UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

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Producción de biodiesel a partir de microalgas nativas cultivadas en un fotobiorreactor a escala laboratorio

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RESUMEN

La producción de biocombustibles se ha expandido en la última década y la demanda de biodiesel ha aumentado en todo el mundo. El biodiesel puede ser producido a partir de lípidos que se encuentran en las células de microalgas debido a su alto metabolismo fotosintético. Este proceso de producción de biodiesel representa los biocombustibles de tercera generación, que tienen un gran potencial para la producción de energía renovable de manera sostenible. Por lo tanto, el objetivo de esta investigación es producir biodiesel a partir de microalgas *Chlorella sp.* nativa cultivada en un fotobiorreactor a escala de laboratorio para maximizar el contenido de lípidos. Un tubular fotobiorreactor de 10 L (FBR) se instaló con un flujo de aire ascendente de 3 L min^{-1}. Luz artificial fue proporcionada por cinco lámparas blancas fluorescentes frías de 20 W con 12 horas de luz y oscuridad, respectivamente. Se observó que el contenido de lípidos más alto fue de 38% con una concentración celular óptima de $4{,}5 \times 10^{6}$ células ml^{-1},cuando se limitó las fuentes de nitrógeno. Un pH neutro de 7 aseguró una tasa de remoción de amonio de -5,2 mg L^{-1} d^{-1} y la tasa de producción de nitrato de 9,9 mg L^{-1} d^{-1}. Los lípidos a partir de microalgas fueron convertidos en biodiesel por un proceso de transesterificación, utilizando un catalizador homogéneo KOH y metanol. La presencia de ésteres metílicos (biodiesel) fue confirmada por Cromatografía de Capa Fina (CCF) utilizando un solvente mezcla compuesto de éter de petróleo, éter dietílico y ácido acético (80: 19: 1) y una cámara de yodo para revelar las placas de CCF. Biodiesel de soya fue elaborado a partir de aceite de soya comercial como un control positivo para el biodiesel de microalgas. A partir de un contenido total de lípidos de 27,4% (v / v), se obtuvo 6 ml de biodiesel de microalgas. El factor de retardo relativo (R_f) obtenido fue 0.76 para biodiesel producido a partir de microalgas e indica lo cercano que es este valor en comparación a otros expuestos en la literatura. De esta manera, la cepa nativa *Chlorella sp.* podría ser una fuente potencial para la producción de biodiesel.

Palabras Clave: microalgas, lípidos, transesterificación, biodiesel.

Abstract

Biofuel production has expanded in the last decade and the demand for biodiesel has increased worldwide. Biodiesel can be produced from lipids found in microalgae cells due to its high photosynthetic metabolism. This process of biodiesel production represents the third-generation fuels, which have a great potential for renewable energy production in a sustainable way. Thus, the goal of this research was to produce biodiesel from native microalgae *Chlorella sp* cultivated on a laboratory scale photobioreactor to maximize the lipid content. A 10 L tubular bench-scale photobioreactor (T PBR) was installed with an upward airflow of 3 L min^{-1}. Lightning was provided by five white fluorescent cold 20 W lamps with 12 hours of light and darkness, respectively. The highest lipid content of 38% with an optimal cellular concentration of 4.5×10^6 cells mL^{-1} was observed when a deprived N-concentration medium was supplied to the culture. A neutral pH of 7 ensured an ammonium's removal rate of -5.2 mg L^{-1} d^{-1} and a nitrate's production rate of 9.9 mg L^{-1} d^{-1}. The lipids from microalgae were converted into biodiesel by a transesterification process utilizing a homogeneous catalyst KOH and methanol. The presence of methyl esters (biodiesel) was confirmed by Thin Layer Chromatography (TLC) utilizing a solvent mixture composed of petroleum ether, diethyl ether and acetic acid (80:19:1) and an iodine chamber for reveling TLC plates. Soja biodiesel from a commercial soja oil was produced as a positive biodiesel control for microalgae biodiesel. From a total lipid content of 27.4 % (v/v), 6 mL of microalgae biodiesel was obtained. The relative retardation factor (R_f) was calculated 0.76 for biodiesel produced from microalgae and indicated how close is this value from the values reported in the literatures. Hence, native strain *Chlorella sp.* could be a potential source for biodiesel production.

Keywords: microalgae**,** lipids, tranesterification, biodiesel, Thin Later Chromatography (TLC)

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Introduction

Continuously usage of petroleum-sourced fuels is now broadly recognized as unsustainable and environmental unfriendly because of the depleting supplies and release of Green House Gases (GHG) into the atmosphere (Hu *et al.*, 2015). According to the statistics presented by the World Energy Council, North America and Asia are the regions that consume the largest amount of energy worldwide, 34.8 % and 32.5 % respectively (WEC, 2015). Latin American countries that consume the major quantity of petroleum barrels per day are Argentina (770), Brazil (3003) and Venezuela (746). In 2010, Ecuador consumed 243 thousand of petroleum barrels per day, while during 2013; Ecuadorian population consumed 254 thousand of petroleum barrels per day (U.S. Energy Information Administration, 2015). Evidentially, the global consumption of fossil fuels tends to increase, and eventually the diminishing of these is warrantied.

The increase of crude oil prices and environmental degradation such as accumulation of GHG in the atmosphere and oil spills; have forced governments, industries, scientists and researchers worldwide to find out alternative energy sources. Biofuels have received considerable attention in recent years, as they are primarily produced from non-toxic, biodegradable and renewable resources, and they do not affect the environment, since its use leads to a reduction in harmful CO_2 emissions, hydrocarbons, particulate matter (PM), and elimination of SO_x emissions (Gouveia and Oliveira, 2009). As claimed by Alam *et al.,* biofuel production from renewable sources is considered to be one of the most sustainable alternatives to fossil fuels, it is viable for environmental and economic sustainability because it maintains natural resources through energy efficient methods (Alam *et al.*, 2015). Biodiesel is currently produced from oil crops, waste cooking oil or animal fat. These sources are the candidates to reduce the consumption of fossil fuels, but in truth they are not viable enough to satisfy

even a small fraction of the total demand of fossil fuels (Hu *et al.*, 2015). In Brazil ethanol is produced from sugar cane, in USA ethanol is mainly produced from maize and Europe produces an estimated of 8% of the global biodiesel from domestically grown rapeseed (SAASTA, 2013).

