

# Universidad de Huelva

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**Metabolic and molecular approaches for the integrated  
production of bioenergetic precursors in microalgae**

**Memoria para optar al grado de doctora  
presentada por:**

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Fecha de lectura: 20 de enero de 2023

Bajo la dirección de los doctores:

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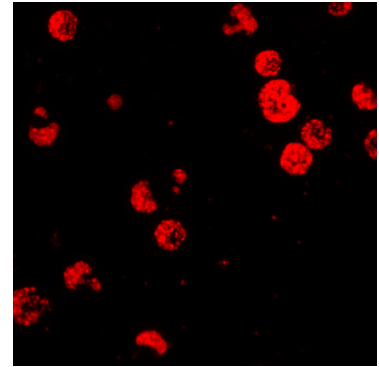
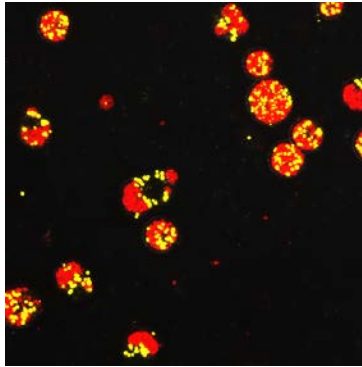
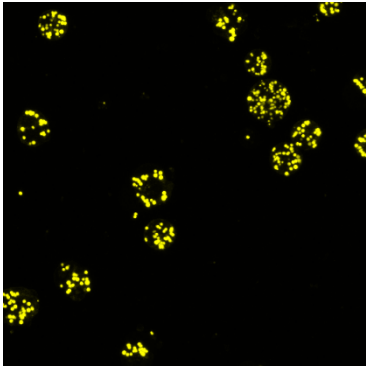
Javier Vigara Fernández

**Huelva, 2023**





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# UNIVERSIDAD DE HUELVA

FACULTAD DE CIENCIAS EXPERIMENTALES  
DEPARTAMENTO DE QUÍMICA “PROFESOR JOSÉ CARLOS VÍLchez MARTÍN”



Universidad  
de Huelva

TESIS DOCTORAL

**“HERRAMIENTAS METABÓLICAS Y MOLECULARES PARA LA  
PRODUCCIÓN INTEGRADA DE PRECURSORES ENERGÉTICOS EN  
MICROALGAS”**

**“METABOLIC AND MOLECULAR APPROACHES FOR THE  
INTEGRATED PRODUCTION OF ENERGETIC PRECURSORS IN  
MICROALGAE”**

PROGRAMA DE DOCTORADO  
CIENCIA Y TECNOLOGÍA INDUSTRIAL Y AMBIENTAL

MEMORIA PRESENTADA PARA OPTAR AL GRADO DE DOCTORA POR:

**Rocío Rengel Domínguez**

Trabajo presentado bajo la dirección de:

**Dra. Rosa María León Bañares**  
**Dr. Javier Vígara Fernández**

Huelva, 2022



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# Abstract



## Abstract

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Microalgae are photosynthetic microorganisms with a great ability of adapting to adverse conditions, developing regulation mechanisms to maintain optimal carbon/nitrogen ratios in the cell for survival. When these microorganisms are submitted to environmental stress conditions, the carbon initially devoted to the synthesis of structures is deviated towards the production of storage compounds.

This doctoral thesis is framed within the response of the microalga *Chlamydomonas reinhardtii* to the absence of nitrogen in the culture medium. Under this nutrient deficiency, the assimilated carbon is relocated to the synthesis of the main reserve compounds: triacylglycerols (TAG) and starch. These compounds are of high interest due to their potential as precursors in the production of energetic compounds, such as biofuels or chemical building blocks in the industry. Other bioactive compounds that are synthesized by microalgae are carotenoids, widely used in pharmacological and pigment industry for their antioxidant properties.

Therefore, scale-up processes where microalgae biomass is used for the production of several compounds for commercialization, in a sustainable and integrated way, imply to identify and enhance the key bottlenecks and difficulties in the production stage. To obtain starch and TAG, induction must be made under stress conditions, which limits cell growth because of the production of storage compounds at the expense of natural cell cycle and division.

In this thesis, a first molecular approach is made for the enhancement of neutral lipids in *Chlamydomonas reinhardtii*, overexpressing a gene responsible for the increase of carbon flux upstream the specific neutral lipid pathway (**Chapter 1**). On the other hand, a second genetic engineering approach is followed, overexpressing a gene directly involved in TAG assembly (**Chapter 2**). In addition, the hypothesis whether the simultaneous overexpression of these genes, can synergistically improve the levels previously reached by carbon flux enhancement, is assessed (**Chapter 3**). Finally, the utilization of the microalgal biomass for the simultaneous production of carotenoids and starch under nitrogen starvation, and the use of starch for the production of chemical precursors is studied (**Chapter 4**).

In **Chapter 1**, the effect of the overexpression of the endogenous gene, encoding the chloroplastic acetyl-CoA synthetase (ACS2), from *Chlamydomonas reinhardtii*, is presented. This enzyme is responsible for the direct assimilation of acetate into acetyl-CoA, which, among other metabolic pathways, feeds the route involved in fatty acid synthesis. The results show an increase in starch production under replete conditions, and a rise in acyl-CoA intermediates, which are further incorporated to TAG biosynthesis. Transfer of cultures to nitrogen depletion causes TAG production in the transformant in a greater proportion than the parental line, and higher transcript levels of the ACS2 gene as a consequence of its overexpression.

In **Chapter 2**, the heterologous expression of the gene encoding a type-1 diacylglycerol acyltransferase, from the boraginaceae *Echium pitardii* (*DGAT1*) in *Chlamydomonas reinhardtii*, is studied. This enzyme catalyzes the last step of assembly of an acyl chain in a diacylglycerol backbone to yield a molecule of TAG. The transformant shows a higher amount of total fatty acids, and a significant rise of the neutral lipid fraction under nitrogen depletion conditions. A higher uptake of acetate from the medium, the presence of intracellular glycerol and a slower degradation of the photosynthetic apparatus compared to the parental line, are also observed.

In **Chapter 3**, the generation of *acs* transformants, and the generation of double *acs/dgat* transformants from the single *acs* clones, is achieved with the aim of establishing a comparison between them. Results show a synergistic enhancement in the neutral lipid content in the double transformant, which simultaneously expresses both genes. In addition, the single *acs* and the double *acs/dgat* transformants present a higher amount of carotenoids under replete conditions, a higher content of starch in replete and N-depleted conditions, and a better evolution of photosynthetic efficiency throughout nutrient deficiency.

Finally, in **Chapter 4**, the ability of *Chlamydomonas reinhardtii* to accumulate starch under nitrogen starvation is utilized for the production of the chemical precursors 5'-hydroxymethylfurfural (5'-HMF) and levulinic acid (LA). These intermediates are used in the industry for the synthesis of biofuels, additives, polymers or molecular imaging probes. The application of a Central Composite Design and Response Surface Methodology allowed to determine the influence of the operational conditions involved in the acidic hydrolysis of starch, firstly to glucose, and in a further step, to 5'-HMF and LA. The best temperature, acid percent, DMSO percent and reaction time conditions were selected for each of the three precursors. Moreover, the extraction of carotenoids, prior to hydrolysis, allows to set the basis for an integrated biorefinery process, aiming to produce several compounds of interest from a sole biomass.

## Resumen

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Las microalgas son microorganismos fotosintéticos con una gran capacidad de adaptación a condiciones adversas, desencadenando mecanismos de regulación con los que se mantienen ratios adecuadas de carbono y nitrógeno en la célula para su supervivencia. Cuando estos microorganismos son sometidos a condiciones de estrés ambiental, el carbono inicialmente dirigido a la formación de estructuras es redirigido a la síntesis de compuestos de reserva.

Esta tesis doctoral se enmarca en la respuesta de la microalga *Chlamydomonas reinhardtii* ante la ausencia de nitrógeno en el medio de cultivo. Ante esta carencia nutricional, el carbono asimilado pasa a ser convertido a los dos compuestos principales de reserva: almidón y triacilglicerol (TAG). Estos compuestos son de alto interés debido a su potencial como precursores en la producción de compuestos energéticos, tales como biocombustibles o intermediarios químicos en la industria. Otras biomoléculas de alto valor añadido generadas por las microalgas son los carotenoides, ampliamente utilizados en la industria farmacológica y de pigmentos por su carácter antioxidante.

Así pues, el escalado a procesos sostenibles e integrados donde una única biomasa de microalgas pueda ser empleada para la obtención de varios productos susceptibles de comercialización, pasa por identificar y mejorar los principales cuellos de botella en el proceso de producción. En el caso de la obtención de almidón y TAG, su inducción en condiciones de estrés tiene como inconveniente la limitación en el crecimiento de los cultivos, ya que los recursos disponibles son dedicados a la acumulación de estos compuestos en lugar de la evolución y división natural de la célula.

En el presente trabajo, se lleva a cabo por un lado, la mejora para la obtención de lípidos neutros mediante manipulación genética, sobreexpresando en *Chlamydomonas reinhardtii* un gen responsable del aumento del flujo de carbono aguas arriba de la ruta metabólica específica de síntesis de lípidos neutros (**Capítulo 1**), y por otro lado, sobreexpresando un gen directamente implicado en el ensamblaje de los triacilglicerol (**Capítulo 2**). Además, se aborda la hipótesis de que la expresión simultánea de ambos genes puede llevar a una mejora superior a la que tendría sólo mejorar el aumento del flujo de carbono hacia la ruta (**Capítulo 3**). Por último, se estudia el aprovechamiento de la biomasa microalgal para la producción simultánea de carotenoides y almidón en carencia de nitrógeno y su utilización en la obtención de precursores químicos de interés industrial (**Capítulo 4**).

En el **Capítulo 1**, se estudia el efecto de la sobreexpresión del gen endógeno que codifica la acetil-CoA sintetasa (*ACS2*) cloroplástica en *Chlamydomonas*, responsable de la producción, en un solo paso, de acetil-CoA desde acetato. El acetil-CoA, entre otros destinos metabólicos, alimenta la ruta de síntesis de ácidos grasos. Los

resultados muestran un aumento en la síntesis de almidón en condiciones de cultivo repletas, además de un aumento de los intermediarios acil-CoA, los cuales son posteriormente incorporados a la ruta de síntesis *de novo* de triacilglicerol. El paso de los cultivos a condiciones de carencia de nitrógeno provoca la síntesis de TAG en mayor proporción que la especie parental, además de detectarse mayores niveles de transcrito del gen ACS2 como consecuencia de su sobreexpresión.

En el **Capítulo 2**, se aborda la expresión heteróloga en *Chlamydomonas reinhardtii* de un gen que codifica una diacilglicerol aciltransferasa de tipo 1 (DGAT1), procedente de la especie boraginácea *Echium pitardii*, que es responsable del último paso de ensamblaje de un acilo al esqueleto de diacilglicerol para formar una molécula de TAG. Se comprobó que el transformante posee mayor cantidad de ácidos grasos totales y un aumento significativo de la fracción de ácidos grasos neutros en condiciones de carencia de nitrógeno, además de un mayor consumo de acetato y presencia de glicerol intracelular con respecto a la especie parental. También se observa una degradación más lenta del aparato fotosintético.

En el **Capítulo 3**, se realiza la obtención de transformantes *acs*, y a partir de éstos, la obtención de transformantes *acs/dgat*, con el fin de establecer una comparativa entre el transformante simple y el doble, que sobreexpresa simultáneamente ambos genes. Los resultados muestran un aumento sinérgico en lípidos neutros en el transformante doble seleccionado, mejorando tanto a la especie parental inicial como al transformante simple *acs* del que procede. Las dos especies transformadas presentan además una mayor cantidad de carotenoides y una mayor síntesis de almidón en medio repleto y carente de nitrógeno, y de nuevo, una mejor evolución de la eficiencia fotosintética en el transcurso de la carencia nutricional.

Finalmente, en el **Capítulo 4**, se utiliza la capacidad de acumulación de almidón de *Chlamydomonas reinhardtii* bajo deficiencia de nitrógeno para la producción de los precursores químicos 5'-hidroximetilfurfural (5'-HMF) y ácido levulínico (LA). Estos intermediarios son ampliamente utilizados en industria para la síntesis de biocombustibles, aditivos, polímeros o sondas de diagnóstico por imagen. La aplicación de un diseño experimental y la metodología de Superficie de Respuesta, permitió determinar la influencia de los parámetros experimentales implicados en la hidrólisis ácida de almidón, en un primer paso a glucosa y posteriormente, a 5'-HMF y LA. Se seleccionaron las condiciones de temperatura, porcentaje de ácido, porcentaje de DMSO y tiempo de reacción para favorecer la síntesis de cada uno de los tres precursores. Además, la extracción de carotenoides previa a la hidrólisis, permite implementar las bases para un proceso de biorefinería integrada para la producción de varios compuestos a partir de una misma biomasa.

# Introduction



## 1. Microalgae: a major source of biocompounds.

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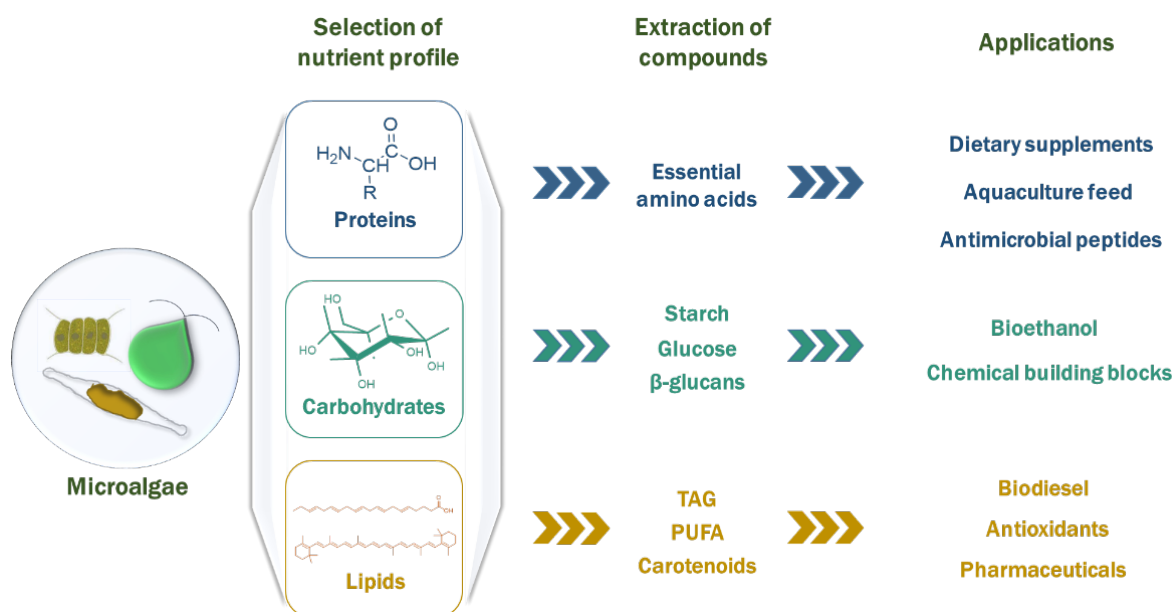
The term microalga refers to unicellular microorganisms which can perform oxygenic photosynthesis, and catalyze atmospheric CO<sub>2</sub> fixation into organic matter (Prasad et al., 2021). They comprise thousands of different strains, including prokaryotic cyanobacteria as well as eukaryotic algae, evolved from primary cyanobacterium endosymbiosis. The presence of chlorophyll a in the former and chlorophyll a, b, and phycobilins in the latter make possible the photosynthetic process (Falkowski et al., 2004).

Microalgae are extended worldwide, in both freshwater and marine environments, and they are capable of adapting to harsh conditions, from extreme temperatures to high salinities or the presence of heavy metals. Several parameters influence culture status, such as light intensity, pH, temperature or nutrient availability. Regulation of these conditions plays a crucial role in growth rates and carbon/nitrogen ratio within the cells, favouring a variety of profiles of carbohydrates, lipid and protein contents suitable for commercial exploitation.

Current commercialization of microalgal-derived products entailed a market value superior to USD 4.3 billion in 2020 (Loke-Show 2022), being in continuous expansion as more scale-up processes are optimized for the production of compounds of interest. Figure 1 presents the most common metabolites extracted from algae and their applications.

Human dietary supplementation by algae is widely extended due to the excellent nutrient profile of certain strains. *Spirulina (Arthrospira) maxima* is a cyanobacterium with a high content in protein, vitamin B<sub>12</sub>, and  $\gamma$ -linolenic acid, which production exceeds 3000 tons per year and can be found in multiple formats (Batista et al., 2013). *Chlorella spp.* are chlorophytes that can reach up to 57% of their dry weight in protein, among other valued compounds as carotenes,  $\beta$ -glucans and vitamins (Bleakley & Hayes, 2017).

Regarding aquaculture, both living microalgae and dry biomass have been revealed to be a good food source in the early stages of growth of bivalves and fish by optimizing diets, setting concentrations of mixed species, stock densities, or nutrients (Deng et al., 2022). Furthermore, they are being tested for the successful delivery of antimicrobial peptides, aiming to progressively reduce the abuse of traditional antibiotics (Cuesta et al., 2021; Kwon et al., 2019).

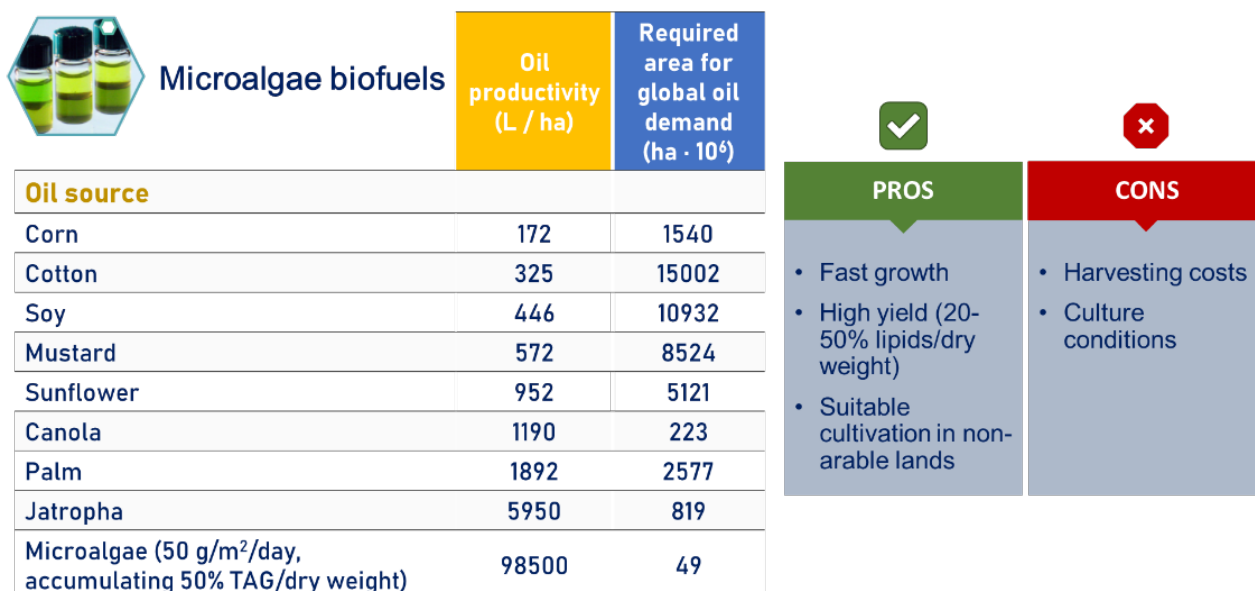


**Figure 1. Main compounds of interest extracted from microalgae and their applications.**

Biodiesel production is one of the fields most intensively researched for substitution of fossil fuels and reduction of carbon footprint. Microalgal ability to accumulate starch and lipids makes them a potential candidate, within renewable sources, for the production of bioethanol via fermentation processes, or biodiesel via transesterification reactions (Raheem et al., 2018). Bioethanol is obtained after carbohydrate extraction and sugar monomerization from algae by hydrolysis, and further fermentation by yeast. Lignocellulosic residues from superior crops have been traditionally used for this purpose, and microalgae have arisen as a suitable alternative, considering that cell disruption and acidic hydrolysis steps are accomplished more easily due to the absence of lignin (Sanchez-Rizza et al., 2017). Species as *Tetraselmis sp.*, *Skeletonema sp.* or *Chlorella sorokiniana* have been tested, reaching yields of 40% of bioethanol per g of reducing sugar (Constantino et al., 2021). With respect to biodiesel, microalgae can store triacylglycerols (TAG) in high percentages within the cell, which can be extracted and transesterified at mild temperatures to obtain the derived methyl esters (Chisti, 2007). Figure 2 depicts estimated oil production of several crops and microalgae-based oil, showing the high yield that makes microalgae a competitive alternative. Other advantages in comparison with superior plants consist in their faster growth, reaching exponential phase within days, and the fact that they can be cultured in open ponds or bioreactors which can be installed in non-cultivable lands. By contrast, biomass harvesting costs and the necessity of applying moderate stress to rise starch or lipid productivity, hamper the overall profit of the process and is still under improvement (B. Chen et al., 2017).

