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Exploitation of microalgae biomass under an integrated biorefinery approach

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As known, microalgae are an appealing source of chemicals and high-value compounds which find application in nutraceuticals, cosmetics and pharmaceutics. Fatty acids (FA), in particular, have drawn attention to the possibility of employing them as a source of biodiesel alternatively to fossil fuels. In addition, several lipid derivatives have been found in microalgae and may be employed in several biotechnological applications. Hydroxy fatty acids can be substrates for several industrial applications thanks to their functionalization, which increases their reactivity and, for this reason, can be used as functional building blocks to produce a multitude of bio-based materials. Recently, a promising method for the chemical modification of unsaturated-FAs (U-FA) has appeared. In fact, U-FA may be modified by members of the hydratase enzyme family to produce saturated and unsaturated hydroxy fatty acids with high stereo- and regio-selectivity. These enzymes are able to introduce a water molecule to the double bond present in the free fatty acids (FFA) Oleic Acid (OA), Linoleic Acid (LA), producing 10-hydroxy fatty acids (10-hydroxy-FAs).

Furthermore, the carbohydrate component of the microalgal biomass may be converted into furfuryl compounds and, in particular in 5-hydroxyl methyl furfural (5-HMF). This is one of the chemical bio-compound different from petroleum-derived ones with the highest added value and may be obtained through lignocellulosic biomasses or hexoses sugars through acid catalysis. It is defined platform molecule because it is the precursor of several compounds for the chemical industry.

In this work, we aimed to optimize a circular bioprocess by performing, starting from the same biomass, two different processes: the biotransformation of microalgal FFAs through the employment of a genetically modified *E. coli* on one side, and the conversion of the remaining biomass in furfuryl products. The first process allowed the production of very interesting lipid derivatives with biotechnological applications, including 10 hydroxy-stearic acid and 10-hydroxy-octadecenoic acid. The second process was obtained through heterogeneous catalysis based on niobium phosphate.

This procedure represents a high-innovative application of microalgal biomass and allows the simultaneous exploitation of FAs and carbohydrates. This may result in an increase in the commercial value of microalgal biomass.

* 1. Introduction

Microalgae are an interesting biomass due to several reasons such as their ability to growth in not-arable lands, in wastewaters or seawaters (Lima et al., 2019), and their ability to photosynthesize, namely converting the solar radiation in energy and organic carbon starting from inorganic carbon sources such as atmospheric CO2. There are different species of microalgae and several of them find application in various industrial sectors due to the high-value compounds that they produce, especially if triggered by stressors (Arena et al., 2021). Although several benefit, microalgal cultivation may be economically not convenient; for this reason, in the last years attempts to make algal cultivation more sustainable were made. In particular, microalgal biomass may be considered as a potential source of several products, in an integrated biorefinery view. In this approach, several streams of the biomass are exploited for the parallel production of numerous products. Examples of biorefinery processes may involve biomass previously employed in the treatment of sewage wastewaters or biomass residual of high-value products extraction. In this sense, the goal is the use of waste to obtain value-added products.

In this work we exploited two streams deriving from the treatment of the microalgal biomass: the oil fraction and the carbohydrates one. The oil fraction was obtained after ball milling of microalgae and solvent extraction. The remaining biomass of the process was constituted by cellular debris containing the carbohydrate fraction.

The oil fraction of microalgae is generally studied for its use as biodiesel. This application is more appealing if microalgal oil is mainly composed by saturated FAs, and in general biodiesel is not considered an added-value product which may make sustainable algal production at high-scale. In this work, we propose to employ microalgal oil fraction for an enzymatic whole cell biocatalysis performed by enzyme of the hydratase family to produce hydroxy fatty acids using oleic and linoleic acid as substrates. These enzymes introduce a water molecule to the double bond present in the OA and LA, producing this interesting molecule which may be employed for the production of aliphatic polyesters, biodegradable biopolymers (Biundo et al., 2023). In particular, in this work the enzyme oleate hydratase from *Elizabethkingia meningoseptica* (Em\_OhyA) was used.

