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Moringa oleifera Lam. as a Bioflocculant for Harvesting Microalgae Grown on Agricultural Wastewaters for Feed Production

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Abstract: Harvesting impacts the costs of microalgae production and affects the characteristics of the final product. Therefore, this study evaluated *Moringa oleifera* seed powder (MP) as a bioflocculant compared to two chemicals (Aluminium Sulphate—AS and Iron Chloride—IC) to harvest a mixed microalgae culture (*Chlorella vulgaris* and *Desmodesmus* sp.) grown on digestate. MP was the most stable flocculant but resulted in the lowest harvesting efficiency of 75%, compared to 94% for AS and 100% for IC. Process parameters such as pH, duration of mixing, grinding method for obtaining the powder, and granulometry had no significant effect on the harvesting efficiency of MP, reinforcing that this is a robust flocculant. The use of a water extraction step increased the harvesting efficiency of MP to 91%, albeit with the need for a higher dosage of flocculant. The algae harvested with MP complied with maximum tolerable levels for swine, cattle, and poultry regarding most trace elements. Nevertheless, all algae samples had Fe and Al contents above the recommended levels, possibly due to the entrapment of metal-rich digestate particles. Therefore, more attention should be paid to the final composition of algae when proposing flocculation as a harvesting method for feed production.

Keywords: *Moringa oleifera*; bioflocculation; harvest; microalgae; biomass composition; trace elements

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1. Introduction

Interest in microalgae has been increasing in recent years as a renewable and sustainable raw material for food and feed production. The European market for microalgae in food and feed was worth US$1.7 billion in 2018, with a projected annual growth of 5% between 2019 and 2027 [1]. Microalgae can be grown in freshwater, seawater, and nutrient-rich residual streams wastewater due to their rapid growth and ability to adapt to harsh conditions [2,3]. Using nutrient-rich agricultural residual streams, such as digestate, results in a decreased cost of microalgae production while providing high biomass yields in a more renewable and sustainable process than the usual commercial microalgal facilities [4]. Moreover, the use of excess digestate (i.e., digestate not used for land application) for microalgal cultivation enables the local creation of value from a stream that would otherwise be subjected to treatments for discharge or transported elsewhere. Digestate is not yet commercially used as a nutrient source for algae cultivation and only plant-based digestates are currently allowed for feed application [5]; nevertheless, the need for local protein production and the incentives in the EU for the increase in biogas production capacity as a response to the current energy crisis are significant incentives for the wide adoption of this technology.

Despite these advantages, algae biomass production is still expensive, and harvesting represents up to 15% of the total production cost, besides resulting in a high energy expenditure depending on the chosen technology [6]. Flocculation has a lower cost than

membrane filtration and lower energy expenditure than centrifugation, even if associated with these operations for further dewatering of the harvested biomass [6]. The use of flocculation for harvesting algae grown on agricultural wastewaters has the further advantage of improving the quality of the spent growing medium to dischargeable levels. Chemical salts such as aluminium sulfate, iron (III) chloride, and iron sulfate are the most widely used for coagulation/flocculation in water treatment [7]. However, when used for the harvesting of microalgae, chemical salts can result in high metal contents in the final biomass [8,9].Therefore, the choice of flocculant is very important and depends on the intended end-use of the biomass, as the flocculant/coagulant to be used should not contaminate or affect algae biomass quality; otherwise, this may result in additional costs for the purification of the produced biomass [9].

Bio-flocculants are naturally sourced flocculants of biological origin and are considered a sustainable alternative to commonly used chemical products due to their eco-friendly aspects for algae harvesting [9–11], especially for food and feed applications. One such bio-flocculant is the powder obtained from the seed of *Moringa*, a plant originally from South Asia that is capable of growing in arid regions. The *Moringa* genus has 13 species known in the literature, and more than one of the species has coagulation/flocculation activity that has been tested in previous studies [12–14]. *Moringa oleifera* Lam., the most cultivated and known species of the *Moringa* genus, is called "Miracle Tree" since every part of the plant can be used in different fields such as wastewater treatment, pharmacy, food, and feed [15–17]. It has also been shown to be effective for microalgae harvesting, as its seeds contain a protein that can be used as a natural organic flocculant [18,19]. In addition, it was shown to reduce bacterial content by 99.9% [20], an important aspect of the food and feed industry.

Various studies have been conducted indicating that *Moringa oleifera* seed can be used for harvesting different microalgae species. The study by Teixeira et al. (2012) [18] *Chlorella vulgaris* found a flocculation efficiency of 89% when 1000 mg L^{-1} *Moringa oleifera* seed flour was used. Yang et al. (2021) [21] obtained a harvesting efficiency of around 95% for *C. vulgaris* and *Scenedesmus obliquus* upon addition of, respectively, 600 mg L^{-1} and 400 mg L^{-1} of *M.oleifera* seed powder. Badruddin et al. (2016) [22] evaluated *Moringa oleifera* seeds before and after oil extraction to harvest *Nannochloropsis oculata*. By adding 5000 mg L^{-1} of *M.oleifera* seed after oil extraction, a 93.77% flocculation efficiency was obtained, while 70.56% was obtained with 4000 mg L^{-1} using the non-extracted seeds.