Research has begun to focus on alternative biomass-derived fuels. On this way, one promising source of biomass for alternative fuel production is microalgae, which can be used as a fuel to supplement coal in generation of electricity (Richardson *et al.*, 2010). From worldwide total biodiesel production, just a simple fraction of 2.42% comes from miocroalgae because its production is still being studied. Main algae biodiesel producers are The Netherlands, UK, USA, Canada and Argentina (Torres *et al.,* 2013).

Currently, microalgae are promoted as an ideal third generation biofuel feedstock due to its fast growth rate, greenhouse gas fixation ability (CO_2 fixation) and high capacity of lipids production (Alam *et al.*, 2015). Microalgae are converters of solar energy capable of producing double time the biomass per unit area contrasted to terrestrial plants. Even so, microalgae are efficient biological factories able to convert zero energy –CO_2 gas emissions- into valuable oils and biomass as feedstocks to produce biofuels (Ghayal and Pandya, 2013).

There are several species of microalgae that biologists have previously categorized depending by their pigmentation, life cycle and basic cellular structure (Ghayal and Pandya, 2013). Main potential species for biodiesel production include *Chlorella* and *Scenedesmus* because they can double their biomasses within 24 hours and the oil contents are in the range of 20-30 % (dry wt.) (Alam *et al.*, 2015). Oil productivity, which is the mass of oil produced per unit volume of microalgae, duplicate

per day (Hu *et al.*, 2015). Microalgae with optimal growth-rates and content of lipids are suitable options for producing biodiesel.

Biodiesel is a mixture of monoalkyl esters of long chain fatty acids, which can be transesterified from algae lipids (Yusuf Chisti, 2007). The most significant distinguishing characteristic of algae oil is its yield, and hence its biodiesel yield. As stated by Ahmad *et al.,* the yield per acre of oil from algae is over 200 times the yield from the best-performing plant or vegetable oils such as maize and sugar cane(2013). There are several methods used to extract oil from algae including chemical solvents, mechanical systems, thermal and plasma techniques (Li *et al.,* 2007). In the case of the former one, algae oil can be extracted by using suitable solvents such as chloroform and methanol. Transesterification is the most common method that leads to monoalkyl esters of algae oil (Ahmad *et al.*, 2013). This process requires a homogeneous catalyst (KOH) and methanol to perform successfully the reaction.

The objective of this study was to assess the biodiesel production from native Ecuadorian microalgae. Optimized cultivation techniques were applied to improve the growth rate of microalgae and to maximize the total content of lipids. The effect of injecting CO_2 to the photobioreactor was evaluated in the culture. Simultaneously, a weekly monitoring was conducted during the reactor operation time to evaluate nutrient assimilation, biomass production and lipids production. Finally, biodiesel was produced by tranesterification process using a chemical homogeneous catalyst (KOH) and methanol. Presence of methyl esters compounds (biodiesel) was confirmed by Thin Layer Chromatography employing silica plates revealed with an iodine chamber.

Materials and Methods

Chemicals.

Sodium nitrate, calcium chloride dehydrate, dipotassium phosphate, monopotassium phosphate, sodium chloride, sodium bicarbonate and proteose peptone were obtained from Reactivos H.V.O (Quito, Ecuador). Magnesium sulfate heptahydrate was acquired from Representaciones Vamarth (Quito, Ecuador). For lipids extraction and tranesterification process, chloroform, hexane, methanol, potassium hydroxide, petroleum ether, dietylether and acetic acid were obtained from Reactivos H.V.O (Quito, Ecuador). All the reagents used in this study were of analytical grade and were used as received.

Microalgae strain cultivation.

The native microalgae was donated from a *Chlorella sp.* strain from “ESPE”, Escuela Politécnica del Ejército. Microalgae were cultivated in a 10 L tubular photobioreactor (T PBR) with a 3 L min^{-1} constant aeration rate, provided by an aquarium pump. Optimal cultivation conditions were assured by offering artificial light with 5 fluorescent 20W OSRAM tubes with 12 hours photoperiods.

Nutrient & fertilizer medium.

The composition of the nutrient medium was (g L^{-1}): $NaNO_3$ (0.25), $CaCl_2 \bullet 2 H_2O$ (0.025), $MgSO_4 \bullet 7 H_2O$ (0.075), K_2HPO_4 (0.075), KH_2PO_4 (0.175), NaCl (0.025), Proteose Peptone (1) and $NaHCO_3$ (0.1). When needed, fertilizer medium was added to the tubular photobioreactor for improving the culture conditions and the composition was: 5 mL of salts solution which include (g): NH_4Cl (100), $MgSO_4 \bullet 7 H_2O$ (4), $CaCl_2$

• 2 H_2O (2), 5 mL of phosphate solution (K_2HPO_4 and KH_2PO_4), 1 mL of Sueoka's high salt medium, 1 mL of an 8% sodium bicarbonate solution and 1g of Agroquality fertilizer. Both mediums were sterilized in the autoclave during 15 minutes at 121ºC prior adding to the T PBR.

CO_2 injection.

A CO_2 air Aluminum cylinder tank provided carbon dioxide (CO_2) to the tubular photobioreactor. The inlet flow was regulated by a gas proportioner instrument (Model G, AALBORG). A mixture of 95% air and 5% CO_2 , was injected to the photobioreactor using a homogeneous Model G gas proportioner blended.

Tubular photobioreactor monitoring (T PBR).

Daily monitoring included taking out 400 mL of medium from the T PBR and measuring in-situ parameters. Dissolved Oxygen (DO), temperature, pH and conductivity were measured with a Thermo Scientific Orion 5-Star portable multiparameter meter (Thermo Scientific, Beverly, MA 01915, USA). Afterwards, 400 mL of fresh nutrient medium were added.

Once a week, microalgae nutrients assimilation was analyzed. NH_4^+-N and NO_3^--N concentrations were determined using Orion ion-selective electrodes, respectively. Chemical oxygen demand (COD) and phosphates were measured by a colorimetric method using a Spectronic 20D+ spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA). Samples for biological oxygen demand (BOD) test were incubated into the Oxitop Box – Thermostat box with forced air circulation for 20 (±0,5 °C) during 5 days. Concurrently, cell density, cellular concentration and total content of lipids were measured every week.

