

EVALUATION OF THE FLOCCULATION EFFICIENCY OF *Chlorella vulgaris* MEDIATED BY *Moringa oleifera* SEED UNDER DIFFERENT FORMS: FLOUR, SEED CAKE AND EXTRACTS OF FLOUR AND CAKE

C. M. L. Lapa Teixeira* and P. C. N. Teixeira

Microalgae Biotechnology Laboratory, Instituto Nacional de Tecnologia, Av. Venezuela 82, Sala 716, Saúde, CEP: 20.081-312, Rio de Janeiro - RJ, Brazil.

Phone: + 55 21 21231262

E-mail: claudia.teixeira@int.gov.br; pcn.teixeira@gmail.com

(Submitted: July 25, 2015 ; Revised: November 23, 2015 ; Accepted: January 4, 2016)

Abstract - Flocculation as a pre-separation method can help make production of biodiesel from microalgae economically feasible. In a previous study, *Moringa oleifera* seed flour (1 g.L^{-1}) was shown to be a very efficient flocculant for *Chlorella vulgaris*, a microalga with high potential for biodiesel production. In this study, several aspects of *C vulgaris* flocculation mediated by *Moringa* were investigated in order to optimize the separation of this biomass. Flocculation efficiency was the same with seeds from different origins and lots. The stationary growth stage was best for harvesting *C vulgaris* cells to carry out flocculation efficiently (93%). The use of flour extracts and cake extracts generated the best cost-benefit ratio (flocculation efficiency from 78 to 97% with a saving in mass of seed of 75%). The highest efficiency was reached with extracts prepared with seawater and NaCl solutions which have high salt concentration. Reasonable stability of the extract allows its use for up to two weeks, provided it is kept at low temperature ($4 \text{ }^{\circ}\text{C}$).

Keywords: *Moringaoleifera*; Microalgae; *Chlorella vulgaris*; Biomass separation; Flocculation.

INTRODUCTION

The use of biofuels has increased in the worldwide energy mix, with some countries mandating minimum blending of biodiesel with petroleum diesel. In Brazil this level is 8%, while in the USA and European Union it reaches 10% and 7%, respectively. To face the challenge of biodiesel production, plants and/or residual oils have been used as raw materials. However, with the aim of increasing production efficiency and diversifying the feedstock, microalgae have been indicated as a very promising raw material for biodiesel production (Mata *et al.*, 2010; Veillette *et al.*, 2012).

In spite of having several advantages in relation to oleaginous plants, microalgae biomass production has a higher production cost than those of oleaginous. The biomass separation process constitutes one of the main components of the biomass production cost (Uduman, 2010). Hence, there is a need to find cheaper biomass separation methods.

Centrifugation is the most efficient biomass separation process, but because of its high cost and energy demand (Benemann and Oswald, 1996; Molina *et al.*, 2003) it is applicable only in the production of biocompounds with very high commercial value. In the search for less expensive processes, filtration could be a choice, but this process has the serious

*To whom correspondence should be addressed

drawback of fouling the filter. Therefore, it can only be used with filamentous microalgae, which in general have low lipid content and are not suitable for biodiesel production.

Flocculation has been considered as a pre-separation process that diminishes the volume for further processing. There are different types of flocculation depending on the flocculant agent and method. Autoflocculation, for example, can occur during growth near the stationary phase, when the pH rises beyond 9 (Spilling *et al.*, 2011; Sukenik and Shelef, 1984). This strategy of raising pH is used to flocculate microalgae (Horiuchi *et al.*, 2003; Nguyen *et al.*, 2014; Wu *et al.*, 2012); however it is necessary to change the pH of the medium after the separation of the biomass in order to make possible its use in a next cultivation. The presence of certain bacteria in the cell suspension also promotes flocculation through the production of EPS (extracellular polymeric substances), which explains the flocculation observed in some cultures of microalgae and can be used as a flocculation method (Kim *et al.*, 2011; Lee *et al.*, 2009); on the other hand, this kind of flocculation has some constraints, such as for example, the relatively high cost for the purification of the biopolymers (EPS), and in the case of direct use of the bacterial suspension the need of an organic carbon source in the medium to sustain the bacterial growth. Certain microalgae have a strong tendency to flocculate and were used to promote flocculation of other microalgae (Salim *et al.*, 2011; Salim *et al.*, 2012), but additional costs for a separate cultivation system for cultivation of the flocculating microalga should be considered.

The use of flocculant agents has been considered (Wyatt *et al.*, 2012; Vandamme *et al.*, 2013; Mandik *et al.*, 2015), but their toxicity potential must be taken into account. Alum salts flocculate cells very efficiently (Papazi *et al.*, 2010) but they can cause serious health problems (Platt *et al.*, 2007). Some natural flocculants have been tested to overcome this problem. Chitosan has proved to be very efficient (Ahmad *et al.*, 2011; Chen *et al.*, 2014), but it works only at low pH, while the pH in microalgal cultures is relatively high. Besides, its price is prohibitive mainly for biomass production for biofuel generation. Starch and its cationic derivatives are an alternative and have been evaluated (Gerde *et al.*, 2014; Vandamme *et al.*, 2010). Among natural flocculants, Moringa seeds have great potential for flocculating cells of *Chlorella vulgaris*, as already demonstrated in our previous work (Teixeira *et al.*, 2012) and in more recent publications (Hamid *et al.*, 2014; Udom *et al.*, 2013). Moringa has been called a “Miracle Tree”, since it

has several important uses: its seeds are used to treat water and effluents, its leaves for food, the seed oil to produce biodiesel, and its flowers for food and cosmetics (Amaglo *et al.*, 2010). It grows in the wild in some regions of Brazil, especially the Northeast (Oliveira *et al.*, 1999).

