RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Multiple applications of recombinant *Moringa oleifera* coagulant protein for the large-scale water treatment applications

Asalapuram Ramachandran Pavankumar,[ab]* Johan Norén,[a] Lakhvinder Singh,[a] Naveenkumar Chandappa Gowda[b]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Provision of safe drinking water, devoid of aetiologies is an all-time challenge due to the usage of unsafe chemicals in most of the water treatment processes. In this regard, proteins of *Moringa oleifera* (MO) provide most promising solutions, as its crude seed extracts are being used as natural 'water clarifier' in drinking and wastewater treatments. Among MO seed proteins, a 6.5 kDa coagulant protein (MO coagulant protein; MOCP) has gained importance because of its antimicrobial and water clarification properties. Considering the biomedical and commercial values of MO plant, in this study, the cDNA coding for MOCP was cloned and over expressed in *E. coli* BL21 (DE3). After bench-scale experiments, for the first time, the recombinant protein production was scaled up to 7L bioreactors in Luria Broth supplemented with 1% glucose. About 42mg of *Moringa oleifera* coagulating recombinant protein (MOCRP; 6.5 kDa) was purified using superparamagnetic iron oxide nanoparticles (SPION) from 1 litre of the over expressed *E. coli* cell cultures. The purified MOCRP revealed an efficient coagulation activity (~66%) even with 0.1 mg/L; while very good antimicrobial response was recorded against a set of Gram-negative and Gram-positive microorganisms with as low as 10mg/L. Thus, we report the over expression and purification of this dual-function protein (MOCRP) from moringa for the first time, using bioreactors with an economical and user-friendly approach for large-scale applications.

Introduction

Infections most commonly spread through consumption of unsafe or unhygienic drinking water or food. Globally such infections cause more than 1.8 million of mortality every year and among which, 88% of the cases are attributable to consumption of unhygienic water.[1] Biohazardous and non-degradable genotoxic compounds of chemical/mineral waste released by the pharmaceuticals, leather and textile industries, etc. contribute to high degree of waterborne infections initiating adverse ecological effects to human and animals.[2] Such physiological adverse health effects leads, not only impending to risks like reproductive impairment, cancers, neurological disorders, but also helps in spreading the multi drug resistant bacteria in drinking[3,4] and surface waters.[5] Though it becomes the priority to provide 'safe drinking water', the technology exists today and is much debated due to the usage of chemicals like aluminum sulfate [alum; $Al_2(SO_4)_3$], chlorine, potassium permanganate (KMnO4), ferric sulphate [$Fe_2(SO_4)_3$], polyethylene terephthalate (PET), etc. that indirectly contributes to several serious health problems on prolonged usage. [6-9]

Using natural or biocompatible materials to adopt indigenous solutions can potentially reduce such complications. In this scenario, the seed extracts/powders of a tropical multipurpose tree, *Moringa oleifera* (MO) is being used in several countries either to clean drinking water[10-14] or to treat wastewater.[15-17] The proteins or chemical components present in MO crude seed extracts (MOCE) possessing coagulation (flocculation or water clarification), antimicrobial and pollutant removal activities[12,14,15,18,19] makes it as a convenient natural agent for water treatment.[20-22] Since crude seed extract is reported to increase the organic content that changes water quality (including odour, colour and taste), it is recommended that the treated water be consumed earlier.[15] Among the components of MOCE, a 6.5 kDa size *Moringa oleifera* coagulant protein (MOCP) possessing antimicrobial and coagulation activities is widely appreciated in water treatment protocols.[20,22-24] Though a couple of attempts have been made to over express and purify MOCP, they are expensive and tedious for large-scale applications.[25,26] Hence, the focus of this study was to over express MOCP in *E. coli* (BL21DE3) and purify its recombinant form (*Moringa oleifera* coagulant recombinant protein; MOCRP) using inexpensive magnetic nanoparticles[23-25] to address large-scale water treatment approaches. In this study, we scaled-up the over expression of MOCRP using bioreactors and show its proficient coagulation and antimicrobial activities and propose its applicability for large-scale water treatment proposals.

