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Microalgal Cell Disruption Through Fenton Reaction: Experiments, Modeling and Remarks on its Effect on the Extracted Lipids Composition

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A novel cell disruption technique, based on the use of Fenton reaction, for the improvement of lipid extraction from microalgae has been recently presented in the literature. In this work, a mathematical model is proposed to quantitatively interpret the relevant experimental results obtained so far. Model results and experimental data are successfully compared. Moreover, a mechanistic explanation of the relevant phenomena underlying the positive effects of disruption on the composition of fatty acids methyl esters, obtained through trans-esterification of the extracted lipids, is presented.

1. Introduction

The potential exploitation of microalgae as renewable resource for the production of biofuels is receiving a rising interest mostly driven by the global concerns related to the increase of CO_2 levels in the atmosphere (Concas et al., 2014). The development of effective and sustainable processes for the extraction of lipids from microalgae cells is crucial in facilitating the industrial-scale production of biofuels from microalgae (Piemonte et al., 2014). The extraction of algal lipids can be performed starting either from wet or dry microalgal biomass. However, since drying of microalgal biomass is economically prohibitive, lipid extraction from wet biomass is generally preferred for industrial applications. On the other hand, in order to extract lipids directly from the wet biomass, the latter one should be subjected to a pre-treatment aimed to break the cell wall and favour the subsequent release of intracellular lipids into the liquid bulk. This way, when the disrupted biomass is contacted with solvents, lipids released from the algal cell are transferred to the solvent phase due to their hydrophobicity and can be later collected by evaporating the solvent. Several cell disruption techniques, based on chemical or physical methods, have been so far proposed in the literature. The physical techniques include ball milling, microwaving, ultra-sonication, cavitation, laser treatment and osmotic shocks. Nevertheless, most of these methods are very difficult to scale up and might involve high energy consumption (McMillan et al., 2013). On the other hand, chemical methods for cell disruption rely on selective interaction of certain chemicals with the components of cell wall. When compared with physical methods, the chemical ones are less energy consuming while frequently showing higher disruption yields and furthermore, are simpler to scale-up. However, even these methods show some significant drawbacks deriving from the cost of chemicals. Moreover, the latter ones might even attack the valuable products (i.e. lipids) thus vanishing the whole process. Recently, Steriti et al. (2014) have proposed a novel cell disruption technique based on the use of Fenton reaction for the improvement of lipid extraction from microalgae. The experimental results have shown that, when disruption was performed under suitable operating conditions, the lipids extracted from *C. vulgaris* were more than doubled with respect to the case where a classical approach was adopted. Moreover, the treatment resulted in a significant improvement of the quality of fatty acid methyl esters (FAMEs) obtained by trans-

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esterification of extracted lipids. These results, together with the extreme simplicity, cheapness and low energy consumptions, are very promising in view of the industrial transposition of the proposed technique. To this aim, the use of suitable mathematical models might represent a valuable tool to design and control possible industrial size reactors. Along these lines, a simple but exhaustive model to quantitatively describe the effects of contact time and reactants concentration on the amount of lipids extractable from microalgae, is presented in this work.

2. Materials and Methods

2.1 Microorganism and culture conditions

A fresh water strain of *Chlorella vulgaris* was cultured in a modified Kolkwitz growth medium, under continuous flux of 100 % (v/v) CO_2 within a 6 L helical tubular photobioreactor coupled with a degasser system as described in the literature (Concas et al., 2012). A light intensity of 100 μE m^{-2} s^{-1} for a light-dark photoperiod of 12 h was provided by fluorescent lamps. Once the culture reached the stationary growth phase the photobioreactor was operated in fed-batch mode. The withdrawals made during the operation in fed-batch mode were used for the cell disruption experiments.