In our previous study (Teixeira *et al.*, 2012), it was observed that flocculation of *Chlorella vulgaris* was greatly enhanced by the use of Moringa seed flour (from 3 to 90%) and the conditions of flour concentration (1 g.L⁻¹), sedimentation time (120 min) and pH (7.04-9.24) of the cell suspension were established for maximization of the flocculation efficiency. This study had the aim of evaluating recovery of *Chlorella vulgaris* cells by flocculation using Moringa seeds in other forms, as extract and seed cake, besides flour, as a strategy for lowering costs since seed cake is a residue from biodiesel production using oil from Moringa seed and the use of extracts may represent an economy of mass of flocculant.

MATERIALS AND METHODS

Microalga

The freshwater microalga *Chlorella vulgaris* CCMA-UFSCar 012 was kindly provided by Prof Armando from the Federal University of São Carlos (Brazil). Its isolate is kept in the collection of microalgae cultures of the Department of Botany, Federal University of São Carlos (CCMA-UFSCar, WDCM 835).

C. vulgaris was cultured in WC modified medium (Guillard and Lorenzen, 1972). The cultivations were carried out in 500-mL Erlenmeyer flasks with continuous agitation of 160 rpm in an orbital shaker (Marconi MACFT-140), in a controlled environment at 25±2 °C. White fluorescent light was used, which provided 60 μmol photons m⁻² s⁻¹ of light intensity in the region of the flasks.

New fresh suspensions were prepared using inocula taken from the stock culture in the exponential growth phase in such a way that an optical density of 0.05 was obtained to begin the growth. In general, the cell suspension reached a biomass concentration of 0.43-0.65 g.L⁻¹ (on a dry weight basis), which corresponds to an optical density (OD) at 730 nm of 0.86-1.30 in 20 days. The cell suspension was used after pH adjustment, if necessary, to 8.5–8.7 by the addition of 1-M HCl or 1-M NaOH. In the set of experiments where the influence of the biomass concentration and growth stage was evaluated, suspensions of different growth stages were used, as well as

diluted suspensions (with the supernatant of the cell suspension of OD 1.2 or WC) from the suspension in the stationary growth stage.

Preparation of Moringa Seed Flour and Moringa Seed Cake

Dry pods of *Moringa oleifera* from Sergipe state and from Paraná state were used. The seeds from Sergipe were donated by Dr. Gabriel Francisco da Silva from the Federal University of Sergipe (Brazil) and those from Paraná (Brazil) were donated by Marianna Ferreira (Itaipu Binacional company). Seeds were shelled and ground with a mortar and pestle and then sequentially sieved through both 860 μm and 420 μm pore sieves to obtain the flour, except in the experiments carried out to evaluate the influence of flour granulometry on flocculation efficiency, where sieves of 175, 250, 420 and 860 μm were used separately in a Vibratory Sieve Shaker (Bertel) to generate flour of different particle sizes.

The Moringa seed cake was provided by Dr. Gabriel Francisco da Silva from the Federal University of Sergipe. It was obtained as a residue during the oil extraction, carried out using a Komet vegetable oil expeller (CA59G).

Preparation of the Extracts

Moringa seed flour or Moringa seed cake was added to the medium to reach 50 g.L^{-1} . Ultrasound and agitation on a magnetic plate for 15 minutes, at 25 °C, were used for extraction followed by centrifugation with a Fanem Excelsa Baby centrifuge at 3500 rpm for 15 minutes. A subsequent filtration step (through a 0.45- μm membrane) was used in some cases. Depending on the experiment different extraction media were used: distilled water, WC medium, saline solution (NaCl) at 0.6 M and seawater (salinity of 35PSU). Seawater at different salinities (20, 25, 30, 35, 40, 45 and 50) and saline solution at different molarities (0.2, 0.4, 0.6, 0.8 and 1 M) were used as extraction media. In order to lower or to increase the salinity of the seawater, distilled water or NaCl was added, respectively.

Flocculation Assays

After the addition of the seed flour (to attain 1 g.L^{-1}) or seed cake (to attain 1 g.L^{-1}) or extracts (1 mL to 99 mL of cell suspension), the suspensions (maintained in Erlenmeyer flasks) were incubated for 15 minutes at 100 rpm on an orbital shaker (Marconi MACFT-140). Then 25 mL of each culture were

transferred to cylinders in triplicate to evaluate flocculation. Before and after the incubation period and at 120 minutes of standing time, suspension aliquots were withdrawn for OD measurement at 730 nm. The aliquots were taken from the middle of the suspension column.

Flocculation efficiency of the Moringa treatment was evaluated using the OD decrease and percent OD decrease. The OD decrease was calculated as the difference of the OD of the suspension obtained after incubation (initial OD) and the OD of the suspension measured at 120 minutes of observation (final OD). Percent OD decrease was calculated as the ratio between the OD decrease and initial OD. The OD decrease was used to represent flocculation efficiency when employing the same cell suspension in the evaluations, as the initial OD was the same. The use of the OD decrease is advantageous because it can be monitored by plotting error bars, which are very important to evaluate the results. The percent OD decrease does not permit this because the percent results are nonparametric samples and standard deviation is not applicable to nonparametric analysis (Dixon, 1983; Siegel, 1988); therefore, percent OD decrease was used (in addition to OD decrease) only in the experiments where the cell suspensions used had different initial OD, since comparisons using only the OD decrease would generate distortions in the evaluations and consequently the conclusions.

Cell suspensions without the addition of Moringa and treated the same as the treated ones were used as control samples. Each situation was tested in triplicate and the results of OD decrease or percent OD decrease presented correspond to the average of the three tests.

RESULTS AND DISCUSSION

At first, seed aspects such as lot and origin, and cell suspension characteristics such as growth phase (not included in the previous article) were investigated using only Moringa seed flour. After that, the use of seeds in the form of cake and extracts (of seed flour and seed cake) was studied in order to optimize the relation cost benefit of recovering *C. vulgaris* cells through flocculation mediated by Moringa seeds.