Materials and Methods

Construction and expression of MOCRP

The cDNA coding for $MO_{2.1}$ (Swissprot, P24303) was prepared according to the requirements of QIAprep® kit and the ChampionTM pET Directional TOPO® expression system. The regenerative primers (5′- CACCCGGATCCATGCARGG NCCNGGNCGNCARCCNGAYTTYCA-3′ and 5′-GTGG CCCAAGCTTTTANGTRCTNGGDATRTTNGANGCNACNC GRTACAT-3′) were used to get an amplicon of 200 bp, through touchdown PCR conditions of denaturation-2 min, 32 times of 98 °C (10 sec), 65 to 55 °C (30 sec), 72°C (30 sec), final extension - 72 °C (10 min). The recombinant protein was cloned in kanamycin resistant pET200/D-TOPO cloning system and expressed in 'BL 21 star (DE3) one shot cells'. The positive recombinant constructs were cultured in LB medium containing 1% glucose at 37 °C (180 rpm) for overnight. The 6.5kDa protein was over expressed by inducing the cell cultures with 1mM IPTG at 0.8 O.D. (optical density) measured at 600nm.

Scaling up of MOCRP production using bioreactors

All growth conditions of shake-flask (e.g. pH, temperature, culture media, growth supplements, etc.) were extended to bioreactor studies and the cell growth, including protein production were optimized accordingly. Since this could be the first attempt to scale up the process, a simple batch process was selected instead of a fed-batch process. The protein production was scaled-up in 7L bioreactor vessel (Belach Bioteknik) from 500 mL shake flask experiments according to the protocol followed by Gustavsson et al.[28-29] with little modifications. Five liters of LB media was prepared in 7L reactor vessel and sterilized in situ after adjusting the pH to 7.2, while the filter sterilized glucose solution was supplemented to make up a final concentration of 1%. About 5% of *E. coli* BL21 transformants obtained from the shake-flask were seeded to get an initial O.D. of 0.1 in the bioreactor. The cells were induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at O.D. 3.0 and harvested after 6h of reactor time. The pH was kept constant at 7.0 during the cultivation through automatic titration with 25% NH_4OH (v/v) and 5M HCl. The dissolved oxygen tension (DOT) was maintained above 30% throughout the experiment by manually increasing the stirrer speed and the airflow. Sterile antifoam was manually added when necessary to avoid excess foaming.

Protein purification using magnetic nanoparticles and confirmation by Western blotting analysis

As the protein forms homodimers[37], 1mM of Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was added to cell-lysis buffer to obtain monomers of MOCRP. The cell pellets were suspended in lysis buffer and were disrupted using a French pressure. The MOCRP was purified by superparamagnetic ironoxide nanoparticles (SPION), according to the simple protocol suggested by Okoli et al.[23,24] Electrophoresis was performed[30] with 25 µg of proteins, transferred to PVDF membrane and confirmed the presence of 6.5 kDa MOCRP by Western blotting.[31,32]

Water clarification analysis

The coagulation activity was estimated from 10µg/mL of MOCRP, MOCE and MOCP in 1mL of 1% kaolin-water suspension.[20,37] Absorbance at 500 nm was recorded at every 30 min up to 90min and the percentage of activity was calculated by the following formula:

$$\% \text{ of activity} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Subsequently, coagulation efficiency of MOCP and MOCRP was estimated at different protein concentrations by varying between 0.01-10 µg/mL.

Evaluation of antimicrobial activity

The overnight cultures of *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Serratia mirabilis* and *Staphylococcus aureus* were diluted to 0.1 O.D. at A_{600nm} in 200 µL of 10 times diluted nutrient broth in a 96-well micro titre plate. About 10 and 50 mg/L of and MOCRP were added to the bacterial cultures; while the controls were grown without these proteins. Growth patterns were studied by measuring their absorbance at 600nm (Multiscan, Thermo Bioanalysis) at every 60 min time interval for 4 h by incubating the 96-well micro titre plates at 37°C (100 rpm). The growth curves were plotted against the concentration of each protein to study its antimicrobial activity.

Results

Purification of MOCRP using user-friendly magnetic nanoparticles

The PCR amplicons of ~200 bp coding to cDNA of $MO_{2.1}$ (gene for MOCP) was successfully cloned into TOPO vector and the plasmid was transformed into high expression vector, *E. coli* BL21(DE3) to produce MOCRP in the presence of kanamycin. A few randomly analyzed positive clones revealed over expression of MOCRP at 14 KDa (Fig. 1A), which was further reduced to monomer (6.5 kDa) using 1 mM TCEP (Fig. 1B). As can be seen in Fig. 1C, MOCRP was efficiently purified using SPIONs and the purified protein was confirmed by Western blotting analysis at 6.5 kDa (Fig. 1D).