Another application based on carbohydrate accumulation is the production of chemical intermediates, used for further polymerization or derivatization processes in industrial manufacturing. Substances such as

levulinic acid, 5'-hydroxymethylfurfural, lactic acid, formic acid, or sugar alcohols are also obtained for their processing into paint additives, insecticides, fuel additives or solvents (Kohli et al., 2019). Microalgae have been tested to be used as feedstock for the production of these platform chemicals, instead of traditional degradation of hemicelluloses from lignocellulosic materials (García-Domínguez et al., 2013) or synthesis from the petrochemical industry.



**Figure 2. Comparison of oil productivities and required land areas between traditional crops and microalgae.** Adapted from Shuba & Kifle, 2018.

Circular economy implies the renovation and recycling of materials, giving them an added value. Water is a precious commodity, starting to be in short supply in regions where drought seasons are becoming frequent. Domestic use and manufacturing processes also reduce its availability. Regarding this, microalgae can significantly help in water recovery through bioremediation processes. Two key aspects can be considered. On the one hand, the capability of growing in wastewater or other agro-industrial wastes because of their high content in N, P, or HCO<sub>3</sub><sup>-</sup>, where microalgae can feed from these nutrients and use these residues as if they were culture media. Carbon and nitrogen removal decreases the possibility of eutrophication once water is delivered back to the environment (Deviram et al., 2020). On the other hand, microalgae have the ability to accumulate heavy metals in a range that does not compromise their survival through ion exchange and complex formation mechanisms, assisting in their removal from contaminated areas (León-Vaz et al., 2021; Priya et al., 2022).

Last of all, microalgae serve as feedstock of other bioactive and high-added value compounds, which exhibit anti-cancer, or anti-inflammatory effects. Species such as *Phaeodactylum tricornutum* are enriched in polyunsaturated fatty acids (PUFA), predominantly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These PUFA are involved in the prevention of cardiac diseases, helping to reduce cholesterol levels and

control inflammation (Chew et al., 2017). Moreover, carotenoids such as astaxanthin, lutein or  $\beta$ -carotene, extracted from *Dunaliella salina* or *Haematococcus pluvialis*, are valued for their strong antioxidant character and potential ability to prevent carcinogenesis (Koyande et al., 2019; Sathasivam et al., 2019), and can be found in sunscreen or skin-care products.

## 2. Genetic engineering of microalgae. Molecular approaches to produce high-added value compounds.

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Although microalgae are extensively used for the production of diverse biocompounds, there are limitations that hinder productivity and their potential of substituting the conventional source of these compounds. As previously mentioned, one of the key factors regarding the synthesis of high-added value compounds, e.g. carotenoids, and TAG, is that their induction within the cell is often achieved by setting harsh culture conditions, mainly nutrient deprivation or high light intensities. In this sense, genetic engineering represents a powerful instrument that allows the enhancement of product quality and productivity to a significant degree. Molecular Biology has rapidly evolved to be a fine-editing technique, utilized to obtain tailored microalgae strains that comply with the desired characteristics.

### 2.1 Methods for genetic transformation of microalgae.

There are currently several approaches to genetically engineer either the nucleus or chloroplast of microalgae, each of them better suited for certain strains depending on the cell structure, as most of them need a transient disruption of the cell wall and nucleus envelope to achieve DNA integration. Table 1 presents principal methods successfully applied on different phyla of microalgae.

Glass beads transformation consists in the agitation of algal suspension and the DNA construct with small glass spheres, assisted by the membrane fusion agent polyethylene glycol (PEG), to facilitate the insertion of the genetic material into the cell. It was first described for the transformation of *Chlamydomonas reinhardtii* (Kindle, 1990), and it works more efficiently in microalgae with a less rigid cell wall. Although it is a relatively inexpensive method, parameters such as vortexing time or incubation period with PEG (which results toxic for the cells in prolonged expositions), have to be taken into account to achieve higher efficiencies (Sreenikethanam et al., 2022). Silicon carbon whiskers are also used for agitation methods and seem to result in higher cell viability after transformation. However, their health risk associated with exposure make them less utilized than glass beads (Potvin & Zhang, 2010).

SPECIES	METHOD	REFERENCE
<b>Chlorophyceae</b>		
<i>Chlamydomonas reinhardtii</i>	Glass beads	(Kindle, 1990) (Díaz-Santos et al., 2013)
	Electroporation	(Yang et al., 2019)
	SiCa whiskers	(Dunahay et al., 1993)
	Bombardment	(Debuchy et al., 1989)
	<i>Agrobacterium</i>	(Kumar et al., 2004)
	CRISPR	(Guzmán-Zapata et al., 2019)
<i>Tetraselmis chuii</i>	<i>Agrobacterium</i>	(Úbeda-Mínguez et al., 2015)
<i>Chlorella vulgaris</i>	Glass beads	(Tokunaga et al., 2019)
<i>Dunaliella salina</i>	Electroporation	(Geng et al., 2003) (Sun et al., 2005)
	Bombardment	(Tan et al., 2005)
	CRISPR	(Ortega-Escalante et al., 2019)
<b>Eustigmatophyceae</b>		
<i>Nannochloropsis gaditana</i>	Electroporation	(Dolch et al., 2017)
	CRISPR	(Ajjawi et al., 2017)
<b>Dinoflagellates</b>		
<i>Amphidinium sp.</i>	SiCa whiskers	(Ten Lohuis & Miller, 1998)
<b>Diatoms</b>		
<i>Phaeodactylum tricornutum</i>		(Apt et al., 1996)
	Bombardment	(Zaslavskaja et al., 2000)
		(Watanabe et al., 2018)
	Conjugation	(Karas et al., 2015)
	CRISPR	(A. Sharma et al., 2018)
<i>Thalassiosira pseudonana</i>	Conjugation	(Karas et al., 2015)

**Table 1. Examples of genetically engineered microalgae.** Adapted from Molina-Márquez et al., 2021

Biolistic method implies bombardment of the cells with tungsten or gold particles coated with the DNA of interest. A high-pressure gene gun is then used to shoot towards the algal suspension (Bhatia & Bera, 2015). This protocol has been broadly used in diatoms, and increases efficiency in chloroplast transformation, delivering several copies of the construct through the membranes (Pratheesh & Vineetha, 2015). By contrast, cell viability is low compared with other procedures and the material cost is higher.

Electroporation allows transformation of microalgae by applying an electric pulse that creates a temporary disruption on the membrane for the DNA to be inserted. Despite being a protocol that requires low amounts of genetic material and is easy to accomplish (Potvin & Zhang, 2010), it becomes difficult to perform on marine species, where NaCl causes an interference with the pulse, reducing efficiency. In these cases, a desalting pretreatment is required. *Dunaliella salina*, *Nannochloropsis oculata* or *Phaeodactylum tricornutum* have been successfully transformed with this technique (Geng et al., 2004).

*Agrobacterium tumefaciens* is a bacterium popularly used for plant transformation, which transfers the T-DNA that includes the gene of interest due to the action of its virulent proteins. Microalgae transformation has been achieved by co-cultivation with previously-induced *Agrobacterium* cells, which are further eliminated to recover the algal clones. Species of biotechnological interest such as *Haematococcus pluvialis*, *Isochrysis galbana* or *Chlamydomonas reinhardtii* have been transformed with this protocol (Doron et al., 2016).

In the recent years, CRISPR/Cas technology has arisen as a novel methodology that overcomes some of the issues concerning traditional techniques, as random integration of the genes. This genome-editing technique, based on the response of bacteria and archaea to bacterial/viral infection, permits the recognition of a desired sequence of the genome. A specific guide RNA is designed to hybridize with the sequence, and the latter is cleaved due to the nuclease activity of the Cas ribonucleoprotein complex. The fragments can be then repaired by different homologous or non-homologous mechanisms, creating insertions of a specific gene or its knockout by deletion (Shin et al., 2016). The added value that CRISPR provides in this field is the possibility of accurately editing the genome *in vivo*, reason for which it is a system intensively explored for therapeutical applications. However, some aspects of this technique still have to be improved, including elimination of off-target events or the appearance of premature stop codons after non-homologous recombinations (Nymark et al., 2016; Wilbie et al., 2019).

## **2.2 Accumulation of high-added value compounds in microalgae by molecular approaches.**

There are numerous examples of genetic engineering applied to microalgae, aiming to enhance the synthesis of products of high interest in the industry. Carotenoids, which play a photoprotective role in photosynthetic organisms, act as pigments and are valued for their antioxidant properties, have been a major target in molecular approaches. Most of the enzymes involved in the carotenoid biosynthetic pathway have been studied through overexpression or knockout of their encoding genes, achieving higher amounts of carotenes and their derivatives, or qualitative changes in the pigment profile of the strains, synthesizing carotenoids that are not usually present in the parental lines (Couso et al. 2011; León et al. 2007). RNA interference, through transcriptional and translational downregulation mechanisms has also been tested, resulting in this case in transformants with low levels of the targeted pigments (Vila et al. 2008).

Microalgae have been used as a platform for the production of therapeutical molecules. Overexpression of genes encoding terpenoid-related synthesis in *Phaeodactylum tricornutum*, has enhanced the production of betulin, an interesting precursor for HIV and cancer treatments (D'Adamo et al., 2019). Expression of *Plasmodium* antigens fused to starch granules in *Chlamydomonas reinhardtii*, has successfully induced immune response in rabbits after injection of the purified protein, showing a potential strategy for the production of a vaccine against malaria (Dauvillée et al., 2010), and chloroplast expression of the fused proteins VP1 from the foot and mouth virus disease and Cholera toxin B, have resulted in increased protein accumulation in comparison with previous expression in *Arabidopsis* (Sun et al., 2003).

Multimiomics approach, although it is not a molecular engineering technique itself, provides valuable information about genes or microorganisms involved in environmental processes. For instance, metagenomic analysis has revealed which mechanisms are plausible in microalgae-bacteria consortia for efficient wastewater treatment and biohydrogen production (Perera et al., 2019; Shetty et al., 2019).

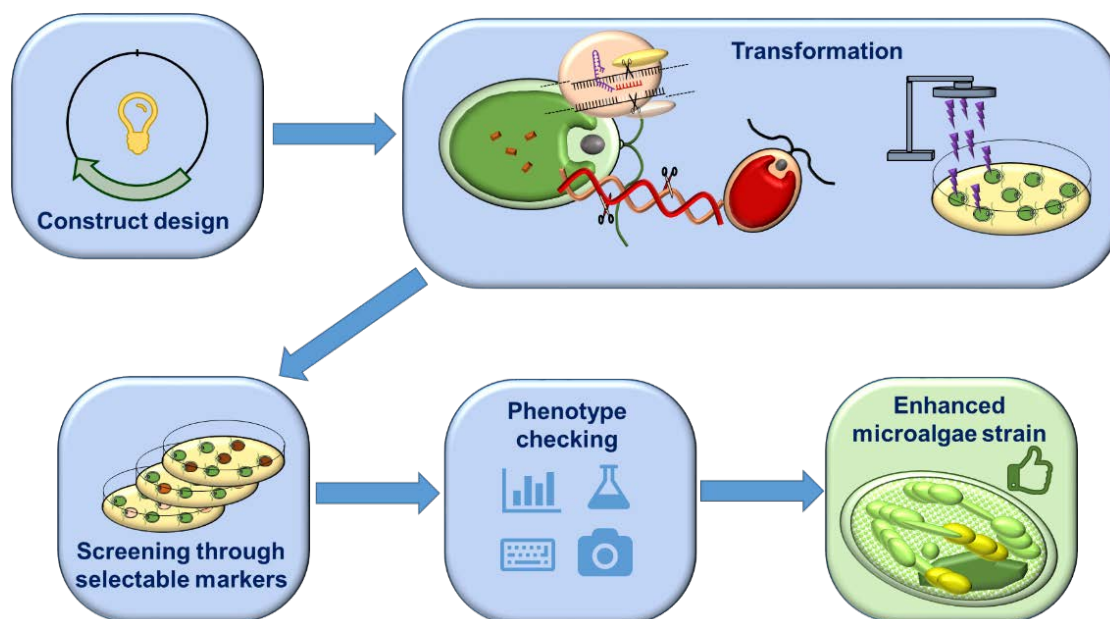
Other examples include: feed supplementation, such as phytase synthesis in *Chlamydomonas* to increase phosphorus availability in poultry grain (Yoon et al., 2011); or increased hydrogen production, by overexpression of hexose symporters (Doebbe et al., 2007). An important application of molecular engineering in microalgae, biofuel production, will be discussed below.

### 2.3 Main difficulties to assess in genetic engineering of microalgae.

Despite the fact that transformation methods are simple to perform, it does not guarantee a sufficient and stable insertion of the DNA in the genome of the microalga. Nuclear transformation using tools different from CRISPR/Cas systems, implies random insertion of the gene within the genome, hence positional effect is one of the factors that influence the phenotype. In addition, foreign genes are prone to be silenced at transcriptional or translational levels, resulting in insertion loss or invariable and reverted phenotypes (León-Bañares et al., 2004). On the other hand, chloroplast transformation is less suitable for proteins that require further steps of glycosylation or secretion (Rasala & Mayfield, 2015). Figure 3 depicts current methodology for generation of microalgae transformants.

Construct design is a key aspect that needs to be considered in microalgae engineering. Strong and constitutive promoters, preferably from the same species, enhance the expression of the selection markers and the gene of interest. Marker genes based on auxotrophic growth and herbicide/antibiotic resistance are the most frequent (Doron et al., 2016; Sizova et al., 2001). Moreover, expression increases when the gene of interest and the reporter gene are fused in a sole plasmid and under the same reading frame, which also makes easier the subsequent screening of the transformants (León-Bañares et al. 2004; Molina-Márquez et al. 2020). When inserting heterologous genes, sequence adaptation to the codon usage of the host organism is also

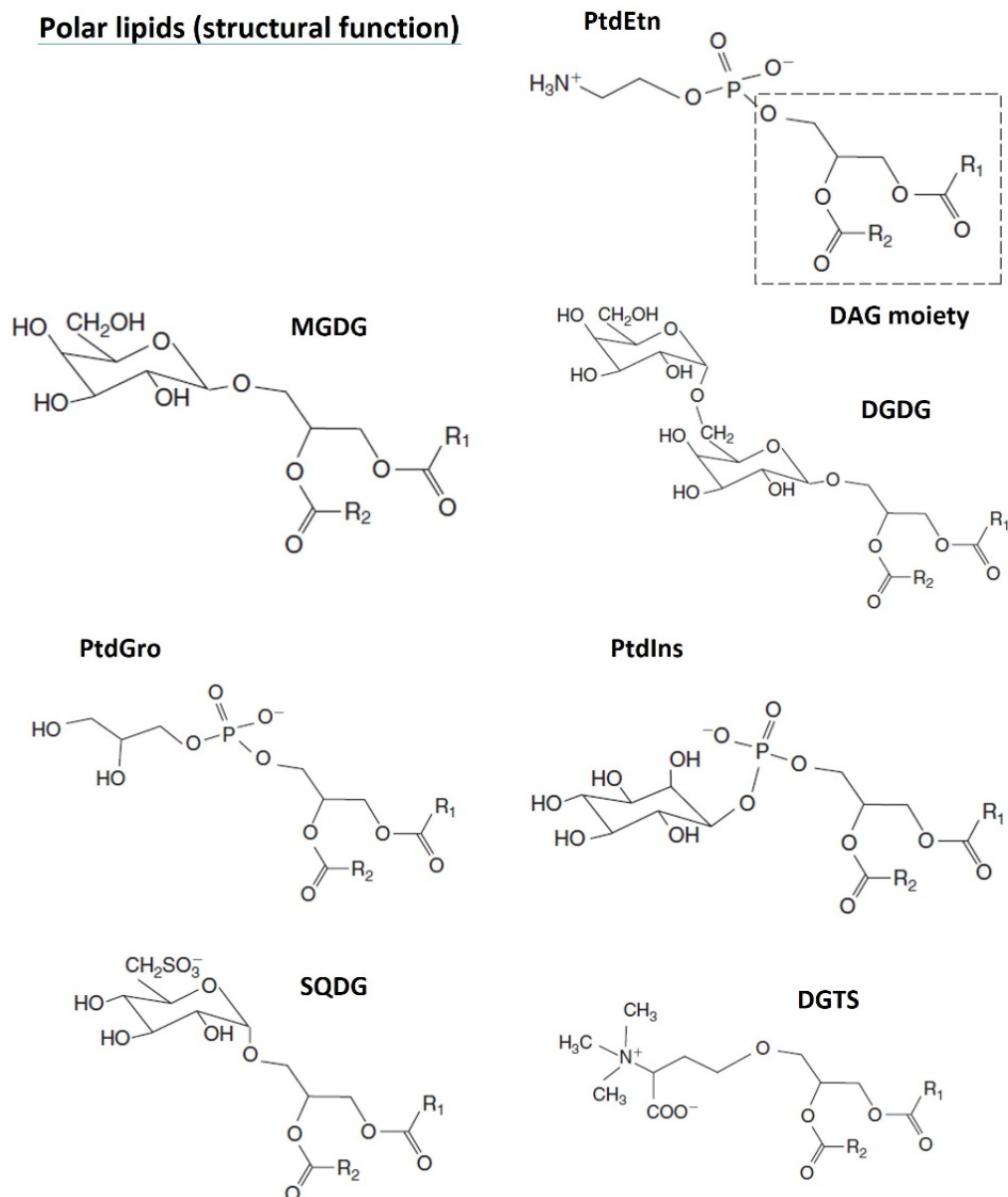
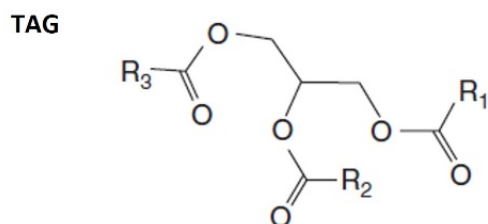
recommended for decreasing silencing mechanisms (Barrera & Mayfield, 2013). The addition of other elements such as introns or fragments of untranslated regions have been shown to provide better transcript levels.