Evaluation of the Fluctuation of the Flocculation Efficiency Throughout Experiments with Seed Flour

Results of flocculation efficiency from all the experiments carried out with Moringa seeds from

Sergipe, used in the form of seed flour at 1 g.L^{-1} are shown in Figure 1. It is important to emphasize that the concentration of seed flour of 1 g.L^{-1} was used in this work because in our previous paper (Teixeira *et al.* 2012) this concentration promoted the highest biomass recovery. Values of the OD decrease (Figure 1A) from 0.86 to 1.18 and values from 70 to 96% in terms of percent OD decrease (Figure 1B) can be observed. Figure 1A also presents the initial OD for each experiment and it can be observed that, in spite of the variation of initial OD of 0.94-1.30 (0.47 to 0.65 g.L^{-1} in terms of concentration), there is no correlation between it and flocculation efficiency. The percent OD decreases observed represent recovered biomass values from 0.43 to 0.59 g.L^{-1} .

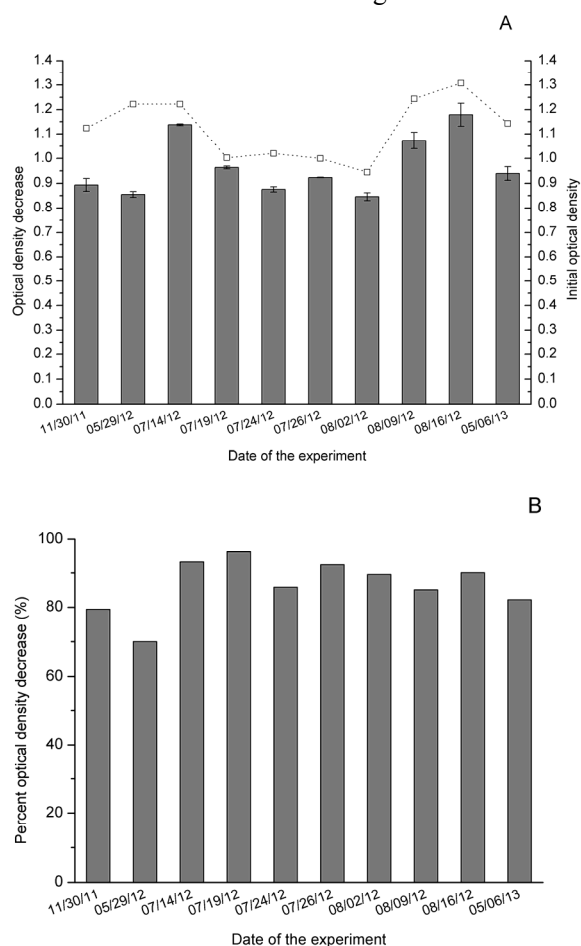


Figure 1: (A) OD decrease and (B) percent OD decrease obtained during the flocculation experiments of *C. vulgaris* cells mediated by Moringa seed flour. Rectangles represent the initial OD.

The percent OD decrease was used to represent these results because they represent different cell suspensions, with different initial OD. The OD decrease was also used to evaluate flocculation effi-

ciency in order to make more appropriate comparisons, since this kind of representation can (unlike percent OD decrease) be monitored by error bars (as already explained).

Since two different lots of Moringa seeds were used in these experiments, the OD decrease was assessed in a new experiment using these lots and the same cell suspension. In this case, no statistically significant difference was observed (0.640 ± 0.029 ; 0.641 ± 0.009), indicating that the differences observed in Figure 1 can be attributed to the varied conditions of the cells and the medium surrounding them. So, it is probable that, in experiments performed on different days, different Zeta potentials and/or different concentrations of extracellular substances interfered in the flocculation efficiency. Different flocculation efficiencies in experiments with Moringa due to Zeta potential differences were reported by Ndabigengesere *et al.* (1995). Accordingly, aspects specifically involving the seed, such as the time passed since arrival at the laboratory or preservation conditions did not interfere in the flocculation efficiency of Moringa as shown in Figure 1.

Seeds from the other state (Paraná) of Brazil were also tested with the aim of verifying whether seeds from regions with different climate could exhibit different flocculation efficiencies. The state of Paraná is located in southern Brazil and has a humid subtropical climate, while Sergipe has a tropical and semi-arid climate. In this experiment, no difference in terms of OD decrease was observed (0.882 ± 0.010 and 0.886 ± 0.013), indicating that indeed the difference observed with time is probably related to the aspects of the sample other than growing conditions of the plants from which the seeds were obtained. Additionally, this result shows that seeds from either of these states can be used without interfering with the expected efficiency of flocculation promoted by Moringa.

These results indicate that differences in conditions of harvesting and storage of the seeds probably do not interfere in Moringa's flocculation efficiency and therefore the Moringa's main active ingredient is relatively quite stable.

Effect of Growth Stage and Biomass Concentration on Flocculation Efficiency of Seed Flour

In order to determine the best cell growth stage to separate biomass through flocculation, we tested different cell suspensions whose cultivation had started on different days, so that their growth stages were different on the day of experimentation. Five cell suspensions were tested, which had initial OD

values around 1.2, 1.0, 0.8, 0.6 and 0.4 (ODs of 1.2 and 1.0, ODs of 0.8 and 0.6, and an OD of 0.4 correspond to stationary, linear and exponential growth stages, respectively).

It can be seen that both the OD decrease (Figure 2A) and percent OD decrease (Figure 2B) rose according to the growth stage and that the highest value was found with cell suspensions in the stationary growth phase, indicating this phase as the best to collect the cells. It should be mentioned that different results in terms of OD decrease among suspensions with different initial ODs can occur (which was observed here), so in these cases, what really indicates differences in flocculation efficiency is the percent OD decrease, which, as can be observed in Figure 2B, also increased as a function of the growth phase.

