

## *Moringa oleifera* seed protein isolate as an alternative for purifying turbid water

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### ABSTRACT

The *Moringa oleifera* oil industries extract oil from the seeds, the de-oiled *M. oleifera* seed meal (DMSM) thus produced is discarded as waste. In the current study, DMSM was used to prepare *M. oleifera* seed protein isolate (MPI). The coagulation ability of MPI to remove turbidity in synthetic turbid water (STW), as well as real mud water (RMW), was evaluated. The results of the jar test, particle size analysis and scanning electron microscopy with STW proved MPI to be a good coagulant. The MPI reduced the turbidity in STW by more than 97% at a very low concentration (15 mg L<sup>-1</sup>). Further, the coagulation efficiency of MPI in RMW was also evaluated. A comparative study demonstrated that both MPI and aluminum sulfate (alum) could reduce >97% of the turbidity at the same concentration (15 mg L<sup>-1</sup>). An increase of pH in MPI treated RMW from 6.9 to 7.1 and a significant decrease of pH in alum treated RMW from 6.9 to 5.8 suggested that usage of lime and soda ash could be avoided for increasing pH after MPI treatment unlike alum treated municipal water. MPI being a natural coagulant, showed a marginal decrease from 1 to 0.5 mg L<sup>-1</sup> in the chemical oxygen demand. The minimum inhibitory concentration and minimum bactericidal concentration values for MPI were found to be 1 and 2 mg mL<sup>-1</sup> respectively against *Escherichia coli* (ATCC 25922 – American Type Culture Collection, Virginia, USA). These results suggested that, in place of alum, MPI can be used as a cost-effective, natural, water treating agent in regions where people cannot afford clean and safe water.

**Keywords:** *Moringa oleifera*; Protein isolate; Natural coagulant; Turbidity; Mud water

### 1. Introduction

*Moringa oleifera*, commonly known as drumstick tree or horseradish tree, are tropical plants native to South East Asian countries and African countries [1]. They can grow in arid, semi-arid and sandy riverbeds. They have an estimated genome size of 315 Mbp and contain more than 100 annotated heat shock proteins, which make them resistant to drought and hot temperatures [2]. Almost every part of

the tree has nutritional and pharmaceutical value. The pods and leaves of *M. oleifera* are part of the native diet. Seeds of *M. oleifera* are traditionally used to clarify high turbid waters [3–5].

Freshwater from river streams and lakes undergo alum (potassium aluminum sulfate) treatment before they are supplied for household consumption. Alum acts as a coagulant and reduces the turbidity of the water. The drawbacks of alum are its pH lowering activity [6] and the absorption

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of aluminum into the body in both prenatal and neonatal stages. The propensity to absorb and retain aluminum in the body grows higher after the age of 60. Aluminum accumulates in bones and neurons of the central nervous system [7]. The extensive exposure of aluminum for decades may lead to Alzheimer's disease [8].

Extracts and protein preparations from *M. oleifera* seeds are proven to be good coagulants, which can be used as an alternative to alum [9–11]. Water-extracts and NaCl-extracts (NE) of *M. oleifera* seeds have been explored extensively for this purpose and it was reported that they not only reduce turbidity but also microbial count, hardness, apparent color and can chelate heavy metals [12–15]. *M. oleifera* seed powder and seed extracts have been used to treat effluents from various industries like electroplating industries for the removal of heavy metals such as lead, copper, zinc, chromium, cobalt [16], coffee industries for coffee fermentation wastewater treatment [17], paper industries for enhancing the primary treatment of effluent [18] and tannery industries for reducing the turbidity of wastewater [19]. The major disadvantage of using seed extracts is that they increase the chemical oxygen demand (COD) in water [10,20,21]. However, the coagulant protein of *M. oleifera* seed a cationic protein of 6.5 kDa having 60 amino acids, does not increase the COD of treated water [20]. Later, many proteins of various molecular weights having coagulant activity have been isolated and purified from *M. oleifera*. Agrawal et al. [22] isolated and purified a 66 kDa protein from *M. oleifera* seed using ion-exchange chromatography, showed a comparable coagulation activity with other small molecular weight (6–16 kDa) cationic coagulant peptides isolated from *M. oleifera*. Globulin fractionation of *M. oleifera* seed powder containing several cationic coagulant proteins with a molecular weight ranging from 0.9 to 12.4 kDa showed good coagulation properties in purifying low turbidity water without increasing the organic matter at a concentration of 13 mg L<sup>-1</sup> [23]. A mixture of active coagulant proteins isolated and purified using a cross-flow filtration method from *M. oleifera* with a molecular weight ranging from 1 to 6.5 kDa showed good coagulation property and removed 96% of the turbidity from low turbidity river water at a very low concentration [24]. Ghebremichael et al. [25] isolated and purified a thermoresistant, a cationic protein having a molecular weight less than 6.5 kDa using ion-exchange chromatography, which showed both coagulation and antimicrobial activity. Although purified single protein preparations are effective in removing turbidity from wastewater without increasing the organic load, they are economically non-viable, and therefore cannot be scaled up for large scale water purification.

