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Recovery of Lutein from Microalgae Biomass: Development of a Process for *Scenedesmus almeriensis* Biomass

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In this work an optimized method for the extraction of lutein from microalgae biomass is presented. It has been developed using dry biomass of the lutein-rich microalga *Scenedesmus almeriensis*. The method comprises three steps, cell disruption, alkaline treatment, and solvent extraction, and renders a carotenoid extract rich in lutein. The results demonstrate that cell disruption is necessary and that the best option among the treatments tested with regard to industrial applications is the use of a bead mill with alumina in a 1:1 w/w proportion as disintegrating agent for 5 min. With regard to the alkaline treatment, the optimal conditions were obtained using 4% w/v KOH with a biomass concentration of 100 g/L for 5 min. Longer alkaline treatments or the use of higher KOH concentrations reduced the yield of the process. Finally, extraction with hexane is optimized. Using a 1:1 ratio hexane to sample volume, a total of eight extraction steps are necessary to recover 99% of lutein contained in the processed biomass. However, the optimal number of extraction steps is six, 95% of the lutein being recovered. In summary, the developed method allows the efficient recovery of lutein from microalgae biomass, it being a scaleable and industrially applicable method.

KEYWORDS: Extraction; lutein; microalgae; *Scenedesmus almeriensis*

INTRODUCTION

Lutein is a xanthophyllic compound recommended to prevent some types of cancer (*1*−*3*), cardiovascular diseases (*4*), and retinal degeneration (*5, 6*). Lutein is also a food colorant allowed by the European Union and reported as E 161 b. Sales of lutein as a feed additive in the United States amount to about $150 million per year. Although lutein is present in fruits and vegetables, the estimated daily intake of 1.5 mg/day (*7*) does not meet the recommended daily needs (*8*); thus, the consumption of lutein supplements is recommendable in some cases. The current commercial source of pure lutein is marigold (*Tagetes erecta* L.). However, the lutein content of marigold flowers is low, 0.03% dry wt, which makes alternative lutein-rich sources interesting. Several microalgae have been proposed as potentially useful to produce lutein, sych as *Muriellopsis* sp. (*9*), *Chlorella zofingensis* (*10*), or *Chlorella protothecoides* (*11*). Recently, a new lutein-rich microalgae strain, *Scenedesmus almeriensis*, has been also proposed as a lutein source (*12*). Compared to higher plants, microalgae have several advantages because they can be cultivated in bioreactors on a large scale, thus being a continuous and reliable source of lutein (*13*−*15*).

Although lutein is currently separated and purified from marigold flowers by a saponification−extraction−recrystallization method (*16*), no industrial processes have been proposed considering microalgae biomass as raw material. Some work has been done with microalgae on the optimization of separate operations as disruption of algal cells (*17*−*20*), extraction of carotenoids with organic solvents (*20*−*25*), and/or saponification of vegetable biomass (*26*−*29*). On the other hand, the few studies dealing with the overall process of lutein recovery from microalgae biomass are developed at a very small scale because they are oriented to analytical purposes (*23, 30*). These methods require much time and high volumes of solvents and disregard the importance of cell disruption in the yield of the process.

In the present paper a complete process for the recovery of lutein from the lutein-rich new strain *S. almeriensis* is developed. The method takes into account the existence of a hard cell wall in this strain, and thus a cell disruption step is included. In addition, an alkaline treatment is done to complete cell disruption and help to remove ionizable lipids. The last stage is a multistep solvent extraction procedure, which is then optimized to minimize the number of extractions and therefore the amount of solvent used. The optimized method allows the obtainment of a final lutein-rich extract that could be used for commercial purposes.