Since Muyibi and Evison (1995) and Nkurunziza

et al. (2009) observed that the flocculation efficiency of turbid water mediated by *Moringa* depended on the water turbidity, the result found could have been affected by the biomass concentration. Moreover, certain substances and ions of the extracellular medium could be also responsible for the differences in flocculation efficiency, since their concentrations change during cultivation as a result of cell metabolism. Therefore, we determined flocculation efficiency in cell suspensions of different biomass concentrations but of the same growth stage. These cell suspensions were prepared by diluting a cell suspension from the late stationary phase of OD around 1.2 (0.6 g.L^{-1}) so as to obtain the same OD values used in the previous experiment (around 0.4, 0.6, 0.8 and 1.0), and as diluent the supernatant of the cell suspension of OD 1.2 was used to avoid introducing new compounds in

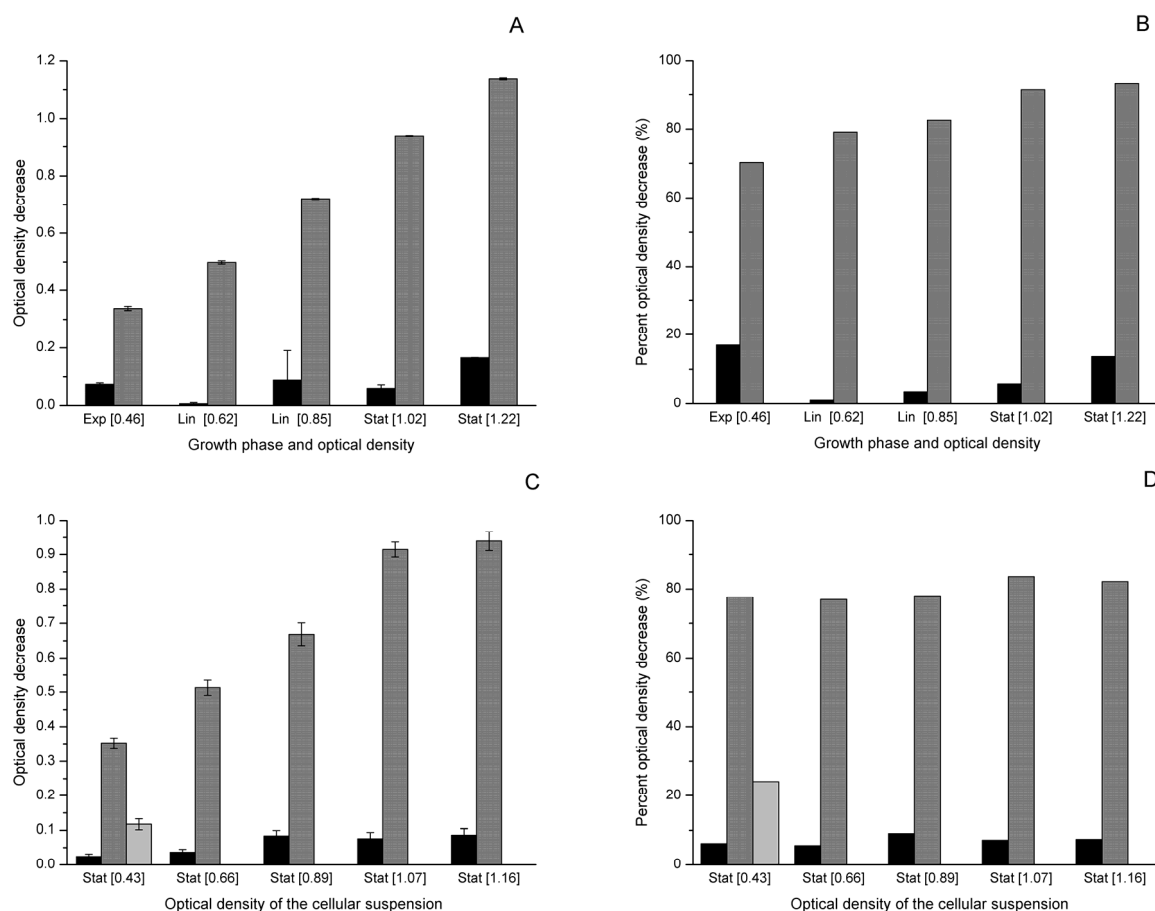


Figure 2: Effect of cell suspension growth phase and optical density on the *Moringa* flocculation efficiency evaluated as (A) OD decrease; (B) percent OD decrease. The growth phases exponential, linear and stationary were abbreviated as *Exp*, *Lin*, and *Stat*, respectively. Effect of initial OD on the *Moringa* flocculation efficiency evaluated as (C) OD decrease and (D) percent OD decrease. In *gray*: treated cells; *black*: non treated (control); *light gray*: cells resuspended in WC before treatment.

the cell suspension to be tested. In addition, to verify whether a dilution using fresh medium could interfere in the results, another cell suspension of OD around 0.4 was tested, which was prepared using WC for dilution.

In Figure 2C, differences in the OD decrease can be observed among the suspensions with different initial ODs, but in this case the result does not indicate differences of flocculation efficiency (as discussed above). It is corroborated by the results shown in Figure 2D, where practically no difference in percent OD decrease was observed when the initial OD increased. Hence biomass concentration did not interfere in the flocculation efficiency and the large increase observed in Figure 2A between cell suspensions of different growth stages and concentration was mainly due to other aspects of the cell suspension (instead of biomass concentration), probably differences in the cell membrane related to the growth phase and perhaps the presence of substances in the extracellular medium that would help the flocculation mediated by Moringa. Flocculation has been observed (Lee *et al.* 2009) of coccolithophorid alga, *Pleurochrysis carterae*, promoted by EPS from microorganisms in nutrient deprivation (which occurs in the stationary phase). The much lower percent OD decrease of the cell suspension with OD of 0.4 (diluted with WC) reinforces the hypothesis that substances from the extracellular medium produced during cell growth (of the microalga as well as of contaminant microorganisms present in the culture) influence the flocculation efficiency of Moringa.

