Antimicrobial efficacy and *in vivo* toxicity studies of a quaternized biopolymeric flocculant

Gurpreet Kaur Khaira, Abhijit Ganguli and Moushumi Ghosh

ABSTRACT

This study evaluated the antibacterial spectrum and safety of a chemically modified biopolymeric flocculant (TMB) against waterborne pathogens. The biopolymer previously characterized as polysaccharide with flocculating activity is produced extracellularly by the bacterium *Klebsiella terrigena*. The amino sugars on the polymer were chemically modified by quaternization, which resulted in *N*,*N*,*N* trimethyl biopolymer (TMB). Quaternization was effective in imparting biocidal activity to TMB against five selected waterborne pathogens, namely, *Aeromonas hydrophila, Yersinia enterocolitica, Salmonella typhimurium, Listeria monocytogenes* and *Escherichia coli* O157:H7. 99.999% inactivation was achieved with *S. typhimurium* at a dose of 60 μg ml^{-1} of TMB within 60 min at the ambient temperature, followed by other pathogens. Haemotological, histopathological and general examinations indicated no adverse effects in Swiss albino mice fed with the quaternized biopolymer (120 mg kg^{-1} body weight^{-1} day^{-1}) over a period of 30 days. These results suggested that TMB was tolerated well without any signs of toxicity and may have potential application as a safe, antimicrobial bioflocculant for both removing and inactivating waterborne pathogens.

Key words | antimicrobial, bioflocculant, *K. terrigena*, quaternized, toxicity

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ABBREVIATIONS

BHI	brain heart infusion
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MIC	minimum inhibitory concentration
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
TMB	*N*,*N*,*N* trimethyl biopolymer

INTRODUCTION

Infectious diseases through transmission of waterborne pathogenic bacteria assume considerable significance in developing countries with approximately 5.5 million cases of hospitalization each year (WHO/UNICEF 2010). Eradication of waterborne pathogens requires potent and/or specific antimicrobial systems, which are safe as well as effective in this regard. Polymers, especially of biological origin, have been extensively studied in the recent past as important alternatives for water treatment because of their biodegradability, particular structure, physico-chemical characteristics, chemical stability and high reactivity (Vroman & Tighzert 2009). These polymers have excellent selectivity towards aromatic compounds and metals, resulting from the presence of chemical reactive groups (hydroxyl, acetamido or amino functions) in polymer chains (Brostow *et al.* 2009; Nwodo *et al.* 2012). The use of polymeric materials with antimicrobial properties, in particular, has gathered considerable interest due to their enormous scope of modification and wide gamut of application (Siedenbiedel & Tiller 2012).

Among the polymeric materials, cationic polymers containing quaternary ammonium groups have proven to be effective antimicrobials and biocides (Mcbain *et al.* 2004; Moore *et al.* 2008; Siedenbiedel & Tiller 2012). A complete characterization of the biopolymer structure is

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important for making chemical modifications; to produce 'customized' biopolymers with a unique combination of mechanical, chemical and biological properties. To this end, very few biologically produced polymers have been thoroughly characterized limiting the generation of chemically modified biopolymers (Rehm 2010). In the recent past, we have extensively characterized an exocellular biopolymer produced by a strain of *Klebsiella terrigena* (Ghosh *et al.* 2009a, b). The biopolymer possesses unique attributes in terms of robustness and high flocculating activity against a wide range of colloid particles and waterborne pathogens. We envisaged that rendering an antimicrobial function to the same may enable inactivation and simultaneous removal of these pathogens during water treatment. Therefore, in the present study, the biopolymer has been chemically modified, and interestingly, the quaternized biopolymer possessed excellent antibacterial activity against all the selected waterborne pathogens. The oral toxicity profile of the chemically modified biopolymer determined in mice is reported.

MATERIALS AND METHODS

All the chemicals and reagents were purchased from Sigma-Aldrich Company (USA) and were of the highest grade available commercially.

The industrial wastewater isolate, *K. terrigena* (Accession number EU082029), previously reported to produce extracellular biopolymeric flocculant was used for harvesting biopolymer as described by Ghosh *et al.* (2009a).

Synthesis and characterization of *N,N,N* trimethyl biopolymeric derivative (TMB)

Purified biopolymer (150 mg) was dissolved in dimethylsulfate (2.4 ml) and deionized water (0.6 ml). The solution was then filtered to eliminate the impurities. Sodium hydroxide (0.18 mg) and sodium chloride (0.132 mg) were added to the resulting suspension. The solution was stirred at an ambient temperature for 6 h. The product was precipitated using acetone, filtered (0.8 μm-pore size) and vacuum dried. White precipitates obtained were redissolved in deionized water (20 ml), subjected to dialysis using a dialysis tubing (Cellulose, MWCO 12000) for 1 day, and lyophilized to obtain powder (4.5 mg) (Belalia *et al.* 2008).

