Alvaro Mendoza*, Gemma Vicente, L. F. Bautista and Victoria Morales

Opportunities for *Nannochloropsis gaditana* biomass through the isolation of its components and biodiesel production

Abstract: Biomass from oleaginous microorganisms is an attractive source of materials used for the production of renewable fuels and industrial products due to its high productivity and the fact that it does not compete with human food. To ensure the economic feasibility and environmental sustainability of microbial biomass as feedstock, it is necessary to integrate its production and processing into the biorefinery concept. To achieve this goal, biodiesel production and fractionation of the whole biomass into different types of compounds (lipids, proteins, etc.) and further processing of each fraction must be performed. In the present work, the use of a microbial biomass source, the microalga *Nannochloropsis gaditana*, has been assessed as potential biorefinery feedstock.

Keywords: biobased products; biofuels; biorefinery; microalga; oleaginous microorganisms.

DOI 10.1515/gps-2014-0094
Received November 19, 2014; accepted March 2, 2015

1 Introduction

Energy and food production capacity are two of the most important human challenges for the future. The current and future demand for energy and the limited non-renewable fossil fuels make the search for new energy sources mandatory [1].

One possibility to produce energy is the use of biomass; depending on the feedstock, this option also involves associated problems, such as land degradation, deforestation, compaction, etc. [2]. Another handicap is the increase in food prices caused by rising demand when the biomass is itself a food resource [3]. So, nowadays, a safe, reliable, and efficient method for generating renewable biofuels reducing fossil fuel dependence is a major goal that must be met.

Biomass from oleaginous microorganisms is an attractive source of material for the production of renewable fuels and industrial products because a high productivity can be achieved [4]. In this category, microorganisms with lipid content above 20% wt. are included [5]. Some species of yeast, fungi and bacteria (heterotrophic microorganisms), and microalgae (eutrophic microorganism) are included in this category [6]. The oleaginous microorganisms do not compete with human food and can be grown on land unsuitable for agriculture [7].

The use of microorganisms in the production of fatty acid methyl esters (FAMEs) used as biodiesel has been investigated in the last decade [8, 9]. However, this biodiesel is still not cost-competitive with conventional diesel [10]. Some studies suggest that the production of microalgal biofuels is relatively close to being profitable, other studies show that “fuel only” is not economically feasible [11]. The processes should be developed integrally to ensure its viability, with the use of the total biomass being the most suitable way to get an efficient process [12].

Some studies have examined the possibility of producing other energy vectors, such as bioethanol or biogas [13–15] but it involves loss of compounds that may have a higher value by themselves, such as carotenoids and astaxanthins, among others [16].

Biomass fractionation without damaging other fractions is one of the critical points for the development of biorefineries [17], and there are few studies about the extraction of more fractions as one of microalgal metabolites [18]. If these fractions are not separated during biodiesel production, they would be lost as a residue or, as biomass with less value, fractions that are greater than the product generated in many cases [13].

The possibility of isolating high value compounds, other than lipids, has been studied in previous works

***Corresponding author: Alvaro Mendoza,** Energy and Chemical Technology/Chemical and Environmental Technology/Mechanical Technology and Analytical Chemistry, Universidad Rey Juan Carlos, C/ Tulipán s/n, 28933 Móstoles, Madrid, Spain, e-mail: alvaro.mendoza@urjc.es
Gemma Vicente, L. F. Bautista and Victoria Morales: Energy and Chemical Technology/Chemical and Environmental Technology/Mechanical Technology and Analytical Chemistry, Universidad Rey Juan Carlos, C/ Tulipán s/n, 28933 Móstoles, Madrid, Spain

[19, 20] although the concentration of these compounds is usually low and the large amount of residual biomass is not valorized. Carotenes are one of the lipids that have gained high interest. Carotenes are not suitable for biodiesel production; nevertheless, they have a high added value due to their photo-protective and antioxidant properties, their use as food colorants, or their ability to improve the stability of other oils [21, 22].

