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Easy reuse of magnetic cross-linked enzyme aggregates of lipase B from *Candida antarctica* to obtain biodiesel from *Chlorella vulgaris* lipids

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In this work, magnetic cross-linked enzyme aggregates (mCLEAs) of CALB (lipase B from *Candida antarctica*) were prepared and characterized. Moreover, a method for an easy, sustainable and economic extraction of lipids from nitrogen-starved cells of *Chlorella vulgaris* var L3 was developed. Then, the extracted lipids (oils and free fatty acids, FFAs) were converted to biodiesel using mCLEAs and chemical acid catalysis. Among several lipid extraction methods, saponification was selected given the amount of wet microalgal biomass it can process per unit of time, its low market value, and because it allows for the use of less toxic solvents. A biodiesel conversion of 80.2 ± 4.4% was obtained by chemical catalysis (1 h at 100°C) using FFAs and methanol as the alkyl donor. However, a biodiesel conversion of more than 90% (3 h at 30°C) was obtained using mCLEAs and methanol. Both chemical and enzymatic catalysts gave biodiesel with similar fatty acid alkyl ester (FAAE) composition. Methanol, at 15% (v/v) or higher concentration, caused a decrease of lipase activity and a concomitant increase in the size of mCLEA aggregates (up to 2 μm), as measured by dynamic light scattering (DLS). The magnetic character of the novel biocatalyst permits its easy recovery and reuse, for at least ten consecutive catalytic cycles (retaining 90% of the initial biodiesel conversion), using mild reaction conditions and environmentally-friendly solvents.

[**Key words:** Biodiesel; *Chlorella vulgaris*; Magnetic cross-linked enzyme aggregates; Lipase B from *Candida antarctica*; Methanol]

Increased global industrialization and improved living conditions have led to an exponential increase in fossil fuel consumption from the beginning of the 20th century to the present. Nevertheless, the depletion of oil reserves and the environmental problems caused by the burning of fuels and the consequential greenhouse gas emissions have increased the interest for greener alternatives (1).

In this regard, biodiesel is a clean-burning diesel fuel composed of a mixture of long-chain fatty acid alkyl esters (FAAEs) which is typically produced from non-toxic, renewable biological sources, such as vegetable oils, microbial oils, animal fats, or even used cooking oils (2,3). This renewable fuel has been considered the best candidate for diesel fuel substitution given that it can be used in any compression-ignition engine without modification requirements. Moreover, biodiesel produces fewer emissions than mineral diesel in terms of unburnt hydrocarbons, carbon monoxide, sulphur content and particulate matter (2).

At present, biodiesel is mainly obtained from conventionally grown edible oils and non-edible plant oils (4). However, social and environmental issues, such as the high demand for water, land, and fertilizers, have limited its production (1). In addition, non-edible plant oils have operational problems due to the additional pretreatment required as a consequence of their high concentration of free fatty acids (FFAs). As result of these drawbacks, nowadays the search for a more sustainable biodiesel feedstock continues and focuses on microbial oils, mainly from microalgae (3,5,6).

Microalgae are very versatile microorganisms that can serve as a sustainable and economic source to obtain biofuels (1). They are good candidates for biofuel production because of their higher photosynthetic efficiency and biomass production than other alternative sources (7,8). Given their high photosynthetic rates, microalgae do not only serve as an effective carbon sequestration platform, but they do also accumulate lipids -up to 77% of dry cell mass-in their biomass (9).

In spite of the potential for microalgae-based biodiesel, its production on an industrial scale still faces many challenges in order to become economically feasible. From the production chain perspective, the critical issues are mainly the use of suitable microalgal species, the development of high-efficiency photobioreactors, the improvement of culturing strategies, the extraction of lipids and their further transesterification methodology, preferentially from wet biomass (1,10). On the other hand, biorefineries,

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which use every component of the raw biomass material, could help probably to lower the cost of production (11).

Biodiesel is commonly obtained by using alkaline catalysts, such as sodium or potassium hydroxides, since these catalysts give high yield in short reaction times (11). However, alkaline catalysis is problematic when oils with high content of FFAs, such as non-edible vegetable oils, microalgae oils and waste cooking oils, are used. This is mainly due to the formation of soaps, which decreases the reaction yield and increases the purification costs, resulting in a more environmentally-unfriendly process (2,11). On the other hand, acid catalysts can also transform both FFAs and triacylglycerides (TAGs) into esters (biodiesel). However, the reaction of transesterification with an acid catalyst is much slower than with an alkaline one, and the use of more methanol is required. In addition to this, acid catalysts are highly corrosive and so are seldom used on an industrial scale (11).