As there are many coagulant proteins in *M. oleifera* seeds [22,23], the preparation of whole seed protein isolate would result in consortia of coagulant proteins whose activity may be better than single pure protein. Our previous study has shown that the de-oiled *M. oleifera* seed meal (DMSM) is a rich source of proteins that account for 62.76% w/v. Therefore, the DMSM may be an economical source for the preparation of *M. oleifera* seed protein isolate (MPI) [26]. In the previous study, we have shown the preparation of MPI from DMSM [27]. In the current study, we have demonstrated the coagulation efficiency of the MPI in synthetic

turbid water (STW) and real mud water (RMW). Further, we have also characterized the MPI in terms of its protein content and studied the kinetics of coagulation. We have also characterized the coagulated sludge of STW and evaluated the effects of MPI on water qualities like COD and microbial load. The preparation of MPI is a cost-effective process, and this natural and safe product is on par with alum in purifying water.

## 2. Materials and methods

### 2.1. Raw materials and chemicals

DMSM was procured from a local market in Mysuru, India. Hexane was purchased from Qualigens Fine Chemicals, Mumbai, India. Kaolin was purchased from Sigma-Aldrich, St. Louis, MO, USA. Media for microbiology work was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All other chemicals were obtained from Sisco Research Laboratories (SRL), Mumbai, India.

### 2.2. Instrumentation

Turbidity was determined using a Turbidimeter (Lovibond® TB10IR TurbiCheck meter). Jar tests were conducted in a flocculator or multiple spindle stirrer supplied by Quesst International, Bangalore, India. Particle size was determined using MicroTrac S3500 Bluewave particle size and shape analyzer (MicroTrac, USA, equipped with Flex software for data capture and analysis). Scanning electron microscopy (SEM) was carried out on LEO 435VP (LEO Electron Microscopy Ltd., Cambridge, UK) equipped with an SEM coating system – Poleron E5100.

### 2.3. Preparation of *M. oleifera* seed protein isolate

DMSM was obtained as dry cakes. They were cleaned and broken into small chunks manually. The seed meal was processed according to the method described by Govardhan et al. [28], to get *M. oleifera* seed flour (moisture content ≤2%), and it was stored in an airtight container at 4°C. MPI was prepared, according to the method described by Jain et al. [27]. Briefly, the DMSM was treated with hexane until the oil content was reduced below 0.5%. The DMSM was air-dried at room temperature (25°C–28°C) for about 24 h and ground to a fine flour in a precision laboratory mill (Quadrumat – Brabender, Buisburg, Germany) fitted with a stainless-steel control sieve (100 µm mesh). De-oiled *M. oleifera* seed powder thus obtained after milling, was stored at room temperature in an airtight container. DMSM was then extracted in 0.4 M sodium chloride solution with flour to solvent ratio of 1:20 (m:v) for 10 min at 55°C. The resulting solution was centrifuged at 6,000 rpm for 30 min. The supernatant containing most of the protein from *M. oleifera* seed meal (MSM) was diluted 6-fold with reverse osmosis purified water. The resulting solution was again centrifuged at 6,000 rpm for 1 h. MPI formed the pellet, which was dried, powdered and stored in an airtight container at room temperature. The protein content of MPI was quantified through the determination of total nitrogen by the Kjeldahl method, as described in AOAC [29].

#### 2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The molecular mass determination under reducing conditions was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a vertical system [30]. A 12% polyacrylamide gel prepared in 1.5 M Tris-HCl buffer, pH 8.8, containing 1% SDS was used. Protein samples of MSM, NE of *M. oleifera* seed powder, MPI were dissolved in 0.5 M Tris-HCl buffer, pH 6.8 containing 2% SDS, 1% dithiothreitol and boiled at 95°C for 5 min before electrophoresis. After the gel run, protein bands were stained with Coomassie Blue R-250.

#### 2.5. Preparation of STW

STW was prepared, according to Katayon et al. [31] with slight modification. Kaolin (5 g) was suspended in 1 L of distilled water. The solution was stirred for 1 h on a magnetic stirrer for uniform dispersion and incubated for 24 h at room temperature for complete hydration of particles. The supernatant was carefully separated from the settled kaolin particles.