Although maximum flocculation efficiency was achieved for cells at late stationary phase it is still possible to attain a reasonable (around 80%) flocculation efficiency when harvesting cells in the exponential phase, which might be advantageous because of the shorter time of cultivation; on the other hand, the time of cultivation required for maximization of target products production should be considered.

Flocculation Efficiency of the Moringa Extracts of Seed and Cake Prepared in Different Ways and Moringa Seed Cake

The use of Moringa seeds as flour was very efficient as well as practical because of the simplicity of the flocculation method. As the dosage of seed flour for maximum efficiency is high (1 g.L^{-1}), the use of seed flour or seed cake in the form of extracts could be a way of saving flocculant and at same time it might allow recycling of the medium after biomass separation. In addition, seed cake was also tested because, as a residue (of the oil extraction), it may

help decrease the cost of flocculation mediated by Moringa seed.

Different methods of preparing the seed flour extracts were assessed. Extractions using a magnetic plate and ultrasound bath as well as using only centrifugation after the extraction process or filtration after centrifugation were carried out. WC was used as extraction medium since it is the medium of the cell suspension in which *C. vulgaris* is grown. Since in preliminary experiments (results not shown) we observed that a 100-fold dilution of the Moringa seed extract at 50 g.L^{-1} in the cell suspension was able to generate the same result as that observed with seed flour at 1 g.L^{-1} , these values of proportion of the dilution in the cell suspension and extract concentration were used, respectively, in these experiments with extracts. The results for OD decrease were: 0.014 ± 0.001 , 0.893 ± 0.026 , 0.789 ± 0.019 , 0.789 ± 0.028 , 0.852 ± 0.017 , 0.890 ± 0.065 , for control, seed flour and for extraction by magnetic agitation, magnetic agitation followed by filtration, ultrasonic agitation, and ultrasonic agitation followed by filtration, respectively. There was only a small difference between the two modes of preparation (magnetic agitation or ultrasonic agitation, with ultrasound being slightly more efficient), and between extracts and seed flour. Filtration did not affect the flocculation efficiency of the Moringa seed extract.

In spite of the small difference of flocculation efficiency observed between magnetic and ultrasonic agitation, in the rest of the experiments ultrasonic agitation was used for the preparation of extracts of seed flour and seed cake because of its practicality. The filtration step was maintained to facilitate withdrawing the extract aliquot because a greasy material appeared on the surface of the extract during the extraction process.

According to Ghebremichael *et al* (2005), Moringa saline extracts had higher flocculation efficiency than aqueous extracts; therefore, to evaluate the possibility of enhancing the flocculation efficiency by using a medium with higher salinity than WC (used in the previous experiments), a saline solution (1M NaCl) was used as medium for the extractions. Freshwater and seawater were also used for comparison because of their higher availability in certain situations, and the use of seawater enables a saving of water and NaCl in relation to the saline solution and water. In these experiments, extract of seed cake was used besides extract of seed flour and their flocculation efficiencies were compared to the seed cake and seed flour flocculation efficiencies, respectively. WC was included as a medium for extraction for the seed flour assay in order to verify if the use of a very low

salinity medium composed of inorganic salts would interfere in the flocculation efficiency.

As can be observed in Figure 3, the medium with highest salinity employed generated the highest flocculation efficiency. Since Gassenschmidt *et al.* (1995), Ghebremichael *et al.* (2005), and Ndabigengesere *et al.* (1995) demonstrated that peptides are involved in the flocculation mechanism of *Moringa*'s flocculation action, this result can be explained by the salting-in or salting-out effect, and a peptide might be involved in the flocculating effect of *Moringa* on the *C. vulgaris* cells. Furthermore, this peptide might also be present in the seed cake since the extraction in a expeller extracts mainly oil and the great majority of its constituent proteins remains in the residual mass. Further evidence of the presence and participation of protein (or peptides) in the observed effect of *Moringa* is that heating of the extract reduced the flocculation efficiency (measured as OD decrease). The seed flour extract was heated to 50 °C for 15 minutes and the OD decrease diminished by 50% in comparison to the non-heated extract. However, since the flocculation efficiency (measured as OD decrease) of the heated extract was higher than the control (i.e., it is still significant) it can be inferred from this result that some flocculating agent that either was not protein (or peptide) or a heat-resistant protein was left in the extract. Thermoresistance in the seed extract of *Moringa* was observed in treatment at temperatures from 60 to 100 °C for 0.5 and 5 hours (Ghebremichael *et al.*, 2005).

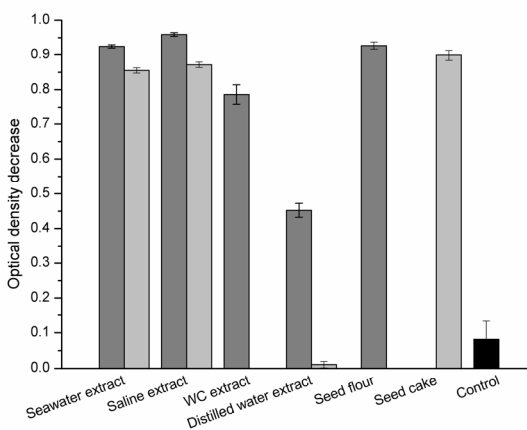


Figure 3: Effect of the medium used to prepare the *Moringa* extracts of seed flour (dark gray columns) and seed cake (light gray columns) on the flocculation efficiency. Black column represents the control.