MATERIALS AND METHODS

Microorganism and Culture Conditions. The microalga *S. almeriensis* was isolated in fresh water from a greenhouse located in

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Almería, Spain. This strain was identified as new by the "Experimental Phycology and Culture Collection of Algae - SAG" and deposited in the Culture Collection of Algae and Protozoa of the Centre for Hydrology and Ecology, Ambleside, U.K., code CCAP 276/24. Cells were produced in an industrial size outdoor tubular photobioreactor (3.000 L), in continuous mode at 0.4 L/day dilution rate, in March. The cultures were performed at pH 8.0 by on-demand injection of CO_2 and at 30 °C by passing thermostated water through a heat exchanger located inside the reactor. The biomass was daily harvested by centrifugation and then lyophilized and stored at −18 °C. This batch of lyophilized biomass had 1.04% dry wt of lutein as measured by the analytical method described at the end of this section and was used as raw material.

Cell Disruption Methods. Three different methods were used for cell disruption: mortar, bead mill, and ultrasound. A mortar laboratory with a 125 mL volume was the first disruption method. The bead mill used had a volume of 2 L and a rotation speed of 120 rpm, with ceramic beads of 28 mm diameter. The ultrasound equipment used was a Pselecta Ultrasons unit. Samples of 50 g of biomass were used with alumina (aluminum oxide, type A-5, Sigma-Aldrich Inc., St. Louis, MO) as abrasion agent added in a 1:1 biomass to alumina ratio used in the experiments as specified next. A total of five cell disruption methods were essayed: (a) M + A (the biomass was ground in a laboratory mortar with alumina), (b) BM (the biomass was treated in a bead mill using beads of 28 mm diameter), (c) BM + A (the biomass was treated with alumina in a bead mill using beads of 28 mm diameter), (d) U (the biomass was treated in the ultrasonic unit), and (e) U + A (the biomass was ground with alumina in a mortar and then introduced in the ultrasonic unit). The experiments were done at room temperature (20−22 °C) for 5, 15, 40, and 60 min. In addition to these methods, a control sample of lyophilized biomass without additional cell disruption was also processed for comparison. The efficiency of each disruption method was evaluated by taking a sample from the processed biomass and applying the analytical method described at the end of the section without the initial cell wall breaking step. The results shown are the proportion of lutein recovered after each disruption method compared to the amount recovered with the analytical procedure.

Alkaline Treatment. The alkaline treatment was carried out in 100 mL flasks with magnetic agitation at 300 rpm. The experiments were prepared to make a 50 mL volume sample, thus filling a half of the flasks. The biomass used was disrupted using the best method found among the described in the former subsection. The variables studied were the biomass concentration, potassium hydroxide concentration and process time. The biomass concentration in the reaction mixture was tested between 20 and 150 g/L. The experiments were prepared by weighing the amount of biomass necessary for the concentration requested and then adding potassium hydroxide solution in water to complete 50 mL. The concentration of the KOH solution varied 4% to 24% w/v. The experiments were done at room temperature (20−22 °C) to avoid degradation of lutein and under continuous agitation to enhance the reaction between biomass and the alkaline agent in order to achieve as homogeneous a mixture as possible, especially when working with biomass concentrations substantially greater than the experiments described in the literature (*9, 28, 31, 32*). The reaction flasks were opaque, and the experiments were performed under argon atmosphere to prevent the degradation of lutein. Samples were collected and analyzed at various intervals during 80 min. The efficiency of each alkaline treatment was evaluated by applying the analytical method without disruption or the saponification steps. The results are given as the proportion of lutein recovered after each alkaline treatment compared to the amount recovered with the complete analytical procedure.

Extraction Methods. A repeated extraction with hexane in a 1:1 v/v proportion was used to recover the carotenoids from the reaction mixture. The sample used used was disrupted and treated with alkali using the best combination of methods found in the two previous subsections. In each extraction step, 5 mL of the sample was put in contact with 5 mL of hexane plus 0.1 mL of ethanol to prevent the formation of emulsions (*33*). The resulting mixture was homogenized for 5 min and then centrifuged at 3500 rpm for 3 min using a Labofuge 200 centrifuge. The supernatant hexanic phase was then collected. The lutein recovery of each step was measured by drying the hexane in a N_2 stream, dissolving in acetone, and injecting in a HPLC as described next. The variable studied was the number of extraction steps.

