energreen


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The selection of a suitable species is an important aspect in order to ensure the efficiency of a process to obtain bio-energy from microalgae culture (Griffiths & Harrison 2009). The species of *Chlorella* genus are known as ideal candidates for this objective due to their high productivity when are cultivated on an industrial scale. The calorific value of this genus can reach 29 kJ/g, a value very close to the 39 kJ/g obtained from rapeseed oil. This fact shows the potential of this group of microalgae as a source of biodiesel (Illman et al., 2000). The composition of *Chlorella* includes proteins, carbohydrates, lipids and fibre in variable proportions, making its biomass a versatile raw material that can be used to obtain different types of energy, such as diesel, bioethanol, methane and hydrogen. However, numerous nutritional and environmental factors applied along the culture can radically alter the composition of the biomass and its energetic value.

As part of its activities focused on the **Development of microalgae culture strategies** to obtain high lipid biomass content, **NEIKER** carried out a study trying to know the effect of different cultivation conditions (nitrogen and carbon dioxide applied, media composition and level of irradiance) on the growth and lipid biomass content.. The purpose was to select the best conditions in order to produce high energy value biomass from this species.

The results obtained show that the lipid content of *C. vulgaris* NK08, cultivated under non-restrictive growth conditions, does not exceed 16% of dry weight, containing a fraction of neutral lipids or triglycerides (TAGs) that is less than 5% of the biomass dry weight..

The lipid content can be increased by **25%** by submitting the crops to progressive nutritional limitation (nitrogen deficiency). Although the increase in total lipid content was not as great as it was shown by other strains of the genus, it was seen that the limitation of nitrogen caused a gradual modification in the composition of the lipids, with a progressive increase in the neutral lipid fraction. After 5 days of depletion, the TAGs content reached to 12% of dry weight (**Fig**. **1**). Taking in mind that this is the right fraction to transform into biodiesel, we can assume that nitrogen limitation is an effective strategy for improving the oil content of this species.

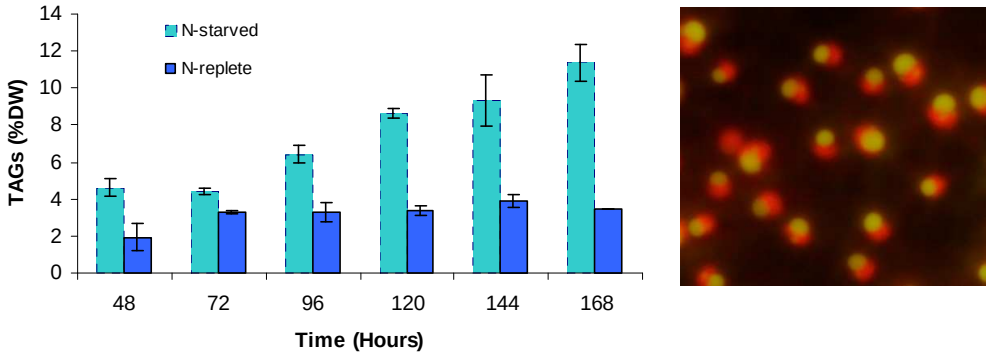


Figure 1. Evolution over time of the TAG content (% dry weight) for *C.vulgaris*-NK08 crops growing under non-restrictive conditions (N-replete) and with nitrogen limitation (N-depleted) (A). Combined microscope image shows the Nile Red lipid marker, showing the accumulation of neutral lipids (yellow fluorescence) in the nitrogen limited cells (B).

Nutritional limitation of crops has often been regarded as an unsuitable strategy to improve the lipid production of many microalgae, due to the fact that the lipid accumulation process is inversely proportional to the final crops productivity.
In the case of C. *vulgaris* NK08, the limitation imposed also led to a reduction in the final yield of biomass (1.6 times less than crops without depletion). However, the final yield of the product of interest was 1.8 times higher than crops undergoing limitation (51 mg TAGs $L^{-1}d^{-1}$ compared with 28.6 mg TAG $L^{-1}d^{-1}$ in crops without limitation). In summary, , the growth restriction of *C. vulgaris* NK08 due to nutritional limitation is compensated by the increase in the TAGs cell content.. Therefore, induction by nitrogen limitation can be considered as a suitable strategy to improve the lipid content of the strain.