2.2 Cell disruption

Once the culture in the photobioreactor reached the stationary growth phase, microalgae were first harvested and then centrifuged to obtain a concentrated pellet of wet biomass. The exact weight of dry biomass contained in the wet pellets was evaluated by means of a suitable calibration line obtained by gravimetrically evaluating the wet weight of biomass obtained after centrifugation and its corresponding dry weight after drying at 105 °C for 24 h (Steriti et al., 2014). Next, wet pellets containing known amounts of dry biomass were subjected to the cell disruption procedure which consisted of contacting them with selected volumes of the disrupting solution within a falcon flask that was then sealed and continuously shaken at 300 rpm for certain periods of time. The disrupting medium consisted of an aqueous solution of H_2O_2 and $FeSO_4$, i.e. the Fenton reactant. A number of experiments were performed by varying the H_2O_2 concentration in the range between zero to 5.8 mol L^{-1}, while keeping constant the concentration of $FeSO_4$ in the disruption solution at 0.024 mol L^{-1}. Contact times ranging from 0 to 5 min were investigated. Once the desired contact time was elapsed, the disruption reaction was suddenly quenched by diluting the entire reacting mixture ten times of its original volume with ethanol.

2.3 Lipid extraction and fatty acid methyl esters analysis.

Neutral lipid extraction was performed directly on the wet disrupted biomass through ethanol and hexane according to the method proposed by Steriti et al. (2014). The percent weight of lipids extracted from the dry biomass was then obtained as the ratio between the weight of lipid obtained and the original dry weight of microalgae which was subjected to the extraction process. It should be noted here that the adopted technique allows the selective extraction of “non degraded” and “non peroxidized lipids”, i.e. only the category of lipids which is useful for producing biofuels. The fatty acid methyl esters composition of extracted lipids was determined according to the European regulation/commission regulation EEC n° 2568/1991 after transesterification with methanol-acetyl chloride (Steriti et al., 2014).

3. Mathematical model

It is well known from the literature that the addition of H_2O_2 to aqueous solutions containing Fe^{2+} ions can trigger the so called Fenton’s reaction which involves the key reactive steps reported in Table 1.

Table 1. Steps and constant rates of the Fenton reaction (Concas et al., 2015)

ID	Reaction	Rate constant	Value	Units
R1	$Fe^{2+} + H_2O_2 \xrightarrow{k_1} Fe^{3+} + OH^{\bullet} + OH^-$	k_1	70	L mol^{-1} s^{-1}
R2	$OH^{\bullet} + H_2O_2 \xrightarrow{k_2} H_2O + HO_2^{\bullet}$	k_2	3.3 x 10^7	L mol^{-1} s^{-1}
R3	$H_2O_2 + Fe^{3+} \xrightarrow{k_3} Fe^{2+} + HO_2^{\bullet} + H^+$	k_3	0.01	L mol^{-1} s^{-1}
R4	$Fe^{3+} + HO_2^{\bullet} \xrightarrow{k_4} Fe^{2+} + O_2 + H^+$	k_4	1.2 x 10^6	L mol^{-1} s^{-1}
R5	$Fe^{2+} + HO_2^{\bullet} \xrightarrow{k_5} Fe^{3+} + HO_2^-$	k_5	1.3 x 10^6	L mol^{-1} s^{-1}
R6	$OH^{\bullet} + Fe^{2+} \xrightarrow{k_6} Fe^{3+} + OH^-$	k_6	3.2 x 10^8	L mol^{-1} s^{-1}

As it can be observed, the reaction steps lead to the production of free radicals such as $OH^\bullet$ and $HO_2^\bullet$ which, due to their high instability and reactivity, are capable to oxidize the organic compounds in solution, thus provoking their degradation. According to the same mechanism, the $OH^\bullet$ radicals produced by the Fenton's reactive chain may react with several organic compounds constituting the cell wall of algae, thus leading to their degradation and the consequent disruption of the protective cell wall followed by the release of intracellular lipids. In order to model such phenomenon, a suitable lumped reaction scheme has been adopted to describe the overall disruption process (Concas et al., 2015):

