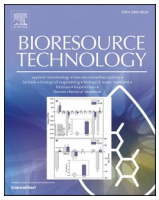
Bioresource Technology 351 (2022) 127035

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech



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

Rocío Rengel [a], Inmaculada Giraldez [b], Manuel J. Díaz [b], Trinidad García [b], Javier Vigara [a], Rosa León [a,*]

[a] Laboratory of Biochemistry, Center for Natural Resources, Health and Environment (RENSMA), University of Huelva, Avda. de las Fuerzas Armadas s/n, 21071 Huelva, Spain
[b] Research Center in Technology of Products and Chemical Processes, PRO2TECS-Chemical Engineering Department, Campus El Carmen, University of Huelva, Huelva, Spain

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

* Corresponding author at: Rosa León. Laboratory of Biochemistry, Center for Natural Resources, Health and Environment (RENSMA), University of Huelva, Avda. Fuerzas Armadas s/n, 21071 Huelva, Spain.
E-mail address: rleon@uhu.es (R. León).

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

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 strengthen 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^+), 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_2. *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 $m^{-2}s^{-1}$. For induction of starch accumulation, cells were grown until ~~at~~ 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^{-1}, 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_2 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_2SO_4 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 biphase 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^{-1}. 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_2 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^{-1} of sulfuric acid at a flow rate of 0.6 mL min^{-1}, 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^{-1} in a TGA/DSC2 thermogravimetric analyzer (Mettler-Toledo, Greifensee, Switzerland). Weight loss was monitorized 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).

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

In this equation, X_n is the normalized value for each independent variable; X is the experimental value found for each variable; $\overline{X}$ is the mean of the experimental values; and X_{max} and X_{min} 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.

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

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 $[(X_{ni})_{max} - (X_{ni})_{min}]$, represents the value range of the independent variables and $\{Z(X_{ni})_{max}] - Z[(X_{ni})_{min}]\}$ 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 $\{Z(X_{ni})_{max}]_{max} - Z[(X_{ni})_{min}]_{min}\}$, 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^{-1} 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 Trebouxinophyceae 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^{-1} and 45 mg g^{-1} of DW in the case of *C. sorokiniana* and *Picochlorum* sp., respectively, 60 and 63 mg g^{-1} for *C. reinhardtii* 704 strain and *Dunaliella salina,* respectively, and 110 mg g^{-1} 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 Trebouxinophycea, *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^{-1} 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^{-1}. 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

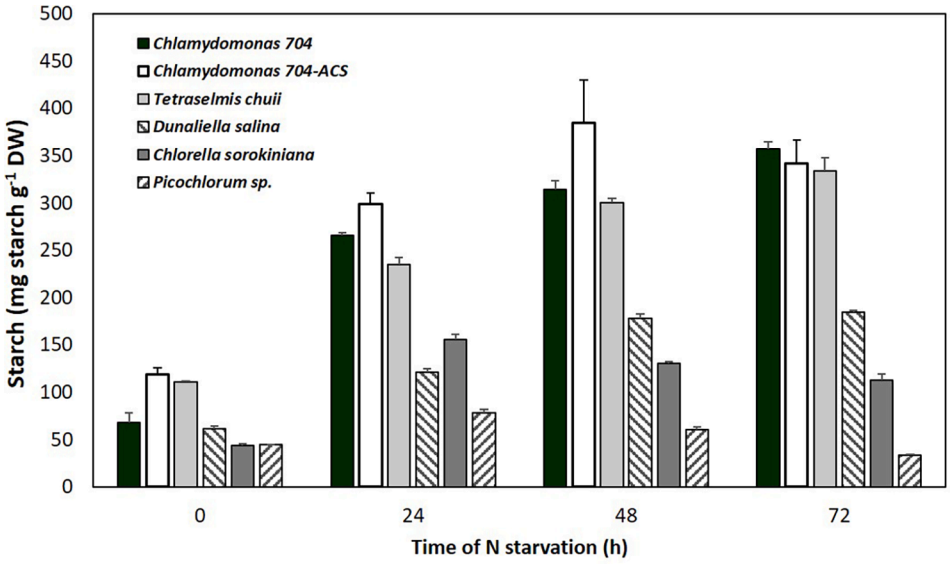


Fig. 1. Time-course evolution of starch accumulation in cultures of different chlorophyte microalgae cultured under nitrogen deprivation. Cells were grown until mid-log phase and transferred to N-depleted media. After 72 h of incubation, cells were pelleted and freeze-dried. Starch was enzymatically 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^{-1} DW. Violaxanthin and neoxanthin experimented a small decrease from 1.3 to 1.05 mg g^{-1} DW and from 0.6 to 0.5 mg g^{-1} 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^{-1} 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^{-1} 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 and 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

