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Evaluation of a Two-Phase Extraction System of Carbohydrates and Proteins from *Chlorella vulgaris* Utex 1803

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Microalgae are a valuable source of high-value products and biofuels, however the high-energy cost required for the extraction of their metabolites has kept questioning on possible industrial upgrading. The aim of this study was to evaluate the effect of temperature, solvent/biomass, NaOH concentration and thermal pre-treatment of the biomass in a 2-cycle carbohydrate and protein extraction system.
Results shown that best conditions for carbohydrates extraction are achieved at a solvent concentration of 3.67 M, 55°C and a solvent/biomass ratio of 30mL/g. On the other side, the best conditions for protein were 3 M, 85°C and 45 mL/g. The efficiencies achieved under these conditions were 95% for carbohydrates and 98% for proteins. Using the best extraction conditions for each metabolite a thermal pre-treatment was performed at 25°C, 75°C and 105ºC. Results indicate that highest efficiencies were achieved with dry biomass pre-treated at 105°C, with values of 95% for carbohydrates and 98% for proteins.

1. Introduction

Microalgae are considered one of the most promising feedstock for the pharmaceutical, food and biofuel industries due to its rapid growth, high CO_2 fixation and do not compete with the use of arable land and drinking water (Chen et al., 2013); however, the high costs associated with harvesting, drying and extraction compared with the low selling price of some products can jeopardize the viability of these systems (Garcia-Cuadra, et al, 2012). This problem has presented as an unique opportunity for research and development of novel low-cost affordable processes for recovering high value metabolites such as isoprenoids, alkaloids, toxins, polysaccharides, polyunsaturated fatty acids, enzymes, non-ribosomal peptides, carbohydrates and proteins(Serive et al., 2012).
Chlorella vulgaris is one of the most commercially used microalgae due to their levels of carbohydrates (33-50%), protein (20-60%) and lipids (10-20%) (Ho et al, 2012.). Carbohydrates are a major by-product of carbon fixation metabolism and is accumulated specially on plastids as reserve (mainly as starch) or in the cell wall (in the form of cellulose, pectin and sulfated polysaccharides) (Chen et al, 2013); however, the metabolism of both cellulose and starch varies significantly between genera and species (Rangel et al, 2004; Rizmani-Yazdi et al, 2011).
one of the most used methods for the recovery of carbohydrates is the removal (physical or mechanical) assisted under alkaline conditions which has proven a favorable alternative due to the low temperature and pressure applied providing a lower production cost compared to other methods of pretreatment. According with Harun et al (2011) the maximum yield was 35% of the total carbohydrates, working at 120°C, 0.75% w/v NaOH for 30 minutes. It is necessary to highlight that highly concentrated alkaline media leads to several adverse effects such as reduction of protein digestibility and damage of certain amino acids (lysine, cysteine)

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(Sari et al. , 2013). Moreover, the temperature is another important, factor because it can act positively on heat resistant molecules but can also degrade thermally labile molecules such as chlorophyll (Serive et al, 2012.). The main objective of this work was to evaluate a two-phase extraction system to obtain both carbohydrate and protein using variables such as temperature, concentration of alkaline medium (NaOH) and the solvent/biomass ratio, to improve selectivity in as for obtaining these two metabolites.

2. Methodology

2.1 Microalgae culturing

Chlorella vulgaris UTEX 1803 was purchased form the Culture Collection of Algae at University of Texas UTEX, the algae was maintained on 500 mL Bold Basal Culture Media (BBM) (Bischoff and Bold 1963) on 1000 mL flask and mixed using filtered air (0.2 μm membrane filter) with 1% (v/v) of CO_2. After 20 days of culture the biomass was centrifuged at 3400 rpm for 15 minutes and dried at 105°C, over 24 hours.

2.2 Metabolites extraction experiments

A 3^3 Central composite Design was applied using STATISTICA® 7.0 to evaluate the effect of Molarity, solvent/biomass ratio and temperature of extraction (Table 1). 1 g of dried biomass was used on each of the experiments; the extraction process lasted 20 minutes on a thermal bath using the temperatures listed for each experiment. After, extraction the biomass was removed by centrifuge. Once removed the wet biomass was subjected to a second extraction process.

Experiment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Molarity	1	3	1	3	2	1	3	1	3	2	2	0,327	3,673	2	2
Solvent(mL)/Biomass (g)	15	45	45	15	30	45	15	15	45	30	30	30	30	4,9	55
Temperature °C	25	25	85	85	55	25	25	85	85	4,8	105	55	55	55	55

Figure 1: Design of Experiments for extraction of carbohydrates and proteins.