Cellular concentration.

Cellular concentration was determined by using a Neubauer counting chamber. To set up this test, 40 μL of microalgae sample was diluted in 160 μL of lugol's solution. This solution helps to immobilize the cells. 10 μL of the prepared mixture were added to the counting chamber. Cell counting was carried out in 40X lens of a Leica CME microscope. Cellular density was calculated by using Eq. 1.

$$Cellular\ concentration\ [\#\ cells\ mL^{-1}] = Average\ cells\ \#\ \times dilution\ factor\ \times 10^{4} \quad \text{Eq.1}$$

Biomass and lipids extraction.

For biomass determination, a 45 mL sample was centrifuged at 5000 rpm for 10 minutes. Biomass was dried by placing it into the oven at 105ºC overnight. Total biomass was determined by weight difference (Eq. 2). Lipid extraction was achieved by a solvent extraction method based on chloroform and methanol. Dried biomass was grinded and transferred to 15 mL falcon tubes. 2 mL of chloroform ($CHCl_3$) and 1 mL of methanol (CH_3OH) were added and centrifuged at 5000 rpm for 10 minutes. The upper layer (supernatant) was the one that contained the extracted lipids. This layer was transferred into another falcon tube where 5 mL of distilled water was added. When centrifugation was done in the last falcon tube, three layers were distinguished: upper layer-water, intermediate layer- organic particles and bottom layer- chloroform and lipids mixture. The bottom layer was transferred into previously weighed digestion tubes. This process was repeated from 3 to 5 times until obtaining a clear supernatant. Finally, when chloroform was totally evaporated, digestion tubes with dry lipids were weighed. Total content of lipids was calculated with Eq. 3 & 4.

$$Cellular\ Density = \frac{falcon\ tube\ with\ dry\ biomass - empty\ falcon\ tube}{centrifugation\ volume} \quad \text{Eq. 2}$$

$$Dry\ lipids\ (g) = glass\ tube\ with\ lipids\ - glass\ tube \qquad \text{Eq.3}$$

$$Lipidis\ content\ \%\ \left(\frac{w}{w}\right) = \frac{Dry\ lipids\ (g)}{Dry\ biomass\ (g)} \times 100 \qquad \text{Eq. 4}$$

Transesterification.

Transesterification was accomplished in presence of a homogeneous catalyst (KOH) and methanol according to the protocol described by Geris *et al* (2007). Previous extracted lipids were added (approx. 2 g) to the solution of KOH and methanol in agitation for 1.5 hours. This mixture was placed into a separatory funnel and distilled water was added to wash the biodiesel produced. The washing biodiesel process started with the removal of the aqueous phase. Subsequently, 15 mL of NaCl saturated solution was added to the separatory funnel. This process was repeated until the aqueous phase was totally cleaned, and pH measurements were conducted until obtaining neutral values (7-6). Biodiesel (upper phase) was transferred into a test tube to determine the total volume of biodiesel obtained. Magnesium sulfate anhydrous was added until biodiesel turned apparently white. In order to confirm the effectiveness of the transesterification process commercial soja oil was employed to produce soja biodiesel as a positive control.

Thin Layer Chromatography (TLC) & R_f values.

TLC plates EMD Millipore Company were prepared by marking two reference points at the bottom and upper part (solvent front) of the plate. The solvent mixture consisted on petroleum ether (8 mL), ethyl ether (1.9 mL) and acetic acid (0.1 mL). With a capillary tube, biodiesel was placed at the starting point (bottom part) of the TLC plate. The TLC plate was stood in a shallow layer of the prepared solvent mixture in a covered beaker until the solvent reached the “solvent front”. Ultimately, the TLC

plate was revealed with an iodine chamber. TLC plates for soja biodiesel produced from commercial oil were also run as a reference.

R_f values are the measurements of the travelled distance by the solvent, and the distance travelled by individual spots. R_f values were calculated using Eq. 5.

$$R_f = \frac{distance\ travelled\ by\ component}{distance\ travelled\ by\ solvent} \quad \text{Eq. 5}$$

Results and Discussion

Optimization of microalgae cultivation.

Nutrient medium was used to cultivate *Chlorella sp.* (Fig. 1), which provided many elements for the growth of microalgae such as C, O, H, N, K, Ca, Mg, P, and trace elements. The initial concentration of nitrate (NO_3^-) was 180 mg L^{-1}. Ammonium concentration (NH_4^+) was provided from protesoe peptone and its initial concentration was 177.8 mg L^{-1}. In the case of P, the initial phosphates (PO_4^{3-}) concentration was 162.1 mg L^{-1}.

Fig 2 illustrates the assimilation of nitrogen and phosphorous by native *Chlorella sp.* strain cultivated in the tubular photobioreactor as a function of operation time. Ammonium absorption started at the second week even faster than nitrate production. According to *Zhu et al.,* the absorption and utilization of nitrogen have the following order: ammonium > urea > nitrate > nitrite (2013).

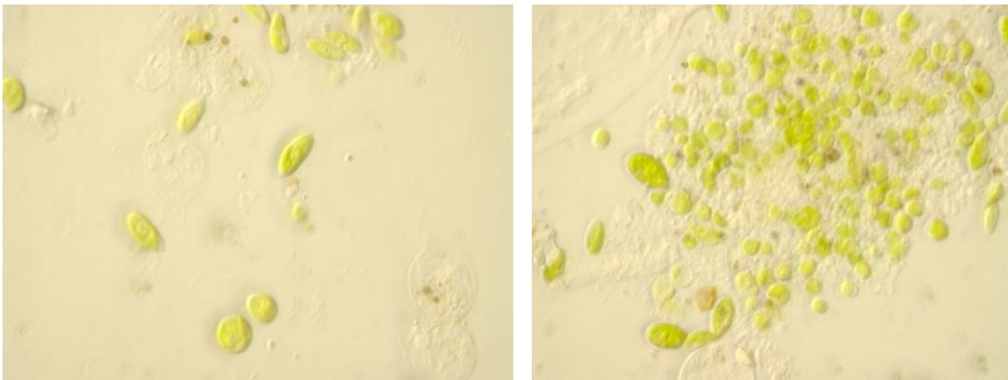

Figure 1: Native Ecuadorian microalgae strain *Chlorella sp*.