Even though several studies have evaluated *M. oleifera* for microalgae flocculation with harvesting efficiencies above 80% [10,18,21–23], the lack of important information, such as the ratio of flocculant to algal biomass and the use of different methodologies for powder preparation, hamper the comparison of these works and the drawing of more general conclusions about the efficacy of this flocculant. Moreover, only a couple of studies have been carried out using *M. oleifera* as a flocculant for harvesting microalgae grown on digestate [24,25] and these have observed the need for a lower amount of flocculant than usually reported for *M. oleifera* for microalgal harvesting. Considering that the high amount of flocculant is the most significant parameter affecting *Moringa*'s applicability for microalgae harvesting [21], it is of importance to further investigate this flocculant for the harvest of microalgae grown on wastewater. Finally, feed safety aspects of microalgae grown on digestate and harvested with *M. oleifera*, with special attention to potentially toxic trace elements, have not yet been addressed in the literature.

Therefore, in this study, the efficiency of *M. oleifera* seed powder for harvesting a mixed microalgae culture grown in digestate was assessed compared to commonly used metallic flocculants. The influence of parameters such as dosage, rotation speed, pH, and bioflocculant preparation method was investigated using the same algal sample to allow for proper comparison between the different used conditions. A discussion on the influence of the growing medium on the harvesting efficiency was conducted and the harvested biomass was characterized for its metal content to assess its feed safety, which has not been addressed in previous studies.

2. Materials and Methods

2.1. Algal Strain and Culture Conditions

The freshwater microalgae species *Desmodesmus* sp. (DCG 0442) and *Chlorella vulgaris* (DCG 0446) were obtained from the BCCM/DCG diatom culture collection (Ghent University, Belgium). The mixed culture (50% of each species based on cell count) was acclimatized to digestate, as described by Seelam et al. [26]. The acclimatized culture, after several cycles of inoculation into increasing concentrations of digestate, consisted mostly of *Desmodesmus* sp. cells (>80%). This culture was inoculated in a bench photobioreactor (Labfors 5 Lux LED, Infors AG, Bottmingen, Switzerland) with a working volume of 3 L at an initial concentration of 2000 living phototrophic cells μL^{-1}. Paper-filtered digestate (10 µm pore size) from food-waste origin at 10% (*v/v*) was used as a source of nutrients. Details on digestate composition and treatment conditions are described by Seelam et al. [26]. A sinusoidal light pattern was used with a maximum intensity of 100 µmol m^{-2} s^{-1} (average intensity of 40 µmol m^{-2} s^{-1}) and a light/dark photoperiod of 16:8 h. The bioreactor was maintained at ambient temperature (18–21 °C), and the pH was set to 7.8 and controlled by automatic CO_2 injection. The algal culture was cultured for 14 days and then transferred to a plastic bottle and kept at 4 °C before further use in the flocculation experiments.

2.2. DNA Analysis of Moringa oleifera

DNA analysis was performed to confirm that the plant used was *M. oleifera*. Total gDNA was isolated using a Qiagen DNeasy Plant Mini Kit (Qiagen Sciences, Valencia, CA, USA) according to the manufacturer's protocol. Two markers were used: *rbcL* (plastid DNA), amplified using universal primers *rbcL-F* and *rbcL-R* [27] and *trnH-psbA* (intergeneric spacer), amplified using *trnH-psbA* F and *trnH-psbA* R [28]. PCR was implemented using 5x Hot FIREPol® Blend Master Mix (Solis Biodyne, Estonia) according to manufacturer's protocol. PCR amplification was performed adjusted to the following conditions: initial denaturation at 95 °C for 12 min, 35 amplification cycles (denaturation at 94 °C for 15 s, annealing at 54 °C for 50 s, and extension at 72 °C for 3 min), and final extension step at 72 °C for 7 min. Isolated and purified PCR products were directly sequenced. The phylogenetic tree was generated from the nucleotide sequences in the dataset using the Maximum Likelihood method based on the Tamura 3-parameter method [29] for *trnH-psbA* and the Jukes-Cantor model [30] for *rbcL*, respectively. Aligned sequences were evaluated with bootstrap analysis (1000 replicates).

2.3. Preparation of Moringa oleifera Seed Powders and Water Extract

The dried seeds were deshelled by hand, and the best seeds (beige, no signs of disease) were selected as a flocculant. The seeds were ground manually using a mortar or automatically with a planetary micro mill (Fritsch Pulverisette 7 premium line) at 800 rpm and 20 s. The ground seeds were passed through a 0.6-mm steel sieve to obtain fine homogenous powders. Alternatively, two other stainless-steel sieves (1.0 mm and 0.15 mm) were used to investigate the effect of the granulometry of the seed powder on flocculation. The *M. oleifera* seed powders were stored in closed plastic tubes and kept at 4 °C to maintain freshness and dryness before further use. Finally, a water extract from *M. oleifera* seed power was also prepared following the method described by Baharuddin et al. [22], i.e., 2 g of seed powder was dissolved in 100 mL distilled water. This solution was stirred for 30 min at room temperature. Then, the solution was filtered 2 times with a Whatman n°1 paper filter (Merck, Rahway, NJ, USA) and immediately used in the flocculation experiments at different dosages.

