Production of bioethanol from biomass of microalgae *Dunaliella tertiolecta*

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***Abstract*—** *Biofuel production from microalgae biomass appears as a promising long term alternative. Dunaliella tertiolecta is a microalgae with high tolerance to salinity, temperature, and light, making it relatively easy to grow. The aim of this study was to establish a pilot-scale culture to evaluate the biomass yield and bioethanol production. The cell culture of D. tertiolecta was started in 20 ml tubes and escalated to 20 L containers. The biomass yield was 0.153 g L^{-1} of dry basis (db) and its characterization showed protein (37% db) as major component followed by carbohydrates (35.6), lipids (13% db) and ash (6.5%). The carbohydrate fraction was composed of starch (27.1% db) and fiber (8.5 %) and its neutral sugar characterization yield glucose (91% molar). The main components of the lipid fraction were linolenic and palmitic acids. The biomass was subjected to an acid pre-treatment for the saccharification of complex carbohydrates, and the hydrolyzed biomass was fermented by Saccharomyces cerevisiae. It was possible to produce 0.615 ml g^{-1} of ethanol. In conclusion, D. tertiolecta has the potential for bioethanol production, making it a promising option for the biofuels future.*

***Keywords*— biofuels, microalgae, bioethanol, fermentation, composition.**

I. INTRODUCTION

Currently, biofuels are being highly studied due to the interest of reducing the emissions of green house gases that affect the environment and humans. Oceans absorbs great concentrations of the CO_2 released into the atmosphere, which causes a decrease in pH and impacts in negative changes to the marine ecosystem (Mata et al., 2010).

The increase in fossil fuel prices and the attempts to mitigate environmental pollution, have evolved in new energy resources, but the development of new process is needed particularly in the production of liquid biofuels such as bioethanol (Vertés et al., 2010).

The bioethanol is gaining popularity among other fuels by the wide variety of raw materials that can be used for production. The main producers of bioethanol used as raw material sugar cane and cereals rich in sugar and starch, such as corn (Gazzoni, 2009). However, the growth of the biofuel industry in the world is limited by the availability of land suitable for agriculture and biomass yields per hectare. Therefore, obtaining biofuel from microalgae biomass appears as a promising alternative for long term, this is because the algae is able to grow practically anywhere where enough sunlight and water is guaranteed. Other positive aspects is that these microorganisms might achieve a complete growth cycle in a few days, do not compete for agricultural areas with the food industry because do not require large areas or fertile land for the propagation. Microalgae biofuel also contributes to environmental improvement by capturing CO_2 (Yun et al., 1997; Benjumea et al., 2009; Zah et al., 2010).

Microalgaes are photosynthetic organisms with the ability to transform solar energy into macro molecules for chemical energy storage, such as carbohydrates, proteins and lipids (De Schamphelaire and Vestraete, 2009).

One of the uses of microalgae is the production of secondary metabolites, which can be used as antibiotics, algaecides, toxins, pharmaceutical active compounds, and also for extraction of macromolecules such as lipids, proteins and nucleic acids (Kumar and Verma, 2009). On the other hand, the components of biomass can also be used in the production of biofuels such as biodiesel, bioethanol and biogas, among others (Demirbas, 2010).

Dunaliella tertiolecta presents high tolerance to changes in salinity, temperature and light, so its cultivation is relatively easy compared to other sensitive species. Once this species rise the adaptation, its rate of growth is very high improving its yield (Chen et al., 2011a).

In this research, *D. tertiolecta* culture was established on a pilot basis, and the biomass was collected and characterized previously to ethanolic fermentation with *Saccharomyces cerevisiae*.

II. MATERIAL AND METHOD

2.1 Biomass cultivation

All material was immersed in 2 M HCl for 24 h, rinsed with distilled water, and finally autoclaved. Seawater recollected from Altata bay (Sinaloa, Mexico) was subsequently filtered through a sieve (100 μm), activated carbon (5 μm) and nylon membrane (0.45 μm). The salinity of the water was measured indirectly and adjusted with distilled water to 33 $g \cdot L^{-1}$ by measuring total soluble solids using a refractometer (Mettler Toledo RE40D). Nutrient solutions for the f/2 medium were prepared according to Guillard and Ryther (1962) for 20 L of culture. Four liters of *D. tertiolecta* culture provided by the Institute of Marine Sciences and Limnology, UNAM (Mazatlan, Sinaloa, Mexico) were used as inoculum. The cultivation was maintained under controlled conditions; temperature of 23 to 24 °C with 12 h light and 12 h darkness cycles and connected to an aeration system with filters of activated carbon and silica. *D. tertiolecta* biomass was recovered by centrifugation at 4 000 rpm and 10 °C, then washed with 0.5 M ammonium formate and lyophilized for preservation.