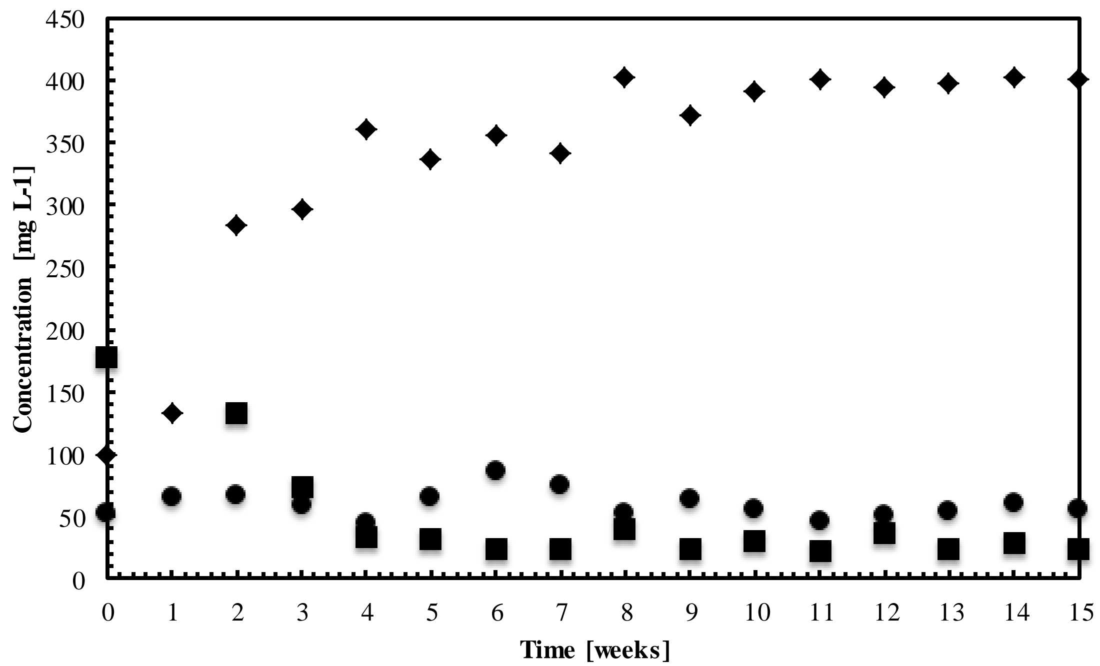

Figure 2: (◆) Nitrate production, (■) ammonium and (•) phosphate assimilation of *Chlorella sp.* strain cultivated in a 10 L T PBR with a 3 L min^{-1} constant aeration rate and 5 fluorescent artificial light 20W OSRAM tubes with 12 hours photoperiods.

Thus, ammonium is directly used to synthesize amino acid while the other nitrogen sources have to be converted to ammonium and then amino acids can be synthesized (Zhu *et al.*, 2013). It was also found that microalgae grow properly using sodium nitrate ($NaNO_3$) as a nitrogen source as illustrated by an optimal biomass productivity and lipid content of 30% (Yusuf Chisti, 2007). The Hydraulic Retention Time (HRT) in the tubular photobioreactor was 20 days hence significant nutrient's removal was observable at the end of this period.

As exposed in Figure 2, there is an evident NO_3-N production and NH_4^+-N removal. Nitrogen balance was done in order to determine the initial and final N-concentration during the HRT. Initial N-concentration was 178.9 mg L^{-1} and after 20 operating days, the concentration was 123.6 mg L^{-1}, with a difference of 55.2 mg L^{-1}. N-concentration for all period is exposed in table 1. None period presented the same N-concentration at the beginning and at the end of every 20 days. For every case there was a difference of N-concentration which indicates that part of the nitrogen was incorporated into microalgae biomass, considering that 5-10% of it is nitrogen (Zaimes and Khanna, 2013). During microalgae cultivation N and P became limiting since they both played a role in controlling the growth rate and lipid production of microalgae.

Table 1: Nitrogen balance for the photobioreactor during operation time

Period	**mg N-NO3 L^{-1}**	**mg N-NH4 L^{-1}**	**mg N L^{-1}**	**Difference [mg N L^{-1}]**	**Incorporated in Biomass [%]**
Day 1	40,6	138,3	178,9		
Day 20	67,1	56,6	123,7	55,2	7,5
Day 40	80,3	19,1	99,4	79,5	11,7
Day 60	84,0	18,7	102,8	76,2	11,0
Day 80	88,9	27,9	116,8	62,2	9,6
Day 100	90,4	18,4	108,8	70,2	10,3

Fertilizer medium was used since week 6 due to poor conditions of microalgae in the photobioreactor. This fertilizer medium is rich in P-source but short in N-source, for all that, low cellular concentration of 1.02×10^6 cells mL^{-1} measured. In fact at this point, a maximum concentration of PO_4^{3-} of 86.2 mg L^{-1} was registered. At the end of the monitoring time, nutrients' concentrations were almost constant. For instance, Zhu and coworkers in their study about cultivation microalgae for biodiesel found that a possible reason for the final T PBR's stationary state is traceable to the low concentration of microalgae cells and limiting nutrient conditions (Zhu *et al.*, 2013).

Effect of CO_2 injection in microalgae growth.

The effect of CO_2 injection in microalgae growth was evaluated in the tubular photobioreactor. Throughout week 5, a mixture of air (95%) and CO_2 (5%) was supplied to the T PBR. As established by Zhu et al, maximum photosynthetic efficiency is often achieved with CO_2 concentrations from 1% to 5% (by volume) (Zhu et al., 2013). In this study, the nutrient medium used already had a C-source, which was $NaHCO_3$ (0.1 mg L^{-1}). Zhu's *et al* study demonstrated that $NaHCO_3$ can be used as a buffering agent to control the pH (2013). However, in this study the CO_2 injected did not have a positive effect in microalgae growth. In fact, as illustrated in Fig. 3 the pH decreased to acid values. In addition, low concentrations of phosphates were assimilated, while fewer nitrates were produced as demonstrated in Fig. 2. However ammonium was normally assimilated.