From the debris of this process, it is possible to obtain other products through a different process. Indeed, the cellular debris are rich in carbohydrate polymers. These may be hydrolyzed in simple sugars by acidic catalysis and subsequently transformed in furfurilic compounds by an isomerization followed by a dehydration step. In particular, the cellular matrix releases glucose which isomerizes in fructose, which in turn is dehydrated in 5-HMF. 5-HMF is a bio-based product which has been defined “sleeping giant” of the chemical industry (Tong et al., 2010), because it is involved in several processes and may be precursor for polymers, pharmaceuticals, agrochemicals, flavours and fuel components. The described reaction is catalyzed by homogeneous acidic catalysis, but in this work a solid catalyst was employed. Solid catalysts have several advantages compared to homogeneous mineral acids, which are toxic and highly polluting. A very effective solid acidic catalyst is niobium phosphate, which presents a very high acidity. Niobium catalysts were already shown to be effective in converting fructose into 5-HMF (García-López et al., 2021), while microalgae have been previously converted into 5-HMF (Jeong, 2021). This work describes a proof-of-concept process which combines the production of two molecules with a good added-value starting from an autochthonous microalgal species, *Chlorella sp*. CW2. The process may be used to make the cultivation of microalgal biomass sustainable and may help in spreading the microalgal industrial sector thanks to the abatement of costs.

* 1. Materials and methods
     1. Algal growth

Microalga *Chlorella sp*. CW2 was previously isolated from the activated sludge obtained from a real wastewater treatment plant and molecularly characterized (Lima et al., 2022). The strain was kept in liquid medium. For this work, a commercial fertilizer (Spray-feed, Pavoni) diluted in H2O was employed at the concentration of 3 g L-1. A pre-culture of the microalgae was set up by inoculating 50 mL of sample from a culture flask in 500 mL of fresh medium. When cells were in late exponential phase (after about 10 cultivation days), the cell suspension were used to inoculate a bubble column photobioreactor with the volume of 5 L. Algae were cultivated for 11 days under a photon flux density of about 250 μmol m-2 s-1. Light intensity was measured with a Delta Ohm-HD 9021 quantometer equipped with a Photosynthetic Active Radiation (PAR) probe (Delta Ohm LP 9021 PAR). The cultivation was repeated four times to harvest enough biomass for the following treatments. The growth was followed by reading the absorbance at 750 nm with a Cary Uv-Vis (Agilent, US) spectrophotometer and by correlating the value with concentration in dry weight through a calibration curve. At the end of the cultivation, biomass was collected, harvested by centrifugation, lyophilized for 24 h in a bench lyophilizator (Alpha 1-2 LDplus, Christ, DE) and homogenised.

* + 1. Microalgal cells treatment to obtain oil and carbohydrate fractions

Microalgal triglycerides were extracted from microalgal biomass through a solid-liquid extraction. Briefly, approximately 2 g of dry biomass suspended in 30 mL NaCl 1M water solution were mechanically lysed by ball milling (Retsch PM 100, DE) for 25 min at 500 rpm. The mixture was subsequently extracted with about 100 mL of Chloroform/Methanol 1:1. The lower phase, containing oil dissolved in chloroform, was collected. The entire procedure was repeated twice and the organic phases were pooled together. The mixture was evaporated in a rotavapor. The upper phase, containing methanol, water and cellular debris was centrifuged to collect the solid fraction. The cellular debris was then rinsed twice with water and dried in an oven over night at 60°C and stored till further analysis and treatment.

* + 1. Preparation of free fatty acids mixture from microalgal oil and whole-cell biocatalyst

The free fatty acid (FFA) mixture was obtained from the triglyceride extracted from the microalgae through a hydrolysis reaction. Briefly, 100 mg of oils were mixed with 2 mL of 20% (w/v) KOH in Ethanol/water 7:1 and incubated for 1 h at 80 °C. Then, the solution was acidified with 100 µl of H2SO4 20% (v/v). 1 mL of ethyl acetate was added to the mixture to extract the FFAs. This mixture was used as substrate for the biocatalytic reaction.

The biocatalyst used in the reaction was *Escherichia coli* BL21 (DE3) whole-cells containing the recombinant enzyme *Elizabethkingia meningoseptica* oleate hydratase (Em\_OhyA) which was expressed as previously described (Biundo et al., 2023). Briefly, after the growth and the expression of the recombinant enzyme, the cells were centrifuged and the pellet stored at -4°C for subsequent use.

* + 1. Analysis of fatty acid content in oils and quantification

The analysis of FAs was performed on microalgae following the National Renewable Energy Laboratory (NREL) method. It was performed by the simultaneous transesterification and extraction of fatty acid methyl esters (FAMEs) and subsequently gas chromatography analysis. Briefly, approximately 10 mg of microalgal biomass were weighed in a HPLC-vial and mixed with 200 µl of chloroform/methanol (2:1, v/v) solution, 300 µl of HCl 0.6 M in methanol and 25 µl of internal standard (pentanoic acid, 10 mg mL-1). The vials were tightly sealed and put in a water bath at 85°C for 1 h. After cooling down at room temperature for 15 min, 1 mL of hexane was added, vortexed and left at room temperature for 1 h. Then, the upper phase was transferred in a new vial and 1 µl was analyzed by gas chromatography using a GC 7890A System (Agilent Technology, USA) equipped with a flame ionization detector (FID) and a capillary column Omegawax 250 (Agilent Technologies, USA). The initial temperature was 50 °C, increased to 220 °C as working temperature. Total analytic time was 79.5 min and argon was used as carrier. The quantification of lipid was performed by comparing samples chromatograms with the standard. Supelco 37-Component FAME Mix (Sigma-Aldrich, USA) was used as standard. Analysis were performed in triplicate and average value retained.