**Figure 3. Schematic workflow for genetic engineering of microalgae.**

### 3. Fatty acid and neutral lipid biosynthesis in green microalgae. Metabolism and genetic engineering.

To adequately undertake the molecular enhancement of microalgae for tailor-made applications, a good understanding of the involved metabolic pathways and principal regulatory elements is needed. Lipid biosynthesis has been extensively studied in superior plants, which share some of the mechanisms presented by green microalgae. In *Chlamydomonas*, as well as in most of chlorophytes, the majority of lipid content is devoted to form membrane structures, which delimit both organelles and individual cells, with a high proportion located in the plastids. Main lipid classes are presented in Figure 4. Polar lipids are amphipathic, with a hydrophobic tail of two acyl chains attached to a glycerol backbone, conforming a common diacylglycerol moiety present in all glycerolipids; and a hydrophilic head composed by a glycosylated or phosphate derivative in the remnant position of the glycerol chain. This double behavior allows the adequate folding of membranes (Ohlrogge & Browse, 1995). In *Chlamydomonas*, chloroplasts are composed of mono- and digalactosyl diacylglycerols, phosphatidylglycerol and sulfoquinovosyl diacylglycerol. In the extrachloroplast space, phosphatidyl

**Polar lipids (structural function)****Neutral lipids (storage function)**

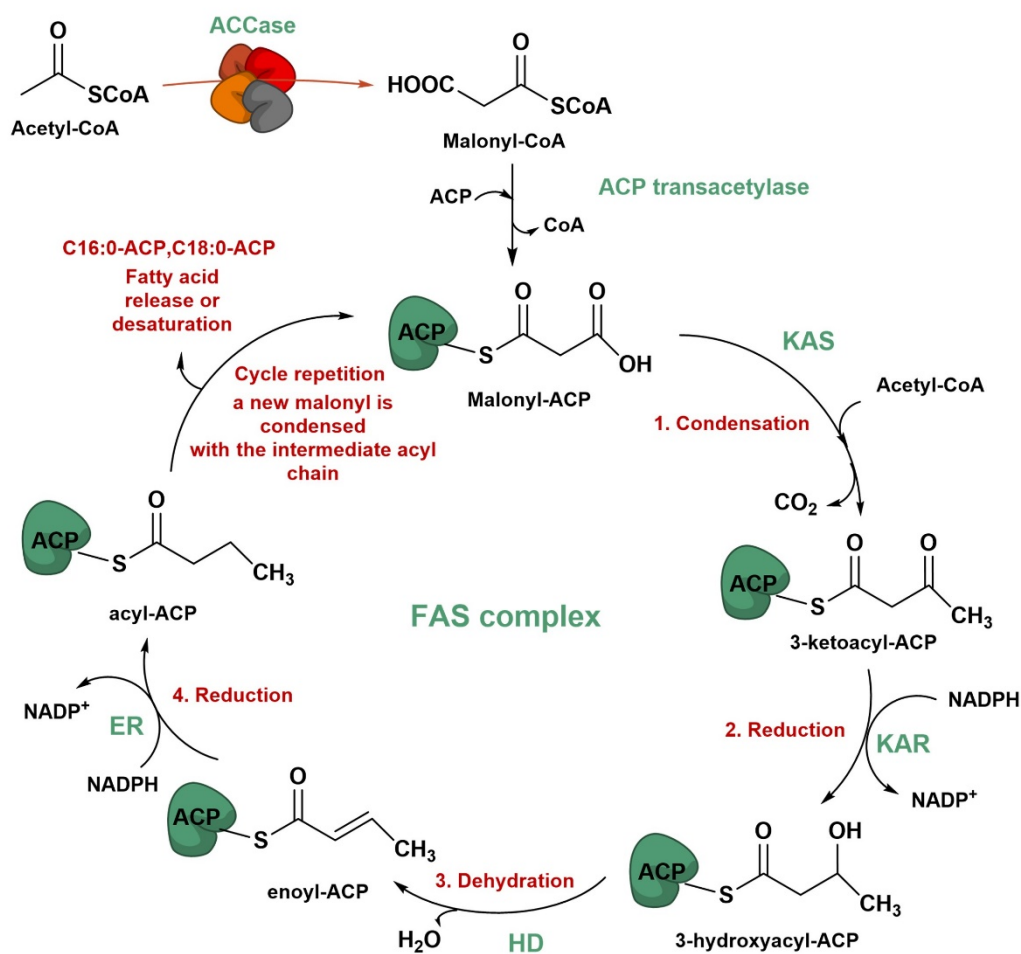
**Figure 4. Lipid classes in *Chlamydomonas*.** Adapted from Harris 2009. PtdEtn: phosphatidyl ethanolamine; DAG moiety: diacylglycerol backbone, common to the presented lipidic species; MGDG: monogalactosyl diacylglycerol; DGDG: digalactosyl diacylglycerol; PtdGro: phosphatidylglycerol; PtdIns: phosphatidyl inositol; SQDG: sulfoquinovosyl diacylglycerol; DGTS: diacylglycerol trimethylhomoserine; TAG: triacylglycerol.

ethanolamine and diacylglyceryl trimethylhomoserine are the predominant lipid species, whereas phosphatidyl inositol exists in a minor proportion (Li-Beisson et al., 2019). Polar lipids profile varies between microalgal species. For instance, the presence in *Chlamydomonas* of the aforementioned diacylglyceryl trimethylhomoserine, instead of phosphatidylcholine, reveals that there are different mechanisms for the synthesis of these compounds, which are not fully understood (Sakurai et al., 2014). On the other hand, neutral lipids as triacylglycerol (TAG), are synthesized for storage purposes, and induced under stress conditions (Terashima, 2017). Other lipid structures to take into account are terpenoids, which include sterols, essential for cell signaling and membrane fluidity; and the previously mentioned carotenoids, with photoprotective activity (Manning, 2022).

### 3.1 Fatty acid biosynthesis.

Fatty acid chains are the primary building blocks needed for subsequent assembly into polar or neutral lipid structures. Acetyl-CoA is a central metabolite that is required for the initiation of the process, whose synthesis can be achieved by different enzymes and predicted to occur simultaneously in various cellular compartments (Courchesne et al., 2009). This metabolic process is depicted in Figure 5.

Acetyl-CoA carboxylase (ACCase) catalyzes the first committed step in the route. In most chlorophyta, ACCase is a heteromeric enzyme consisting of 4 subunits that perform the carboxylation of acetyl-CoA (2 carbons) into the 3-carbon intermediate malonyl-CoA (Huerlimann & Heimann, 2013). Malonyl-CoA is then transferred to the acyl carrier protein (ACP) via ACP-transacylase, which allows the binding of fatty acid intermediates during the synthesis, and enters fatty acid synthase (FAS) complex as malonyl-ACP (Baba & Shiraiwa, 2013). FAS is a multimeric complex composed of four enzymes, which together perform sequenced cycles of elongation. First, 3-ketoacyl-ACP synthase (KAS) condenses malonyl-ACP and acetyl-CoA into a 4-carbon intermediate, 3-ketoacyl-ACP, releasing a CO<sub>2</sub> molecule in the process. The keto group of this precursor is reduced to a hydroxyl group by the action of ketoacyl-ACP reductase (KAR), then dehydrated to form a double bond catalyzed by hydroxyacyl-ACP dehydratase (HD), and finally reduced to a saturated chain the by action of enoyl-ACP reductase (ER). At this point, the synthesized chain has been subsequently increased to yield a 4-carbon precursor. The cycle is repeated up to the formation of a saturated fatty acid of 16 carbons (palmitic acid, C16:0), or 18 carbons (stearic acid, C18:0) (Li-Beisson et al., 2019).



**Figure 5. Fatty acid biosynthesis.** ACCase: acetyl-CoA carboxylase; ACP: acyl carrier protein; FAS: fatty acid synthase; KAS: ketoacyl-ACP synthase; KAR: ketoacyl-ACP reductase; HD: hydroxyacyl-ACP dehydratase; ER: enoyl-ACP reductase.

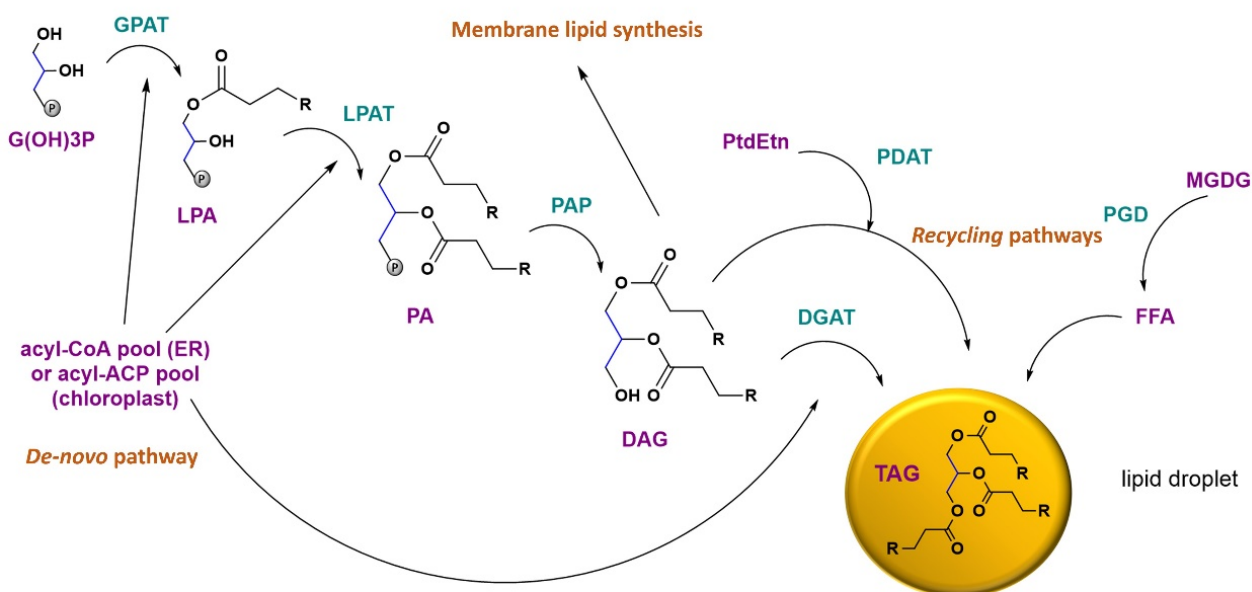
Once this point in the pathway is achieved, the C16:0 or C18:0 acyl-ACP chains can be used for further plastid lipid synthesis, released as saturated fatty acids, or in the case of C18:0-ACP be desaturated to C18:1-ACP by the action of a stearoyl-ACP desaturase. These substrates, C16:0, C18:0 and C18:1, are modified in the chloroplast or exported to the endoplasmic reticulum to increase either carbon number by the action of elongases, or number of desaturations by the action of desaturases (Bellou et al., 2014). Polyunsaturated fatty acids (PUFA), contain from 2 to 6 unsaturations depending on the microalgal species, yielding diverse fatty acid profiles. In *Chlamydomonas*, the main PUFA associated to plastid structures are C16:4, C18:3 (both n-3 and n-6 isomers) and C18:4 (Siout et al., 2011).

### 3.2 TAG assembly.

One of metabolic fates of fatty acids in microalgae, is their esterification into triacylglycerols to act, besides starch, as a carbon storage products under unfavourable conditions, which include limitation of N, P or S (Ran

et al., 2019). TAG production can be performed by *de-novo* synthesis or membrane recycling, in which these structures supply the required chains to form the lipidic droplets. Figure 6 depicts the main mechanisms involved in TAG generation.

*De-novo* TAG synthesis, also known as the *Kennedy pathway*, is an acyl-CoA dependent metabolic route, as different acyltransferases sequentially perform acylations of a glycerol-3-phosphate backbone. In mammals and yeast, TAG production is achieved at the endoplasmic reticulum (ER) membranes. By contrast, in microalgae, both plastidial and ER locations have been found for the involved enzymes. However, there are still many whose subcellular location has been predicted rather than experimentally demonstrated, requiring further studies (Terashima, 2017). In the case of *Chlamydomonas*, it has been reported that the majority of the TAG backbones have a chloroplastic origin, and lipid droplets can be found in the plastids and the cytosol (Fan et al., 2011). In the chloroplast, newly synthesized acyl-ACP chains are used for TAG synthesis, whereas in the ER pathway, acyl chains are incorporated through long chain acyl-CoA synthetase, which activates free fatty acids that have been previously exported from the chloroplast (Bai et al., 2022).



**Figure 6. TAG assembly pathways in eukaryotic microalgae.** Blue captions indicate enzymes and purple captions substrates. ER: endoplasmic reticulum; G(OH)3P: glycerol-3-phosphate; GPAT: glycerol-3-phosphate acyltransferase; LPA: lysophosphatidic acid; LPAT: lysophosphatidic acyltransferase; PA: phosphatidic acid; PAP: phosphatidic acid phosphatase; DAG: diacylglycerol; DGAT: diacylglycerol acyltransferase; TAG: triacylglycerol; PtdEtn: phosphatidyl ethanolamine; PDAT: phospholipid diacylglycerol acyltransferase ; MGDG: monogalactosyl diacylglycerol; PGD: plastid galactoglycerolipid degradation; FFA: free fatty acids.

The committed steps in the route are: two consecutive acylations, the first one performed in the *sn-1* position, catalyzed by glycerol-3-phosphate acyltransferase (GPAT); and the second performed in the *sn-2* position, by lysophosphatidic acyltransferase (LPAT). After these esterifications, the phosphate group in the *sn-3* position is removed from the glycerol backbone by phosphatidic phosphatase (PAP), to yield diacylglycerol (DAG), a key branching point within the pathway, which can go through different routes to produce membrane-related lipid classes. DAG has been proposed to be a substrate for the synthesis of diacylglycerol trimethylhomoserine and phosphatidyl ethanolamine in the ER, and sulfoquinovosyl diacylglycerol and monogalactosyl diacylglycerol in the plastid (Li-Beisson et al., 2015).

Finally, the acylation of the *sn-3* position of DAG is performed by the action of diacylglycerol acyltransferase (DGAT). Its key role as the enzyme responsible of yielding TAG by *de-novo* synthesis, converts it as one of the genes of study in this thesis. One type-1, and five type-2 (also known as DGTT), isoforms of membrane-bound DGAT enzymes have been distinguished in *Chlamydomonas reinhardtii*, which lack of similarity in their structures, despite sharing the same catalytic activity (Chen & Smith, 2012). In addition, a soluble type-3 DGAT, similar to the one located in plants, has been recently identified in the chloroplast (Bagnato et al., 2017).

The existence of several isoforms of DGAT points at different localizations and functionality within subcellular compartments. Generation of knockout mutants of each of the 5 isoforms of DGAT2 by RNAi silencing, resulted in different lipid patterns, detecting significant oil decreases only in *dgat2-1* and *dgat2-5* mutants (Deng et al., 2012a). Substrate preferences also differ between DGAT enzymes. For instance, polyunsaturated acyl-CoAs are the preferred source for DGAT2-1, while DGAT2-2 favoured the incorporation of monounsaturated acyl-CoAs (Liu et al., 2016).

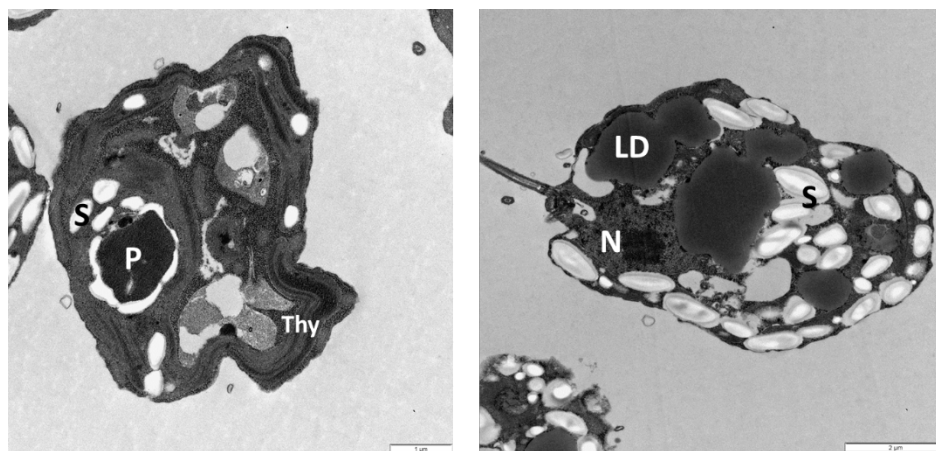
TAG can be also produced by recycling mechanisms, using membrane lipids as the source of the acyl chains instead of synthesizing them *de-novo*, reason why they are also considered acyl-CoA independent mechanisms. In this sense, two main enzymatic reactions have been identified: the transacylation by phospholipid:diacylglycerol acyltransferase (PDAT), which transfers a fatty acid located in the *sn-2* position of a membrane lipid, to the *sn-3* position of a DAG molecule (Bates, 2016); and lipase activity by plastid galactolipid degradation enzyme (PGD), which preferentially releases C18:1 fatty acids from the membrane lipid MGDG to be incorporated into TAG (Li et al., 2012).

The resulting TAG molecules are stored in lipid droplets, surrounded by a membrane lipidic layer, carotenoids and proteins as the major lipid droplet protein, that allow maintenance of the structures (Moellering & Benning, 2010). Fatty acid profile of the TAG fraction in *Chlamydomonas* has been studied, showing an enrichment in C16:0, C18:1 and C18:2 (Siaut et al., 2011). When environmental conditions change into a more

favourable status, carbon remobilization occurs and TAGs are degraded to release fatty acids by the action of lipases (Cagnon et al., 2013).

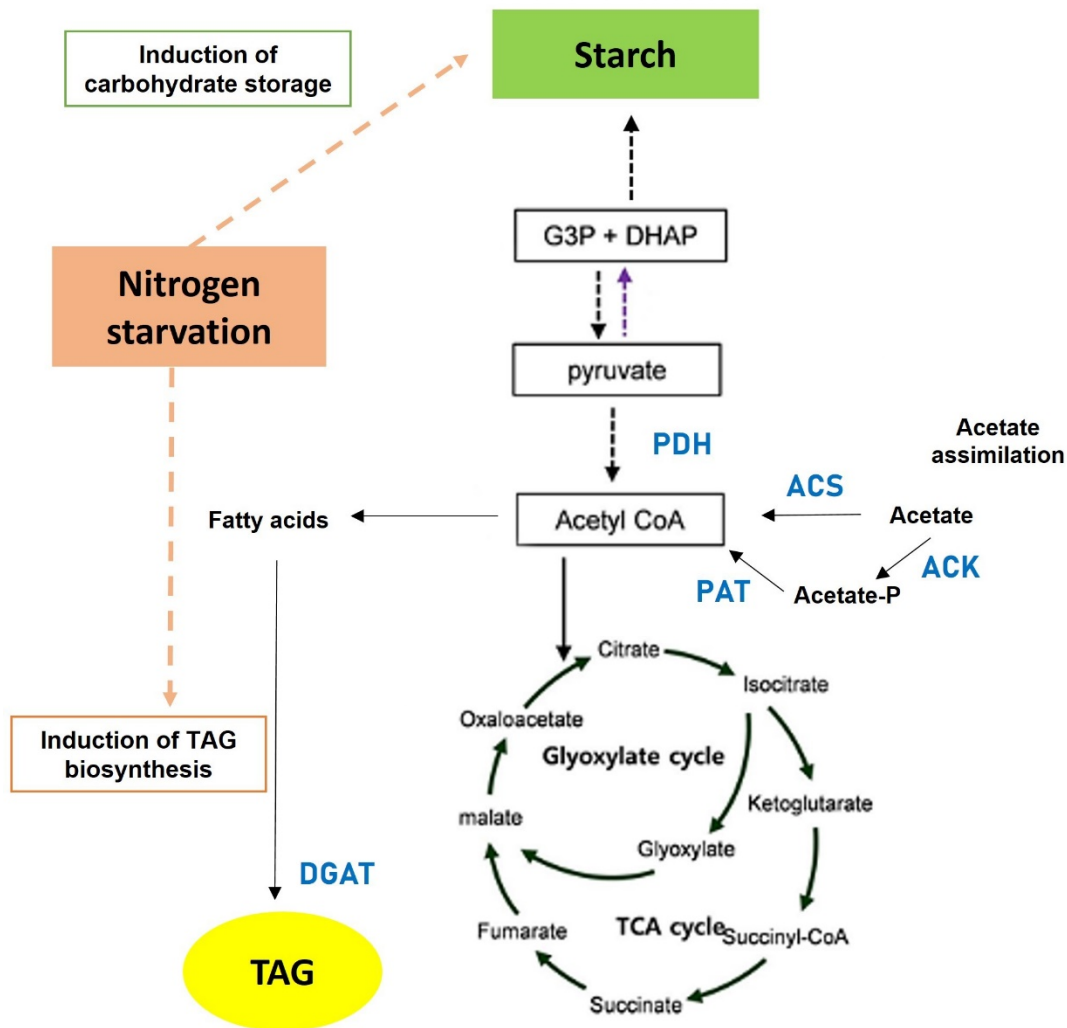
### 3.3 Carbon partitioning and competing pathways under nitrogen starvation.

Carbon and nitrogen metabolism play a critical role in their distribution between macronutrients and relocation under stress factors. Nitrogen deficiency is the stress condition most used for these physiological studies. Microalgae biochemical composition changes dramatically from replete to N-depleted conditions, reducing protein levels and inducing the accumulation of the two main carbon sinks used as reserves, starch and TAG, as shown in Figure 7. Lipids, which account for approximately 20% of dry weight in species of *Chlamydomonas*, can reach up to 60% of microalgal dry weight under nitrogen limitation. Same behavior is observed for starch (Li-Beisson et al., 2015; P. Sharma et al., 2018). In addition, photosynthetic efficiency declines due to the oxidative stress caused by depletion conditions, and combined with the carbon shift from structural to energetic purposes, causes the arrest of the cell cycle (Ran et al., 2019). Subsequently, other events such as respiration, autophagy or gametogenesis are upregulated, whereas chlorophyll or membrane lipid levels are decreased. Overall transcription processes are also reduced under nitrogen limitation (Juergens et al., 2015).