2.4. Effects of Dosage on Harvesting Efficiency

Two chemical flocculants were also assessed for their harvesting efficiencies in comparison to *M. oleifera* seed powder (MP), aluminium sulphate (AS—$Al_2(SO_4)_3$ $18H_2O$, Chem-Lab, Belgium), and iron (III) chloride (IC—$FeCl_3$ $6H_2O$, Merck, Rahway, NJ, USA). The three flocculants (MP, AS, and IC) were tested in nine different doses (g L^{-1}), i.e., 0.01,

0.1, 0.2, 0.3, 0.4, 0.5, 1, 2.5, and 5. The flocculant in a powder form was added to 100 mL flasks containing 100 mL of algae sample with a standard initial optical density (OD_{680}) in the range of 1.2–1.4, corresponding to an algal dry matter of approximately 0.5 g L^{-1}. The samples were mixed in a shaker (GFL orbital shaker type 3015, Hannover, FRG) for 5 min at 150 rpm for the coagulation step, followed by a flocculation step at 70 rpm for 20 min, and then left to rest (sedimentation step). The used flocculation protocol was developed from preliminary experiments using AS. Samples were taken every 20 min during the resting phase from the middle of the liquid column in the flasks, and their OD_{680} was measured in a spectrophotometer (Jenway 6400, Chelmsford, UK). The formula applied for the calculation of the harvesting efficiency is shown in Equation (1):

$$\text{Harvesting efficiency } (\%) = \frac{\text{Abs}_0 - \text{Abs}_t}{\text{Abs}_0} \times 100 \quad (1)$$

Abs_0 in the OD_{680} of the algal culture was measured before flocculant addition, and Abs_t is the OD_{680} in the middle of the liquid column on time t of the harvesting process.

2.5. Effect of pH on the Harvesting Efficiency

Stock solutions of 2 g L^{-1} AS and 1 g L^{-1} IC were prepared to investigate the effect of pH on harvesting efficiency. The pH of the samples was adjusted with the AS and IC stock solutions to values of 4.8, 5.8, and 6.8. For MP, 1M HCl and 1M NaOH stock solution were prepared and used for adjusting the pH of the algal culture to values varying from 3 to 11 before the addition of the MP. Cultures with pH adjustment but without MP addition were used as control. Other experimental conditions were the same as described in the previous sections.

2.6. Effect of Rotation Speed and Duration on Flocculation Efficiency

Seed powder at 0.5 mg L^{-1} was added to an algal culture of pH 8, and a lower flocculation speed of 30 rpm was tested. Then, six different combinations of coagulation/flocculation durations (min) were tested, i.e., 5/10, 5/20, 10/10, 10/20, 20/10, and 20/20. Other experimental conditions were the same as described in the previous sections. Flocculation efficiency was calculated with Equation (1).

2.7. Characterization of the Harvested Microalgae

To determine its heavy metal content, the harvested biomass was dried at 60 °C until a constant mass was reached. Then, 0.1 g of dry samples were pre-digested with 10 mL HNO_3 for 30 min, followed by 30 min in an ultrasonic water bath. The samples were then subjected to microwave digestion (UltraWAVE, Milestone, Milan, Italy), and the total metal concentrations in the digested samples were analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian Vista MPX, Kissimmee, FL, USA).

2.8. Statistical Analysis

All experiments were conducted as independent triplicates. The results from the triplicates were compared using a one-way ANOVA followed by a Tukey HSD test ($p < 0.050$) after checking for the normality of the residues using the software Statistica® (version 7.0). Error bars were plotted based on the standard deviations obtained from the experimental triplicates.

3. Results and Discussion

3.1. Moringa oleifera Identification

The *Moringa oleifera* seeds used in the present study were sourced from a local shop in India. The DNA sequence analysis was carried out to ensure that the used seeds belonged to the M. oleifera species. The integrity of the isolated DNA was determined using agarose gel (1.5%) electrophoresis and was found to be of good quality (Supplementary Material Figure S1). The Maximum Likelihood tree was generated using the identified trnH-psbA and rbcL

sequences (Supplementary Material Figure S2). The phylogenetic tree of the rbcL and trnh-psbA (Supplementary Material Figure S3) showed that the used seeds belonged most closely to the *Moringa oleifera* species and were well separated from the genera Theobroma, Melochia, Hibiscus, Dianthus, and Maerua (Supplementary Material Figure S3). In this study, mainly trnH-psbA was found to be efficient for the molecular analysis of *Moringa* sp. since the ratio of the branch was higher than 95%.

3.2. Effect of Flocculant Dosage on Harvesting Efficiency

The effectiveness of *M. oleifera* seed powder (MP) to harvest a mixed culture of *Desmodesmus* sp. and *C. vulgaris* grown on digestate was investigated with varying dosages from 0.01 g L^{-1} to 5 g L^{-1}. Aluminium sulfate (AS) was chosen as a positive control as a widely used chemical coagulant, but likely unsuitable for feed application due to toxicity effects [31]; the use of iron (III) chloride (IC) for microalgae harvesting has been increasing and this flocculant can be compatible with feed applications within a limited concentration in the final product [32]. The two chemical coagulants were tested in the same range of dosages as *M. oleifera*; a negative control without the addition of flocculants was also included. The obtained flocculation efficiencies after 60 min of sedimentation are shown in Figure 1.

