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INVESTIGATING THE BACTERIAL INACTIVATION POTENTIAL OF PURIFIED OKRA *(Hibiscus esculentus)* SEED PROTEINS IN WATER PURIFICATION

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Abstract
The ability of purified okra protein (POP) as coagulant and as disinfectant material in comparison with aluminium sulphate (AS) in water treatment was assessed. A laboratory jar test experiments and Colilert-18/Quanti-Tray method of bacterial analysis were conducted using POP as coagulant in treating river water. The results show an excellent dual performance function of POP against the conventional coagulant, AS in drinking water treatment. It was observed that a marked inactivation of approximately 100% of faecal and E-coli count in raw water was achieved with POP and zero regrowth of bacteria after 72-hour post treatment. However, there was regrowth in total coliform count as a result of the presence of other microbes other than E-coli and faecal coliform in the system. In all cases AS showed a reduced performance against the two indicator organisms achieving only 93% with remarkable regrowth of E-coli and faecal coliform after prolonged storage time in the clarified water. Turbidity removal was also noted to be approximately similar, 92% across all coagulants tested. Therefore, the use of POP in water treatment could improve access to clean water in developing countries and could help in reducing the import of water treatment chemicals.

Keywords: Okra, water treatment, inactivation, coliform, protein

1. Introduction

In conventional water treatment processes, a disinfecting unit is used to eliminate pathogens using chlorine-based compounds (Farre *et al*., 2012). Disinfection process eliminates pathogens in water during treatment thereby improving the quality of water and offers the consumers with palatable and safe drinking water. However, this unit could be very expensive to maintain especially in rural areas where access to clean drinking water is a big challenge. Consequently, the absence of such a critical unit process has increased the risk of exposing people to various waterborne diseases. Recently, several naturally-occurring extracts have been studied for their antimicrobial properties (Shaheed *et al*., 2009; Dubreuil, 2013). Most notable among them is Moringa oleifera (MO) with very outstanding performance (Jahn *et al.*, 1986; Beltrán-Heredia *et al*., 2012; Kansal and Kumari, 2014). Similarly, there are many other naturally-occurring materials of animal origin such as chitosan and sodium alginate that have shown excellent results in water treatment (Kawamura, 1991; Saranya *et al.*, 2013). However, in many developing countries, natural extracts have successfully been used in treating ailments such as diarrheal disease (Dubreuil, 2013). Ingale and Gandhi (2016) revealed a successful use of MO aqueous extract in treating epilepsy and anxiety in patients. The presence of antimicrobial agents in natural plant extracts are well established and have been investigated against some isolated microbes (Ebrahimzadeh *et al.*, 2009; Gothandam, 2010; Bindhu and Umadevi, 2013), e.g. Cryptosporidium parvum oocysts as reported by Petersen et al. (2016). However, despite its widely acclaimed applications in folk medicine Matthews *et al*. (2009), there is a little information regarding the use of natural extracts as a disinfectant in water treatment.

Therefore, it is important to understand the potential of natural extracts in water treatment especially their antimicrobial properties. One important locally available material that is worthy of further investigation is Okra (OK) seed as presented here.

OK is a plant widely grown in Nigeria and many other tropical regions of the world for its nutrients and ease of cultivation. It can grow under different environmental conditions and reach maturity within three months of planting. OK seed is a major source of protein, vitamin, calcium and oil; it also cures ulcers and providing relief from haemorrhoids (Abidi *et al.*, 2014). The OK pod contains carbohydrate and mucilaginous substances that is capable of removing turbidity during the treatment of tannery and industrial wastewaters (Agarwal *et al.* 2003). In addition, Jones and Bridgeman (2016) showed that OK seed extracted with 1 M NaCl concentration can remove up to 98% turbidity in a polluted water. OK is one of the most consumed traditional vegetables, eaten fried, boiled or steamed, and may be added to salads, soups and stews. The extracted mucilage of OK pod is used as a suspending agent and as a pharmaceutical adjuvant in paracetamol and other drug delivery (Zaharuddin *et al.* 2014). It is also widely used in cosmetic, pharmaceutical and food industries as preservative.

Therefore, the aim of this paper was to investigate the antimicrobial impact of purified OK seed proteins against pathogens in water treatment.