#### 2.6. Coagulation/flocculation test (jar test)

Jar tests were conducted on a 6-paddle flocculator supplied by Quesst International, Bangalore, India. Jar test conditions were working volume: 400 mL; stage 1:150 rpm for 3 min, and stage 2:30 rpm for 15 min. MPI was used at concentrations ranging from 0–25 mg L<sup>-1</sup>. Alum at a concentration of 15 mg L<sup>-1</sup> was taken as a positive control for coagulation/flocculation. Water samples were allowed to settle for 30 and 60 min. These conditions were maintained throughout the work.

#### 2.7. Kinetics of turbidity reduction

STW was prepared, and the jar test was carried out, as explained previously. Water was treated with 15 mg L<sup>-1</sup> of MPI. The rate at which the residual turbidity reduced after MPI treatment during sedimentation was studied by plotting residual turbidity vs. time in two systems, namely bulk (400 mL) and vial (15 mL). In a bulk system (400 mL), the sample (15 mL) was drawn from the top of the solution at regular intervals of 10 min using a pipette. During each draw, the movement of the pipette into and out of the system disturbed the sedimentation rate. To overcome this problem, we used the sample vial (15 mL), which went into the turbidimeter and sedimentation took place inside the turbidimeter. The residual turbidity was then measured *in-situ* at regular intervals without disturbing the sedimentation.

#### 2.8. Particle size analysis

STW was prepared, and the jar test was carried out, as explained above. STW was treated with 15 mg L<sup>-1</sup> of MPI. The particle size of the sludge obtained after treatment was analyzed on particle size analyzer. The wet samples were inserted as a suspension into the flow sink of particle size analyzer and the suspension was dispersed throughout the flow system. The measurement was initiated using the

software. The data were compared with the data obtained from the sludge of untreated water.

#### 2.9. Scanning electron microscopy

After the MPI treatment of STW, the sludge was examined by a SEM after drying into powder and gold sputtering. The SEM image of treated and untreated STW was analyzed.

#### 2.10. Preparation of RMW

The soil was collected from the CSIR-Central Food Technological Research Institute garden, Mysore, India. The collected soil was sieved (mesh No. 35) using a sieve to obtain fine soil particles. RMW was prepared by mixing 50 g of fine soil with 1 L of tap water and allowing the larger particles to settle down for 3–4 h and then decanting. The turbidity of prepared RMW was maintained between 100–150 NTU.

#### 2.11. Measurement of COD of water

The COD of MPI treated and untreated RMW samples were determined by closed reflux colorimetric method as described in APHA [32].

#### 2.12. Antibacterial properties of MPI

Antibacterial activity of the MPI was studied in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, according to Delelegn et al. [33], against a pure culture of *Escherichia coli* ATCC 25922 (American Type Culture Collection, Virginia, USA). MPI was dissolved in 0.4 M NaCl solution. Tubes with a 0.4 M NaCl solution devoid of extract were used as the control, and tubes with an equal quantity of sterile water served as a negative control to check the effect of the solvent on bacterial growth. All the tubes were inoculated with 10<sup>5</sup> cells of bacterial culture and incubated at 37°C for 24 h. The inhibition of growth was confirmed in triplicates. MIC was defined as the concentration at or above which no growth of the organism was observed. For MBC, the cultures from the tubes showing no growth were spot inoculated on nutrient agar, and it was incubated at 37°C for 24 h. The lowest concentration showing no growth on solid medium after incubation was referred to as MBC.

#### 2.13. Effect of MPI treatment on the microbiological quality of water

Microbiological quality of RMW treated with MPI was evaluated using the most probable number (MPN) method according to Delelegn et al. [33]. The typical *E. coli* colonies were counted after plating 1 ml sample on to HiChrome *E. coli* agar plates and incubating them at 37°C for 48 h.

#### 2.14. Statistical analysis

Data were analyzed for statistical significance using GraphPad Prism 5. A significance level of a *p*-value of less than 0.05 was used.

### 3. Results and discussion

#### 3.1. Preparation of MPI and characterization

MPI was prepared and the total yield was calculated. The total protein concentration of MPI was determined using the Kjeldahl method. The yield of MPI from DMSM was 55% and the protein concentration in MPI was >90%. Later, coagulant proteins were compared among MSM, NE of *M. oleifera* seed powder and MPI using SDS-PAGE. The MPI included most coagulant proteins from MSM and NE (Fig. 1). SDS-PAGE showed three major proteins in MPI with molecular weights approximately equal to 6.5, 14 and 29 kDa, as reported by Jain et al. [27]. Ndabigengesere et al. [20] reported an active coagulant which had a molecular weight of 13 kDa and was a dimer of 6.5 kDa.

