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Harvesting *Chlorella variabilis* Biomass Using *Moringa oleifera* Seed-induced Sedimentation

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Authors' contributions

This work was carried out in collaboration between the two authors. Author CNO designed the study, did some of the experiments, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author IE carried out most of the experimental work and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate the efficacy of using *Moringa oleifera* seed powder, filtered cold water extract, and autoclaved cold water extract to induce sedimentation of *Chlorella variabilis* NIES 2541 cells without pH adjustment.
Place and Duration of Study: Department of Plant Science and Biotechnology, University of Nigeria, Nsukka between October, 2017 and July, 2018.
Methodology: Three sets of dry seeds of *Moringa oleifera* were prepared namely: (a) powdered seed, (b) Cold water extract of the seed which was prepared by soaking powdered seeds in cold water for 30 minutes, and filtering the extract through cheese cloth, and (c) autoclaved extract which was prepared by autoclaving the extract obtained from (b) for 20 minutes at 121°C. *Chlorella variabilis* was cultivated in BG11 medium and different concentrations of these *Moringa* seed samples were added to the culture broth, mixed and allowed to sediment. The sedimentation rates were monitored at 30 minutes intervals by taking samples from the top and measuring the optical density at 680 nm.
Results: In all the three cases, the rate of sedimentation increased with increase in the

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concentration of the *Moringa* seed used. In comparison with seed powder, use of cold water extract resulted in significant decrease in the sedimentation rate ($P<0.05$). However, more than 60% sedimentation was achieved by addition of extract from 10 g/l seed powder and incubating for only 30 minutes. Autoclaving the extract did not result in significant decrease in the efficacy of sedimentation ($P>0.05$). More than 70% sedimentation of *Chlorella variabilis* culture with an optical density of 3.5 was achieved in 30 minutes by addition of 7 g/l of autoclaved seed extract.
Conclusion: Although using *Moringa* seed powder resulted in the highest rate of cell sedimentation, autoclaved seed extract can still be used for efficient harvesting of *Chlorella variabilis*.

Keywords: Chlorella variabilis; Moringa oleifera; seed powder; cold water extract; harvesting of microalgae; biomass sedimentation.

1. INTRODUCTION

Cultivation of microalgae has been increasing steadily due to their various applications such as in wastewater treatment [1-5], biodiesel oil production [6-12] as well as in production of antioxidants [13-16]. Microalgae are also used in soil bioremediation [17], production of single cell protein [18,19], carbon dioxide fixation [20] and in treatment of water and effluent from dyeing industries [21,22]. Although, microalgae have these various applications, the cost of harvesting microalgae biomass after cultivation is capital intensive and represents a significant percentage of the total production costs [23]. Several methods have been developed for harvesting microalgae biomass and these include filtration of the culture [24], centrifugation [25], microbial flocculation [26], flotation [27] or by sedimentation [25]. Natural sedimentation is hardly enough for harvesting microalgae biomass and there is usually a need to add some flocculants. The use of various inorganic and organic flocculants have been investigated and these include metal salts such as Aluminium sulfate, Aluminium chloride, Ferric chloride and Ferric sulphate [23,28,29], and polyethylene oxide [29]. Papazi et al. [30] also tested the ability of 12 salts to sediment *Chlorella minutissima* cells in culture. Among all these flocculants, natural organic flocculants are preferred because they are environmentally friendly and some of them are edible. Some authors have worked on the use of organic flocculants such as chitosan [28,31-33] and even microbial flocculant [26]. Seeds of *Moringa oleifera* have been extensively investigated as a flocculant in water treatment and removal of dye effluent from industries [22,34]. Recently, some researchers have reported the use *M. oleifera* seeds in various forms to harvest microalgae since it is cheap, easily available and non-toxic. Teixeira and Teixeira [35] used seed cake, seed flour and extract from cake and flour to flocculate *Chlorella vulgaris*. Hamid et al. [36] compared the abilities of *M. oleifera* seed flour, protein powder and alum to flocculate *Chlorella sp.* cells for the purpose of harvesting them. Udom et al. [37,38] compared the effectiveness of various flocculants (alum, ferric chloride), cationic polymer (Zetag 8819), anionic polymer (E-38), *Moringa oleifera* and *Opuntia ficus-indica* cactus for harvesting microalgae grown in semi continuous culture in a photobioreactor under natural light. They investigated the cost-effectiveness of each flocculating agent. Hamid et al. [36] harvested microalgae from aquaculture waste water as a phytoremediation method using *M. oleifera*. In most of these previous experiments, either rigorous extraction steps were used or the pH of the media was adjusted to either highly alkaline [39] or acidic level. The process of extraction adds to the cost of harvesting the cells while the method of pH adjustment is not suitable for continuous culture operations where only a fraction of biomass is harvested, and the residual biomass serves as inoculum for the subsequent operation.

