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Evaluation of Dissolved Organic Carbon (DOC) as a Measure of Cell Wall Degradation During Enzymatic Treatment of Microalgae

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The feasibility of using Dissolved Organic Carbon (DOC) measurements as a means to assess the efficacy of an enzyme-assisted pretreatment on the recovery of lipids from microalgae was investigated. Attention was focused on *Nannochloropsis* sp., a marine microalga of great biotechnological interest for its ability to accumulate large amounts of lipids and other valuable compounds.
The enzymatic pretreatment was carried out using two commercial enzyme preparations, one (CEL) rich in cellulase and the other (GMA) rich in galactomannase. Experiments were performed according to a fractional two-level factorial design. The factors studied were temperature (15–75 °C), pH (2–8), pretreatment time (30–270 min), CEL dosage (0–20 mg/g) and GMA dosage (0–2 mg/g). DOC was determined by a TOC analyzer and used as the response variable. Under the experimental design conditions, temperature, pH, pretreatment time and CEL dosage were found to be statistically significant ($p < 0.05$), with the former factor being the most influential. No significant interactions were observed between the main factors, indicating that each of them exerted its effect independently of the others. A good correlation was also found between the measured DOC values and the yields of lipid extraction from the enzymatically treated biomass, demonstrating that DOC measurements can be used to quantify the enzyme-induced degradation of algal cell walls.

1. Introduction

Microalgae are considered one of the most promising alternative sources of lipids 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 (Makareviciene et al., 2013). Currently, a major obstacle to the industrial development of microalgal processes is represented by the high energy consumption associated with the extraction of lipids from the cells. The reason is that microalgae have thick and highly resistant cell walls that must be broken or at least damaged to allow recovery of algal components. Treatments such as ultrasonication, high-pressure homogenization and bead beating can be used to improve the recovery of algal components but they are energy intensive (Günerken et al., 2015) and potentially capable of damaging the components to be extracted (Hammed et al., 2013). Recently, attempts have been made to replace them with milder treatments, such as those based on the use of cell wall degrading enzymes. Enzymatic treatments have many important advantages over traditional methods, including environmental compatibility, reusability and high specificity for target cell wall components (Sander and Murthy, 2010). However, few studies have so far been performed in this field and definitive conclusions on the effectiveness of these treatments cannot still be drawn.
An important measure of the efficacy of enzymatic pretreatments is represented by the degree of hydrolysis (Hammed et al., 2013). Commonly, this parameter is calculated from the amount of reducing sugars released (Karray et al., 2015). A weakness of this method is that it does not take into consideration organic compounds other than sugars that could be produced during the treatment. Some researchers have proposed the use of Dissolved Organic Carbon (DOC) to estimate the degree of hydrolysis of cell wall of plants or yeasts after thermal, acid or enzymatic treatments (He et al., 2006). In contrast to the determination of reducing sugars,

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DOC measurements account for the presence of all soluble hydrolysis products that are released into the aqueous phase (Popov et al., 2016).
In this contribution we investigate the feasibility of using DOC values as a means to assess the effectiveness of an enzyme-assisted pretreatment of microalgae. Attention was focused on *Nannochloropsis* sp., a marine microalga of great biotechnological interest for its ability to accumulate large amounts of lipids (Perin et al., 2014) and other valuable compounds, such as the omega-3 polyunsaturated fatty acid EPA and the carotenoids astaxanthin and zeaxanthin (Leu and Boussiba, 2014). Because of its rigid and highly structured cell wall (Yao et al., 2012), *Nannochloropsis* shows unusual resistance to degradation and therefore represents a suitable model organism for testing the efficacy of enzymatic treatments.
Two commercial enzyme preparations, one rich in cellulase and the other rich in galactomannanase, were used for treating *Nannochloropsis*. In addition to their main enzyme activities, these preparations contain other side activities that are expected to improve the effectiveness of the pretreatment (Prévot et al., 2013). Furthermore, their relatively low cost makes them particularly attractive for large-scale biotechnological applications.