#### 3.2. Coagulation/flocculation test (jar test)

STW was prepared in the range of 350–400 NTU. The optimum concentration of MPI and sedimentation time for turbidity removal was found to be 15 mg L<sup>-1</sup> and 30 min, respectively (Fig. 2). Though other concentrations of MPI, such as 10, 20, and 25 mg L<sup>-1</sup> at a sedimentation time of 30 and 60 min, were also effective in removing the turbidity, 15 mg L<sup>-1</sup> was effective and quicker than others. At 15 mg L<sup>-1</sup> concentration and 30 min of sedimentation time both alum and MPI reduced the turbidity of STW to significant levels (>97%).

#### 3.3. Kinetics of turbidity reduction

STW had an initial turbidity of 180 NTU. The no-treatment control showed a reduction of turbidity to only 175 NTU after 120 min (Fig. 3a). In the vial system, the residual turbidity reduced to 20 NTU in half an hour and to 7 NTU in 120 min (Fig. 3b). The reduction in turbidity followed the first-order decay with a rate (decay) constant of 0.09 min<sup>-1</sup> and started to plateau at 9.2 NTU. The half-life of the decay was 7.4 min as the initial turbidity was reduced to half in 7.4 min. In the bulk system, the residual turbidity

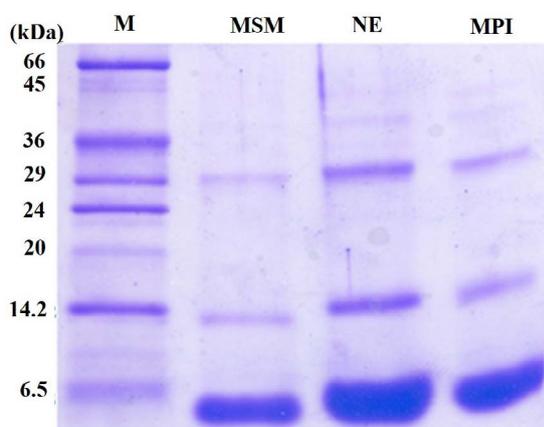


Fig. 1. SDS-PAGE profile of protein marker (M); *M. oleifera* seed meal (MSM), NaCl extract of *M. oleifera* seed meal (NE) and *M. Oleifera* seed protein isolate (MPI).

reduced to only 90 NTU in half an hour and reached approximately 12.5 NTU in 120 mins (Fig. 3c). An initial delay in the reduction of turbidity was observed in the bulk system which lasted for 12 min and then followed the first-order decay with a rate (decay) constant of 0.04 min<sup>-1</sup> and plateaued to 10.8 NTU. The half-life of the decay here was 17.21 min. The variation in results between the two systems may not be due to the volume, but maybe due to samples drawn from different depths and the disturbance in sedimentation in the bulk system. These results show that MPI can be used as an alternative for alum for a quick reduction in turbidity in water.

#### 3.4. Particle size analysis

Particle size analysis showed a single peak with a median particle size of 13.17  $\mu\text{m}$  diameter for sediments of untreated water, whereas, two peaks were found for sediments of treated water, one at 14.38  $\mu\text{m}$  (80%) and another at 221.2  $\mu\text{m}$  (20%) median particle size. Further, the number of particles at 13–14  $\mu\text{m}$  median size was reduced, and the reduced number reflected in the  $\sim 220 \mu\text{m}$  peaks, showing the coagulation of particles (Fig. 4). Although two peaks were obtained in particle size analysis, one may argue that the peak at 221.2  $\mu\text{m}$  may be of that of MPI. It should be noted that the concentration of MPI used is very low compared to the kaolin particles in synthetic turbid solutions. Hence MPI cannot contribute to 20% of the particles.

#### 3.5. Scanning electron microscopy

SEM was carried out to strengthen the coagulating activity of MPI. From SEM data, it is evident that the kaolin particles aggregate together in the presence of MPI. Figs. 5a and b show SEM images of sediment from untreated water containing kaolin particles (magnification of 10 and 2  $\mu\text{m}$  respectively). The particles are randomly distributed and are small. Figs. 5c and d show SEM images of sediment from MPI treated water (magnification of 10 and 2  $\mu\text{m}$  respectively). The particles have formed aggregates and are larger. Aggregates are formed due to coagulation between MPI and many small kaolin particles to give particles of larger size.

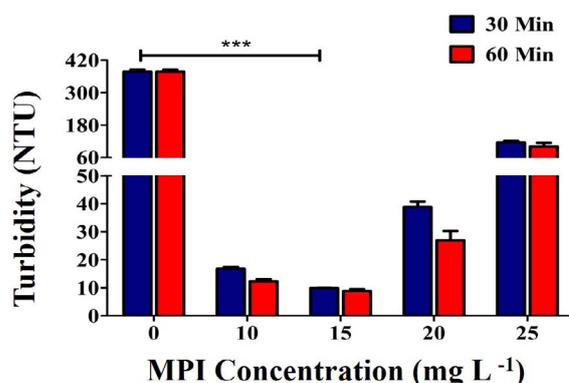


Fig. 2. Optimization of MPI concentration and sedimentation time for turbidity reduction in synthetic turbid water.