* + 1. Biocatalytic reaction on FFA from *Chlorella sp. CW2* and analysis

The biocatalytic conversion of unsaturated FFA to hydroxy-FFA (HFFA) was carried out at 37 °C and 200 rpm for 24 h in 50 mL unbaffled Erlenmeyer flasks containing 10 mL of 50 mM NaCitrate buffer pH 6 supplemented with 0.4 mM Tween 20 and 10 mg mL-1 wet cell weight (WCW) biocatalyst and 21 mg microalgae-derived FFA. The reaction was stopped by the addition of 4 mL EtOAc for liquid-liquid extraction of FFA and HFFA mixture. An aliquot of 1.4 mL was collected, out of which 40 µL were separated into a new vial, evaporated at 80 °C and stored at -20 °C until further analysis. The mixture of FFA and HFFA present in the collected samples at the end of the bioreactions was analyzed by HPLC after converting them into phenacyl esters through derivatization with 2-bromoacetophenone catalyzed by triethylamine, as previously described (work submitted) by using a Waters Alliance 2695 separation module (Waters, MA, USA) equipped with a Kinetex EVO C18 column (Phenomenex, 150 mm x 4.6 mm, 100 Å, 5 μm) coupled to a Waters 2996 UV detector set at 242 nm.

* + 1. Catalytic treatment of *Chlorella sp. CW2* debris to obtain furans and analysis

A stainless-steel autoclave (Tefic Biotech Co. Limited, Xi’an, China) was used as reactor to perform the catalysic process. Inside the reactor, a 50-mL PTFE chamber was placed, in which, in a typical experiment, 24 mL of water and 40 mg algal de-fatted biomass (dried cellular debris, see section 2.2) were placed. The heterogeneous catalytic reactions were carried out in the presence of commercial powder of NbOPO4·nH2O provided from Companhia Brasileira de Metalurgia e Mineração (CBMM) and used without further treatments. The reactor was placed in an oil bath set at a fixed temperature and magnetically stirred (LLG-uniSTIRRER 7). Variables studied were the pretreatment of the biomass, reaction temperature and mass ratio catalyst/biomass. After the experiments, aliquots of each batch were filtered and 5-HMF was analyzed using a HPLC Dionex UltiMate 3000 equipped with a column Rezex ROA-Organic acid H+ operating at 60 °C and using 0.6 mL·min-1 of a 5 mM H2SO4 aqueous solution as eluent.

* 1. Results and discussion

In Figure 1, a typical growth curve is shown as the variation of cell concentration in g L-1 over time. The culture displayed the typical lag and exponential phase, followed by the start of the stationary phase. At this point, each culture was harvested to avoid change of composition due to the starvation of nutrients, naturally occurring during the stationary phase.

After the cultivation, the biomass was collected and lyophilized. Due to its high energy-requirements, lyophilization is performed only to optimally preserve the biomass and to have a correct quantification of the employed sample for the experiment. In view of an industrial or semi-industrial process aimed at the biorefining of the microalgal biomass, lyophilization may be avoided and the process may be applied directly on the wet biomass by obtaining the same result (data not shown).

Samples of the dried biomass were analyzed for the qualitative and quantitative content in FA. The results are reported as FAME content. Results, reported in Figure 2, showed that in the microalgal biomass there was a predominance of poly unsaturated FAs, and only C16:0, palmitic acid, had a high content, compared to other both saturated and monounsaturated FAs. In particular, amongst polyunsaturated FAs, C18:3n3 linolenic acid and C18:2n6 linoleic acid were the most abundant.

Figure 1: A typical growth curve of the microalga Chlorella sp. CW2, cultivated in a solution of commercial fertilizer. At the start of the stationary phase, the cultures were harvested and the biomass collected by centrifugation.

Figure 2: Fatty acid content of the microalgal cells of Chlorella sp. CW2. FAME stands for fatty acid methyl esters. Standard deviation is reported as error bar.