NMR analysis

^{1}H-NMR spectra of biopolymer and TMB were recorded using Bruker Avance II (400 MHz) spectrometer.

Compositional analysis

The total sugars, neutral sugar, uronic acids, amino sugar content and pyruvic acid of the quaternized biopolymer were performed as described by Yokoi *et al.* (1997). Elemental analysis was carried out with a 2400 II elemental analyser (Perkin Elmer Company, Bedford, MA, USA).

Determination of antibacterial activity

Five commonly existing waterborne pathogens, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Salmonella typhimurium* ATCC 25315, *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* O157: H7 ATCC 32150 were used for determining antimicrobial efficacy of the quaternized biopolymeric flocculant. The indicator cultures were grown in brain heart infusion (BHI) broth at 37 °C with shaking (120 rpm) prior to the experiment.

MIC determination

Minimal inhibitory concentrations (MICs) were determined by microtiter broth dilution method, performed using a sterile 96 well-microtitre plate reader (Bioscreen C, Thermolabsystems, Helsinki, Finland) (Andrews 2001; Raafat *et al.* 2008). The indicator strains were grown in the respective broth at 37 °C to an optical density of 1 at 600 nm and subsequently diluted in the same medium to about 10^7 CFU ml^{-1}. Briefly, serial two-fold dilutions of TMB solutions were prepared in the appropriate culture medium in microtitre plates. Final TMB concentrations used were 1–100 μg ml^{-1}. Each well of the microtitre plate then received 100 μl of the inoculated medium, and the plates were incubated at 37 °C for up to 24 h. MIC was

defined as the lowest concentration of compound required to completely inhibit microbial growth after incubation.

Waterborne pathogens removal assay from water

Cultures of *A. hydrophila, Y. enterocolitica, S. typhimurium, L. monocytogenes* and *E. coli* O157:H7 were grown overnight in BHI broth, harvested by centrifugation. Cell pellets of all the cultures were washed three times with sterile tap water, mixed, resuspended in sterile tap water, mixed in equal proportions and spiked in 1 l of sterile tap water held in Erlenmeyer flasks (three replicates). TMB in different doses was then added to each replicate, mixed thoroughly by hand rotation and allowed to stand at ambient temperature for 10, 30 and 60 min. Control experiments comprised similar combinations but lacked TMB. In parallel experiments, chlorine was added at different concentrations prepared from 10 mg l^{-1} of stock solution. The bacterial cell count was done using the colony count method. The efficiency of the TMB and chlorine in destroying bacteria was evaluated by comparing the initial and final bacterial count after treatment.

Toxicity assessment

Oral toxicity

TMB solutions were prepared with sterile distilled water and kept under refrigeration until use. The toxicity studies were carried out in accordance with the OECD guidelines 423 (OECD 2001). Twenty-five pathogen-free male Swiss mice, 8 weeks old, were acclimatized to a 12/12 h light/dark cycle for 1 week. They were housed in a polystyrene cage, allowed free access to feed and sterile tap water, divided into five groups of five animals per cage and identified. TMB and native biopolymer were administered orally at a dose of 25, 75, 120 and 140 mg kg^{-1} in distilled water for 30 days. The control group received sterile distilled water. The animals were observed for mortality and morbidity such as convulsions, tremors, grip strength and pupil dilation. All animals were weighed before treatment, weekly during treatment and at the end of the study. The feed consumption was recorded weekly.

Haematology

At the end of the experiment, animals were anaesthetized in an ether chamber, and blood was collected by cardiac puncture. Analysis of red blood cell count (RBC), haematocrit (Hct), haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and differential cell count were conducted.

Histology

Necropsies were performed on all study animals; the liver and kidney were analysed macroscopically. In order to microscopically examine the tissues, the latter were fixed in aqueous Bouin, processed, embedded in paraplast, sectioned to a thickness of 7 μm, and stained with haematoxylin and eosin. Histological analysis of organs was done as described by Gauthier *et al.* (2011).

Statistical analysis

Data were presented as mean ± SD of three independent experiments. During the 30-day oral toxicity, treated and control groups were compared by ANOVA with significance $p \leq 0.05$, using Statistica 11.01, 2012.