2.3 Chlorophyll removal

Temperature is an important factor, as it can degrade thermally labile molecules such as chlorophylls (Serive, et al., 2012), thus generating overestimation in the protein extracts (Slocombe et al., 2013), changes in color and molecular structure (Schwenzfeier et al., 2011). To reduce this problem culminated 20 minutes of heat treatment, samples were centrifuged at 3400 rpm for 15 minutes and the supernatant was passed through a vertical column of internal diameter 1.4 cm and height 12.5 cm filled with activated carbon .

2.4 Protein and carbohydrates quantification

Protein was quantified using Lowry method (Lowry et al., 1951), briefly, 1 mL of chlorophyll-less extract is mixed with 1.4 mL of Solution A-B-C, after 20 minutes, 0.2 mL of Folin reactive is added into the mixture. The final solution was measured using a spectrophotometer at 750 nm.
Carbohydrates were measured using phenol-sulfuric acid method (Dubois et al., 1956). Briefly, 1 mL of chlorophyll-less extract is mixed with 0.5 mL of 5% phenol, after homogenization, 2.5 mL of 95% sulfuric acid is added to the mixture. The sample was measured using a spectrophotometer at 480 nm.

3. Results and discussion

3.1 Carbohydrate extraction

According to Pareto charts (Figure 1) the most important variable for the first and second extraction is solvent/biomass, while the molarity, temperature and the interactions between them do not represent significant variations in the extraction results.

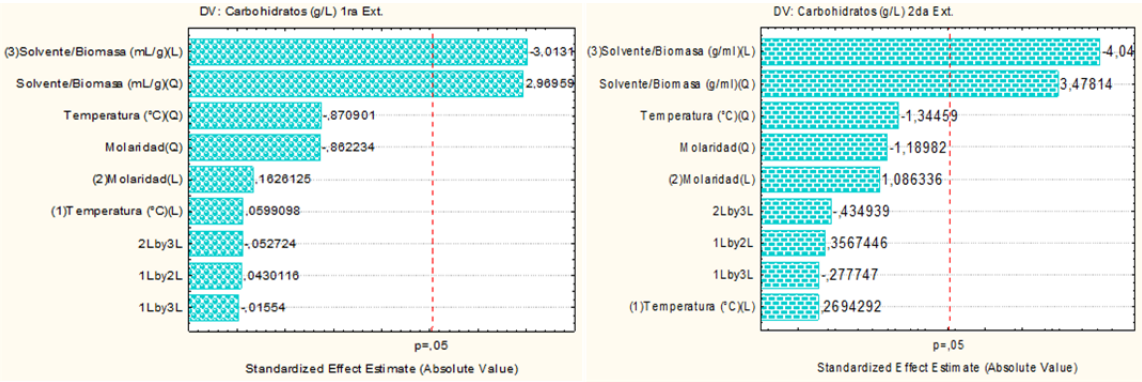

Figure 1: Pareto Charts for first (left) and second (right) extraction of carbohydrates.

Surface response from the first extraction (Figure 2) shows that by using a concentration of 2M, 55°C and 15 mL/g solvent/biomass the concentration of carbohydrates recovered was 59.5 g/L, this corresponds to 89.22% of the initial biomass, this values are higher to those reported by Zhou et al (2011), where the value achieved corresponds to 83% under 180°C, 0.4mol/L $MgCl_2$ and an extraction time 10 minutes. Finally for the second extraction up to 10 g/L of carbohydrates (20% of the biomass) can be obtained under 60°C, 2.5M and 15 mL/g solvent/biomass ratio. It is noteworthy that the maximum amount of carbohydrates obtained on the second extraction is lower than in the first extraction.

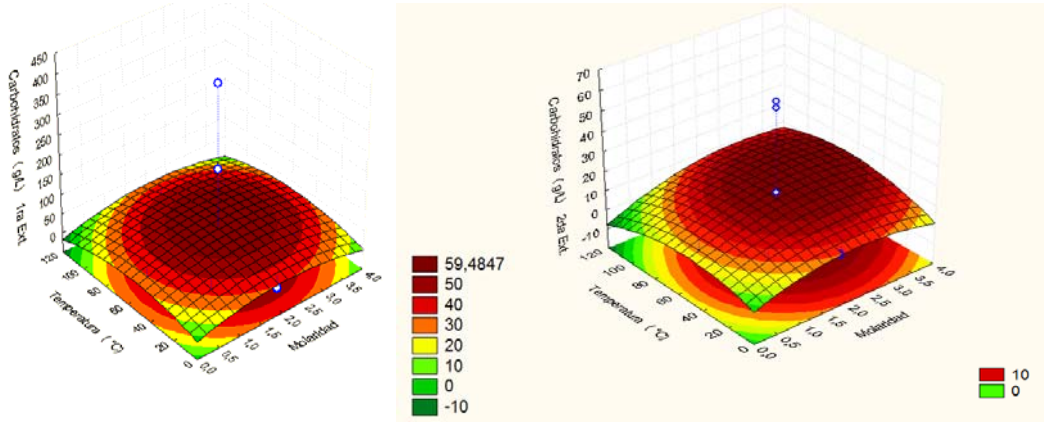

Figure 2: Surface response for first (left) and second (right) extraction of carbohydrates.