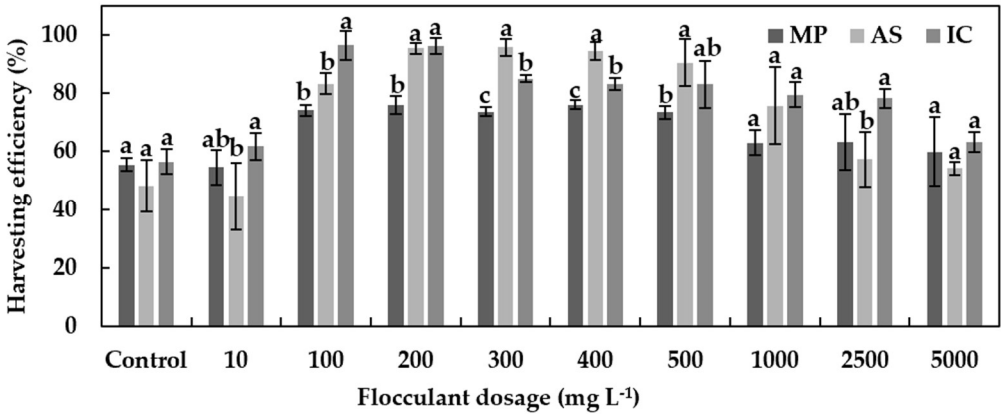

Figure 1. Effect of nine dosages of *Moringa oleifera* seed powder (MP), aluminium sulfate (AS), and iron (III) chloride (IC) on the harvesting efficiency of a mixed culture of *Desmodesmus* sp. and *C. vulgaris* (500 mg L^{-1}) after a 60-min sedimentation time. Control without the addition of flocculants was also carried out in the same conditions as the assays with the addition of flocculants. The rotation speed and time used were 150 rpm for 5 min, followed by 70 rpm for 20 min in an orbital shaker. Columns represent the average values, and error bars represent the standard deviations of triplicates. Different letters indicate significant differences between the flocculants for a same dosage after a one-way ANOVA followed by a Tukey test ($p < 0.05$).

Interestingly, the negative control, without the addition of flocculants, had an average harvesting efficiency of 53%, indicating that the used culture is a suitable candidate for harvesting by sedimentation and that the addition of a low dosage of flocculants could be a strategy for improving the sedimentation rate and, consequently, the economics of the process. Therefore, the addition of 10 mg L^{-1} of flocculants, resulting in a biomass/flocculant ratio of 50, was assessed to test whether this small addition would suffice to improve the sedimentation rates of the *Desmodesmus-Chlorella* culture. As shown in Figure 1, the addition of 10 mg L^{-1} of flocculants did not affect the harvesting efficiency of the culture and yielded similar results to the negative control ($p > 0.05$).

When using higher dosages of flocculants, between 100 mg L^{-1} and 5000 mg L^{-1}, MP resulted in lower maximum harvesting efficiency, of 76%, compared to 95–96% obtained with the chemical flocculants. There was no significant difference in harvesting efficiency between the tested dosages ($p > 0.05$). Even though *M. oleifera* yielded the lowest harvesting efficiencies, a longer settling time might have resulted in better yields. While

both chemical coagulants resulted in immediate floc sedimentation, the samples harvested with *M. oleifera* had increasing harvesting efficiencies over time (Figure S4). Moreover, the flocculation conditions were not optimized for MP in this experiment, which could result in underperformance; this was further investigated in the following sections.

When comparing the chemical flocculants, the highest harvesting efficiency obtained with IC was 96% with the addition of 100 mg L^{-1}, while AS reached the same harvesting efficiency with a higher dose of 200 mg L^{-1}. To better compare the two tested chemical coagulants, the molar concentration in terms of their cations (Fe^{3+} and Al^{3+}) was calculated for the 100 mg L^{-1} dosage, yielding 3.7×10^{-4} mol L^{-1} and 3.0×10^{-4} mol L^{-1}, respectively. This small change in molar loading may account for the lower harvesting efficiency found for Al^{3+} at 100 mg. L^{-1}. Nevertheless, the effect of pH was also investigated, as pH is an important parameter in flocculation/coagulation [33–35], and the addition of both chemical flocculants influenced the final pH of the microalgae suspension due to their acidic nature. Therefore, a new experiment was conducted in which, instead of adding a fixed amount of flocculant, the final pH was used as the target value, i.e., the pH adjustment of the samples was performed by adding a solution of AS (pH 3.9) or IC (pH 2.6) to reach the final pH values of 4.8, 5.8, and 6.8. Since the pH of the AS solution was higher than that of the IC solution, it was always necessary to add more AS to reach a given pH value. Nevertheless, IC resulted in the highest harvesting efficiency of 99% at a pH of 5.8, while AS could not reach efficiencies higher even with a further addition to reach a final pH of 4.8 (Figure S5). Therefore, IC proved to be a better coagulant than AS in the tested conditions, with a higher maximum harvesting efficiency obtained at a lower dosage.

