J Appl Phycol (2012) 24:557–563
DOI 10.1007/s10811-011-9773-1

Evaluation of *Moringa oleifera* seed flour as a flocculating agent for potential biodiesel producer microalgae

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Received: 31 July 2011 / Revised and accepted: 12 December 2011 / Published online: 6 January 2012
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Abstract Microalgal biofuel alternatives have been hindered by their cost and energy intensive production. In the microalgal harvesting process, the intermediate step of flocculation shows potential in drastically reducing the need for costly centrifugation processes. *Moringa oleifera* seeds, which have been used for water treatment due to their high flocculation potential, low cost and low toxicity, are presented in this paper as strong candidate for flocculating *Chlorella vulgaris,* a microalgae with high biodiesel production potential. Early results of our group showed a very high flocculation (around 85% of biomass recovery). The aim of this work was to investigate the influence of *Moringa oleifera* seed flour concentration, sedimentation time and pH on the flocculation efficiency. Cell suspensions treated with *Moringa* seed flour (1 g L^{-1}) had their flocculation significantly increased with the rise of pH, reaching 89% of flocculation in 120 min at pH 9.2. Sedimentation time of 120 min and a concentration of 0.6 g L^{-1} proved to be ample for substantial flocculation efficiency. In spite of the need for more research to ensure the economic viability and sustainability of this process, these results corroborate *Moringa oleifera* seeds as a strong candidate as a bioflocculant for *Chlorella vulgaris* cells and indicate optimal pH range of its action.

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Keywords Bioflocculant · Chlorella vulgaris · Flocculation · Moringaceae · pH

Introduction

Microalgal biodiesel production currently remains a poor cost competitor to conventional fuel sources. Utilizing dry biomass, its production process requires the separation of the microalgal biomass from the culture medium. In a recent 2009 algal industry survey, production costs, more specifically extraction and harvesting, were considered as the number one challenge facing algal biofuel (ABO 2009). The separation process presents high capital, operational costs and energy demands (Wijffels and Barbosa 2010). The current methods used for separation are each limited in their ability to provide cost-effective solutions for biodiesel production (Schenk et al. 2008).

At present, the most commonly used separation methods are filtration and centrifugation. Filtration is effective for microalgal cells which are relatively large, such as *Arthrospira* sp. (Papazi et al. 2010), but is unable to separate the biomass from the cultivation medium for cells of smaller dimensions. Centrifugation, on the other hand, successfully separates the microalgae efficiently, independent of size, although this process involves the exposure of the microalgae cells to high gravitational forces and shear stresses that can damage the cell structure. Also, this method unfortunately consumes a great deal of energy (Benemann and Oswald 1996) and is costly (Knuckey et al. 2006); thus, it is suitable only for products with high commercial value (Becker 1995).

Processes such as electrocoagulation (Poelman et al. 1997) and bioflocculation (Lee et al. 2009; Oh et al. 2001) are being investigated for more cost-effective and efficient alternatives. These approaches have shown encouraging

results, allowing for rapid processing of large quantities of culture, greatly reducing the volume of culture needing to be centrifuged. Coagulation, which utilizes a method that either adjusts the pH or adds electrolytes, and flocculation, which uses the addition of cationic polymers (Papazi et al. 2010), have produced a viable alternative to explore the economic feasibility of the production of microalgal biofuel.

Coagulants/flocculants have been used worldwide for water treatment. Among the most used flocculants, aluminum salts flocculate microalgae well (Henderson et al. 2006); however, due to their adverse health effects to both animals and humans, they are not suitable for use with microalgal biomass. *Moringa oleifera* seeds, on the other hand, do not pose the same health risks (Ndabigengesere and Narasiah 1998) as aluminum and ferric salts yet maintain a high flocculation potential in water treatment, attaining 92–97% flocculation (Muyibi and Evison 1995). If available locally, they are less expensive, making them a viable alternative in water and wastewater treatment, as well as a possible flocculant agent in the microalgae biomass separation process.