**Figure 7. TEM pictures of *Chlamydomonas reinhardtii* under nitrogen replete conditions (left) and nitrogen starvation (right).** LD: lipid droplet; N: nucleus; P: pyrenoid; S: starch granule; Thy: thylakoid membrane.

Regulatory mechanisms that control carbon flux towards starch and TAG are not fully elucidated, but these pathways seem to compete for carbon supply under nitrogen starvation. This was demonstrated in BAFJ15 mutants of *Chlamydomonas*, whose inability to synthesize starch resulted in a dramatic increase in neutral lipids (Li et al., 2010). However, the origin of the assimilated carbon is not clear. In mixotrophic cultures, where inorganic carbon fixation and organic uptake from the medium are simultaneously present, there are studies pointing at photosynthetic fixed carbon as the preferred source for lipids (Li et al., 2010), but there are also other



**Figure 8. Simplified relationship between acetate assimilation, acetyl-CoA pools, and TAG/starch synthesis under nitrogen starvation.** Adapted from Koo et al. 2017. G3P: glyceraldehyde-3-P; DHAP: dihydroxyacetone-P; PDH: pyruvate dehydrogenase; ACS: acetyl-CoA synthetase; ACK: acetate kinase; PAT: phosphate acetyltransferase; FA: fatty acid; TAG: triacylglycerol; DGAT: dicacylglycerol acyltransferase; TCA: tricarboxylic acid cycle.

works that estimated a 75% of the carbon used for fatty acid synthesis and 70% for subsequent TAG assembly coming from acetate assimilation. The remaining 30% of the carbon content would be supplied by membrane turnover (Takeuchi & Benning, 2019). However, there is a general consensus in the preference of starch accumulation over TAG in the first stages of deprivation. When the former reaches an optimal intracellular level, the latter starts increasing its synthetic rates (Fan et al., 2012).

Consequently, substrate availability is a key factor involved in TAG accumulation. A schematic representation of carbon flux under nitrogen starvation is presented in Figure 8. Acetyl-CoA intervenes in fatty acid synthesis, whose maximum levels always seem to precede maximum TAG concentrations (Avidan et al., 2015), and it is also utilized for the biosynthesis of isoprenoids and amino acids, and for acetylation processes

performed in post-translational modifications (Shtaida et al., 2015). In the plastids, acetyl-CoA can be synthesized from different substrates. On the one hand, pyruvate dehydrogenase (PDH) catalyzes the conversion from pyruvate to acetyl-CoA. PDH is an enzymatic complex which has been found to be upregulated in early stages of N-deprivation for some green algae, as *Chlorella denticata* (Avidan et al., 2015). On the other hand, acetyl-CoA can be produced from acetate, the main carbon source used for *Chlamydomonas* in mixotrophic conditions, via two distinct pathways: acetyl-CoA synthetase (ACS), which transforms acetate into acetyl-CoA in a single step; and the system acetate kinase/phosphate acetyltransferase (ACK/PAT), in which ACK catalyzes a first step of phosphorylation of acetate into acetyl-P, and PAT incorporates CoA and removes the phosphate group to finally yield acetyl-CoA (Wase et al., 2014).

As acetyl-CoA is an important precursor supplying fatty acid synthesis, its production by ACS, and effect on lipid accumulation have been objectives of study in this thesis. There are three ACS isoforms potentially identified in *Chlamydomonas reinhardtii* (Harris, 2009), with cytosolic and plastidial locations. ACS transcript levels have been measured in N-depletion under phototrophic and mixotrophic conditions, and were found to be upregulated in all of them, together with one of the isoforms of diacylglycerol acyltransferase (Ramanan et al., 2013). Moreover, lipid content has been linearly improved in starchless mutants of *Chlamydomonas* with increasing acetate concentrations in mixotrophic cultures up to 50 mM (Ramanan et al., 2013).

All things considered, nitrogen starvation triggers an intricate response in microalgae, with multiple metabolic pathways affected to adapt to the unfavourable conditions. Identifying the limiting steps influencing the involved routes, is an indispensable condition for the improvement of TAG biosynthesis.

### **3.4 Genetic engineering to improve neutral lipid content in microalgae.**

As previously mentioned, microalgae are a potential source of TAG and PUFA for subsequent biodiesel production and nutrient supplementation, respectively. The principal difficulties to overcome at laboratory scale are the decrease of biomass productivity under stress conditions at which neutral lipids biosynthesis is induced (Singh & Dhar, 2011). Molecular approaches under study aim to concurrently increase TAG and affect growth to a small extent. Table 2 presents several studies following different strategies to increase lipid content.

Overexpression of the enzymes which are directly involved in fatty acid or TAG biosynthesis are the most studied. Outcomes are variable depending on the species, including cases where no significant change in lipid content was detectable (Dunahay et al., 1996; La Russa et al., 2012). Other strategies successfully accomplished are overexpression of transcription factors, alteration of lipid trafficking, or knockdown of lipid catabolism, which hinders TAG degradation.

Factors as the choice of a good promoter and gene insertion can also affect the observed phenotype (Zhang et al., 2022). However, microalgae are still a platform able to produce complex metabolites within a single species (Pierobon et al., 2018).

	Type of manipulation	Results	Ref.
<b>Fatty acid biosynthesis</b>			
<i>Cyclotella cryptica</i>	Overexpression of ACCase gene	No effect	(Dunahay et al., 1996)
<i>Scenedesmus quadricauda</i>	Overexpression of ACCase gene	1.6-fold improvement in fatty acid content	(Gomma et al., 2015)
<i>Nannochloropsis oceanica</i>	Overexpression of MAT	36% improvement in lipid content	(J.W. Chen et al., 2017)
<i>Phaeodactylum tricorutum</i>	Overexpression of KAS	No effect	(Fan et al., 2018)
<b>TAG assembly</b>			
<i>Chlamydomonas reinhardtii</i>	Overexpression of LPAAT	20% improvement in lipid content	(Yamaoka et al., 2016)
<i>Phaeodactylum tricorutum</i>	Expression of GPAT under the Pt211 promoter	2.6-fold improvement in lipid content	(Zou et al., 2018)
<i>Phaeodactylum tricorutum</i>	Overexpression of DGAT1	35% improvement in neutral lipid content	(Niu et al., 2013)
<i>Tetraselmis chui</i>	Overexpression of DGAT1 from <i>Echium pitardii</i>	2-fold improvement in TAG content	(Úbeda-Mínguez et al., 2017)
<i>Chlamydomonas reinhardtii</i>	Overexpression of DGAT2	29-fold improvement in TAG content	(Iwai et al., 2014)
<i>Nannochloropsis oceanica</i>	Overexpression of DGAT2	78% improvement in TAG content	(Zienkiewicz et al., 2017)
<i>Chlorella minutissima</i>	Overexpression of G3PDH, GPAT, LPAAT, PAP, and DGAT	2-fold improvement in neutral lipid content	(Hsieh et al., 2012)
<i>Nannochloropsis salina</i>	Recombinant expression of DGAT1	74.9% improvement in TAG content	(Beacham & Ali, 2016)
<i>Phaeodactylum tricorutum</i>	Overexpression of DGAT2	35% improvement in TAG content	(Niu et al., 2013)
<i>Chlamydomonas reinhardtii</i>	Overexpression of DGAT2-1; DGAT2-5	20% and 44% improvement in lipid content, respectively	(Deng et al., 2012)
<b>Substrates upstream TAG pathway</b>			
<i>Phaeodactylum tricorutum</i>	Silencing of PDK	82% improvement in neutral lipids	(Ma et al., 2014)
<i>Chlamydomonas reinhardtii</i>	Overexpression of malic enzyme (ME)	18% improvement in total lipid content	(Kim et al., 2019)
<i>Schizochytrium</i>	Overexpression of acetyl-CoA synthetase (ACS)	11.3% improvement in fatty acid content	(Yan et al., 2013)
<b>Lipid catabolism</b>			
<i>Thalassiosira pseudonana</i>	Deletion of multifunctional lipase	3.3-fold improvement in total lipids	(Trentacoste et al., 2013)
<b>Transcription factors</b>			
<i>Nannochloropsis gaditana</i>	Knockout of ZnCys transcription factor	2-fold improvement in total lipids	(Ajjawi et al., 2017)
<i>Chlamydomonas reinhardtii</i>	Overexpression of <i>dof</i> transcription factor	2-fold improvement in total lipids	(Ibáñez-Salazar et al., 2014)

**Table 2. Molecular approaches for lipid enhancement in microalgae.** Adapted from Naghshbandi et al. 2019.

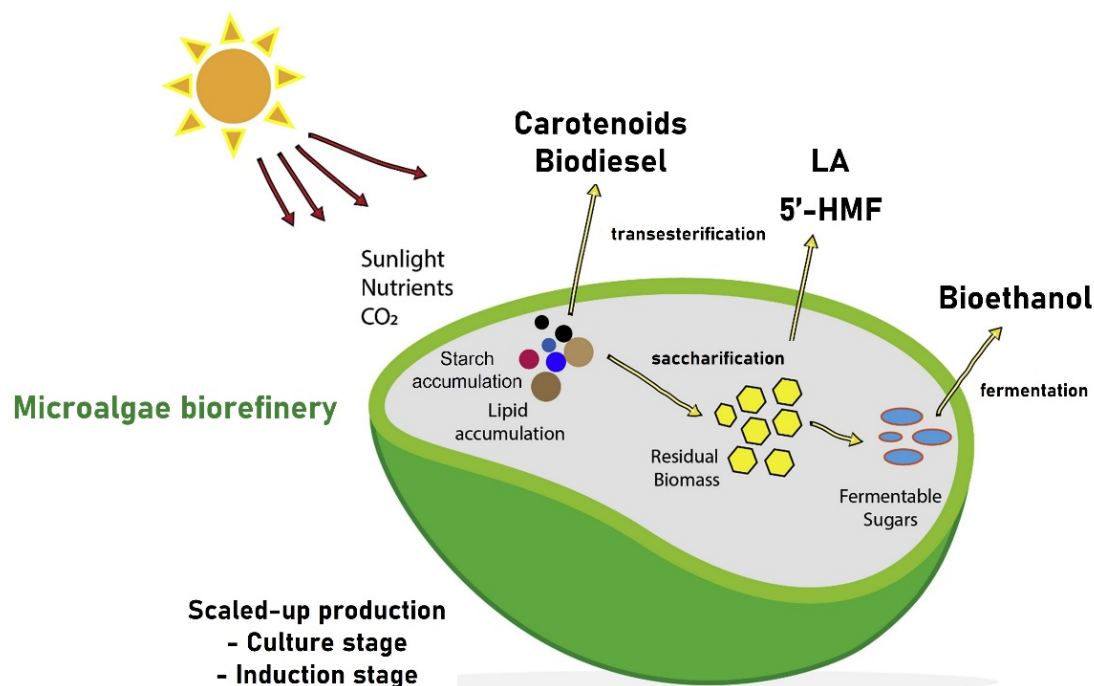
## 4. Integral production of high-added value compounds using microalgae biomass as feedstock.

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Taking into account the broad potential of microalgae to produce high-added value compounds, there are currently major efforts aiming to set a scaled-up, staged production of products from a sole biomass. Green biorefineries using microalgae as raw material, as presented in Figure 9, could be set selecting the strains with the best biochemical profile for the desired applications. Productivity would be enhanced by culture conditions and genetic engineering (Khan & Fu, 2020). As compounds such as TAG and starch are better induced by nitrogen starvation, some authors propose a 2-stage cultivation process, where the first step would be devoted to produce biomass by optimal conditions; and the second step would be the induction stage, where the compounds of interest are synthesized (Peng et al., 2019). Open ponds and photobioreactors are the two main choices for large scale cultivation. However, the design needs optimization, as ponds are less costly and easier to operate, but offer less control over the biomass productivity and higher risk of contamination than bioreactors (Chew et al., 2017).

The following step in large-scale production is biomass harvesting, which is one of the bottlenecks in scale-up processes, as the operational costs are elevated. Processes as air flotation, filtration or flocculation can reduce the necessity of centrifugation (Cuevas-Castillo et al., 2020). In this sense, there are studies applying genetic engineering to induce the self-flocculation ability of microalgae by expression of proteins involved in cell adhesion (Díaz-Santos et al., 2016; Lowder & Herbert, 2015). This would allow to avoid the utilization of metallic cations or pH increase for flocculation, usually more expensive and not suitable for food applications.

Once algal biomass is collected, it can be extracted in different fractions, which allow to obtain the macronutrients or metabolites of interest. Lipids and carotenoids can be isolated using organic solvents, whereas carbohydrates remain in the solid fraction and can be hydrolyzed in acidic conditions (Monte et al., 2020).

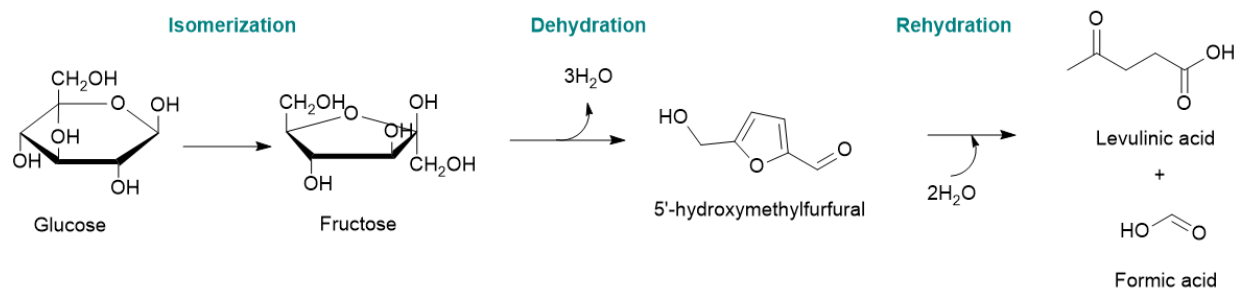


**Figure 9. Simultaneous production of high-added value products and precursors from microalgae.** Adapted from Deviram et al. 2020.

Previous studies have tested microalgae as the raw material for the integrated extraction of compounds of interest. Different scenarios for the concurrent production of carotenoids and fertilizers from *Dunaliella salina* and *Haematococcus pluvialis* have been simulated, predicting results that are economically viable when waste and centrifugation needs are reduced by water recycling, among other factors (Thomassen et al., 2016). Scaled-up cultivation of *Chlorella* in semiarid regions for the production of proteins, can increase its profit a 61% with the simultaneous extraction and commercialization of chlorophylls and fatty acids (AlMahri et al., 2019).

In this thesis, the ability of *Chlamydomonas reinhardtii* to synthesize starch under nitrogen starvation, has been explored for the production of platform chemicals. Starch saccharification can be performed to an extent that glucose monomers are dehydrated and rearranged to form 5'-hydroxymethylfurfural (5'-HMF), as shown in Figure 10. This molecule, which is also known as a contaminant in honey synthesized by gradual hexose degradation, is valued in industry as a precursor for the biofuel dimethylfurfural (DMF), or 2,5-furandicarboxylic acid (FDA), a promising raw material for substitution of the polymer polyethylene terephthalate (PET), traditionally used for the production of packaging (Rosatella et al., 2011). If the hydrolysis goes under harsher conditions or in aqueous systems, 5'-HMF is rehydrated, yielding levulinic acid (LA) and formic acid (Hayes et al., 2008). LA is an intermediate used for manufacturing of  $\gamma$ -valerolactone, a green solvent; and 5-aminolevulinic acid, used as a fluorescent probe in diagnostic imaging, among others (Jeong & Kim, 2020).

Acidic hydrolysis of microalgae-derived starch to yield 5'-HMF and LA has been recently studied. Green microalgae, as *Chlorella sorokiniana* or *Dunaliella salina*, have shown conversion rates to 5'-HMF and LA up to 52% and 44%, respectively (Amoah et al., 2019; Rihko-Struckmann et al., 2020). Addition of catalyzers, zeolites for instance, can enhance 5'-HMF conversion yields by favoring glucose isomerization to fructose (Wang et al., 2016).



**Figure 10. Synthesis of 5'-hydroxymethylfurfural from glucose dehydration, and further rehydration to levulinic acid.**

As nitrogen starvation induces the accumulation of starch and TAG, the same biomass can be processed to isolate these precursors. Simultaneous commercialization along with co-products of high-added value as carotenoids, would improve overall productivity and compensate harvesting costs.

## 5. References.

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# Objectives



## Objectives.

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Global demand for high-added value compounds and exhaustion of raw materials and fossil fuels has led to the bioprospecting of new alternatives in their synthesis. Microalgae can be a suitable platform for the production of these energetic precursors, optimizing the conditions to counteract the effects of culturing under nitrogen starvation. To assess this potential, the following objectives have been set in this thesis:

- Achieve the enhancement in neutral lipid synthesis by a molecular approach that increases a main precursor upstream the TAG assembly pathway, acetyl-CoA, by overexpression of an endogenous gene encoding a chloroplastic acetyl-CoA synthetase (*ACS2*) in *Chlamydomonas reinhardtii* (**Chapter 1**).
- Improve neutral lipid synthesis by a molecular approach that enhances the specific TAG assembly pathway, by heterologous expression of a gene encoding a type-1 diacylglycerol acyltransferase (*DGAT1*) in *Chlamydomonas reinhardtii* (**Chapter 2**).
- Verify whether the simultaneous overexpression of *ACS2/DGAT1* leads to a synergistic effect on lipid accumulation in *Chlamydomonas reinhardtii* the by combination of an adequate supply of precursors and higher availability of the key limiting enzyme of TAG biosynthetic pathway (**Chapter 3**).
- Demonstrate that *Chlamydomonas reinhardtii*, can be used as a feedstock for integral production of high-added value products and precursors, such as carotenoids, 5'-hydroxymethylfurfural and levulinic acid. (**Chapter 4**).



# Results and Discussion





## CHAPTERS 1, 2 Y 3: ARTÍCULOS

Los artículos que forman parte de los capítulos 1, 2 y 3, debido a restricciones relativas a derechos de autor, han sido retirados de la tesis. En sustitución de éstos, ofrecemos la siguiente información: referencia bibliográfica, enlace al artículo si estuviera publicado y resumen.

- Rengel, R., Smith, R. T., Haslam, R. P., Sayanova, O., Vila, M., & León, R. (2018). Overexpression of acetyl-CoA synthetase (ACS) enhances the biosynthesis of neutral lipids and starch in the green microalga *Chlamydomonas reinhardtii*. *Algal Research*, 31, 183–193. <https://doi.org/10.1016/j.algal.2018.02.009>

Enlace al texto completo: <https://doi.org/10.1016/j.algal.2018.02.009>

Resumen:

Genetic engineering can be the solution to achieve the economically feasible production of microalgal based biofuels and other bulk materials. A good number of microalgal species can grow mixotrophically using acetate as carbon source. Moreover, experimental evidence suggests that the biosynthesis of acetyl-CoA could be a limiting step in the complex multifactor-dependent biosynthesis of acylglycerides and point to acetyl-CoA synthetase (ACS) as a key enzyme in the process. In order to test this hypothesis we have engineered the model chlorophyte *Chlamydomonas reinhardtii* to overexpress the endogenous chloroplastic acetyl-CoA synthetase, ACS2. Expression of the ACS2 encoding gene under the control of the strong constitutive *RBCS2* promoter in nitrogen-replete cultures resulted in a 2-fold increase in starch content and 60% higher acyl-CoA pool compared to the parental line. Under nitrogen deprivation, the *Cr-acs2* transformant shows 6-fold higher levels of ACS2 transcript and a 2.4-fold higher accumulation of triacylglycerol (TAG) than the untransformed control. Analysis of lipid species and fatty acid profiles in the *Cr-acs2* transformant revealed a higher content than the parental strain in the major glycolipids and suggests that the enhanced synthesis of triacylglycerol in the transformant is not achieved at the expense of membrane lipids, but is due to an increase in the carbon flux towards the synthesis of acetyl-CoA in the chloroplast. These data demonstrate the potential of engineering the chloroplastic ACS to increase the carbon flux towards the synthesis of fatty acids as an alternative strategy to enhance the biosynthesis of lipids in microalgae.

- Rengel, R., et al.: Enhancement of lipid synthesis in the microalga *Chlamydomonas reinhardtii* by heterologous expression of a type-1 diacylglycerol acyltransferase. (Artículo en proceso de publicación).