Optimal doses for all tested flocculants were in a similar range and lower than 500 mg L^{-1}, resulting in a biomass/additive ratio between 1 and 5. Interestingly, the harvesting efficiency decreased at high doses for both chemicals. For *M. oleifera* seed powder, the harvesting efficiency was not altered, but more loose flocs were observed at higher dosages, resulting in higher standard deviations in the obtained results (Figure 1). This has also been observed in previous studies and is attributed to a destabilizing effect when excess flocculant molecules are present in the solution [36]. Nevertheless, *M. oleifera* seed powder had a good performance over a wide range of concentrations, indicating a less pronounced destabilizing effect that can come as an advantage in industrial processes, as it would result in a more robust process with a lower risk of reduced performance due to a dosage error.

3.3. Optimization of Harvesting Conditions for M. oleifera Seed Powder

3.3.1. *Effect of pH*

The success of harvesting microalgae by flocculation depends on many parameters, such as the chemical structure of the flocculants, their molecular weight, the way the flocculant interacts with the microalgae, the pH of the algae culture, the algae species, and the cell size [37]. Therefore, several of these parameters were further investigated to optimize the harvesting efficiency of M. oleifera seed powder for the used algal suspension. It has been reported in previous studies that pH is a significant parameter when using MP as a flocculation agent [18,38]. Therefore, a range of pH values varying from acidic to basic was tested to assess its effect on the harvesting efficiency of MP, and the results are shown in Figure 2. The initial pH of the algae sample was 7, and it was used as a control without any additions during the experiment.

At the end of 60 min, MP was effective in both acidic and alkaline conditions, with harvesting efficiencies ranging from 74% to 89% at pH values between 3 and 11 and no significant difference between the tested conditions ($p > 0.05$). This can be explained by the high isoelectric point of *M. oleifera*, which only shows anionic properties at pH > 11 [39–41]. However, previous studies have reported different results with *M. oleifera* seed powder. For instance, Teixeira et al. [18] observed significantly higher harvesting efficiencies at pH > 7, and Behera & Balasubramanian [23] found maximum efficiencies at a pH value of 9.5. This discrepancy may be attributed to the auto-flocculation properties at acidic pH values observed for the algal culture used in the present study. As shown in Figure 2,

when the pH decreased from 7 to 6, a significant increase in harvesting efficiency was observed even in the absence of MP. While Behera & Balasubramanian [23] did not report the harvesting efficiency of their algal culture in the absence of flocculants, Teixeira et al. [18] did not observe any auto-flocculation of the used *Chlorella* suspension at acidic pH values, which can explain their lower harvesting efficiencies at lower pH values. Even though in the present study both *Desmodesmus* sp. and *C. vulgaris* were present, *Desmodesmus* was dominant in the cultures used for the flocculation experiments. It has been shown that *Scenedesmus*, a closely related genus to *Desmodesmus*, has auto-flocculation properties at acidic pH values due to the neutralization of its cell wall charges by protons [42], which can explain the observed results in the present study.

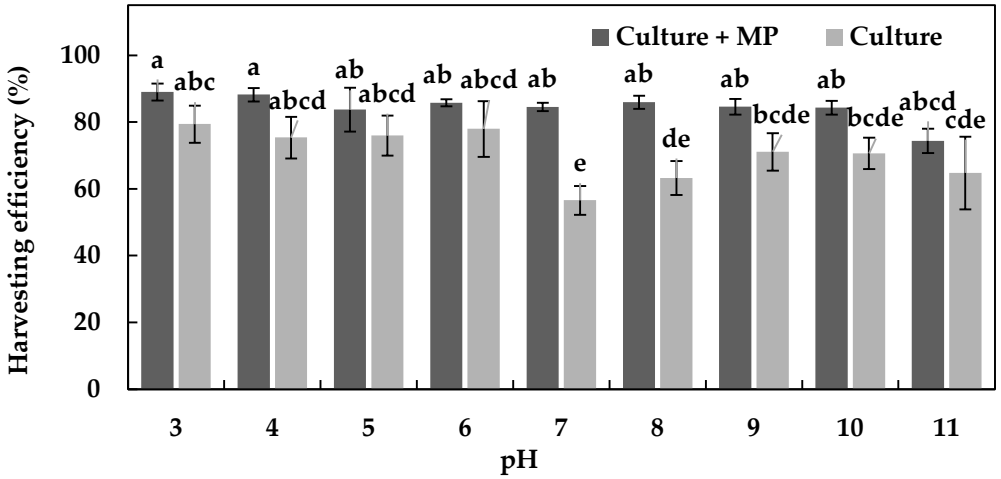

Figure 2. Effect of different pH values on the harvesting efficiency of a mixed culture of *Desmodesmus* sp. and *C. vulgaris* (500 mg L^{-1}) with (Culture + MP) and without (Culture) MP after a 60-min sedimentation time (seed powder concentration: 500 mg L^{-1}). Different letters indicate significant differences between the pH values after a one-way ANOVA followed by a Tukey test ($p < 0.05$). The rotation speed and time used were 150 rpm for 5 min, followed by 70 rpm for 20 min in an orbital shaker. Columns represent the average values, and error bars represent the standard deviations of triplicates.

