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Production of Hydroxy Fatty Acids and 5-Hydroxy Methyl Furfural from Microalgal Biomass: An Integrated Biorefinery Perspective Involving Chemical and Enzymatic Conversion

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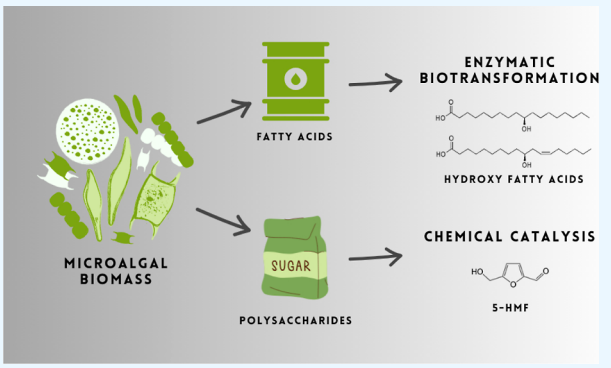
ABSTRACT: A novel biorefinery process utilizing microalgal biomass has been developed, focusing on the enzymatic biotransformation of microalgal fatty acids in hydroxy fatty acids (HFAs) and the chemical conversion of the cellular debris to 5-hydroxymethyl furfural (5-HMF). First, the process was demonstrated using the dry biomass of the microalgal strains *Chlorella* sp. *CW2*, and *Chlorella* sp. Barcarello and *Nannochloropsis gaditana* obtained a C18:1 substrate reduction of approximately 68.7, 83.4, and 71.5% and a maximum 5-HMF yield of 28.6 ± 1.4, 35.2 ± 5.4, and 25.2 ± 1.5%, respectively. Further optimization of the process was performed on the wet biomass of the microalga *Chlorella* sp. *CW2* by using intensified process operations, achieving the production of double-functionalized HFAs. The described process yields building blocks for the chemical industry starting from microalgal biomass, potentially sourced from the biological treatment of wastewaters. The enhanced sustainability and reduced operational costs provided by this innovative biorefinery approach represent significant advancements in the microalgal industry.



INTRODUCTION

Microalgae are photosynthetic microorganisms of high scientific interest for several reasons. First, they have a high photosynthetic efficiency, making them more productive in comparison to land plants.[1] They may be grown in seawater or wastewaters,[2] not competing with food crops and may be used as a lipid source for biofuel production. Although there is a growing demand for microalgal biomass, its production is still industrially limited because of its high production costs. In order to decrease the expenses and increase the feasibility of microalgae production and manufacturing, integrated biorefineries have been recently proposed.[3,4]

In an integrated biorefinery, microalgal main components (proteins, lipids, and carbohydrates) are extracted, separated, and utilized for several purposes.[5] The biomass, furthermore, may also be grown in wastewater, performing its biotreatment while decreasing the concentration of pollutant compounds such as organic carbon, nitrogen, and phosphorus. The biorefinery concept ensures that every fraction of the harvested microalgal biomass is utilized, with the aim of creating a zero-waste process. In some experimental cases proposed in the literature, the microalgal biomass grown in wastewaters is used for enhanced biomass, lipid, and high-value compound production.[6,7] More recently, microalgal biorefineries were proposed for enhancing lipid production by using tobacco industry wastewater[8] or for obtaining functional pigments, biodiesel, and biofertilizers from hydroponic wastewaters.[9]

The lipidic fraction of microalgal biomass, primarily composed of triglycerides, has several applications. In the energy sector, these are used as the source of biofuels, while in pharmaceutical and cosmetic industries, they are valuable ingredients for various products.[10,11] Microalgal lipids can be functionalized, and in particular, an −OH residue may be added along the aliphatic chain of fatty acids, thereby generating hydroxy fatty acids (HFAs). HFAs are a class of compounds of growing interest with several biotechnological applications due to the proprieties given by the hydroxyl group, which, for

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example, allows the formation of polymers. HFAs can be extracted from plants with a low yield of productivity, also due to seasonality. The most abundant HFA in nature is ricinoleic acid, the main component of castor oil. The latter can also pose health issues due to the presence of toxins and allergens.[12] However, ricinoleic acid is one of the main HFAs used in the industry as is or on its saturated form produced after hydrogenation and originates from 12-hydroxystearic acid. This is used as a surfactant but also as a substrate for the creation of waxes, polymers, nylons, cosmetics, and additives.[13] HFAs may also be produced from unsaturated fatty acids by enzymatic hydration of a double bond along the chain. The enzymatic reaction is regioselective and stereoselective. In particular, *Elizabethkingia meningoseptica* oleate hydratase (Em_OhyA) can add a water molecule to the Δ9 unsaturation of fatty acids, catalyzing the conversion in (*R*)-10-hydroxy fatty acids (10-HFA).[14] The chemical reaction would provide a mixture of HFA isomers, which could result in a time- and resource-consuming method for the purification of each component.[15] HFAs can also be naturally found within the microalgal cell; for example, the presence of the algaenan-associated fatty acid 15-OH-$C_{32:0}$ has been reported in the *Nannochloropsis* genus.[16] HFAs were found also in macroalgae; for instance, a mixture of 4 isomeric hydroxy C18 was found in *Tricleocarpa jejuensis*, a red alga, which also showed algicidal proprieties.[17] Microalgal lipids may be also used as substrates for the production of HFAs starting from unsaturated fatty acids, as shown by Eser et al., which obtained several compounds starting from the fatty acids of *Nannochloropsis gaditana* and *Pavlova lutheri* by using recombinant hydratases.[18] Anyway, the use of microalgal fatty acids with Em_OhyA has yet to be demonstrated. Similar to lipids, the carbohydrate fraction of the microalgal cell may also be used as a substrate. Microalgal carbohydrates, found in accumulation products like starch or within the cell wall, can be valorized through processes such as anaerobic digestion or fermentation.[19] Recently, the use of these carbohydrates as a substrate for the production of furanic compounds was proposed.[20,21]