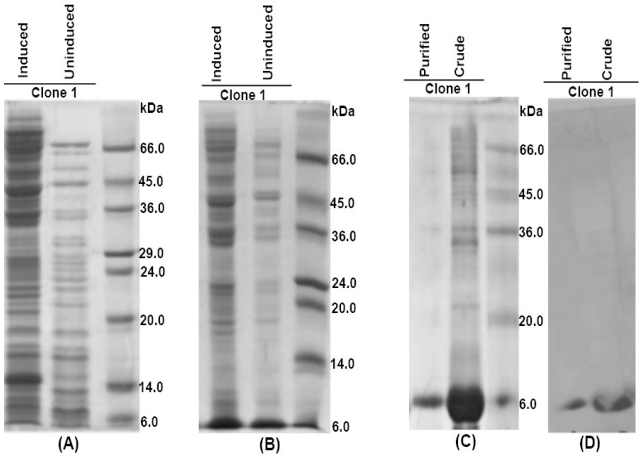

Figure 1. Production and purification of MOCRP
(A) Protein profiles of whole-cell lysates of induced and uninduced clone resolved on 10% SDS-PAGE revealing 14 kDa homodimer form of MOCRP. (B) Homodimers reduced to 6.5 kDa monomers by the addition of 1mM TCEP in lysis buffer. (C) The purified MOCRP at 6.5kDa, using magnetic nanoparticles. (D) Confirmation of MOCRP at 6.5kDa on PVDF membrane using anti-His antibody.

After preliminary small-scale experiments, the SPIONs were used to purify the protein at large scales appreciating its cost effectiveness.

Large-scale production of MOCRP using bioreactors

After the shake flask experiments, protocols were scaled-up by maintaining the oxygen supply, pH and temperature, which are often considered to be the most influential parameters in *E. coli* cultivations. About 11g/L of wet biomass was obtained by bioreactor studies, compared to ~1.5g/L from shake flask experiments. From this biomass ~42mg/L of MOCRP were purified compared to 0.6mg/L from shake flask cultivations. The amount of target protein (MOCRP) per wet cell weight is approximately 9 times higher in the bioreactor case indicating higher cell populations with significant amount of over expressed protein. As a consequence, the cost to produce MOCRP could be significantly reduced by using bioreactors instead of shake flasks.

Efficiency of MOCRP in water clarification

Preliminary experiments with 10µg/mL of MOCRP, MOCP and MOCE revealed a turbidity reduction of about 85%, 81% and 75%, respectively. This indicates the superiority of MOCRP over MOCP and MOCE, against 1% kaolin solution (Fig. 2).

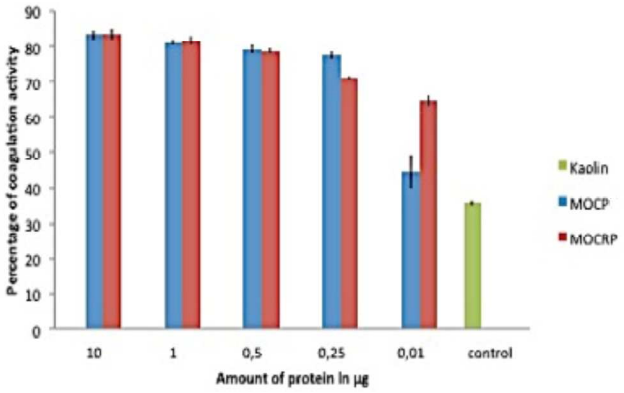

Figure 2. Percentage of turbidity reduction kinetics of MOCP, MOCRP and MOCE

Initially, the experiments were performed using 10µg/mL of MOCP, MOCRP and MOCE. The bar chart shows about 85% of turbidity reduction for MOCRP compared to that of MOCE and MOCP, ~75% and ~81% respectively. Different protein concentrations from 0.01µg/mL to 10µg/mL of MOCRP and MOCP were investigated against 1% kaolin solution to identify an optimal concentration for the coagulation activity. Even 0. 01 µg/mL of MOCRP revealed about 66% of activity, while the MOCP show only 39%, which was comparable to that of kaolin.