2. Materials and methods

Good quality seeds of OK were purchased at Marama, a local market in Hawul local government area of Borno State, Nigeria. The seeds were harvested from matured OK plants and were manually threshed to access the kernels. The kernels were then sorted, packaged and labelled, then transported to Civil Engineering Laboratory at the University of Birmingham, United Kingdom for laboratory processing, preparation and analysis. The seeds were washed with laboratory tap water to remove contaminants and foreign materials such as stones, plant debris to ensure the quality of the seeds. The cleaned seeds were then dried in an oven (Memmert type, UF 30 115 VOLT, Germany), at 60ºC for six hours before grinding into fine powder. Each seed powder was preserved in a plastic container in a refrigerator at 4°C (standard temperature for protein storage) until used.

2.1 Lipid extraction

The ground seed powders (212 and 300 µm) were defatted using high-grade hexane in an electro-thermal Soxhlet extractor (QUICKFIT England, EX5/53). 20 grams of the seed powder was used during the extraction. About 1% w/v of seed sample in 2 litres of solvent volume (hexane) was used and heated to 60 °C. The process was performed continually for 8 hours with each complete cycle taking approximately 2-3 minutes. The residues obtained from the extraction thimble were removed and dried overnight at room temperature of 19 ± 2 °C. The dried residue was ground again into a fine powder using a pestle and mortar and was used in the

subsequent purification processes. Each of these samples was kept in a plastic container at 4 °C in a refrigerator until use.

2.2 Purification of Bio-active proteins

A Hi-Trap Q HP (1 ml) anion column, (GE Healthcare, Sweden) was used for the purification of the protein from the seeds of the Hibiscus following the procedures of Jones and Bridgeman, (2016). The column was connected to a pump (Watson-Marlow Breeder pump 323, UK) and the pump head adjusted to a flow rate of 1 ml per minute. The preservatives were washed with 10 ml of DI water, followed by 10 column volume of 1 M of NaCl was dissolved in a phosphate buffer. The column was then equilibrated with the phosphate buffer 10 CV before loading the protein in order to bring the column to the required pH of 6.5. 5g of oil-free powder was dissolved in 0.1 M phosphate buffer and mixed thoroughly for one hour using a magnetic stirrer (Stuart Scientific, UK) to form a suspension. The suspension was centrifuged at 20,000 rpm at 4 °C for 40 minutes before decanting the supernatant using Heraeus Megafuge16 (Thermo Scientific, Germany). The supernatant was filtered through a 0.45 µm membrane filter before injecting into a IEX column. The filtered supernatant was injected using the peristaltic pump onto the ion exchange column to separate the protein of interest from the contaminants. A sample of the product of the protein separation was taken and loaded into the Column at a flow rate of 1 ml per minute, where the protein of interest was bound to the Column matrix throughout the loading process while the weakly-bound contaminants were washed away with the equilibrating (initial or starting buffer using 10 CV). The bound proteins of interest were eluted, beginning with 0.3, 0.5 and 1.0 M of NaCl-phosphate buffers and various fractions were collected. The Column was washed with another 10 CV of starting buffer before loading another sample to prevent Column blockage and contamination of subsequent sample to be purified. The collected fractions were analyzed for absorbance using a spectrophotometer (Varian Carey 50 probe UV-visible, Australia) and coagulation activity of the purified proteins using a standard jar tester (Phipps and Bird, 7790-900B USA).

2.3 Collection of water sample

All the waters used in this study were river waters collected from Bourn Brook canal, adjacent to the University of Birmingham Train station. Water samples were collected in a set of one-litre (1 L) plastic containers and allowed to settle naturally before conducting any test. Water samples were kept in a refrigerator at 4 °C before the tests for bacterial inactivation potential of the seed extracts.

2.4 Jar test experiments

Jar tests were conducted using a standard apparatus comprising 6, 1L beakers (Phipps and Bird, 7790-900B USA) jar tester to evaluate the optimum coagulant dose for the coagulation tests. For effective dispersion of the coagulant, the collected water was rapidly mixed at 200 rpm for 1 min during which various doses of the coagulants were added into the beakers. The mixing speed was

reduced to 30 rpm for a further 30 minutes to simulate the flocculation stage. The suspension was then allowed to stand undisturbed to facilitate settlement for 1 hr. A final 10 ml sample of this treated waters were drawn at 3cm depth from the top surface of the waters in the beakers using a syringe, and the turbidity of the waters were then measured using a turbidity meter (HI 93703, Hanna). All experiments were conducted at room temperature (19 ºC) for a minimum of three repeated tests. Turbidity measurements were conducted with, ±0.3 NTU accuracy throughout the whole measurement range. The reduction due to inactivation of total coliforms, faecal coliform and E-coli was investigated with purified protein doses range from 0.74-1.48 mg/l. In addition, the calculated percentage reduction in total coliform count, faecal coliform and E-coli was also compared with the standard log removal value (LRV). Statistical analysis was performed using analysis of variance (ANOVA) on 2016 EXCEL spread sheet.