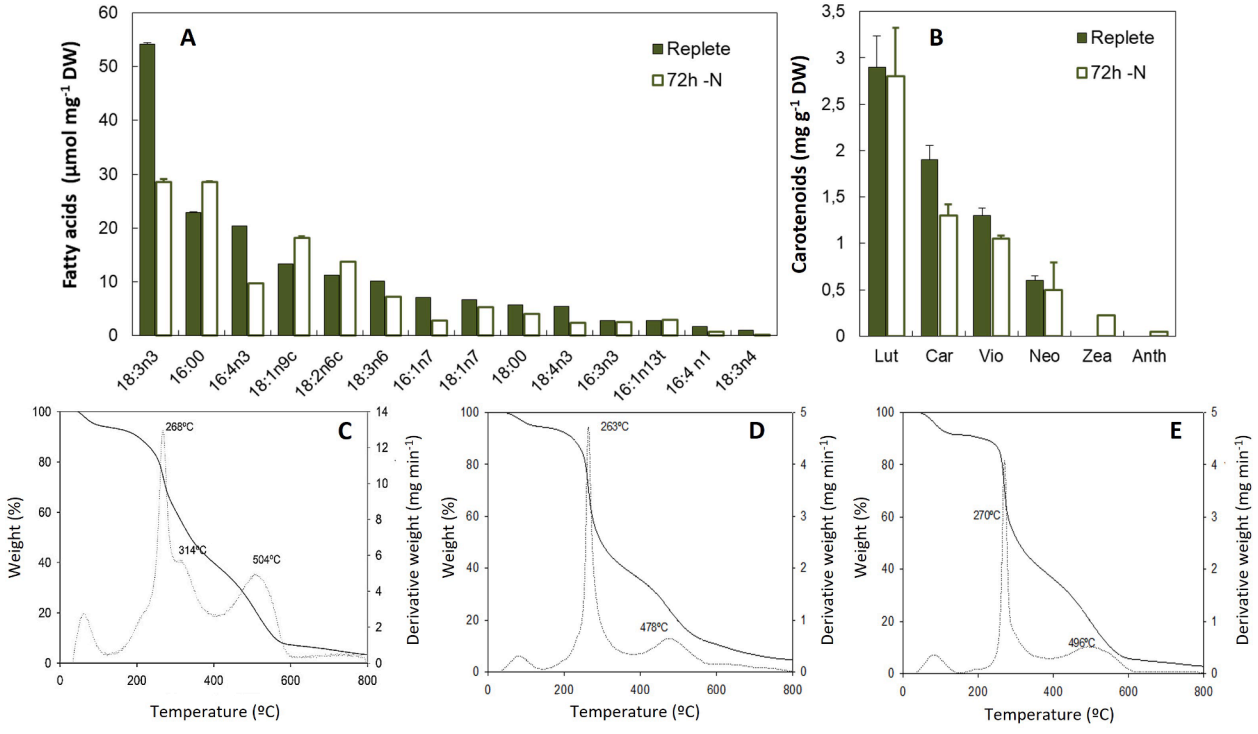


Fig. 2. Characterization of *C. reinhardtii* biomass cultured in nutrient replete and N-deprived growth media for 72 h. Fatty acid (A), carotenoid profile (B) and thermogravimetric analysis of replete (C) and nitrogen starved algal biomass before (D) and after (E) pigment extraction. Antheraxanthin (Anth); 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^{-1} 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^{-1} 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^{-1} 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^{-1}) and maltose (up to a concentration of 52,6 mg g^{-1}) 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 5́-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.