Microalgal carbohydrates can be utilized after preliminary lipid extraction as a valuable source of furans, particularly 5-HMF. In fact, 5-HMF obtained from biomass is a biobased renewable material which is an alternative to petroleum-based equivalent.[22] The production of 5-HMF from biomass is a sustainable and reliable process, as previously discussed.[23] In the conversion process already proposed, microalgal cells were subjected to chemical catalysis for obtaining 5-hydroxymethyl furfural (5-HMF). 5-HMF is derived from the hydrolysis of complex sugars in glucose, followed by isomerization into fructose and dehydration. The reaction is catalyzed by acidity, with solid catalysts, particularly niobium-based catalysts, proposed as a greener alternative to mineral acids. 5-HMF is a chemical platform, serving as a biobased substrate for the synthesis of polymers, fragrances, pharmaceuticals, fuels, additives, etc.[24] Although the use of microalgal biomass for the production of furans has been suggested, this reaction has yet to be integrated into a biorefinery process.

In this work, we propose a proof-of concept biorefinery process for the use of microalgal biomass for two key purposes: (i) the production of 10-HFA from the fatty acids of three microalgal strains through Em_OhyA enzymatic catalysis and (ii) the use of the defatted microalgal biomass residuals as substrates for the chemical production of 5-HMF. The process was tested on three microalgal strains, *N. gaditana*, *Chlorella* sp. Barcarello, and *Chlorella* sp. *CW2*. The proposed process was also further optimized on the alga *Chlorella* sp. *CW2*, effective in civil water remediation,[25] with a focus on process intensification. This work proposes an innovative multiproduct biorefinery utilizing microalgal biomass to produce multiple high-value compounds. By potentially sourcing biomass from wastewater treatment, this approach supports a circular economy with a minimum environmental impact and enhances the viability of microalgal industries.

MATERIALS AND METHODS

Algal Growth. Microalga *Chlorella* sp. *CW2* and *Chlorella* sp. Barcarello were previously isolated and molecularly characterized;[25] *N. gaditana* (CCAP 849/5) was obtained from the Scottish Association for Marine Science. For cultivation in a 5 L bubble column photobioreactor, a commercial fertilizer (Sprayfeed, Pavoni) was used at a concentration of 3 g L^{-1}. The cultivation lasted 11 days with 250 μmol m^{-2} s^{-1} provided light intensity, measured with a Delta Ohm-HD 9021 quantometer equipped with a Photosynthetic Active Radiation (PAR) probe (Delta Ohm LP 9021 PAR). Several batch cultivations were performed in order to obtain enough biomass for subsequent treatments. At the end of each cultivation, biomass was harvested by centrifugation, lyophilized for 24 h in a bench lyophilizator (Alpha 1−2 LDplus, Christ, DE), and homogenized. In one case, the biomass of *Chlorella* sp. *CW2* was harvested and directly used.

Extraction of Lipidic and Carbohydrate Fractions. Before extraction, 4 g of lyophilized microalgal biomass in batches of 400 mg was mixed with 30 mL of water and disaggregated using a ball mill (Retsch PM 100, DE) for 30 min at 500 rpm. Then, the batches were mixed, and 100 mL of a 1:1 mol ratio mixture of chloroform/methanol ($CHCl_3$/CH_3OH) was added. Successively, the two phases were separated by using a liquid−liquid extractor, previously described.[26] The phase containing total lipids and chloroform was then evaporated in a rotavapor in order to obtain total lipids. It is worth noting that this method does not necessarily extract all of the lipidic content of the cells and was later further optimized. From the other phase composed of methanol, water, and debris, the solid residuals were then collected by centrifugation. Before further analysis and treatment, the debris was rinsed twice with water and dried for 12 h at 60 °C.

A second procedure for the extraction of triglycerides was set up and applied only to the microalga *Chlorella* sp. *CW2*. It consisted of the use of approximately 10 g of wet microalgal biomass (humidity approximately 90%) directly after the harvesting. The biomass of *Chlorella* sp. *CW2* was dispersed in 150 mL of water and mechanically disrupted by ball milling (Retsch PM 100, DE) at 500 rpm for 25 min. The biomass suspension was then added with 75 mL of ethyl acetate and manually mixed in a funnel. The upper organic phase containing total lipids dissolved in ethyl acetate was then collected. The procedure was repeated twice, and the extracted phases were pooled together. The lower water phase, containing cellular debris, was centrifuged (4500 rpm, 10 min) to collect the solid fraction, which was then rinsed twice and stored without further processing until further analysis and treatment.

Preparation of the Free Fatty Acid Mixture from Microalgal Lipids and the Whole-Cell Biocatalyst. After the extraction of total lipids from microalgae, the saponification reaction was performed in order to obtain FFAs. The reaction was carried out with a solution of 20% (w/v) KOH in ethanol/water 7:1 and 100 μL of H_2SO_4 20% (v/v). At the end of the