Resumen:

Microalgae represent a valuable alternative for the synthesis of biofuels and other high-added value compounds, which production can be optimized by engineering approaches. *Chlamydomonas reinhardtii* was transformed using a construct containing a diacylglycerol acyltransferase (DGAT1) gene from the Boraginaceae *Echium pitardii*, which encodes the enzyme that catalyzes the assembly of the last fatty acid needed for the synthesis of a triacylglycerol molecule. The selected *dgat* transformant showed a 1.7-fold increase in the content of both, total and neutral, lipids compared to the parental line. Under N-deprivation, the transformant exhibited faster acetate uptake, higher accumulation of neutral lipids and higher glycerol internal pools, showing that overexpression of DGAT1 has a great impact not only on TAG accumulation but on lipid homeostasis and carbon metabolism in general. The transformant also showed a better performance in photosynthetic efficiency. These results show the potential of engineered microalgae for the production of sustainable alternatives to carbonbased fossils.

- Rengel, R., et al.: Synergistic effect of overexpression of enzymes involved in TAG biosynthesis and carbon supply in the green microalga *Chlamydomonas reinhardtii*. (Artículo en proceso de publicación).

Resumen:

Microalgae are studied as a promising source for the production of biofuels and a variety of bioactive compounds. However, it is important to assess the factors influencing overall productivity, such as culture conditions and potential bottlenecks within the metabolic routes involved in the synthesis of the desired compounds. In this work, *Chlamydomonas reinhardtii* has been genetically engineered to overexpress a gene encoding an endogenous acetyl-CoA synthetase (ACS2), and subsequently transformed to simultaneously overexpress a heterologous gene encoding a type-1 diacylglycerol acyltransferase (DGAT1). Under nitrogen starvation, the single transformant *acs* showed a 40% increase in total and a 36% in neutral lipids content, compared to the parental line. In the double *acs/dgat* transformant, the accumulation reached also a 40% higher amount of total lipids, and improved its neutral lipids a 68% the content of the parental line, and a 24% the content of the *acs* transformant. The *acs* and *acs/dgat* clones also showed higher carotenoid content, higher starch accumulation at the early stages of N-deprivation, and a better resistance to the degradation of the photosynthetic apparatus, in comparison with the parental line. These results demonstrate that the simultaneous enhancement of the precursor supply and overexpression of a key enzyme catalyzing the last step of the biosynthetic pathway, can improve lipid productivity in microalgae.

## Chapter 4: Simultaneous production of carotenoids and chemical building blocks precursors from chlorophyta microalgae

This chapter has been published as: **Rengel, R.**, Giraldez, I., Díaz, M. J., García, T., Vígara, J., & León, R. (2022). Simultaneous Production of Carotenoids and Chemical Building Blocks Precursors from Chlorophyta Microalgae. *Bioresource Technology*, 127035. <https://doi.org/10.1016/J.BIORTECH.2022.127035>



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## Simultaneous production of carotenoids and chemical building blocks precursors from chlorophyta microalgae

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### HIGHLIGHTS

- Microalgae can be utilized as feedstock for platform chemicals.
- Co-production of carotenoids can make profitable the generation of HMF and LA.
- Natural Carotenoids are increasingly-demanded high-value compounds.
- 5'-hydroxymethylfurfural and levulinic acid were synthesized via starch hydrolysis.
- Operational conditions were optimized performing Multiple Regression Analysis.

### ARTICLE INFO

#### Keywords:

Microalgae  
Biomass  
Starch  
5'-hydroxymethylfurfural  
Levulinic acid

### ABSTRACT

Replacement of fossil fuels has to be accompanied by the incorporation of bio-based procedures for the production of fine chemicals. With this aim, the microalga *Chlamydomonas reinhardtii* was selected for its ability to accumulate starch, an environmentally-friendly alternative source of chemical building blocks, such as 5'-hydroxymethylfurfural or levulinic acid. The content of appreciated lipophilic coproducts was assessed in the selected microalga cultured at different nutritional conditions; and the parameters for the acidic hydrolysis of the algal biomass, obtained after pigments extraction, were optimized using a Central Composite Design. Response Surface Methodology predicted that the optimal hydrolysis conditions were elevated temperature, high DMSO % and short hydrolysis time for glucose. LA was favored at long times and high acid % and 5'-HMF at lower acid % and high DMSO %. *Chlamydomonas* can therefore be used as a sustainable feedstock for the simultaneous production of high-added value lipophilic compounds and platform chemicals.

### 1. Introduction

Microalgae are versatile fast-growing unicellular photosynthetic microorganisms able to produce a variety of bulk and high-added value compounds, including new bioactive unexplored ones (Khan et al. 2018; Schüler et al. 2017). Their potential ability to reduce greenhouse gas emissions, and to serve as feedstock for the production of carbon-neutral third-generation biofuels, has made them the center of increasing attention (Enamala et al. 2018; Khan and Fu 2020).

The capability of oleaginous microalgal species to accumulate

neutral lipids, mainly as triacylglycerols (TAG), which can be transesterified to produce biodiesel is, without doubt, the aspect that has attracted higher attention (Chen et al. 2017; Li-Beisson et al. 2019). However, algal biomass has also been studied for the production of biogas, bio-oil, biohydrogen and bioethanol (Constantino et al. 2021). In fact, some microalgae species are able to accumulate high concentrations of not cellulosic carbohydrates which can be an interesting alternative source for the production of bioethanol and other biofuels (Sanchez Rizza et al. 2017).

The need to replace fossil fuels in the transport sector is a global

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<https://doi.org/10.1016/j.biortech.2022.127035>

Received 3 February 2022; Received in revised form 15 March 2022; Accepted 16 March 2022

Available online 18 March 2022

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priority. However, this replacement has to be accompanied by the progressive substitution of usual procedures for the production of fine chemicals, traditionally based in the petrochemical synthesis, by alternative sustainable platforms. One of the novel sustainable materials that has been proposed as a promising precursor for the synthesis of valuable chemical building blocks is 5'-hydroxymethylfurfural (5'-HMF), which can be obtained by acid dehydration of carbohydrates (Rosatella et al. 2011). Levulinic acid (LA), formed by rehydration of 5'-HMF, is also a platform chemical for the production of biofuel, paint additives, green solvents or pharmaceuticals (Pileidis and Titirici 2016; Rihko-Struckmann et al. 2020). 5'-HMF, LA and other furanic derivatives can therefore be transformed into a variety of commercially interesting chemical precursors, connecting bio-based platforms with the chemical synthesis (Kohli et al. 2019; Yu and Tsang 2017).

The best 5'-HMF yields are obtained using fructose as substrate, however it is desirable to optimize the production of 5'-HMF using cheap raw substrates or agro-industrial wastes as starting materials. Extensive research has been devoted to investigate the production of 5'-HMF or other furanic compounds from ligno-cellulosic material (García-Domínguez et al. 2015), food waste (Esteban and Ladero 2018) and other waste biomass (Menegazzo et al. 2018). However, the use of algal biomass as substrate for the production of furanic compounds has been poorly explored (Amoah et al. 2019; Jeong and Kim 2021b; Wang et al. 2016).

Many microalgae species respond to nutrient starvation or other stressing conditions, which limit the assimilation of nutrients, by accumulating the excess of carbon as storage reserves in the form of neutral lipids, such as triacylglycerides (TAG), or high molecular weight-carbohydrates, such as starch. Both triacylglycerols and starch are hydrocarbon compounds which do not have nitrogen, sulfur or phosphate in their molecules. Some higher plants also accumulate starch as carbon and energy storages, especially in the seeds. This is the case of cereals, which have been the main source of carbohydrates for the fermentative production of first generation bioethanol. However, using microalgal biomass has many advantages over crop cultures. They grow about 10 times faster than higher plants, and can be grown in arid lands with saline or waste-water without competing for cultivable land with edible species (Chen et al. 2017; Chisti, 2007). Microalgae also present advantages in comparison with agro-industrial wastes, because they can reach very high concentrations of carbohydrates, they have a simple cellular structure and lack lignin (Khan et al. 2018).

Moreover, the production of starch can be easily combined with the production of other high added-value compounds, such as carotenoids, which accumulation can also be induced by stressing conditions in many species. The simultaneous production of high value co-products, such as pigments, vitamins or anti-oxidants, has been proposed as a solution to get the economic feasible production of microalgal biofuels conforming to the current concept of a microalgal-based biorefinery (Schüler et al. 2017). Carotenoids are extensively used as dietary supplements and food colorants in aquaculture or poultry feed and have an increasing demand in human nutrition and cosmetics. Although most of the commercialized carotenoids are synthetic, consumer's preferences for natural additives have strengthened the market of the natural carotenoids that was valued at \$1.24 billion in 2016 and is expected to grow until reaching a global value \$1.9 billion in 2028, especially for lutein, *b*-carotene, lycopene and astaxanthin, being microalgae one of the best source of natural carotenoids. (Gong and Bassi, 2016).

In this paper a collection of robust fast-growing green microalgae was screened for their capacity to accumulate starch. The content of commercially appreciated lipophilic coproducts in the selected microalgae was assessed. Additionally, the best conditions to produce 5'-HMF and LA by acidic hydrolysis of the algal remnant biomass obtained after the extraction of the pigments, were studied using a Central Composite Design (CCD).

## 2. Materials and methods

### 2.1. Strains and culture conditions

*Chlamydomonas reinhardtii* wild type strain 704 (cw15, arg7, mt<sup>+</sup>), which was kindly provided by Dr. Emilio Fernández from the University of Córdoba; *Chlamydomonas reinhardtii* ACS strain, in which the chloroplastic Acetyl-CoA synthetase encoding gene has been overexpressed by genetic manipulation as previously described (Rengel et al. 2018), and *Chlorella sorokiniana* 211–32, which was retrieved from the algal collection of the Institute of Plant Biochemistry and Photosynthesis (IBVF, Seville), were cultured photomixotrophically in TAP (Tris-Acetate-Phosphate) medium at 70 rpm. *Dunaliella salina* CCAP 19/18 was cultured in mineral liquid medium (Johnson et al. 1968) and bubbled with air containing 3% (v/v) CO<sub>2</sub>. *Tetraselmis chuii* (strain 8–6, from IBVF, Seville) and *Picochlorum* sp., which was isolated from the marshlands of the Odiel River (De la Vega et al. 2011), were cultured in F/2 medium (Guillard 1975). All cultures were maintained in a thermostated chamber, at 25 °C illuminated with cool white light from fluorescent lamps at a set intensity of 100 μE m<sup>-2</sup>s<sup>-1</sup>. For induction of starch accumulation, cells were grown until the middle of their exponential phase and harvested by centrifugation, washed and cultured in N-free medium for 3–4 days.

### 2.2. Extraction and quantification of total chlorophylls and carotenoids

For the extraction of pigments, 50 mg of lyophilized microalgal biomass were successively treated with 2x3 mL of 80% ethanol and heated at 70 °C for 15 min. Cells were pelleted by centrifugation for 10 min at 4400 rpm, and the supernatant was collected for determination of total chlorophylls and carotenoids, that were chromatographically separated and analyzed in a Merck Hitachi HPLC-DAD, using a RP-18 column and two mobile phases: 100% ethyl acetate (solvent A) and 9:1 acetonitrile:water (solvent B). The program consisted on a gradient of 0–60% A (0–16 min), 60% A (16–30 min) and 100% A (30–35 min). Flow rate was set on 1 mL min<sup>-1</sup>, and the injected volume of the samples was 100 μL. Pigments were identified and quantified by comparison with commercial standards purchased from Sigma-Aldrich (Munich, Germany) or DHI Lab (Hørsholm, Denmark).

### 2.3. Determination of dry weight

For dry weight determination, 30 mL of microalgal cultures were filtered through GF/F Whatman glass-fiber filters, which were previously tared, and washed with 0.5 M ammonium formate. The filters were introduced in a drying oven overnight at 100 °C before being weighed. Measurements were performed in triplicate.

### 2.4. Extraction and fractionation of fatty acids

For extraction of total lipids (TL), the protocol described by Abida et al. 2015 was followed with some modifications. 5–10 mg of lyophilized biomass were dissolved in 4 mL of boiling ethanol for 5 min and subsequently extracted with 2 mL of methanol and 8 mL of chloroform, being stirred at room temperature for 1 h. Samples were filtered, evaporated under N<sub>2</sub> gas and resuspended in 100 μL of chloroform. An aliquot of 50 μL of the lipidic extract was then taken for fractionation on Discovery® DSC-Si SPE Cartridges (Supelco, USA). Neutral lipids (NL) were eluted in chloroform:acetic acid 100:1 (v/v). TL and NL fractions were methylated with 1 mL of a methylation mix consisting of MeOH:toluene:H<sub>2</sub>SO<sub>4</sub> 70:28:2 and incubated at 85 °C for 90 min. Samples were cooled before adding 1 mL of heptane and 1 mL of NaCl 1% to induce biphasic formation. Tubes were vortexed and centrifuged 5 min at 1000 rpm. Fatty acid methyl esters (FAMES) were recovered in the heptane phase, evaporated with nitrogen flux and resuspended in 50 μL of heptane + 0.01% (w/v) butylated hydroxytoluene (BHT) to avoid oxidation.

## 2.5. GC-FID quantification of fatty acid methyl esters

FAMES were determined by an Agilent 7890A gas chromatograph, using an Agilent DB-23 column (30 m × 0.25 mm × 0.25 μm) coupled with a flame ionization detector. The volume of injection was 1 μL, and the oven program consisted of an initial hold at 150 °C for 2 min and then a temperature ramp up to 250 °C at a rate of 10 °C min<sup>-1</sup>. The temperature was then held for 5 min for a total time of 17 min. C17:0 and C23:0 were used as internal standards.

## 2.6. Acidic hydrolysis of microalgal biomass

Samples consisting of 30–35 mg of depigmented algal biomass were incubated with the hydrolysis reaction mix, which consisted of different concentrations of sulfuric acid (volume range 0–4%) and 3:1 DMSO: NaCl-saturated water (volume range 0–75%) up to a final volume of 1 mL, as detailed in the experimental design (Table 1). Samples with the corresponding reaction mix were placed in glass vials and sealed after purging with N<sub>2</sub> gas. Reactions were heated and stirred at 150 rpm at the indicated temperatures and times. Hydrolysates were cooled at room temperature and filtered with nylon 0.45 μm membranes prior to HPLC analysis.

## 2.7. HPLC-RI analysis of carbohydrates and furfurals

The concentration of the main hydrolysis products (glucose, LA and 5'-HMF) in the reaction mix was determined by HPLC. The Chromatographic determination was performed using an Agilent 1100 HPLC equipped with refractive index detector and an ion-exchange resin BioRad Aminex HPX-87H column under the following conditions: the column temperature was set at 50 °C, and mobile phase consisted on 0.005 mol L<sup>-1</sup> of sulfuric acid at a flow rate of 0.6 mL min<sup>-1</sup>; The volume injected was 5 μL.

## 2.8. Determination of total carbohydrates and starch

The content of total carbohydrates was determined as described in DuBois et al. 1956, using phenol-sulfuric acid reagent and glucose as standard for a calibration curve. Starch was determined by an α-amylase/amyloglucosidase enzymatic method, using the K-TSTA kit from Megazyme (Ireland) and 5 mL of algal culture as previously described (Rengel et al. 2018). The intracellular content of free glucose was measured using the starch kit.

## 2.9. Thermogravimetric assay (TGA).

15 mg of lyophilized algal biomass obtained from standard and 72 h-N-deprived cultures were heated from 0 °C to 800 °C at a rate of 20 °C min<sup>-1</sup> in a TGA/DSC2 thermogravimetric analyzer (Mettler-Toledo, Greifensee, Switzerland). Weight loss was monitored throughout the analysis, and first derivatives of the loss curve were calculated for the detected thermal events.

## 2.10. Elemental nitrogen analysis and protein determination

To quantify the nitrogen content of *C. reinhardtii* grown in N-replete and N-deprived conditions, the samples were lyophilized and analyzed in a LECO TRUSPEC CHNS MICRO. The protein content was estimated

using a coefficient of 4.78 (Lourenço et al. 2004). All measures were done in triplicate.

## 2.11. Central Composite Design for optimization of the acidic hydrolysis conditions

An extended Central Composite Factorial Design was chosen to optimize the hydrolysis conditions for the starch-enriched depigmented algal biomass with a low number of reaction tests. The ranges for the four independent variables: temperature (T), reaction time (ti), percentage of sulfuric acid (Sa) and the percentage of dimethylsulfoxide (DMSO) in the final reaction mixture, were selected on the basis of the optimal intervals used for the processing of similar biomass (Amoah et al. 2019; Pulidindi and Kim 2018; Rosatella et al. 2011) and considered at five levels (three main and two extended levels). In this way, the total number of experiments required for the optimization was reduced to 30, calculated as previously reported (Palma et al. 2021). To facilitate the direct comparison of the selected independent variables (operating parameters), their values have been normalized (-1 to +1) using Eq. (1).

$$X_n = \frac{X - \bar{X}}{(X_{\max} - X_{\min})/2} \quad (1)$$

In this equation, X<sub>n</sub> is the normalized value for each independent variable; X is the experimental value found for each variable;  $\bar{X}$  is the mean of the experimental values; and X<sub>max</sub> and X<sub>min</sub> are the maximum and minimum values, respectively.

A second-degree polynomial equation (Eq. (2)) has been used to correlate the independent parameters (T, ti, Sa, DMSO), denoted as X, with the concentration of the hydrolysis products (Glucose, LA, 5'-HMF), denoted as Y.

$$Y = a_0 + \sum_{i=1}^n b_i X_{mi} + \sum_{i=1}^n c_i X_{ni}^2 + \sum_{i=1}^n \sum_{j=1}^n d_{ij} X_{ni} X_{nj} (i < j) \quad (2)$$

The coefficients for the equations were obtained using the software Statistica 10.0 (StatSoft, Inc., Tulsa, IK, USA) and the data experimentally acquired. The values for the independent variables showed in each term of the equation are those determined as statistically significant. Therefore, Student's *t*-test was applied to exclude coefficients surpassing a significance level of 0.05.

For each value of an independent variable (experimental condition), the influence of the other variables on the production of the considered compound can vary; for this reason the average change in the dependent variable was determined using Eq. (3), where [(X<sub>ni</sub>)<sub>max</sub> - (X<sub>ni</sub>)<sub>min</sub>], represents the value range of the independent variables and {Z(X<sub>ni</sub>)<sub>max</sub> - Z[(X<sub>ni</sub>)<sub>min</sub>]} represents the value range of the dependent variables.

$$\frac{\int_{(X_{ni})_{\min}}^{(X_{ni})_{\max}} [Z(X_{ni})_{\max} - Z(X_{ni})_{\min}] dX_{ni}}{[(X_{ni})_{\max} - (X_{ni})_{\min}]} \quad (3)$$

The difference between the expression {Z(X<sub>ni</sub>)<sub>max</sub>]<sub>max</sub> - Z[(X<sub>ni</sub>)<sub>min</sub>]<sub>min</sub>}, which establishes the maximum possible difference between the maximum and the minimum values of the considered compound, and the Eq. (3) (DZi) is shown in Eq. (4). This difference can be used to quantify the relative influence of the independent variables on each dependent variable.

**Table 1**

**Comparison of the main components of *C. reinhardtii* cells cultured in nitrogen replete medium (+N) or subjected to nitrogen starvation for 72 h (-N). All results are expressed in mg g<sup>-1</sup> DW. TC, Total carbohydrates; TL, Total lipids; NL, Neutral lipids; TP, Total proteins. Errors indicate standard deviation.**

	TC	Starch	TL	NL	TP	Carotenoids	Chlorophylls
+N	231 ± 3.6	46.8 ± 4.0	167.5 ± 5.4	58.8 ± 0.5	393.8 ± 2.2	6.7 ± 0.1	46.6 ± 3.4
-N	438.9 ± 4.1	356.7 ± 8.2	128.4 ± 5.8	102.9 ± 2.1	135.9 ± 0.6	5.9 ± 0.1	23.6 ± 1.2

$$DZ_i = \{ [Z(X_{ni})_{max}]_{max} - [Z(X_{ni})_{min}]_{min} \} - \frac{\int_{(X_{ni})_{min}}^{(X_{ni})_{max}} [Z(X_{ni})_{max} - Z(X_{ni})_{min}] dX_{ni}}{[(X_{ni})_{max} - (X_{ni})_{min}]} \quad (4)$$

### 3. Results and discussion

#### 3.1. Screening microalgae for their ability to accumulate starch under nitrogen starvation

Six green microalgal strains of different classes were chosen to be studied for their ability to accumulate starch. The chlorophycean *C. reinhardtii* (704 and ACS strains) and *D. salina*; *T. chuii* (class Prasinophyceae), and *C. sorokiniana* and *Picochlorum* sp., from the Trebouxiophyceae class, were cultured in their corresponding nutrient-replete media as described in section 2.1 until the exponential phase of growth. The microalgae were then collected by centrifugation, washed and transferred to nitrogen-free medium. The transgenic strain *C. reinhardtii* ACS, in which the Acetyl-CoA synthetase, the main enzyme involved in the assimilation of acetate, is overexpressed has also been included in the screening. Starch accumulation in the nitrogen-starved cultures was followed during three days (Fig. 1).