	Normalized Variables				Variable values				Experimental values		
Exp	T	ti	Sa	DMSO	T (°C)	ti (min)	Sa (%)	DMSO (%)	Glucose (mg g^{-1})	LA (mg g^{-1})	5′-HMF (mg g^{-1})
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^{-1}. 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^2 and Snedecoŕs F values have been calculated. The R^2 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, $[(X_{ni})_{max} - (X_{ni})_{min}]$, and the maximum possible difference between the maximum and minimum values of the considered compound, $\{Z(X_{ni})_{max}]_{max} - Z[(X_{ni})_{min}]_{min}\}$.

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,

A

Polynomial Parameter Correlation Equations
Glucose = 125.7 + 53.0401 T + 56.5182 ti + 46.3129 Sa - 36.1829 DMSO + 12.23 T ti + 28.5166 T Sa – 35.8573 T DMSO
LA = 2.4962 - 9.1952 T + 3.9829 ti + 8.1742 Sa + 4.7802 T^2 + 4.2901 Sa^2 - 8.1841 T ti - 10.7982 T Sa + 7.2039 ti Sa
5'-HMF = 0.01 - 11.4262 T + 7.2610 T^2 + 3.2565 ti^2 + 1.6179 Sa^2 + 3.6058 $DMSO^2$ -2.8074T ti -5.5134 ti Sa + 2.6884 Sa DMSO
TOTAL = 148.3575 + 27.1531 T + 52.2089 ti + 43.5935 Sa - 37.5464 DMSO + 11.1194 T^2 + 28.8156 T Sa + 34.8484 T DMSO

B

	Individual Influences			
	Temperature influence	Time influence	H_2SO_4 influence	DMSO influence
Glucose	27.06%	19.90%	18.98%	19.02%
LA	32.25%	23.61%	30.14%	0.00%
5'-HMF	48.11%	10.50%	16.77%	10.62%
TOTAL	23.41%	19.20%	24.45%	22.95%

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^{-1} 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^2 and Snedecoŕs 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 *Chlorococcum* 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 –

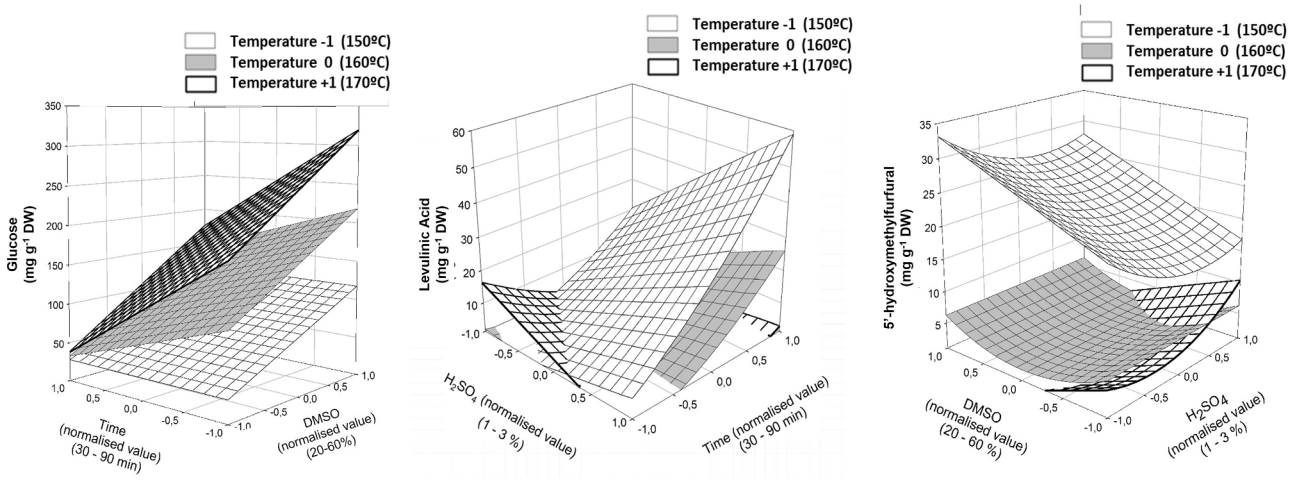


Fig. 4. Response Surface plots for each target compound. Response surface plots, representing the variation of Glucose (A), LA (B) and 5′-HMF (C) as a function of the most influential operational parameters (normalized values) for each compound.