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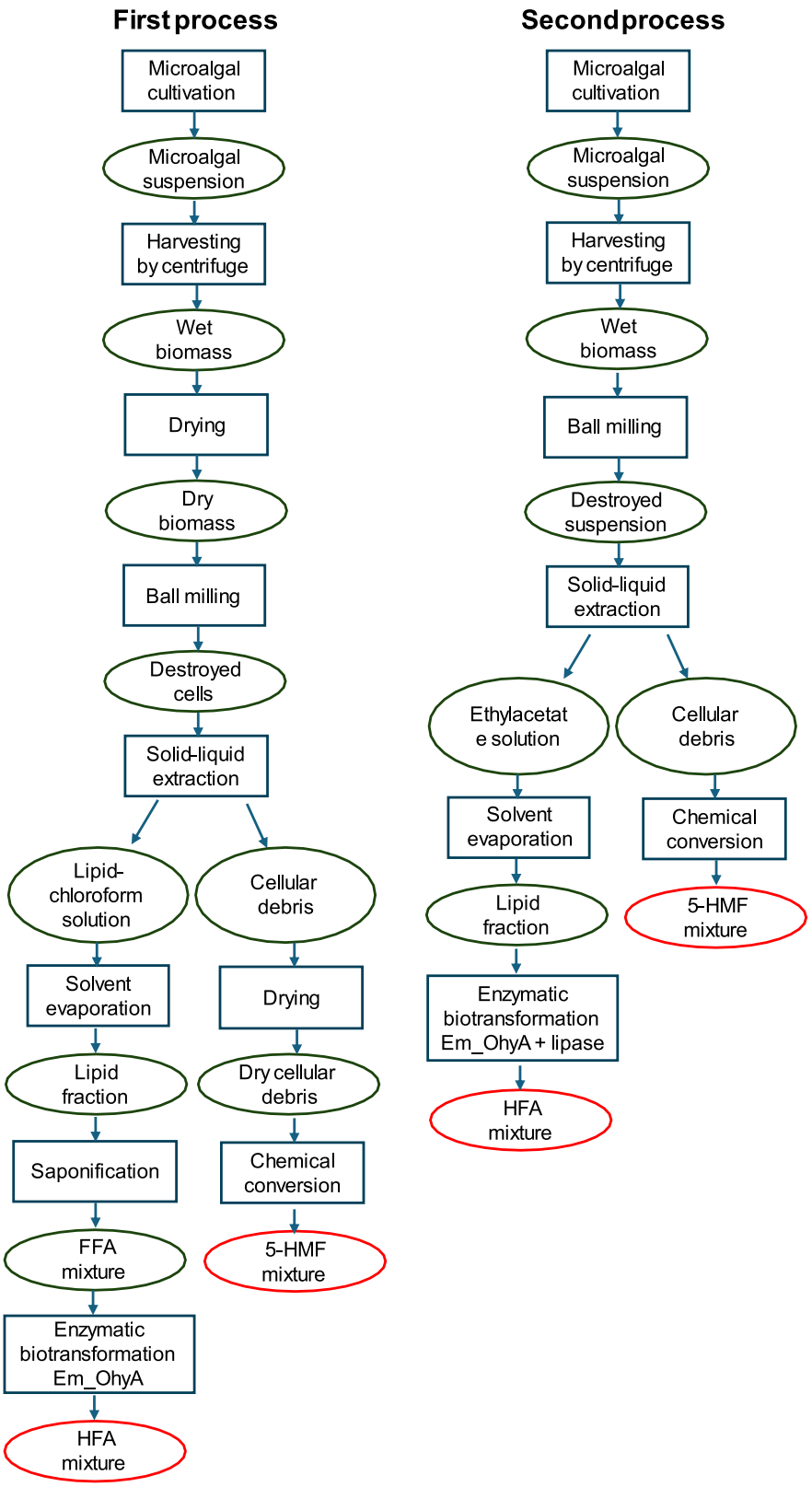

Figure 1. Simplified block diagram describing the implemented biorefinery process.

reaction, FFAs were extracted with 1 mL of ethyl acetate. This mixture was used as a substrate for the biocatalytic reaction as described before.[26]

Escherichia coli BL21 (DE3) whole cells containing the recombinant enzyme *E. meningoseptica* oleate hydratase (Em_OhyA), which was expressed as previously described,[14] were used as the biocatalyst. After the growth and the expression of the recombinant enzyme, the cells were centrifuged (4000 rpm, 10 min), and the pellet was stored at −4 °C for subsequent use.

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Characterization of Microalgal Biomass. The analysis of total lipids and FAs was performed on microalgae following the National Renewable Energy Laboratory (NREL) method[27] in the presence of an internal control. It was performed by the simultaneous transesterification and extraction of fatty acid methyl esters (FAMEs) and subsequently analyzed by gas chromatography using a 7890A System (Agilent Technology) equipped with an Omegawax 250 capillary column and a flame ionization detector. Details about the procedure were described before.[26] The analysis was done directly on the dry microalgal biomass or on the microalgal oil previously extracted. Analysis were performed in triplicate, and the average value was retained.

The oil extraction yields were calculated as

$$Y_{oil} = [\text{sum of all the fatty acids present in the sample}] / [\text{amount of biomass}] \times 100 \quad (1)$$

The total carbohydrate content in the biomass was determined by the Dubois method with some adaptations[28] as described before,[29] both on dry and on wet defatted biomass. The Dubois methodology consents to a quantitative analysis of the sugars, but not a qualitative one, thanks to a colorimetric reaction given by furans obtained through strong acid catalysis with sulfuric acid.

Biocatalytic Reaction on FFAs from Three Microalgae and Analysis. Unsaturated FFAs were converted into HFAs in 50 mL unbaffled Erlenmeyer flasks. The reaction was run at 37 °C and 200 rpm for 24 h. A 10 mL solution was composed of 50 mM sodium citrate buffer, pH 6, with 0.4 mM Tween 20, 10 mg mL^{-1} wet cell weight biocatalyst, and 21 mg of microalgae-derived FFA. The reaction was stopped by the addition of ethyl acetate solvent (4 mL).