3.2 Protein extraction

Pareto charts (figure 3) show that for the first and second extraction the most important variables are the temperature and solvent/biomass ratio, however unlike the first extraction lower temperature ranges improve the extraction of proteins. It is important to note that the influence of the solvent/biomass ratio is constant, demonstrating that it is not necessary to use high concentrations of solvent.

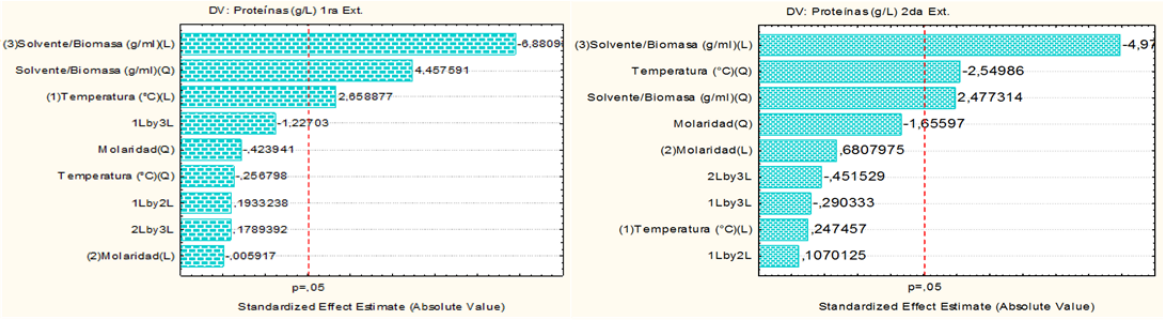

Figure 3: Pareto Charts for first (left) and second (right) extraction of proteins.

Response surface from the first extraction of proteins (Figure 4) shown that the highest concentration achieved was 30 g/L (45% of initial proteins using a solution of 3M, a temperature between 100-110°C and a solvent/biomass ratio of 15 mL/g. This results are higher than those reported by Barbarino & Lourenco (2005), where most initial extraction of proteins corresponding to 24%, working under conditions of 95 ° C, and 0.1 N NaOH for 10 minutes.

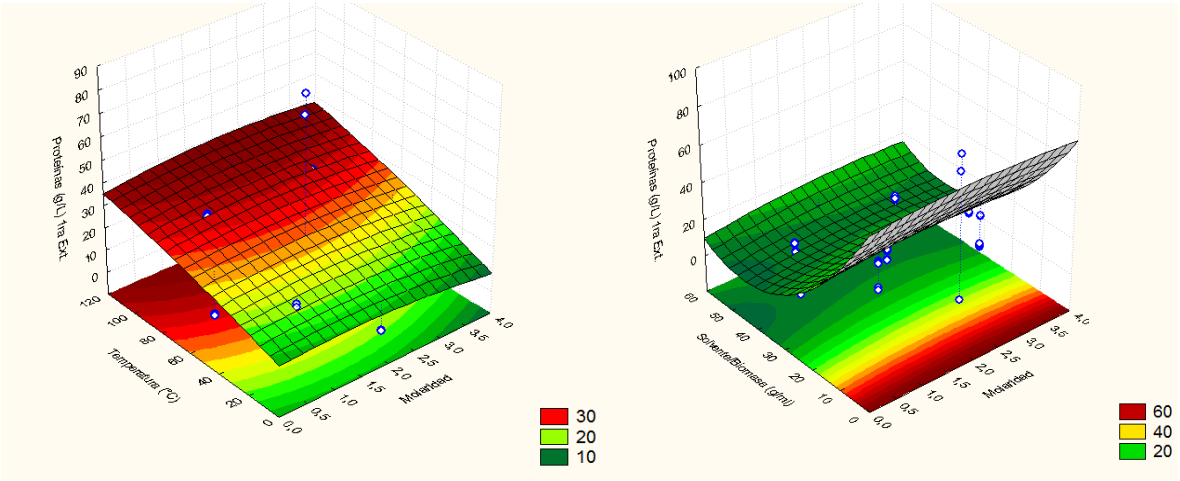

Figure 4: Surface response for first extraction of proteins, Molarity/Temperature (left) and Molarity/ solvent/biomass (right).

