HARVESTING CHLORELLA VULGARIS BY NATURAL INCREASE IN PH - A NEW ASPECT OF THE CULTURE IN WASTEWATER MEDIUM

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Abstract - The harvesting of microalgae Chlorella vulgaris 211-19 is investigated by slow and natural increase in pH (natural flocculation). Effects of medium composition on harvesting are particularly investigated. Experiments are carried out in two media differing in nitrogen nutrients: a Sueoka based medium with ammonium (NH_4+) and a BBM based medium with nitrate added to the wastewater medium. It is found that one of these two media allows natural flocculation more easily. It is because natural flocculation in the waste water medium requires much higher Ca_2+ and Mg_2+ concentrations to generate cell aggregates than artificial flocculation due to increase in pH by soda addition (for example [Ca_2+] natural=136 mg/L (3,4 mM) whereas [Ca_2+] artificial=34 mg/L (0,85 mM)). Harvested microalgae cells have been pre-concentrated up to 19 gDM/L (DM: Dry Matter) by calcium phosphates increase and up to 33 gDM/L by magnesium compounds.

Key words - dewatering; harvesting, pH-induced flocculation; natural flocculation, treatment of wastewater by microalgae.

1. Introduction

Harvesting is a critical step of microalgae exploitation as it can represent 20 to 30% of the overall process cost [1-5]. Centrifugation, the standard technique used for niche markets [6-8], should be excluded for mass markets because too much expensive and energy consuming in favour of flocculation or membranes processes for instance according to the dewatering rate needed [8].

Microalgae can be harvested and pre-concentrated by the flocculation methods used in wastewater treatment based on aluminium or iron salts, or polyelectrolyte addition [9–11]. Several other methods have also been investigated, including bioflocculation by microalgal polymers and/or bacterial exopolysaccharides [12–14], electrocoagulation [15], electroflocculation [16] or combined flocculation and flotation [17]. Some conditions can lead to the natural flocculation of microalgae with no or limited intervention. Thus, the synthesis of extracellular organic matter (probably exopolysaccharides) during a limited growth period due to nutrients deprivation could generate bridging between microalgal cells. Precipitation of salts contained in the culture medium at high pH (8.5 to 10.5) with suited medium composition can lead to cells precipitation [18-19], and some microalgal cells have even the ability to autoflocculate at a moderate pH [20].

Natural flocculation by slow, natural rise in pH is known for several decades [20]. This phenomenon can be observed in favourable conditions when microalgae grow with no CO_2 input [22] leading to a pH increase because of the photosynthesis and/or the stripping by air bubbling of dissolved CO_2. In such conditions, pH may reach the solubility limit of some salts [23]. According to the culture medium composition, natural flocculation by pH increase was associated to the precipitation either of calcium compounds, mainly phosphates or carbonate, or magnesium hydroxide. With recovery rate greater than 80%, maximal cells concentration generally is around 20 g DM/L and sometimes up to 30-35g/L [20, 21, 24, 25]. Invoked mechanisms include charge neutralization by positively charged precipitates, sweep flocculation and weighting effects [26, 27].

Flocculation of *Chlorella vulgaris* cells will be investigated in two actual culture media with respectively ammonium (Sueoka based medium) and nitrate (Bold Basal based medium) as nitrogen source. Nitrate based media are usually encountered to grow algae. Ammonium based media was also considered as *Chlorella vulgaris* is able to metabolize such nitrogen source, and ammonium is found in some livestock manure [28]. Ammonium based media are also useful to make all of its components highly assimilated by microalgae in order to avoid mineral accumulation when the culture system is recycled into the medium supernatant after cells harvesting [29]. Minimal ions concentrations are firstly determined by a quick, artificial pH increase obtained by NaOH addition into the culture medium enriched in magnesium or calcium. In a second time, natural flocculation of *Chlorella vulgaris* obtained through a slow, natural pH increase resulting from CO_2 depletion by photosynthesis is evaluated as a potential harvesting technique in real conditions, either by the action of calcium phosphate, or magnesium compounds. In these conditions, minimal ion concentrations obtained in the first part are tested and updated if required [5, 30].

2. Experimental

2.1. Strain & growth conditions

Chlorella vulgaris is a eukaryotic unicellular green freshwater alga [31]. Its cells are spherical or ellipsoidal with a mean diameter of around 4–5µm. The strain used in the study was *C. vulgaris* 211–19 (SAG, Germany), chosen for its ability to assimilate both ammonium (NH_4^+) and nitrate ions (NO_3^-), with a preference for ammonium [32, 33]. The strain was, therefore, grown in two media. The first one, with NH_4^+ ions as nitrogen source, was adapted from the autotrophic Sueoka's medium [34] described by Harris [35]. The second was a mBB medium with NO_3^- as the nitrogen source add in wastewater medium [36]. The nutrient concentrations in the media given in Table 1 were adjusted so as to reach a biomass concentration of 2 g DM L^{-1} (DM, dry matter) in a batch culture without mineral limitation.