At pH values of 7 and 8, which is the common pH range used when growing algae on digestate, MP addition increased the harvesting efficiency by 36% and 50%, resulting in a final harvesting efficiency of around 85%, indicating that M. oleifera is an adequate flocculant for the proposed application in this study without the need for pH adjustment.

3.3.1.1. Effect of Flocculation Speed and Coagulation/Flocculation Duration

The harvesting process occurs when coagulation and flocculation events complement each other. The coagulation process requires a fast and short mixing process for the algal cells and flocculants to come in contact and interact in the suspension, whereas a slow but long mixing process is required for the aggregated particles to come together and settle [43]. Therefore, the speed and mixing time used for these two phases was investigated to optimize the harvesting efficiency of the *M. oleifera* powder.

Initially, a slower flocculation rotation speed of 30 rpm was compared to the previously used 70 rpm, resulting in an improvement of 12% in the harvesting efficiency (data not shown). The reduction in flocculation speed would also result in energy savings in the process, so the new flocculation speed of 30 rpm was chosen, and different coagulation and flocculation durations were tested to improve the harvesting efficiency. It was observed that the shorter time of 10 min, instead of 20 min, at the reduced flocculation speed of 30 rpm was sufficient for achieving maximal flocculation efficiency when coagulation time and speed were kept the same, i.e., 5 min and 150 rpm (Figure S6). Longer coagulation times were detrimental to the overall harvesting efficiency, possibly due to perturbation of floc formation, while the duration of the flocculation phase did not seem to affect the process.

3.4. Effect of Preparation of *M. oleifera* Flocculant on the Harvesting Efficiency

As the adjustment of process conditions and pH values was unable to increase the maximum harvesting efficiencies found of 81–86%, different preparation procedures for the *M. oleifera* flocculant were tested to possibly reduce the amount of flocculant needed while improving the harvesting efficiency obtained. Initially, two parameters that have not been investigated before, i.e., method of grinding and powder granulometry, were assessed to understand their impact on the flocculation efficiency of MP.

MP obtained both by manually grinding with a mortar and automatically milling with a ball mill were compared, and the results indicated that the milling process only slightly affected the flocculation efficiency of *M. oleifera* (Figure 3). The flocculation efficiency at the lowest dose (100 mg L^{-1}) was significantly reduced ($p < 0.05$) by 13% when using the powder produced with the automatic mill, but the maximum harvesting efficiency was obtained when using doses higher than 200 mg L^{-1}. This result suggests that the proteins responsible for flocculation are well protected within the seed and are not easily subjected to physical denaturation, which is an advantage when upscaling the production of flocculant for microalgae harvesting from this seed. *Moringa* seeds contain ~35–40 oil, commercially called 'Ben oil' [44,45], and it has been reported that the lipid cells surround the protein molecules [45], which could explain the observed resistance to denaturation.

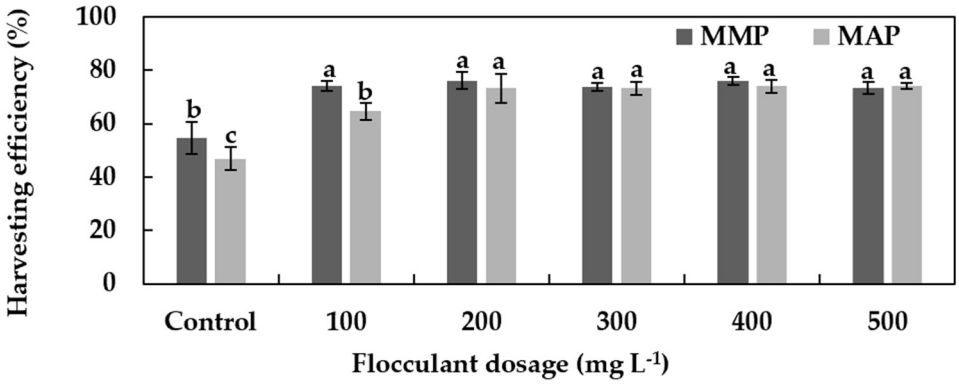

Figure 3. Effect of *M. oleifera* seed powder prepared with different grinding methods, manually-ground powder (MMP) and automatically-ground powder (MAP), on the harvesting efficiency of a mixed culture of *Desmodesmus* sp. and *C. vulgaris* (500 mg L^{-1}) after a 60-min sedimentation time. Control without the addition of flocculants was also carried out in the same conditions as the assays with the addition of flocculants. The rotation speed and time used were 150 rpm for 5 min, followed by 70 rpm for 20 min in an orbital shaker. Columns represent the average values, and error bars represent the standard deviations of triplicates. Different letters indicate significant differences between different dosages of *Moringa* powders using two different grinding methods after a one-way ANOVA followed by a Tukey test ($p < 0.05$).

The powder produced with the automatic mill was then tested after sieving through three different grid sizes (1.00 mm, 0.60 mm, 0.15 mm) to assess whether a finer or coarser granulometry would positively impact the flocculation efficiency of the used powder. However, no significant difference was observed between the different granulometries at the end of the experiment (Figure S7). It may be that the proteins responsible for flocculation are highly soluble in the algal culture, and therefore even a coarser granulometry enables sufficient contact between the solution and the proteins to result in high harvesting efficiency.