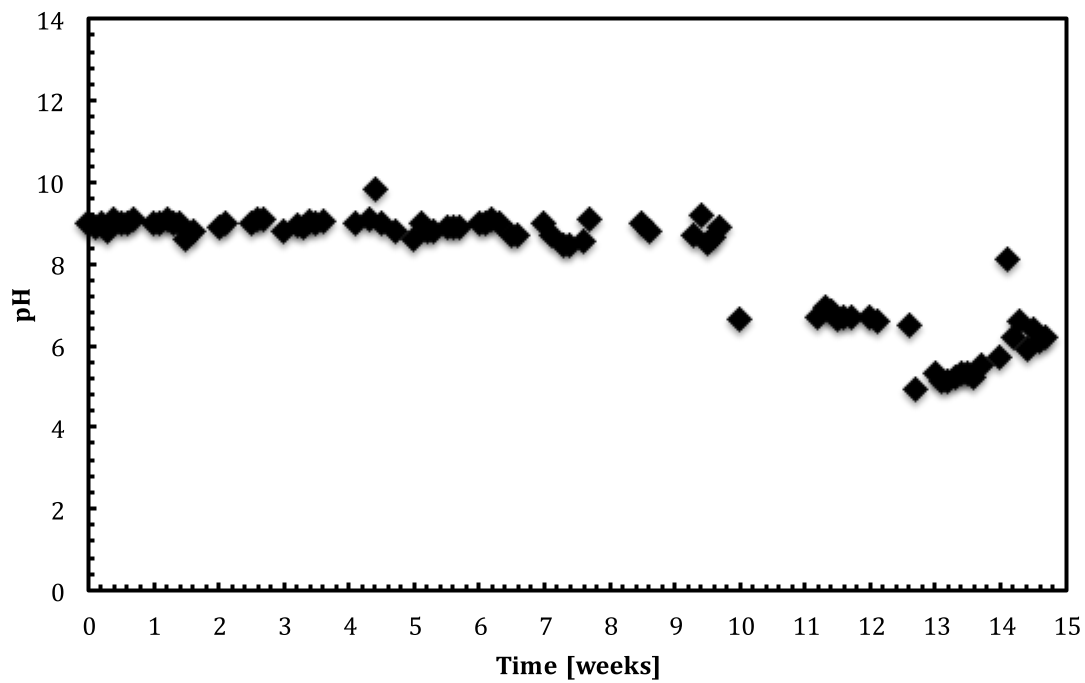

Figure 3: pH monitoring for *Chlorella sp.* strain cultivated in a 10 L T PBR with a 3 L min^{-1} constant aeration rate and 5 fluorescent artificial light 20W OSRAM tubes with 12 hours photoperiods.

pH is another main factor influencing the cultivation of microalgae. When beginning with the monitoring (Fig. 3) the pH of the cultivation was 9, while an optimal growth pH range is from 6 to 7 (Yusuf Chisti, 2007). As described by Zhu et al, when pH > 8.3 the most abundant ion present in the cultivation is HCO_3^- and when the pH <5 the majority of dissolved inorganic carbon is CO_2 (2013). Therefore the predominant ion when pH was 9, was HCO_3^- because of the high concentration of $NaHCO_3^-$.

Daily monitoring of operational parameters.

Daily monitoring of operational parameters in the photobioreactor included pH, DO, temperature and conductivity measurements. pH is one of the factors influencing the cultivation of microalgae in the photobioreactor. As illustrated in Fig. 3 when beginning with the monitoring, the pH of the cultivation was 9, while according to the literature for an optimal growth, the pH values typically range from 6 to 7 (Yusuf Chisti, 2007). As described by Zhu et al, when pH > 8.3 the most abundant ion present in the cultivation is HCO_3^- and when the pH <5 the majority of dissolved inorganic carbon is CO_2 (2013). Therefore, in the cultivation medium evaluated in this study, HCO_3^- was the predominant ion when pH was 9 because of the high concentration of $NaHCO_3^-$.

Temperature varied from 19-25 ºC (Fig. 4), the ideal temperature for microalgae culture ranges between 15-27 ºC (Pruvost *et al.*, 2015). Dissolved Oxygen values oscillated between 5-7 mg L^{-1} (Fig. 4) and are the appropriated for the culture of microalgae. Low oxygen concentrations (< 4 mg L^{-1}) can have deleterious effects on microalgae causing significant decrease in photosynthetic performance (Haas *et al.*, 2014). DO over 8 mg L^{-1} is toxic for *Chlorella sp.* because it causes oxygen tension during periods of high light intensity (morning times) leading to photo-oxidation which reduces the yield of the culture (Dormido *et al.*, 2014).