$$y_{OH/X} OH^\bullet + X \xrightarrow{k_d} y_{L/X} LH + DX \quad (1)$$

The simplified reaction above states that the $OH^\bullet$ radicals react with the algal cells (X) with constant rate k_d to produce disrupted cells (DX), and generic lipids (LH), respectively. The yield $y_{OH/X}$ takes into account that several molecules of $OH^\bullet$ might be needed to produce the rupture of one mole of algal biomass while the coefficient $y_{L/X}$ represents the moles of lipid released for each mole of microalgae cells being disrupted. Once released in the liquid bulk, lipids (LH) can react with $OH^\bullet$ radicals to initiate a reactive chain which finally leads to the production of lipid peroxides (LO_2H), water and other degradation products (Table 2). The initiation step of lipid peroxidation consists of the abstraction of one weakly bonded hydrogen atom from the unsaturated lipid (LH) by a free radical $OH^\bullet$ which leads to the conversion of the lipid molecule into a lipid radical ($L^\bullet$), as shown in the following reaction:

$$OH^\bullet + LH \xrightarrow{k_{L1}} L^\bullet + H_2O \quad (2)$$

The radical reaction chain, once triggered, proceeds according to the steps of propagation and termination shown in Table 2, where the corresponding reaction constants are also reported.

Table 2. Steps of the lipid peroxidation reactive chain and related rate constants (Concas et al., 2015)

ID	Reaction	Rate constant	Value	Units
R7	$L^\bullet + O_2 \xrightarrow{k_{L2}} LO_2^\bullet$	k_{L2}	3.0 – 4.6 x 10^8	L mol^{-1} s^{-1}
R8	$LO_2^\bullet + LH \xrightarrow{k_{L3}} LO_2H + L^\bullet$	k_{L3}	1.9 x 10^1	L mol^{-1} s^{-1}
R9	$L^\bullet + L^\bullet \xrightarrow{k_{L4}} NRS_1$	k_{L4}	6.6 x 10^4	L mol^{-1} s^{-1}
R10	$L^\bullet + LO_2^\bullet \xrightarrow{k_{L5}} NRS_2$	k_{L5}	1.0 x 10^5	L mol^{-1} s^{-1}
R11	$LO_2^\bullet + LO_2^\bullet \xrightarrow{k_{L5}} NRS_3$	k_{L6}	6.6 x 10^4	L mol^{-1} s^{-1}

* Non reactive species

According to the approach usually adopted in the literature, the algal disruption and lipid peroxidation reactions above are typically considered to be governed by second order kinetics and thus the following material balances can be written to quantitatively describe the time evolution of algae, lipids and lipid radicals concentration in the bulk liquid:

$$\frac{d[X]}{dt} = -k_d\left[OH^\bullet\right][X] \quad (3)$$

$$\frac{d[LH]}{dt} = y_{L/X} k_d\left[OH^\bullet\right][X] - k_{L1}\left[OH^\bullet\right][LH] - k_{L3}\left[LO_2^\bullet\right][LH] \quad (4)$$

$$\frac{d\left[L^\bullet\right]}{dt} = k_{L1}\left[OH^\bullet\right][LH] - k_{L2}\left[L^\bullet\right][O_2] + k_{L3}\left[LO_2^\bullet\right][LH] - k_{L4}\left[L^\bullet\right]^2 - k_{L5}\left[L^\bullet\right]\left[LO_2^\bullet\right] \quad (5)$$

$$\frac{d\left[LO_2^\bullet\right]}{dt} = k_{L2}\left[L^\bullet\right][O_2] - k_{L3}\left[LO_2^\bullet\right][LH] - k_{L5}\left[L^\bullet\right]\left[LO_2^\bullet\right] - k_{L6}\left[LO_2^\bullet\right]^2 \quad (6)$$

$$\frac{d\left[O_2\right]}{dt} = -k_{L2}\left[L^\bullet\right][O_2] \quad (7)$$

along with the following initial conditions: [X] = 3.97x10^{-3} (mol L^{-1}), [LH] = 0 (mol L^{-1}), [$L^\bullet$] = 0 (mol L^{-1}), [$LO_2^\bullet$] = 0 (mol L^{-1}) and [O_2] = 2.58x10^3 (mol L^{-1}) at t=0. In order to solve *Eq*s. (3-7) the concentration of $OH^\bullet$ radicals at each integration time should be known. To this aim the system of equations describing the mass balances of all the species appearing in the Fenton's reaction chain of Table 1 should be coupled to