Moringa oleifera is one of the 14 species of tropical plants in family Moringaceae; all of which have coagulant properties (Jahn 1988). *Moringa oleifera* is the most widespread species in its family, occurring in Asia, Africa and America. It is abundant in the tropic and subtropic regions. *Moringa* is a multi-purpose plant, with its leaves, seeds and flowers being used as food (Mendieta-Araica et al. 2011) and phytochemicals (Amaglo et al. 2010), its seed in water and wastewater treatment (Gassenschmidt et al. 1995; Ghebremichael et al. 2005) and the seed's oil in cosmetics (Kleiman et al. 2008) and as lubricant (Mani et al. 2007), as well as have been indicated as a good raw material for biodiesel production (Rashid et al. 2008). Currently, the largest producer of *Moringa*, with an annual production of 1.1 to 1.3 million tons, is India, cultivated from an area of 380 km² (Talreja 2010).

Moringa seed active compounds are peptides of molecular weight ranging from 6 to 20 kDa, with an isoelectric pH value between 9 and 10 or higher (Gassenschmidt et al. 1995; Ndabigengesere et al. 1995; Santos et al. 2005). It was reported that in saline extract, this compound is a polyelectrolyte with a molecular weight around 3.0 kDa (Okuda et al. 2001), though Ghebremichael et al. (2005) correlated the flocculation effect observed with a peptide obtained in saline extract and Santos et al. (2005) purified and characterized a new lectin extracted in 0.15-M NaCl solution from *Moringa* seeds which has flocculating activity. The flocculation potential of *Moringa* has been confirmed with the jar test (Ghebremichael et al. 2005; Sánchez-Martín et al. 2010; Okuda et al. 2001) as well as in cuvettes in a spectrophotometer (Ghebremichael et al. 2005).

Preliminary results from our group showed that *Moringa* seed flour was effective in the flocculation of microalgae such as *Chlorella vulgaris* and *Scenedesmus* sp—strong candidates for biodiesel production (Mata et al. 2010). Using milled seeds in a concentration of 1 g L^{-1} and 240 min of sedimentation time, biomass recovery reached 84% and 72% for *Chlorella* and *Scenedesmus*, respectively (data not published).

In this study, we intend to further investigate the seeds of *M. oleifera* as a flocculant agent for *Chlorella vulgaris* by evaluating its flocculation efficiency in the following sedimentation parameters—pH, sedimentation time and *Moringa* seed flour concentration.

Materials and methods

The freshwater microalga *Chlorella vulgaris* (Chlorophyceae) was obtained from UFSCar culture collection (Federal University of São Carlos, São Paulo, Brazil) and cultured in WC medium (Guillard and Lorenzen 1972).

Cultures were grown in 500-mL Erlenmeyer flasks with continuous agitation of 160 rpm on a orbital shaker, in a controlled environment at 25±1°C. Cultures were continually illuminated with 24 μmol photons m^{-2} s^{-1} white fluorescent light.

Inocula were taken from the stock culture in the exponential growth phase and diluted with fresh medium to reach an initial optical density of 0.05 to begin the growth. Approximately 20 days were necessary to reach the optical density (OD_{730}) range of 0.90–1.06 indicative of the late exponential growth phase cultures used in the experiments.

The dry pods of *Moringa oleifera* were collected from trees of Sergipe State. The seeds were provided by Dr. Gabriel Francisco da Silva from the Federal University of Sergipe. The seeds were shelled and ground with a mortar and pestle and then sequentially sieved through both 860- and 420-μm pore sieves in order to obtain the seed flour used in the flocculation assays.

Flocculation assays

The influence of sedimentation time on flocculation efficiency was evaluated using *Moringa* seed flour concentration of 1.0 g L^{-1} and sedimentation time range of 20 to 240 min. To evaluate the influence of *Moringa* seed flour concentration, a sedimentation time of 120 min and concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g L^{-1} of the milled seeds were used. Cell suspension pH was adjusted to 8.5–8.7 (range commonly found in these cultures in the late exponential phase) when necessary by the addition of 1-M HCl or 1-M NaOH.

In investigating the effect of pH, the culture was divided, and the pH was adjusted to different values by the addition of 1-M HCl or 1-M NaOH, ranging from approximately 4.0 to 9.3—so as to obtain five cell suspensions with different pH, including acid and alkaline ranges, and to cover the typical pH

range of microalgae cell suspensions. The *Moringa* seed flour was added to the cultures in Erlenmeyer flasks to obtain a 1 g L^{-1} concentration. These experiments were carried out on three different days.