The solvent allowed to extract both the FFA and HFA. An aliquot of 40 μL of the solution was evaporated at 80 °C and stored at −20 °C before further analysis by HPLC, following the method previously described.[14]

One-Pot Biocatalytic Conversion of Oils into Hydroxy Fatty Acids. Biocatalytic transformation was carried out in one pot containing 504 U of lipase from *Candida rugosa* (Sigma-Aldrich, USA) in 50 mM sodium citrate pH 6, 0.4 mM Tween 20, and 10 g L^{-1} whole-cell biocatalyst containing the recombinantly produced Em_OhyA.

The biocatalytic reaction began with the addition of 1% (v/v) previously extracted algal oil. The bioreactor was incubated at 37 °C and stirred at 200 rpm for 24 h. The products were then mixed with 200 mL of ethyl acetate and centrifuged at 4000 rpm for 20 min to separate the organic phase containing FFAs and 10-HFA.

The organic mixture was collected and dried over anhydrous sodium sulfate to obtain a mixture containing 10-HFA. Samples of the obtained samples were derivatized and analyzed by GC−MS as previously described.[14]

Catalytic Treatment of *Chlorella* sp. *CW2* Debris to Obtain Furans and Analysis. The catalytic reactions were performed in a stainless steel autoclave (Tefic Biotech Co. Limited, Xi'an, China). 24 mL of water, 40 mg of algal dried cellular debris, and 80 mg of catalyst were placed in the 50 mL PTFE chamber inside the reactor. The reactor was heated in an oil bath at 210 °C and was magnetically stirred using an LLG-uniSTIRRER. Some experiments were performed in the presence of a biphasic system in the contemporary presence of 12 mL of aqueous suspension and 12 mL of methyl isobutyl ketone (MIBK) together with the substrate and the solid catalyst as described previously.[21] This consisted of a commercial powder of $NbOPO_4 \cdot nH_2O$ supplied by Companhia Brasileira de Metallurgia e Mineração (CBMM). After the experiments, the reactor was cooled down for 20 min, and samples from each batch of both aqueous phase and organic phase were filtered and analyzed by HPLC as explained previously.[21]

An improved version of the process used wet biomass that had already been treated with ethyl acetate to remove lipids, which served as a substrate for the reactions. An equivalent quantity based on carbohydrate content, estimated using the Dubois method, was added, which involved incorporating 500 mg of wet biomass from *Chlorella* sp. *CW2* (this is the quantity containing the same amount of carbohydrates compared to the reactions performed with dry biomass), along with water, catalyst, and, in some experiments, the MIBK.

The catalytic performance in terms of the percentage of 5-HMF yield (Y) is defined as follows

$$Y = [\text{5-HMF}]/[\text{totalsugars}] \times 100 \quad (2)$$

where [total sugars] corresponds to the concentration of total sugars analyzed by the Dubois method and present in the reacting biomass and [5-HMF] is the concentration of 5-HMF measured at the end of the catalytic experiment. All the catalytic tests were performed in triplicate, and average values of the results together with their fluctuations were reported in the figures.

Statistical Analysis. All of the tests and analyses were performed in triplicate. An average value is reported together with the standard deviation as an error bar.

Operations and Optimization Process. The proof-of-concept process described in this work was first tested on the dry biomasses of three microalgal species, namely, *Chlorella* sp. *CW2*, *N. gaditana,* and *Chlorella* sp. Barcarello, to assess the possibility to use fatty acids and carbohydrates from different species in a biorefinery process. In the first process, represented on the left of Figure 1, after microalgal harvesting, the biomass was dried and destroyed to facilitate the subsequent extraction steps. Dry biomass was extracted through solid−liquid extraction with chloroform−methanol as solvents, and the lipid fraction was separated from the organic phase through evaporation. The obtained lipids were saponified into FFAs to obtain substrates for the biotransformation reaction mediated by Em_OhyA enzyme which produces HFAs. Cellular debris, after a further drying step, was used in the catalytic reaction yielding 5-HMF. The second process, depicted on the right side of Figure 1, was a revised version of the first one aimed at optimizing the environmental and energetic impact of the process. In this case, only the biomass of *Chlorella* sp. *CW2* was tested, and any step of drying was removed from the process. After harvesting, the biomass was disrupted, and lipid extraction was performed with ethyl acetate as a solvent. The extracted lipids were subsequently used for a combined biotransformation involving the use of lipase and Em_OhyA enzymes to produce HFAs. By doing so, the saponification step was avoided. Furthermore, cellular debris was used without further drying in the catalytic reaction for the production of 5-HMF.

RESULTS AND DISCUSSION

Lipid Composition of the Microalgal Biomass. Dry biomass of microalgae *Chlorella* sp. *CW2*, *N. gaditana,* and *Chlorella* sp. Barcarello contained, respectively, 5.6 ± 1.7%, 10.1 ± 2.7%, and 6.8 ± 0.1% of fatty acids (Table 1). As shown in Figure 2, in *Chlorella* sp. *CW2*, the most abundant fatty acid was

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Table 1. Microalgal Biomass Content in Fatty Acids Calculated as FAMEs according to the NREL Procedure[27]

	fatty acids (%)
Chlorella sp. CW2	5.6 ± 1.7
N. gaditana	10.1 ± 2.7
Chlorella sp. Barcarello	6.8 ± 0.1

palmitic acid (C16:0), followed by linolenic, linoleic, and oleic acids (C18:3, C18:2, and C18:1). In *N. gaditana*, the most abundant fatty acids were palmitic and palmitoleic acids (C16:0 and C16:1), followed by eicosapentaenoic acid (EPA). In *Chlorella* sp. Barcarello, the most abundant fatty acids were linoleic, linolenic, and palmitic acids (C18:2, C18:3, and C16:0). Oleic acid (C18:1), the main substrate for the enzyme Em_OhyA, was present in all of the analyzed biomasses. Previous studies have reported similar results for these strains.[25]

Biocatalytic Reaction of Microalgal Substrates. For the valorization of the dry biomass of three microalgae strains, a lipid extraction was first conducted, which yielded 4.1%, 5.5%, and 13.3%, respectively, for *Chlorella* sp. *CW2*, *N. gaditana*, and *Chlorella* sp. Barcarello on dry biomass. This extraction was performed with a classic chloroform/methanol solid−liquid extraction starting from the dry biomass in a single batch. A preparative saponification step to convert triglycerides in FFAs was performed on the extracted total lipids from the microalgal biomass, as described in the Materials and Methods section and in Figure 1.