In the present study, the ability of *M. oleifera* seed powder, filtrate from cold aqueous suspension of seed powder and autoclaved filtrate were compared for their ability to flocculate *Chlorella variabilis* cells without any pH adjustment.

2. MATERIALS AND METHODS

2.1 Materials

Moringa oleifera pods were harvested from the Botanical Garden, Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. *Chlorella variabilis* NIES-2541 stock culture was obtained from the Department of Microbiology University of Nigeria, Nsukka.

2.2 Preparation of *Moringa oleifera* Seed

The seeds were removed from the pods and the outer shells were removed by hand. Only healthy seeds were selected, ground in porcelain mortar and used for sedimentation experiments (Fig. 1). Three sets of dry seeds of *Moringa oleifera* were prepared namely: (a) powdered seed, (b) powdered seeds were soaked in cold water for 30 minutes, and the extract was filtered through cheese cloth, and (c) the extract obtained from (b) was autoclaved for 20 minutes at 121°C.

2.3 Sedimentation with Seed Powder

Chlorella variabilis NIES-2541 stock was maintained in BG11 medium. The stock culture was revived and cultured in BG 11 medium under photoautotrophic condition for two weeks in 500 ml Erlenmeyer flasks. The cultures were mixed by intermittent manual shaking three times daily. The culture was illuminated at an intensity of 100 $\mu molm^{-2}s^{-1}$ using a 32-W white bulbs (ASTRA NU-PARK, CHINA). Three grams of dry *M. oleifera* seeds were ground to fine powdery paste with mortar and pestle. The powder was suspended in distilled water to a concentration of 50 g/l. Various volumes corresponding to different concentrations (1-5 g/l) of the *M. oleifera* suspension was added into labelled test tubes. Corresponding volumes of algal biomass with optical density of 5.2 at 680 nm were dispensed into each test tube to make a total volume of 10 ml. The mixture was inverted severally to mix and then allowed to stand undisturbed on a test tube rack. One milliliter sample was withdrawn from the upper layer of each test tube every 30 minutes for a period of 180 minutes. At the end, each sample was diluted with 9 ml of distilled water and the optical density was read at 680 nm. Each experiment was performed three times and the average values were plotted.

2.4 Sedimentation with Cold Water Extract of *Moringa oleifera* Seeds

Three grams of dry *M. oleifera* seeds were ground to fine powdery paste with mortar and pestle. Two grams of the powder was suspended in 40 ml of distilled water inside 100 ml conical flask and manually shaken intermittently for 30 minutes to extract the active ingredients. The suspension was filtered through a double folded cheese cloth and various volumes (0.2 to 1.0 ml) of the clear supernatant were dispensed into labeled test tubes. Appropriate volumes of fully grown *C. variabilis* culture (9.8 - 9 ml) with an optical density of 5.2 were dispensed into the