the equations above. In this regard, it should be pointed out that, since the rate constants k_3, k_4 and k_5 are order of magnitude smaller than k_2 and k_6 (Table 1), the reactions R3, R4 and R5 can be neglected. Moreover, since OH• is a highly reactive free radical with an extremely short life time of nanoseconds, the pseudo steady state assumption can be invoked. Therefore, the concentration of the $OH^•$ radicals can be evaluated for each integration time as follows:

$$\left[OH^{\bullet}\right]=\frac{k_1\left[Fe^{2+}\right]\left[H_2O_2\right]}{k_2\left[H_2O_2\right]+k_6\left[Fe^{2+}\right]+y_{OH/X}k_d\left[X\right]+k_{L1}\left[LH\right]} \tag{8}$$

As it can be observed from *Eq.* (8), the concentrations of Fe^{2+} and H_2O_2 should be known at each integration time in order to evaluate the current concentration of $OH^•$. To this aim the material balances related to Fe^{2+} and H_2O_2 must be coupled to the equations so far reported. In this regard it should be noted that k_1 and k_3, and k_4 and k_5 are much lower than k_2 and k_6, respectively (Table 1), so that the material balances for Fe^{2+} and H_2O_2 can be written as follows:

$$\frac{d\left[H_2O_2\right]}{dt}=-k_2\left[OH^{\bullet}\right]\left[H_2O_2\right] \tag{9}$$

$$\frac{d\left[Fe^{2+}\right]}{dt}=-k_6\left[OH^{\bullet}\right]\left[Fe^{2+}\right] \tag{10}$$

along with the initial conditions: $[H_2O_2] = [H_2O_2]_0$, in the range 0 – 5.8 (mol L^{-1}) and $[Fe^{2+}] = 2.4x10^{-2}$ (mol L^{-1}) at t = 0. Finally, in order to evaluate the percentage of extracted lipids, the following assumptions have been considered. Specifically, given the high hydrophobic character of lipids, it can be reasonably assumed that all the lipids liberated from microalgal cells during the disruption reaction, and not degraded at the end of the disruption process, could be effectively transferred to the organic solvent phase and then recovered. On the other hand, as shown by Steriti et al. (2014), when solvent extraction was performed on undisrupted microalgae, the weight of extracted lipids was equal to about the 7%wt by unit weight of dried microalgal biomass subjected to the extractive procedure. On the basis of such considerations, it can be reasonably inferred that, once the disruption reaction is terminated, the entire amount of lipids LH liberated from the disrupted biomass and not degraded by hydroxyl radicals, can be recovered in the subsequent solvent extraction step. In the case where a residual undisrupted biomass X remained in solution after the disruption phase was terminated, the lipids correspondingly recovered are of about 7 %wt of its dry weight. For this reason, the weight percentage of lipids recovered (η_L %wt) from the microalgal biomass subjected to the disruption procedure under the different operating conditions can be evaluated as follows:

$$\eta_L\%=\frac{\left[LH\right]MW_L+0.07\left[X\right]MW_X}{\left[X\right]^0 MW_X}\cdot 100 \tag{11}$$

The system of ordinary differential equations (3-7) and (9-10) was numerically integrated as an initial value problem through the subroutine DIVPAG of the International Mathematics and Statistics Library (IMSL) while tuning of model parameters values to fit the experimental data was carried out through a Fortran optimization subroutine based on the least-squares method.

4. Results and discussion

Several disruption experiments were performed where contact time and concentration of disruption reactant were suitably varied in order to identify their corresponding values at which the amount of extractant lipids is maximized. The effect of the contact time variation on the amount of extracted lipids when using a disruption solution containing 0.5 mol L^{-1} of H_2O_2 and 0.024 mol L^{-1} of $FeSO_4$ is shown in Figure 1a. It can be observed that when no disruption treatment was performed, i.e. the contact time was zero, extracted lipids were about 7 %wt by dry weight of biomass. Nevertheless, when the lipid extraction was preceded by a disruption treatment, the amount of extracted lipids was increased if the contact time was augmented up to 3 min when a maximum value of extracted lipids equal to 17.4% was achieved. However, when the disruption procedure was protracted for more than 3 min, a decrease of the extracted lipids was observed, as a result of the more pronounced lipid peroxidation phenomena taking place. In Figure 1a, the comparison between experimental data and model results is also shown. While the values of the main rate constants used to perform the simulations are shown in Tables 1 and 2, the remaining