The FFAs were used as a substrate for the biocatalytic reaction involving the whole cells of *E. coli* carrying the recombinant enzyme Em_OhyA. Through this process, a water molecule can be inserted into the Δ9 double bond present in unsaturated fatty acids, obtaining (*R*)-10-hydroxy fatty acids (10-HFAs).[30] As the enzyme has a high specificity for oleic acid, its content was used as a marker to monitor the reaction advancement and analyzed after the biocatalytic reaction as described in the Materials and Methods section.

The residual initial substrate oleic acid observed after the reaction was 31.3 ± 2.5, 16.6 ± 1.5, and 28.5 ± 1.4% compared to the initial one, respectively, for *Chlorella* sp. *CW2*, *N. gaditana*, and *Chlorella* sp. Barcarello (Figure 3), equivalent to an approximate conversion of 68.7, 83.4, and 71.5% of the initial amount. The decrease of the C18:1 substrate was compared with control reactions using *E. coli* cells containing an empty vector, which confirmed that the decrease is related to its conversion in 10-HFA, suggesting that the FFA contained in each microalgal species can be used for the biotransformation mediated by Em_OhyA enzyme. Only a few works investigated lipids from microalgae for transformation in HFAs. Although Eser at al. used fatty acids from two microalgae, *N. gaditana* and *P. lutheri*, with recombinant hydratases, they neither catalyze the formation of 10-HFA nor tested the process on *Chlorella* strains.[18]

Valorization of the Cellular Debris: Conversion of Algal Polysaccharides to 5-HMF by Chemical Catalysis. From the perspective of a circular economy and biorefinery, the exhausted biomass obtained after the extraction of lipids was utilized in a subsequent valorization, aimed at producing furans and, in particular, 5-HMF. The residual biomass still contains valuable biomolecules including carbohydrates, which can be further exploited. A scheme of the process was shown in previous works.[20] For the reactions, a solid acidic catalyst was used, namely, $NbPO_4$, which was chosen because of the strong acid features and the presence of both Lewis and Brønsted acid sites as previously shown.[29] This allows performing all the steps necessary for the conversion of carbohydrates in 5-HMF: (i) hydrolysis of complex carbohydrates in a glucose monomer catalyzed by Lewis acid sites, (ii) isomerization of glucose in fructose catalyzed by Lewis acid sites, and (iii) dehydration of fructose in 5-HMF catalyzed by Brønsted acid sites.

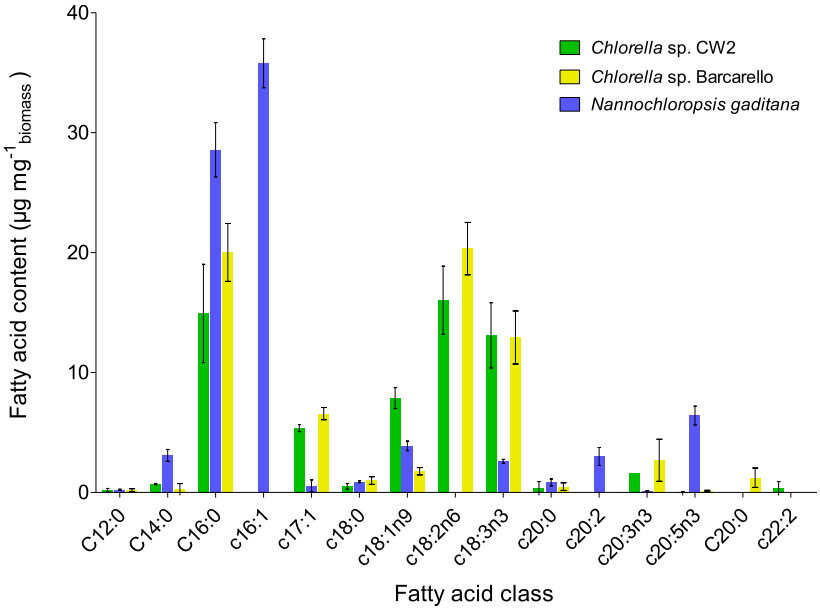

Figure 2. Fatty acid content of the microalgal cells (dry biomass) of *Chlorella* sp. *CW2*, *N. gaditana*, and *Chlorella* sp. Barcarello. Analysis was done on dry biomass. Standard deviation is reported as error bar. C12:0: lauric acid; C14:0: myristic acid; C14: myristoleic acid; C16:0: palmitic acid; C16: palmitoleic acid; C17:1: *cis*-10-heptadecanoic acid; C18:0: stearic acid; C18:1n9: 9-elaidic acid; C18:1n6: 6-elaidic acid; C18:2n6: linoleic acid; c18:3n3: linolenic acid; C20:0: arachidonic acid; C20:2: *cis*-11,14-eicosadienoic acid; C20:3n3: eicosatrienoic acid; C20:5n3: eicosapentaenoic acid; C22:0: behenic acid; and C22:2: docosadienoic acid.