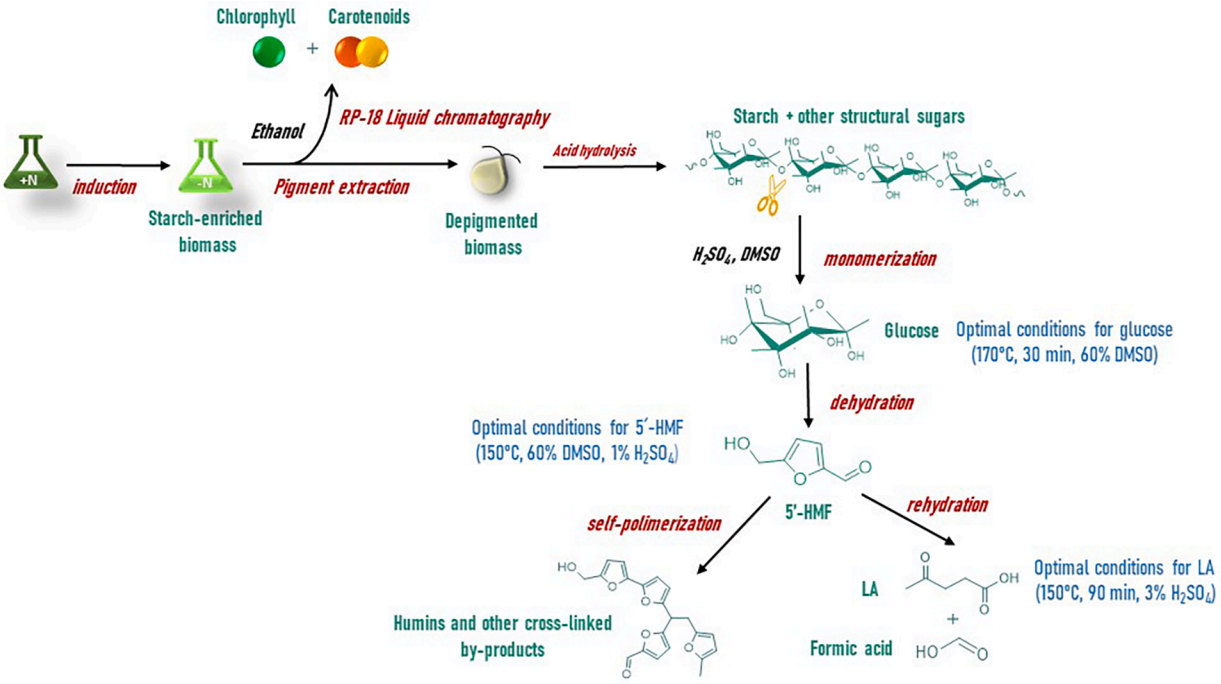


Fig. 5. Scheme of the overall workflow for the production of glucose, LA and 5′-HMF from algal biomass. Optimal conditions predicted by Response Surface Methodology for each target product have been included.

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

References

Abida, H., Dolch, L.-J., Meï, C., Villanova, V., Conte, M., Block, M.A., Finazzi, G., Bastien, O., Tirichine, L., Bowler, C., Rébeillé, F., Petroutsos, D., Jouhet, J., Maréchal, E., 2015. Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in Phaeodactylum tricornutum. Plant Physiol. 167 (1), 118–136.

Akien, G.R., Qi, L., Horváth, I.T., 2012. Molecular mapping of the acid catalysed dehydration of fructose. Chem Commun. 48 (47), 5850.

Amoah, J., Hasunuma, T., Ogino, C., Kondo, A., 2019. 5-Hydroxymethylfurfural production from salt-induced photoautotrophically cultivated Chlorella sorokiniana. Biochem Eng J. 142, 117–123. https://doi.org/10.1016/j.bej.2018.11.014.

Batista, A.P., Gouveia, L., Bandarra, N.M., Franco, J.M., Raymundo, A., 2013. Comparison of microalgal biomass profiles as novel functional ingredient for food products. Algal Res. 2 (2), 164–173. https://doi.org/10.1016/j.algal.2013.01.004.