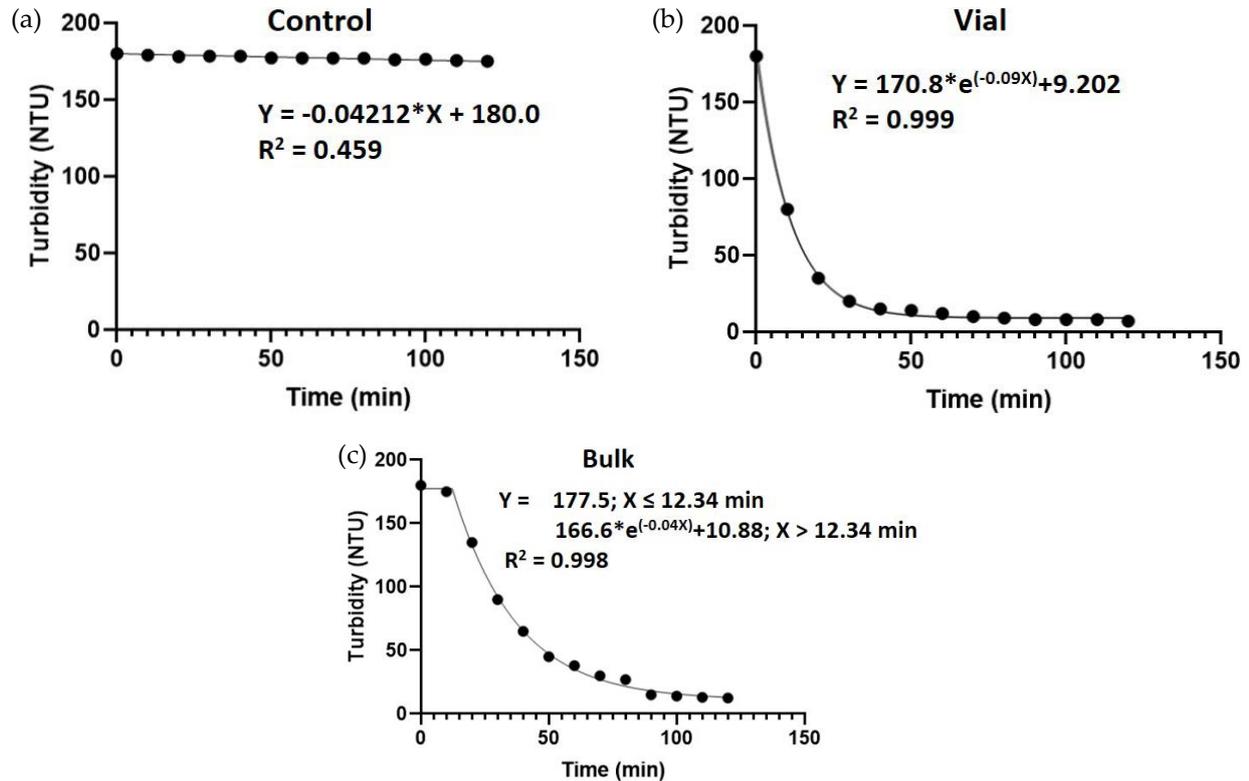


Fig. 3. Kinetics of residual turbidity reduction after MPI treatment in synthetic turbid water (a) control – synthetic turbid water with initial turbidity of 180 NTU, turbidity reduced only by 5 units after 120 min. (b) Synthetic turbid water in 15 mL vial with initial turbidity of 180 NTU treated with MPI, follows a first order decay in reduction of turbidity. (c) Synthetic turbid water in 400 mL bulk system with initial turbidity of 180 NTU treated with MPI, follows a first order decay in reduction of turbidity after a small initial delay.

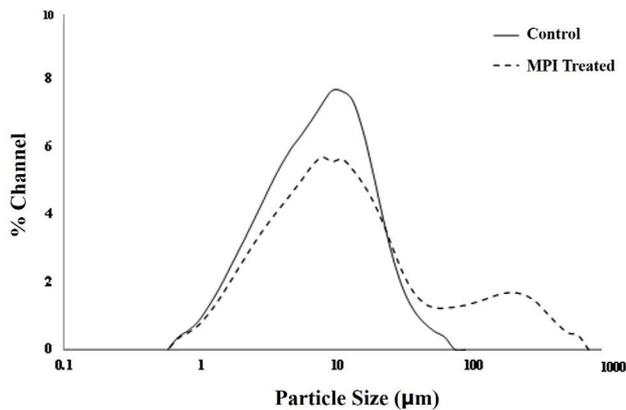


Fig. 4. Particle size analysis of sludge before and after MPI treatment in synthetic turbid water.