The results revealed the presence of substrates for the oleate hydratase employed for the production of 10-hydroxy fatty acids. These enzymes are able to introduce a water molecule to the Δ9 double bond present in unsaturated fatty acids, producing 10-hydroxy fatty acids (10-hydroxy-FAs) (Engleder et al., 2015). Although the enzyme may use different FFAs as substrates, it has a high specificity for the oleic acid, and therefore in this work it was considered as a marker for the biotransformation reaction. Considering that the enzyme is able to catalyze the bioreaction only on FFA as substrate, a preparative step of hydrolysis was performed on the extracted triglyceride from the microalgal biomass, as described in section 2.2. The mixture of FFAs was then employed as substrate for a biocatalytic reaction employing the whole cells of *E. coli* containing the recombinant enzyme Em\_OhyA. To assess the advancement of the reaction, the content of C18:1 was used as marker and analyzed (as shown in section 2.5) after the biocatalytic reaction, which was conducted for 24 h at 37°C and 200 rpm. The relative consumption compared to a blank reaction conducted without catalyst is shown in Figure 3, which showed a high reduction of the substrate, correspondent to the 81% of the initial amount. The presence of the desired product was confirmed by HPLC analysis, as described in section 2.5 (data not shown).

Figure 3: Relative abatement of the substrate oleic acid after the reaction of biotransformation in 10-hydroxy fatty acid.

As far as the de-fatted biomass is concerned, it was employed for the catalytic hydrothermal transformation of the carbohydrate content in 5-HMF in batch reactions. Several reaction conditions were tested, as reported in Table 1. In all the experiment a pretreatment, useful to release simple sugars from the biomass, was inserted before the catalytic step, aimed instead to the conversion of hexose sugars into 5-HMF. Catalytic test in experiments A and B were carried out with 40 mg of catalyst at 180°C, while the other reactions with 80 mg of catalyst at 210°C. Results, expressed as produced mmol of 5-HMF, are reported in Figure 4.

Table 1: Conditions of the experiments conducted on the defatted biomass of Chlorella sp. CW2.

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| Experiment | Pretreatment | Catalysis | Employed biomass |
| A | Acetic Acid 0.1 M, 120°C, 2 h | 4,5 h, 100% cat, 180 °C | CW2d |
| B | heterogeneous catalyst in water, 120°C, 2 h | 4,5 h, 100% cat, 180 °C | CW2d |
| C | Water, 120°C, 2 h | 4,5 h, 200% cat, 210 °C | CW2d |
| D | Acetic Acid 0.1 M, 120°C, 2 h | 4,5 h, 200% cat, 210 °C | CW2d |
| E | heterogeneous catalyst in water, 120°C, 2 h | 4,5 h, 200% cat, 210 °C | CW2d |

From the results shown in Figure 4, it appears evident that experiments A to D failed in the production of 5-HMF compared to experiment E. This occurred both if the pretreatment was conducted in water or in diluted acetic acid. In the same way, even at the lowest tested temperature of 180°C together with the lowest catalyst concentration of 100% (A and B) or at higher temperature and catalyst concentration (C and D) the effect was similar. The yield substantially increased when the catalyst was inserted directly in the reaction mixture (E), with a preliminary step at 120°C for 2 hour and a catalytic step of 4.5 h at 210°C. The optimal condition to obtain the best results, in terms of yield to 5-HMF amongst the various tests performed using defatted biomass and 80 mg NbOPO4 nH2O catalyst were a preliminary step at 120°C for 2 h, followed by a catalytic step of 4.5 h at 210°C.

Figure 4: Amount of 5-HMF produced in batch hydrothermal reaction catalysed by NbOPO4 nH2O catalyst.

* 1. Conclusions

In this work, we set up a proof-of concept process demonstrating that it is possible to employ the biomass of an autochthonous strain of *Chlorella sp*. in a biorefinery approach. The biomass was extracted from its content of triglycerides, which were saponificated in FA. The oleic acid contained in the FA mixture was employed for a biotransformation reaction for the production of 10-hydroxy FA. The cellular debris obtained as a side-stream from the extraction of oils from the biomass were instead exploited for their content in sugars and converted through a catalytic heterogeneous reaction in 5-HMF. The best tested conditions for these conversions were identified in a preliminary step at 120°C for 2 h, followed by a catalytic step of 4.5 h at 210°C, both with 80 mg of catalyst (equivalent to 200% compared to the initial biomass). Both the obtained products have a good added-value and may be used to make the cultivation of biomass sustainable. This approach may help spreading the microalgal industrial sector thanks to the abatement of costs, although a careful economic analysis should be done in order to properly evaluate the proposed technology.

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