2.5 Antimicrobial tests

The experiment of testing for the antimicrobial effects of the OK extracts and the purified protein samples was conducted using the Colilert-18/Quanti-Tray (IDEX Inc., UK) for total coliform, faecal count and E-coli detection because of its ease of operation, flexibility, accuracy and speed. After the jar test experiments, Colilert-18 powder was added to both the raw and the treated water samples in a 100 ml sterilized vessel. The mixture there from was thoroughly shaken to completely dissolve the powder. The content was then poured into a tray, sealed and incubated at 35 °C for 18 hours to check for coliform and E-coli counts and 44.5°C for 18 hours for the assessment of faecal count. After the incubation period, samples were taken for the positive wells count. A yellow coloration was an indication of the presence of coliform. To observe for the presence of either the E-coli or faecal count, the tray was transferred to a Longwave Ultraviolet 365 nm lamp spectrolite 160 (Spectronics Cooperation, USA). The number of positive wells that fluoresce were counted. A Fluoresce well is an indicator of either E-coli or faecal coliform, this was differentiated based on the temperature used during the incubation period. The numbers of all the small and large positive wells were then read from the most probable number (MPN) comparative chart provided by the manufacturer, and the results presented as MPN/100ml. To determine the re-growth of bacteria after treatment, final treated water was tested for bacterial re-growth after 72 hours in terms of coliform count, E-coli, and faecal coliform to assess total antimicrobial inactivation potential of the extracts in water treatment. The test was repeated on the treated water which was kept in a disinfected environment to investigate bacterial regrowth and the effect of residual extracts and protein over the 72-hour period.

3. Results and Discussion

Tables 1 and 2 show the results for initial bacterial inactivation with purified okra protein (POP) and then aluminum sulphate (AS) in raw water and their results after lengthy storage time before and after treatment.

The raw water contained a bacterial colony of 517.2/100 ml for the total coliform count, 90.8/100 ml for E-coli and 114.5/100 ml for the faecal count. The results show a significant

($p<0.05$) decrease in bacteria count using 0.74 and 1.48 mg/l doses of POP and 15 mg/l of AS as a control. The percentage reduction in the total coliform count was approximately between 95 and 100% with either dose of the purified proteins while AS produced a maximum reduction in coliform count of 93%. Most notably, the 1.48 mg/l dose recorded the best removal efficiency in the coliform count of approximately 100% with POP. In all cases, the standard LRV of total coliform was 2.7−log (99.9%). With 15 mg/l dose of AS, the LRV was <1−log (90%). It was observed that most of the bacteria in the raw water are sensitive to the purified proteins at a higher dose.

The inactivation of E-coli and faecal species recorded almost 100% performance across all the samples using both 0.74 mg/l and 1.48 mg/l doses while AS achieved only 78 and 76% removal of E-coli and faecal coliforms respectively.

Table 1 Removal of Coliform, E-coli and faecal count in water treatment using POP and AS

		POP (mg/l)		
AS (mg/l)				
Parameters	Raw water	0.74	1.48	15
Initial turbidity (NTU)	11.9	1.0	0.9	0.92
Number of positive wells for coliform:				
♠ Large wells	49	17	8	23
♠ Small wells	27	3	1	5
Total coliform count (MPN/100ml)	517.2	24.1	9.7	36.8
Percentage reduction in coliform count (%)		95.34	98.13	93
LRV (%)		90	99	90
Number of positive wells for E-coli:				
♠ Large wells	40	–	1	15
♠ Small wells	7	–	1	3
E-coli count (MPN/100ml)	90.8	0	2.0	21.1
Percentage reduction in E-coli count (%)		100	99.61	78
LRV (%)		99	99	90
Number of positive wells for faecal coliform:				
♠ Large wells	43	–	–	18
♠ Small wells	9	–	–	4
Total faecal count (MPN/100ml)	114.5	0	0	26.9
Percentage reduction in faecal count (%)		100	100	76
LRV (%)		99	99	90

Table 2 The regrowth of coliform, E-coli and faecal count after 72 hour post treatment with POP