2.2 Composition analysis

The AOAC method (988.05; Micro Kjeldahl) was used for protein determination. Briefly, 0.1 g sample was weighed, then 1.5 g of catalyst mixture (5% $CuSO_4$ and 95% K_2SO_4) and 5 ml of H_2SO_4 were added. It was boiled in the digester micro kjeldahl then distilled (Labconco Rapid Still I) and mixed with 40% NaOH. The distillate was titrated with 0.1 N HCl and the protein content was calculated using a protein nitrogen factor of 6.25. Ashes were determined by the gravimetric method AOAC (923.03). The sample was calcined into a muffle at 550 °C for 3 h, then it was cooled into a desiccator previously to weighing. Mineral content was analyzed from the ashes according to the AOAC (955.06) method. The individual determination of each mineral was performed by atomic absorption spectrophotometry (Varian Model SpectrAA-20). The minerals Ca, Mg, Fe and Zn were determined by lamps at specific wavelengths, while the Cu and K were measured by flame emission.

Lipids were extracted from the biomass by mixing with 8 ml of hexane for 30 min at 60 °C. Upper phase was separated by spinning, transferred to a preweighed flask and rotovapped for lipid concentration. The content was calculated by weight difference.

Total dietary fiber assay was determined according to AOAC (Method 985.29) with the commercial kit from Megazyme. First, 0.6 g of sample was weighed by duplicated in flasks and 50 ml of phosphate buffer (pH 6.0) were added. The enzymatic hydrolysis was performed with thermostable α-amylase (150 U) in a boiling water bath for 30 min at 100 °C, protease (35 U, pH 7.5) and amyloglucosidase (40 U, pH 4.5) both during 30 min at 60 ° C. The solubilized dietary fiber was subsequently mixed with 300 ml of ethanol (60 °C) for precipitation, after 2 h was recovered by filtration in crucibles with 1 g of celite and oven-dried (70 °C). After cooled and weighed; the crucibles were used for protein and ash quantification. Total dietary fiber was determined by gravimetry. For starch determination, 30 mg of biomass was mixed with 30 U of α-amylase and 20 min boiled in water bath for 20 min. Four ml of 200 mM sodium acetate and 20 U amyloglucosidase were added and the samples incubated for 30 min at 50 °C. The volume was adjusted at 10 ml with distilled water and insoluble material was pelleted by spinning (3,000 xg for 10 min). The supernatant was diluted and aliquotes were used for glucose assay by GODOP method (glucose oxidase, peroxidase and 4-aminoantipyrine in hydroxybenzoic acid buffer and sodium azide) (Megazyme). The absorbance was measured at 510 nm using a spectrophotometer (Varian Cary 1E) and calculations based in glucose solutions of known concentration.

Lipids were extracted by the method of Folch et al. (1957). Extracted lipids were derivatized to methyl esters following the AOAC (969.33). A gas chromatograph (Varian CP-3800) with FID detector (250 °C) and a capillary column 30 m x 0.32 mm was used. The injector and detector temperature were 250 and 275 °C, respectively. Identification and quantification of fatty acids were performed by comparing the retention times of a standard mixture of 37 methylesters fatty acids C4-C24 (Supelco).

The composition of neutral sugars was determined by the method of alditol acetates (Albersheim et al., 1967). Approximately 2 mg of sample were weighed and hydrolysed for 1 h at 121 °C (dry heat) with 500 μl of 2 N trifluoroacetic acid (TFA) containing 100 $\mu L \cdot ml^{-1}$ myo-inositol. The TFA soluble supernatants were separated from the solid fraction by centrifugation and dried, follow by the transformation to alditol acetates (Blakeney et al., 1983). For the analysis, the samples

were injected into a gas chromatograph Varian CP-3800 with FID detector (250 °C), a DB-23 capillary column 30 m x 0.25 mm (210 °C) and helium as carrier (3 $ml \cdot min^{-1}$). The calculation was performed from a calibration curve with known concentrations of rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and myo-inositol as an internal standard (all standards were Sigma®).