2. Materials and methods

2.1 Chemicals, enzymes and microalgae

Methanol, chloroform, sodium chloride, 2-propanol, hydrochloric acid (37 % v/v), n-hexane, sodium hydroxide and potassium hydrogen phthalate were purchased from Carlo Erba (Milano, Italy).
Cellulyve® 50LC (CEL) and Feedlyve® GMA (GMA) were from Lyven SA (Colombelles, France). These preparations were selected on the basis of their major enzyme activities, as reported in Zuorro et al. (2014). CEL was rich in 1,4-β-cellobiosidase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21), while GMA contained galactomannanase (EC 3.2.1.15) as main enzyme component. To obtain the desired dosage, an appropriate amount of each preparation was added to water and the solution was gently stirred for a few minutes.
Nannochloropsis sp. was obtained as a lyophilized powder from DISPAA of the University of Firenze (Italy).

2.2 DOC measurement

A TOC–L analyzer (Shimadzu, Japan) was used for DOC measurements. Before analysis, liquid samples were passed through a 0.45-μm nylon filter and diluted with double-distilled water. The DOC content was determined in terms of total carbon using a calibration curve obtained with standard solutions of potassium hydrogen phthalate. The contribution of the enzymes to the TOC content of each sample was determined and subtracted from the value obtained for the whole liquid sample.

2.3 Determination of total lipid content

Total lipid content was determined using the chloroform–methanol–water solvent system as reported by Ma et al. (2013) with slight modifications. Specifically, a known amount of microalgal biomass was mixed with chloroform/methanol (2:1, v/v) and shaken for 60 minutes at 37°C. After centrifugation at 12,000 × g for 10 min, the supernatant was collected and combined with a 1% sodium chloride solution (20 % of the total volume). The chloroform layer was then removed and evaporated under vacuum. The lower layer was washed with two volumes of chloroform and vacuum evaporated at 40 °C in a rotary evaporator. The biomass was extracted three times and the lipid content was calculated as the sum of the values obtained in each step.

2.4 Enzymatic pretreatment of microalgae and lipid extraction

The enzymatic pretreatment of *Nannochloropsis* was performed in 20-mL screw-capped glass flasks as described by Zuorro et al. (2013). In a typical experiment, 0.2 g of microalgae and an appropriate amount of the enzyme solution were loaded into the flasks and magnetically stirred in a thermostated water bath (±0.1 °C). The pH of the solution was adjusted by adding small amounts of 0.1 N HCl or 0.1 N NaOH and monitored during the treatment. At the end of the treatment, the flask content was centrifuged at 12,000 × g for 10 min. The supernatant was separated and collected for TOC analysis. Lipid extraction was carried out at room temperature by adding 10 mL of n-hexane/2-propanol (3:2, v/v) to the treated biomass. After 30-min stirring, the suspension was centrifuged at 12,000 × g for 5 min. The amount of extracted lipids was determined gravimetrically after solvent evaporation.

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, mg g^{-1}) and GMA dosage (D_2, mg g^{-1}) on the enzymatic treatment (Zuorro, 2015). The CCD consisted of a half fraction of the full 2^5 factorial design augmented by six central points and two axial points at ±α for each factor, for a total of 32 runs. To ensure the rotatability of the design space, the value of α was taken as $(2^{5-1})^{1/4} = 2$ (Montgomery, 2012). Furthermore, the run order was randomized to minimize the effects of uncontrolled factors.