Since saline and seawater extracts resulted in better results for both the seed flour and seed cake, the best NaCl concentration in the medium and the best salinity for preparation of the saline and seawater extracts, respectively, were assayed. The OD decreases

of the control samples for different molarities and different salinities were 0.024 ± 0.004 and 0.111 ± 0.002 , respectively. For saline extract the best NaCl molar concentration (Figure 4A) tested for seed flour was 0.6 M and the best salinity for the seawater (Figure 4B) was 35 PSU, although the differences among different salinities and among different molarities were low. For seed cake extracts the differences of flocculation efficiency were still lower among different salinities and molarities, but for the molarity 1 M the flocculation efficiency was slightly higher than for the other molarities. The highest flocculation efficiency for saline extract of seed flour was found at a NaCl concentration that was in accordance with the value found for seawater salinity that generates the higher flocculation efficiency, since the concentration 0.6 M presents a salinity of 35 PSU. The result for the extract in seawater is very interesting because the higher flocculation efficiency occurred at the natural salinity (35 PSU), which eliminates the need to add more salt or dilute the seawater.

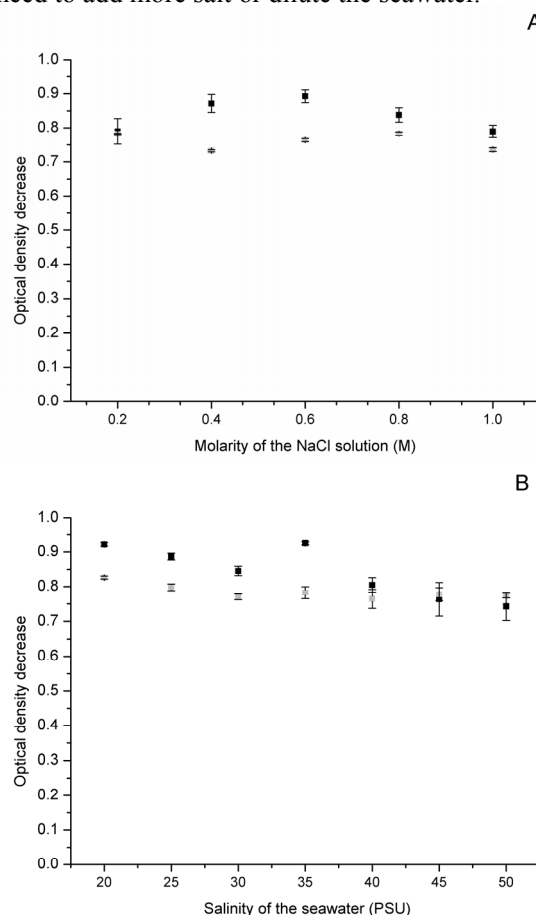


Figure 4: Effect of (A) of the molarity of the saline solution and (B) salinity of the seawater used to prepare the extracts of seed flour (dark gray rectangles) and seed cake (light gray rectangles) on the *Moringa* flocculation efficiency.

The influence of granulometry of seed flour used for the preparation of the saline extracts was also investigated. Granulometries of 175 and 860 μm , the extreme values tested before, were chosen. The results showed a very small difference of OD decrease in the two different particle sizes (0.867 ± 0.009 and 0.822 ± 0.007 for 175 and 860 μm , respectively). As in the case of seed flour, the grinding of the seeds can be done in such a way that a large range of granulometries can be used without diminishing the flocculation efficiency of Moringa extract.

Effect of the Moringa Seed Flour Extract Volume on the Flocculation Efficiency

Different volumes of saline extract (0.6 M) were tested in order to minimize the volume of extract used to flocculate cells. Figure 5 indicates that, for a volume of extract of 0.5 mL the efficiency is still very high but decreases drastically below this value. Therefore, a volume of extract of 0.5 mL for each 100 mL of cell suspension is the minimum for an efficient flocculation.

Since the extract concentration used in this experiment was $50 \text{ g}\cdot\text{L}^{-1}$, the volume of 0.5 mL (used for 99.5 mL of cell suspension) contained a seed mass of 0.025 g, which in comparison to 0.100 g, mass of seed flour required for maximum efficiency flocculation for each 100 mL of cell suspension, represents a saving in mass of seed of 75%. Consequently, the use of Moringa seed in the form of extract is more convenient from an economical point of view than flour.

Determination of the Stability of the Moringa Extract

An important aspect to be tested is the stability of the extract in order to optimize its preparation. A saline extract (0.6 M) was prepared and used on the same day and then 7, 14, 21 and 28 days later (maintained in a refrigerator at a temperature of 4 °C). As can be observed in Figure 6, after 14 days the extract maintained its high efficiency, represented by high percent OD decrease (89%) and in the third week the flocculation efficiency declined to an unsatisfactory level (24%). The percent OD decrease for control samples were 11, 4, 8, 4 and 2%, for day 0, day 7, day 14, day 21 and day 28, respectively. Thus, two weeks after preparation of the extract is the limit for good efficiency. In this experiment, percent OD decrease was used to infer flocculation efficiency because different cell suspensions were used (as they were performed on different days) and there was no

need to use additional OD decrease to make the comparisons because it became evident that day 14 was the limit for using this extract as there was a very large difference between the percent OD decrease for day 14 and for day 21.

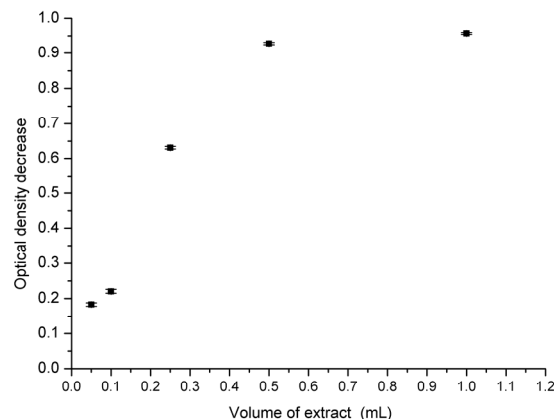


Figure 5: Effect of the volume of extract added to the cell suspension on the flocculation efficiency of Moringa extract of seed flour.