Chen, B., Wan, C., Mehmood, M.A., Chang, J.S., Bai, F., Zhao, X., 2017. Manipulating environmental stresses and stress tolerance of microalgae for enhanced production of lipids and value-added products–A review. Bioresour Technol. 244, 1198–1206. https://doi.org/10.1016/j.biortech.2017.05.170.

Chisti, Y. Biodiesel from microalgae. 2007. Biotechnol. Adv. 25, 294-306. http://doi.org/10.1016/j.biotechadv.2007.02.001.

Constantino, A., Rodrigues, B., Leon, R., Barros, R., Raposo, S., 2021. Alternative chemo-enzymatic hydrolysis strategy applied to different microalgae species for bioethanol production. Algal Research 56, 102329.

Cuevas-Castillo, G.A., Navarro-Pineda, F.S., Baz Rodríguez, S.A., Sacramento Rivero, J. C., 2020. Advances on the processing of microalgal biomass for energy-driven biorefineries. Renewable and Sustainable Energy Reviews 125, 109606.

De la Vega, M., Díaz, E., Vila, M., León, R., 2011. Isolation of a new strain of Picochlorum sp and characterization of its potential biotechnological applications. Biotechnol Prog. 27 (6), 1535–1543. https://doi.org/10.1002/btpr.686.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal Chem. 28 (3), 350–356. https://doi.org/10.1021/ac60111a017.

Enamala, M.K., Enamala, S., Chavali, M., Donepudi, J., Yadavalli, R., Kolapalli, B., Aradhyula, T.V., Velpuri, J., Kuppam, C., 2018. Production of biofuels from microalgae - A review on cultivation, harvesting, lipid extraction, and numerous applications of microalgae. Renew Sustain Energy Rev. 94, 49–68.

Esteban, J., Ladero, M., 2018. Food waste as a source of value-added chemicals and materials: a biorefinery perspective. Int J Food Sci Technol. 53 (5), 1095–1108. https://doi.org/10.1111/ijfs.13726.

García-Domínguez, M.T., García-Domínguez, J.C., López, F., de Diego, C.M., Díaz, M.J., 2015. Maximizing furfural concentration from wheat straw and Eucalyptus globulus by nonisothermal autohydrolysis. Environ Prog Sustain Energy. 34 (4), 1236–1242. https://doi.org/10.1002/ep.12099.

Girisuta, B., Janssen, L.P.B.M., Heeres, H.J., 2006. A kinetic study on the decomposition of 5-hydroxymethylfurfural into levulinic acid. Green Chem. 8 (8), 701. https://doi.org/10.1039/b518176c.

Gong, M., Bassi, A., 2016. Carotenoids from microalgae: A review of recent developments. Biotechnol Adv. 34 (8), 1396–1412. https://doi.org/10.1016/j.biotechadv.2016.10.005.

Guillard, R.R.L., 1975. In: Culture of Marine Invertebrate Animals. Springer US, Boston, MA, pp. 29–60.

Hayes, D.J., Fitzpatrick, S., Hayes, M.H.B., Ross, J.R.H., 2005. The Biofine Process–Production of Levulinic Acid, Furfural, and Formic Acid from Lignocellulosic Feedstocks. In: Kamm, B., Gruber, P.R., Kamm, M. (Eds.), Biorefineries-Industrial Processes and Products. Wiley-VCH Verlag GmbH, Weinheim, Germany, pp. 139–164.

Jackson, D.S., 1991. Solubility Behavior of Granular Corn Starches in Methyl Sulfoxide (DMSO) as Measured by High Performance Size Exclusion Chromatography. Starch - Stärke 43 (11), 422–427. https://doi.org/10.1002/star.19910431103.

Jeong, G.T., Ra, C.H., Hong, Y.K., Kim, J.K., Kong, I.S., Kim, S.K., Park, D.H., 2015. Conversion of red-algae Gracilaria verrucosa to sugars, levulinic acid and 5-hydroxymethylfurfural. Bioprocess Biosyst Eng. 38 (2), 207–217. https://doi.org/10.1007/s00449-014-1259-5.