To further test the water solubility of the proteins responsible for flocculation in the *M. oleifera* seed, the seed powder was dissolved in water, and the solution was filtered before being mixed with the algae suspension at different doses (Figure 4). A harvesting efficiency between 88% and 91%, the highest one observed in this study, was obtained at 2–3 g L^{-1} of MP-equivalent. As a result, it was confirmed that the flocculant proteins were indeed water-soluble, and the previous dissolution possibly increased their ability to

interact with the algal cells, improving the harvesting efficiency of the process. However, a higher dosage was needed to achieve similar flocculation as the one obtained with the seed powder. Previous studies have shown better protein extraction from the seed powder when using a saline solution than water [23,46,47].

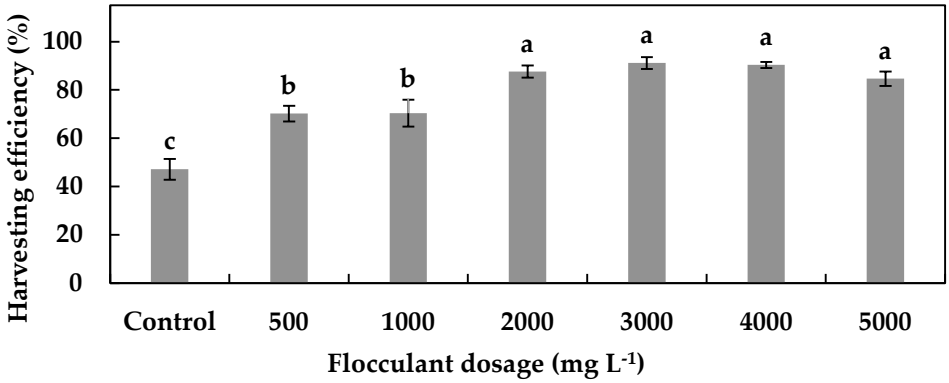

Figure 4. Effect of six different doses of *M. oleifera* seed powder water extract (20 g L^{-1}) on the harvesting efficiency of a mixed culture of *Chlorella* and *Desmodesmus* (500 mg L^{-1}) after a 60-min sedimentation time. Control without the addition of flocculants was also carried out in the same conditions as the assays with the addition of flocculants. The rotation speed and time used were 150 rpm for 5 min, followed by 30 rpm for 10 min in an orbital shaker. Columns represent the average values, and error bars represent the standard deviations of triplicates. Different letters indicate significant differences between the different dosages of dissolved *M. oleifera* powder after a one-way ANOVA followed by a Tukey test ($p < 0.05$).

3.5. Effect of Flocculants on the Composition of the Harvested Biomass

Bioflocculants are usually proposed for the harvesting of microalgae as an alternative to more traditional chemical flocculants, which are said to contaminate the final biomass with their metallic ions. However, studies usually do not conduct a composition analysis of the harvested biomass to prove (or refute) this statement. Especially with *M. oleifera*, no trace element analyses have been carried out in the harvested biomass to assess its possible further use in food or feed applications. Therefore, the algae biomass harvested with the three tested flocculants (AS, IC, and MP) were characterized for their content of trace elements, and the results are shown in Table 1. These are compared against the maximum tolerable levels (MTL) for swine, cattle, and poultry, which are defined as the amount of a certain mineral that can be fed for a defined period without impairing animal health or performance [48].

Table 1. Maximum tolerable levels (MTL) of micronutrients in livestock feed [48 and the content of these elements in the harvest microalgae with the different flocculants (MP—*M. oleifera* seed powder; AS—aluminium sulfate; IC—iron (III) chloride).

Biomass	Mn	Zn	Cu	Cd	Pb	Fe	Al
	mg kg^{-1}						
Algae + MP	130.5	284.2	95.4	0.3	8	10,672.2	4614
Algae + AS	37.5	141.4	35.9	BQL	BQL	4844.6	29,687
Algae + IC	30.9	44.3	9.5	BQL	BQL	47,240.4	1727.2
MTL							
Swine	1000	1000	100	10	10	3000	1000
Cattle	2000	500	25	10	100	500	1000
Poultry	2000	500	250	10	10	500	1000

BQL—below quantification level.

Fe and Al content in all algae samples characterized were above the recommended values. For Fe, the sample harvested with MP had a much higher content than the sample harvested with AS, while only trace amounts of Fe were found in the seed powder; a similar observation can be made for Al, as the algae harvested with MP had more than double the amount of Al found in the samples harvest with IC. Previous research has indicated that MP does not neutralize the charges of the algae cell wall but rather interacts with algae cells by forming bridges that connect clusters of cells, forming flocs. This bridge flocculation results in larger flocs with higher moisture content [21], which may lead to higher retention of trace elements from the medium in the biomass upon drying. The algae used in the present study were grown on digestate, a medium rich in several trace elements; the used digestate, for instance, had an Al content of 2.6 mg kg^{-1} and a Fe content of 8.1 mg kg^{-1}, while usual commercial media used for algae cultivation contain no Al and about 1 mg kg^{-1} of Fe. As the algae used in the present study were grown on digestate, a medium rich in several trace elements, digestate particles were likely trapped in the algae-MP flocs and might have been responsible for the high Al and Fe contents found in this sample.