The levels of each factor were chosen to cover a range of values of practical interest. They are reported in Table 1 in both actual (X_i) and coded (x_i) values. The latter were obtained using the following equation:

$$x_i = \frac{X_i - X_{i,0}}{\Delta X_i} \quad (1)$$

where $X_{i,0}$ is the actual value of the i-th factor at the centre-point level and ΔX_i is the step change value for that factor. The DOC of the liquid at the end of the treatment, expressed as the concentration of organic carbon in the aqueous phase (y, mg L^{-1}) was used as the response variable. The design of experiments was performed with the Design-Expert® software (version 7.0, Stat-Ease Inc., Minneapolis, MN, USA). The experimental design layout and the observed DOC values are presented in Table 2.

Table 1: Actual and coded levels of the factors used in the experimental design

Factor	Unit	Factor level				
		–2	–1	0	+1	+2
Temperature (T)	°C	15	30	45	60	75
pH	–	2	3.5	5	6.5	8
Pretreatment time (P)	min	30	90	150	210	270
CEL dosage (D_1)	mg g^{-1}	0	5	10	15	20
GMA dosage (D_2)	mg g^{-1}	0	0.5	1	1.5	2

Table 2: Experimental design layout and observed (y_{exp}) and calculated (y_{calc}) DOC values. SO is the standard order and RO the run order of experiments

SO	RO	x_1	x_2	x_3	x_4	x_5	y_{exp} (mg L^{-1})	y_{calc} (mg L^{-1})
1	13	–1	–1	–1	–1	+1	587.0	605.3
2	28	+1	–1	–1	–1	–1	712.7	764.1
3	17	–1	+1	–1	–1	–1	639.9	650.2
4	29	+1	+1	–1	–1	–1	809.4	809.1
5	24	–1	–1	+1	–1	–1	711.8	653.9
6	9	+1	–1	–1	–1	–1	788.9	812.8
7	18	–1	–1	+1	–1	–1	638.0	698.8
8	31	+1	+1	+1	–1	–1	823.2	857.7
9	21	–1	–1	–1	–1	-1	662.1	663.2
10	22	+1	–1	–1	+1	+1	794.1	822.1
11	14	–1	–1	–1	–1	–1	754.0	708.2
12	15	+1	+1	–1	–1	–1	937.3	867.0
13	19	–1	–1	1	+1	+1	675.4	711.9
14	26	+1	–1	–1	+1	–1	817.6	870.7
15	1	–1	–1	1	+1	–1	668.9	747.2
16	30	+1	+1	1	+1	+1	917.0	915.7
17	2	–2	0	0	0	0	626.0	629.6
18	23	+2	0	0	0	0	947.7	947.3
19	11	0	–2	0	0	0	680.1	631.7
20	10	0	+2	0	0	0	730.7	721.6
21	8	0	0	–2	0	0	680.1	739.8
22	27	0	0	+2	0	0	899.7	837.0
23	20	0	0	0	–2	0	762.2	730.5
24	16	0	0	0	+2	0	852.1	846.4
25	4	0	0	0	0	–2	744.3	788.4
26	3	0	0	0	0	+2	840.3	788.4
27	5	0	0	0	0	0	817.0	788.4
28	12	0	0	0	0	0	792.6	788.4
29	32	0	0	0	0	0	776.9	788.4
30	6	0	0	0	0	0	781.5	788.4
31	25	0	0	0	0	0	845.6	788.4
32	7	0	0	0	0	0	844.9	788.4

Table 3: Estimates of the regression coefficients of model equation with their corresponding standard errors (SE), 95 % confidence intervals (CI) and p-values

Coefficient	Effect	Estimate	SE	Low CI	High CI	p-value
β_0	–	788.42	10.25	767.34	809.49	<0.0001
β_1	T	79.43	9.36	60.19	98.67	<0.0001
β_2	pH	22.46	9.36	3.22	41.70	0.0239
β_3	P	24.31	9.36	5.07	43.55	0.0153
β_4	D_1	28.98	9.36	9.74	48.22	0.0047
β_{22}	pH × pH	–27.95	8.37	–45.16	–10.74	0.0026

3. Results and discussion

Total lipid content of *Nannochloropsis* sp. was 53.25 ± 1.25 mg/100 mg, which represents a significantly high value compared with other microalgal species (Griffiths and Harrison, 2009).