Apart from lipids, these microorganisms contain other compounds, such as proteins that can be used in animal feed and human food, antioxidants, or biofertilizer [23]. Some algae species have, in their composition, large amounts of protein, and in some cases, their quality is similar to other rich protein foods, thus making them an interesting source of protein for the increasing future requirements [24]. Some previous studies have investigated the combined benefits of anaerobic digestion and amino acid extraction from microalgae [25].

In the search for a sustainable alternative, the biorefinery concept has been developed, wherein the full use of biomass for energy, chemicals, and materials is performed through integrated processes [26, 27].

In this work, the production of biodiesel and the isolation of high added value products from lipids and other fractions like proteins are studied. A microbial biomass source, the alga *Nannochloropsis gaditana*, has been assessed as potential biorefinery feedstock.

2 Materials and methods

2.1 Materials

Lyophilized biomass of N. gaditana was purchased from Easy Algae (Cádiz, Spain). The extraction solvent, methanol (>99%), was purchased from Scharlab (Madrid, Spain). Analytical grade 95% to 97% sulfuric acid (Scharlab, Spain) was used as catalyst. All other chemicals were reagent grade or higher.

2.2 Biomass characterization

Under reflux at 75°C, 1 g of lyophilized *N. gaditana* biomass with 20 ml of methanol was processed and magnetic stirred at 300 rpm during 20 min in a nitrogen atmosphere [28]. After reflux, the samples were filtered in a ceramic filter with porosity grade 4 (Pobel) under vacuum, separating two fractions: solid fraction and liquid fraction. The solid fraction was dried at 110°C for 1 h in an oven to eliminate solvent remains. This fraction was subsequently used for protein isolation. The liquid fraction (oil and solvent) was subjected to an evaporation process at 110°C under vacuum (Laborota 4000, Heidolph, Germany) to remove the solvent excess. The resultant oil was used to isolate different lipids and produce biodiesel.

Proteins were measured using a modified version of the Lowry method [29]. The amino acid composition was determined using a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd., UK). Total carbohydrates were determined according to the phenol-sulfuric acid method using D-glucose as standard [30] and UV detection at 490 nm. Elemental composition (C, N, and H) was measured using a Vario EL III Element Analyzer (Elementar Analysensysteme GmbH, Germany).

2.3 Lipid isolation

The isolation of lipid fractions was conducted by a silica preparative chromatographic column. About 40 g of silica fumed (Sigma-Aldrich) was mixed with 30 ml of chloroform and compacted in a glass column (45 cm high and 3 cm width). The different lipid fractions were separated by polarity using *n*-hexane as mobile phase until complete fractionation was achieved. Furthermore, the separated fractions were identified with TLC.

2.4 Biodiesel production

Reactions with extracted lipid and with the saponifiable lipid fraction obtained from chromatographic column were carried out at 65°C in a 0.1 ace round-bottom pressure flask reactor (Sigma-Aldrich) stirred autoclave, and equipped with temperature controller and pressure gauge. These experiments were performed by under stirring (900 rpm) at autogenous pressure. Then, 1 g of oil with 19 ml of methanol and 1.75 g of sulfuric acid were all placed into the reactor. After 4 h, the reaction mixture was collected, washed with a mixture of *n*-hexane and diethyl ether, and immediately washed three times with water to eliminate nonreactant compounds and sulfuric acid. Finally, organic fraction (biodiesel and organic solvents) was subjected to an evaporation process, at 110°C under vacumm (Laborota 4000, Heidolph, Germany), in order to remove the solvent. Finally, 0.1 g of the resulting FAMEs was dissolved in 0.15 ml of *n*-hexane and a 1 µl sample was analyzed by TLC [5].

2.5 Protein isolation

Isolation of proteins was performed following the Gerde process [31] using raw (without lipid extraction) and spent biomass.

3 Results and discussion

3.1 Biomass characterization

Results of the biomass characterization are shown in Table 1. The lipid fraction was 36.7%, while the carbohydrate and protein contents were 15.7% and 33.8%, respectively. The elemental analysis used to calculate the C, H, and N ratios gave typical biomass contents.