model parameters and initial conditions are reported in Table 3. It should be noted from Table 3 that the lipid yield value $y_{L/x}$ (mol_{lipids} /$mol_{biomass}$), which represents the moles of lipids released for each mole of dry biomass being disrupted, has been evaluated by assuming average lipid content of *C. vulgaris* equal to 22 %wt through the relationship reported by Concas et al., (2015). All the parameters values appearing in Table 3 were taken from the literature or experimentally evaluated except for the reaction rate constant of the lipid peroxidation initiation reaction k_{L1} (L mol^{-1} s^{-1}) and the yield $y_{OH/x}$ representing the moles of OH• radicals needed to oxidize one mole of microalgal biomass.

Table 3. Model parameters

Symbol	Value	Units	Reference
k_d	5.00×10^8	L mol^{-1} s^{-1}	Kang et al., 2002
k_{L1}	5.44×10^7	L mol^{-1} s^{-1}	Tuned parameter
$y_{L/X}$	6.99	mol mol^{-1}	Mujtaba et al., 2012
$y_{OH/X}$	2.27×10^4	mol mol^{-1}	Tuned parameter

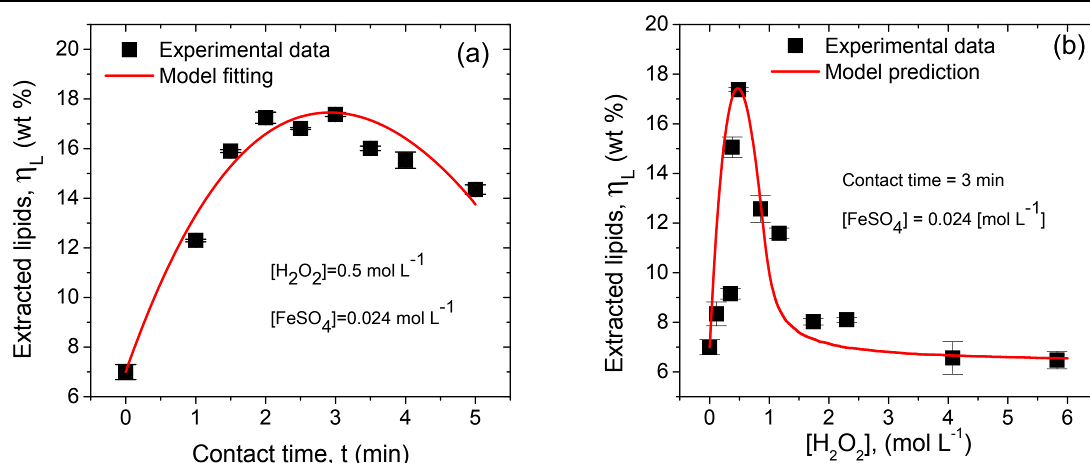

Figure 1. Comparison of model results and experimental data in terms of lipids extracted after cell disruption performed by varying (a) the contact time and (b) the H_2O_2 concentration.