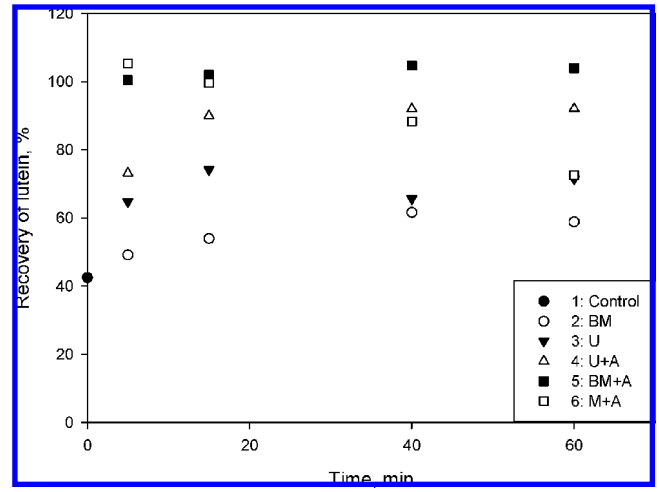

Figure 1. Recovery of lutein from lyophilized biomass of *Scenedesmus almeriensis* as a function of the process time for the following cell disruption methods: 1, control (no cell disruption); 2, BM (bead mill); 3, U (ultrasound); 4, U + A (mortar with alumina + ultrasound); 5, BM + A (bead mill with alumina); 6, M + A (mortar with alumina).

Analytical Methods. The carotenoid profile was quantified by HPLC after an analytical extraction procedure. Solid samples were treated using a modification of the method proposed by Del Campo et al. (*9*). For this, 10 mg was ground in a mortar with alumina and then extracted three to four times (or after the extract was clear) with 1 mL of acetone. Acetone extracts were then evaporated under nitrogen gas, and the residue was redissolved in pure ethyl ether. The sample was then saponified by adding the same volume of KOH in MeOH 4% w/v, instead of the 2% w/v proposed by Del Campo et al. (*9*). This modification is described under Results. The mixture was stirred and then allowed to react for 15 min at 0 °C in darkness under nitrogen gas. To stop the reaction and to remove excess alkali, 2 mL of 10% NaCl was added. After separation of the phases, ether was evaporated under nitrogen. The remaining pigment, redissolved in pure acetone, was ready for HPLC. The efficiency of cell disruption methods of the scaled up process was evaluated by performing the analysis without the grinding step.

The efficiency of the scaled up alkaline treatments was assessed by taking aliquots of 1 mL that were successively extracted with hexane until no color was observed in the hexanic phase. The hexane was then evaporated under nitrogen gas and the remaining pigment redissolved in 1 mL of pure acetone and centrifuged to discard particulate material. The extract obtained was ready for HPLC analysis. The analysis of hexanic samples from the scaled up extraction process was done by drying 1 mL of sample under a nitrogen stream and redissolving in acetone.

The HPLC procedure was as described by Minguez-Mosquera et al. (*32*), as modified by Del Campo et al. (*9*) and then by Cerón et al. (*34*) using a Shimadzu SPD-M10AV high-performance liquid chromatograph, different column and removing salts. Separation was performed on an Lichrosphere RP-18 5 μm column (4.6 × 150 mm). The eluents used were (A) water/methanol (2:8, v/v) and (B) acetone/methanol (1:1, v/v). The pigments were eluted at a rate of 1 mL/min and detected by measuring absorbance at 360−700 nm. Lutein was quantified by integration at 450 nm. Standards of β-carotene and lutein were provided by Sigma Chemical Co. (St. Louis, MO), and violaxanthin was from DHI.