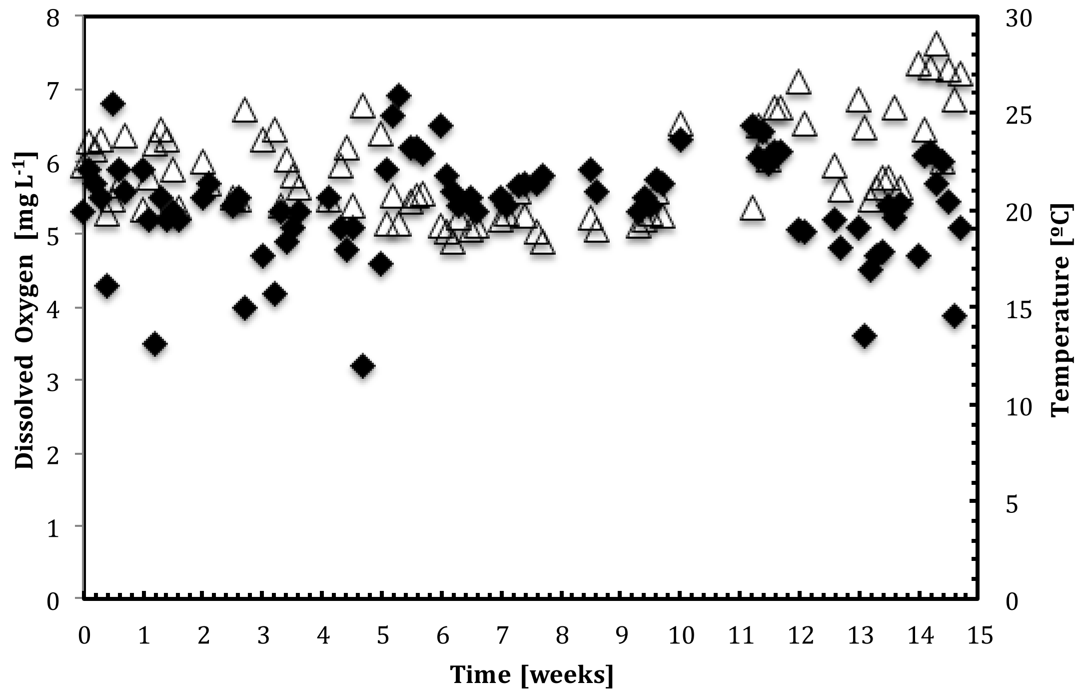

Figure 4: Dissolved Oxygen (◆) and Temperature (Δ) monitored for *Chlorella sp.* strain cultivated in a 10 L T PBR with a 3 L min^{-1} constant aeration rate and 5 fluorescent artificial light 20W OSRAM tubes with 12 hours photoperiods.

In the photobioreactor, conductivity had values within 1500-2000 μS cm^{-1} during first operation time while using nutrient medium. When fertilizer medium was added to the culture (day 80), conductivity values were mainly constant between 1000 and 1500 μS cm^{-1} (Fig 5). Conductivity indicates the total ions present on the culture. As nutrient medium is abundantly composed by salts and organic compounds, conductivity increases (Mostafa *et al.*, 2012).

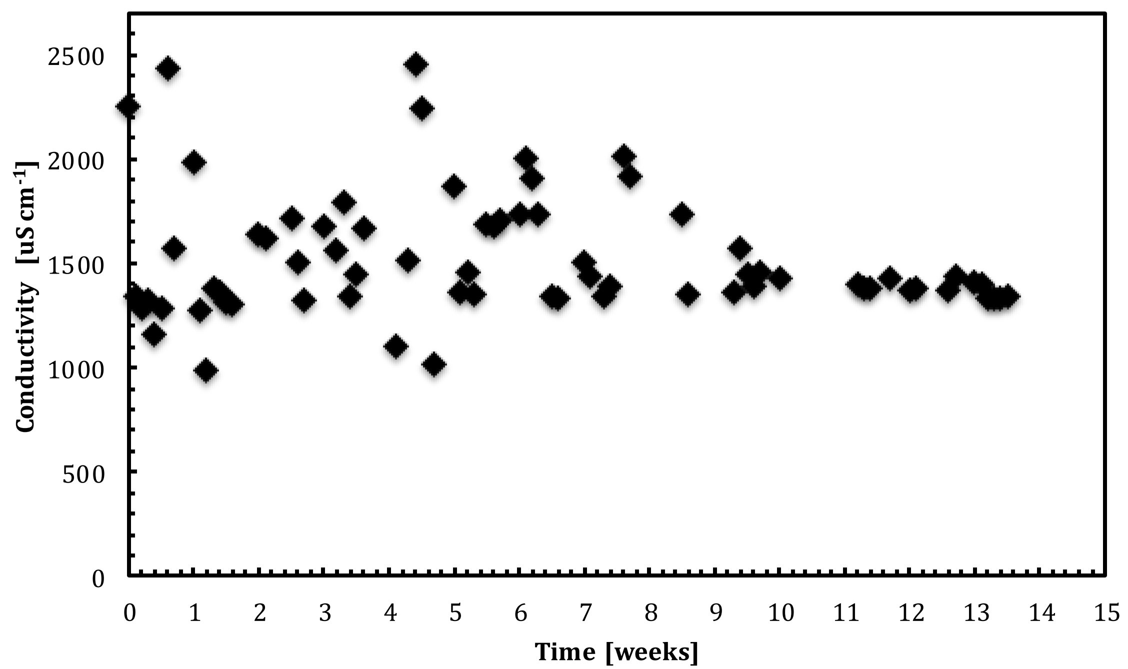

Figure 5: Conductivity (◆) monitoring for *Chlorella sp.* strain cultivated in a 10 L T PBR with a 3 L min^{-1} constant aeration rate and 5 fluorescent artificial light 20W OSRAM tubes with 12 hours photoperiods.

Cellular concentration, lipid content and biomass production.

Cellular concentration measurement are a good indicator of cells growth and presented an initial concentration of 1.8×10^6 cells mL^{-1}. As shown in figure 6, during the first 3 weeks cellular concentration was not constant. The second week presented less concentration than the initial one, while in the third and fourth week showed an increment on cells growth. However, during CO_2 injection (week 5) there was a remarkable decline on cells growth, showing a concentration of 1.0×10^6 cells mL^{-1}. Fertilizer medium improved the culture growth and straightaway, the reported cellular concentration was 4.2×10^6 cells mL^{-1}. Since week 6 cellular concentration remained partially constant within a range of 3.5×10^6 - 4.5×10^6 cells mL^{-1}.

Air enriched with CO_2 is considered to upgrade microalgae growth because they incorporate it into the photosynthesis process, providing energy and higher capability for cells to duplicate (Tsuzuki *et al.*, 1990). In contrast, cellular concentration in this experiment decreased when CO_2 was injected to the photobioreactor. This is attributable to the low pH values registered during week 5. When fertilizer medium was supplied, cellular concentration values incremented considerably. As stated by Zhu et al., cellular growth increases with increasing concentration of P since this element plays an important role in metabolic processes of microalgae such as transduction and energy conversion during photosynthesis (2013).

An important part of this study was is to determine the total lipids content or triacylglycerol (TAG) because these are used for biodiesel production. Figures 6 & 7 show the lipids content according to cellular and biomass concentration, respectively. There is a notorious relationship between cellular concentration and content of lipids. The initial value for lipids content was 9.0 %(v/v). During week 5, when the lowest cellular concentration was measured, the lipids content was 10.3 %(v/v). A meaningful

increment on the content of lipids was appreciated since week 6, when fertilizer medium was supplied. In week 8, the highest cellular concentration was registered (4.2×10^6 cells mL^{-1}) as well as the highest content of lipids 38.4 %(v/v). Subsequently, microalgae culture reached an optimal content of lipids within 25-30% (v/v). Microalgae oil content for *Chlorella sp.* varies from 28-32 % (v/v) (Andruleviciute *et al.*, 2014).