All cell suspensions (with and without *Moringa*) were agitated for 15 min at 100 rpm on an orbital shaker. Cell suspensions without *Moringa* seed flour were used as a control group. pH was measured after incubation time to determine whether it had changed during incubation.

Experiments using aluminum sulphate (1 g L^{-1}) as a flocculant in place of *Moringa* seed flour were carried out using the same conditions employed with *Moringa* seeds (1 g L^{-1}) for comparisons of flocculation efficiency.

To further investigate the changes in pH and OD after incubation time, additional experiments were conducted: pH: 1 g L^{-1} *Moringa* seed suspensions prepared in WC medium with and without Tris buffer (without cells) had their pH adjusted to pH 4.03 and incubated in exactly the same manner as the cell suspensions used in the assays reported previously—15 min and at 100 rpm. The pH was measured after the incubation time. WC media with and without Tris buffer and without *Moringa* were used as controls; OD: *Moringa* seed flour at a concentration of 1 g L^{-1} was added to the WC medium (without cells) at the same pH values used in the experiments with the cells. OD_{730} readings were done before and after the incubation time (carried out in the same conditions to the experiments of flocculation efficiency evaluation).

All experiments were carried out in triplicate.

Determination of the flocculation efficiency

After the incubation period, 20 mL of the cultures was transferred to tubes to evaluate flocculation. As in Papazi et al. (2010), tubes were chosen over the jar test in this work because they reduce the sample volume requirements.

OD_{730} measurements were done before and after the incubation period and designated times after culture transfer to the tubes. The aliquot withdrawn for OD measurement was from the middle of the suspension column. Flocculation efficiency was represented as OD decrease and percent OD decrease. OD decrease was calculated as the difference of the OD obtained after the incubation time (OD initial) and the OD value measured after different times of observation (OD final). Percent OD decrease was calculated as a ratio between the OD decrease and the OD initial. Photographs were taken for a visual representation of adding *Moringa* seed, to serve as a qualitative evaluation.

Results

The results of "OD decrease" (Fig. 1) showed that at a sedimentation time of 240 min, the flocculation efficiency

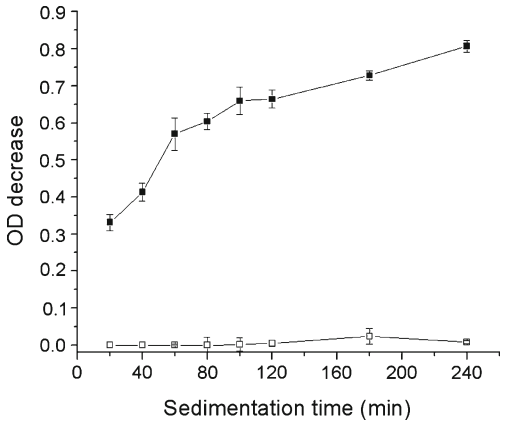

Fig. 1 Effect of different sedimentation times on the flocculation efficiency of *Chlorella vulgaris* treated with *Moringa oleifera* seed flour in a concentration of 1 g L^{-1}. Treated samples (*filled squares*) and control samples (*empty squares*)

of the *Moringa* seed tended to reach a maximum, producing a 96 percent OD decrease; still at 120 min, a reasonable efficiency of 80% was attained and chosen for the experimental evaluations. Control samples (without *Moringa*) did not show a significant OD decrease compared to the treated samples.

There was very little increase in flocculation efficiency over the *Moringa* seed flour concentration range of 0.6 to 1.0 g L^{-1} (Fig. 2). However, due to the small sample volumes, in order to facilitate the assays in the following investigations, 1 g L^{-1} was used instead of 0.6 g L^{-1}.