Jeong, G.T., Kim, S.K., 2020. Valorization of thermochemical conversion of lipid-extracted microalgae to levulinic acid. Bioresour Technol. 313 (May), 123684 https://doi.org/10.1016/j.biortech.2020.123684.

Jeong, G.-T., Kim, S.-K., 2021a. Statistical optimization of levulinic acid and formic acid production from lipid-extracted residue of Chlorella vulgaris. J Environ Chem Eng. 9 (2), 105142.

Jeong, G.-T., Kim, S.-K., 2021b. Thermochemical conversion of defatted microalgae Scenedesmus obliquus into levulinic and formic acids. Fuel 283, 118907. https://doi.org/10.1016/j.fuel.2020.118907.

Johnson, M.K., Johnson, E.J., MacElroy, R.D., Speer, H.L., Bruff, B.S., 1968. Effects of Salts on the Halophilic Alga Dunaliella viridis. J Bacteriol. 95 (4), 1461–1468. https://doi.org/10.1128/jb.95.4.1461-1468.1968.

Juergens, M. T., Deshpande, R. R., Lucker, B. F., Park, J.-J., Wang, H., Gargouri, M., … Shachar-Hill, Y., 2015. The Regulation of Photosynthetic Structure and Function during Nitrogen Deprivation in Chlamydomonas reinhardtii. *Plant Physiol. 167*(2), 558–573. https://doi.org/10.1104/pp.114.250530.

Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microb Cell Fact. 17 (1), 1–21. https://doi.org/10.1186/s12934-018-0879-x.

Khan, S., Fu, P., 2020. Biotechnological perspectives on algae: a viable option for next generation biofuels. Curr Opin Biotechnol. 62, 146–152. https://doi.org/10.1016/j.copbio.2019.09.020.

Kim, T.-H., Oh, Y.-K., Lee, J.W., Chang, Y.K., 2017. Levulinate production from algal cell hydrolysis using in situ transesterification. Algal Research 26, 431–435.

Kohli, K., Prajapati, R., Sharma, B.K., 2019. Bio-based chemicals from renewable biomass for integrated biorefineries. Energies 12 (2). https://doi.org/10.3390/en12020233.

Li-Beisson, Y., Thelen, J.J., Fedosejevs, E., Harwood, J.L., 2019. The lipid biochemistry of eukaryotic algae. Progress in Lipid Research 74, 31–68.

Libessart, N., Maddelein, M.-L., van den Koornhuyse, N., Decq, A., Delrue, B., Mouille, G., D'Hulst, C., Ball, S., 1995. Storage, Photosynthesis, and Growth: The Conditional Nature of Mutations Affecting Starch Synthesis and Structure in Chlamydomonas. Plant Cell. 7 (8), 1117.

Liu, J., Pan, Y., Yao, C., Wang, H., Cao, X., Xue, S., 2015. Determination of ash content and concomitant acquisition of cell compositions in microalgae via thermogravimetric (TG) analysis. Algal Res. 12, 149–155. https://doi.org/10.1016/j.algal.2015.08.018.

Lourenço, S.O., Barbarino, E., Lavín, P.L., Lanfer Marquez, U.M., Aidar, E., 2004. Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. Eur J Phycol. 39 (1), 17–32. https://doi.org/10.1080/0967026032000157156.

Menegazzo, F., Ghedini, E., Signoretto, M., 2018. 5-Hydroxymethylfurfural (HMF) Production from Real Biomasses. Molecules 23 (9), 2201. https://doi.org/10.3390/molecules23092201.

Morales-Sánchez, D., Schulze, P.S.C., Kiron, V., Wijffels, R.H., 2020. Production of carbohydrates, lipids and polyunsaturated fatty acids (PUFA) by the polar marine microalga Chlamydomonas malina RCC2488. Algal Res. 50 (April), 102016 https://doi.org/10.1016/j.algal.2020.102016.

Mukherjee, A., Dumont, M.-J., Raghavan, V., 2015. Review: Sustainable production of hydroxymethylfurfural and levulinic acid: Challenges and opportunities. Biomass Bioenergy 72, 143–183. https://doi.org/10.1016/j.biombioe.2014.11.007.