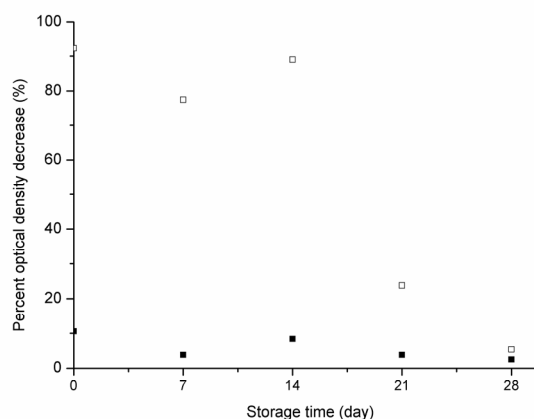


Figure 6: Dependence of the flocculation efficiency of the Moringa extract of seed flour on the date of preparation (evaluation of the extract's stability). Black rectangles represent the controls.

We have not found any literature reference to the effect of the storage of saline extract of Moringa seeds on its flocculation efficiency. In aqueous solution, the active principle deteriorated after third day upon storage under room temperature (Katayon *et al.*, 2004; Levick, 2005) and after the fifth day at 3 °C (Katayon *et al.*, 2004). In the present study a longer time was obtained for the preservation of the flocculant agent and it is possible that different compounds with different stabilities are present in the extract depending on the media used for its preparation.

CONCLUSIONS

It is possible to use *Moringa* seeds of different origins and lots. Seed flour and seed cake extracts perform very well, with saline and seawater being the most efficient media. Agitation on a magnetic plate or ultrasound can be employed to prepare the extracts. The best phase to harvest cells is the stationary one, but concentrated suspension of previous phases can also be used. The comparatively low mass of *Moringa* seeds necessary to attain high flocculation efficiency in the case of the extract, in addition to its stability for two weeks, is a comparative advantage in relation to use of the flour. Besides, the use of seed cake has an advantage over seed flour because this is a residue from *Moringa* seed oil production.

ACKNOWLEDGEMENTS

The authors would like to thank Ana Lucia Barbosa de Souza and Diego Filócomo for technical assistance; Prof Gabriel Francisco da Silva from the Federal University of Sergipe (Brazil) and Marianna Ferreira (Itaipu Binacional Company) for providing the seeds of *Moringa oleifera* and Prof. Armando Vieira from the Federal University of São Carlos (Brazil) for providing the culture of *Chlorella vulgaris*. The research leading to this paper was partially funded by the Financer of Research and Projects (Finep) and National Counsel of Technological and Scientific Development (CNPq).

REFERENCES

- Ahmad, A. L., Yasin, N. H. M., Derek, C. J. C., Lim, J. K., Optimization of microalgae coagulation process using chitosan. *Chemical Engineering Journal*, 173, 879-882 (2011).
- Amaglo, K. N., Bennett, R. N., Curto, R. B. L., Rosa, E. A. S., Turco, V. L., Giuffrida, A., Curto, A. L., Crea, F., Timpo, G. M., Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L., grown in Ghana. *Food Chemistry*, 122, 1047-1054 (2010).
- Benemann, J., Oswald, W. J., Systems and economic analysis of microalgae ponds for conversion of CO₂ to biomass. U.S. Department of, Energy, 105-109 (1996).
- Chen, G., Zhao, L., Qi, Y., Cui, Y. L., Chitosan and its derivatives applied in harvesting microalgae for Biodiesel production: An outlook. *J. of Nanomaterials*, Article ID 217537, 9 pages (2014).
- Dixon, W. J., Massey Jr., F. J., Introduction to Statistical Analysis. 4th Ed., McGraw-Hill Book Co., New York (1983).
- Gassenschmidt, U., Jany, K. D., Tauscher, B., Niebergall, H., Isolation and characterization of a flocculating protein from *Moringa oleifera* Lam. *Biochimica et Biophysica Acta*, 1243, 477-481 (1995).
- Gerde, J. A., Yao, L., Lio, J., Zhiyou, W., Wang, T., Microalgae flocculation: Impact of flocculant type, algae species and cell concentration. *Algal Research*, 3, 30-35 (2014).
- Ghebremichael, K. A., Gunaratna, K. R., Henriksson, H., Brumer, H., Dalhammar, G., A simple purification and activity assay of the coagulant protein from *Moringa oleifera* seed. *Water Research*, 39, 2338-2344 (2005).
- Guillard, R. R. L., Lorenzen, C. J., Yellow-green algae with chlorophyllide. *J. Applied Phycology*, 8, 10-14 (1972).
- Hamid, S. H. A., Lananan, F., Din, W. N. S., Lam, S. S., Khatoun, H., Harvesting microalgae, *Chlorella* sp. By bioflocculation of *Moringa oleifera* seed derivatives from aquaculture wastewater phytoremediation. *International Biodeterioration and Biodegradation*, 95, 270-275 (2014).
- Horiuchi, J. I., Ohba, I., Tada, K., Kobayashi, M., Kanno, T., Kishimoto, M., Effective cell harvesting of the halotolerant microalgae *Dunaliella tertiolecta* with pH control. *J. Bioscience Bioengineering*, 95, 412-415 (2003).
- Lee, A. K., Lewis, D. M., Ashman, P. J., Microbial flocculation, a potentially low cost harvesting technique for marine microalgae for the production of biodiesel. *J. Applied Phycology*, 21, 559-567 (2009).
- Levicki, K., M.sc. Thesis Royal Inst. of Technol. (KTH), Stockholm, SE (2005).
- Katayon, S., Megat Mohd Noor, M. J., Asma, M., Thamer, A. M., Liew Abdullah, A. G., Idris, A., Suleyman, A. M., Aminuddin, M. B., Khor, B. C., Effects of storage duration and temperature of *Moringa oleifera* stock solution on its performance in coagulation. *International J. of Engineering and Technology*, 1, 146-151 (2004).
- Kim, D. G., La, H. J., Ahn, C. Y., Park, Y. H., Oh, H. M., Harvest of *Scenedesmus* sp. with bioflocculant and reuse of culture medium for subsequent high-density cultures. *Bioresource Technology*, 102, 3163-3168 (2011).
- Mandik, Y. I., Cheirsilp, B., Boonsawang, P., Praertsan, P., Optimization of flocculation efficiency