Table 1: Composition (% wt.) of *Nannochloropsis gaditana*.

Fraction	*N. gaditana*
Lipids	36.7±2.0
Proteins	33.8±3.6
Carbohydrates	15.7±3.5
Others	13.8
Elemental composition	
Carbon (% wt.)	49.8±0.2
Hydrogen (% wt.)	8.1±0.1
Nitrogen (% wt.)	6.9±0.1

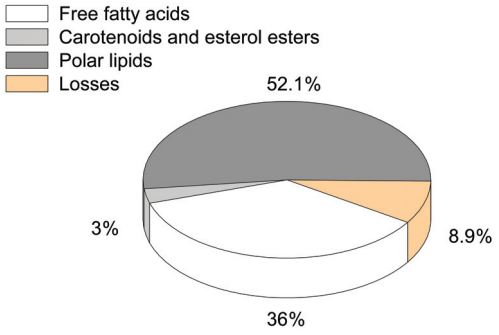

Figure 2: Ratio of isolated lipids in silica chromatographic column from *Nannochloropsis gaditana* oil.

3.2 Lipid isolation

The fraction of lipid extracted from the biomass was introduced in the silica chromatographic column (Figure 1) in order to obtain the different lipids from the mixture. The results are shown in Figure 2, which summarizes the carotenoids; esterol esters; free fatty acids; mono-, di- and triglycerides; and polar lipids from microalgal biomass. A great separation of lipids was achieved using the column because losses were only 8.88%. The purity of these isolated fractions was close to 100%.

Carotenoids and sterol esters may be used directly in food, while free fatty acid and saponifiable lipids (mono-, di-, and triglycerides and polar lipids) can be used to produce biodiesel.

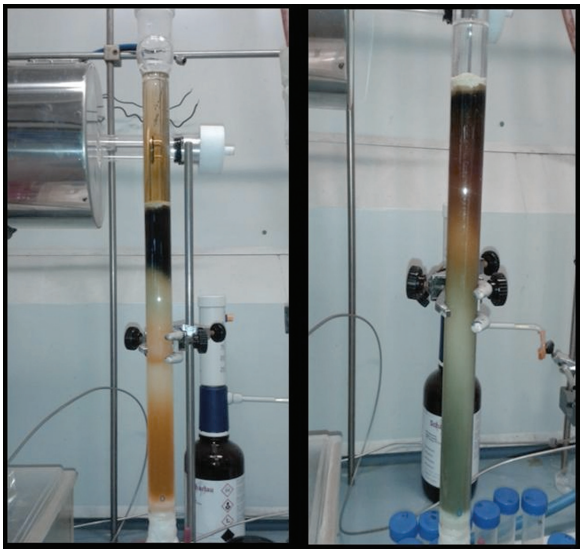

Figure 1: Isolation of lipids by silica chromatographic column for *Nannochloropsis gaditana* oil.

3.3 Biodiesel production

Biodiesel composition obtained in the reaction with the lipid extracted fraction, along with the free fatty acids and with the saponifiable lipid fractions obtained using the chromatographic column, is shown in Table 2. The composition analyses of the biodiesel produced in the reaction using the lipid extracted fraction showed that the ester content was slightly lower (96.15%) than that included in the EN 14214 standard (96.5%). The main impurities were free fatty acids and polar lipids. However, a higher content of FAME (98.3%) was achieved in the reaction using free fatty acid and saponifiable lipids obtained using the chromatographic column. Thus, the isolated free fatty acids and saponifiable compounds increased the ester content compared with the reaction performed with the full extracted oil. This fact makes it possible to avoid a subsequent purification stage, as the ester content achieved is above the reference value of 96.5% based on the EN14214 standard [32].

3.4 Protein isolation

The proteins present in the residual biomass after lipid extraction were isolated and the total amount was estimated. These results, summarized in Table 3, show a

Table 2: FAME production from *Nannochloropsis gaditana*.