An experiment with varying concentrations from 0.01µg/mL of MOCRP revealed about 66% of turbidity reduction, while MOCP show only about 39% (Fig. 2). Based on the results, 0.01-0.25µg/mL of MOCRP shows very good coagulation activity and hence this could be the optimal concentration. However, either of the purified proteins did not show considerable increase in the activity when the amount of protein increased beyond 10 µg/mL, which could be an optimal concentration for the turbidity reduction experiments.

Efficient antimicrobial activity of MOCRP against enteric bacteria

Antimicrobial assay was performed with the different concentrations of MOCRP and MOCP against a set of 5 bacterial strains comprising Gram-positive and Gram-negative bacteria (*Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Serratia mirabilis* and *Staphylococcus aureus*). Even 10µg/mL of MOCRP and MOCP disclosed a significant antimicrobial activity that can be comparable with the activity of 50µg/mL protein concentration. As can be seen in Fig. 3 (A-E), all the strains tend to show almost complete susceptibility of 3-4 h, which is considered to be critical for any bacterial growth. The bacterial strains under investigation tended to grow very slowly up to 30-40% after 9 h of incubation. As can be seen in the Fig. 3 B and E, in the case of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, there was no significant difference observed between 10 and 50µg/mL of either MOCRP or MOCP. However, for some reason, *Salmonella typhimurium* show better inhibition against 10µg/mL of protein rather 50µg/mL (Fig. 3C). In the other hand, *Bacillus Subtilis* (Fig. 3A) and *Serratia mirabilis* (Fig. 3D) revealed a dose dependent susceptibility against the antimicrobial proteins.

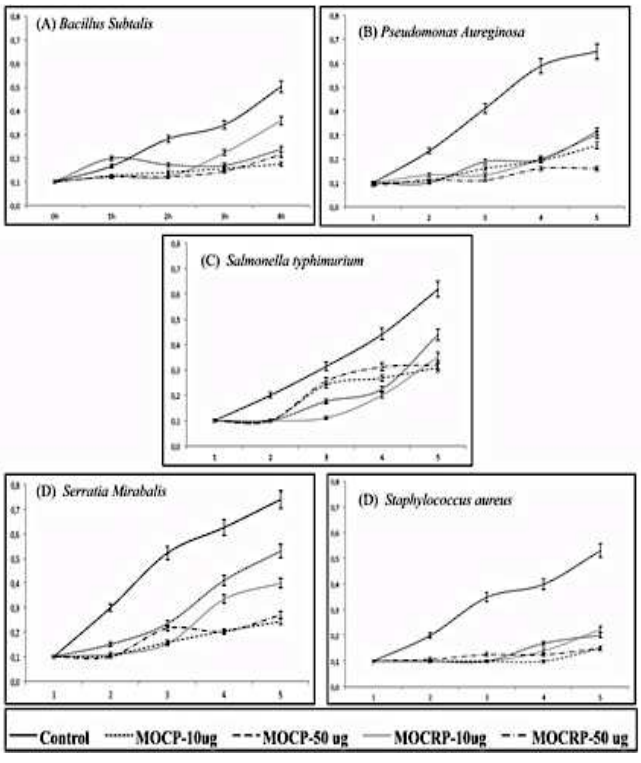

Figure 3. Antimicrobial activity of MOCRP and MOCP against five species of bacteria
Growth profiles of the five different species of enteric pathogens cultured

in NB for 4h of incubation at 37 ºC showed potent susceptibility against MOCRP and MOCP. The graph is plotted against time (X-axis) and the optical density (O.D.) of the bacterial cultures (Y-axis). Black lines indicates the growth profile of control strains (without antimicrobial protein); Red dotted and continuous lines refers to 10µg and 50 µg/mL of MOCRP; while Green dotted and continuous lines indicates 10µg and 50 µg/mL of MOCP, respectively. In all the cases 50µg/mL of MOCRP and MOCP efficiently control the bacterial growth less than 0.3 O.D., while their controls grew up 0.6-0.8 O.D. However, even 10µg/mL of the protein expressed either equivalent or comparable antimicrobial activity with that of 50µg/mL, except *Serratia*.

Discussion

Flocculation and antimicrobial activities of *Moringa oleifera* coagulant protein (MOCP) are well investigated among other useful properties of the protein. In order to explore such advantages including industrial production of the protein for large-scale water treatment through an eco friendly solution, instead of using harmful chemicals in water treatment.