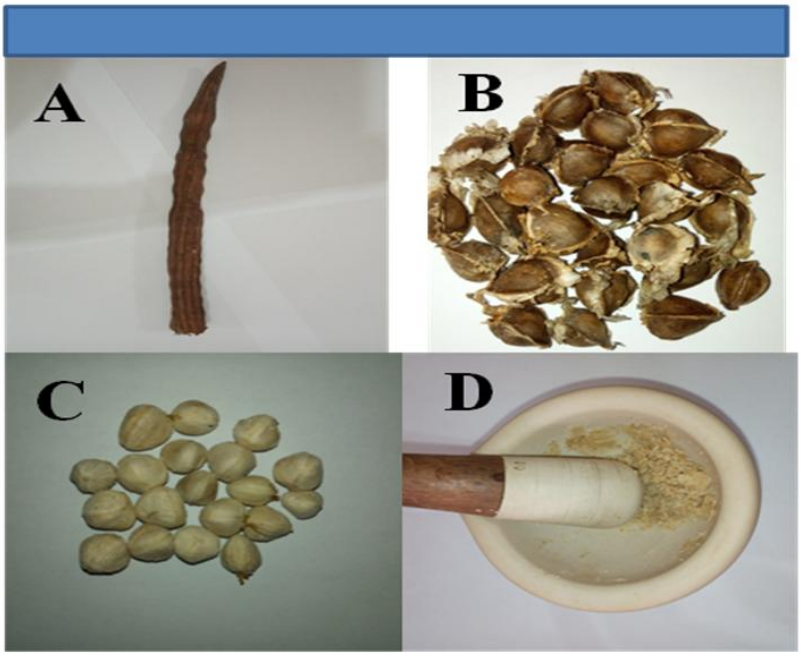

Fig. 1. Photographs of *Moringa* used in this study
A = Moringa pod; B = Moringa seeds; C = Moringa seeds after shelling, and D = Grinding of the shelled Moringa seeds

corresponding labeled test tubes. Each test tube was inverted gently several times to mix. The mixture was allowed to stand undisturbed for 180 minutes. One milliliter sample was withdrawn from the top of each test tube every 30 minutes for a period of 180 minutes. At the end, each sample was diluted with 9 ml of distilled water and the optical density read at 680nm. Each experiment was performed three times and the average values were plotted.

2.5 Sedimentation with Autoclaved *M. oleifera* Seed Filtrate

Three grams of dry *M. oleifera* seeds were ground to fine powdery paste with mortar and pestle. Two grams of the powder was suspended in 40 ml of distilled water inside 100 ml conical flask and manually shaken intermittently for 30 minutes. The suspension was filtered through a double folded cheese cloth and the filtrate was autoclaved at 121°C for 20 minutes. After cooling to room temperature, various volumes (0.2 to 1.0 ml) of the autoclaved filtrate were dispensed into labelled test tubes. Appropriate volumes of fully grown *C. variabilis* culture (9.8 - 9 ml) with an optical density of 3.5 was dispensed into the corresponding test tubes and inverted gently several times to mix. The mixture was allowed to stand undisturbed and one milliliter sample was withdrawn from the top of each test tube every 30 minutes for a period of 180 minutes. At the end, the samples were diluted with 9 ml of distilled water and the optical density read at 680 nm. Each experiment was performed three times and the average values were plotted.

2.6 Percentage Sedimentation

The percentage of *Chlorella variabilis* NIES-2541 cells sedimented by different concentrations of the filtrate or powdered *M. oleifera* seeds after 30 minutes of incubation was calculated using the formula:

$$\text{Percentage sedimentation} = [(I\ OD_{680} - FOD_{680}) / I\ OD_{680}] \times 100$$

Where I OD = Initial optical density of the algal culture used

F OD = Final optical density of the algal culture after incubating for 30 minutes with *M. oleifera* seed extract or powder.

2.7 Statistical Analysis

All the experiments were performed in three replicates and the results were presented as means of the three values. Analysis of Variance (single classification) was used to test for significant differences among the means of the treatments.