During the evaluations of pH effect on the flocculation efficiency, an increase of initial OD in the treated samples was observed when compared to the non-treated ones, showing significant differences in the lower pH range (Fig. 3). The results of a follow-up experiment are also shown in Fig. 3. The same tendency of increasing OD at lower pH was observed without cells, showing that this effect is derived from *Moringa* seed flour. Also of note, a

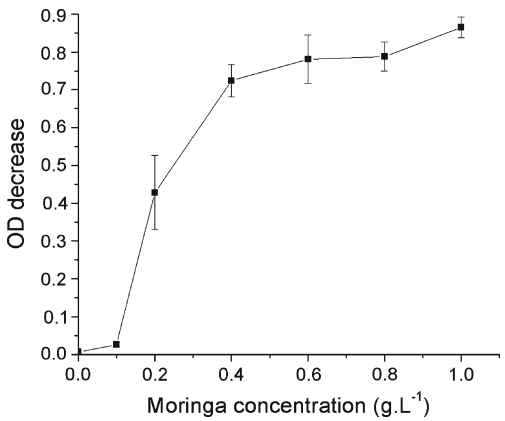

Fig. 2 Effect of different *Moringa oleifera* seed flour concentrations on the flocculation efficiency of *Chlorella vulgaris* at 120-min sedimentation time

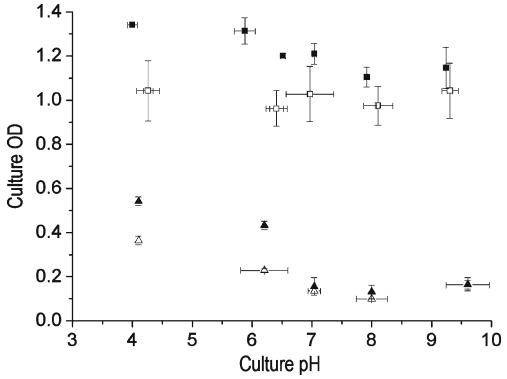

Fig. 3 Effect of different pH on OD of suspensions of *Moringa oleifera* seed flour in a concentration of 1 g L^{-1} in the presence or absence of *Chlorella vulgaris* suspension. Samples treated with *Moringa* in the presence of cells (*filled squares*), samples non-treated with Moringa in the presence of cells (*empty squares*), samples just after the addition of *Moringa* seed flour in the absence of cells (*empty triangles*) and samples after incubation time in the absence of cells (*filled triangles*)

milky appearance in the *Moringa* suspensions at pH 4.10 and 6.20 was observed, most distinctly at pH 4.10.

As this OD increase interferes in the results of the decrease OD calculations, it was necessary to show the results of flocculation efficiency depending on the pH on an "OD culture" graph (Fig. 4a, b) instead of on an "OD decrease" one. There was no difference between initial OD and 2-h sedimentation OD in the non-treated samples, whereas in the treated samples, there was a difference which was higher at the alkaline pH.

In spite of observing an OD increase in the cultures at acidic pH, calculation of "% OD decrease" was done for all results in order to discuss flocculation efficiency at different pH. The % OD decrease results presented in Table 1 show that pH affected this parameter, with enhanced % OD decrease at higher pH; the control samples had negligible % OD decrease in relation to the treated ones.

pH values were adjusted in both treated and non-treated cell suspensions to attain determined values. However, pH variations after incubation were observed mainly in the case of suspensions with *Moringa* in the acidic range (Fig. 5). It is possible to observe that at pH 4.03, there was an increase in pH in non-treated suspensions though not as pronounced as that seen in the treated cultures. On the other hand, in the alkaline range, more specifically at pH 10.26, a pH decrease was observed in both types of suspension: treated and non-treated.

The Tris buffer effect may explain this result in part as this buffer makes up part of the cultivation medium. However, the greatest pH increase in *Moringa*-treated suspensions in relation to non-treated ones observed around pH