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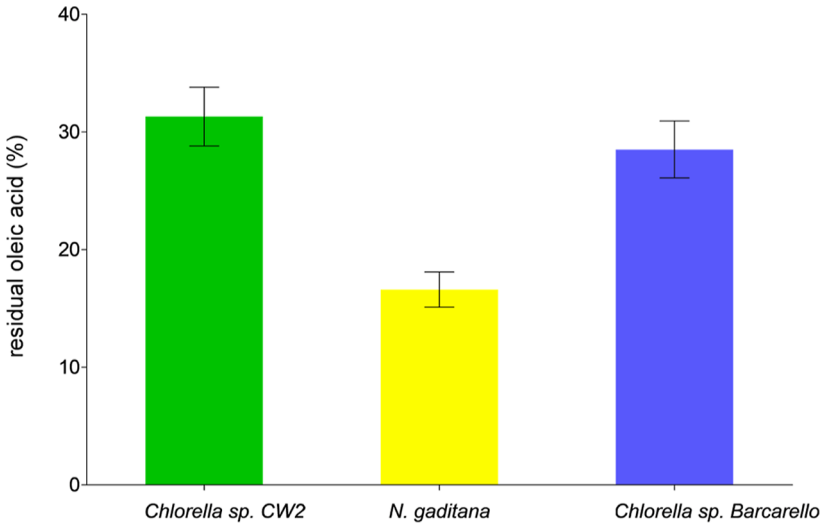

Figure 3. Residual oleic acid obtained from three different microalgae after the reaction of biotransformation in 10-hydroxy fatty acids with Em_OhyA enzyme.

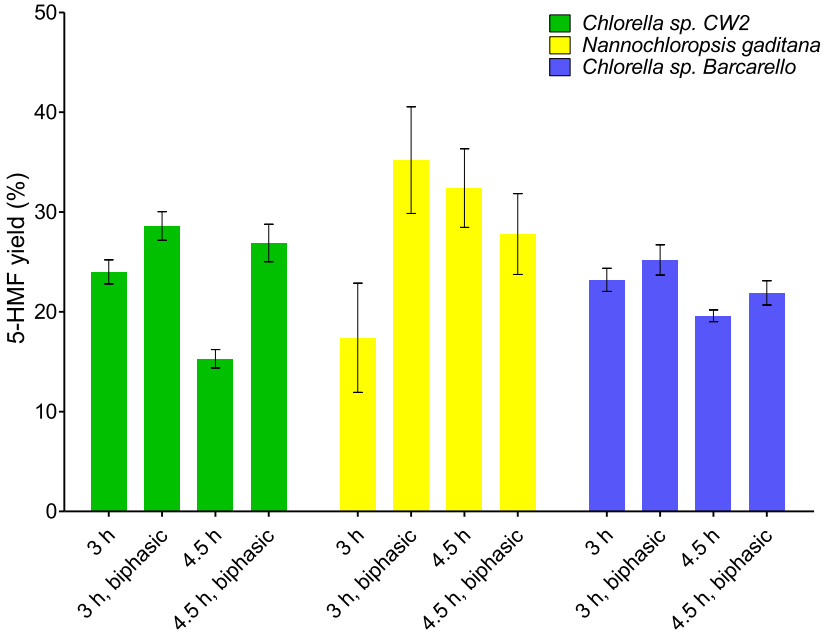

Figure 4. Yield of 5-HMF from total sugars contained in microalgal cellular debris after catalytic conversion under different conditions using niobium phosphate as a catalyst. In some cases, methyl isobutyl ketone was used as a cosolvent. Green: *Chlorella* sp. CW2; Yellow: *N. gaditana*; and blue: *Chlorella* sp. Barcarello.

The defatted biomass was first characterized for its carbohydrate content, which serves as a substrate for the catalytic reaction to produce 5-HMF. The carbohydrate content was found to be 11.1 ± 1.9%, 10.9 ± 0.2%, and 11.6 ± 1.8% on the dry defatted biomass for *Chlorella* sp. *CW2*, *N. gaditana,* and *Chlorella* sp. Barcarello, respectively.

Samples of defatted biomass were employed in catalytic experiments in the presence of $NbPO_4$ catalyst to obtain 5-HMF. In all the tested conditions, the catalytic reactions achieved satisfying yields, spanning from 15.3 to 32.4% (Figure 4). Apart from 5-HMF, the reaction may yield several other compounds which may be derived directly from the sugars or as byproducts resulting from the hydrolysis of 5-HMF (levulinic and formic acid) or from the condensation of residual sugars and furans (humins). These side reactions were previously described[20] and justified the discrepancy between the conversion of sugars and the yield in 5-HMF. These results represent an improvement compared to previous works starting from whole biomass.[20,21] Between 3 and 4.5 h, the best reaction time was 3 h when residual biomass was from *Chlorella* sp. *CW2* and *Chlorella* sp. Barcarello and for 4.5 h when starting from *N. gaditana*. These differences reflect the diversity of the microalgal structure, and in particular of the cell−wall. When the sugars are less available, for example, in the form of cellulose, previous reactions of hydrolysis are needed in order to release them. If the sugars are more available, e.g., in the form of starch or of free sugars, the reaction optimum most likely occurs after shorter reaction times.