3. RESULTS AND DISCUSSION

Various concentrations of powdered *Moringa oleifera* seeds were either used directly (powdered) or mixed with 20 ml of distilled water, extracted for 30 minutes under shaking, and filtered. The effects of addition of the powder or filtrate to the culture broth on sedimentation of *Chlorella variabilis* NIES-2541cells are shown in Fig. 2. The results showed that the rate of cell sedimentation, as measured by a decrease in the optical density of the upper phase, was dependent on the concentration of the *M. oleifera* seed powder or filtrate. When 1 g/l of the powder was added directly, the optical density decreased from 5.2 to 2.1 in 180 minutes. However, by increasing the concentration to 5 g/l, the sedimentation rate increased significantly and the optical density decreased to 1.02 after 90 minutes. In other words, about 80% of the *Chlorella* cells can be harvested through sedimentation by adding 5 g/l *M. oleifera* seed powder to the culture. However, since the powder sediments with the cells, separation of the seed powder from the cells can impose a technical challenge. Thus the effect of adding filtered extract to the culture broth on cell sedimentation was investigated. As shown in Fig. 2, addition of filtrate also induced flocculation, and thus sedimentation of the cells in a concentration-dependent manner. The optical density decreased from 5.2 to 2.1 (about 60% decrease) when an extract from 5 g/l seed was added. Although, the percentage sedimentation obtained in the present experiment was lower than that of other workers [35,39] the extraction procedures used here and extraction time were different. The algal species were also not the same and the medium pH was not adjusted in the present experiment. The moisture content and particle size of the *Moringa* seed powder were not also the same with that of other workers. All these factors affect the efficacy of *Moringa* seed sedimentation of microalgae cells.

Various concentrations of the seed powder or filtrate were added to *C. variabilis* culture in test tubes. The test tubes were inverted several times to mix the content and then left to stand on a test rack undisturbed. One milliliter sample was withdrawn from the upper layer of the test tubes to measure the optical density at 680 nm at 30 min interval.

The effects of higher concentrations of the *M. oleifera* seed powder and extracts on cell sedimentation were investigated and the results are shown in Fig. 3. The rates of sedimentation were also concentration dependent. However, increasing the *M. oleifera* seed powder concentration from 6 g/l to 10 g/l, did not result in any significant difference ($p < 0.05$) in the amount of sedimented cells after 90 minutes of incubation. More than 80% sedimentation was obtained in the cultures treated with *M. oleifera* seed powders higher than 6%. When filtrates of *M. oleifera* seed extracts were used, 37%, 54%, and 62% sedimentations were obtained for 6 g/l, 8 g/l and 10 g/l, respectively. These were lower than the corresponding values obtained when *M. oleifera* seed powder was used. However, it is important to note that by adding extract from 10 g/l *M. oleifera* seed powder to *Chlorella variabilis* culture and prolonging the incubation time to 180 minutes, as high as 80% of the cells sedimented and thus efficiently harvested.

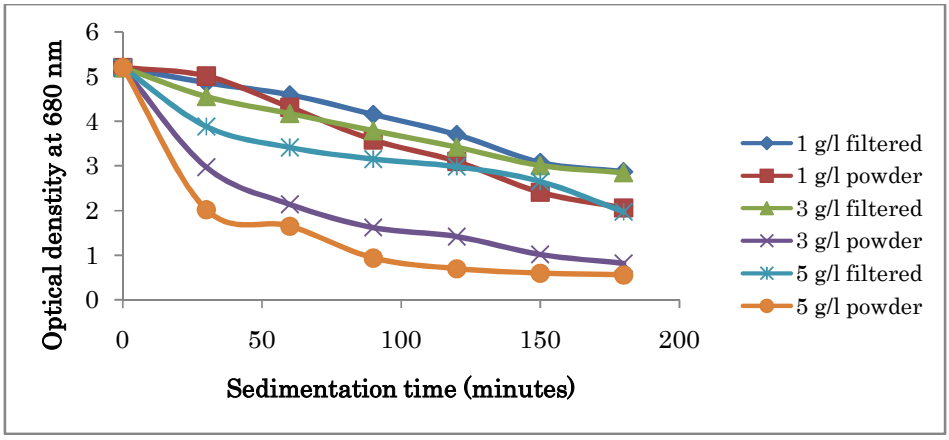

Fig. 2. Effect of various concentrations of powdered and filtered *M. oleifera* seed extract on sedimentation of *Chlorella variabilis* cells