Table 1. Biomass, total lipid and triglycerides (TAGs) content according to the biomass (% DW) and TAGs productivities in batch cultures of *C. vulgaris* NK08 growing under non-restrictive nutritional conditions and under nitrogen limiting conditions.

	Non limited	Limited
Biomass (g DW^{-1}) L^{-1}	5.1 ± 0.1	2.8 ± 0.1
Lipids (% DW)	16.9 ± 0.5	22.5 ± 0.2
TAGs (% DW)	3.4 ± 0.1	11.5 ± 0.1
Productivity (mg TAG $L^{-1}d^{-1}$)	28.6 ± 2.3	51 ± 3.1

In addition, the nitrate initial concentration used as supplement the culture medium can be adjusted to optimize the cell density achieved before the induction of neutral lipids storage. The maximum yields were obtained using intermediate doses of nitrate (0.6 g L^{-1} of KNO_3).

In order to achieve the above-mentioned yields, CO_2 must be provided during the whole cultivation cycle. During the culture initial stage, when there is no nutritional restriction in the growing medium, the provision of CO_2 provoked fast nitrogen consumption and increased the growth rate, generating an increase of biomass yield per unit of volume. Once the nitrogen was consumed, the provision of CO_2 also led favored to an accumulation of TAGs. This shows that, although cell growth stops after depletion of the nitrogen in the culture medium, the cells retain the capacity to continue the assimilation of available carbon and incorporate it as reserve of lipids.

Analyzing the effect of irradiance, the results showed that the photosynthetic efficiency of this species is stable over a range of light intensity (250-500 µmol photons $m^{-2}s^{-1}$). Bearing in mind the productivity values achieved and the limited irradiation demand of *Chlorella*

NK08, this strain could be suitable for use in regions whose climate showed low irradiation, although the effect of temperature on growth should also be taken into account.

In addition, tests were also carried out to identify the effect of changing the ratios of the main nutrients of the culture medium (N: P: Mg: Fe). The results showed that while the conditions established for the nitrogen were maintained, the yield achieved for the strain was fairly stable over a wide range of ratios.

In order to assess the energy potential of the biomass obtained in all growth conditions tested, **CENER** carried out chemical characterization (proximal composition). The results obtained confirmed that the composition of the biomass is also stable in the different conditions tested, containing 16-18% FAMEs (Fatty Acid Methyl Esters). However, the carbohydrates are the largest fraction in the *Chlorella* biomass. This fact indicates that, despite of the accumulation of neutral lipids, starch could be the main reserve fraction of this strain.

In the framework of the **Evaluation of Extraction Systems and Transformation into Biodiesel**, **CENER** has carried out a series of activities focused on the optimization of the extraction of oils from *C. vulgaris* NK08, using conventional methods with organic solvents. This was achieved with a number of multifactorial experiments that included the study of the following variables: extraction time, working temperature, number of re-extractions, type of solvents and the solute/solvent ratio.

The results obtained led us to conclude that the best yields for lipid extraction were obtained by directly stirring the sample in the presence of a solvent (**Fig. 2A**). With regard to the extraction conditions, the best results were obtained with two successive 30-minute extractions and using a solute/solvent ratio of 3:40 (p/v). The residue pellet obtained after extraction was quite faded when compared to the biomass before extraction (**Fig. 2C**), indicating that the extraction was apparently efficient.

After evaporating the solvent with an evaporator, an oleic fraction was obtained with a reasonably solid appearance (**Fig. 2B**).