RESULTS

The analytical procedure described under Materials and Methods for solid samples was assumed to extract 100% of the carotenoids present in the sample and thus used as a reference

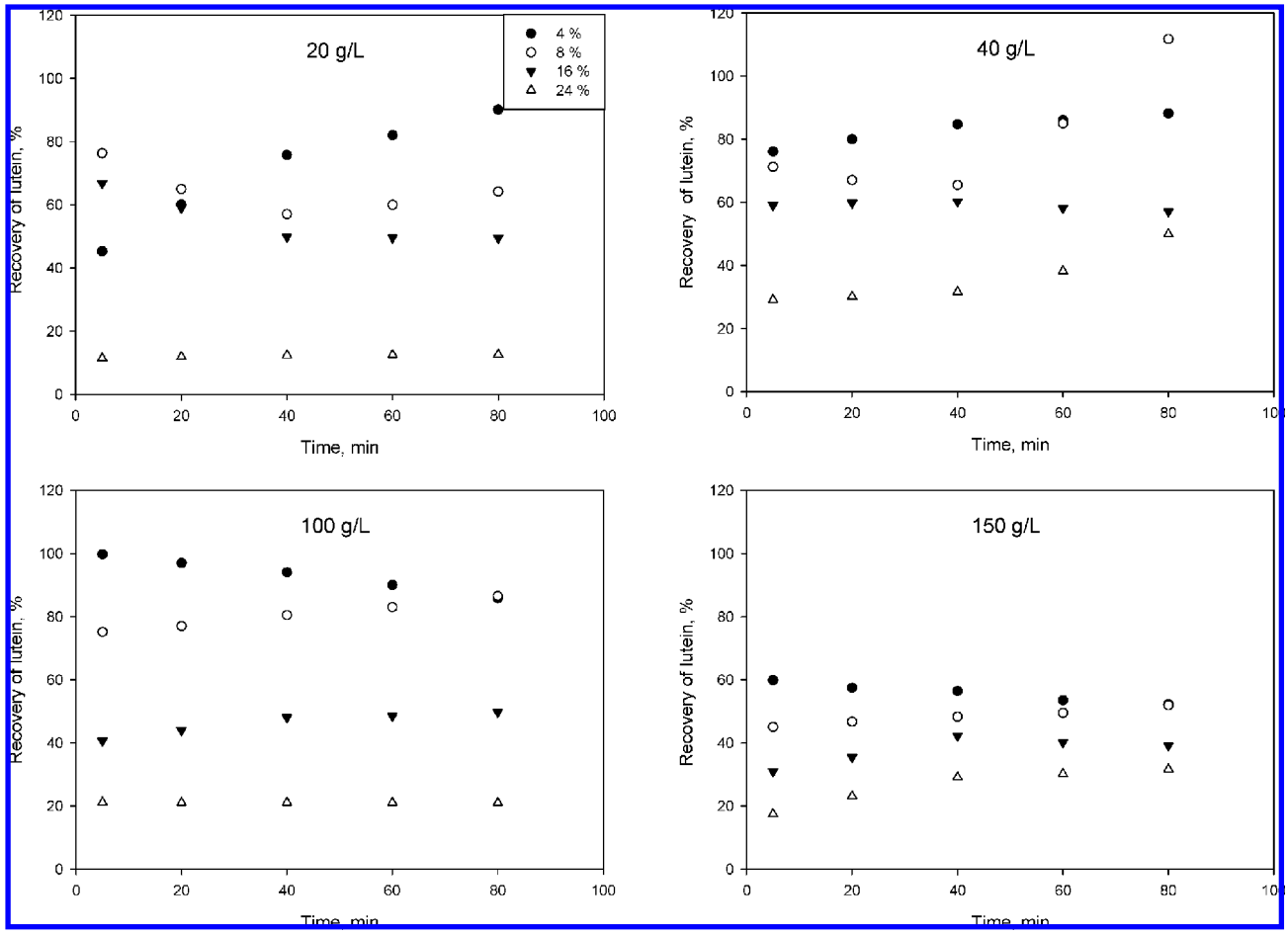

Figure 2. Recovery of lutein as a function of process time after the alkaline treatment carried out in different conditions of biomass concentration (values shown at the top of each plot) and KOH concentration (% KOH w/v, shown in the legend).

