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Effect of Thermal Pre-Treatment on Fermentable Sugar Production of *Chlorella Vulgaris*

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Microalgae are microorganisms with a great potential, since they contain large amounts of metabolites to the development of products such as bio-ethanol. However, to achieve the release of these compounds is necessary to carry out different types of pre-treatment to the algal biomass in a manner which would improve the outcome of the cell disruption for the release of these compounds.
This paper analyzed the effect of thermal pre-treatment to the biomass of *Chlorella vulgaris* for obtaining fermentable sugars using three different temperatures (105, 75 and 25 ºC). The alga was cultivated in bioreactors type airlift in the total absence of light for 16 days using as substrate wastewater production of ethanol from sugar cane molasses; It was then assessed the cell disruption by means of Organosolv and acid hydrolysis in order to free up the fermentable sugars.
Through the OrganoSolv process were obtained 0.165 g of fermentable sugars/g biomass and 0.128 g of fermentable sugars/g of biomass and through the method of acid hydrolysis at a temperature of heat pretreatment of 75 ºC for the two cases.
The use of micro-organisms presents a good overview for the development of bioethanol from sugars of microalgae, that already Pose a scenario of a distillery of 100 m^3 /day ethanol at 96 % purity would be up to 1,300 m^3/day of distillery as a substrate for biomass of *C. vulgaris*, which would give a large amount of sugars for the development of approximately 1,600 L/d ethanol.

1. Introduction

The large scale production of wastewater is an inevitable consequence of modern societies, which has left a mark on global biogeochemical cycles, especially nitrogen and phosphorus (Aslan, and Kapdan, 2006), in addition, high concentrations of carbon and other nutrients have saturated the capacity of ecosystems to deal with these effluents (Boursier et al. 2005) (Olguin, 2003); to control this problem, there have been a number of environmental regulations that regulate the levels of organic load, nitrogen and phosphorus that may contain water and treated (Garcia et al, 2000).
The bioethanol production process produces a type of wastewater known as vinasse, which are generated in high volumes (between 9 and 14 L/L ethanol produced) (Travieso et al, 2008), and are characterized by low pH, strong smell , a high chemical oxygen demand COD, high biochemical oxygen demand BOD, high amounts of organic matter, nutrients such as nitrogen, phosphorus and potassium, and a dark brown color which difficult the photosynthesis of native aquatic flora, therefore water quality deteriorates and is detrimental to life exists therein (Singh and Dhar, 2006) (Satyawali and Balakrishnan, 2008).In Colombia, it takes one million one hundred thousand liters of bioethanol to meet the demand from cities like Bogota, Medellin, Cali and Barranquilla where is mandatory to use this blend ethanol with gasoline (Fedebiocombustibles, 2013), so it cab produce about ten million liters of vinasse under a realistic scenario. A large part of this stillage are used as fertilizer, such as Ecocarbovin developed by the ICP-Ecopetrol , however this use is limited by the type of soil and the amount of nutrients that crops need (Ferreira, and Montenegro, 1987), therefore it is necessary to carry certain amount of storage ponds for further treatment, as these generate great environmental damage if are discharged directly. Due to the

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growing demand for bioethanol in the Colombian market, the treatment of vinasse as the main by-product, has become one of the problems of this industry, therefore it is necessary to develop new and more efficient methods to dispose this kind of waste.

Microalgae are unicellular microorganisms that are now seen as a valuable source of products for food, high-value lipids, biofuels, and various products with industrial interest; these microorganisms are able to rapidly consume nutrients and organic or inorganic carbon, for which they have been studied for the degradation of pollutants such as phenols, aromatic compounds and recalcitrant biopolymers as melanoidins (Maynard *et al,* 1999), this process is known as phytoremediation (Olguin, 2003), this process has applications such as: (a) removal of nutrients in sewage and effluent rich in organic matter, (b) industrial wastewater treatment with trace metals and acids, (c) CO_2 sequestration, (d) processing and degradation of xenobiotics and (e) detection of toxic compounds using algae-based sensors (Olguin, 2003) (Singh and Patel, 2012) (Olguin et al, 2001).

