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# Enhanced Lipid Extraction from Unbroken Microalgal Cells Using Enzymes

Antonio Zuorro*[a], Roberto Lavecchia[a], Gianluca Maffei[a], Francesco Marra[a], Selenia Miglietta[b], Alberto Petrangeli[a], Giuseppe Familiari[b], Teodoro Valente[a]

[a]Dipartimento di Ingegneria Chimica, Materiali e Ambiente, Università "La Sapienza", Roma (Italy)
[b]Dipartimento di Scienze Anatomiche, Istologiche, Medico-legali e dell'Apparato Locomotore, Università "La Sapienza", Roma (Italy)
antonio.zuorro@uniroma1.it

The marine microalga *Nannochloropsis* sp. was chosen as a model organism to investigate the feasibility of using cell wall-degrading enzymes to enhance the recovery of intracellular lipids. An enzyme cocktail containing galactomannanase, 1,4-β-cellobiosidase and β-glucosidase as main components was prepared from commercial enzyme preparations. The effects of pretreatment time (P), enzyme dosage (D), pH and temperature (T) on the amount of extracted lipids were investigated using response surface methodology. Under the best conditions (P = 90 min, D = 1.3 mg $g^{-1}$, pH = 5, T = 36 °C) over 70 % of the lipids present in the microalga were recovered. SEM and TEM characterization of enzyme-treated microalgae showed extensive cell damage with significant disruption of the cell wall and release of algal material. Overall, the results obtained strongly support the use of commercial enzyme preparations to improve lipid recovery from microalgae and provide useful information on the influence of process conditions on the treatment efficiency.

## 1. Introduction

Due to the high lipid content, easy adaptability to growth conditions and the possibility of growing either in fresh or salt water, microalgae are considered one of the most promising alternative sources of lipids for biodiesel production (Makareviciene et al., 2013). Furthermore, they can utilize low-cost carbon substrates or wastewaters from various industrial activities as a source of nutrients (Vidotti et al., 2014), suggesting the possibility of integrating their production with the treatment of waste effluents. However, at the current stage of development, biodiesel production from microalgae is not economically viable (Lam and Lee, 2012). One of the major bottlenecks in the overall process is represented by the high energy consumption associated with the extraction of lipids from the algal cells. This is due to the fact that microalgae have thick and highly resistant cell walls that must be broken to allow lipid recovery. High-pressure homogenization, bead beating, ultrasonication and microwave are the most common methods of cell disruption (Lee et al., 2012). These treatments allow good lipid recovery but are energy intensive and potentially capable of damaging other valuable non-lipid components. For these reasons, alternative methods of microalgal cell disruption are actively being investigated.
Enzymatic treatment of microalgae is an attractive but still little-explored method of lipid extraction (Gerken et al., 2013). It is based on the selective degradation of cell-wall components by specific enzymes. Although the method is mild and environmentally friendly, there are two major limitations to its large-scale implementation. The first is the high cost of enzymes and the second is the lack of information on the influence of process variables on the efficiency of lipid recovery and, thus, on the possibility of optimizing the treatment.
The aim of this contribution was to investigate the suitability of commercial enzyme preparations of relatively low cost to enhance the recovery of lipids from microalgae. The marine microalga *Nannochloropsis* sp. was used as a model organism. This microalga is of great industrial interest because of its ability to accumulate large amounts of lipids (Perin et al., 2014) and other valuable components, such as the carotenoids astaxanthin and zeaxanthin and the omega-3 polyunsaturated fatty acid EPA (Leu and Boussiba, 2014).

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However, it shows unusual resistance to mechanical and chemical treatments due to the presence in its outer cell layer of algaenan, a non-hydrolyzable aliphatic component forming hard structures that resemble cutan, the major structural component of the cuticle of plants (Boom et al., 2005). This makes the extraction of intracellular components from the microalga a challenging and energy-consuming process.