Affordable large-scale production of MOCP

This study has shown the potential applications of MOCP (and its recombinant form, MOCRP) like antimicrobial and coagulation properties to propose it as an appealing tool for ecofriendly water treatment approach. The pET200/D-TOPO vector allowed expression of recombinant protein with an N-terminal tag containing Xpress™ epitope with a His-tag enabled the removal of tag after protein purification. However, this system requires a specific PCR primer consisting of a stop codon to terminate the N-terminal peptide and second ribosome binding site (AGGAGG) 9-10 base pairs 5' of the initiation ATG codon of the protein of interest. Though MOCRP was anticipated at 6.5 KDa, the protein was found at 14 KDa, which was further reduced it to its monomeric form. Though several studies reported MOCP at 14kDa as a homodimer, a couple of reports are important pertaining to the expression of recombinant protein expression studies (25,26, 37). In 2003, Brion et al.[25] cloned the cDNA coding region of this *Moringa oleifera* flocculent protein, in *E. coli* to study its aggregation activity with montmorrillonite particles by purifying the protein with nickel affinity chromatography. In another study, Suarez et al.[26], produced recombinants using synthesized double-stranded oligonucleotides that expressed the protein fused with intein and then purified $MO_{2.1}$ with a chitin-linked (closed ellipse) bead column., Implementation of laborious and tedious protocols in the both cases complicate their adoptability for large-scale applications. Therefore, we undertook the challenge to develop a simple protocol to over express MOCP and to check its suitability for large-scale applications. The expressed protein was purified using SPION considering its advantages of separating with an external magnet, less time consuming, cost effectiveness and can be operated by semiskilled personnel. Based on our experiments, we consider, if the yield per wet cell weight can be maintained even in a fed-batch process, as compared to the current batch process, the production cost can be lowered even further. In addition it might be possible to improve the yield through process optimization since this was the first attempt to produce MOCRP using a bioreactor.

Efficiency of MOCRP in terms of coagulation and antimicrobial activities

Even 0.01µg/mL of MOCRP showed turbidity reduction of ~66% indicated its competence over the similar approaches,[26,27] where they have used 10µg/mL (0.01mg/mL) of the purified protein. According to this study, we propose to clean about 4200L of turbid water from 42mg of MOCRP (at the concentration of 0.01µg/L), which could possibly be obtained from 1L of the culture. It is noteworthy that Saurez et al. [26] and Brion et al.[27], used about 5mg/mL and 0.1mg/mL of purified FLO ($MO_{2.1}$), respectively, to show antimicrobial activity. This is about 10 times higher than the MOCRP, produced in this study. Though the other three strains (*Bacillus* spp., *Pseudomonas* spp. and *Staphylococcus* spp.) were found to be susceptible for MOCRP at least for 4 h, *Salmonella* (Fig. 3C) and *Serratia* (Fig. 3D) show a very little growth. Since these bacterial species shares homologies with each other, they might be expected to behave similar.[36] However mere differences between growth profiles of the bacteria could be due to the heterogeneity between the populations of MO seeds[20] compared to that of MOCRP. Nevertheless, the time of 3-4 h is crucial not only for water treatment protocols but also for the investigated antimicrobial assays. Bacterial growth restriction at the early stages could efficiently control the growth of bacteria and this time also sufficient to remove microbial contaminants, and other aetiologies in any process development.

Conclusions

We have explored an informative and constructive solution for over expression of MOCRP and its purification using using user-friendly magnetic nanoparticles. Development of cost effective and simple protein purification protocol with very limited steps makes this technique possible to be operated by even semi-skilled personnel. Subsequently, 5L bioreactor cultivations, efficiency of MOCRP compared to that of MOCE and MOCP shows the advantages the MOCRP. The MOCRP produced in this study is advantageous in terms of flocculation and antimicrobial activities compared to that of MOCP. Hence, we suggest that MOCRP can be used for the large-scale water treatments, possibly as a natural solution.

Acknowledgements

ARP and LS acknowledge European Commission's Erasmus Mundus External Cooperation Window (EURINDIA) for the financial support. The authors also thank Prof. Gen Larsson, Dr. GK Rajarao and Dr. Andres Veide for permission to use the laboratory facilities and technical help throughout the study.