Nitrate-based media are widely used to grow algae, and the ammonium-based medium was tested because it is highly assimilated by the microalga and can, therefore, be recycled in the photobioreactor (PBR) after cell harvesting without causing accumulation of minerals [37, 38]. The protocol allows recovery of a microalgae suspension with a

final biomass concentration of 0.8g DML^{-1}. All of cultures are added Hutner's solution as nutrient matter [39].

Table 1. *Ionic composition of the two culture media ($mg.L^{-1}$).*

	Na^+	K^+	NH_4^+	Mg^{2+}	Ca^{2+}	Cl^-	NO_3^-	HCO_3^-	SO_4^{2-}	PO_4^{3-}
Sueoka based(*)	230	28,7	243,9	13,8	6,8	493,1	-	610	54,6	69,8
BBM based(*)	541,5	28,7	-	13,8	6,8	28,7	840,4	610	54,6	69,8

(*)0,5 mL.L-1 of Hutner's trace elements solution [Hutner et al. 1950]) is also added to the solution

2.2. Flocculation experiments

The required minimal concentrations of Ca^{2+} and Mg^{2+} for flocculation were firstly estimated by sharply increasing the pH by NaOH addition. Chemical flocculation by base addition is often considered as a surrogate technique of autoflocculation [20, 25, 26]. Autoflocculation was then tested in the two media. As no flocculation was observed after 9h in the initial media, experiments were repeated in one medium doped with magnesium or calcium in order to determine the minimal concentrations of [Ca^{2+}] min and [Mg^{2+}] min for flocculation. Chemical flocculation and autoflocculation tests were run at T = 24 ± 1ºC in test tubes containing 120mL of a 0.4g DM L^{-1}microalgal suspension obtained by twice diluting the 0.8g DM L^{-1} harvest with fresh, cell-free culture medium. Test tubes were placed in front of a light panel similar to that used for the microalga culture in PBR.

Ca^{2+} or Mg^{2+} concentrations in the osmosed water medium were increased when required by adding small volumes of stock solutions of $MgSO_4.7\ H_2O$ or $CaCl_2.2\ H_2O$ at 50g L^{-1} in increments of 20–30 mg L^{-1}, each compound is essentially separative added in osmosed water which consist of *Chlorella vulgaris* filtered from its culture. Efficient mixing was achieved by a magnetic stirrer set at 500 rpm for at least 10 min. This chemical flocculation tests for estimating the minimal concentration of Ca^{2+} or Mg^{2+} were performed by fast addition of 1N NaOH solution with stirring under a light intensity of 150µmol$m^{-2}s^{-1}$ until the pH reached 11.8. It was considered that the minimum concentration corresponded to a settling efficiency of 80%. As cells settled, the suspension clarified so that the fraction of the intensity emitted by the light panel that was transmitted through the culture increased. Thus the settling efficiency was evaluated with a photovoltaic cell placed 5 cm below the suspension surface. All experiments on a given medium were performed in triplicate on withdrawals taken from the same culture over a total period of 4 days (one experiment per day)

2.3. Settling efficiency and cell concentration in the aggregate zone

The performance of cell recovery by flocculation was evaluated using two criteria: (1) settling efficiency and (2) estimated cell density in the aggregate zone.

Settling efficiency E was taken as the percentage of flocculated cells, and was computed according to the Beer–Lambert law as [41]:

$$E = \frac{OD_{682i} - OD_{682s}}{OD_{682i}} \quad (1)$$

Where OD_{682i} and OD_{682s} are, respectively, the optical densities of the processed suspension and supernatant measured at 682 nm with a Lambda 2S spectrophotometer (PerkinElmer) [40]. OD_{682s} was measured 20 min after the beginning of chemical flocculation tests or after the total decantation of cells for autoflocculation tests.

Floc density was estimated by computing the cell concentration in the flocculated zone by mass balance as [41]

$$C_{est} = C_i + \frac{V_i}{V_f}.C_i.E\ (gDML^{-1}) \quad (2)$$

where V_i is the initial volume of the suspension (mL), V_f the volume of the cell aggregate zone after settling (mL), and C_i the cell concentration in the processed suspension (g L^{-1}).

2.4. Analysis

Cell concentration is expressed on a DM basis. The stability of the PBR operation was monitored by checking the constancy of the cell concentration in the PBR, and by assay of the total chlorophylls and carotenoids. Total chlorophylls are roughly proportional to the cell content, but the measure is available more quickly. A change in the carotenoid-to-chlorophyll ratio reveals some stress in the culture.