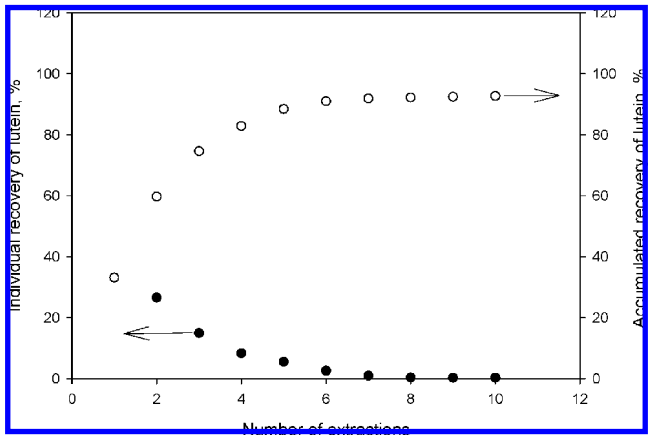

Figure 3. Recovery of lutein after alkaline treatment by repeated extraction with 1:1 v/v hexane. Solid symbols represent the proportion of lutein initially present that has been recovered in step *n*. Open symbols show the accumulated recovery after *n* extraction steps.

to evaluate the performance of the different steps of the scaled-up process. Early during this research it became evident that the method originally proposed by Del Campo et al. (*9*) for *C. zofingiensis* did not extract the carotenoids from *S. almeriensis* completely because the yields obtained in the preparative scale were higher than the analytical results. The analytical method was reassessed, and the saponification step with 2% w/v KOH in MeOH was identified as the limiting step. To overcome this, the method was re-evaluated using concentrations of KOH in MeOH between 4 and 24% w/v, and the results obtained showed

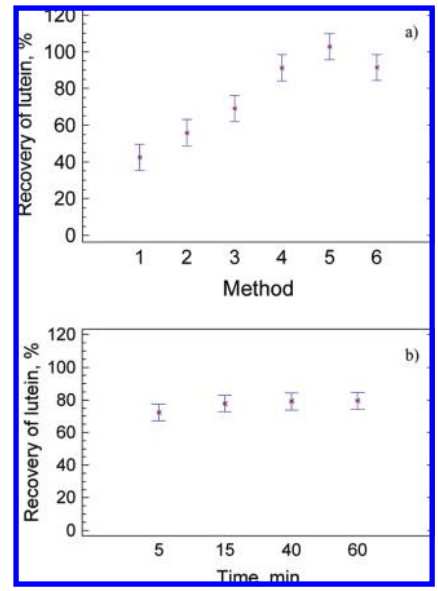

Figure 4. ANOVA of the effect of the cell disruption method (**a**) and process time (**b**) in the recovery of lutein from the lyophilized biomass of *Scenedesmus almeriensis*. Overlapping bars indicate no significant difference for a 95% confidence level.

that the recovery of carotenoids was optimal with 4% w/v KOH in MeOH. In these conditions, the batch of biomass of *S. almeriensis* used in this study had a 1.04% dry wt content of lutein, 0.3% dry wt violaxanthin, and 0.2% dry wt β-carotene, as well as other carotenoids under 0.1% (neoxanthin and