## 2. Materials and methods

### 2.1 Chemicals, enzymes and microalgae

Methanol, 2-propanol, chloroform, n-hexane and sodium chloride were purchased from Carlo Erba (Milano, Italy). Glutaraldehyde, uranyl acetate, lead citrate and epoxy resin Embed-812 were from SIC (Roma, Italy), osmium tetroxide from Agar Scientific (Stansted, UK), propylene oxide from BDH Italia (Milano, Italy).
Feedlyve® GMA (Fe-GMA) and Cellulyve® 50LC (Ce-50LC) were from Lyven SA (Colombelles, France). Fe-GMA contained galactomannanase (EC 3.2.1.15) as main enzyme component, while Ce-50LC was rich in 1,4-β-cellobiosidase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). They were mixed in the ratio of 9:1 (w/w) to obtain an enzyme preparation with high cell wall-degrading activity.
*Nannochloropsis* sp. was obtained as a lyophilized powder from DISPAA (University of Firenze, Italy). The growth conditions, medium composition and reactor configuration are given in Bondioli et al. (2012).

### 2.2 Determination of lipid content

The lipid content of *Nannochloropsis* was determined using the chloroform–methanol–water solvent system as reported by Ma et al. (2013). To ensure complete lipid recovery, the biomass was re-extracted three times and the lipid content was calculated as the sum of the values obtained in each step.

### 2.3 Electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to investigate the morphological changes of microalgal cells due to the enzymatic treatment. Microalgal samples for SEM were smeared on poly-L-lysine coated slides, fixed in 2.5 % glutaraldehyde and post-fixed in 4 % osmium tetroxide followed by sequential dehydration with increasing concentrations of ethanol. The slides were cut with a diamond blade, dried in carbon dioxide, platinum-coated and observed using a Hitachi S-4000 SEM operated at 15 kV. Samples for TEM were fixed, post-fixed and dehydrated as described above, immersed in propylene oxide for solvent substitution and embedded in Embed-812 epoxy resin. A Leica EM UC6 ultramicrotome was used to obtain ultrathin cell sections (70–80 nm), which were stained with 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 recovery of lipids from *Nannochloropsis* was studied in batch mode. Different organic solvents were preliminarily screened for their ability to recover lipids from the enzyme-treated microalga, following the procedure described elsewhere (Zuorro et al., 2014b). The mixture n-hexane/2-propanol (3:2, v/v) was the most effective solvent. In these experiments, known amounts of microalgae and the enzyme solution were loaded into screw-top glass flasks and kept under agitation for the required time. Then, the flask content was centrifuged at 10,000 × g for 5 min and the recovered biomass extracted for 1 h at 25 °C with the solvent. The amount of extracted lipids was determined gravimetrically after solvent evaporation.

### 2.5 Experimental design and statistical analysis

A central composite design (CCD) was used to evaluate the effects of pretreatment time (P), enzyme dosage (D), pH and temperature (T) on the amount of extracted lipids (*y*). The CCD consisted of a full $2^4$ factorial design, eight axial points at distance ±α from the design centre and six replicated central points, for a total of 30 runs. To ensure the rotatability of the design space, the value of α was taken as $(16)^{1/4} = 2$ (Montgomery, 2012). Furthermore, the run order was randomized to minimize the effects of uncontrolled factors. Actual and coded factor levels are reported in Table 1. Coded values were obtained from the following equations:

$$x_1 = \frac{P - 90}{30} \tag{1}$$

$$x_2 = \frac{D - 1}{0.5} \tag{2}$$

$$x_3 = \frac{pH - 4}{2} \tag{3}$$

$$x_4 = \frac{T - 45}{15} \tag{4}$$

The experimental design layout and the observed amounts of extracted lipids are presented in Table 2.
The Design-Expert® software (version 7.0, Stat-Ease Inc., Minneapolis, MN, USA) was used for the design and analysis of experiments.