Biomass dry weight was determined by gravimetry. The sample was filtered through a rinsed glass fiber filter (Whatman GF/F), pre-dried, and weighed. The filter (sample filtered) was dried for 24 h at 105ºC, cooled in a desiccator, and weighed again. Measurement was computed as the average of a triplicate, and the experimental error was estimated as the average absolute deviation of the experimental values. Pigments were extracted with pure methanol, incubated for 45 min at 45ºC, and centrifuged. The total chlorophyll (Chl-t) and carotenoid contents were determined according to Ritchie's equation [40] from the measurement of absorbances at 652 and 665 nm.

3. Results and discussion

3.1. Estimation of minimal Mg^{2+} and Ca^{2+}concentrations for flocculation in model mediumby increasing artificial pH

To determine the required minimum concentration toenable flocculation with magnesium and calcium, the cells are harvested after culture 0.8gMS.L^{-1}, and resuspended in a volume of osmosed water such that the final concentration of either one 0.4gMS.L^{-1}. The control of concentration is performed by measuring of pigments and pigments calibration/cell concentration curves.

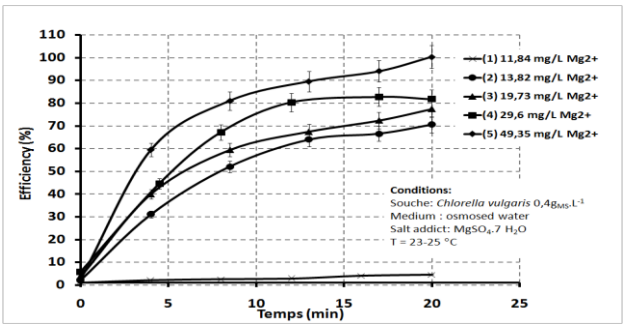

Figure 1. *Influence of the Magnesium quantity on induced flocculation in osmosed water medium*

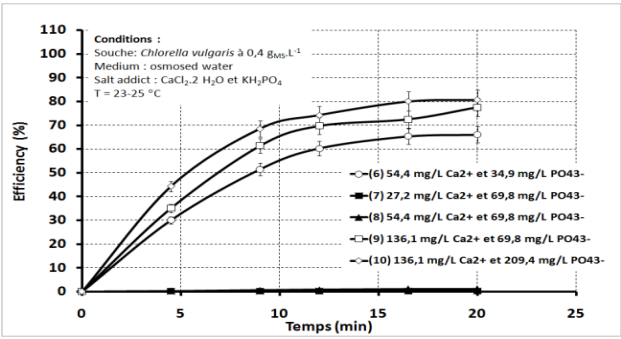

Figure 2. *Influence of the Magnesium quantity on induced flocculation in osmosed water medium*

The minimal concentrations of calcium or magnesium ions in the osmosed water medium, $[Ca^{2+}]$ min and $[Mg^{2+}]$ min, required to flocculate cells were estimated by sharply increasing the pH to 11,8 by adding a small volume of 1N NaOH to a harvest sample. With $[Mg^{2+}]$ at 13.84mg. L^{-1} the settling efficiency (E) was reached to 70% and with $[Ca^{2+}]$ at 136.1mg.L^{-1} also 69.8mg.L^{-1} PO_4^{-3} so E is up 80%.

3.2. Flocculation tests in Sueoka (NH_4^+) and BBM (NO_3^-) media

3.2.1. Chemical flocculation

Requiring the minimal concentration of Mg^{2+} and $Ca^{2+}$for flocculating easily of microalgae was done in two real culture's medium by addition of NaOH and this action was established in osmosed water one. The results are summarized in Table 2.

Table 2. *Flocculation efficiency (E) and cell concentration in the aggregate zone (C_{est}) determined at the minimal requirements in Mg^{2+} or Ca^{2+} ($[PO_4^{3-}]$ = 69.8mg.L^{-1}, concentration of cell = 0.4gDM.L^{-1}, artificial increase in pH up to 11,8 by NaOH 1N addition)*

	SUEOKA based medium (NH_4^+)		BBM based medium (NO_3^-)	
	$[Mg^{2+}]_{min}$ = 24,2 mg.L^{-1} (1 mM)	$[Ca^{2+}]_{min}$ = 34 mg.L^{-1} (0,85 mM)	$[Mg^{2+}]_{min}$ = 44.9 mg.L^{-1} (1,85 mM)	$[Ca^{2+}]_{min}$ = 34 mg.L^{-1} (0,85 mM)
V_f (mL)	3,5 ± 0,5	6 ± 0,5	13 ± 0,5	5 ± 0,5
E (%)	84	74	87	83
C_{est} (g DM.L^{-1})	10,3 ± 1.6	5,5 ± 0,4	3,2 ± 0.1	7,2 ± 0,8