As it can be observed from Figure 1a, the experimental behavior is well captured by the proposed model, thus confirming that the assumptions made about the chemical physical mechanisms affecting the percent amount of extracted lipids after disruption are consistent with the experimental evidence. To test the predictive model capability, further experimental data, obtained by performing disruption with growing concentrations of H_2O_2, while keeping fixed the contact time at the value of 3 min, were simulated. Specifically, in such experiments the concentration of $FeSO_4$ was kept at 0.024 mol L^{-1}, while the concentration of H_2O_2 was varied within the range 0 - 5.8 mol L^{-1}. As it can be seen from Figure 1b, the resulting experimental data are quite well predicted by the proposed model. Therefore, the model represents the first step towards the development of a design and control tool which allows optimizing the investigated technique, thus avoiding the undesired oxidation of extracted lipids. In this regard it should be clarified that, while the lipid oxidation phenomena can be minimized when operating under optimal conditions, in any case, a certain amount of lipids released from the algal cell after disruption is subjected to the attack of hydroxyl radicals and thus is irreversibly degraded to useless products. While from one hand such phenomenon might be seen as a drawback of the technique, from the other one it is probably the main responsible of the significant improvement of the quality of the biodiesel obtained from microalgae lipids observed by Steriti et al. (2014), as a result of the disruption treatment. In fact, as it can be observed from Figure 2a, the analysis of the FAMEs obtained through trans-esterification of the lipids, highlighted that the disruption treatment provoked a dramatic reduction of the relative content of undesired polyunsaturated fatty acids and, in particular, a significant decrease of linolenic fatty acid (C18:3) with respect to the case where disruption was not performed (Steriti et al., 2014). The above characteristics make the biodiesel obtainable from disrupted biomass more stable than the corresponding one from undisrupted biomass. Most probably, such improvement was due just to the fact that an aliquot of algal lipids released from the cell during disruption are oxidized by the OH• radicals. In fact, the latter ones

attack preferably the polyunsaturated fatty acids, including the linolenic one, since they display multiple double bonds where the hydrogen atoms can be easily abstracted by the OH• radicals produced by the Fenton reaction. Therefore, while from one hand the peroxidation reactions can actually provoke a degradation of lipids liberated by cells, from the other side they involve mainly the most undesired category of lipids thus providing a better quality of the resulting oil in view of its exploitation for producing biofuels.

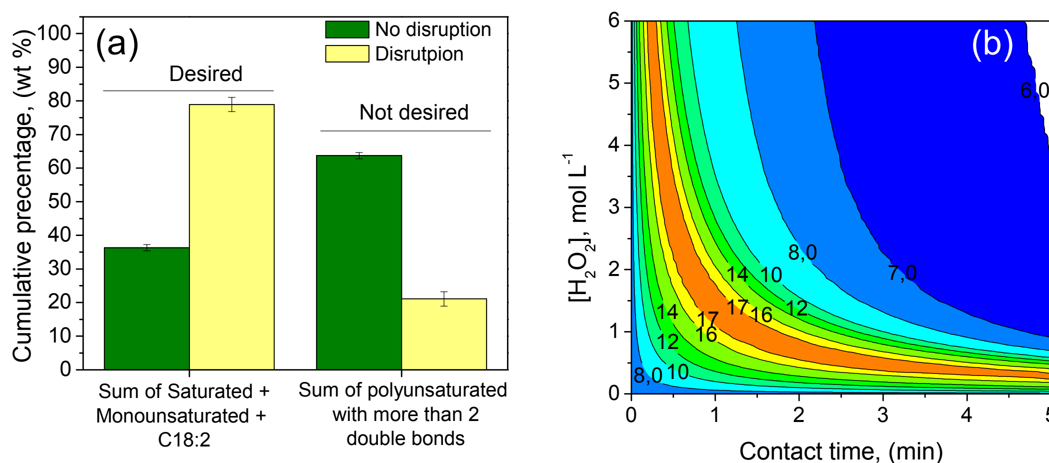

Figure 2. Comparison between FAMEs obtained from un-disrupted microalgae and after disruption performed under the optimal conditions (a) and (b) optimization map obtained through the model.

Finally it should be noted that the proposed model would permit to obtain optimization maps showing the achievable lipid extraction yields as function of the adopted operating parameters. For instance, the optimization map shown in Figure 2b would allow one to choose the best couple of values of H_2O_2 concentration and contact time through which the maximum lipid extraction can be achieved.

5. Concluding remarks

A mathematical model for the simulation of the effect of a cell disruption treatment based on Fenton reaction on the amount of lipids extractable from *C. vulgaris* is proposed. By comparing model results with literature experimental data a good matching is obtained. Moreover, a possible explanation of how the disruption treatment may have influenced the improvement of FAMES composition observed in the literature, is proposed. The model might represent the first step towards the development of a software tool useful to optimize the implementation of the disruption technique at the industrial scale.

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