2.3 Bioethanol production

Biomass was hydrolyzed with sulfuric acid 1% (v/v) at 120 °C for 15 min (ratio 1:100). The hydrolyzed was filtered with a fiberglass filter in order to remove the solid residue. The saccharified product was neutralized with sodium hydroxide (NaOH) 2 M and then autoclaved at 120 °C for 15 min.

Conditioning of *Saccharomyces cerevisiae* was made with a suspension 10% (w/v) of lyophilized yeast in sterile distilled water. In 900 ml of distilled water 12 g tryptone, 24 g yeast extract and 12 ml of glycerol were dissolved, while in 100 ml of distilled water, 9.4 g and 2.2 g of monobasic potassium phosphate. Both solutions were autoclaved and mixed (Harun et al., 2009).

To prepare inoculum for the fermentation, 200 ml of Terrific Broth (TB) medium was transferred to a flask and 6 ml of the yeast suspension was added and incubated for 24 h (200 rpm, 30°C). The incubation time was 21 to 24 h, it was previously determined by measuring the optical density (OD) at 620 nm (Varian Cary 1E) each 3 h. OD readings and the incubation time were used for the kinetics of growth. The yeast was recovered by centrifugation at 600 x *g* for 2 min, the supernatant was removed and 3 washes (1% phosphoric acid) were needed to remove residual sugars (Harun and Danquah, 2011).

Kinetic curve for ethanol production was generated to determine the maximum rate production during the fermentation process. Sampling was performed every 3 h (2 ml aliquots), until 66 h of incubation. Prior to chromatographic determination of ethanol, the samples were centrifuged at 5 000 rpm for 5 min and filtered with 0.45 µm nylon membrane, then the sample was placed in vials and 0.2% internal standard (1-propanol) was added, it was placed in an incubator at 60 °C for 15 min and 1 ml of headspace gas vial and injected into the gas chromatograph. The ethanol concentration was calculated using a calibration curve with known standards of ethanol and 1-propanol.

The ethanol assay was performed with a chromatograph equipped with injector (200 °C) and FID detector (225 °C).The initial column (DB-23, 30 m x 0.25 mm and 0.25 µm film thickness) temperature was 40 °C for 1.6 min then at 200 °C at rate of 30 $°C \cdot min^{-1}$. The injection volume was 1 µl with a split of 30. Helium was used as carrier gas at a flow rate of 3 $ml \cdot min^{-1}$ with an initial pressure of 6.5 psi and a ramp up to 11.6 psi, the total run time was 12 min.

III. RESULTS AND DISCUSSION

3.1 Proximal analysis

Culture yields of *D. tertiolecta* were 0.153±0.038 g of dry biomass (db) per liter of culture, much minor amount than a previous study where reported up to 0.5 $g \cdot L^{-1}$ (Chisti, 2007). The marked difference in yield may be caused by the mineral content of the biomass, however the previous work does not report the mineral remotion from biomass before dehydration, in consequence, sea-water minerals and probably the own culture medium might cause yield over-estimation. In this research we performed exhaustive biomass washing with ammonium formate to minimize mineral interference. Previous reports reached up to 60% in mineral content when not removed (Mageswaran and Sivasubramaniam, 1984).

The proximal characterization of *D. tertiolecta* showed that proteins had the highest proportion to a value of 37.1% db (Table 1); this value is consistent with previous studies reporting from 32 to 53% protein content (Vásquez-Suarez et al., 2007). The ash content was 6.5%, indicating the success of washing and the culture remotion (Table 1). Little variation was found in the mineral concentration of *D. tertiolecta* with respect to the species *Spirulina platensis*, *Chlorella vulgaris* and *Isochrisis galbana* studied by Tokusoglu and Üunal (2003), and agreed with the mineral composition of algaes (Globblelaar, 2004), indicating an adequate culture growth of *D. tertiolecta* (Table 2).

TABLE 1
PROXIMAL COMPOSITION (DRY BASIS) OF THE BIOMASS OF *D. tertiolecta*.