C. vulgaris has been proposed for the treatment of effluents due to its easy adaptability and rapid growth (Travieso *et al,* 2008). In the treatment of vinasse, Valderrama et al (2002) used cultures of *C. vulgaris* supplemented for 4 days for another 6 days macrophytes achieving a reduction in color of 52 %. Travieso et al (Travieso et al, 1999) (Travieso et al, 2008) used cultures of *C. vulgaris* SR/2 after an anaerobic pretreatment stillage, resulting in an excellent culture medium for the growth of algae. However to date there are no reported work in which local stillage are evaluated as a culture medium for the growth of microalgae, or the quality of the biomass produced from these with intent to obtain value added products.

The aim of this study is to evaluate the effect of thermal pretreatment on microalgae biomass as a way for improvement of carbohydrate extraction using *Chlorella vulgaris* UTEX 1803 towards the production of biofuels and value-added products .

2. Methodology

2.1 Culture conditions

Chlorella vulgaris UTEX 1803 was obteined from the collection of algal cultures at the University of Texas (Austin, TX, USA). Initially, the strain was maintained in laboratory-scale photobioreactors using *Bold Basal* culture medium (BBM) whose composition is (M): $NaNO_3$ (2,94 X 10^{-3}), $MgSO_4.7H_2O$ (3,04 X10^{-4}) NaCl (4,28 X 10^{-4}), K_2HPO_4 (4,31 X 10^{-4}), KH_2PO_4 (1,29 X 10^{-3}), $CaCl_2.2H_2O$ (1,70 X 10^{-4}) and micronutrients (M) $ZnSO_4.7H_2O$ (3,07 X 10^{-5}), $MnCl_2.4H_2O$ (7,28 X 10^{-6}), MoO_3 (4,93 X 10^{-6}), $CuSO_4.5H_2O$ (6,29 X 10^{-6}), $Co(NO_3)_2.6H_2O$ (1,68 X 10^{-6}), H_3BO_3 (1,85 X 10^{-4}), EDTA (1,71 X 10^{-4}), KOH (5,53 X 10^{-4}), $FeSO_4.7H_2O$ (1,79 X 10^{-5}). 2.5 L Cylindrical airlift reactors (14 cm of internal diameter and 35 cm height) with 2 L of culture were used. The culture was mixed using filtered air (0.2 μm membrane filter) flow of 0.6 L/min without any additional CO_2 supply.

2.2 Vinasse production

The vinasse used was obtained from fermented molasses by evaporation without recirculation in the laboratory school of chemical engineering at the Universidad Industrial de Santander.

For fermentation, 45 Kg of comercial molasses were diluted in 151 L of water to about 18° Brix, the mixture was pasteurized at a temperature of 80 ° C for 1 h, then cooled to 40 °C and pH adjusted to 4.2 by adding concentrated sulfuric acid (95 %). The inoculum was prepared using 20 liters of the mixture and adding ammonium chloride (144 g) , magnesium sulphate (24 g), urea (24 g) and phosphate rock (10 g) used for the activation of 500 g of commercial yeast Saccharomyces cerevisiae (Levapan) . The inoculum was added to the diluted molasses tank and is aerated for one hour. The fermentation was conducted for 3 days. Elapsed wort was evaporated at 94 °C in two stages, each of about 2 h.

2.3 Experimental setup

Cylindrical airlift reactors with an internal diameter of 14 and 35 cm height with a culture volume of 2 L (1.5 L of vinnase and 0.5 L of inoculum) were used. The reactors were attached to an aeration system with flow of 0.6 L/min.

Due to the dark color which prevents the passage of light uniformly, the reactors were coated with aluminum foil to prevent totally the passage of light, thus allowing a 0:24 h light: dark cycle. Each experiment was performed in triplicate for 18 days, consisting of an original and two replicates for each of the treatments. After the 18 days the biomass produced was recovered through were centrifuged at 3,600 rpm for 20 min.