The basal starch content presented high variability among the microalga species studied. It was 43 mg g<sup>-1</sup> and 45 mg g<sup>-1</sup> of DW in the case of *C. sorokiniana* and *Picochlorum* sp., respectively, 60 and 63 mg g<sup>-1</sup> for *C. reinhardtii* 704 strain and *Dunaliella salina*, respectively, and 110 mg g<sup>-1</sup> in the case of *C. reinhardtii* ACS and *T. chuii*, which represents more than 10% of their dry weight.

In all the studied strains, transference to N-deprived medium induced the accumulation of starch. This is typical of chlorophytes, as well as of land plants, which are able to produce starch as carbon storage polysaccharide under unfavourable nutrient conditions. However, the ability to accumulate starch reserves is highly dependent of the species and of the culture conditions. The highest amounts were obtained for the two strains of *C. reinhardtii* which reached starch contents around 35% of their dry weight after 3 days of N starvation, followed by *Tetraselmis*, which accumulated 30% of starch after the same time of N deprivation. *Dunaliella salina* showed intermediate values, with a 15% of starch content after 3 days of induction. The robust, fast growing, *Chlorella sorokiniana* also reached starch values of about 15% after two days without N, while the other Trebouxiophyceae, *Picochlorum* sp, reached a

starch content of 7% after 24 h of induction, value which was reduced to 5% of its dry weight at 3 days of N-starvation.

Interestingly, the transgenic strain of *Chlamydomonas* (ACS), despite having higher basal starch content when cultured in replete medium, reached a starch content similar to that of the wild strain, after 3 days of N starvation. This strain has previously shown to have higher ability to accumulate TAG under nitrogen deprivation (Rengel et al. 2018), however it does not seem to have a superior ability than the wild type strain to accumulate starch under nitrogen limitation. *Chlamydomonas* 704, which is a fast growing strain with a well-known metabolism, was therefore selected for further analysis.

#### 3.2. Characterization of *Chlamydomonas reinhardtii* under N-starved conditions

The biochemical composition of the chosen microalga was studied under N starvation, focusing mainly on its lipidic and carotenoid profiles (Table 1). We observed that, in addition to starch, important quantities of neutral lipids are stored in N deprived *C. reinhardtii* cells. In these conditions, total carbohydrates showed a 2-fold rise, whilst starch content experimented a 7.6-fold increase, from 46.8 to 356.7 mg g<sup>-1</sup> DW, coming to suppose more than 80% of total carbohydrates. This means that carbohydrates other than starch, which are mainly part of glycoproteins and glycolipids, decrease from 184 to 82 mg g<sup>-1</sup>. The total content of lipids experimented a decrease of a 23.4% under nitrogen deprivation, while the content in neutral lipids was practically duplicated. During nitrogen starvation, the cell division is stopped in the microalga *Chlamydomonas reinhardtii*. The cells continue growing and increasing their cell volume, but the number of cells is maintained constant. This growth is accompanied by an important turnover of membrane lipids to boost the accumulation of neutral lipids (TAG), which are packed into oil bodies, while polar lipids, such as monogalactosyldiacylglycerol (MGDG), phosphatidylglycerol (PG), sulfoquinovosyldiacylglycerol (SQDG) or phosphatidylethanolamine (PE) suffer an important reduction, as has been previously demonstrated (Rengel et al. 2018). The major nitrogenous component, the proteins, experimented a strong decrease of nearly 3-fold. Regarding the pigments, carotenoids content gently decreased (12%), whereas chlorophylls dramatically declined by half. This biochemical profile is consistent with the response of the microalgae to nitrogen deprivation. When the nitrogen source is

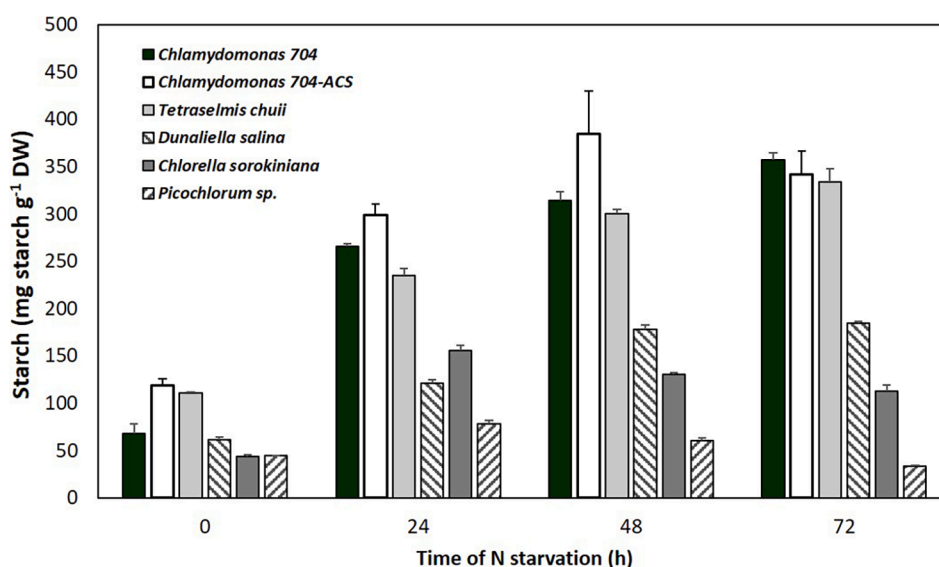


Fig. 1. Time-course evolution of starch accumulation in cultures of different chlorophyte microalgae cultured under nitrogen deprivation. Cells were grown until mid-log phase and transferred to N-depleted media. After 72 h of incubation, cells were pelleted and freeze-dried. Starch was enzymatically determined. Error bars represent standard deviation (n = 3).

completely exhausted, carbon relocation is triggered, inducing the biosynthesis of storage compounds, mainly starch and TAG (Li-Beisson et al. 2019, Morales-Sánchez et al. 2020). Chlorophyll degradation is accompanied by induction of the biosynthesis of some carotenoids, such as zeaxanthin and antheraxanthin, which have been reported to contribute to cell photoprotection and to avoid photooxidative damage under stress conditions (Juergens et al. 2015).

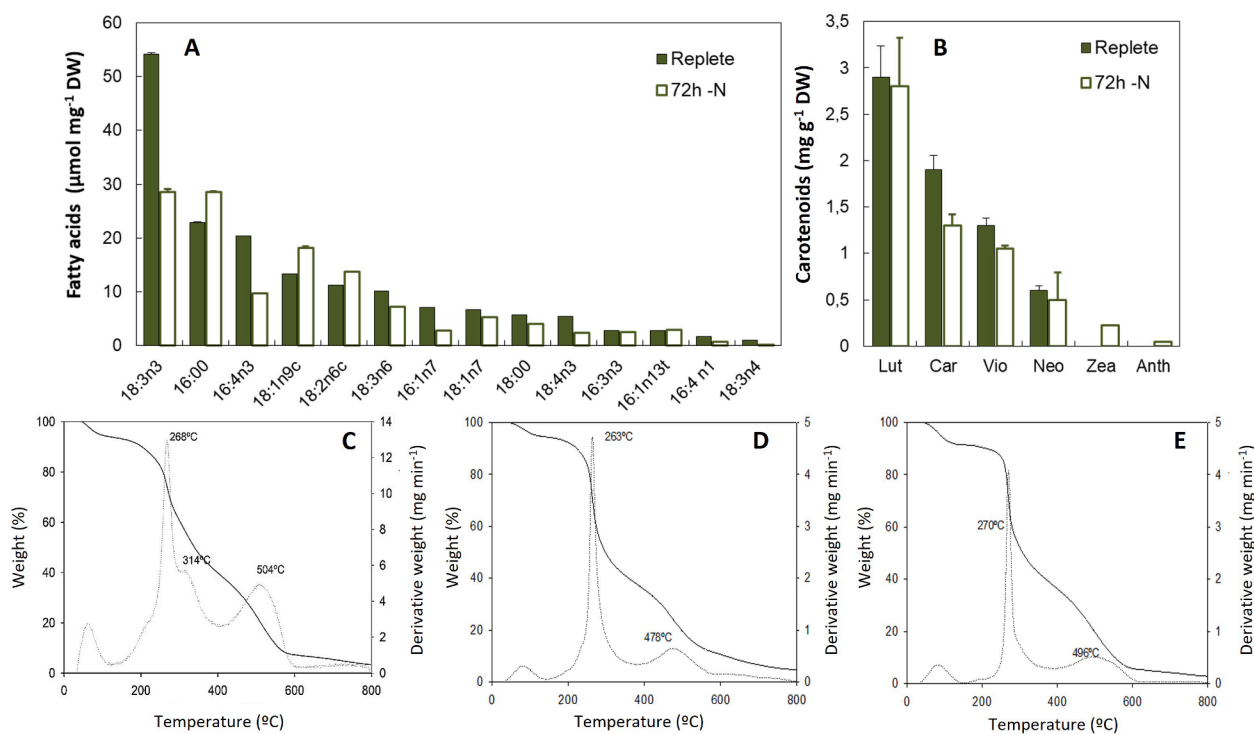
A more detailed study on the fatty acid (Fig. 2A) and the carotenoid composition (Fig. 2B) of *Chlamydomonas* cells grown in standard and N-deprived conditions revealed that the lack of nitrogen source causes some modifications in these profiles. The content in palmitic (C16:0) and oleic (C18:1) acids, fatty acids typical of neutral storage lipids, is increased in N starved cells, while polyunsaturated fatty acids, such as 18:3n3, 16:4n3 or 18:4n3, which are commonly found in membrane lipids, suffer a strong decline under nitrogen deprivation. The chlorophyll content experimented a strong decrease after transference of the cultures to N-deprived medium, reaching the half of the value in N-replete medium. With respect to carotenoids, lutein was the only pigment that maintained its intracellular level in stress conditions, with a value of 2.9 mg g<sup>-1</sup> DW. Violaxanthin and neoxanthin experimented a small decrease from 1.3 to 1.05 mg g<sup>-1</sup> DW and from 0.6 to 0.5 mg g<sup>-1</sup> DW, respectively. The content of β-carotene, which is part of the core complexes of photosynthetic systems bounded to the chlorophyll, was strongly reduced when cells were transferred to depleted medium, from 1.9 to 1.31 mg g<sup>-1</sup> DW. On the other hand, zeaxanthin and antheraxanthin, which were undetected in N-replete cultures, reached values of 0.22 and 0.05 mg g<sup>-1</sup> DW in N-starved medium, respectively.

Additionally, the selected microalga, cultured with and without nitrogen, and the remnant biomass obtained after the extraction of pigments were studied by thermogravimetric analysis (TGA) (Fig. 2C-E). The lyophilized samples were heated from 0 to 800 °C and the variations experimented in the algal mass were registered in thermograms, which represent the weight loss (%) with the temperature (TG curve), and its corresponding derivative thermogravimetric (DTG) plot. Three main

peaks, which correspond to the thermal decomposition of the different components of the complex mixture of biomolecules in the microalgal cells, can be observed at the temperature intervals of 90–110 °C; 260–314 °C and 478–496 °C.

The first decline in weight occurs between 50 and 150 °C, corresponding to the loss of free and loosely bound water molecules by evaporation and represents less than 10% of the total biomass weight, in agreement with previous reports (Batista et al. 2013). The major weight loss, can be observed in the range 263–314 °C. In the nitrogen replete biomass (Fig. 2C), this peak is observed at 268 °C with an additional shoulder at 314 °C, which is not detected in the DTG curves of the N-starved cultures and depigmented cells (Fig. 2D and 2E). In these samples, the major peak appears at 263 °C and 270 °C, respectively. This thermal event is generally associated with the decomposition of proteins, lipids and carbohydrates (Liu et al. 2015; Pane et al. 2001). The disappearance of the shoulder at 314 °C in N deprived cells can be due to the decomposition of part of the proteins, which content is drastically reduced in N starved algal cells, or modifications in the starch composition, which is enriched in the unbranched amylose fraction under N starvation, as it has been reported that nitrogen starvation in *Chlamydomonas* triggers decrease in amylopectin fraction, from 95% to 70% (Libessart et al. 1995). Curiously, the strong increase in starch in the N starved cells is not reflected in the TGA curves, probably because it is covered up by the reduction of proteins with similar temperature range of thermal decomposition. Finally, a third weight loss at temperatures higher than 450 °C indicates the presence of additional heat resistant compounds. This last peak is larger in N replete biomass (Fig. 2C) and could be related to the decomposition of proteins, which have been reported to contribute to the TG-DTG curves also in this range of temperature. On the other hand, the decrease observed in the decomposition rate of the depigmented biomass (Fig. 2E) could represent a lower complexity and an easier bond cleavage due to carotenoid and chlorophyll extraction prior to hydrolysis.

Similar thermograms have been observed in other microalgae, such



**Fig. 2.** Characterization of *C. reinhardtii* biomass cultured in nutrient replete and N-deprived growth media for 72 h. Fatty acid (A), carotenoid profile (B) and thermogravimetric analysis of replete (C) and nitrogen starved algal biomass before (D) and after (E) pigment extraction. Antheraxanthin (Anth); Lutein (Lut), Neoxanthin (Neo); Violaxanthin (Vio); Zeaxanthin (Zea); β-carotene (Car). Error bars represent standard deviation (n = 3). Thermograms show weight loss (solid line) and its first derivative (dash line) curves.

as *Tetraselmis subcordiformis* and *Arthrospira platensis*, that tend to accumulate carbohydrates and lipids when transferred to N-depleted cultures (Liu et al. 2015). Samide and Tutunaru (2017) tested the thermal stability of chlorophyllic extracts from plant and seaweed mixtures and obtained TG curves that showed an interval between 330 and 500 °C for the main decomposition stage.

This TGA analysis confirms that algal biomass is a complex mix of heterogeneous components and allows to corroborate that the gross composition of N starved cells before and after the extraction of pigments is not significantly different. In addition, the biochemical characterization of the algal biomass cultured with and without nitrogen, confirms that nitrogen deprivation can induce the accumulation of starch as potential substrate for the synthesis of 5'-HMF and LA and allows the simultaneous production of lutein or zeaxanthin, highly demanded for their antioxidant properties, which might improve the yield of the overall process.

### 3.3. Processing the depigmented algal biomass: Experimental design for acidic hydrolysis

The chlorophyte microalga *C. reinhardtii*, with a great ability to accumulate high quantities of starch under N starvation, was selected to study the simultaneous production of carotenoids and a starch-enriched residue, which can be submitted to acidic hydrolysis for the production of 5'-HMF or LA. Around 50 mg of lyophilized biomass from a nitrogen-starved *C. reinhardtii* culture was treated with ethanol for the extraction of pigments (carotenoids and chlorophylls) as detailed in section 2.2. The starch content of the pigment-less biomass was characterized prior to acidic hydrolysis, yielding mean values around 17 mg, which represents about 57% of the depigmented biomass.

The starch-enriched residue could be hydrolyzed into glucose and converted into 5'-HMF and/or LA by further acidic hydrolysis. To optimize the conditions for starch hydrolysis and explore the main generated

products (Glucose, 5'-HMF and LA), an extended Central Composite Factorial Design was performed as detailed in 2.11 section. The influence on the process of four independent operational variables: temperature (T), reaction time (ti); Sulfuric Acid concentration (Sa) and Dimethylsulfoxide concentration (DMSO) was analyzed.

The values of the independent variables used in this study ranged between 140 and 180 °C for the temperature (T), between 0 and 120 min for the reaction time (ti), between 0 and 4% for the concentration sulfuric acid (Ac) and between 0 and 75% for the concentration of DMSO (DMSO) (Table 2). A total of 30 hydrolysis reactions were performed at the experimental conditions detailed in Table 2. After cooling and filtering, the composition of the reaction mixtures was analyzed by HPLC-RI as detailed in 2.7. The concentrations of the main hydrolysis products (Glu, 5'-HMF, LA) related to the depigmented biomass, obtained for each experimental condition, are shown in Table 2.

Results showed high variability in the amounts of glucose, 5'-HMF and LA obtained for the different conditions tested through the design. The maximum concentration of glucose, 387.2 mg g<sup>-1</sup> DW, was obtained at 170 °C, 90 min, 3% of sulfuric acid and 20% of DMSO (Experiment n° 8). The maximum LA amount (70.1 mg g<sup>-1</sup> DW) was achieved in Experiment n° 14, at 150 °C, 90 min, 3% of acid and 20% of DMSO. The highest concentration of 5'-HMF was observed in Experiment n° 21 at 140 °C, 60 min, 2% of sulfuric acid and 40% of DMSO, with a value of 48.9 mg g<sup>-1</sup> DW. The absence of sulfuric acid in the reaction mixture (Experiment n° 25), produced small amounts of glucose and no LA or 5'-HMF, confirming the essential role of the acidic conditions to catalyze the cleavage of starch bonds and to further produce the compounds of interest. In addition to glucose, LA and 5'-HMF, low quantities of other sugars, such as arabinose (below 1 mg g<sup>-1</sup>) and maltose (up to a concentration of 52,6 mg g<sup>-1</sup>) were detected in the reactions with low overall productivity, indicating suboptimal hydrolysis of the carbohydrates Formic acid, which is besides levulinic acid, a rehydration product of 5'-HMF, was also obtained in the reactions in

**Table 2**

**Central Composite Design and concentration of the main hydrolysis products obtained under the selected experimental design.** Parameters: Temperature (T); Time (ti); Sulfuric Acid (Sa) and Dimethylsulfoxide (DMSO). Results are related to depigmented biomass.

Exp	Normalized Variables				Variable values				Experimental values		
	T	ti	Sa	DMSO	T (°C)	ti (min)	Sa (%)	DMSO (%)	Glucose (mg g <sup>-1</sup> )	LA (mg g <sup>-1</sup> )	5'-HMF (mg g <sup>-1</sup> )
1	-1	-1	-1	1	150	30	1	60	10.56	0.0	16.3
2	-1	-1	1	-1	150	30	3	20	21.3	22.9	30.3
3	-1	1	-1	-1	150	90	1	60	119.0	9.9	40.8
4	-1	1	1	1	150	90	3	20	113.4	67.7	23.4
5	1	-1	-1	-1	170	30	1	20	88.6	10.8	10.2
6	1	-1	1	1	170	30	3	60	94.6	0.8	21.2
7	1	1	-1	1	170	90	1	60	62.4	0.5	4.4
8	1	1	1	-1	170	90	3	20	387.2	0.7	0.8
9	0	0	0	0	160	60	2	40	127.1	0.8	0.0
10	0	0	0	0	160	60	2	40	99.4	0.5	0.0
11	-1	-1	-1	-1	150	30	1	20	9.1	4.9	24.7
12	-1	-1	1	1	150	30	3	60	80.2	12.1	37.2
13	-1	1	-1	1	150	90	1	60	104.3	3.9	38.0
14	-1	1	1	-1	150	90	3	20	97.1	70.1	20.3
15	1	-1	-1	1	170	30	1	60	6.8	10.5	0.0
16	1	-1	1	-1	170	30	3	20	200.6	0.6	9.6
17	1	1	-1	-1	170	90	1	20	224.3	0.7	3.7
18	1	1	1	1	170	90	3	60	225.2	1.8	1.3
19	0	0	0	0	160	60	2	40	127.1	1.1	0.0
20	0	0	0	0	160	60	2	40	126.9	1.0	0.0
21	-2	0	0	0	140	60	2	40	22.1	31.4	48.9
22	2	0	0	0	180	60	2	40	291.2	3.7	1.6
23	0	-2	0	0	160	0	2	40	3.58	0.6	0
24	0	2	0	0	160	120	2	40	271.2	2.0	9.3
25	0	0	-2	0	160	60	0	40	4.68	0.4	0
26	0	0	2	0	160	60	4	40	263.2	30.8	3.0
27	0	0	0	-2	160	60	2	0	275.6	0.6	9.5
28	0	0	0	2	160	60	2	75	66.3	0.6	11.8
29	0	0	0	0	160	60	2	40	89.4	0.6	0.0
30	0	0	0	0	160	60	2	40	158.4	0.8	0.0

which LA was produced, at amounts lower than 3 mg g<sup>-1</sup>. The low values of formic acid detected can be due to its decomposition into gaseous species, as has been previously reported (Zhang et al., 2015). Other compounds, such as insoluble humins, or other polymerization products which are usually obtained by self-condensation of furfurals have not been determined. More detailed explanations for these results from are discussed in section 3.5.