Component	Content (% or g· 100g^{-1} of biomass)
Protein	37.2 ± 2.80
Carbohydrates	
Starch	27.1 ± 0.10
Fiber	8.5 ± 1.10
Ashes	6.5 ± 0.80
Lipids	13.0 ± 0.30

TABLE 2
COMPOSITION OF MINERALS IN THE BIOMASS OF *D. tertiolecta*

Mineral	mg of mineral· g^{-1} biomass
Sodium (Na)	11.16 ± 0.10
Potassium (K)	4.29 ± 0.03
Calcium (Ca)	2.27 ± 0.09
Magnesium (Mg)	2.43 ± 0.08
Iron (Fe)	1.37 ± 0.02
Manganese (Mn)	0.03 ± 0.00
Zinc (Zn)	0.10 ± 0.00
Cupper (Cu)	0.02 ± 0.00

The lipid concentration was 13%, therefore it was considered low because for viable production of biodiesel, the lipid content must range from 20-50% (Brennan and Owende, 2010); while Mata et al. (2010) mentioned a wider range up to 20-70%. However the lipid content in microalgae biomass might be modificated by changing culture condition, like temperature, salinity and nitrogen source (Wu and Hsieh, 2008). This result reveals the need of further studies focused to improve the lipid content in *D. tertiolecta* biomass. The main fatty acids in *D. tertiolecta* were palmitic acid (C16:0) and linolenic acid (C18:3, cis-9,12,15) with 19.08 ± 1.59 and 36.53 ± 1.18 respectively, which resulted into a typical behavior in this species of microalgae as reported in previous studies (Volkman et al., 1989; Gouveia and Oliveira, 2009; Chen et al., 2011b).

From the total carbohydrate content for *D. tertiolecta* biomass (35.6%), glucose was the major neutral sugar component with 91% of the molar proportion. Other sugars were found in lower concentrations (5%), as shown in Figure 1. This content was higher to previously reported by Brown et al. (1997) for five species of *Clorophytes*, including *D. tertiolecta*, where they determined less than the 60% of glucose content, while values of sugars like galactose, rhamnose, mannose and fucose, were higher to our findings in the present study. For the neutral sugar arabinose, similar concentrations were obtained. The carbohydrate composition varies according to microalgae specie (Williams and Laurens, 2010). The high glucose content in the biomass is due to the starch formed in the cell chloroplast, since this polysaccharide is conformed of chains of α-1,4-glucose rearranged in linear (amylose) o branched (amylopectin) structures. For fermentable sugars releasing, it was necessary to carry out an hydrolysis of the biomass, process known as a saccharification (Harun and Danquah, 2011; Ho et al., 2013). Previous report of 37% of carbohydrate content in biomass of *C. vulgaris*, was named suitable for bioethanol production (Hirano et al, 1997; Brennan and Owende, 2010), therefore our results might be considered favorable for this purpose.

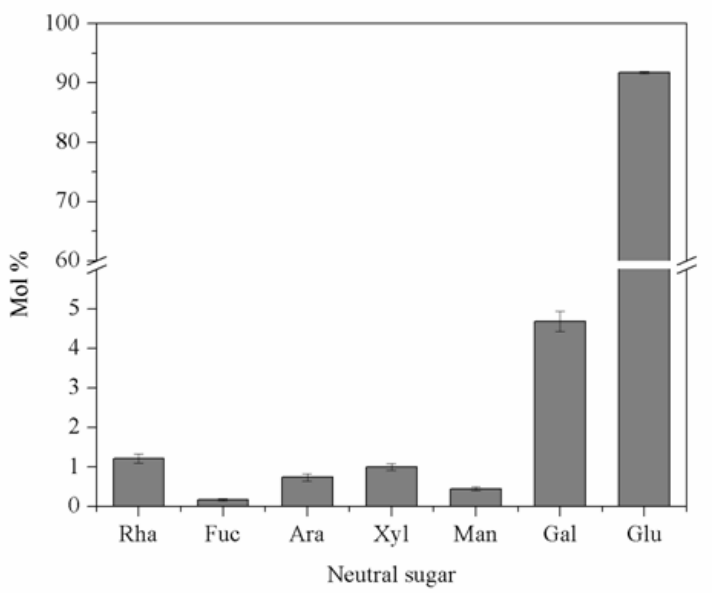

FIGURE 1. NEUTRAL SUGAR COMPOSITION OF *D.* TERTIOLECTA DETERMINED BY GAS CHROMATOGRAPHY. THE BARS INDICATE STANDARD DEVIATION (N=4). RHA, RAMNOSE; FUC, FUCOSE; ARA, ARABINOSE; XYL, XYLOSE; MAN, MANNOSE; GAL, GALACTOSE; GLU, GLUCOSE.