Palma, A., Loaiza, J.M., Díaz, M.J., García, J.C., Giráldez, I., López, F., 2021. Tagasaste, leucaena and paulownia: three industrial crops for energy and hemicelluloses production. Biotechnol Biofuels 14 (1), 89. https://doi.org/10.1186/s13068-021-01930-0.

Pane, L., Franceschi, E., De Nuccio, L., Carli, A., 2001. Applications of thermal analysis on the marine phytoplankton, Tetraselmis suecica. In *J Therm Anal Calorim.* 66, 145–154. https://doi.org/10.1023/A:1012443800271.

Pileidis, F.D., Titirici, M.M., 2016. Levulinic Acid Biorefineries: New Challenges for Efficient Utilization of Biomass. ChemSusChem. 9 (6), 562–582. https://doi.org/10.1002/cssc.201501405.

Pulidindi, I., Kim, T., 2018. Conversion of Levulinic Acid from Various Herbaceous Biomass Species Using Hydrochloric Acid and Effects of Particle Size and Delignification. Energies 11 (3), 621. https://doi.org/10.3390/en11030621.

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 Res. 31 (January), 183–193. https://doi.org/10.1016/j.algal.2018.02.009.

Rihko-Struckmann, L.K., Oluyinka, O., Sahni, A., McBride, K., Fachet, M., Ludwig, K., Sundmacher, K., 2020. Transformation of remnant algal biomass to 5-HMF and levulinic acid: Influence of a biphasic solvent system. RSC Adv. 10 (42), 24753–24763. https://doi.org/10.1039/d0ra02784g.

Rosatella, A.A., Simeonov, S.P., Frade, R.F.M., Afonso, C.A.M., 2011. 5-Hydroxymethylfurfural (HMF) as a building block platform: Biological properties, synthesis and synthetic applications. Green Chem. 13 (4), 754–793. https://doi.org/10.1039/c0gc00401d.

Samide, A., Tutunaru, B., 2017. Thermal behavior of the chlorophyll extract from a mixture of plants and seaweed. J Therm Anal Calorim. 127 (1), 597–604. https://doi.org/10.1007/s10973-016-5490-y.

Sanchez Rizza, L., Sanz Smachetti, M.E., Do Nascimento, M., Salerno, G.L., Curatti, L., 2017. Bioprospecting for native microalgae as an alternative source of sugars for the production of bioethanol. Algal Res. 22, 140–147. https://doi.org/10.1016/j.algal.2016.12.021.

Schüler, L.M., Schulze, P.S.C., Pereira, H., Barreira, L., León, R., Varela, J., 2017. Trends and strategies to enhance triacylglycerols and high-value compounds in microalgae. Algal Res. 25, 263–273. https://doi.org/10.1016/j.algal.2017.05.025.

Tongtummachat, T., Akkarawatkhoosith, N., Kaewchada, A., Jaree, A., 2020. Conversion of Glucose to 5-Hydroxymethylfurfural in a Microreactor. Front Chem. 7, 1–9. https://doi.org/10.3389/fchem.2019.00951.

Vasudevan, V., Mushrif, S.H., 2015. Insights into the solvation of glucose in water, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF) and N, N-dimethylformamide (DMF) and its possible implications on the conversion of glucose to platform chemicals. RSC Adv. 5 (27), 20756–20763. https://doi.org/10.1039/C4RA15123B.

Wang, J.J., Tan, Z.C., Zhu, C.C., Miao, G., Kong, L.Z., Sun, Y.H., 2016. One-pot catalytic conversion of microalgae (Chlorococcum sp.) into 5-hydroxymethylfurfural over the commercial H-ZSM-5 zeolite. Green Chem. 18 (2), 452–460. https://doi.org/10.1039/c5gc01850a.

Yu, I.K.M., Tsang, D.C.W., 2017. Conversion of biomass to hydroxymethylfurfural: A review of catalytic systems and underlying mechanisms. Bioresour Technol. 238, 716–732. https://doi.org/10.1016/j.biortech.2017.04.026.

Zhang, X., Hewetson, B.B., Mosier, N.S., 2015. Kinetics of Maleic Acid and Aluminum Chloride Catalyzed Dehydration and Degradation of Glucose. Energy Fuels 29, 2387–2393. https://doi.org/10.1021/ef502461s.