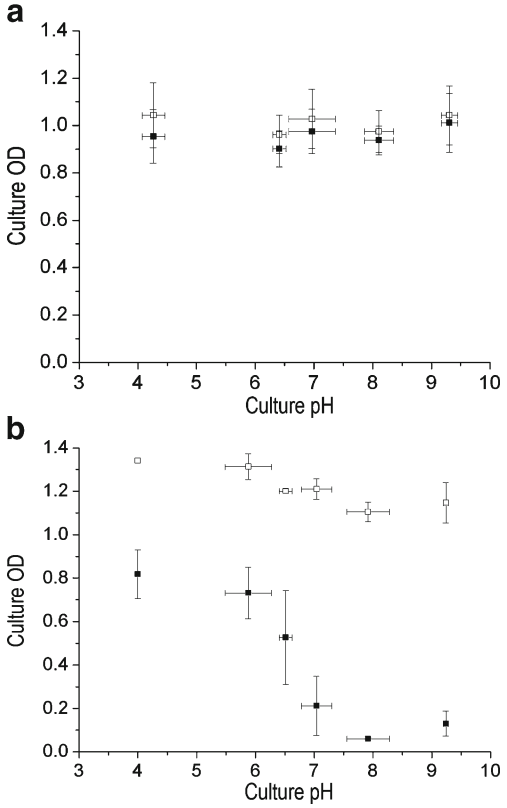

Fig. 4 Effect of different pH on flocculation efficiency of *Chlorella vulgaris* suspension treated with *Moringa oleifera* seed flour in a concentration of 1 g L^{-1} at a sedimentation time of 120 min: **a** control samples and **b** *Moringa*-treated samples. Initial OD (*empty squares*) and OD at a sedimentation time of 120 min (*filled squares*)

4 indicates that *Moringa* seed compounds also play a role in this effect. In *Moringa* suspension adjusted to pH 4 in the medium with and without Tris buffer, pH increments of 1.97 and 1.94 after the incubation time were observed, respectively. In the absence of *Moringa* (control sample), a much lower pH increase was observed: 0.37 and 0.94 in the absence and presence of the Tris buffer, respectively.

The photographs reveal the large difference between flocculation effect of the suspensions treated and non-treated with *Moringa* at pH of 4.26 and 9.31 (Fig. 6a, b). At pH 9.31, the effect was higher than at pH 4.26. Non-

Table 1 Flocculation of *Chlorella vulgaris* cells of different pH cultures for 120 min of sedimentation

Non-treated suspension		Treated suspension	
Culture pH	OD decrease (%)	Culture pH	OD decrease (%)
4.26	8.5	4.00	39.0
6.41	6.2	6.51	56.1
6.97	5.1	7.04	82.4
8.10	3.8	7.91	81.4
9.31	3.0	9.24	88.6

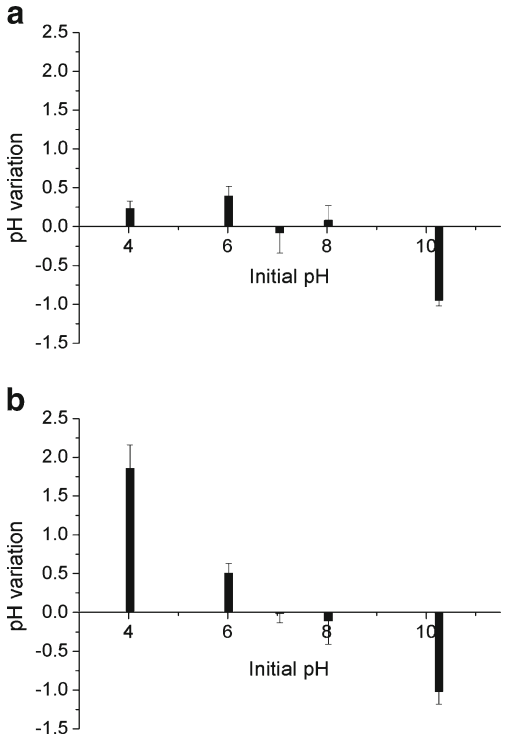

Fig. 5 pH variations of the cell suspensions non-treated (**a**) and treated (**b**) with *Moringa oleifera* seed flour after incubation time as a function of initial pH