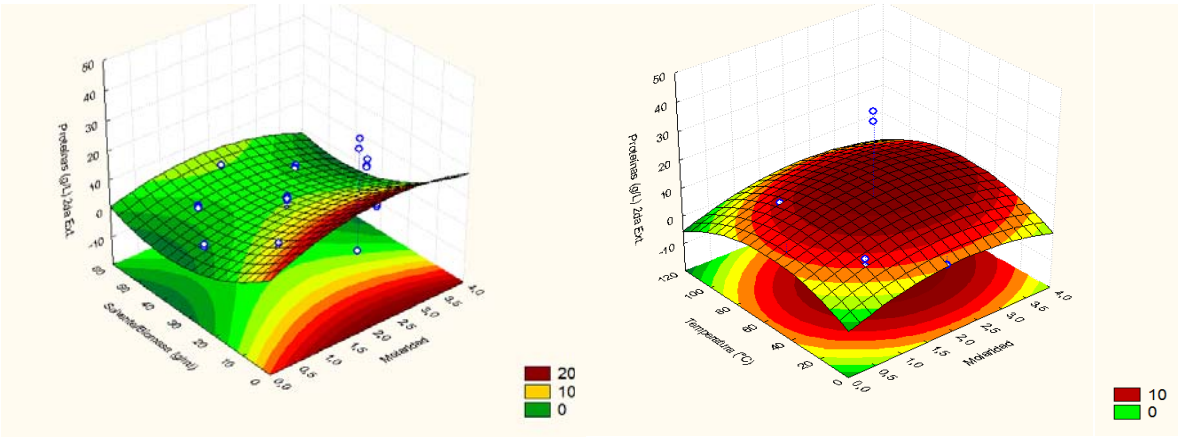

Figure 5: Surface response for second extraction of proteins, Molarity:solvent/biomass ratio (left) and Molarity: temperature (right).

3.3 Thermal pre-treatment efficiency

With the aim of improving the extraction efficiency of both carbohydrates and protein, a new sample of fresh biomass was subjected to a thermal pretreatment (25 and 75 ° C for 24 h) other than the initial (105 ° C, 24 h). Figure 6 shows a comparison of the results obtained for each pre-treatment temperature. it can be seen that the highest concentration of protein (98%) is obtained under a thermal pretreatment of 105°C, however it should be noted that under room temperature (25ºC) the protein concentration is very close (89%), this result despite not being the highest value posess a great advantage in terms of energy savings, as the drying of biomass represents 90% of the total energy of the process (Lardon et al., 2009). Finally, thermal pretreatment conditions for the extraction of carbohydrates are similar to those reported for proteins, since the test with thermal pretreatment at 105°C obtained the highest values (95%).

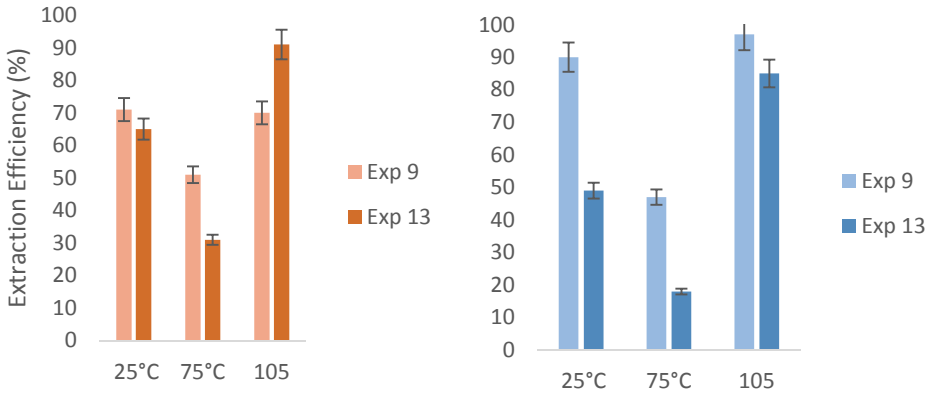

Figure 6: Efficiency of thermal pre-treatment on carbohydrates (left) and proteins (right)

4. Conclusions

According with the results lower solvent/biomass ratio allows higher initial extraction of carbohydrates, while at higher temperatures (>55ºC) a higher amount of protein are extracted. Also it was found that Larger concentrations of carbohydrates (95%) and proteins (98%) are obtained in the first extraction step; in the other hand, biomass thermal pretreated at 105°C has an average extraction efficiency of 91% for carbohydrates and proteins, however the results obtained with the biomass without thermal pretreatment (25ºC) seems promising because represents a decrease in the total cost of treatment of biomass and therefore the total cost of the process, however it is necessary to determine how they could exploit this advantage with the proposed extraction method or with other methods

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