3.2.2. Natural flocculation

In this part, cell suspensions at 0.4 gDM.L^{-1} were left under a light intensity of 500µmolm^{-2}.s^{-1} with air bubbling, but with no CO_2 input, to let the pH rise slowly by photosynthesis and the stripping of dissolved carbon dioxide. Both $[Ca^{2+}]$ and $[Mg^{2+}]$ in the media were below the minimal values estimated by NaOH precipitation. Cells were thus not expected to precipitate. Flocculation tests were, however, carried out first without supplementing the media with $[Ca^{2+}]$ or $[Mg^{2+}]$ in order to test the capacity of the pH to increase 'naturally' (i.e. with bubbling and illumination, but with no nutrient input) in the media. The minimal concentrations of $[Ca^{2+}]$ and $[Mg^{2+}]$ inducing cell flocculation and decantation were then determined. The various autoflocculation tests performance is summarized in Table 3.

These results suggest that the natural flocculation of *C. vulgaris* cannot be obtained in an ammonium-based culture medium with low salinity. In contrast, with BBM medium plus Mg^{2+} and Ca^{2+} addition at 1000mg.L^{-1} and 120mg.L^{-1} respectively, the pH increased naturally up to 10.8 in 8h and the flocculation of microalgae would be occurred.

Table 3. *Experimental design of natural flocculation assays (C. vulgaris c_i = 0,40 gDM.L^{-1}; L = 159 µmol.m^{-2}.s^{-1}).*

No of assay	Medium[1]	pH increase	Concentrations (mg.L^{-1})			Flocculation occurrence
			Mg^{2+}	Ca^{2+}	PO_4^{3-}	
1	Sueoka	Natural	13,8	6,8	70	-
2	Sueoka + Ca^{2+}	*Id.*	*id.*	61	*id.*	-
3	*Id.*	Chemical	*id.*	*id.*	*id.*	Yes
4	BBM	Natural	13,8	6,8	69,8	-
5	BBM + Ca^{2+}	*id.*	*id.*	34	*id.*	-
6	BBM ++ Ca^{2+}	*id.*	*id.*	120	*id.*	Yes
7	BBM + Mg^{2+}	*id.*	44,9	6,8	*id.*	-
8	BBM ++ Mg^{2+}	*id.*	1000	*id.*	*id.*	Yes

[1]"+ Ca^{2+}/Mg^{2+}" means that medium is complemented up to the minimal concentration in Ca^{2+} or Mg^{2+} required for flocculation by NaOH addition, and "++ Ca^{2+}/Mg^{2+}" that the medium is dopedmuch above the minimal concentration required for flocculation by NaOH addition.

4. Conclusions

Magnesium or Calcium minimal concentrations found with NaOH induced flocculation were in accordance with those already published for the same or other strains. However, they were far below the effective minimal concentrations required to induce natural flocculation and natural or artificial flocculation mechanisms were different.

Observed natural flocculation could be able to appear only under specific conditions. In particular, the culture medium should have nitrate ions as nitrogen source. The results suggest that natural flocculation by slow pH increase with no CO_2 provision should be envisaged only for strains cultivated in wastewater in which calcium and magnesium concentrations are above the required minimum. Moreover, the wastewater medium is potentially rich in mineral salts, particularly Ca^{2+} and Mg^{2+}ions that could cause the precipitations induced flocculation of microalgae. Our next papers will focus on mechanism of flocculation and culture of microalgae in wastewater medium.

The freshwater microalgae *Chlorella vulgaris* 211-19 is harvested by slow and natural increase in pH (natural flocculation). Effect of medium composition is particularly investigated. Experiments are carried out in two media with different nitrogen nutrients, a Sueoka based medium with ammonium and a BBM based medium with nitrate. It is found that none of the media allows natural flocculation. However, natural flocculation in the NO_3^- based medium becomes possible if either Ca^{2+} or Mg^{2+} concentrations are increased, but it remains impossible in the NH_4^+ medium. Natural flocculation requires much higher Ca^{2+} and Mg^{2+} concentrations to generate cells aggregates than artificial increase in pH by soda addition (for example $[Ca^{2+}]_{natural}$=136 mg/L (3,4 mM) whereas $[Ca^{2+}]_{artificial}$=34 mg/L (0,85 mM)). Cells have been pre-concentrated up to 19 gDM/L by calcium phosphates induced natural flocculation and up to 33 gDM/L by that induced by magnesium compounds.

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