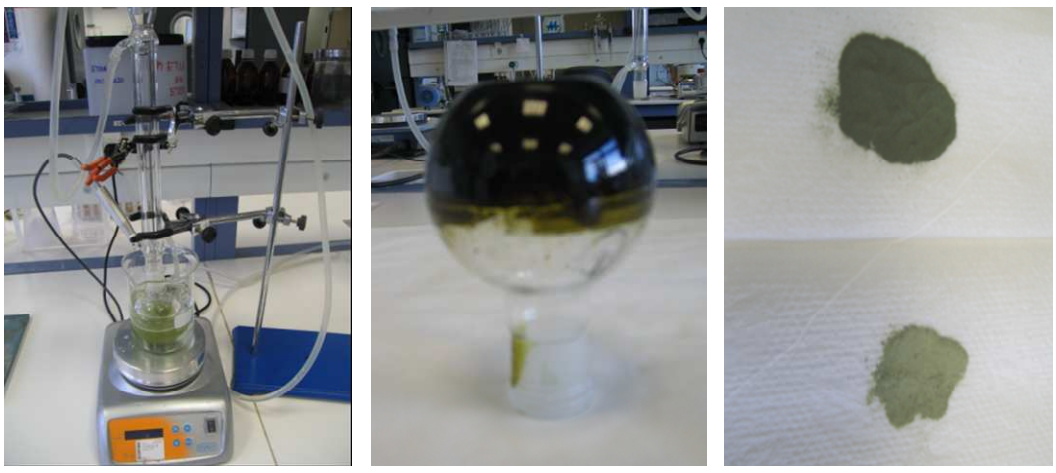
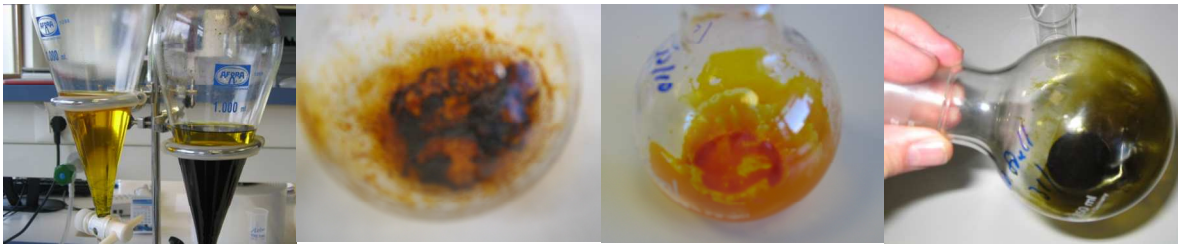


Figure 2. (A) Lipid extraction system used, (B) Solid consistency of the lipids extracted (C) *C. vulgaris* NK08 biomass before and after extraction with solvents.

Two different procedures were used to transform the oils extracted into biodiesel.

On the one hand, **CENER** evaluated the efficiency of different conventional procedures, including (1) alkaline trans-esterification (sodium methylate catalyzer), (2) acid esterification (sulphuric acid catalyzer) and (3) by reaction with acetyl chloride in methanol of the saponifiable fraction after having separated the unsaponifiable fraction (**Fig. 3A-B**). The most satisfactory results were obtained with the third method, allowing us to obtain a biodiesel of fluid and liquid appearance

(**Fig. 3D**) with a high percentage of FAMEs (greater than 90%). We were also able to obtain an unsaponifiable fraction from which other high-added-value compounds were obtained.

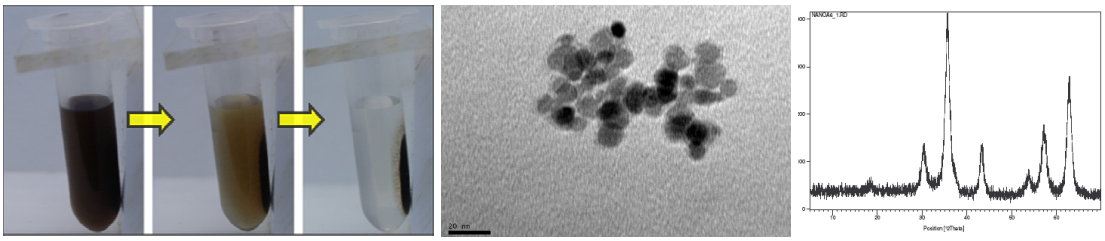


(A) (B) (C) (D)

Figure 3. Separation of the saponifiable (A) and unsaponifiable fractions (B) and (C) after evaporation, and the Biodiesel obtained (D).

