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Optimization of enzyme-assisted lipid extraction from *Nannochloropsis* microalgae

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ABSTRACT

Cell wall disruption is one of the most critical and energy-consuming steps in the overall process of lipid extraction from microalgae. In this paper we investigate a multi-enzyme pretreatment based on the use of cellulase (CEL) and mannanase (GMA) for the recovery of lipids from the marine microalga *Nannochloropsis* sp. A central composite design coupled with response surface methodology was used to optimize the treatment and evaluate the effect of temperature (T=15–75 °C), pH (2–8), pretreatment time (P=30–270 min), CEL dosage (D_1=0–20 mg/g) and GMA dosage (D_2=0–2 mg/g) on lipid extraction. About 90% of lipids were recovered from *Nannochloropsis* cells pretreated under optimal process conditions (T=53 °C, pH=4.4, P=210 min, D_1=13.8 mg/g and D_1=1.5 mg/g). FTIR spectra and TEM images of *Nannochloropsis* cells showed that the enzymatic treatment produced significant alterations in the algal cell wall structure, with extensive loss of cell boundaries and release of intracellular material. Overall, the results obtained indicate that the proposed approach can significantly improve the recovery of lipids from *Nannochloropsis* sp., further supporting the use of enzymes in microalgal processes.

1. Introduction

Microalgae are considered an attractive lipid feedstock for biodiesel production due to their high lipid content, easy adaptability to growth conditions and the fact that they can be cultivated without competing with agriculture for land, water and nutrients [1] Currently, however, commercial production of biodiesel from microalgae is not competitive with that from traditional sources [2,3]. One of the factors hindering the large-scale use of microalgae as a lipid source is the high energy consumption associated with the recovery of lipids [4]. In fact, in most microalgal species, lipids are located inside the cell, which must be disrupted to allow extraction. The hardness of algal walls and their organization into a complex multi-layered structure make disruption an energy-intensive process [5]. Common methods of cell disruption involve the use of mechanical (*e.g.*, ultrasonication, high-pressure homogenization, bead beating) or chemical (*e.g.*, alkali, acid, detergent) means. In addition to requiring a large consumption of energy or chemicals, these treatments may cause damage to the most easily degradable algal components, such as proteins and carotenoids, that could be co-extracted with lipids in a biorefinery perspective [6,7].

The above considerations have prompted efforts to find alternative methods of cell disruption, such as those based on the use of cell wall degrading enzymes [8]. Enzyme-based treatments rely on the ability of some hydrolyzing enzymes to degrade structural cell wall components, thereby facilitating the release of intracellular compounds. Compared to other methods of cell disruption, the use of enzymes results in higher selectivity, milder treatment conditions and better product quality.

Enzymatic treatments are generally performed by the addition of purified enzymes or enzyme preparations [9–11] or, in some cases, by using an enzymatic extract from another microorganism [12]. Recently, a new enzymatic method, known as autolysis or self-digestion and based on the disruption of the cell by endogenous algal enzymes, has been proposed by Demuez et al. [13].

The potential of enzymatic cell disruption in biorefinery applications, particularly in terms of process feasibility, disruption efficiency and specific energy requirements, has been analyzed in some recent review papers [13–15]. An examination of published studies indicates that the selection of an appropriate enzyme and the determination of optimal process conditions are key factors for the design of an effective enzymatic treatment. In particular, the type and dosage of the enzyme largely affect the process costs and effectiveness. The choice of a suitable enzyme is strictly related to the characteristics of the algal wall which, in turn, depend on the microalgae species, the growth conditions and the harvesting and dewatering steps. In some cases, the use of enzyme mixtures has been proved to provide better results than the application of

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Table 1
Main enzyme components and activities of the enzyme preparations used (T_{OPT} and pH_{OPT} are the optimal temperature and pH values provided by the manufacturer).

Enzyme preparation	Code	Biological source	Main enzyme component	T_{OPT} (°C)	pH_{OPT}	Activity (U g^{-1})
Cellulyve® 50LC	CEL	*Trichoderma reseei*	Cellulase (EC 3.2.1.4)	55	4.5	≥240
Feedlyve® GMA	GMA	*Aspergillus niger*	Endo-β-1,4-mannanase (EC 3.2.1.78)	50	5.0	≥10,000

single enzymes [13]. However, very few studies have investigated the possibility of using rigorous methods like, for example, the mixture design approach, to formulate optimal enzyme mixtures for the recovery of lipids from microalgae [16] or, more in general, the hydrolysis of cellulosic biomass [17]. As regards the treatment conditions, temperature, pH, enzyme dosage, hydrolysis time and agitation speed are the most influential variables [14]. Some of them may exhibit synergistic or antagonistic interactions, with important implications on the disruption efficiency [18].