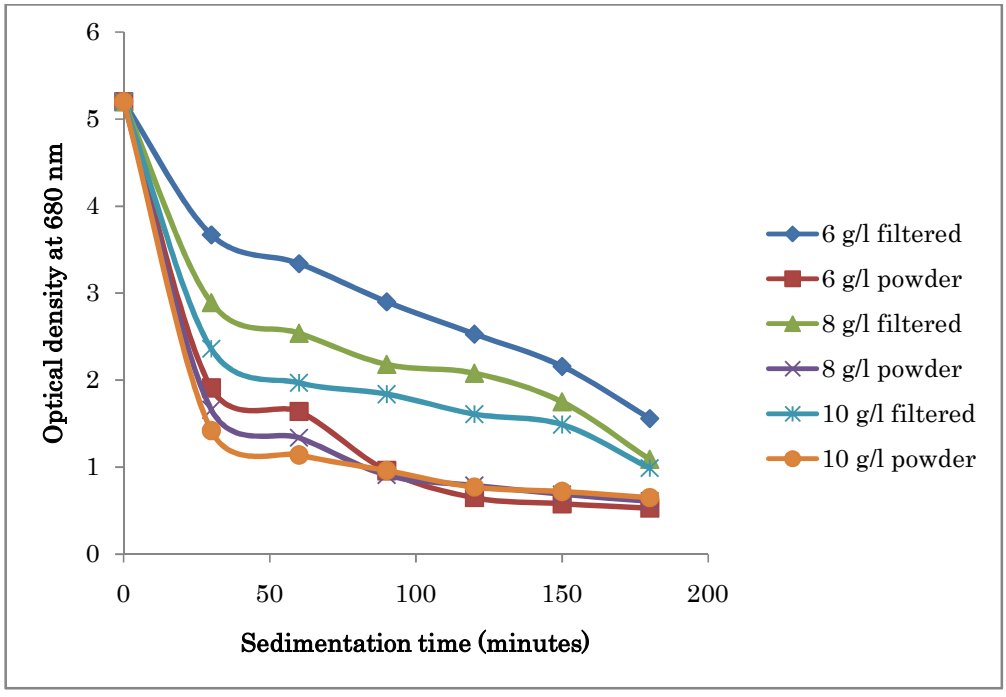

Fig. 3. Effect of concentrations of powdered and filtered *M. oleifera* seed extract on sedimentation of *Chlorella variabilis* cells

The experimental procedures were the same as explained above (Fig. 2) but here higher concentrations of *M. oleifera* seed filtrate or powder were used.

Photographs of test tubes containing cells of *Chlorella variabilis* during the sedimentation experiments are shown in Fig. 4. The photographs show that in comparison with the control, the optical densities of the culture containing *Moringa* seed powder (SD) or *Moringa* seed extract (SE) decreased with time. Furthermore, the decrease in the optical density of the culture with seed powder was higher than the one with the seed extract while in both cases, the decrease increased with increase in the concentration of *Moringa* seed added.

A comparison of the percentage sedimentation of *Chlorella variabilis* culture after 30 minutes treatment with *M. oleifera* seed powder and filtrate is shown in Fig. 5. For the short incubation time of 30 minutes, about 74% of the cells can be harvested by addition of 10 g/l of *M. oleifera* seed powder. However, with 5 g/l, only about 60% of the cells sedimented after 30 minutes of incubation. In the case of extract, there was almost linear relationship between the filtrate concentration and percentage cell sedimentation after 30 minutes. It is worthy to note that addition of extract from 10 g/l resulted in 56% sedimentation. Although, the use of extract in place of powder resulted in a significant decrease in the sedimentation ($p < 0.05$) for all the concentrations tested, the advantage of using the extract is that there is no need for separation of the seed debris from the cells after sedimentation. Although *M. oleifera* seed is edible and has been reported to have many therapeutic values, depending on the intended microalgae cell usage, it may be very necessary to separate the seed debris because of