3.6. Applicability of Moringa as a Flocculant for Microalgae Grown on Digestate

This study proposed using the seed powder of *Moringa oleifera* as a bioflocculant for harvesting green microalgae grown on digestate for feed production. A dosage between 100–500 mg L^{-1} was found to give the highest harvesting efficiencies (around 80%), representing a ratio of microalgae: flocculant between 5:1 and 1:1. This is similar to the ratio found by Yang et al. [21] of 1:1 for *C. vulgaris* and 1.5:1 for *S. obliquus*, and is also in the same range of several other studies, including one of the first reports of *Moringa* as a bioflocculant for microalgae harvesting [18].

The amount of *Moringa* seed powder needed for achieving high harvesting efficiencies greatly surpasses that of other bioflocculants by a factor of 10 or more; this has been considered a disadvantage as it results in high costs with flocculant addition and a greater amount of generated "sludge" [21]. Such conclusions are made taking into consideration that the seed powder would be used only as a flocculant with no added value for the final product. Nevertheless, *Moringa* seed has been recently shown as a good protein source and a beneficial additive to animal feed [49,50]; the used *Moringa* seed powder in this study, for instance, had 39% protein in its composition. Moreover, the use of a mixture of microalgae and *Moringa* leaves was shown to have synergistic effects as a feed for goats [51], and a similar result could potentially be found when evaluating the use of a mixture of microalgae with *Moringa* seeds. Therefore, the final obtained product of microalgae-*Moringa* could potentially have a higher nutritional value than only the microalgae itself.

Furthermore, the use of *Moringa* seed powder as a flocculant enabled the reuse of the cultivation water and had a positive effect on the biosynthesis of polyunsaturated fatty acids, especially α-linolenic acid (C18:3 ω3), which is one of the essential fatty acids that need to be externally acquired in the human diet [52], possibly further increasing the added value of using *Moringa* as a bioflocculant for microalgae harvesting. Finally, even though Yang et al. [21] calculated increased cost when using *Moringa*, the environmental impacts of the alternatives should be considered. This study showed that a simple mill grinding was effective in upscaling the production of the used seed powder and that different granulometries did not impact the harvesting efficiency, resulting in a low-energy and zero-chemical process for the production of the flocculant. The production of tannin-based flocculants, identified by Yang et al. as the most promising of the ones tested in their study, includes several process steps, including the use of a chemical modification with significant environmental impact [53].

One point of attention in this study was the high content of Fe and Al in the microalgae grown on digestate and harvested with *Moringa* seed powder. Since the algae biomass would likely be only one component in a feed formulation, the high Fe and Al contents would become diluted in the complete feed and would not pose any problems for animal nutrition. Nevertheless, the high final contents of these metals in the algae cultivated in digestate and harvested with MP should be a point of attention in future studies.

4. Conclusions

The optimum dose of 0.5 g L^{-1} MP (biomass/flocculant ratio of 1) resulted in a harvesting efficiency of around 80%, while the use of water extract of MP at 3 g L^{-1} (biomass/flocculant ratio of 0.17) resulted in a harvesting efficiency of 91%. Therefore, *Moringa oleifera* seed powder was found to be an effective flocculant for harvesting a mixed algal culture grown on digestate, especially when using a water-extraction step during the flocculant preparation. The robustness of this flocculant makes it attractive for industrial applications, but more studies are needed to evaluate its economic viability and environmental impacts. Finally, the characterization of the trace element composition of the final biomass proved to be of importance and can help to guide further developments in the field aiming at obtaining high harvesting efficiencies together with high-quality biomass.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app122412968/s1, Figure S1. 1.5 % agarose gel electrophoresis results of gDNA isolated from the seed samples of *Moringa sp.* obtained from India. Figure S2. 1.5% agarose gel electrophoresis of PCR analysis of isolated seed samples of *Moringa* using *rbcL* and *trnH-psbA* primers. Figure S3. Harvesting efficiency of *Moringa oleifera* seed powder (MP), aluminium sulfate (AS), and iron (III) chloride (IC) added to a mixed culture of *Desmodesmus* and *Chlorella* (500 mg L^{-1}) at 200 mg L^{-1}. Figure S4. Influence of pH values in the harvesting efficiency of aluminium sulfate (AS) and iron (III) chloride (IC). The different pH values were obtained by the addition of either AS or IC solutions to the microalgae suspension, resulting in different final concentrations of each flocculant for a given pH. Figure S5. Effect of different duration combinations for the coagulation and flocculation steps when using *M. oleifera* seed powder for the harvest of a mixed culture of *Chlorella* and *Desmodesmus* (seed powder concentration of 500 mg L^{-1}, pH 8, coagulation and flocculation rotation speeds of 150 rpm and 30 rpm, respectively). The effect of sedimentation duration (20, 40, or 60 min) on the harvesting efficiency is also shown. Different letters indicate significant differences between the flocculation times after a one-way ANOVA followed by a Tukey test ($p < 0.05$). Figure S6. Effect of three different granulometries of *Moringa oleifera* seed power on the flocculation efficiency at 60 min sedimentation (seed powder concentration: 0.5 g L^{-1}, the rotation speed and time used were 150 rpm for 5 min followed by 30 rpm for 10 min).

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