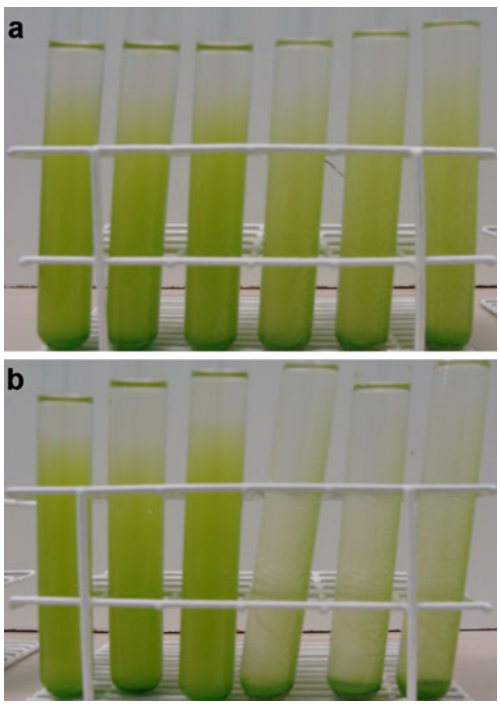

Fig. 6 Photograph of suspensions in pH 4.26 (**a**) and in pH 9.31 (**b**). The three suspensions located at the *left* are the controls, and the three at the right, the treated ones

Table 2 Flocculation efficiency of aluminum sulphate and *Moringa* seed flour at 10- and 30-min sedimentation time and concentration of 1 g L^{-1}

Sample	OD decrease (%) in 10 min	OD decrease (%) in 30 min
Control	0.0	1.0
Treated with aluminum sulphate	86.2	93.8
Treated with *Moringa* seed flour	17.8	47. 7

treated suspensions showed lower sedimentation in comparison to the treated ones.

Aluminum sulphate demonstrated only a slightly higher flocculation (around 95%) in comparison to *Moringa* seed flour (around 87%) in a sedimentation time of 120 min. However, in a shorter time of 10 and 30 min, it was possible to detect higher differences of "% OD decrease" (Table 2) between the cell suspensions treated with aluminum sulphate and that treated with *Moringa* seed flour. Control suspensions did not show an appreciable % OD decrease.

Discussion

Microalgal biofuel production has, until today, failed to become a cost-competitive alternative to traditional fuel sources. Unlike aquaculture where microalgae are used directly in conjunction with the growth medium, the production of biodiesel requires a separation of the microalgal biomass from the culture medium. The harvesting of biomass by filtration has proven to have serious limitations due to the small size (3–30 mm diameter) of the microalgal cells and the relatively dilute suspensions (< 0.5-kg m^{-3} dry biomass) in commercial culture; however, centrifugation, although effective, is extremely costly and energy intensive; its energy demand has been estimated at 3,000 kWh t^{-1} (Benemann and Oswald 1996). An intermediate flocculation step has been proposed to reduce costs in this particularly important aspect of the microalgae biomass production process for its use as a raw material for biofuel generation.

Moringa oleifera seeds have demonstrated to be a very suitable flocculant for microalgae biomass, reaching flocculant activity comparable to aluminum sulphate, as well as having various other advantages such as safety. Aluminum sulphate had a 72% flocculation efficiency for *Chlorella vulgaris* at 2 mg L^{-1}, with a settling time of 10 min (Oh et al. 2001), and with *Chlorella* sp., it was 80% in a concentration of 8 mg L^{-1} and a settling time of 30 min (Liu et al. 1999). Moreover, aluminum sulphate at the same concentration of *M. oleifera* seeds attained the same flocculation efficiency in less time while reaching higher flocculation efficiency. On the other hand, *Moringa* seeds, in comparison to aluminum sulphate, have low to zero toxicity, do not result in corrosion problems and produce a

much smaller volume of sludge, which is not hazardous (Ndabigengesere and Narasiah 1998). Furthermore, the coagulation efficiency of *M. oleifera* was found to be independent of storage temperature and container (Katayon et al. 2006).

The economic viability of *Moringa* seeds as a flocculant agent is difficult to discuss. However, one would reason that as it is used as a low-cost alternative in water and wastewater treatment in developing countries, it would serve as a low-cost flocculant in biofuel production from microalgae. *Moringa* grows well in tropic and subtropic regions, and its cultivation presents great advantages as it is a fast growing tree whose dry pods can be harvested after just one year of growing (Ndabigengesere and Narasiah 1998). Most importantly, the production potential appears economically feasible as it can be produced locally, reducing importation and generating farm and employment income.