	Methyl esters	Free fatty acids	Polar lipids
Extracted lipids	96.15±0.8	1.39±0.2	2.46±0.5
Saponifiable lipids from column	98.28±0.9	1.7±0.3	–

Table 3: Total protein amount of the fraction and amount of protein isolated from *Nannochloropsis gaditana*.

	% Total fraction respect total biomass	% Total protein respect total fraction	% Total protein isolated respect total biomass
Spent biomass	61.43±1.25	31.79±0.19	1.96

substantially different behavior in the protein extraction process. About 61.43% wt. of the initial biomass was obtained after lipid extraction (column 1). This fraction was used to isolate the proteins, and the total protein isolated from this fraction relative to the initial biomass was 1.96% wt. (column 3).

These results could be attributed to various reasons. On the one hand, the method of lipid extraction with organic solvents may extract soluble proteins in these solvents [33]. On the other hand, the large number of proteins bound to other compounds, such as lipids or carbohydrates, especially in the case of microalgae, make the separation more difficult and, thus, less efficient than using other biomass.

Once proteins were isolated, amino acid composition was analyzed. Results from chromatograms of the amino acid of the proteins isolated from non-defatted biomass and defatted biomass are shown in Figures 3 and 4, respectively. These results show that most of the amino acids are present in the recovered biomass, mainly glutamine (49.68%), alanine (19.94%) and proline (7.21%), including some with high nutritive value, such as threonine (1.8%), valine (1.5%), isoleucine (0.26%), leucine (0.36%), phenylalanine (0.43%), or lysine (2.42%). Decrease of some amino acids, such as valine, alanine or leucine, may cause its hydrophobic behavior resulting in increased solubility in organic solvent use during the lipid extraction process.

4 Conclusions

Biodiesel production and the isolation of the resulting biomass compounds have been studied. The processes

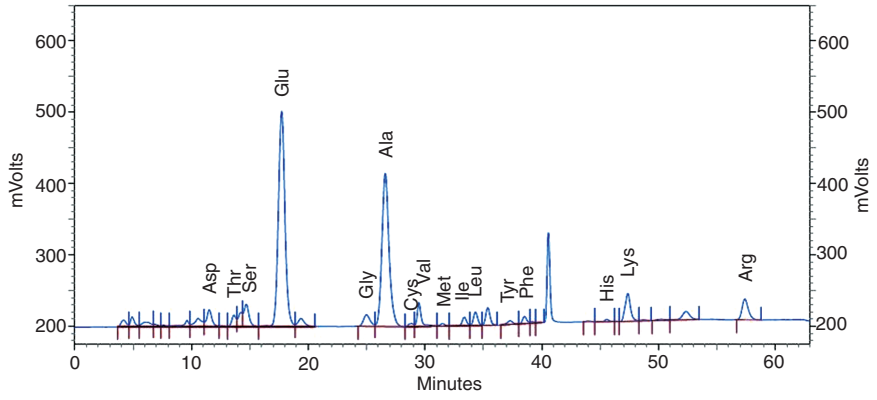

Figure 3: Chromatogram of amino acids of proteins isolated from *Nannochloropsis gaditana*.

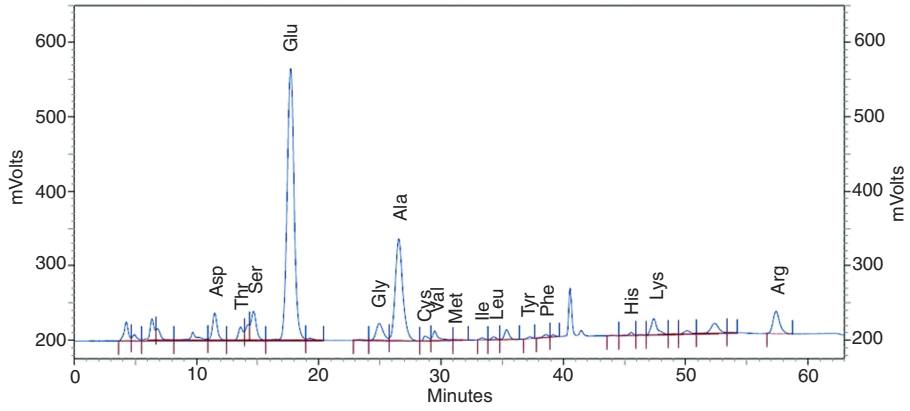

Figure 4: Chromatogram of amino acids of proteins isolated from spent biomass from *Nannochloropsis gaditana*.