A preliminary control experiment was carried out without enzymes by setting the treatment conditions at their centre-point values (T = 45 °C, pH = 5, P = 5 min). The resulting DOC was 419.0 ± 50.3 mg L^{-1}. In the presence of enzymes, DOC values ranging from 587.0 to 947.7 mg L^{-1} (mean value: 773.4 mg L^{-1}) were observed (Table 2). In the factorial space, the maximum DOC value (937.3 mg L^{-1}) was achieved at T = 60 °C, pH = 6.5, P = 90 min, D_1 = 15 mg g^{-1} and D_2 = 0.5 mg g^{-1}. These results clearly attest the effectiveness of the enzymatic treatment and the possibility of using DOC as a measure of the effects of enzymes on cell wall degradation. A similar conclusion was reached by He et al. (2006), who found a significant increase in the DOC of the liquid after the enzymatic hydrolysis of potato samples rich in carbohydrates and proteins. The ability of the enzyme preparations used in this study to degrade the cell wall of *Nannochloropsis* sp. was confirmed by previous observations with a scanning electron microscope (SEM) and a transmission electron microscope (TEM), which revealed extensive disruption of the microalgal cell wall and the release of intracellular material (Zuorro et al., 2014).

The results of CCD experiments summarized in Table 2 were analysed by different empirical models (linear, two-factor interaction, quadratic and cubic) following the procedure described by Zuorro (2015). The best result was obtained using the 2nd-order polynomial equation:

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

where y is the process response, x_i are the coded independent variables, β_0 is the intercept and β_i, β_{ii} and β_{ij} are the linear, pure quadratic and interaction regression coefficients, respectively.

A stepwise regression method was used to iteratively add and remove model terms from Eq. (2) based on their statistical significance. From this procedure, the following equation was derived:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{22} x_2^2 \quad (3)$$

The six model coefficients, together with their standard errors, 95 % confidence intervals and p-values, are listed in Table 3. Eq. (3) provided a fairly good fit to the data, with coefficient of determination (R^2) and adjusted-R^2 of 0.802 and 0.764, respectively. An analysis of residuals showed no apparent violations of basic ANOVA assumptions, that is, normally distributed errors with constant variance and independent of one another (Zuorro, 2014).

As can be seen in Table 3, four out of the five factors, namely, T, pH, P and D_1, were statistically significant, their effects on the response variable being all positive and increasing in the order: pH < P < D_1 < T. pH affected the response through both a linear and a quadratic term. Finally, there were no statistically significant interactions between factors, implying that each factor exerted its effect independently of the others. The fact that GMA dosage did not affect in a statistically significant manner the process response could be due to the fact that hemicelluloses, although essential in maintaining the architectural integrity of algal cell walls, are present in very low amounts (Razeghifard, 2013). Accordingly, their degradation can be expected to increase the DOC value of the liquid at the end of the treatment only to a limited extent.

To better appreciate the contributions of T, pH, P and D_1 to lipid recovery, 3D response surfaces were generated from Eq. (3). Some representative plots are shown in Figure 1, from which the positive effects of temperature, pretreatment time and CEL dosage on the release of organic compounds into the treatment solution are evident. We also note that pH had a non-monotonic effect on the response variable, with a maximum located at about pH 5, a value close to the optimal pH of the enzyme preparations used.

The extraction yield of lipids from the untreated biomass was 40.73 ± 0.26 %, while that from the enzyme-treated material ranged from 43.43 % to 68.32 %, depending on the operating conditions. Plotting the observed lipid extraction yields (y_{LIP}) against the corresponding DOC values gave the results displayed in Figure 2. A least-square regression analysis of the data provided the following relationship:

$$y_{LIP} = 0.095\ DOC - 17.035 \quad (4)$$

Eq. (4) was also validated by performing an additional experiment under conditions different from those of the CCD (T = 53 °C, pH = 4.2, t = 210 min, D_1 = 14 mg g^{-1}, D_2 = 1.5 mg g^{-1}). The percentage error of prediction was 8.9 %. The good correlation between the two variables (R^2 = 0.813) suggests that the enhanced recovery of lipids is a consequence of the enzyme-induced cell wall degradation, which in turn results in an increase in the DOC values of the liquid phase.