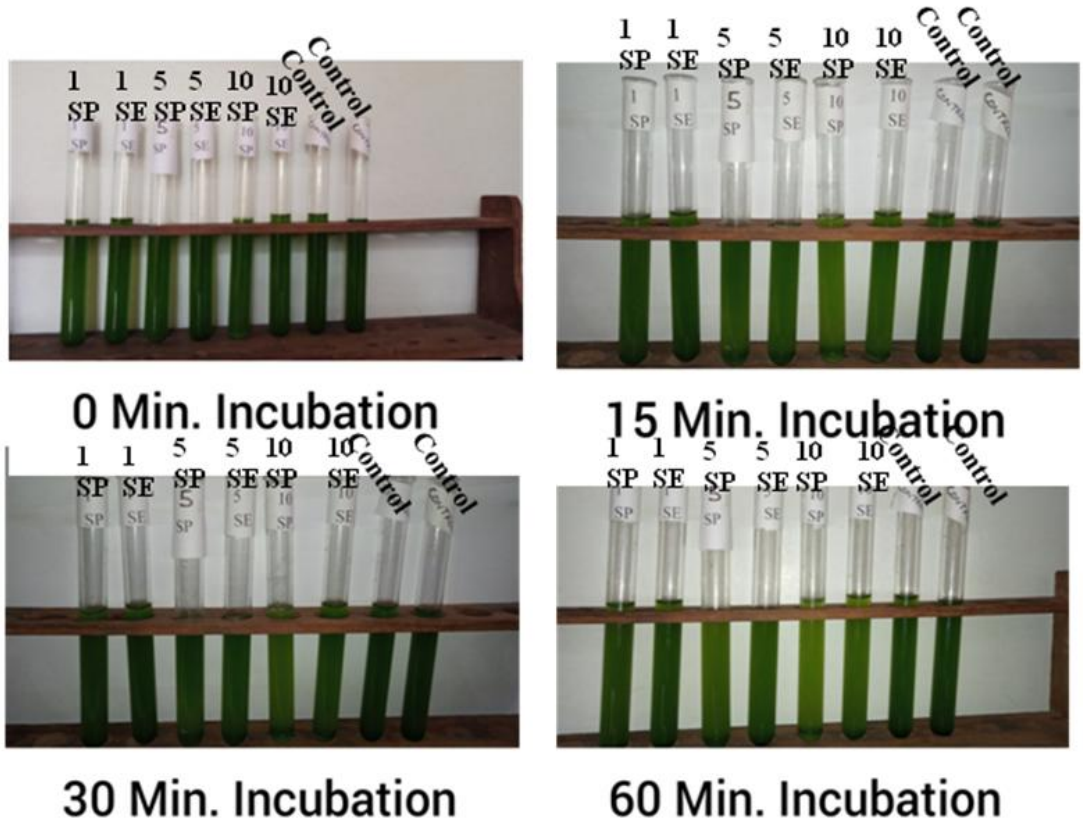

Fig. 4. Photographs of *Chlorella variabilis* culture showing the effects of *Moringa* seed concentration on cell sedimentation

SP = Moringa seed powder; SE = Moringa seed extract. The Figs. (1~10) denote the concentration of Moringa in g/l. The initial optical density (680 nm) of the culture was 8.5

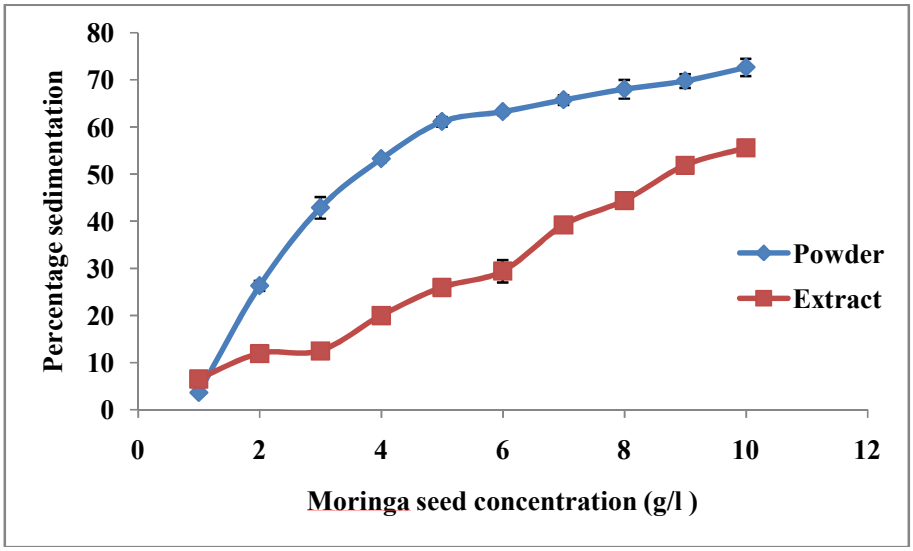

Fig. 5. Comparison of the effects of *M. oleifera* seed powder and filtered seed extract on percentage sedimentation of *Chlorella variabilis* cells after 30 minutes of incubation

