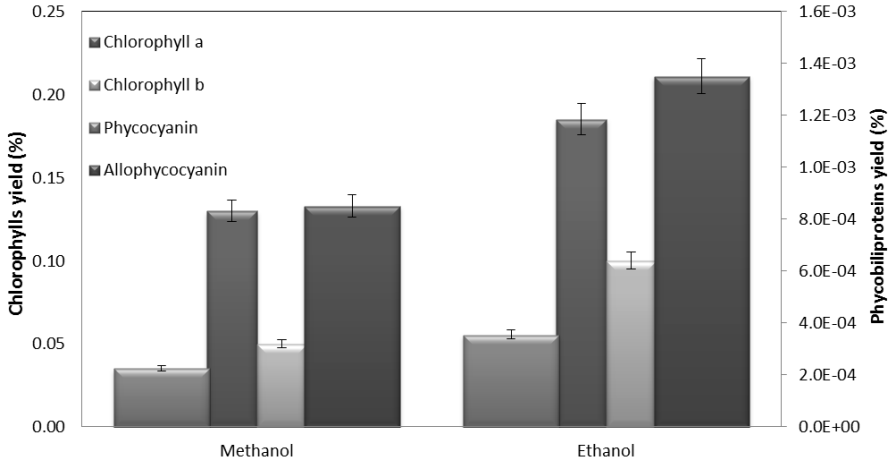

Figure 2: Effect of solvent in pigments and phycobiliproteins extraction from Amphiprora sp. microalgae

Figure 2 shows a comparison of pigments and phycobiliproteins yield using methanol and ethanol, it can be seen that ethanol presents better performance as chlorophylls as phycobiliproteins extraction, although values extracted are low, in a large scale microalgae processing for biofuels the extraction of these components can be significant and benefits in a great way the total process economy owing to high value of pigments extracted.

For reducing sugars production, comparison of routes shows that multifunctional systems are more convenient from the efficiency point of view than HSE and OSE routes (Table 1.), obtaining up to five times more reducing sugars than HSE route, concentration of reducing sugars in sugar cane bagasse, is in the range from 2.58 to 20.45 mg/mL, with an overall average of 10.53 mg/mL, although yield of reducing sugars from microalgae is lower than bagasse, values obtained in this study are comparable to values obtained with other sources as bean dregs waste and cashew apple bagasse. In a microalgae-based topology of biorefinery is attractive the utilization of fermentable sugars for bioethanol production which can be used as reagent for transesterification, organosolv pretreatment, pigments and proteins extraction, or can be purified and valued as biorefinery product for commercialization, distribution and use.

Table 1: Comparison of routes for reducing sugars production from microalgae strain Amphiprora sp.

Route	HSE	OSE	MSE	MSM
Reducing sugars yield (mg/mL)	0.45	1.47	2.5	2.63

Table 2 shows a comparison of solvent-based oil extraction methods and oil extraction efficiency using multifunctional system, lowest efficiency is reached using EHE which can be explained because hexane added to ethanol after first extraction instead of use directly on biomass, HBE, CSE and CBE routes presents similar efficiencies, however taking into account other selection criteria, cyclohexane is several times more expensive than hexane, and HBE route can be performed at room temperature, which is more convenient in a large scale process, SHE route is used as control for total lipid extraction, for this reason presents the highest efficiency, but uses highly toxic solvents. Relative efficiencies using multifunctional routes are higher than solvent based routes with the advantage of a simultaneous reducing sugars production and oil transesterification.

Table 2: Comparison of routes for oil extraction from microalgae strain Amphiprora sp.

Route	HBE	CBE	SHE	CSE	EHE	MSE	MSM
Relative lipid yield (%)	74.52	72.49	100.00	75.58	43.66	89.01	82.32

In order to ensure the presence of biodiesel in multifunctional system, FTIR spectra of hexane phase was compared with FTIR spectra of oil obtained by HBE route (Figure 3), (C=O) bond appears as in biodiesel as lipid spectra, this bond for our lipid extract was found at 1704 cm^{-1} and for the hexane phase after multifunctional route at 1750 cm^{-1}. In this spectrum appears a peak at 3400 cm^{-1}, corresponding to the OH bond that is common in glycerol spectra (Ooi et al., 2001). The shape of this spectra is owing to the origin of the sample which is product of a multifunctional process where there was no a rigorous purification of the products obtained.