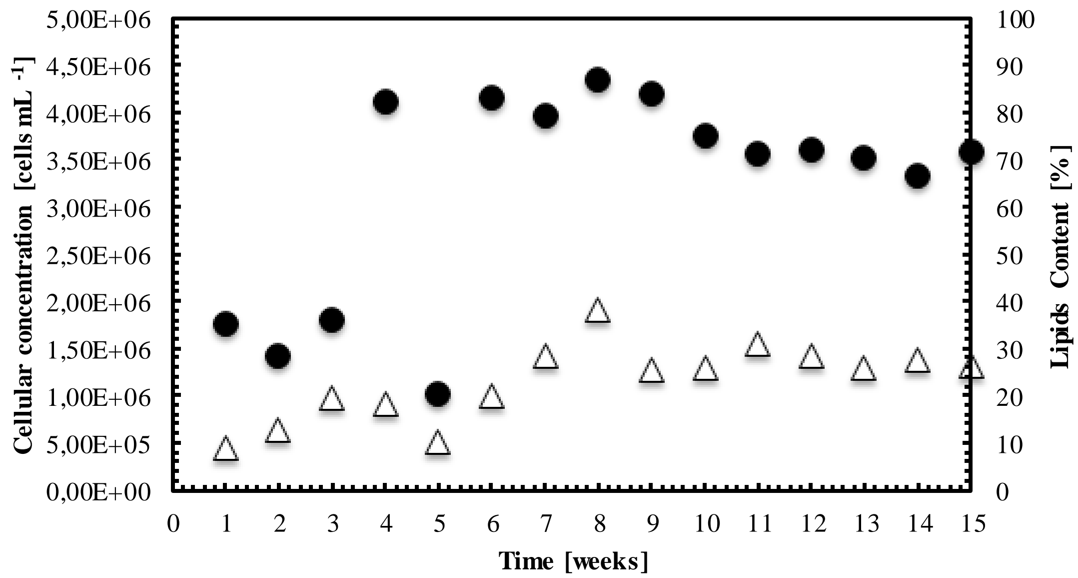

Figure 6: Cellular concentration (•) and lipid content (Δ) for *Chlorella sp.* strain cultivated in a 10 L T PBR with a 3 L min^{-1} constant aeration rate and 5 fluorescent artificial light 20W OSRAM tubes with 12 hours photoperiods.

In the case of the biomass concentration, during the first week the concentration was 0.7 g mL^{-1}. The lowest biomass concentration (0.1 g mL^{-1}) was found during week 5 (Fig. 7). As can be observed, from week 5 to 6, biomass concentration increased by 17.4 % which leads to a final concentration of 0.69 g mL^{-1}. In addition, 0.8 g mL^{-1} was the highest biomass concentration, and it was measured during week 8. Since then, the concentration tended to be constant and oscillated within 0.6- 0.7 g mL^{-1}.

Lipid content in microalgae highly depends on nitrate in the medium. N-Stress is a suitable option for increasing lipid content. The possible reason is that the content of adenosine monophosphate deaminase (AMPD) increases under N-stress conditions, promoting the catalysis of adenosine monophosphate (AMP) to ionsine monophosphate (IMP) and ammonia. Because most of isocitrate dehydrogenase (ICDH) in mitochondrion is dehydrogenase dependent on AMP, reducing AMP would inhibit the activity of ICDH (Zhu *et al.*, 2013). Afterwards, ICDH is used to produce ATP molecules, if ATP is not being produced, ICDH will accumulate in the cell. Therefore, additional lipids stock would be expected.

Consequently, as high lipid production is correlated with nutrient deprivation, it results in diminished photosynthetic efficiency and decreased cellular growth (Chiu *et al.*, 2009). Hence, the two conditions of high lipid content and high biomass productivity are mutually exclusive. When fertilizer medium was provided to the culture, lipids content incremented significantly from 10 to 25% (v/v) (Fig. 7) and even the highest content of lipids was registered 38.4% (v/v), because there was a N-privation. However, the microalgae strain has gained an optimal content of lipids and an ideal growth rate of 1.4×10^5cells mL^{-1} d^{-1}.