Evaluating the effects of pH on the flocculation is an important tool to investigate the mechanism and optimization of its activity. The pH range chosen includes values commonly found in microalgae suspensions and coincides with several studies published of pH influence on the biomass recovery mediated by diverse flocculants (Divakaran and Sivasankara Pillai 2002; Liu et al. 2009; Vandamme et al. 2010). Our flocculation results to the changes in pH are in agreement with a participation of compound(s) which is(are) charged depending on the pH. Similar results were observed by Santos et al. (2005) with lectins isolated from *M. oleifera* seeds, but in this study, the higher efficiency was observed at pH 4. A cationic starch flocculant displayed the same kind of dependence of the pH on its flocculation efficiency observed in our studies (Vandamme et al 2010), whereas with chitosan, an inverse effect has been observed: higher flocculation efficiency in the region of pH 5–7 in relation to the alkaline pH range (Divakaran and Sivasankara Pillai 2002). The results of our study provide a promising outlook as the pH range with the largest effect is exactly that of the typical pH of the *C. vulgaris* cultures. It is noteworthy that the increase of OD in the acidic range observed after the addition of *Moringa* to the cell suspension does not interfere with the conclusion about the pH effect of the flocculation efficiency as the OD decrease observed at alkaline pH was much higher than that at acidic pH.

Our work showed that variations in pH after incubation time were observed mainly in suspensions with *Moringa* in the acidic range. This led us to repeat the experiments several times in order to reach the desired value as well as to carry out other experiments to evaluate these pH changes. Our results with WC medium without Tris buffer further suggest the participation of *Moringa* seed substances in the effect observed.

Our choice to use tubes for flocculation efficiency evaluations is supported by the research of Papazi et al. (2010) where the authors studied ferric and zinc salts as coagulants for *C. minutissima* culture. In our study, 20 mL of cell suspension was used in tubes of 40 mL, as done in their work. We decided upon this method instead of cuvettes (Ghebremichael et al. 2005; Salim et al. 2011) as our early experiments showed unreliable data as the percent OD decrease obtained was very different to that obtained in tubes. We believe that this is due to sampling differences in obtaining OD in tubes and in cuvettes. In the former, an aliquot of the cell suspension is taken from the middle of the medium, and in the latter, the readings of OD are done directly in the cuvette. Since the window of the sample compartment of the spectrophotometer is relatively wide and reaches almost the bottom of the cuvette, OD readings represent regions not evaluated in the tubes. It is important to note that in other sets of flocculation experiments performed in tubes and in the jar test apparatus, similar results were found, which demonstrates that it is possible to make evaluations in tubes without compromising the reliability of results while reducing the culture volume needed to perform the assays.

Other bioflocculants tested for flocculating microalgae, such as cationic starch and chitosan, indicate high biomass recovery (80–90%) with relatively low flocculant concentrations ranging from 1.6 to 70 mg L^{-1} and, in general, 30-min sedimentation time (Bilanovic et al. 1988; Divakaran and Sivasankara Pillai 2002; Liu et al. 1999; Liu et al. 2009; Vandamme et al. 2010). In our study, optimal flocculation efficiency of *Moringa* seeds was observed at a concentration of 1 g L^{-1}, with a sedimentation time of 240 min; however, it was possible to attain a flocculation efficiency of about 80% using 0.6 g L^{-1} in a sedimentation time of 120 min—yet still more than other bioflocculants. Considering that the true concentration of the flocculating agent in the mass of *Moringa* seed used is unknown, it is reasonable to believe that it is present in very low quantities and that the flocculation efficiency of *Moringa* seed coagulants might be comparable to other bioflocculants.

Further studies should include tests to evaluate ways of lowering costs while maintaining efficacy. With regard this, extracts should be obtained in different conditions and tested; for example, it was observed that the flocculation efficiency could be improved using a saline extract instead of an aqueous solution (Okuda et al. 1999) and the dissolved organic carbon was not enhanced (Okuda et al. 2001). It is of utmost importance that the identification of the active compounds be explored as well as the total organic carbon be evaluated as an increase in this parameter has already been found (Sánchez-Martín et al. 2010). In addition, growth evaluations reusing the medium should be done as it opens the possibility to reuse the cultivation medium after biomass separation. Gene cloning of the *Moringa* coagulating proteins in other organisms should also be investigated.

Acknowledgments The authors are grateful to Dr. Gabriel Francisco da Silva from Sergipe Federal University for providing the seeds of *Moringa oleifera* and to Dr. Armando Vieira from São Carlos Federal University for providing the culture of *Chlorella vulgaris*.

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