According to X-ray crystallographic studies of Tunega et al. [34], the structure of relaxed kaolinite contains surface hydroxyl groups. The presence of these hydroxyl groups makes kaolinite electronegative and absorbs cations. When alum is used for water treatment, the aluminum ions ( $\text{Al}^{3+}$ ) interact with the kaolin or clay particles present in water leading to coagulation, flocculation, and sedimentation. The flocculant proteins found in *M. oleifera* seeds are reported to be positively charged [16,31]. The amino acid

sequence of one of the flocculant proteins (6.5 kDa) shows eight amino acids with a positively charged side chain group and one amino acid with a negatively charged side chain group [35]. So, the net charge on the protein at the natural pH of water (6.5–8.5) would be positive. Thus, each protein molecule would interact with kaolin particles and aggregate the particles which flocculates and sediments.

### 3.6. Evaluation of efficacy of MPI in purifying RMW

RMW was prepared in the range of 100–150 NTU. MPI at a concentration of  $15 \text{ mg L}^{-1}$  showed a significant reduction in the turbidity of RMW (>97%), and the color of the RMW changed from brown to colorless. The efficiency of MPI in purifying the RMW was better than water extract and NE of *M. oleifera* seeds at an equivalent protein level of  $15 \text{ mg L}^{-1}$ . MPI showed a 97.25% reduction in residual turbidity while water and NE showed 95.28% and 96.45% reduction, respectively. Although the difference in coagulation activity between seed extracts and MPI is negligible, treatment with MPI is more desirable as the organic load on MPI treated water is lower than the seed extracts treated water. The mean COD of an RMW sample was  $1 \text{ mg L}^{-1}$ . On treatment with MPI, it reduced to  $0.5 \text{ mg L}^{-1}$ , whereas the COD increased to 132.5 and  $160 \text{ mg L}^{-1}$  on treatment with the water and salt extracts, respectively (Fig. 6). Since MPI reduces the COD, it is more suitable