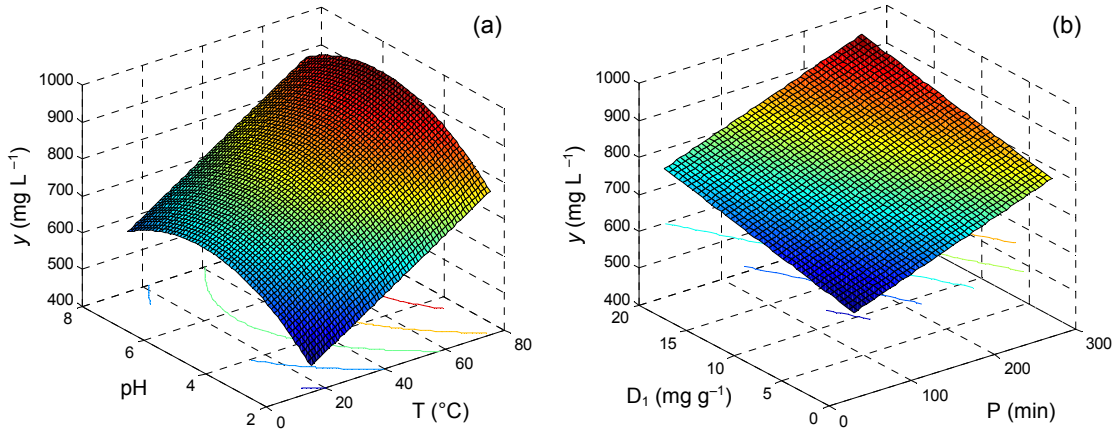

Figure 1: Response surface plots showing the influence of temperature (T), pH, pretreatment time (P) and CEL dosage (D_1) on DOC. For each plot, the of the other factors were set at their central values

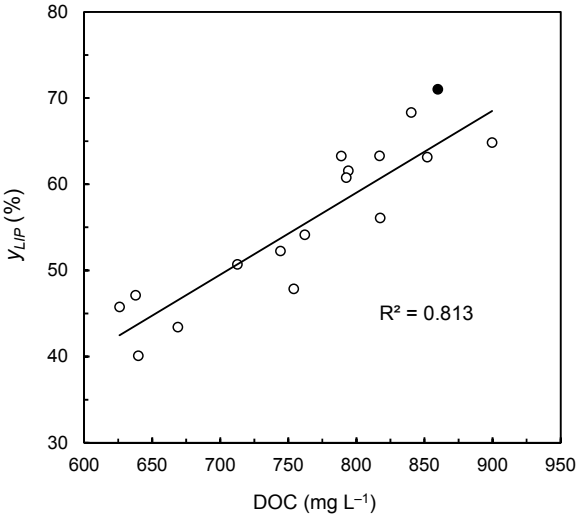

Figure 2: Observed dependence of lipid extraction yields (y_{LIP}) from DOC values (solid symbol: validation point)

4. Conclusions

The results of this study demonstrate that DOC measurements can give useful information on the enzyme-induced degradation of algal cell walls. In particular, we have shown that the enzymatic pretreatment of *Nannochloropsis* sp. results in an increase in the DOC values of the liquid phase and a parallel improvement of lipid recovery. The determination of DOC by a TOC analyzer is very rapid and easy to perform and, combined with other methods for the characterization of the cell wall, could contribute to provide a more complete picture of the effects of enzymatic treatments on microalgae.

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