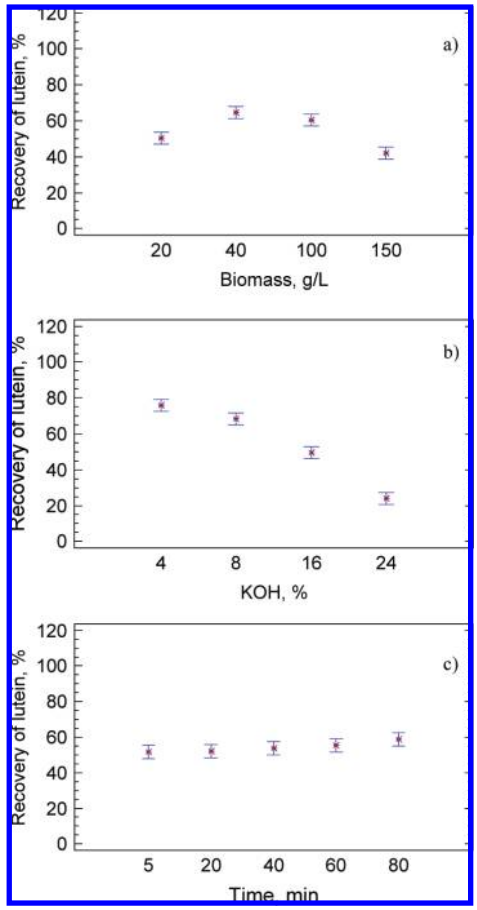

Figure 5. ANOVA of the effect of alkaline treatment in the recovery of lutein from biomass of *Scenedesmus almeriensis* after optimal disruption. The variables shown are (**a**) biomass concentration, (**b**) KOH concentration, and (**c**) processing time.

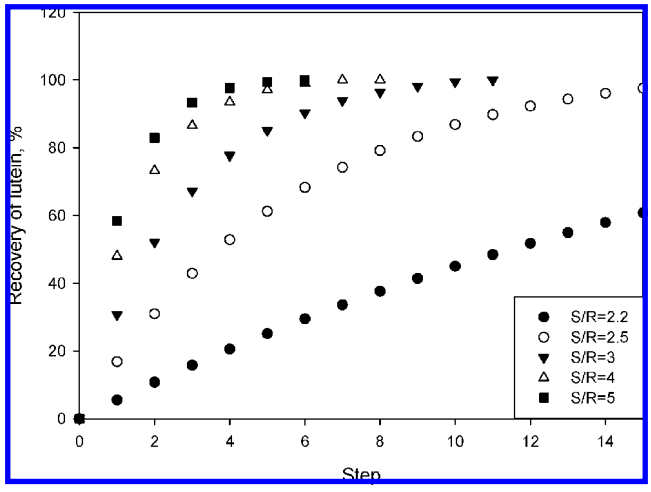

Figure 6. Theoretical recovery of lutein using multistage countecurrent L-L extraction as a function of the number of extraction steps for different solvent to raffinate (S/R) rates.

zeaxanthin). The rest of the results concerning the different steps of the extraction procedure reported next are expressed as percentage of lutein recovery.

The large-scale recovery method proposed consists of three steps: cell disruption, alkaline treatment, and extraction. Hexane was chosen as the extraction solvent because of its wide use in carotenoid extraction and in general in the food industry. The optimization of the cell disruption step was carried out first by studying the influence of the device used as well as the operating conditions including time. Cell disruption was carried out according to six methods based on mortar, bead mill, and ultrasound. During each experiment, samples were withdrawn at different times. The results are shown in **Figure 1** as the percent recovery of lutein obtained after the treatment. As **Figure 1** shows, if a cell disruption step is not performed, only 40% of lutein contained in the biomass is recovered after an alkaline treatment and extraction. The mortar method included was a scaled version of the disruption method originally included in the analytical procedure (*9*) and was tested for comparison purposes, but obviously impractical for a large-scale version. Therefore, the most efficient disruption was the use of a bead mill with alumina, followed in decreasing order by ultrasound plus alumina, ultrasound, and bead mill alone. The recovery yield of lutein remained constant for operation times over 30 min in every case. For the more efficient methods the maximum recovery yield was attained after only 5 min. The optimal conditions were obtained by grinding in a bead mill with alumina for 5 min, resulting in a recovery yield of 98%. These conditions were used as standard for the study of the next two steps of the process.