## 3. Results and discussion

The lipid content of *Nannochloropsis* was 53.2 ± 1.2 wt%. This is a very high value, which confirms the ability of *Nannochloropsis* sp. to accumulate large amounts of lipids when grown under nitrogen starvation conditions (Bondioli et al., 2012) in efficient culture systems (Biondi et al., 2013).
The enzyme-assisted recovery of lipids from the microalga gave the results shown in Table 2, where $x_1$, $x_2$, $x_3$ and $x_4$ denote, respectively, the coded pretreatment time (P), enzyme dosage (D), pH and temperature (T). As can be seen, the amount of extracted lipids ranged from 20.8 to 36.5 wt% or, in terms of percentage yields, from 39.1 to 68.6 %. The maximum recovery was achieved at P = 90 min, D = 1 mg $g^{-1}$, pH = 6 and T = 45 °C. To correlate the experimental data, different empirical models (linear, two-factor interaction, quadratic and cubic) were considered and tested for their statistical significance.

*Table 1: Natural and coded levels of the factors included in the experimental design*

| Factor | Unit | Factor level | | | | |
|---|---|---|---|---|---|---|
| | | –2 | –1 | 0 | +1 | +2 |
| Pretreatment time (P) | min | 30 | 60 | 90 | 120 | 150 |
| Enzyme dosage (D) | mg $g^{-1}$ | 0 | 0.5 | 1 | 1.5 | 2 |
| pH | – | 2 | 4 | 6 | 8 | 10 |
| Temperature (T) | °C | 15 | 30 | 45 | 60 | 75 |

*Table 2: Experimental design layout. $y_{exp}$ and $y_{calc}$ are, respectively, the experimental and calculated amounts of extracted lipids, SO is the standard order and RO the randomized order of runs*

| SO | RO | $x_1$ | $x_2$ | $x_3$ | $x_4$ | $y_{exp}$ (wt%) | $y_{calc}$ (wt%) |
|---|---|---|---|---|---|---|---|
| 1 | 28 | –1 | –1 | –1 | –1 | 34.8 | 32.9 |
| 2 | 29 | +1 | –1 | –1 | –1 | 35.3 | 32.9 |
| 3 | 1 | –1 | +1 | –1 | –1 | 35.0 | 36.3 |
| 4 | 30 | +1 | +1 | –1 | –1 | 36.2 | 36.3 |
| 5 | 2 | –1 | –1 | +1 | –1 | 29.9 | 28.7 |
| 6 | 3 | +1 | –1 | +1 | –1 | 27.1 | 28.7 |
| 7 | 4 | –1 | +1 | +1 | –1 | 32.1 | 32.0 |
| 8 | 5 | +1 | +1 | +1 | –1 | 30.8 | 32.0 |
| 9 | 7 | –1 | –1 | –1 | +1 | 32.3 | 31.4 |
| 10 | 8 | +1 | –1 | –1 | +1 | 32.2 | 31.4 |
| 11 | 9 | –1 | +1 | –1 | +1 | 35.2 | 34.7 |
| 12 | 10 | +1 | +1 | –1 | +1 | 36.3 | 34.7 |
| 13 | 11 | –1 | –1 | +1 | +1 | 22.3 | 22.0 |
| 14 | 12 | +1 | –1 | +1 | +1 | 23.2 | 22.0 |
| 15 | 13 | –1 | +1 | +1 | +1 | 23.0 | 25.4 |
| 16 | 14 | +1 | +1 | +1 | +1 | 25.8 | 25.4 |
| 17 | 18 | –2 | 0 | 0 | 0 | 35.9 | 35.4 |
| 18 | 19 | +2 | 0 | 0 | 0 | 35.6 | 35.4 |
| 19 | 25 | 0 | –2 | 0 | 0 | 23.0 | 26.0 |
| 20 | 23 | 0 | +2 | 0 | 0 | 34.5 | 32.7 |
| 21 | 27 | 0 | 0 | –2 | 0 | 30.0 | 32.8 |
| 22 | 6 | 0 | 0 | +2 | 0 | 20.8 | 19.3 |
| 23 | 17 | 0 | 0 | 0 | –2 | 34.9 | 35.0 |
| 24 | 16 | 0 | 0 | 0 | +2 | 25.8 | 26.8 |
| 25 | 15 | 0 | 0 | 0 | 0 | 35.2 | 35.4 |
| 26 | 20 | 0 | 0 | 0 | 0 | 35.9 | 35.4 |
| 27 | 21 | 0 | 0 | 0 | 0 | 33.2 | 35.4 |
| 28 | 22 | 0 | 0 | 0 | 0 | 34.1 | 35.4 |
| 29 | 24 | 0 | 0 | 0 | 0 | 36.5 | 35.4 |
| 30 | 26 | 0 | 0 | 0 | 0 | 35.2 | 35.4 |