2.4 Biomass quantification

Every 2 days, a sample of 10 mL of each reactor was taken, centrifuged at 3400 rpm for 20 minutes and the supernatant removed, the pellet was resuspended in 10 mL of distilled water, then filtered on preweighed nitrocellulose filters (1 μm). The filters with the samples were taken to oven for 24 hours at 105 °C and then in a desiccator until constant weight.

2.5 Thermal pre-treatment

Temperature is one of the determining factors when take advantage of the different metabolites, as it can act positively on heat-resistant molecules, but also can degrade thermally labile molecules such as chlorophyll (Nguyen et al, 2009); also, drying of biomass represents 84 % of the total energy consumption (Patil et al, 2012); as different extraction methods require several stages prior including biomass drying and evaporation of solvents at high temperatures (Mata et al, 2010), therefore it is necessary investigate the effect of relative humidity in the biomass extraction processes.

Considering the above, the improvement was evaluated in the extraction of carbohydrates using 2 hydrolytic methods previously evaluated by Peñarada et al. (2011).

The biomass obtained was subjected to a heat treatment in an oven, varying the exposure time and temperature (Table 1). Each of the samples was hydrolyzed using one of two methods: Organosolv or acid hydrolysis.

Table 1. Temperature and time of pre-treatment of the biomass

Temperature (ºC)	Time (h)
105	24
75	24
25	0

2.6 Organosolv method

10 g of heat-treated biomass were mixed with 100 mL of prepared solvent (methanol 32 % v/v 0.6 M sulfuric acid as catalyst). The mixture was autoclaved at 15 psi and 121 °C for a period of 4 h.

Acid hydrolysis method

10 g of heat-treated biomass (105, 75 and 25 °C) for each sample were mixed with 100 mL of solvent (0.5 M HCl) in a beaker and mixed on a magnetic stirring plate at 500 rpm and 25 °C for a reaction period of 2 h.

2.7 Filtration and neutralization

Biomass and liquor from each of the hydrolysis were separated by vacuum filtration using filter paper (1 *µ*m). The liquor was neutralized with calcium carbonate. In order to remove acid residues present in the biomass, a cylce of washed with distilled water is recommended; after washing the biomass is dried in an oven at 105 °C for 12 h for the subsequent quantification of sugars. Neutralized liquor was passed through a bed of activated carbon, in order to remove color and thus avoid inconsistencies in the subsequent quantification of carbohydrates.

2.8 Carbohydrate quantification

Phenol-sulfuric acid method was used (Dubois et al, 1956). A sample of 1 mL of each culture was centrifuged at 3400 rpm for 20 min. To the pellet was added 1 mL of 5 % phenol and 5 mL of sulfuric acid at 95.5 %. Finally, each of these samples was transferred to cells and absorbance was measured at 488 nm and 490 nm 485 nm 480 nm to identify xylose, arabinose, fructose, galactose and glucose, respectively.

3. Results and Discussion

3.1 Biomass production

After 18 days was obtained 13.2 g/L with a productivity of 0.73 g/L * d (Figure 1); these values are close to those reported by Liu et al. (2012) who used diluted and pretreated molasses for the heterotrophic production of *Chlorella zofingiensis*. On the basis of these results it is possible to consider using vinasses as culture medium for the production of microalgae at large scale.