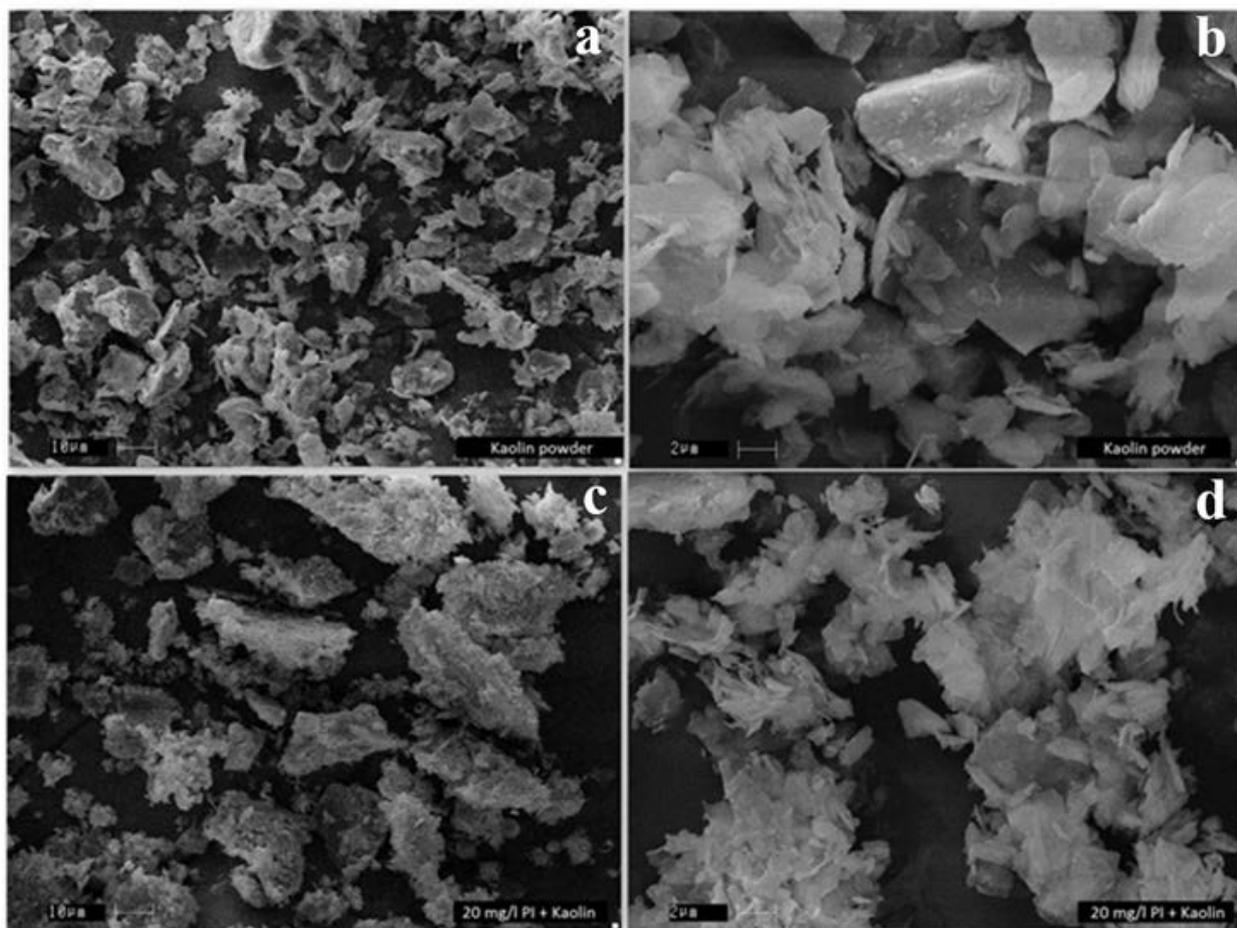


Fig. 5. SEM images of sludge from untreated water containing Kaolin particles resolution of (a) 10  $\mu\text{m}$  and (b) 2  $\mu\text{m}$ . SEM image of sludge from MPI treated water resolution of (c) 10  $\mu\text{m}$  and (d) 2  $\mu\text{m}$ .

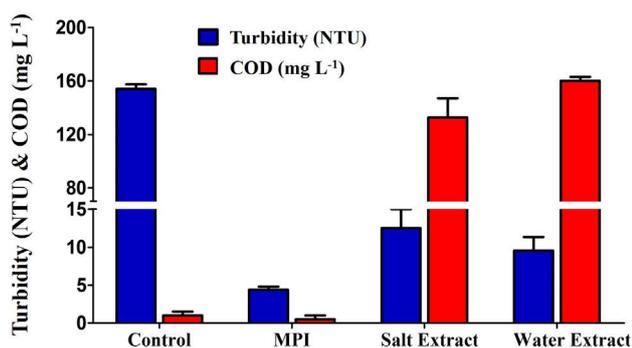


Fig. 6. Comparison of the coagulation efficiency of water extract and NaCl extract of *M. oleifera* seed with MPI in real mud water.

for treating RMW than water extract and salt extract of *M. oleifera* seed powder.

### 3.7. Comparison of MPI's coagulation activity with alum

The coagulation activity of MPI was compared with 15 mg L<sup>-1</sup> of alum in RMW as well as in STW and found that both had similar activity in reducing the turbidity. In the case of STW, both MPI and alum reduced the

turbidity by >97%. Similar results were observed in the case of RMW (Figs. 7a and b). Further, alum and MPI showed a small decrease in organic load in treated water samples. While MPI slightly increased the pH of the treated water from 6.9 to 7.1, alum significantly decreased the pH to 5.8 (Fig. 8). In water treatment plants, it is a routine practice to add lime or soda ash to increase the pH to acceptable levels (6.5–8.5) after treating with alum [28]. The use of MPI will avoid the addition of lime or soda ash, as MPI treatment alone results in water with acceptable pH. Hence, MPI could be a better substitute for alum in water treatment for removing the turbidity.

### 3.8. Antimicrobial activity of MPI

Antibacterial activity of MPI against the pure culture of *E. coli* was evaluated in terms of MIC and MBC values. MPI showed a MIC of 1 mg mL<sup>-1</sup> and MBC values of 2 mg mL<sup>-1</sup> against *E. coli*. At this high concentration, MPI increases the turbidity of the treated water. Hence, MPI cannot be used as antimicrobial in water treatment. However, the antimicrobial activity shown by crude *M. oleifera* seed extracts may be due to the presence of biomolecules like polyphenols in the seed [28,36].