The aim of the present study was to optimize the enzyme-assisted extraction of lipids from *Nannochloropsis* sp. using cellulose/mannanase enzyme mixtures. A rigorous approach based on central composite design and response surface methodology was used to evaluate the effects of the main process variables, including single enzyme dosages, on lipid recovery. *Nannochloropsis* is a marine microalga of great industrial interest because of its ability to accumulate large amounts of lipids and other valuable compounds, such as eicosapentaenoic acid (EPA) and carotenoids [19–21]. However, it shows unusual resistance to mechanical and chemical treatments, which makes the extraction of intracellular components a challenging and energy-consuming process [22,23]. The results of the present study indicate that an optimized multi-enzyme treatment of *Nannochloropsis* microalgae can lead to a significant improvement in lipid extraction. The approach followed can be applied to optimize the enzyme-assisted recovery of any intracellular component from this or other microalgae and identify the contribution of each process variable to the extraction efficiency.

2. Materials and methods

2.1. Chemicals, enzymes and microalgae

Methanol, isopropanol, chloroform, hexane, sodium chloride, hydrochloric acid (37 wt%) and sodium hydroxide, all of analytical grade, were purchased from Carlo Erba (Milano, Italy).

Cellulyve® 50LC (CEL) and Feedlyve® GMA (GMA) were obtained from Lyven SA (Colombelles, France). The main components of the two enzyme preparations and their activities are reported in Table 1. The enzymes were dissolved in distilled water and the pH of the solutions was adjusted to the desired values by adding 0.1 N HCl or NaOH.

Nannochloropsis sp. was provided in lyophilized form by the Department of Agri-Food Production and Environmental Sciences of the University of Florence (Italy). The growth conditions and the reactor configuration used are given in Bondioli et al. [24].

2.2. Determination of total lipid content

Total lipid content of *Nannochloropsis* was determined by the method of Ma et al. [25] with slight modifications. Specifically, 0.2 g of biomass were mixed with 18 ml of chloroform/methanol (2:1, v/v) and stirred at 37 °C for 1 h. Then, the mixture was centrifuged (10,000 × g, 10 min), the supernatant collected and the residual biomass re-extracted two more times under the same conditions. The three supernatants were combined and a 1% sodium chloride solution (20% of the total volume) was added. After 10-min stirring at 37 °C, the mixture was centrifuged (10,000 × g, 10 min), the organic phase recovered and evaporated under vacuum at 40 °C. The lipid content was calculated from the weight of the residue and expressed as grams of lipids per 100 g of dry biomass.

2.3. FTIR and TEM studies

Fourier-transform infrared (FTIR) studies were carried out on dry biomass samples. The samples were quartered as described by Diniz et al. [26] in order to obtain a representative and homogeneous portion of material from the total amount of biomass. Final samples consisted of 5 mg of microalgae. They were stored in sealed plastic bags in the dark until analysis. Measurements were performed on a Bruker Vertex 70 spectrometer (Bruker Optics, Billerica, MA, USA) equipped with a Platinum ATR sampling module. FTIR spectra were collected in duplicate in the mid infrared region (4000–600 cm^{-1}) at a resolution of 3 cm^{-1}.

TEM samples were prepared by smearing a suspension of microalgae on poly-L-lysine coated slides, which were air-dried and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) solution. Post-fixation of the slides was done in 4% osmium tetroxide for 1 h, followed by rinsing in PB and dehydration with increasing concentrations of ethanol (30–100%). After dehydration, the specimens were immersed in propylene oxide for solvent substitution and embedded in Embed-812 epoxy resin. Ultrathin cell sections (70–80 nm) were obtained using a Leica EM UC6 ultramicrotome, post-stained (2% uranyl acetate and Reynolds lead citrate) and examined with a Zeiss EM10 electron microscope operated at 60 kV.

2.4. Enzyme-assisted lipid extraction

The enzyme-assisted extraction of lipids from *Nannochloropsis* was investigated in batch mode following the procedure described elsewhere [27]. Briefly, 0.2 g of microalgae and 10 ml of the aqueous enzyme solution (at a concentration corresponding to a nominal enzyme dosage of 0–20 mg/g) were loaded into 20-ml screw-capped flasks. The flasks were placed in a water bath thermostated at the run temperature (15–75 ± 0.1 °C) and magnetically stirred for the appropriate time (30–270 min). Then, the algal suspension was centrifuged (10,000 × g, 10 min) and the supernatant removed. The resulting biomass was contacted with 10 ml hexane/isopropanol (3:2, v/v) and stirred at room temperature for 30 min. After this time, the suspension was centrifuged (10,000 × g, 5 min) and the amount of extracted lipids determined gravimetrically after solvent evaporation.

Additional experiments were carried out, under the optimum treatment conditions determined from the modeling of the experimental data, by increasing the extraction time up to 6 h (compared to the value of 0.5 h used in central composite design experiments).

2.5. Experimental design

A central composite design (CCD) was used to investigate the effects of temperature (T), pH, pretreatment time (P), CEL dosage (D_1) and GMA dosage (D_2) on lipid extraction. The CCD consisted of a one-half fraction of the full 2^5 factorial design (16 points), augmented by two axial points per factor at distance $\pm\alpha$ from the

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