For the optimization of the large-scale alkaline treatment, the influence of biomass concentration, KOH concentration, and processing time in the recovery of lutein was studied. The biomass concentrations used were 20, 40, 100, and 150 g/L using as alkaline agent KOH in water at concentrations of 4, 8, 16, and 24% w/v, totaling 16 experiments that were carried out for 80 min. Samples were withdrawn at 5, 20, 40, 60, and 80 min to study the influence of time. The data obtained are displayed in **Figure 2**, where it is shown that the recovery of lutein is influenced by the three variables tested and that the optimal results were obtained for a biomass concentration of 100 g/L with 4% KOH and 5 min operation, yielding a 99% recovery of the lutein contained in the disrupted biomass.

The results of the repeated liquid−liquid (L-L) extraction with 1:1 v/v hexane are shown in **Figure 3**. A single step attained only a 30% recovery of the lutein present in the solution obtained after the disruption and alkaline treatment, whereas six consecutive steps recovered 95% of lutein and 99% lutein was recovered for eight extraction steps. Note that the data shown in the figure only approach 96−97% because they are referred to the lutein content initially present in the biomass that has been finally recovered and therefore show the small losses of the disruption and alkaline treatment.

DISCUSSION

A cell disruption step is necessary to recover lutein from *S. almeriensis* biomass because otherwise only 40% of lutein is obtained. **Figure 4** shows a variance analysis of the different disruption methods tested. This allows separation of the variables and statistically significant differences between the experimental sets to be seen. In this sense, there is significant difference between methods 1, 2, and 3 (without the use of alumina as a disintegrating agent) and the methods that use alumina. The confidence bars of these methods (4, ultrasound; 5, bead mill; and 6, mortar) overlap, meaning that all three could be used successfully, although the mortar is difficult to scale. The bead mill has been chosen among these for its higher mean and scalability. On the other hand, time had little influence in the range tested. Therefore, the optimal conditions considered are the use of a bead mill with 1:1 w/w alumina for 5 min.

To extract carotenoids from microalgae biomass it is necessary to perform an alkaline treatment to break ionizable lipids

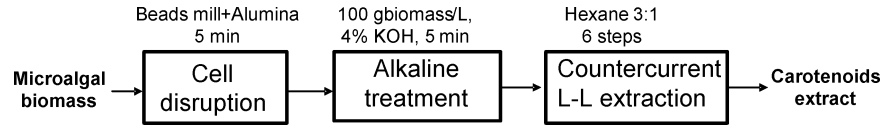

Figure 7. Schematic blockchart of a large-scale process for the recovery of lutein from *Scenedesmus almeriensis*.

(acyl-glycerols), complete cell wall destruction, and free the carotenoid fraction that might appear in esterified form. This allows recovery of the carotenoids in a nonpolar phase that otherwise would also extract many other lipids such as fats and waxes, as well as other nonpolar compounds that, after the alkaline treatment, remain ionized in the aqueous phase. Although most carotenoids appear in microalgae esterified to a certain degree (*35*−*38*), in the case of *S. almeriensis* most of the lutein is present in free form. The literature describes several alkaline treatments that mainly differ in the solvent used [water, methanol, or ethanol (*39*)] and the concentration of the alkaline agent (potassium hydroxide) that varies from 0.4 to 60% w/v (*10, 13, 14, 20, 23, 28, 31, 40*). However, excessive alkalinity can also damage the carotenoids; thus, the concentration of KOH must be optimized for each microalga. The proportion of biomass to alkaline agent is disregarded in the analytical methods, because it is always used in great excess, but it is a factor that must be taken into account for the scale-up.