In addition, the **UPV/EHU** has carried out preliminary studies in parallel with the above to assess the usefulness of the magnetic biocatalysers which has been developed for the enzymatic acquisition of biodiesel. To this end, and up to the present, work has been carried out to obtain nanostructured magnetic support materials, which have been used to obtain CLEAs (*Cross-Linked Enzyme Aggregates*) of lipase. The enzyme used (CALB, lipase B of *Candida antarctica*) was obtained from commercial sources.

The magnetic nanoparticles (MNPs) were obtained by precipitating iron salts in an alkaline medium with mechanical stirring (**Fig. 4A**) and their surfaces were then functionalized with amino groups. The MNPs obtained were characterized structurally using transmission electron microscopy (**Fig. 4B**). X-ray diffraction confirmed their nature as magnetite (**Fig. 4C**), while magnetic characterization showed their superparamagnetic nature, both before and after immobilization of the CALB enzyme.



(A) (B) (C)

Figure 4. (A) Attraction effect of a magnet on the magnetic nanoparticles (MNPs) obtained. The images shown were taken every 20 seconds. (B) Transmission electron microscope image showing that the size of the MNPs is approximately 10 nm. (C) X-ray diffraction spectrum showing that the MNPs are made of magnetite.

The MNPs were also used to obtain CLEAs of CALB lipase and both the hydrolytic and synthetic activity of the same was characterized in aqueous mediums and organic solvents. It was found that the CLEAs were able to catalyze the transesterification to biodiesel reaction of 70% of the oil extracted from *C. vulgaris* NK08 in 20 hours at a temperature of 40°C. After 42 hours of incubation, all the oil had been converted into biodiesel (FAPE). The progress of the reaction was followed by thin layer chromatography (TLC) on silica gel plates (**Fig. 5**). This simple analytical technique allowed us to have monitorized the progress of the esterification reaction, as well as being useful for detecting the free fatty acids, the mono (MG), di (DG) and triglycerides (TG), as well as the photosynthetic chlorophyll pigments (G) and carotenoids (C) present in the oil.

The results obtained are preliminary although they are highly positive because they indicate that the magnetic catalyzers developed are effective for catalyzing the enzymatic production of biodiesel at 40°C. More than this, we should take in mind the high stability of the CLEAs and the fact that they can be recovered in successive catalytic cycles due to their magnetic nature.

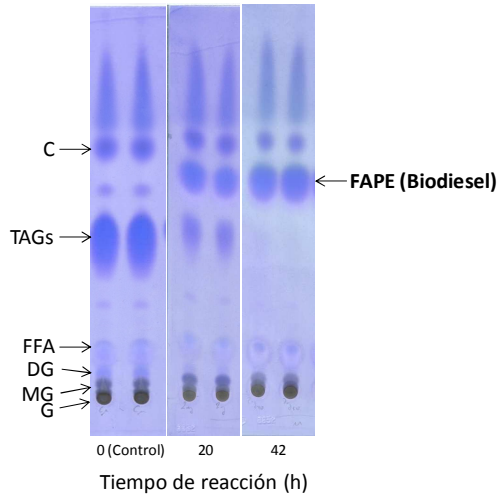


Figura 5. Seguimiento por TLC de la producción de biodiesel (FAPE) a partir de aceites extraídos de *C. vulgaris* NK08. La reacción de transesterificación catalizada por mCLEAs de CALB transcurrió a 40°C en una mezcla de *n*-hexano y 2-propanol. Se observa que después de 42 h de reacción la totalidad de TAGs se han convertido en biodiesel. C, pigmentos carotenoides; TAGs, triglicéridos; FFA, ácidos grasos libres; DG, diglicéridos; MG, monoglicéridos; G, pigmentos verdes.