used demonstrate the feasibility of the isolation with high added value fractions, such as carotenoids and proteins. At the same time, these processes led to the production of biodiesel, thus giving the former more value and making them more economically viable. Moreover, the high purity of the compounds avoids subsequent purification processes.

Acknowledgments: The authors wish to thank the financial support provided by the Universidad Rey Juan Carlos and Oficina de Cooperación Universitaria (Project PRIN13-CC05).

References

[1] Akay G. *Curr Opin Biotech* 2011, 22, S145–S146.
[2] Popp J, Lakner Z, Harangi-Rákos M, Fári M. *Renew Sust Energ Rev* 2014, 32, 559–578.
[3] Scovronick N, Wilkinson P. *Global Environ Chang* 2013, 23, 1587–1593.
[4] Chisti Y. *Biotechnol Adv* 2007, 25, 294–306.
[5] Vicente G, Bautista, LF, Rodríguez R, Gutiérrez FJ, Sádaba I, Ruiz-Vázquez RM, Torres-Martínez S, Garre V. *Biochem Eng* 2009, 48, 22–27.
[6] Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M. *Renew Energ* 2009, 34, 1–5.
[7] Oncel SS. Renew *Sust Energ Rev* 2013, 26, 241–264.
[8] Pinzi S, Leiva D, López-García I, Redel-Macias M, Dorado P. *Biofuel Bioprod Bior* 2014, 8, 126–143.
[9] Tkachenko AF, Tigunova OA, Shulga SM. *Cytol Gen* 2013, 47, 6, 343–348.
[10] Woinaroschy A. *Fuel* 2014, 135, 393–405.
[11] Zhu L. *Renew Sust Energ Rev* 2015, 41, 1376–1384.
[12] Hong-Wei Y, I-Chen H, Chun-Yen Ch, Shih-Hsin H, Duu-Jong L, Jo-Shu Ch. *Bioresour Technol* 2013, 135, 166–174.
[13] Ramos-Suárez JL, Carreras N. *Chem Eng J* 2014, 242, 86–95.
[14] Sialve B, Bernet N, Bernard O. *Biotechnol Adv* 2009, 27, 409–416.
[15] Brennan L, Owende P. *Renew Sust Energ Rev* 2010, 14, 2, 557–577.
[16] Guedes AC, Amaro HM, Malcata FX. *Mar Drugs* 2011, 9, 4, 625–644.
[17] Vanthoor-Koopmans M, Wijffels R H, Barbosa MJ, Eppink MHM. *Bioresour Technol* 2013, 135, 142–149.
[18] Markou G, Nerantzis E. *Biotechnol Adv* 2013, 31, 8, 1532–1542.
[19] Molina Grima E, Belarbi, EH, Acién Fernández FG, Robles Medina A, Chisti Y. *Biotechnol Adv* 2003, 20, 491–515.
[20] Guedes AC, Amaro HM, Malcata FX. *Biotechnol Progr* 2011, 27, 597–613.
[21] Limon P, Malheiro R, Casal S, Acien-Fernandez FG, Fernandez-Sevilla JM, Rodrigues N, Cruz R, Bermejo R, Pereira JA. *Food Chem* 2015, 175, 203–211.
[22] Koller M, Muhr A, Braunegg G. *Algal Research* 2014, 6, 52–63.
[23] Bhattacharjee JK. *Advances in Applied Microbiology*, Oxford: Ohio, 1970, vol. 13, p 139–161.
[24] Spaloare P, Joannis-Cassan C, Duran E, Isambert A. *J Biosci Bioeng* 2006, 101, 87–96.
[25] Ramos-Suarez J L, Garcia Cuadra F, Gabriel Acien F, Carreras N. *Chem Eng J* 2014, 258, 1–9.
[26] Sharma P, Sarker BR, Romagnoli JA. *Comput Chem Eng* 2011, 35, 1767–1781.
[27] Hariskos I, Posten C. *Biotechnol J* 2014, 9, 739–752.
[28] Carrero A, Vicente G, Rodríguez R, Linares M, del Peso GL. *Catal. Today* 2011, 167, 148–153.
[29] López CVG, García M, Fernández FGA, Bustos CS, Chisti Y, Sevilla JMF. *Bioresour Technol*, 101, 7587–91.
[30] DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. *Anal Chem*, 1956, 28, 350–356.
[31] Gerde JA, Wang T, Yao L, Jung S, Johnson LA, Lamsal B. *Algal Research* 2013, 2, 145–153.
[32] Asociación Española de Normalización y certificación, EN 14114, 2003.
[33] Filly A, Fernández X, Minuti M, Visinoni F, Cravotto G, Chermat F. *Food Chem* 2014, 8, 150–193.