Finally, lipases can also catalyze the transformation of both FFAs and TAGs to esters, but at lower temperatures than in alkaline and acid catalysis (30-40°C) (11). In contrast to chemical catalysis, enzymatic production of biodiesel permits the reduction of downstream processing, the use of mild reaction conditions and the easy recovery of glycerol, while avoiding product purification and chemical waste production (11–13). Thus, biotechnological production of biodiesel catalyzed by lipases has received great consideration in recent years (14,15).

These advantages prove that enzyme-catalyzed biodiesel production has a high potential to be an eco-friendly process and a promising alternative to the chemical process. Nevertheless, many drawbacks (e.g., high costs of enzyme, slow reaction rates and enzyme deactivation by solvents) remain to be solved (16,17).

Enzyme immobilization is one way to compensate for the high cost and instability of lipases. Recently, we have developed a method to prepare magnetic cross-linked enzyme aggregates (mCLEAs) of lipase B from *Candida antarctica* (CALB). This method consists of the cross-linking of insolubilized lipase to aminated magnetic nanoparticles (MNPs) by glutaraldehyde (18–20). The resulting mCLEAs fulfil the main benefits of both magnetic biocatalysts and CLEAs, as they show improved thermal and storage stabilities, and can be reused after an easy recovery from the reaction mixture with a magnet. This procedure avoids the use of filtration or centrifugation that often results in the irreversible aggregation of CLEAs and the formation of clusters (clumping) (21). mCLEAs of different enzymes have also been successfully used to hydrolyze starch (22), decolourize recalcitrant dyes (23), obtain biosurfactants (24), or convert other lipids into biodiesel (13,19,25).

The aim of this work was to produce biodiesel from the microalga *Chlorella vulgaris* var L3 by means of an easy and economic method. For that purpose, the efficiency of various extraction methods, such as Soxhlet, shake flask and saponification, were compared. Moreover, the potential feasibility to use the resulting defatted biomass to obtain other added-value co-products was evaluated. Then, the extracted lipids were converted into biodiesel catalyzed by mCLEAs of CALB. The enzymatic reaction was optimized based on temperature, agitation method, concentration and type of substrates and the possibility of reusing the biocatalyst. Finally, catalysis using mCLEAs of CALB was compared to acid catalysis.

MATERIALS AND METHODS

Materials Lipozyme CALB L, the lipase B of *C. antarctica* (CALB, EC 3.1.1.3, containing 19.1 U/mg protein and 7.50 mg protein/ml) was kindly provided by Novozymes (Bagsvaerd, Denmark). 3-Aminopropyltriethoxysilane (APTS), $NaBH_4$, $FeCl_2$, $FeCl_3$, Triton X-100 and standard high purity fatty acid methyl/ethyl esters (FAMEs and FAEEs) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Standard FFAs were purchased from TCI Europe (Zwijndrecht, Belgium). Olive oil (Carbonell), used as a control substrate for transesterification reactions, was acquired at a local supermarket. All other chemicals were supplied by Merck (Darmstadt, Germany).

Microalgae culture Cells of *C. vulgaris* var L3, cultured according to Ikaran et al. (26) using 6 mM sodium nitrate as the sole nitrogen source, were obtained from Neiker-Tecnalia (Arkaute, Spain). After nitrogen depletion, cultures were incubated under the same conditions for seven more days to obtain nitrogen-starved cells (denoted as stressed cultures). Finally, the biomass was harvested by centrifugation and dried in an oven. The potential factor of lipids to obtain biodiesel from microalgal biomass was estimated according to Lepage and Roy (27).

Lipid extraction Soxhlet and Soxtec extraction were tested. Samples (70 g) of dried *C. vulgaris* var. L3 biomass were treated in a 500 ml Soxhlet extractor. The extraction was initially performed using *n*-hexane according to UNE-EN-ISO 659 (i.e., 250 ml of *n*-hexane per 25 g of sample) using a total extraction time of 14 h. After *n*-hexane extraction, two consecutive extractions with chloroform:methanol (2:1, v/v) were carried out during 15 h each. After the first extraction, the sample was ground to facilitate the second solvent extraction. The extracted lipids (denoted as oil 1) showed a solid-like consistency due to the presence of complex lipids (mainly phospholipids and glycolipids) together with other simple lipids. This crude oil was used as substrate for biodiesel production by enzymatic catalysis. The extraction was optimized in order to set-up an optimized standard extraction method. Thus, the mixture of chloroform:methanol (2:1, v/v) was used with two different extraction times (8 and 16 h), and solvent concentration of sample (40 ml per 1–5 g) was studied. Then, the extraction was carried out with Soxhtec equipment (Soxtec Auto Fat Extraction System, FOSS Analytical, Hilleroed, Denmark).