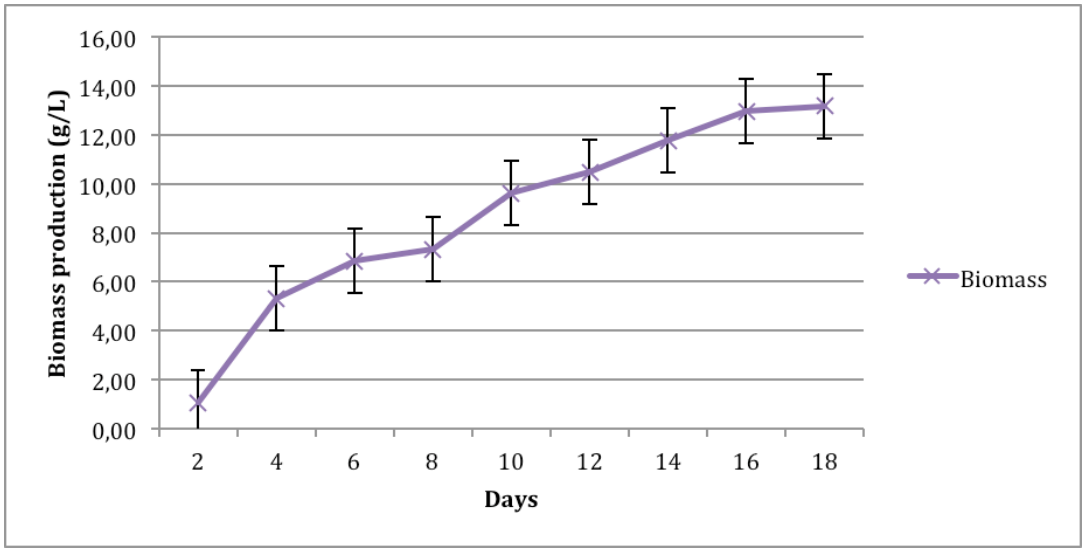

Figure 1. Biomass production in culture media enriched with vinnase.

3.2 Pre-treatments comparison for obtaining carbohydrates

For both methods it was found that at a pretreatment temperature of 75 °C gives the greatest concentration of carbohydrates (0.17 g Carbohydrate/g biomass for OrganoSolv and 0.13 g for acid hydrolysis), followed by 25 to 105 °C respectively (Figure 2).

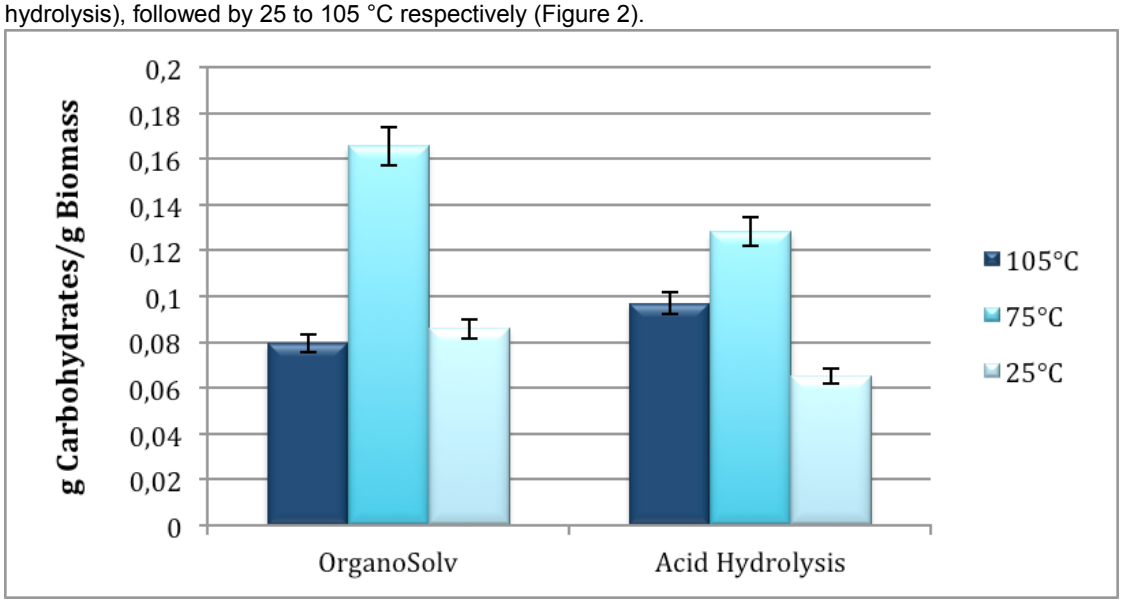

Figure 2. Total sugars released to the pretreated biomass for both Organosolv and acidic hydrolysis methods for each of the pre-heat treatments