*Table 3: Estimates of the coefficients in eq. 6 with the associated standard errors (SE), 95 % confidence intervals (CI) and p-values (p)*

| Coefficient | Effect | Value | SE | low CI | high CI | *p* |
|---|---|---|---|---|---|---|
| $\beta_0$ | – | 35.36 | 0.57 | 34.18 | 36.53 | <0.0001 |
| $\beta_2$ | D | 1.68 | 0.34 | 0.98 | 2.37 | <0.0001 |
| $\beta_3$ | pH | –3.39 | 0.34 | –4.09 | –2.70 | <0.0001 |
| $\beta_4$ | T | –2.04 | 0.34 | –2.73 | –1.34 | <0.0001 |
| $\beta_{22}$ | D × D | –1.50 | 0.31 | –2.15 | –0.86 | <0.0001 |
| $\beta_{33}$ | pH × pH | –2.33 | 0.31 | –2.97 | –1.68 | <0.0001 |
| $\beta_{44}$ | T × T | –1.11 | 0.31 | –1.75 | –0.46 | 0.0018 |
| $\beta_{34}$ | T × pH | –1.27 | 0.41 | –2.13 | –0.42 | 0.0053 |

The best results were obtained by the following second-order polynomial equation:

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \quad (5)$$

where $y$ is the response variable, $x_i$ are the independent variables, $\beta_0$ is the intercept and $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are the linear, quadratic and interaction regression coefficients.

A stepwise regression method was used to identify the statistically significant terms in eq. (5). This procedure consists in the iterative addition and removal of terms from the model equation so as to meet a specified significance level (in our case, $p < 0.05$). Elimination of the non-significant terms led to the following reduced model:

$$y = \beta_0 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{34} x_3 x_4 \quad (6)$$

Eq. (6) provided a very good fit to the data, with coefficient of determination ($R^2$), adjusted-$R^2$ and prediction-$R^2$ of 0.919, 0.893 and 0.747, respectively. The estimates of the model coefficients, together with their standard errors, confidence intervals and *p*-values are listed in Table 3. Analysis of residuals was performed as reported in Zuorro (2014). No violations of basic ANOVA assumptions were present and the lack of fit was not significant. From the coefficient values in Table 3, we see that:

a) Three of the four main factors, namely, enzyme dosage (D), pH and temperature (T), were statistically significant, their effect on lipid recovery increasing in the order: D < T < pH;
b) They all affected the response variable through both linear and quadratic terms;
c) There was a statistically significant interaction between pH and T, indicating that the effect of pH on lipid recovery was dependent on the temperature level considered.

To better appreciate the individual and interactive contributions of factors to lipid recovery, 3D response surfaces were generated from the regression model. More specifically, the response variable was plotted as a function of two factors varying in the factorial part of the design ($-1 \le x_i \le +1$), while setting the others to their center-point values. Some representative plots are shown in Figure 1.