The reactions were also conducted in a biphasic system in the presence of methyl-isobutyl ketone (MIBK), a selective solvent for the desired product 5-HMF successfully used before with another strain of *Chlorella*, where it increased the reaction yield

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by about 60%.[21] The resulting operation is an extractive reaction, in which the reaction occurs in the aqueous phase, while the organic phase constantly solubilizes the product from the reaction medium, avoiding the production of undesired products. In this case, the presence of MIBK optimized the reaction yield only in *Chlorella* sp. *CW2* and *Chlorella* sp. Barcarello. After 3 h, the reaction yield increased from 24.0 ± 1.2 to 28.6 ± 1.4 and from 23.2 ± 1.2 to 25.2 ± 1.5%, respectively, for *Chlorella* sp. *CW2* and *Chlorella* sp. Barcarello, while after 4.5 h, the yield was 26.9 ± 1.9 and 21.9 ± 1.1%, respectively. These results are comparable to and slightly enhanced than those previously reported in the literature, regarding the conversion of remnant biomass from *Haematococcus pluvialis*.[31,32] *H. pluvialis* in the cyst phase accumulates starch-like compounds along with the main product astaxanthin,[33] making its sugars more available for catalysis compared to those contained in the employed strains of *Chlorella*. These strains have low storage carbohydrate levels and contain sugars mainly in recalcitrant form (cellulose) in the cell wall, as previously explained.[21] This makes our results even more remarkable. In *N. gaditana*, instead, the addition of organic solvent increased the reaction yield only after 3 h, from 17.4 ± 5.5 to 35.2 ± 5.3%, while the reaction yield after 4.5 h was almost constant (from 32.4 ± 3.9 to 27.8 ± 4.1%). A constant yield indicates that in the monophasic conditions, the reaction parameters were already optimized. Overall, the results are very similar between the two *Chlorella* strains, while they are slightly different in the *Nannochloropsis* strain. This likely depends on the different cell compositions of the different genus.

Process Optimization: One-Pot Biocatalytic Cascade and Debris Valorization of Wet *Chlorella* sp. *CW2*. With the aim of optimizing and making the biorefinery process greener, the biomass of *Chlorella* sp. *CW2* was used after the harvesting without any additional treatments. 10 g of wet biomass was directly employed for lipid extraction as described in the Materials and Methods section, yielding a total lipid extraction of 4.7%. The extracted oil was analyzed to assess its content in fatty acids, which resulted in 34.6 ± 3.6% of the total oil. Fatty acid profiles are depicted in Figure 5. The profile

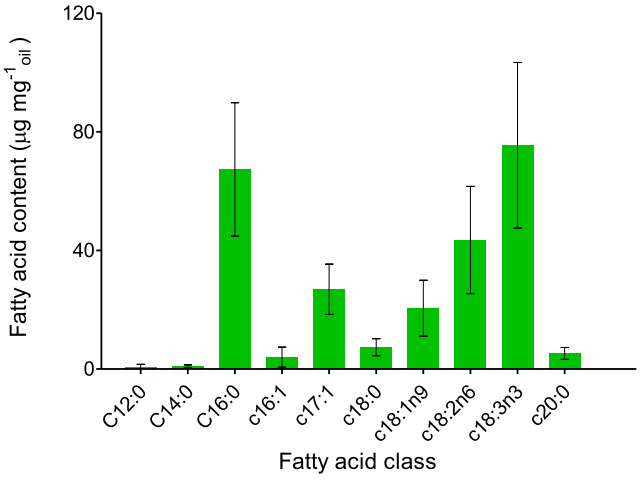

Figure 5. Fatty acid content of the microalgal cells of wet *Chlorella* sp. *CW2*. Analysis was done on extracted lipids. Standard deviation is reported as error bars. C12:0: lauric acid; C14:0: myristic acid; C14: myristoleic acid; C16:0: palmitic acid; C16: palmitoleic acid; C17:1: *cis*-10-heptadecanoic acid; C18:0: stearic acid; C18:1n9: 9-elaidic acid; C18:2n6: linoleic acid; c18:3n3: linolenic acid; and C20:0: arachidonic acid.

confirmed that previously found from the whole biomass reported in Figure 2, showing that the oil extraction from wet biomass was effective and did not alter the profile. Furthermore, the wet biomass had 92.4% water content.

Total lipids were extracted from *Chlorella* sp. *CW2* wet biomass with solid−liquid extraction with ethyl acetate as a greener and safer solvent compared to chloroform−methanol previously used on the dry biomasses. Total lipids, furthermore, were not saponified but were used as extracted in a biocascade enzymatic reaction involving, at the same time, a hydrolysis of triglycerides in FFAs mediated by lipase and the hydration of FFAs containing a Δ9 double bond mediated by the Em_OhyA enzyme. All the different products obtained after the reactions were characterized with GC−MS, and the results are reported in Figure 6.

Commercial lipase from *C. rugosa*, a widely used enzyme for triglyceride hydrolysis since it is highly efficient and active over a wide pH and temperature range, was used for the first step of the biocascade enzymatic reaction.[34] In the samples obtained after the biocascade reactions, a decrease in the amount of substrates for Em_OhyA enzyme (namely, C16:1, C18:1, C18:2, and C18:3) compared to the only treatment with lipase was observed (Figure 6a), indicating that the two enzymes are able to work simultaneously and yield HFAs of a different nature. This is a remarkable result, as in the described process, the algal oil was emulsified with the detergent Tween 20. Usually, the lipase enzyme prefers an aqueous−oil bilayer,[35] which does not meet the OhyA requirements, in need of strictly emulsified oil. The percentage amount of the obtained HFA indicated that the substrate consumption is proportional to the relative abundance of the substrates (Figure 6b), which is already reported in Figure 5. The diversity of the obtained products indicates that microalgal oils may be used to obtain differently functionalized HFAs; the multiple functionalization, including the presence of double bonds, holds interest for the chemical industry, as it creates an ideal substrate for the development of biobased materials such as lubricants and polymers.[14,36]

After characterizing the carbohydrate content of the wet defatted biomass, resulting in 0.8 ± 0.1%, it was utilized in catalytic reactions to obtain 5-HMF as already done for dry biomasses. In this case, yields from 18.9 ± 0.9 to 24.0 ± 1.2% were obtained (Figure 7), with a slight decrease compared to the same reaction realized on dry biomass, probably due to the residual presence of solvent in the treated biomass. In this case, the presence of MIBK after 4.5 h did increase the reaction yield as expected, while after 3 h, the difference falls within the experimental variability, remaining constant. Although biphasic reactions generally increase the reaction yields because of a reduction of side reactions, it can happen that the yields remain constant because no great quantities of side products are yielded.