3.2 Bioethanol production

The saccharified biomass was fermentated during 66 h as seen in Figure 2, and the maximum concentration of ethanol occurred between 30 and 33 h of incubation at a efficiency of 0.6158 ± 0.06 ml ethanol·g^{-1} biomass (db), equal to 0.48 ± 0.05 g ethanol·g^{-1} biomass (db).

The advantage of saccharification was demonstrated by Harun et al. (2010) and Harun and Danquah (2011), when unhydrolyzed biomass used as carbon source for ethanol production resulted in lower productivity as compared with fermentation process over hydrolyzed carbon source. However, it is important to highlight that these differences in fermentative efficiency are also influenced by the microalgae specie, culture conditions and fermentation process.

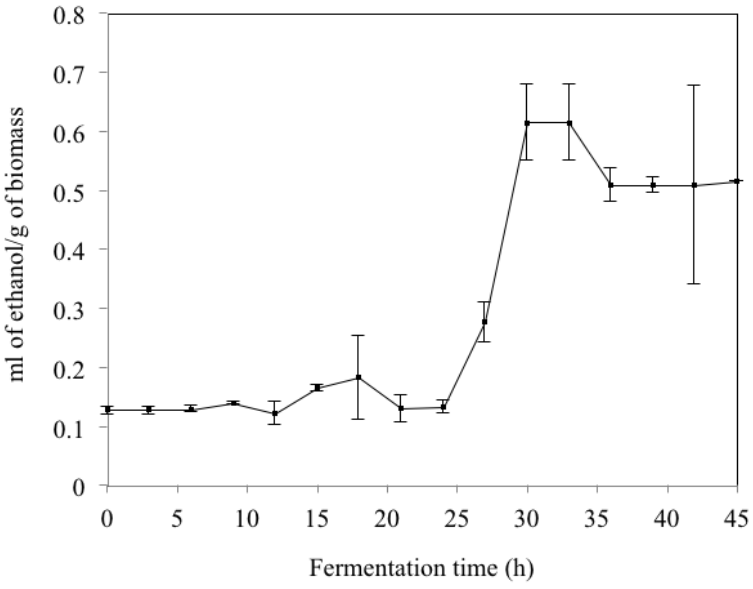

FIGURE 2. KINETICS OF ETHANOL PRODUCTION AT DIFFERENT FERMENTATION TIMES OF *D. TERTIOLECTA* BIOMASS WITH *S. CEREVISIAE*. THE BARS INDICATE STANDARD DEVIATION (N = 3).

IV. CONCLUSION

D. tertiolecta demonstrate good cultivation adaptability and its potential as raw material for the production of biofuels. The amount and fermentability of carbohydrates composing the biomass of *D. tertiolecta* makes it suitable for the bioethanol production, but lipid accumulation at the growing conditions was considered not competitive for biodiesel production.