As can be seen, an increase in enzyme dosage increased lipid recovery, while an increase in pH caused a decrease in lipid recovery. The latter can be attributed to a progressive departure from the optimal pH values of the enzymes present in the mixed preparation, which were all close to 5. High pretreatment temperatures had a negative effect on lipid recovery but there was an optimal temperature region lying roughly between 30 and 40 °C. Since the optimal temperatures of the enzymes used are of about 50 °C, it can be hypothesized that the positive effect of temperature on cell wall-degrading activity is counteracted by some other effect causing a reduction in the extraction efficiency. A possible explanation is that higher temperatures destabilize lipid emulsions, making lipid recovery more difficult (Chabrand and Glatz, 2009). As is known, algal lipids are stored in subcellular compartments, the so-called lipid bodies, from which they must be released to allow extraction (Liang et al., 2012). Of course, coalescence of the released lipids into large droplets favor their recovery by the solvent. Similar destabilizing effects were observed for the pH of the enzyme solution, a variable that, like temperature, can affect emulsion stability (Wu et al., 2009). Finally, under the experimental conditions investigated the pretreatment time was not a significant factor. This may indicate that the enzymatic degradation of cell wall components is mostly accomplished within the first 60 min (the –1 level of the P factor), so that little improvement can be achieved with longer incubation times.

The enzymatic treatment of *Nannochloropsis* was optimized by maximization of the response variable (Eq. 6).

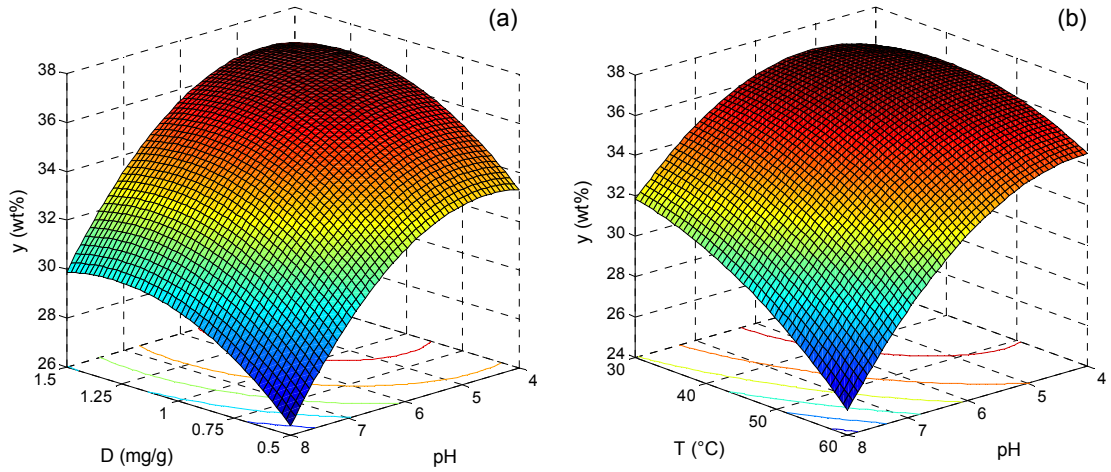


*Figure 1: Response surface plots showing the influence of enzyme dosage (D), pH and temperature (T) on lipid recovery (y) from Nannochloropsis: (a) P = 90 min, T = 45 °C; (b) P = 90 min, D = 1 mg $g^{-1}$*

The search domain was restricted to the factorial part of the design and the pretreatment time was set to its central level (P = 90 min). We obtained: D = 1.3 mg $g^{-1}$, pH = 5, T = 36 °C and $y_{max}$ = 37.3 wt%, which corresponds to a recovery of over 70% of the lipids present in the microalga. Validation experiments performed under the above conditions gave: $y$ = 35.7 ± 2.5 wt% (percentage error: 4.3%), thus confirming the good descriptive and predictive capabilities of the developed model.