In order to statistically determine the relationship between the different operational conditions, experimental data were subjected to multiple regression and modelling as described in section 2.11. The conditions used to carry out the acid hydrolysis have demonstrated to be critical for the composition of the obtained products for either algal (Cuevas-Castillo et al. 2020) or other waste biomass (Menegazzo et al. 2018).

The acidic hydrolysis of different algae species such as *Gracilaria verrucosa*. (Jeong et al. 2015) or *Chlorella sorokiniana* (Amoah et al. 2019), to obtain sugars, levulinic acid and 5'-HMF has been previously studied (Amoah et al. 2019; Jeong et al. 2015). Recently, the use of depigmented *Dunaliella* biomass as substrate for the hydrolytic process (Rihko-Struckmann et al. 2020) has been studied for the production of 5'-HMF. The addition of different catalysts, organic solvents or inorganic salts to the reaction mixture have been shown to improve 5'-HMF yield in algal hydrolysates (Rihko-Struckmann et al. 2020; Rosatella et al. 2011; Yu and Tsang 2017). These authors demonstrated that an organic phase benefits the production of 5'-HMF and avoids collateral reactions that lead to its rehydration. The utilization of biphasic water/organic systems has also shown to minimize the production of humins and other undesirable polymerization products. On the other hand, the presence of water in the reaction mixture has been proven to induce the synthesis of LA (Kim et al. 2017; Mukherjee et al. 2015), as it is itself a rehydration product derived from 5'-HMF. Consequently, as both compounds are of major interest to be used as platform chemicals, the hydrolysis reaction can be optimized according to the desired final product.

### 3.4. Statistical interdependence among selected extracted compounds

The experimental data obtained were used to determine the coefficients for the polynomial equation (Eq. (2)) using the procedure described in section 2.11. The equations found for each of the selected compounds (dependent variable) relative to the normalized independent variables with the set of constant coefficients obtained for each

compound are shown in Fig. 3A. To verify the accuracy of the obtained equations, R<sup>2</sup> and Snedecořs F values have been calculated. The R<sup>2</sup> values obtained for all the equations were higher than 0.90 and F-values were higher than 5, indicating an adequate statistical correlation between experimental and modelled data. In these equations, a positive value in the independent variable means an increase in response. Conversely, a negative value indicates a decrease in response at higher levels of the variable.

To simplify the graphical representation of these equations it was necessary to choose three of the four experimental conditions studied. To select the most significant independent variables, the relative statistical influence of the independent variables (T, ti, Sa and DMSO) on each dependent variable (glucose, 5'-HMF and LA), DZi values were calculated using Eq. (3) and Eq. (4), as indicated in section 2.11. The plots showing the variation of the influence of each variable against the normalized values from -1 to +1 for each dependent variable were constructed and are shown in Fig. 3B. These values allow to weight the influence, as percentages, of each independent variable (experimental conditions) on the variation of each dependent variable (compound of interest and, consequently, estimate their relative influence.

In this way, under each value of an independent variable, the difference between the maximum and minimum values of the studied compound is related to the influence of the independent variables. On the contrary, if the experimental condition plotted had no effect on the synthesis of the compound, then the aforementioned difference should coincide with the height of the plotted rectangle, having the range of values of the independent variable, [(X<sub>ni</sub>)<sub>max</sub> - (X<sub>ni</sub>)<sub>min</sub>], and the maximum possible difference between the maximum and minimum values of the considered compound, {Z[(X<sub>ni</sub>)<sub>max</sub>]<sub>max</sub> - Z[(X<sub>ni</sub>)<sub>min</sub>]<sub>min</sub>}.

The percentages shown in Fig. 3B represent the medium relative influence (DZi) of each independent variable tested, namely temperature, time, sulfuric acid and DMSO on the productivity of glucose, levulinic acid, 5'-HMF and on the total sum of these substances.

From the quantification of the influence of the variables studied on the measured compounds, it can be deduced that: temperature is the most important independent variable for the evolution of the compounds under study; the glucose concentration depends mainly on the process temperature, followed by time and DMSO; levulinic acid is mainly dependent on temperature, although, in this case, followed by the concentration of sulfuric acid and the process time. Similarly, the evolution of 5'-HMF concentration depends mainly on temperature,

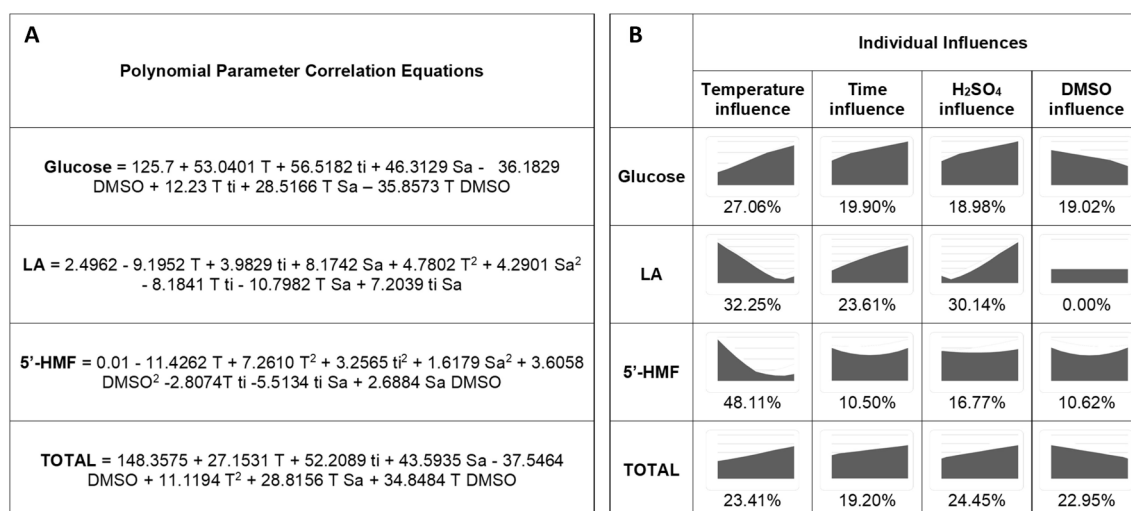


Fig. 3. Equations for each of the studied compounds as a function of the operational variables and relative influence of the different operational conditions. (A) Polynomial equations that predict the concentrations in mg g<sup>-1</sup> DW of each studied compound (Glucose, LA and 5'-HMF) as a function of the independent variables: temperature (T), time (ti), sulfuric acid concentration (Sa) and Dimethylsulfoxide concentration. R<sup>2</sup> and Snedecořs F-value threshold values for the coefficients were set at 0.85 and 5, respectively. (B) relative influence of the experimental condition for each compound, calculated using equations Eq. (3) and Eq. (4). The mean influence (DZi value), in percentage, of each independent variable on the dependent variables is also shown.

with sulfuric acid and DMSO concentration being the second and third most important parameters.

### 3.5. Response surfaces for dependent variables

To visualize the equations of Fig. 3A and represent the influence of each experimental condition (independent variable) on the productivity of the chosen hydrolysis products (dependent variables), a series of response surface plots were constructed for each compound (Fig. 4). For simplicity, only the three independent variables with the highest statistical influence, determined considering the previous analysis, have been plotted. In Fig. 4, the response surface plots over the range of normalized operational variables are displayed. From these surface plots, the optimal values of all dependent variables can be deduced. In addition, a schematic design of the overall process, including the optimal predicted operational conditions is shown in Fig. 5.

Temperature was the most influential variable for all products, being more important for glucose production (Fig. 4A) and less contributive to LA (Fig. 4B) and 5'-HMF (Fig. 4C), specially at higher values. This could be due to a higher rate of the hydrolysis of starch granules at higher temperatures, to yield glucose, which can be easier dehydrated to levulinic and 5'-hydroxymethylfurfural once their substrate is available in the reaction medium. Moreover, the degradation of glucose under high temperatures could consequently explain the negative influence of time on the productivity of this compound and the positive effect on LA as shown on the response surfaces. As the reaction progresses, the monosaccharide levels diminish to favor production of 5'-HMF and LA (Tongtummachat et al. 2020). Thus, the maximum levels of glucose are those coinciding with the highest temperature (170 °C), for 30 min and 60% of DMSO. On the other hand, the negative effect of higher temperatures on the chemical precursors might be due to further degradation of these compounds to humins, which have been reported to be formed by condensation of LA, at harsher conditions (Akien et al. 2012; Hayes et al., 2005).

More acidic conditions and lower temperature have shown to favor the transformation of 5'-HMF to LA (Fig. 4B). Girisuta and coworkers (2006) showed that the yield is highly dependent on the acidity of the medium and not very dependent on temperature. Higher concentrations of LA were obtained by Jeong and Kim (2021a) from *Chlorella vulgaris* biomass increasing acid concentrations. Levulinic acid production is maximized under low temperature, in agreement with the results shown by Kim et al. 2017 on *Chlorella* and *Nannochloropsis* and Jeong and Kim 2020 on *Scenedesmus obliquus*. A balance is therefore necessary for maximum 5'-HMF extraction without excessive degradation to LA when the former is the compound of interest within the process. The highest

amounts of levulinic acid can be found at 150 °C, 90 min and 3% of sulfuric acid.

A similar response can be observed for 5'-HMF (Fig. 4C). The highest amounts of 5'-HMF have been found at the lower temperatures tested. The influence of the amount of DMSO is higher than that calculated for sulfuric acid, although a slight increase with decreasing sulfuric acid can be observed. These data are in agreement with data shown by Wang et al. 2016 on *Chlorococccum* sp. Higher 5'-HMF concentrations can be found under the lowest temperature (150 °C) and high amounts of DMSO (60%). In this sense, dimethylsulfoxide has been shown to improve solubility of starch in biphasic systems, although a little amount of water is necessary to ensure the full dispersion of the granules (Jackson, 1991). Thus, the adequate distribution of the polysaccharide in the mixture can enhance the hydrolysis of glycosidic bonds. In addition, the organic solvent has been reported to compete with water molecules on the solvation shell of glucose interacting by hydrogen bonds (Vasudevan and Mushrif, 2015). Limitation of the mobility of the substrate avoids contact with other molecules in the reaction, which can lead to side condensations to humins, and favors the dehydration to 5'-HMF. This could be reflected by the optimal concentration of 5'-HMF at 60% DMSO in the response surface.

## 4. Conclusions

In this work, *Chlamydomonas reinhardtii* was selected as candidate for the alternative production of 5'-hydroxymethylfurfural and levulinic acid. Algal pigments were previously extracted as high-added value coproducts. Starch-enriched biomass was used as a feedstock for LA and 5'-HMF via acid hydrolysis, performing a Central Composite Design to establish their optimal operational conditions. Glucose maximum values were predicted at higher temperatures and DMSO percentages. LA was optimized at lower temperature, and more acidic conditions. 5'-HMF was favored by lower temperatures and higher acid and DMSO concentrations. *Chlamydomonas* can consequently be used as a sustainable source of carotenoids and chemical building blocks.

### Funding

This research was funded by The European Regional Development Fund through the Spanish State Research Agency (research grant PID 2019-110438RB-C22) and the Andalusian government (I + D + i-JA-PAIDI-Retos projects 2020-PY20\_00728). Funding for open access charge: University of Huelva / CBUA.

### CRediT authorship contribution statement

Rocío Rengel: Investigation, Methodology, Validation, Writing –

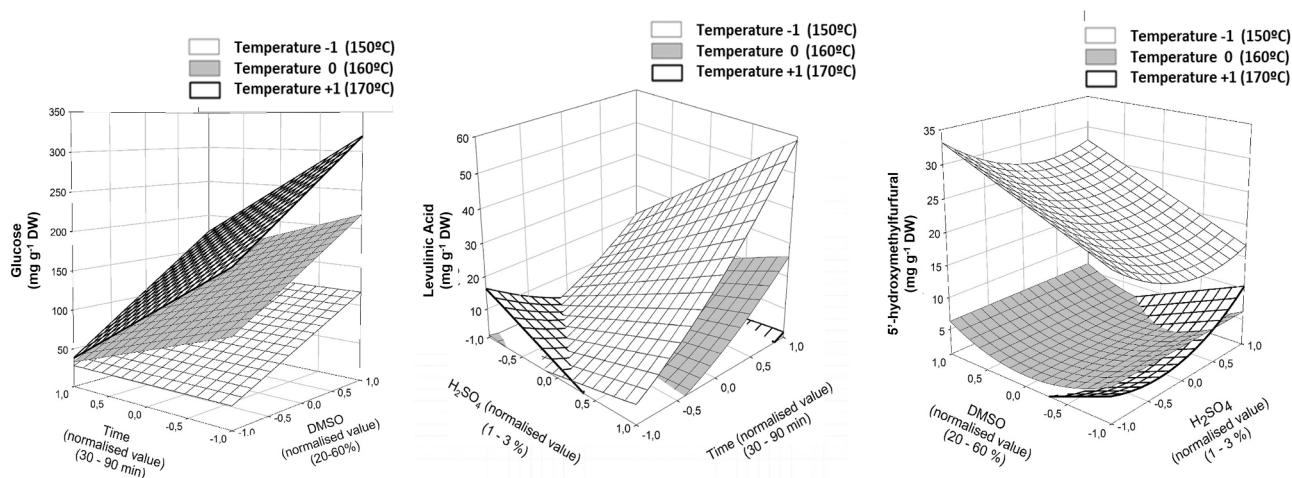


Fig. 4. Response Surface plots for each target compound. Response surface plots, representing the variation of Glucose (A), LA (B) and 5'-HMF (C) as a function of the most influential operational parameters (normalized values) for each compound.

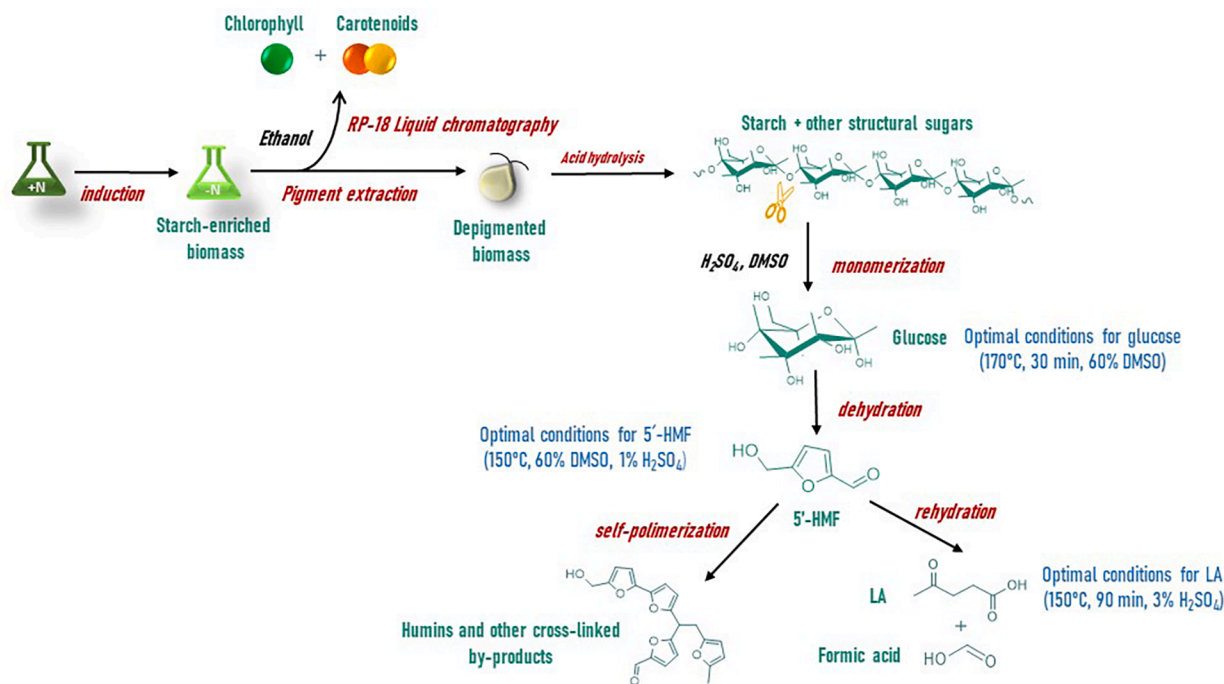


Fig. 5. Scheme of the overall workflow for the production of glucose, LA and 5'-HMF from algal biomass. Optimal conditions predicted by Response Surface Methodology for each target product have been included.

original draft. **Inmaculada Giraldez**: Investigation. **Manuel J. Díaz**: Formal analysis, Methodology, Software, Validation. **Trinidad García**: Investigation. **Javier Vigara**: Funding acquisition, Writing – review & editing. **Rosa León**: Conceptualization, Writing – original draft, Funding acquisition.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgment

Funding for open access charge: Universidad de Huelva / CBUA.

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## Conclusions



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## Chapter 1

1. Overexpression of *acetyl-CoA synthetase (ACS2)* in *Chlamydomonas reinhardtii*, induces an increase of the carbon flux towards triacylglycerol synthesis, reaching values of the acyl-CoA intermediates in the transformant *acs2* a 60% higher than the parental line, and a 2.4-fold increase in triacylglycerol content under nitrogen depletion conditions.
2. Carbon availability is a limiting factor for triacylglycerol *de-novo* biosynthesis, as the rise in acetate concentration in the medium, from 16 to 50 mM, produces an enhancement in the levels of neutral lipids, in both the parental line and the *acs2* transformant. On the contrary, starch depends in a greater extent from photosynthetic carbon fixation under mixotrophic conditions, as in the increase in acetate concentration in the medium, does not induce an improvement of its content in neither of the studied strains.
3. The increase in carbon flux towards triacylglycerol synthesis, due to ACS2 overexpression, produces a decrease in membrane lipid degradation, which is a consequence of nutrient deficiency. Monogalactosyl diacylglycerol fraction declines a 72% in the parental line and a 37% in the *acs2* transformant, whereas in the digalactosyl diacylglycerol fraction, decreases in a small extent in the parental line and increases a 30% in the case of the *acs2* transformant.
4. Overexpression of *acetyl-CoA synthetase* in the *acs2* transformant, induces ACS2 transcript levels up to 6-fold higher than the parental line at 48 hours of nitrogen starvation. On the other hand, the gene encoding the  $\alpha$ -carboxyltransferase subunit of acetyl-CoA carboxylase (ACX1), reaches a 2.8-fold increase on its transcript levels in the transformant, compared with the parental line at 48 hours of N-depletion. However, the expression levels are not induced in neither of the strains under nitrogen depletion conditions, in comparison with replete conditions.

## Chapter 2

5. Heterologous expression of the gene *diacylglycerol acyltransferase (DGAT1)*, from the boraginaceae *Echium pitardii* in the microalga *Chlamydomonas reinhardtii*, produces an enhancement in total fatty acid synthesis of 1.7-fold in the *dgat* transformant, and an increase of 2-fold of the neutral lipid content under replete conditions, and a 1.7-fold increase under nitrogen starvation.
6. Fatty acid profile of the neutral lipid extracts from the *dgat* transformant, at 72 hours of nitrogen deficiency, reveals a major contribution of the fatty acids originating from *de-novo* synthesis, being saturated and monounsaturated fatty acids, the ones that suffer a higher increase. Nonetheless, there is also a supply of polyunsaturated fatty acids from membrane recycling processes.
7. The enhanced synthesis of neutral lipids due to *DGAT1* expression, provokes an increase in acetate consumption in the *dgat* transformant, in comparison with the parental line, with values up to 44% higher at 72 hours of nitrogen depletion. On the other hand, intracellular glycerol levels, which is a substrate necessary for triacylglycerol synthesis, is detected in the *dgat* transformant throughout the nutrient deficiency, while glycerol levels were not detected in the parental line.
8. Under nitrogen starvation, photosynthetic efficiency of the *dgat* transformant declines at a slower rate than the parental line, presenting better values of the photosynthetic parameters related to electron transport and reduction, within the electronic transport chain at 72 hours of induction.