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REFERENCES

[1] AOAC (1998). Official methods of analysis. 16th ed. USA.
[2] Albersheim, P., Nevins, D. J., English P. D. & Karr, A. (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas- liquid chromatography. Carbohydrate Research, 5, 340-345.
[3] Benjumea, H. N., Agudelo, S. J. R. & Rios, L.A. (2009). Biodiesel: Producción, calidad y caracterización. Editorial Universidad de Antioquia.
[4] Blakeney, A. B., Harris, P. J., Henry, R. J. & Stone, B. A. (1983). A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydrate Research, 113(2), 291-299.
[5] Brennan, L. & Owende, P. (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews, 14(2), 557-577.
[6] Brown, M. R., Jeffrey, S. W., Volkman, J. K. & Dunstan, G. A. (1997). Nutritional properties of microalgae for mariculture. Aquaculture, 151(1), 315-331.
[7] Chen, Y., Liu, G. F., Jia, L. S., Gao, Y. H. & Chen, C. P. (2011a). Annual change of outdoor seawater cultivation of *Dunaliella* for biomass energy. Renewable Energy Resources, 29(1), 57-61.
[8] Chen, M., Tang, H., Ma, H., Holland, T. C., Ng, K. S. & Salley, S. O. (2011b). Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology, 102(2), 1649-1655.
[9] Chisti, Y. (2007). Biodiesel from microalgae. Biotechnology Advances, 25, 294-306.
[10] Demirbas, A. & Demirbas, M. F. (2010). Algae Energy: Algae as a new source of biodiesel. Springer, London.
[11] De Schamphelaire, L. & Vestraete, W. (2009). Revival of the biological sunlight-to-biogas energy conversion system. Biotechnology and Bioengineering, 103(2), 296-304.
[12] Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226(1), 497-509.
[13] Gazzoni, D. L. (2009). Biocombustibles y alimentos en América Latina y el Caribe, Publicado por IICA. Pp. 39-57.
[14] Gouveia, L. & Oliveira, A. C. (2009). Microalgae as a raw material for biofuels production. Journal of Industrial Microbiology & Biotechnology, 36(2), 269-274.
[15] Grobbelaar, J. U. (2004). Algal Nutrition–Mineral Nutrition. Handbook of microalgal culture: Biotechnology and Applied Phycology, 95-115.
[16] Guillard, R. R. L. & Ryther, J. H. (1962). Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Canadian Journal of Microbiology, 8(2), 229-239.
[17] Harun, R. & Danquah, M.K. (2011). Influence of acid pre-treatment on microalgal biomass for bioethanol production. Process Biochemistry, 46, 304-309.
[18] Harun, R., Danquah, M.K. & Forde, M.G. (2010). Microalgal biomass as a fermentation feedstock for bioethanol production. Journal of Chemical Technology and Biotecnology, 85(2), 199-203.
[19] Hirano, A., Ueda, R., Hirayama, S. & Ogushi, Y. (1997). CO_2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. Energy, 22(2), 137-142.
[20] Ho, S. H., Huang, S. W., Chen, C. Y., Hasunuma, T., Kondo, A. & Chang, J. S. (2013). Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. Bioresource Technology, 135, 191-198.
[21] Kumar, R. A. & Verma, S. K. (2009). Secondary metabolite production in Cyanobacteria. Algal Biology and Biotechnology, 205-226.
[22] Mageswaran, R. & Sivasubramaniam, S. (1984). Mineral and protein contents of some marine algae from the costal areas of Northern Sri Lanka. Journal of the National Science Council of Sri Lanka, 12(2), 179-189.
[23] Mata, T. M., Martins, A. A. & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews, 14, 217-232.
[24] Megazyme Ltd 2005. Total dietary fibre assay procedure K-TDFR 01/12. Megazyme International Ireland Limited.
[25] Tokuşoglu, Ö. & Üunal, M. K. (2003). Biomass nutrient profiles of three microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrisis galbana*. Journal of Food Science, 68(4), 1144-1148.
[26] Vásquez-Suarez, A., Guevara, M., Salazar, G., Arredondo-Vega, B., Cipriani, R., Lemus, N. & Lodeiros, C. (2007). Crecimiento y composición bioquímica de cuatro cepas de *Dunaliella* para ser utilizadas en acuicultura. Boletín del Centro de Investigaciones Biológicas, 41(2), 181-194.
[27] Vertés, A. A., Qureshi, N., Blanschek, H. P. & Yukawa, H. (2010). Biomass to biofuels: strategies for global industries. Blackwell Publishing Ltd., Oxford, UK.
[28] Volkman, J. K., Jeffrey, S. W., Nichols, P. D., Rogers, G. I. & Garland, C. D. (1989). Fatty acid and lipid composition of 10 species of microalgae used in mariculture. Journal of Experimental Marine Biology and Ecology, 128(3), 219-240.

[29] Williams, P. J. & Laurens, L. M. (2010). Microalgae as biodiesel & biomass feedstocks: review & analysis of the biochemistry, energetics & economics. Energy & Environmental Science, 3(5), 554-590.
[30] Wu, W. T. & Hsieh, C. H. (2008). Cultivation of microalgae for optimal oil production. Journal of Biotechnology, 136, S521-1521.
[31] Yun, Y. S., Lee, S. B., Park, J. M., Lee, C. I. & Yang, J. W. (1997). Carbon dioxide fixation by algal cultivation using wastewater nutrients. Journal of Chemical Technology and Biotechnology, 69(4), 451-455.
[32] Zah, R., Binder, C., Bringezu, S., Reinhard, J. Schimid, A. & Schütz, H. (2010). Future Perspectives of 2nd Generation Biofuels. vdf Hochschulverlag AG an der ETH Zürich. Pp. 107.