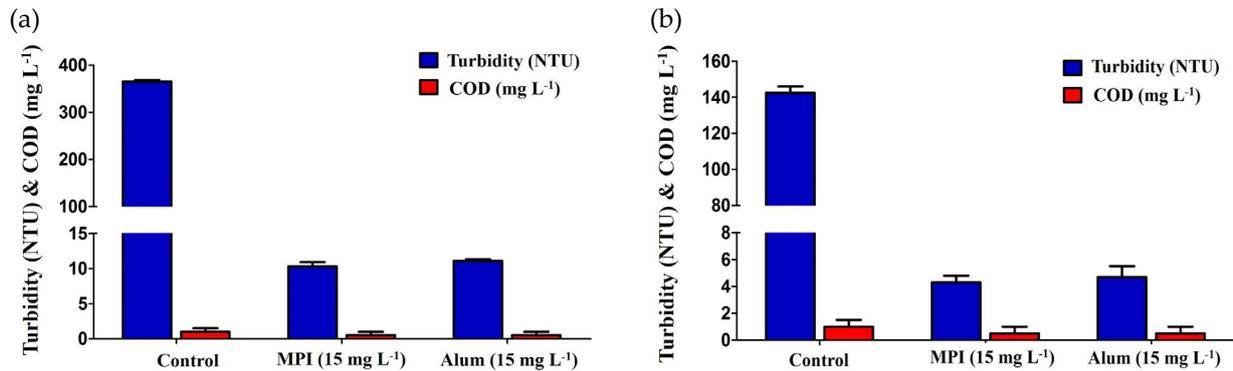


Fig. 7. Comparison of the coagulation efficiency of MPI with alum in (a) synthetic turbid water and (b) real mud water.

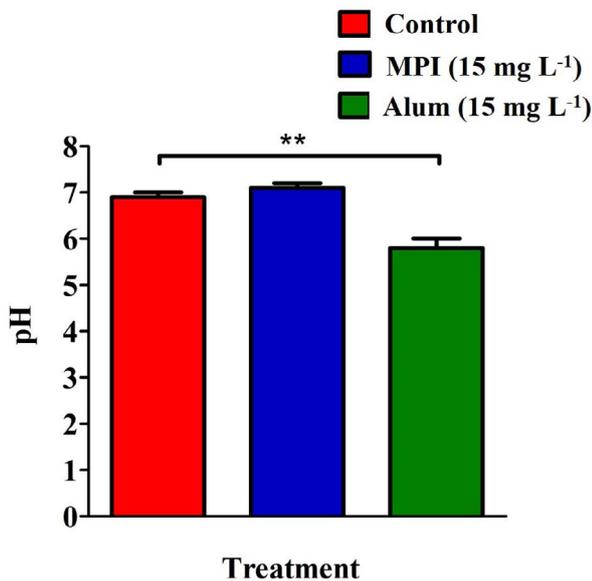


Fig. 8. Effect of MPI and alum on the pH of real mud water.

### 3.9. Effect of MPI treatment on the microbiological quality of water

Although MPI showed higher MIC and MBC values against the pure culture of *E. coli*, at a concentration of 1 mg mL<sup>-1</sup> MPI, the MPN and *E. coli* were significantly ( $p < 0.0001$ ) reduced. At 2 mg mL<sup>-1</sup> MPI, the MPN index was <2/100 mL and *E. coli* were absent in MPI treated RMW. Control samples showed high MPN index values and *E. coli* count (Fig. 9).

## 4. Conclusion

The DMSM produced as a by-product in *M. oleifera* oil industries can be utilized as raw material for producing MPI. The optimum concentration of MPI and the sedimentation time for turbid water treatment was found to be 15 mg L<sup>-1</sup> and 30 min, respectively, at which the turbidity reduced by >97% which is currently used alum. Being a natural coagulant, it did not increase the organic load in the treated water unlike crude extracts (salt and water extracts) of seed powder. The pH increasing activity of MPI could be

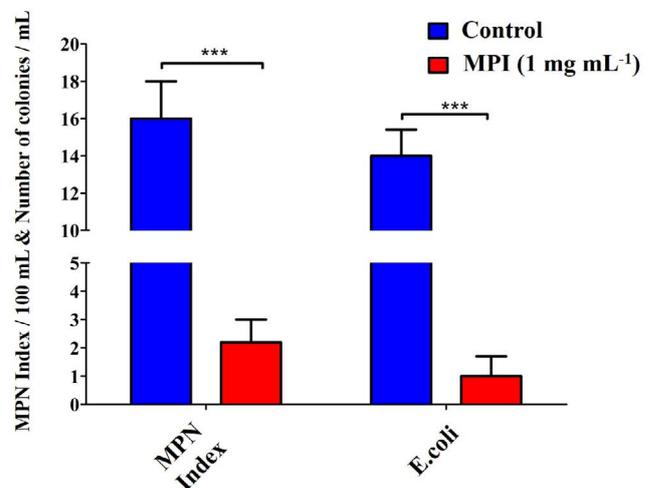


Fig. 9. Microbial quality of MPI treated water.

advantageous over alum to obtain the acceptable pH range of water without the need for the addition of lime or soda ash. Further, MPI was effective in reducing the MPN index of water. Therefore, the use of MPI is a cost-effective way of purifying the turbid water in the regions where people cannot afford clean water.

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