Bionotes

Alvaro Mendoza

Alvaro Mendoza obtained his degree in Environmental Science from the University of Castilla-La Mancha. Currently, he is completing his PhD in the field of biorefineries from oleaginous microorganisms at the Energy and Chemical Technology Department, Rey Juan Carlos University.

Gemma Vicente

Gemma Vicente obtained her BSc and PhD in Chemistry from the Complutense University of Madrid. First, she developed her research career in the Chemical Engineering Department of the Complutense University of Madrid (1994–2001), the Chemical Engineering Department of the Aston University (Birmigham, UK) (1997), and the Crops Research Centre of TEAGASC (1998, 1999) in Carlow (Ireland). During this time, her main research line was the biodiesel production process. Her teaching activity started in the ICAI School of Engineering (Comillas Pontifical University of Madrid) in 2000. She joined Rey Juan Carlos University in 2001, where she is currently an Associate Professor. Her teaching activity has been focused on subjects related to chemical engineering, environmental engineering and energy in a number of degrees, Masters, and PhD courses. She is currently the Director of the Master on Chemical Engineering. Her research interest is now related to the biobased fuel and product production from microorganisms.

L. Fernando Bautista

L. Fernando Bautista is an Associate Professor at Rey Juan Carlos University. He received his BSc degree in Chemistry from the Complutense University of Madrid and obtained his PhD from the same institution. His professional activity started in the R&D Centre of Probisa and then in the Process Department of Técnicas Reunidas where he served as a freelance consultant. He has developed his

research career in the Chemical Engineering Department of the Complutense University of Madrid (1994–2004) and in the Department of Biotechnology at the Technical University of Denmark as a Visiting Researcher (1998–1999). In 2004, he joined Rey Juan Carlos University. His current research interest is focused on process biotechnology applied to new biofuel production and soil bioremediation.

Victoria Morales

Victoria Morales obtained his PhD in 2008 (Rey Juan Carlos University) working on the synthesis and characterization of hybrid organic-inorganic mesostructured materials for supporting sharpless catalysts. He has occupied several teaching positions in the Chemical and Environmental Engineering Group, starting in 2003 as a PhD student, becoming a teaching assistant in 2006, and an Assistant Professor in 2008. He was a visiting researcher at the Ecole Nationale Supérieure de Chimie de Montpellier (France) in 2006 under the supervision of Prof. Daniel Brunel, and at the Department of Chemical Engineering in the University of California at Santa Barbara (USA) in 2009 and 2013 under the supervision of Prof. Brad F. Chmelka. His main research lines are focused on the synthesis, characterization, and catalytic application of organically-functionalized mesostructured materials and biobased fuels as well as products from microalgal microorganisms integrated in the biorefinery concept.