- of lipid-rich marine *Chlorella* sp. biomass and evaluation of its composition in different cultivation modes. *Bioresource Technology*, 182, 89-97 (2015).
- Mata, T. M., Martins, A. A., Caetano, N. S., Microalgae for biodiesel production and other applications: A review. *Renewable Sustainable Energy Review*, 14, 217-232 (2010).
- Molina, E. G., Belarbi, E. H., Fernández, A. F. G., Medina, A. R., Chisti, Y., Recovery of microalgal biomass and metabolites: Process options and economics. *Biotechnology Advances*, 20, 491-515 (2003).
- Muyibi, S. A., Evison, L. M., Optimizing physical parameters affecting coagulation of turbid water with *Moringa oleifera* seeds. *Water Research*, 29, 2689-2695 (1995).
- Ndabigengesere, A., Narasiahn, K., Talbot, B., Active agents and mechanism of coagulation of turbid waters using *Moringa oleifera*. *Water Research*, 29, 703-710 (1995).
- Nguyen, T. D. P., Frappart, M., Jaouen, P., Pruvost, J., Bourseau, P., Harvesting *Chlorella vulgaris* by natural increase in pH: Effect of medium composition. *Environmental Technology*, 35, 1378-1388 (2014).
- Nkurunziza, T., Nduwayezu, J. B., Banadda, E. N., Nhapi, I., The effect of turbidity levels and *Moringa oleifera* concentration on the effectiveness of coagulation in water treatment. *Water Science and Technology*, 59, 1551-1558 (2009).
- Oliveira, J. T. A., Silveira, S. B., Vasconcelos, K. M., Cavada, B. S., Moreira, R. A., Compositional and nutritional attributes of seeds from the multiple purpose tree *Moringa oleifera* Lamarck. *J. of the Science Food Agriculture*, 79, 815-820 (1999).
- Papazi, A., Makridis, P., Divanach, P., Harvesting *Chlorella minutissima* using cell coagulants. *J. of Applied Phycology*, 22, 349-355 (2010).
- Platt, B., Drysdale, A. J., Nday, C. M., Roloff, E. V. L., Drever B. D., Salifoglou, A., Differential toxicity of novel aluminium compounds in hippocampal culture. *Neurotoxicology*, 28, 576-586 (2007).
- Salim, S., Bosma, R., Vermuë, M. H., Wijffels, R. H., Harvesting of microalgae by bio-flocculation. *J. of Applied Phycology*, 23, 849-855 (2011).
- Salim, S., Vermuë, M. H., Wijffels, R. H., Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. *Bioresour. Technol.*, 118, 4955 (2012).
- Siegel, S., Castellan Jr., J. N., *Nonparametric Statistics for the Behavioral Sciences*. 2nd Ed., McGraw-Hill Book Co., New York (1988).
- Spilling, K., Seppälä, J., Tamminen, T., Inducing autoflocculation in the diatom *Phaeodactylum tricorutum* through CO₂ regulation. *J. of Applied Phycology*, 23, 959-966 (2011).
- Sukenik, A., Shelef, G., Algal autoflocculation-verification and proposed mechanism. *Biotechnology Bioengineering*, 26, 142-147 (1984).
- Teixeira, C. M. L. L., Kirsten, F. V., Teixeira, P. C. N., Evaluation of *Moringa oleifera* seed flour as a flocculating agent for potential biodiesel producer microalgae. *J. of Applied Phycology*, 24, 557-563 (2012).
- Udom, I., Zaribaf, B. H., Halfhide, T., Gillie, B., Dalrymple, O., Zhang, Q., Ergas, S. J., Harvesting microalgae grown as wastewater. *Bioresource Technology*, 139, 101-106 (2013).
- Uduman, N., Qi, Y., Danquah, M. K., Forde, G. M., Hoadley, A., Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. *J. Renewable Sustainable Energy*, 2, 012701 (2010).
- Vandamme, D., Foubert, I., Meesschaert, B., Muylaert, K., Flocculation of microalgae using cationic starch. *J. of Applied Phycology*, 22, 525-530 (2010).
- Vandamme, D., Foubert, I., Muylaert, K., Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends in Biotechnology*, 31(4), 233-239 (2013).
- Veillette, M., Chamoumi, M., Nikiema, J., Faucheux, N. and Heitz, M., Productions of biodiesel from microalgae. Available from: <<http://www.intechopen.com/books/advances-in-chemicalengineering/production-of-biodiesel-using-triglycerides-from-microalgae>> (Accessed: July 17, 2015) (2012).
- Wyatt, N. B., Gloe, L. M., Bradey, P. V., Hewson, J. C., Grillet, A. M., Hankins, M. G., Pohl, P. I., Critical conditions for ferric chloride induced flocculation of freshwater algae. *Biotechnology Bioengineering*, 10, 493-501 (2012).
- Wu, Z., Zhu, Y., Huang, W., Zhang, C., Li, T., Zhang, Y., Li, A., Evaluation of flocculation induced by pH increase for harvesting microalgae and reuse of flocculated medium. *Bioresource Technology*, 110, 496-502 (2012).