To evaluate the effects of the treatment on the algal cells, samples of *Nannochloropsis* subjected to enzymatic treatment under optimal conditions and to subsequent lipid extraction were inspected by SEM and TEM. As apparent from Figure 2, pretreatment with cell wall-degrading enzymes induced significant morphological changes in microalgal cells and extensive cell damage. In particular, TEM imaging revealed disruption of large portions of cell wall and the release of algal material. This provides a clear evidence of the effectiveness of the enzymatic treatment. As a result of the enzyme action, the mass transfer resistance associated with the cell walls of *Nannochloropsis* can be expected to be greatly reduced, increasing solvent accessibility to lipid bodies and leading to high lipid recovery efficiencies.

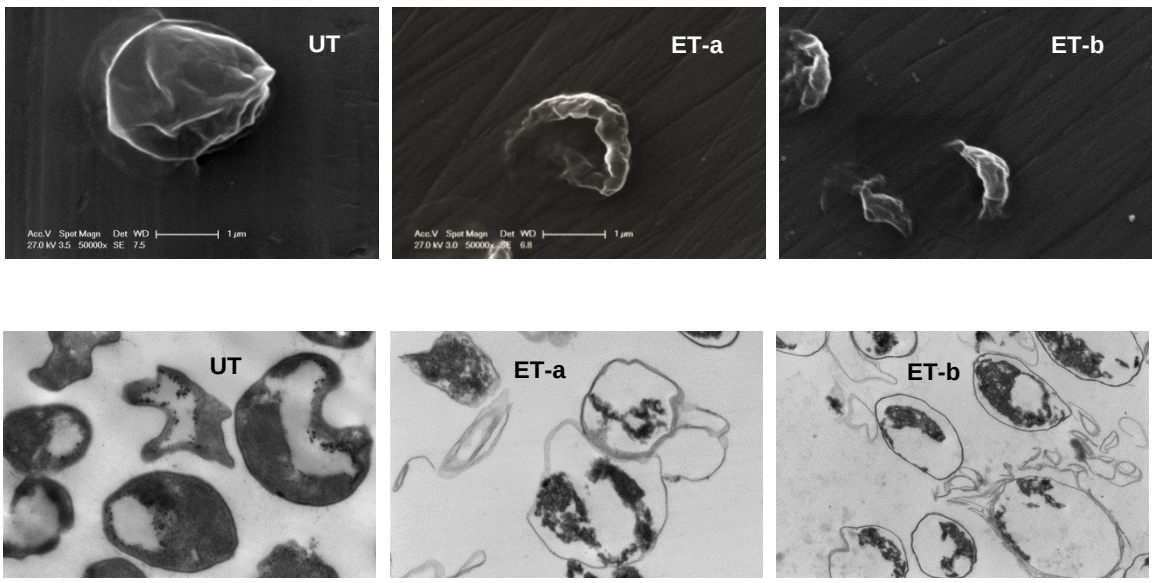


*Figure 2: SEM (upper row) and TEM (bottom row) images of Nannochloropsis cells untreated (UT) and after enzymatic treatment (ET). Treatment conditions: P = 90 min, D = 1.3 mg $g^{-1}$, pH = 5, T = 36 °C*

## 4. Conclusions

The results of this study demonstrate that enzymatic treatment of microalgal cells can be a promising alternative to conventional methods of lipid extraction. In particular, we have shown that very high lipid recovery can be achieved by proper selection of process conditions. The high cost of enzymes, which is one of the major obstacles to the large-scale implementation of these treatments, can be at least partly overcome by using mixtures of commercial enzyme preparations. In this regard, enzymes already proven to be effective in the recovery of bioactives from plant material should be tested at first (Zuorro et al., 2014a).
Future research should focus on the development of optimal enzyme cocktails based on the cell-wall composition of the algal species of interest. The possible recovery and reuse of the enzyme solution is another important issue to be investigated in future studies.

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