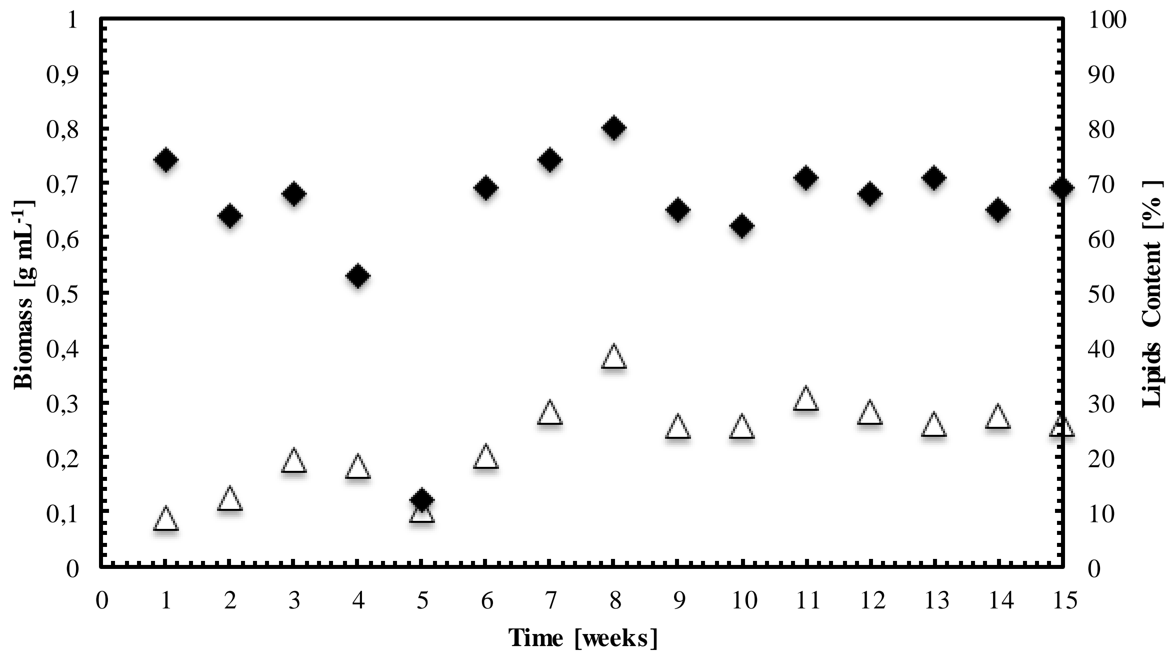

Figure 7:Biomass production (◆) and lipid content (Δ) for *Chlorella sp.* strain cultivated in a 10 L T PBR with a 3 L min^{-1} constant aeration rate and 5 fluorescent artificial light 20W OSRAM tubes with 12 hours photoperiods

Production of biodiesel from transesterification process.

Catalytic transesterification is a common and well-established chemical reaction in which alcohol reacts with triglycerides of fatty acids (microalgae lipids) in the presence of a catalyst (KOH) (Krishnakumar *et al.*, 2008) (Fig. 8). Therefore in this study, transesterification of microalgae TAGs took place in presence of methanol and KOH as a homogeneous catalyst to obtain methyl esters (biodiesel). Several investigators found that the reaction starts very fast and almost 80% of the conversion takes place in the first 5 minutes and after 1 hour nearly 93-98% of triglycerides are converted to ester (Krishnakumar *et al.*, 2008). In the present work, three transesterification trials were made. Table 2 presents the results for the transesterification process including different content of lipids (%), amount of lipids (g) and final volume (mL) of obtained biodiesel. The reaction time was 60 minutes, which ensures total conversion of TAGs into methyl esters. For the first trial, when washing biodiesel, saponification occurred because using KOH conducted to hydrolyze any produced ester. This undesirable saponification reduces the ester performance (Geris *et al.*, 2007). A NaCl saturated solution was used for the next two trials to avoid emulsion formation and impeding biodiesel separation process. The third trial presented the highest amount of lipids (2.2 g) as well as the highest biodiesel volume (6 mL). Biodiesel final volume does not depend on the content of lipids (Ault *et al.*, 2012). As indicated in table 2, first trial had an elevated content of lipids (38.35 %) however, the final biodiesel volume was 2mL, which is the lowest volume between the three trials. Obtained microalgae biodiesel had a color between white and light green and a neutral pH.

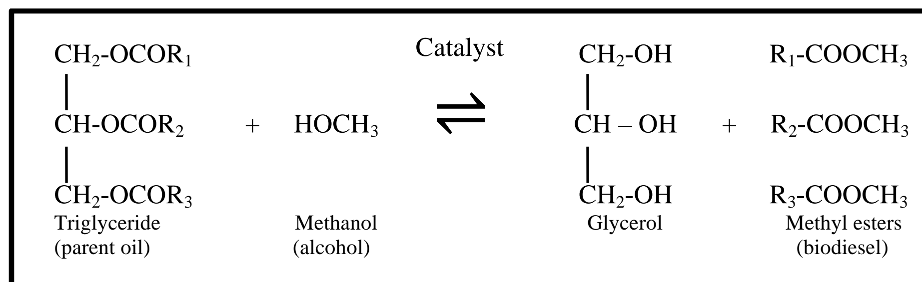

Figure 8: Transestrification of tricyceride (microalgae TAGs) in presence of KOH (homogeneous catalyst) to methyl esters (biodiesel).

Table 2: Tranesterification process including different content of lipids (%), amount of lipids (g) and final volume (mL) of obtained biodiesel

Trial	Content of lipids [%]	Lipids [g]	Biodiesel [mL]
1	38.4	0.9	2
2	30.9	1.2	2.6
3	27.4	2.2	6

The reaction efficiency of the transesterification process was evaluated using TLC plates. The chromatogram analysis confirmed the conversion of triglycerides in methyl esters. Retardation factors (R_f) values calculated in this study are presented in table 3, since these factors are difficult to keep constant from one experiment to another, "Relative R_f values" are generally considered (Ault and Pomeroy, 2012) . According to Geris *et al*, Soja biodiesel has a relative R_f value of 0.83 (2013), which is similar to the R_f value for the soja biodiesel obtained in this study, 0.82. On the same way, Shan *et al.* experiments on biodiesel from microalgae, presented a relative R_f value of 0.7 (Shan *et al.*, 2012). Relative R_f values for this work are reasonably close to the one obtained in Shan *et al.* studies, 0.71 and 0.76 for trial 2 and 3, respectively. Differences between these values are caused by the purity of solvents, the solvent mixtures, TLC plates, amount of methyl ester spotted and temperature (Wegeberg and Felby, 2010).

Table 3: Retardation factor (R_f) determined by using Thin Layer Chromatography (TLC) for three different trials of microalgae biodiesel a Soja biodiesel control

Substance	$\boldsymbol{R_f}$
Microalage biodiesel (trial 1)	0.65
Microalage biodiesel (trial 2)	0.71
Microalage biodiesel (trial 3)	0.76
Soja Biodiesel (control)	0.82

Conclusions

The feasibility of producing biodiesel from native Ecuadorian microalgae was successfully studied based on microalgae culture optimization, total lipid content and the transesterification process. *Chlorella sp.* is a good lipid producer with optimal lipid content, presenting an ideal growth. The optimal microalgae growth was obtained when using fertilizer medium. Nevertheless, it is essential to find out a desirable concentration of $NaHCO_3$ and CO_2 to maintain an optimal pH in the culture medium. Cellular concentration always had optimal values ranging from $3.5 \times 10^6 - 4.5 \times 10^6$ (Saeid and Chojnacka, 2015), lipids content oscillated from 20% to 30% which is optimal for *Chlorella sp.* (Nurachman *et al.*, 2015). It is highly recommended to carry out a Life Cycle Assessment in order to determine if its production is sustainable. The present study provides important information regarding microalgae culture conditions for biodiesel production as the first biofuel from microalgae processed in Ecuador.

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