Notes and references

[a] *Department of Industrial Biotechnology, School of Biotechnology, Royal Institute of Technology (KTH), 10691 Stockholm, Sweden.* [b] *Lab4Life Bio-Research Private Limited, Bangalore, India. E-mail: pavasa@kth.se*

Present address: *ARP: Science for Life Laboratory, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden. LS: JCDM College of Engineering, JCD Vidyapeeth, Sirsa-125055, India. NKCG: Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm.*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

References

1. WHO, 2012. http://www.who.int/water_sanitation_health/diseases/burden/en/ index. html#. Recently accessed on 26th February 2012.
2. U.S. Geological Survey, 2010. http://minerals.usgs.gov/minerals/pubs/. Recently accessed on 26th February 2012.
3. V. Figueira, I. Vaz-Moreira, M. Silva, C. M. Manaia. Diversity and antibiotic resistance of *Aeromonas sp.* in drinking and waste water treatment plants. *Water Res.* 2011, 45, 5599-5611.
4. N. G. H. Taylor, D. W. Verner-Jeffreys, C. Baker-Austin. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance. *Trend. in Ecol. Evol.* 2011, 26, 278-284.
5. N. Masters, A. Wiegand, W. Ahmed, M. Katouli. *Escherichia coli* virulence genes profile of surface waters as an indicator of water quality. *Water res.* 2011, 45, 6321-6333.
6. A. Gadgil. Drinking Water in Developing Countries. *Ann. Rev. Energy Environ.* 1998, 23, 253-286.
7. E. Kim, J. Little, A.N. Chiu. Estimating Exposure to Chemical Contaminants in Drinking Water. *Environ. Sci. Technol.* 2004, 38, 1799-1806.
8. P. Westerhoff, Y. Yoon, S. Snyder, A. Wert. Fate of Endocrine-Disruptor, Pharmaceutical, and Personal Care Product Chemicals during Simulated Drinking Water Treatment Processes. *Environ. Sci. Technol.* 2005*,* 39, 6649-6663.
9. C. Bach, X. Dauchy, M. C. Chagnon, S. Etienne. Chemical compounds and toxicological assessments of drinking water stored in polyethylene terephthalate (PET) bottles: A source of controversy reviewed. *Water Res.* 2011, 46, 571-583.
10. N. Dabigengesere, K. S. Narasiah, B. G. Talbot. Active agents and mechanism of coagulation of turbid water using *Moringa oleifera*. *Water Res.* 1995, 29, 703-710.
11. L. J. Fuglier, The Miracle Tree: *Moringa oleifera*, Natural Nutrition for the Tropics. Church World Service, Dakkar, Senegal. 1999, 68.
12. F. Anwar, S. Latif, M. Ashraf, A.H. Gilani. *Moringa oleifera*: a food plant with multiple medicinal uses. *Phytother Res.* 2007, 21, 17-25.
13. H. Bhuptawat, G. K. Folkard, S. Chaudhari. Innovative physico-chemical treatment of wastewater incorporating *Moringa oleifera* seed coagulant. *J. of Hazard. Mater.* 2007, 142, 477–482
14. R. S. Ferreira, T. H. Napoleao, A.F.S. Santos, R. A. Sa, M.G. Carneiro-da-Cunha, M. M. C. Morais, R.A. Silva-Lucca, M. L. V. Oliva, L. C. B. B. Coelho, P. M. G. Paiva. Coagulant and antibacterial activities of the water-soluble seed lectin from *Moringa oleifera*. *Lett. in Appl. Microbiol.* 2011, 53, 186–192.
15. N. Dabigengesere, K. S. Narasiah. Quality of water treated by coagulation using *Moringa oleifera* seeds. *Water Res.* 32 (1998) 781-791.
16. Y. Chun-Yang, Emerging usage of Plant-Based Coagulants for Water and Wastewater Treatment. *Process Biochem.* 2010, 45, 1437-1444.
17. R. F. Olanrewaju, S.A. Muyibi , T.O. Salawudeen, A. M. Aibinu. An Intelligent Modeling of Coagulant Dosing System for Water Treatment Plants based on Artificial Neural Network. *Aust. Basic App. Sci.* 2012, 6, 93-99.
18. T. Okuda, A. U. Baes, W. Nishijimam, M. Okadam, Coagulation Mechanism Of Salt Solution-Extracted Active Component In *Moringa Oleifera* Seeds. *Water Res.* 2001, 35, 830-834.