Shake flask extraction was tested using biomass samples extracted with *n*-hexane which were ground and homogenized. The flask, with a reflux condenser, was heated at 72°C for 30 min and magnetically stirred at 420 rpm. Samples of 75 mg of dry biomass per ml of solvent (chloroform:methanol 2:1, v/v) were extracted. Then, the preparation was vacuum-filtered to separate the pellet from the solvent. The pellet was collected onto the filter (fraction extracted from microalgae) and subjected to a second extraction under the same conditions. After each extraction, the sample was washed with 50 ml of solvent. The extracted sample (denoted as oil 2), showed a solid consistency (as occurred with oil 1) due to the presence of both saponifiable and unsaponifiable lipids.

Saponification extraction was carried out according to Molina Grima et al. (28). Briefly, fresh biomass was saponified at 65°C with 30 ml ethanol (containing 0.2 g KOH) per g of dry biomass. After 3 h of treatment, the unsaponifiable fraction was extracted twice with *n*-hexane (100 ml each). The saponifiable fraction was converted into FFAs by setting the pH to 3.5 with sulphuric acid. Finally, the extracted preparation of real FFAs (denoted as rFFAs) was washed thrice with *n*-hexane (100 ml each) and used to obtain biodiesel by both acid and enzymatic catalysis.

Model FFAs mixtures A model mixture of FFAs (denoted as mFFAs), with similar composition to that of *C. vulgaris* var L3, was obtained by mixing 28.2% (w/w) palmitic, 2.8% (w/w) oleic, 22.3% (w/w) linoleic and 46.7% (w/w) linolenic acids obtained from commercial sources.

mCLEAs of CALB Magnetic nanoparticles (MNPs) of magnetite and mCLEAs of CALB were obtained as previously reported (19), using ammonium sulfate and glutaraldehyde as precipitating and cross-linking reagents, respectively. The final preparation was thoroughly washed and stored at 4°C in phosphate buffered saline (PBS) (150 mM, pH 4.4) until use. Prior to its first utilization, and before each reutilization in consecutive catalytic cycles, the magnetic biocatalyst was washed and resuspended in the same solvent (e.g., *n*-hexane, methanol, ethanol, 2-propanol) used in the reaction mixture.

Biodiesel production Biodiesel from rFFAs (0.5 g) obtained by acid catalysis was carried out in a 250 ml volumetric flask containing 20 ml of acetyl chloride:methanol (5:95, v/v). The flask, equipped with a reflux condenser, was magnetically stirred at 100°C for 1 h. After cooling, the biodiesel was extracted twice with 5 ml of *n*-hexane and 10 ml aqueous NaCl (0.5%, w/v). Chemically catalyzed biodiesel was only obtained with rFFAs preparations and analyzed by gas chromatography.

The enzymatic (trans)esterification reaction was carried out using mCLEAs of CALB as catalyst in the presence of various alcohols used as alkyl group donors. The production of FAAEs was assessed by analysis of 5 μl samples withdrawn at different times.

For transesterification reactions, both microalgal crude oils (oil 1 or oil 2) and FFAs preparations were used as substrates. Crude microalgal oils (30 mg containing 50% w/w, of unsaponifiable lipids) were mixed with 400 ml of 2-propanol and 100 ml of *n*-hexane to decrease the viscosity and facilitate the characteristically difficult handling of these oils. Reactions were routinely stirred by mechanical agitation in a gyratory mixer (ELMI, Intelli-Mixer RM-2L, Riga, Latvia) from 40°C to 50°C, and initiated by the addition of 1% (w/w) of mCLEAs (in relation to substrate). Initially, the magnetic biocatalyst was washed with *n*-hexane but, due to its high volatility, it was substituted by the alkyl donor used in forthcoming assays. Thus, transesterification reactions were carried out without any cosolvent. In these reactions, using FFAs as substrate, a stoichiometry of 10:1 (mol:mol, alcohol:fatty acid) was used, instead of the molar ratio 3:1 used for oils.

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