	POP (mg/l)		AS (mg/l)	
Parameters	Raw water	0.74	1.48	15
Initial turbidity (NTU)	11.9	1.0	0.9	0.92
Number of positive wells for coliform:				
• Large wells	49	49	49	29
• Small wells	27	48	48	15
Total coliform count (MPN/100ml)	517.2	2419.6	2419.6	68.0
Percentage reduction in coliform count (%)		400	400	46
LRV (%)				
Number of positive wells for E-coli:				
• Large wells	40	–	-	15
• Small wells	7	–	-	9
E-coli count (MPN/100ml)	90.8	0	0	28.4
Percentage reduction in E-coli count (%)		0	0	25
LRV (%)				
Number of positive wells for faecal coliform:				
• Large wells	43	–	–	18
• Small wells	9	–	–	11
Total faecal count (MPN/100ml)	114.5	0	0	35.9
Percentage reduction in faecal count (%)		0	0	26
LRV (%)				

The LRV was approximately >2−log (99%) with the proteins whereas AS recorded a LRV of <1−log (90%) in E-coli and faecal removal in raw water.

The results of the further experiment performed on the regrowth of bacteria after 72-hour treatment in the re-suspended sludge is presented in Table 2. The results revealed a complete bacterial kill of E-coli and faecal coliform after the lapse time of 72 hours in treated water with the purified proteins. The results also showed the presence of strong antibacterial agents in the seed with a high inactivation potential against E-coli and faecal coliform that could achieve the WHO (1970) standard of zero presence of the two indicator organisms. Overall, turbidity removal witnessed was approximately 92% across the treatment process using any of the coagulants in this regard.

This is the first time a purified OK protein from northeastern Nigeria was tested to determine its antimicrobial potential on total coliform, faecal coliform and E-coli count in an untreated water. The protein purification process increased the bacteria inactivation performance of POP using at a low dose. The 1.48 mg/l achieved a result that is WHO compliant due to elimination of organic matter in the seed that may provide a shield to the pathogens in water (Shaheed *et al.*, 2009). The WHO (1970) stipulated a zero presence of E-coli and faecal count per 100 ml in drinking water which has been achieved with POP. In this work, POP showed stronger antimicrobial potential than when 15 mg/l of AS was dosed under the same coagulation condition, indicating their

potential in disinfecting pathogens. It is noteworthy that most of the bacterial colonies present in the raw water were sensitive to the purified active antimicrobial agents of the protein compared with AS. The examination of most faecal coliform and E-coli revealed excellent inactivation performance. The complete inactivation of faecal coliform and E-coli by the purified proteins further solidifies the argument that the presence of saponin, tannin and alkaloids in the seeds could be responsible for the disinfection activity. It is clear that the two indicator organisms are highly sensitive to the antimicrobial agents and were part of the microbes eliminated during the treatment.

The results of the purified POP samples that were used after 3-day storage showed a robust performance against faecal coliform and E-coli bacteria. Even after re-suspension of the sludge, complete inactivation of these organisms was still seen, indicating that both E-coli and faecal coliform can be controlled using purified proteins because they are highly sensitive to the antioxidants. It is also likely that the residual protein in the treated water was able to disinfect secondary microbes that were in contact with the treated water during the 72-hour storage. This result is not in agreement with the work reported by Ndabigengesere and Narasiah, (1998) who observed complete regrowth of bacteria in the re-suspended water sludge post treatment. The zero presence of both faecal and E-coli bacteria after 72-hr post treatment revealed the disinfection potential of POP, hence eliminating the risk of disinfection-by-product formation if the final water is proposed for disinfection with chlorine.

5. Conclusion and recommendation

The inactivation of bacteria in water due to active compounds in POP could be a cost-effective disinfecting material in water treatment having significant inactivation potential against faecal coliforms and E-coli bacteria. The 100% inactivation achieved against bacterial species demonstrated the potential of purified okra protein as a disinfectant in water treatment. Again, the LRV of >2.7 revealed that POP can achieved WHO standard water quality of zero presence of E-coli and faecal coliform. The purification of the proteins enhanced bacterial kill ability especially regarding E-coli and faecal coliform, due to the removal of natural organic compounds in the seeds. Furthermore, the zero regrowth of bacteria that occurred after lengthy storage indicates the ability of POP residue to act as an effective post treatment measure against ingress of pathogens into distribution pipes. Similarly, the coagulation potential of POP was significant, showing competitive result to that obtained with AS. It is essential that natural extract use in water treatment should be purified to improve water quality and reduce the risk of disinfection-by-product formation due to organic matter addition in treated water. Also, POP should be incorporated into rural water supply chain to improve access to clean drinking water.

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