19. J. W. Fahey. *Moringa oleifera*: A Review of the Medical Evidence for Its Nutritional, Therapeutic, and Prophylactic Properties. *Trees for Life*, 2005, 1, 1-5.
20. K .A. Ghebremicahel, K. R. Gunaratna, H. Henriksson, H. Brumer, G. Dalhammar. A simple purification and activity assay for coagulant protein from *Moringa oleifera* seed. *Water Res.* 2005, 39, 2338–2344.
21. A.Y Arku, S.M. Musa, A.L.E. Mofoke, Characterization and Standardization of Crude Moringa Oleifera Seeds for Wastewater Treatment. *J. of Appl. Phytotech. Environ. San*. 2012, 1, 67-74.
22. K. R. Gunaratna, C. Andersson, K. A. Ghebremichael, G. Dalhammar. Purification and immobilization of natural coagulant for water treatment. 2006, 2nd IGC conference proceedings.
23. C. Okoli, A. Fornara, J. Qin, M. S. Toprak, G. Dalhammar, M. Muhammed, K. R. Gunaratna, Characterization of Supermagnetic Iron Oxide Nanoparticles and Its Application in Protein Purification. *Nanosci. Nanotech.* 2011, 1, 1–6.
24. C. Okoli, M. Boutonnet, L. Mariey, S. Järås, K. R. Gunaratna. Application of magnetic iron oxide nanoparticles prepared from microemulsions for protein purification. *J. Chem. Technol. Biotechnol*. 2011, 86, 1386–1393.
25. M. Broin, C. Santaella, S. Cuine, K. Kokou, G. Peltier, T. Joët. Flocculent activity of a recombinant protein from *Moringa oleifera* Lam. Seeds. *Appl. Microbiol. Biotechnol.* 2002, 60, 114–119.
26. M. Suarez, J. M. Entenza, C. Doerries, E. Meyer, L. Bourquin, J. Sutherland, I. Marison, P. Moreillon, N. Mermod. Expression of a Plant-Derived Peptide Harboring Water-Cleaning and Antimicrobial Activities. *Biotechnol. Bioeng.* 2003, 81, 13-20.
27. U. Gassenschmidt, K. D. Jany, B. Tauscher, H. Niebergall. Isolation and characterisation of a flocculating protein from *Moringa oleifera* Lam. *Biochim. Biophys. Acta*. 1995, 124, 477–481.
28. A. P. J. Sweere, K. C. A. M. Luyben, N. W. F. Kossen, Regime analysis and scale-down: Tools to investigate the performance of bioreactors. *Enzyme Microbiol. Technol.* 1987, 6, 386-398.
29. M. Gustavsson, G. Bäcklund, G. Larsson. Optimisation of surface expression using the AIDA autotransporter. *Microbiol. Cell. Factories.* 2011, 10, 72.
30. U. K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, 15, 680-685.
31. J. Sambrook, E. F. Fritsch, T. Maniatis. Molecular cloning: A laboratory manual. 1989, 18.47–18.57.
32. K. L. H. Ambroz. Improving Quantification Accuracy for Western Blots, Image Analysis. 2006, 33-35. www.licor.com./bio/QuantitativeWesterns/pdf/bt0609_LI-COR_final.pdf Recently accessed on 26th February 2012.
33. M. F. Sanner. Python: A Programming Language for Software Integration and Development. *J. Mol. Graphics Mod.* 1999, 17, 57-61.
34. R. K. Prasad. Color removal from distillery spent wash through coagulation using Moringa oleifera seeds: Use of optimum response surface methodology. *J. Hazard. Mater.* 2009, 165, 804–811.
35. M. R. Fahimi, N. W. A. Z. Najib, P. C. Ping, N. Hamidin. Mechanism of turbidity and hardness removal in hard water sources by *Moringa olieifera. Appl. Sci*. 2011, 11, 2947-2953.
36. S. Bascomb, S. P. Lapage, W. R. Willcox, M. A. Curtis. Numerical Classification of the Tribe Klebsielleae. *Gen. Microbiol.*1971, 66, 279-295.
37. A. R. Pavankumar, R. Kayathri, N. A. Murugan, Q. Zhang, V. Srivastava, C. Okoli, V. Bulone, G. K. Rajarao, H. Ågren. Dimerization of flocculent protein from *Moringa oleifera*: experimental evidence and in-silico interpretation. *Biomolcular Structure and Dynamics*. 2013, DOI: 10.1080/07391102.2013.770374.