Variance analysis of the experimental data (**Figure 5**) shows that the recovery of lutein was significantly affected by the concentration of KOH used as well as by the biomass load of the experiment. On the other hand, the time of operation had no significant influence. With regard to the biomass load, the recovery of lutein was low using 20 g/L, because the ratio of potassium hydroxide to biomass was too high. When the biomass concentration was increased to 40 g/L and then to 100 g/L, this ratio decreases and lutein is recovered with a higher yield. However, for the biomass concentration of 150 g/L the lutein yield was lower, probably due to the increase in viscosity, which made agitation difficult and hindered mass transfer, limiting the alkaline attack, or due to the low proportion of alkaline agent, which might have been exhausted, thus limiting the extent of the reaction. With regard to the KOH concentration, the analysis of variance shows a significant difference among all of the experiments, where a higher KOH concentration from 4 to 24% w/v lowered the recovery of lutein. The 2% w/v concentration was not included because it gave poorer results during the optimization of the analytical procedure as explained above. Therefore, the maximum lutein yield was obtained for 4% KOH. From these data it can be concluded that the optimal alkaline treatment conditions are a biomass concentration of 100 g/L with 4% KOH w/v for 5 min. In these conditions a lutein recovery of 98% is obtained.

The final step is the recovery of the carotenoids using extraction with adequate solvents. Solubility of carotenoids is high in organic solvents such as acetone, petroleum ether, benzene, hexane, diethyl ether, chloroform, dichloromethane, ethanol, and methanol. Some of these solvents have been tested for their ability to isolate carotenoids from microbial cells in a number of investigations (*11, 14, 21*−*24, 30, 31, 33, 40*−*45*). However, only hexane was a reasonable choice because it is widely accepted for food processing. The experimental data reported under Results (**Figure 3**) demonstrate that the carotenoids, and particularly lutein, can be recovered by repeated extraction with hexane. An eight-step single-contact extraction attains a lutein recovery of >99%, operating with the same volume of solvent (S) and raffinate (R), whereas a six-step process will yield a 95% recovery. Therefore, a total of 6−8 L of hexane is needed to process 1 L of saponified solution, which is a very large amount. The use of countecurrent contact can greatly decrease the amount of solvent needed and thus improve the economy of the process. The distribution equilibrium of lutein between the hexane phase (solvent, S) and the solution containing the processed biomass (raffinate, R) can be obtained from the data presented, resulting in

$$C_R = 2.1 C_S \quad (1)$$

where C_R is in the raffinate and C_S the equilibrium volumetric concentration of lutein in the solvent. The distribution is proportional because the lutein is in small quantity compared to the bulk of hexane and aqueous solution, and the two phases are immiscible. These data allow estimating how a countercurrent process would perform instead of the repeated single contact. For a 99% lutein recovery a minimum S/R = 2 is needed, which means a 4-fold reduction in the amount of hexane used for the extraction with regard to the single repeated mode. In this condition an infinite number of equilibrium steps is needed but only 17 steps are required for S/R = 2.5, 10 for S/R = 3, and only 7 for S/R = 4, which means half the solvent and one step less. This is shown in **Figure 6**, where it can be seen how the use of a low solvent to raffinate ratio requires many extraction steps, whereas, on the other hand, as few as four steps can be used for a 5:1 solvent to raffinate ratio. For comparison purposes, a 95% lutein recovery can be done in nine countercurrent steps if operating with a solvent ratio S/R = 2,5, in six steps with S/R = 3, or in only four steps if a S/R = 4 is used.

A block diagram of the proposed extraction process is shown in **Figure 7**. According to this diagram from 1 kg of dry biomass a carotenoid extract containing close to 10 g of lutein, in addition to β-carotene and violaxanthin, is obtained. The hexane is removed from the extract by vacuum distillation. The carotenoids are recovered and stabilized by solubilization in olive oil, rendering a final product consisting in 1000 mL of olive oil containing 1% of lutein. This product can be directly used as lutein complement or in the formulation of special foods for animal or human consumption.

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