## Chapter 3

9. The insertion of *DGAT1* in transformants that overexpress the *ACS2* gene, produces a synergistic effect in neutral lipid synthesis, with an increase of a 68% in neutral lipid content in the *acs/dgat* transformant compared to the parental line, and a 24% enhancement in comparison with the single *acs* line, which shows the efficiency of the combination of an adequate carbon flux, and the overexpression of specific enzymes of the pathway. In addition, the *acs* and *acs/dgat* transformants show a 40% more content in total fatty acids with regard to the parental line.

10. *ACS2* overexpression and *ACS2/DGAT1* simultaneous overexpression, lead to an increase in carotenoid content under replete conditions, which could be favored by better carbon assimilation and triacylglycerol assembly in comparison with the parental line.
11. Starch content is greater in the *acs/dgat* transformant than the parental line and the *acs* transformant, at 72 hours of nitrogen depletion, possibly due to a better evolution of its photosynthetic efficiency during the nutritional stress.

## Chapter 4

12. *Chlamydomonas reinhardtii* has been selected for the application of experimental design, for the production of 5'-hydroxymethylfurfural and levulinic acid, as it accumulates starch up to a 35% of its dry weight under nitrogen depletion conditions, which is the maximum value reached by the analyzed strains in the screening. Moreover, the extraction of carotenoids, prior to starch hydrolysis, allows the integrated production of these compounds of interest from a sole biomass.
13. The application of the Central Composite Design and Response Surface Methodology, allows to assess the influence of experimental parameters, in the production of glucose, 5'-hydroxymethylfurfural and levulinic acid, from the hydrolysis of the starch extracted from the microalga. Optimal conditions for starch monomerization to glucose are 170°C, 30 min of reaction time and 60% of DMSO. Regarding 5'-hydroxymethylfurfural, optimal values are 150°C, 60% of DMSO and 1% of H<sub>2</sub>SO<sub>4</sub>. Finally, the established parameters for levulinic acid are 150°C, 90 min of reaction time and 3% of H<sub>2</sub>SO<sub>4</sub>.



## Conclusiones.

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De acuerdo con los objetivos propuestos en la presente tesis doctoral, y los resultados obtenidos en los diferentes capítulos, se enuncian las siguientes conclusiones:

### Capítulo 1

1. La sobreexpresión del gen que codifica la acetil-CoA sintetasa (*ACS2*) en *Chlamydomonas reinhardtii*, provoca un aumento del flujo de carbono hacia la síntesis de triacilglicerol, alcanzando los intermediarios acil-CoA, en medio repleto, valores hasta un 60% mayores que los de la especie parental, y provocando un aumento de 2,4 veces del contenido en triacilglicerol, en condiciones de carencia de nitrógeno.
2. La disponibilidad de carbono es un factor limitante para la síntesis *de novo* de triacilglicerol, ya que el aumento de la concentración de acetato en el medio, de 16 a 50 mM, produce una mejora de los niveles de lípidos neutros, tanto en la especie parental como en el transformante *acs2*. Por el contrario, el almidón depende en mayor medida del carbono fijado fotosintéticamente en condiciones mixotróficas, ya que el aumento de la concentración de acetato en el medio, no provoca un incremento de su contenido en ninguna de las cepas estudiadas.
3. El aumento del flujo de carbono hacia la síntesis de triacilglicerol por la sobreexpresión del gen *ACS2*, genera una menor degradación de los lípidos de membrana, como consecuencia de la carencia de nitrógeno, provocando una reducción del 72% en la fracción de monogalactosil diacilglicerol en la especie parental y del 37% en el transformante *acs2*. En el caso de la fracción de digalactosil diacilglicerol, se reduce en pequeña medida en la especie parental, mientras que aumenta hasta un 30% en el caso del transformante.
4. La sobreexpresión del gen *acetil-CoA sintetasa* en el transformante *acs2* de *Chlamydomonas reinhardtii*, produce niveles de transcrito de los genes *ACS2* hasta 6 veces mayores que en la especie parental, alcanzándose la máxima diferencia de expresión a las 48 horas de carencia de nitrógeno. Por su parte,

el gen que codifica para la subunidad  $\alpha$ -carboxiltransferasa de la acetil-CoA carboxilasa (*ACX1*), alcanza niveles de expresión hasta 2,8 veces más altos que los de la especie parental a las 48 horas de deficiencia de nitrógeno. Sin embargo, los niveles de expresión de este gen no se ven inducidos en ninguna de las dos cepas en dichas condiciones de carencia con respecto a condiciones de medio repleto.

## Capítulo 2

5. La expresión heteróloga del gen *diacilglicerol aciltransferasa (DGAT1)* de la boraginácea *Echium pitardii* en la microalga *Chlamydomonas reinhardtii*, provoca un incremento de la síntesis de ácidos grasos totales de 1,7 veces en el transformante, y un aumento de 2 veces, la cantidad de lípidos neutros tras su cultivo en medio repleto y de 1,7 veces en condiciones de carencia de nitrógeno.
6. El perfil de ácidos grasos en los extractos de lípidos neutros del transformante *dgat* a las 72 horas de carencia de nitrógeno, revela una mayor contribución de los ácidos grasos procedentes del proceso de síntesis *de novo*, siendo los ácidos grasos saturados y monoinsaturados los que sufren un mayor aumento, si bien, también existe aporte de ácidos grasos poliinsaturados procedentes del reciclaje de estructuras de membrana.
7. El incremento de la síntesis de lípidos neutros, generado por la expresión del gen *DGAT1*, produce un aumento del consumo de acetato en el transformante *dgat* en comparación con la especie parental, alcanzando hasta un 44% más de carbono asimilado a las 72 horas de carencia de nitrógeno. Por otra parte, se detectan considerables niveles de glicerol libre intracelular, sustrato necesario para la síntesis de triacilglicerol, en el transformante *dgat* a lo largo del estrés nutricional, a diferencia de lo que ocurre en la cepa parental en la que no hay niveles detectables de glicerol.
8. En condiciones de carencia de nitrógeno, la eficiencia fotosintética del transformante *dgat* disminuye a menor velocidad que la de la especie parental, presentando mejores valores en los parámetros relacionados con los procesos de transporte y reducción de la cadena fotosintética de transporte electrónico a las 72 horas de inducción del estrés.

### Capítulo 3

9. La inserción del gen *DGAT1* en transformantes que sobreexpresan el gen *ACS2*, provoca la mejora sinérgica de la síntesis de lípidos neutros un 68% respecto a la estirpe parental y un 24% respecto al transformante simple *acs*, en condiciones de carencia de nitrógeno, lo que demuestra la eficacia de la combinación de un adecuado suministro de precursores, con la sobreexpresión de enzimas específicas del final de la ruta. Además, los transformantes *acs* y *acs/dgat* presentan un 40% más de ácidos grasos totales que la cepa parental en las mismas condiciones.
10. Tanto la sobreexpresión de *ACS2* como la sobreexpresión simultánea de *ACS2/DGAT1* lleva a un aumento del contenido en carotenoides en medio repleto, el cual podría verse favorecido por la mayor asimilación de carbono y el ensamblaje de triacilgliceroles respecto a la especie parental.
11. El contenido de almidón en el transformante doble *acs/dgat* es mayor que el de la especie parental y el transformante simple *acs*, con valores 1,4 veces más altos tras 72 horas en condiciones de deficiencia de nitrógeno, posiblemente debido a la mejor evolución de su eficiencia fotosintética durante el estrés nutricional.

### Capítulo 4

12. Se ha seleccionado la especie *Chlamydomonas reinhardtii* para la aplicación del estudio experimental de producción de 5'-hidroximetilfurfural y ácido levulínico, al acumular hasta un 35% de su peso seco en almidón en condiciones de carencia de nitrógeno, el valor máximo encontrado entre las especies analizadas en el screening. Además, la extracción de carotenoides, previa al proceso de hidrólisis de almidón, permite la producción integrada de estos compuestos de alto valor añadido, a partir de una misma biomasa.
13. La aplicación del Diseño Central Compuesto y la metodología de Superficie de Respuesta, permiten evaluar la influencia de los parámetros experimentales en la obtención de glucosa, 5'-hidroximetilfurfural y ácido levulínico, a partir de la hidrólisis de almidón procedente de la microalga. Las condiciones óptimas obtenidas para la monomerización de almidón a glucosa son 170°C de temperatura, 30 minutos de reacción y 60% de DMSO. Respecto al 5'-hidroximetilfurfural, las condiciones óptimas establecidas son 150°C, 60% de DMSO y un 1% de H<sub>2</sub>SO<sub>4</sub>. Finalmente, los parámetros óptimos obtenidos para la síntesis de ácido levulínico son 150°C, 90 minutos de reacción y un 3% de H<sub>2</sub>SO<sub>4</sub>.



## Abbreviations



## Abbreviations

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<b>5'-HMF</b>	5'-hydroxymethylfurfural
<b>ACS</b>	Acetyl-CoA synthetase
<b>ANOVA</b>	Analysis of variance
<b>APHVIII</b>	Aminoglycoside 3'-phosphotransferase
<b>BHT</b>	Butylated hydroxymethyltoluene
<b>C-</b>	Negative Control
<b>C+</b>	Positive Control
<b>Cas</b>	CRISPR associated
<b>CRISPR</b>	Clustered Regularly Interspaced Short Palindromic Repeats
<b>DAD</b>	Diode Array Detector
<b>DGAT</b>	Diacylglycerol acyltransferase
<b>DHA</b>	Docosahexanoic acid
<b>DMSO</b>	dimethylsulfoxide
<b>DNA</b>	Desoxyribonucleic acid
<b>DW</b>	Dry weight
<b>EPA</b>	Eicosapentanoic acid
<b>ER</b>	Endoplasmatic reticulum
<b>ESI-MS/MS</b>	Electrospray Ionization – Mass spectrometry
<b>FA</b>	Fatty acid
<b>FAME</b>	Fatty acid Methyl Ester
<b>GC-FID</b>	Gas Chromatography – Flame Ionization Detector
<b>Ha</b>	Hectare
<b>HPLC</b>	High Performance Liquid Chromatography
<b>HSP70A/RBCS2</b>	Heat Shock Protein / Rubisco subunit
<b>LA</b>	Levulinic Acid
<b>LC</b>	Liquid Chromatography
<b>MRM</b>	Multiple Reaction Monitoring
<b>NCBI</b>	National Center for Biotechnology Information
<b>NIST</b>	National Institute of Standards and Technology
<b>PAR</b>	Photosynthetically Active Radiation
<b>PBS</b>	Phosphate Buffered Saline

<b>PCR</b>	Polymerase Chain Reaction
<b>PDH</b>	Pyruvate dehydrogenase
<b>PDK</b>	Pyruvate dehydrogenase kinase
<b>PUFA</b>	Polyunsaturated fatty acid
<b>qPCR</b>	Quantitative PCR
<b>RNA</b>	Ribonucleic acid
<b>SiCa</b>	Silicon Carbide
<b>SPE</b>	Solid Phase Extraction
<b>TAG</b>	Triacylglycerol
<b>TAP</b>	Tris Acetate Phosphate
<b>TCA</b>	Tricarboxylic acid cycle
<b>TEM</b>	Transmission Electronic Microscopy



# Curriculum vitae



## 1. PUBLICATIONS

- **Rengel, R.**, Giraldez, I., Díaz, M. J., García, T., Vígara, J., & León, R. (2022). Simultaneous Production of Carotenoids and Chemical Building Blocks Precursors from Chlorophyta Microalgae. *Bioresource Technology*, 127035. <https://doi.org/10.1016/j.biortech.2022.127035>. JCR Impact Factor (2021): 11.889
- Molina-Márquez, A., **Rengel, R.**, Vila, M., Gómez-villegas, P., León-Vaz, A., Vígara, J., & León, R. (2021). Potential use of probiotics and micro/macro algae as additives and major ingredients in aquafeeds. In E. Abollo & J. Maroto (Eds.), *CETMAR (First*, pp. 123–162). CETMAR.
- Molina-Márquez A., Vila M., **Rengel R.**, Fernández E., García-Maroto F., Vígara J., León R. (2020). Validation of a new multicistronic transcript for the efficient and stable expression of transgenes in microalgae. *International Journal of Molecular Sciences*. 21, 718: 1-14.
- **Rengel R.**, Smith R.T., Haslam R., Sayanova O., Vila M., León R. Overexpression of acetyl-CoA synthetase (ACS) enhances the biosynthesis of neutral lipids and starch in the green microalga *Chlamydomonas reinhardtii* (2018). *Algal Research*. 31: 183-193. <https://doi.org/10.1016/j.algal.2018.02.009>. JCR Impact Factor (2018): 3.723

## 2. WORKS SUBMITTED TO NATIONAL/INTERNATIONAL CONFERENCES OR WORKSHOPS

- **R. Rengel**, A. Molina, T. García, J. Vígara, J. Díaz, R. León. Biotechnological application of nitrogen starvation for the production of chemical precursors from the microalga *Chlamydomonas reinhardtii*. XV National Meeting of the Nitrogen Metabolism Group. Córdoba, Spain. 2022. Poster.
- **R. Rengel**, T. García, J. Vígara, J. Díaz, R. León. Optimization studies for integral production of pigments and chemical building blocks from microalgal biomass. III International Congress of Young Researchers of the Sea. Motril, Granada (Spain). 2021. Poster.
- **R. Rengel**, A. Molina, F. García, D. López, J. Vígara, R. León. Synergistic effect of overexpression of genes involved in tag biosynthesis and substrate supply on the lipid profile of *Chlamydomonas reinhardtii*. II International Congress of Young Researchers of the Sea. Málaga, Spain. 2019. Oral Communication.
- P.Gómez, A. León, A. Molina, **R. Rengel**, M. Vila, I. Giraldez, J. Vígara, R. León. The chemistry of life. European Researchers Night. Huelva. 2019. Oral Communication.
- **R. Rengel**, M. Vila, J. Vígara, R. León. Enhancement of neutral lipids and starch biosynthesis by overexpression of acetyl-coa synthetase (ACS) in the green microalga *Chlamydomonas reinhardtii*. I Congress of Young Researchers of the Sea. Cádiz, Spain. 2018. Poster.
- M. Vila, A. Molina, **R. Rengel**, E. Díaz-Santos, R. León. Simultaneous expression of two promoterless genes in the nucleus of *Chlamydomonas reinhardtii*. 18th International

- Conference on the Cell and Molecular Biology of Chlamydomonas. Washington D.C. (USA). 2018. Poster.
- A.Molina, **R. Rengel**, M. Vila, J. Vigará., R. León. Optimization of the microalgal expression vector phyco69. 18th International Conference on the Cell and Molecular Biology of Chlamydomonas. Washington D.C. (USA). 2018. Poster.
  - **R. Rengel**, Smith R.T., Haslam R.P., Sayanova O., Vila M., Vigará J., León R. Derivation of the carbon flux towards storage compounds by overexpression of plastidial acetyl-coa synthetase (*acs2*) in *Chlamydomonas reinhardtii*. 18th International Conference on the Cell and Molecular Biology of Chlamydomonas. Washington D.C. (USA). 2018. Poster.
  - P.Gómez, A. León, A. Molina, **R. Rengel**, M. Vila, E. García, I. Giráldez, J. Vigará, R. León. The colors of the sea. European Researchers Night. Huelva. 2018. Oral Communication.
  - A.Molina, **R. Rengel**, M. Vila, J. Vigará, R. León. Expression analysis of heterologous genes in *Chlamydomonas* using the polycistronic vector Phyco69. IX Conference on Aquaculture in the South Atlantic Coast. Cartaya (Huelva). 2018. Poster.
  - **R. Rengel**, M. Vila, J. Vigará, R. León. Enhancement of neutral lipids and starch in the chlorophyte microalga *Chlamydomonas reinhardtii* through overexpression of acetyl-coa synthetase (ACS2). IX Conference on Aquaculture in the South Atlantic Coast. Cartaya (Huelva). 2018. Poster.
  - R. León, M. Vila, A. Molina, A. León, P. Gómez, **R. Rengel**, J. Vigará. Design and obtaining functional phytoplankton. European Researchers Night. Huelva. 2017. Conference.
  - **R. Rengel**, M. Vila, R. León. Manipulation of the carbon assimilation pathway in the green microalga *chlamydomonas reinhardtii* by overexpression of acetyl-coa synthetase for the enhancement of lipid and starch biosynthesis. BIOTEC 2017 – National Biotechnology Congress. 2017. Poster.
  - A.Molina, **R. Rengel**, M. Vila, R. León. Validation of a polycistronic expression vector for microalgae. BIOTEC 2017 – National Biotechnology Congress. 2017. Poster.
  - A.Molina, M. Vila, **R. Rengel**, R. León. Enhancement of the carotenoid biosynthesis pathway in the unicellular microalga *Chlamydomonas reinhardtii*. VIII Southern Atlantic Coastal Aquaculture Workshop. Cartaya (Huelva). 2016. Poster.
  - R. León, M. Vila, A. Molina, A. León, P. Gómez, **R. Rengel**, J. Vigará. Microalgae: from auxiliary cultures to source of high added value products. VIII Southern Atlantic Coastal Aquaculture Workshop. Cartaya (Huelva). 2016. Oral Communication.
  - **R. Rengel**, M.C. Romero, J.M. Vega, J. Vigará. Characterization of glutamate dehydrogenase activity in the acidophile microalga *Coccomyxa onubensis*. XIII National Meeting of the Nitrogen Metabolism Group. Villanueva de la Serena, Badajoz. 2016. Poster.
  - M.C. Romero, **R. Rengel**, J.M. Vega, J. Vigará. Effect of abiotic stress on the activity of antioxidant enzymes in the acidophile microalga *Coccomyxa onubensis*. XIII National Meeting of the Nitrogen Metabolism Group. Villanueva de la Serena, Badajoz. 2016. Poster.

- R. León, E. Díaz-Santos, **R. Rengel**, J. Vígara, M. Vila. Acquirementt of genetic engineered functional phytoplankton. XV National and I Iberian Conference on Aquaculture. Huelva, Spain. 2015. Oral Communication.
- A. León, **R. Rengel**, M.C. Romero, J. Vígara. Characterization of ascorbate peroxidase activity in *Dunaliella salina* crude extracts. XXXVII Conference of the Spanish Society for Biochemistry and Molecular Biology. Granada, Spain. 2015. Poster.
- E. Díaz-Santos, M. Vila, M. de la Vega, **R. Rengel**, A. Azogil, R. León, J. Vígara. Bioflocculation induction in microalgae for the improvement of the biomass harvesting system at industrial scale. VII Southern Atlantic Coastal Aquaculture Workshop. 2015. Poster.

### 3. PARTICIPATION IN PROJECTS

- **Nutritional and functional value of microalgae isolated in the coasts of Cádiz and Huelva for their potential application as supplement in animal feed.** May 2022. PI: Antonio León.
- **Adapting microalgae metabolism for the production of colorless bioactive and bioavailable carotenoids** (PID2019-110438RB-C22). Jun 2019. PI: Rosa León.
- **Cross-border network for the development of innovative products in microalgae.** (0055\_ALGARED\_PLUS\_5\_E). Apr 2017-Dec 2019. PI: Rosa León.
- **Recombinant microalgae as platform for the production and supply of oral vaccines and antimicrobial peptides in aquaculture.** Dec 2016-Dec 2019. PI: Rosa León.

### 4. GRANTS

- **Predocctoral European mobility grant (CEIMAR – UNIVERSITY OF HUELVA)** International Campus of Excellence of the Sea (CEIMAR). March-June 2016.

### 5. INTERNATIONAL STAYS

- **Rothamsted Research. Harpenden, UK.** March – Jun 2016. Characterization of mutants of *Chlamydomonas reinhardtii* overexpressing the gene encoding acetyl-CoA synthetase. Supervisor: Olga Sayanova.
- **Rothamsted Research. Harpenden, UK.** July – December 2018. Functional characterization of elongase-encoding genes in yeast, and screening of *Chlamydomonas* mutants overexpressing the gene diacylglycerol acyltransferase.

## 6. TEACHING

- Theoretical and practical teaching in Chemistry, subject of the Degree in Mining Engineering. 4.2 credits. Academic year 2021/2022.
- Practical teaching in Organic Chemistry, subject of the Degree in Chemistry. 6 credits. Academic year 2021/2022.
- Practical teaching in Organic Chemistry II, subject of the Degree in Chemistry. 4.6 credits. Academic year 2021/2022.
- Theoretical and practical teaching in Chemistry, subject of the Degree in Mining Engineering. 5.5 credits. Academic year 2022/2023.