Even with a slight decrease in the overall reaction yields, the reaction is effective, demonstrating that the operation may be successfully conducted on wet biomass, avoiding high-impact operation of biomass drying.

Although some biorefinery processes on microalgal biomass were previously proposed,[37] in this work for the first time, two innovative operations are integrated and optimized in a process involving the biomass of three different microalgal biomasses. The proposed process aims to produce chemical building blocks which may be used in the chemical industry, and for this reason, the microalgal biomass may be derived from the biological treatment of wastewaters. This approach is innovative and may help expand the microalgal industry.

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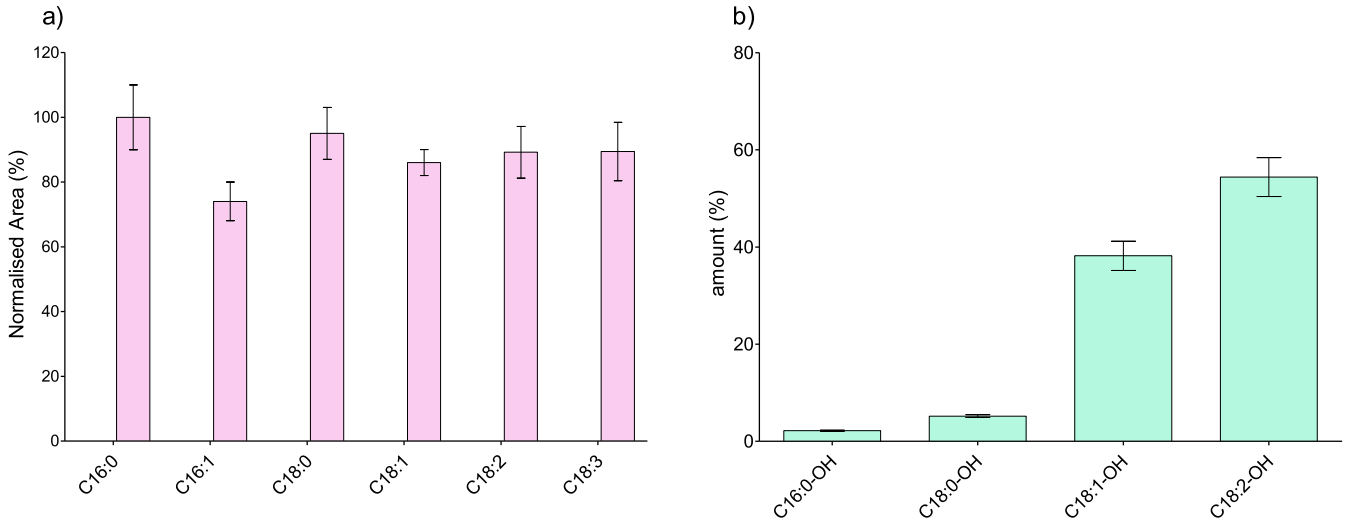

Figure 6. Biocascade reaction yields. (a) Reported values represent the area of the peaks of GC−MS analysis after one-pot reaction, relatively to the sample treated with only lipase and (b) relative abundance to several classes of HFAs obtained after the biocascade reaction on *Chlorella* sp. CW2 fatty acids.

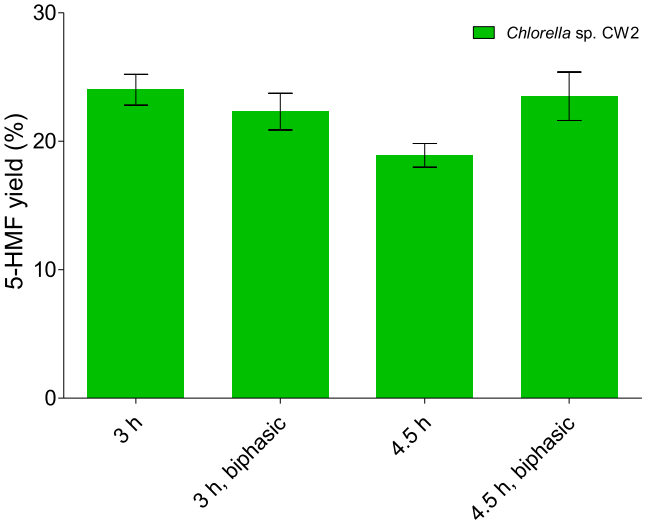

Figure 7. Yield in 5-HMF to total sugars contained in the microalgal cellular debris after catalytic conversion in different conditions with niobium phosphate as the catalyst reaction with methyl-isobutylketone as a cosolvent on *Chlorella* sp. CW2 processed from wet biomass.

CONCLUSIONS

This work demonstrates the potential of using microalgal biomass from three different strains in a biorefinery process involving the biotransformation of fatty acids in HFAs and the chemical catalysis of the residual biomass in 5-HMF. First, dry biomass was employed, yielding a C18:1 substrate conversion of approximately 68.7, 83.4, and 71.5% and a maximum 5-HMF yield of 28.6 ± 1.4, 35.2 ± 5.3, and 25.2 ± 1.5% for *Chlorella* sp. *CW2*, *N. gaditana,* and *Chlorella* sp. Barcarello, respectively. After process optimization on *Chlorella* sp. *CW2*, which involved using wet biomass and a biocascade enzymatic extraction, multiple functionalized HFAs were obtained from the lipid fraction, and the wet defatted biomass was successfully used for 5-HMF catalysis. This work shows a proof-of-concept process for producing chemical building blocks from microalgal biomass potentially sourced from wastewater treatment. This approach could significantly reduce the costs of the microalgal industry.

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Notes

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