the possible effects of *M. oleifera* seed powder on the taste, and activities of the harvested cells. On the other hand, the seed debris after the extraction can potentially be used as feed and food additives. In this study, extraction was done for only 30 minutes with cold water. The extraction yield can be increased by increasing the extraction time, as well as using other treatments such as hot water or other solvents. The use of organic solvents such as ethanol and ethyl acetate may result in a significant increase in the extraction yield. However, it will add to the cost of extraction and the solvents must be evaporated before use, thus adding to the complexity and cost of the process.

Percentage sedimentation was calculated from the formula outlined in line 143 above (subsection 2.6). The blue and red plots are for moringa seed powder and extracts respectively.

The above results have shown that the percentage sedimentation (amount of cells harvested) can be increased by increasing the concentration of the *M. oleifera* seed or prolonging the sedimentation time. The choice would depend on the type of microalgae cell. Increasing the concentration of the *M. oleifera* seed will increase the harvesting cost and the economic feasibility of using very high concentration of the seed depends on the value of the microalgae. On the other hand, prolonging the sedimentation time reduces the culture time if artificial light is used or if the harvesting is done in the day time. However, for open door cultures utilizing solar light, the harvesting can be done at night. Nevertheless, the stress of sedimentation on the cells must be considered. This depends on the type of cells, and there is a need to evaluate the sensitivity of the target cells to long time sedimentation.

In the course of this study, it was found that the extracts were easily contaminated by molds during storage at room temperature. Thus, the effect of autoclaving the extract on the efficacy of sedimentation was investigated. The results showed that the compound responsible for the sedimentation is heat stable and addition of the autoclaved extract resulted in efficient sedimentation of *Chlorella variabilis* cells. As shown in Fig. 6, with an initial optical density of 3.5, addition of autoclaved *M. oleifera* seed extract resulted in the sedimentation of the cells in a concentration dependent manner. After 60 minutes of sedimentation, the optical densities of the cultures treated with autoclaved extracts from 1, 3 and 5 g/l decreased to 2.5, 2.2, and 0.9, respectively. However, there was no significant difference in the optical density of the cultures treated with autoclaved extracts from 7 and 10 g/l. In both cases, the optical density decreased to about 0.52.

The dependence of the percentage sedimentation on the concentration of the seeds used for extraction is shown in Fig. 7. The percentage sedimentation increased almost linearly with increase in the concentration of the seeds used for extraction up to 7 g/l.