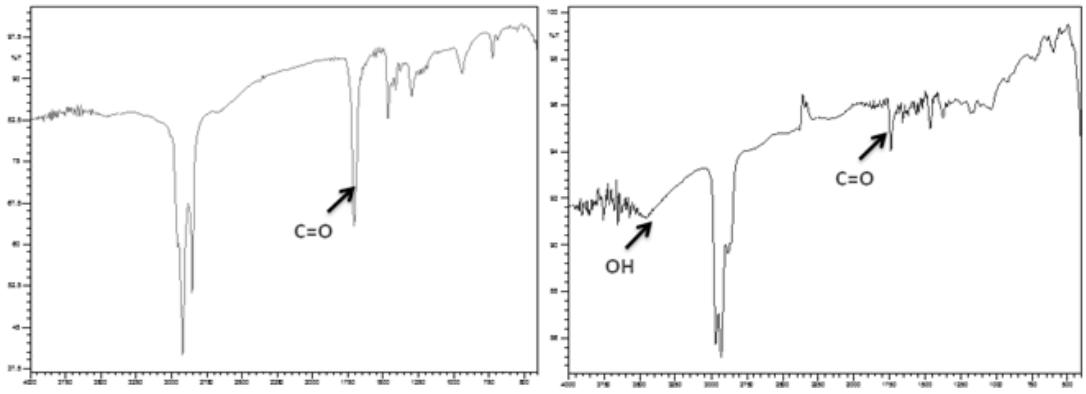

Figure 3: Comparison of FTIR spectra of hydrophobic phase from HBE and multifunctional routes

Table 3 shows a comparison of operating conditions for microalgae oil transesterification and conditions for multifunctional process, altought MSE and MSM routes can be used for transesterification, products separation is limited to hydrophylic/hydrophobic phase, and biodiesel yield is not determined, for this reason is necessary to develop efficient products separation processes and a conventional transesterification step for total FAME yield. Reaction times of MSM and MSE routes are more than five times longer than reaction time of transesterification, and reaction is performed at higher temperature, increasing energy requirements, also, a higher amount of alcohol is required which could impact in energy and environmental issues for a large scale process, for now, multifunctional routes must be taken into account for joint reducing sugars production and lipid extraction but not for microalgae oil transesterification.

Table 3: Comparison of operating conditions for microalgae biodiesel production by route.

Route	Transesterification	MSE	MSM
Alcohol	Methanol	Ethanol	Methanol
Catalyst	NaOH	H_2SO_4	H_2SO_4
Temperature (K)	316	333	333
Alcohol/Oil ratio	14:1	60:1	60:1
Reaction time (h)	0.8	5	4

4. Conclusions

Microalgae biomass of *Amphiprora* sp. strain has the potential of be used for the development of a topology of biorefinery owing to its composition of carbohydrates, lipids, proteins and special substances, experiments in lab scale were performed for obtaining several products, for pigments and high value phycobiliproteins extraction two solvents were evaluated, for all metabolites extraction, use of ethanol increases chlorophylls, phycocyanin and allophycocyanin yield, in comparison to methanol. In addition, four methodologies for reducing sugars production from microalgae strain, organosolv pretreatment, acid hydrolysis, multifunctional process using methanol and multifunctional process using ethanol, higher reducing sugars yield was reached using multifunctional processes obtaining similar yields to other feedstocks as bean dregs waste and cashew apple bagasse.

For lipid extraction, five solvent-based methods and two multifunctional systems were compared from the efficiency point of view, hexane and cyclohexane based methods, solvent extraction with high speed homogenization, continuous reflux solvent extraction, ethanol-hexane method, multifunctional process using methanol and multifunctional process using ethanol, being more convenient for lipid extraction the multifunctional routes, for solvent-based routes, can be concluded that HBE method is more convenient for lipid extraction taking into account not only efficiency but cost of solvents and operation conditions. For microalgae oil transesterification is shown that reaction using basic catalyst

and methanol in an alcohol/oil ratio of 14:1 is more convenient for biodiesel production from oilgae, because multifunctional process is not fully developed for this stage and only was identified the presence of biodiesel but not FAME yield in hydrophobic phase.

Taking into account results, can be defined two routes for production of biodiesel, chlorophylls, phycobiliproteins, crude oil and reducing sugars, one route without the use of multifunctional processes with includes pigments extraction using ethanol, reducing sugars production by organosolv pretreatment, lipid extraction by HBE method and oil transesterification by methanol/NaOH; and a second route composed by pigments extraction using ethanol, multifunctional process by MSE route and biodiesel production from algae oil by methanol/NaOH transesterification. Use of ethanol as a reagent for pigments and reducing sugars production gives the opportunity of the development of an integrated biorefinery by the use of reducing sugars obtained for third generation bioethanol production which can be used in these steps.

5. Acknowledgments

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