Within carbohydrates released by the two methods of hydrolysis glucose is the highest, with values up to 81 % for both Organosolv to acid hydrolysis (Figure 3). The second carbohydrate present in a higher proportion is fructose (between 8 and 12 % for the two methods), the rest consists (about 13 %) of fermentable carbohydrates such as galactose, arabinose and xylose.

Carbohydrate concentration obtained in the pre-treated biomass at 75 °C is higher, which is due to the biomass humidity (approx. 8 %) which improves the contact area for the hydrolysis reaction and also helps the miscibility of solvents for the extraction of sugars in this case being longer.

For biomass pretreated at 25 °C the excess of water contained therein causes the acid concentration decreases significantly causing the contact between it and the biomass is much smaller, leading to the

yield of the reaction and hence the sugar release is low. Finally, for the pre-treatment at 105 °C in the absence of presence of water, the acid concentration will be high causing the disruption process yield is high.

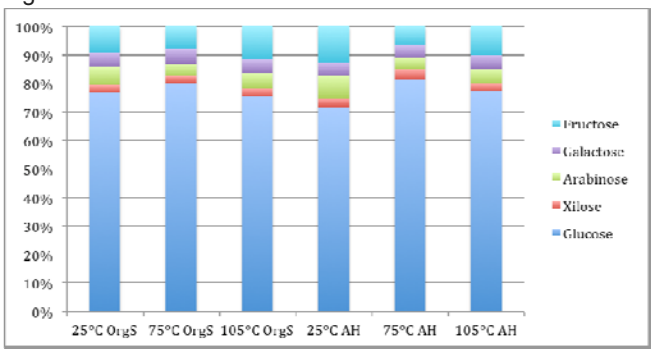

Figure 3. Percentage of fermentable sugars released by Organosolv method for each of the pre-treatments

According to the results, in order to allow a greater release of sugars the biomass must contain a relatively low humidity, so that it should not be completely dried (pre- treatment 105°C with about 0%) and contain high amounts of moisture (Pretreatment 25 °C with about 75 %). Zhou et al. (2011) demonstrated that variations in the concentrations of reactants can cause changes in the amount of released sugars improving or worsening the result of reaction and extraction thereof. Miranda et al. (2012) found that when *Scenedesmus obliquus* is thermally pretreated the water removed by the micropores of the cellulose in the cell wall can cause irreversible changes in its structure, creating amorphous areas in which the voltage between the elasticity is reduced causing fibers in them, which causes more susceptible to attack by acids. The results obtained for various types of cell disruption reached high amounts of biomass sugars released lower yields dehydrated and wet biomass.

4. Conclusions

It was found that the thermal pretreatment has a positive effect on the carbohydrate extraction; with the higher values found in the pretreated biomass at 75 ºC both by the Organosolv as the acid hydrolysis method (Up to 0.17 g of sugars/g biomass and 0.14 g of sugars/g biomass respectively), where the high glucose content of the sugars extracted is due primarily to the composition of the cell wall of the alga, which is composed mostly of cellulose Hemicellulose and starch, which to the degrading generate this single sugar. On the other hand, the acid hydrolysis presents a better picture for cell disruption of *C. vulgaris* because although their performance is a little lower than the Organosolv method, the reaction conditions (25 °C and 1 atm) require low power consumption, Doing more attractive this procedure at the time of producing fermentable sugars. Finally obtaining fermentable sugars for bioethanol production, using vinasses as substrate has a great potential, because in addition to serving as a treatment to wastewater can be used for obtaining high quantities of biomass (2,917 kg by Organosolv and 2402 kg by acid hydrolysis) for further processing to fermentable sugars as well as other value added products.

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