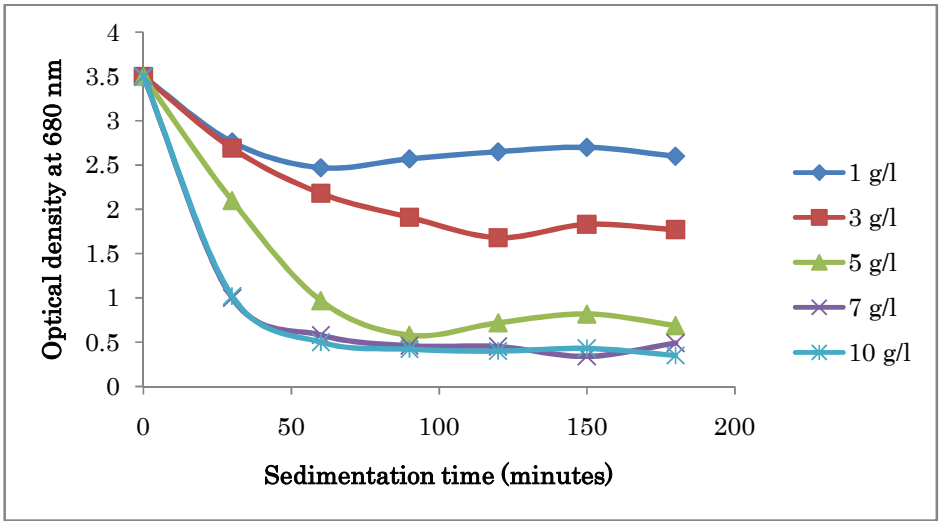

Fig. 6. Effect of autoclaved *Moringa oleifera* seed filtered extract on sedimentation of *Chlorella variabilis* cells. The experimental procedures were the same as those of Fig. 2 except that autoclaved *M. oleifera* filtered extract was used here

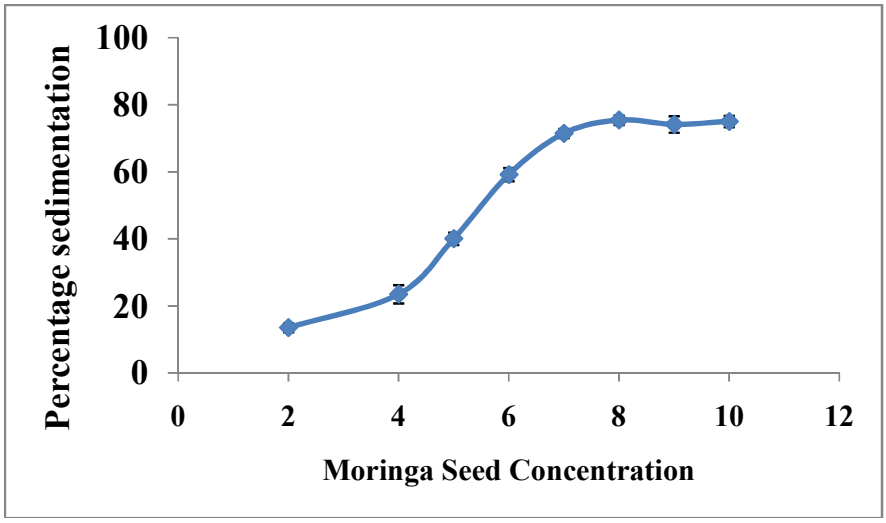

Fig. 7. Effect of autoclaved filtered *M. oleifera seed* extract on percentage sedimentation of *Chorella variabilis* cells after 30 minutes of incubation. Percentage sedimentation was calculated from the formula above (subsection 2.6)

Although the initial cell concentration (OD = 3.5) was lower than the concentration used in Fig. 1 (5.2), it is important to note that even with the autoclaved extracts, the sedimentation rates were very high. Using extracts from 7 g/l, more than 70% of the cells in a culture with optical density of 3.5 sedimented in 30 minutes. This is very significant since it is not necessary to harvest all the cells during microalgae cultivation. The residual cells serve as the seed for the next batch of culture. In fact, depending on the cells and the culture condition, it is recommended that only about 50% of the cells should be harvested at a time. When too much cells are harvested, the culture will experience another lag phase leading to poor light utilization efficiency.

4. CONCLUSION

Morinag oleifera seed powder was very efficient in sedimentation of *Chlorella variabilis* without pH adjustment, and thus can be used to harvest the

cells from the culture broth. Replacing the seed powder with filtered cold water extract of the seed resulted in decrease in the sedimentation rate but high percentage sedimentation was still achieved by increasing the concentration and prolonging the treatment time. Further optimization of the extraction processes requires a better knowledge of the nature of the active ingredients. Okuda et al. [40] reported that the flocculation ingredients are proteins while Bichi [41] noted that they are polyelectrolites. However, the present study suggests that the flocculation-inducing compound in *M. oleifera* seed is apparently heat-stable since autoclaved filtrate of the seed extract was still very efficient in cell sedimentation. This study has demonstrated that *Moringa* seed, which is an environmentally friendly flocculant, can be used for efficient harvesting of microalgae. This will be especially useful in developing microalgae biotechnology in rural areas where *Moringa* grows widely and the seeds are very cheap and easily available.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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