RESULTS

Synthesis and characterization of TMB

Figure 1 depicts the ^{1}H-NMR spectra of the biopolymer and quaternized biopolymer (TMB), respectively. The spectra revealed an intense signal at 3.66 ppm corresponding to the trimethyl ammonium group. Sieval *et al.* (1998) indicated that the peak at 3.6 ppm is assigned to the trimethyl amino group, the peak at 3.1 ppm is assigned to dimethyl amino groups, and the peaks between 4.7 and 5.7 ppm are assigned to ^{1}H protons. The degree of quaternization (DQ) of TMB was determined potentiometrically as 73%.

Both physical and chemical characteristics of the quaternized biopolymer were compared with its native counterpart. The total sugar and total protein content of the biopolymer

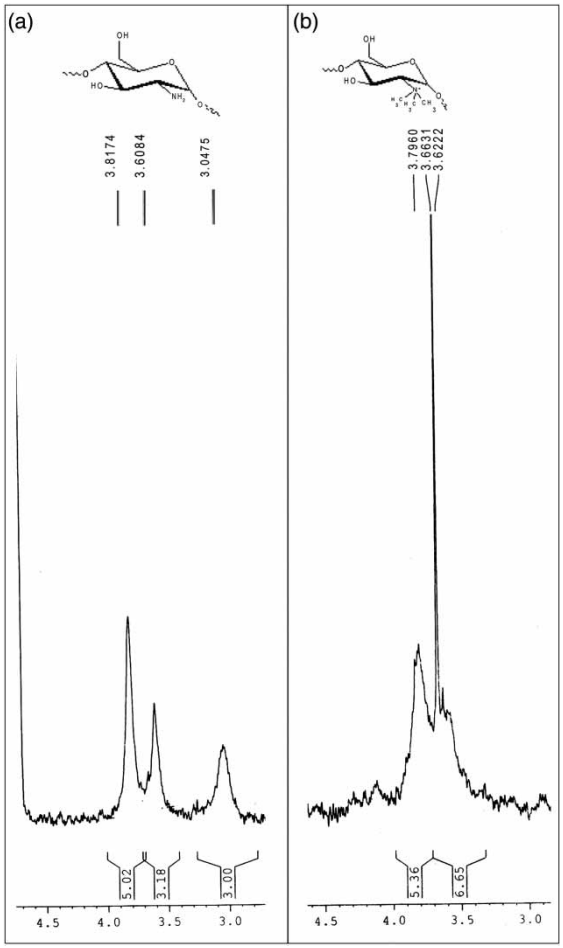

Figure 1 | ^{1}H-NMR spectra of the native biopolymer (a) and quaternized biopolymeric derivative (TMB) (b).

was 66.8 and 2.45% (w/w), respectively, indicating a primarily polysaccharide structure of TMB similar to the native biopolymer. The amino sugars (5.8%), acidic polysaccharides including uronic acid (2.83%) and pyruvic acid (7.4%) did not differ significantly from native biopolymer (results not shown).

Biocidal activity of quaternized biopolymeric flocculant

The inhibitory activity of quaternized biopolymer (TMB) was evaluated through MIC determination against five selected waterborne pathogens. The TMB markedly inhibited the growth of all the pathogens in BHI broth with MIC values ranging from 50 to 90 μg ml^{-1} within contact time of 30–90 min. Highest inactivation of 99.999% (5 log reduction) was achieved against *S. typhimurium* at a dose of 60 μg ml^{-1} in 60 min. Table 1 shows the MIC values of

Table 1 | MIC values of biopolymer and quaternized biopolymer (TMB) tested against *S. typhimurium*, *E. coli*, *A. hydrophila*, *L. monocytogenes* and *Y. enterocolitica*

	MIC (μg ml^{-1})		
Bacteria	**TMB**	**% inactivation**	**Biopolymer**
L. monocytogenes	80	99	>100
E. coli	73	99.9	>100
S. typhimurium	60	99.999	>100
Y. enterocolitica	82	99	>100
A. hydrophila	76	99.9	>100

MIC: minimum inhibitory concentration.

TMB against all the selected waterborne pathogens. However, the antibacterial activity was dependent on type of bacteria, inoculum size and assay conditions. Our investigations indicated that increasing the bacterial inoculum (10^5 to 10^7 CFU ml^{-1}) had no significant effect (more than four-fold) on the MIC of the tested quaternized biopolymer (data not shown). The inhibitory effect was further confirmed by plating treated cultures onto respective selective media and BHI agar; possibilities of injured pathogens were confirmed by parallelly plating the TMB treated pathogens on thin agar layer (TAL) plates (results not shown).

Removal assays

The initial count of spiked waterborne pathogens in tap water samples was 6 log CFU ml^{-1}. Significant reduction ($p < 0.5$) in numbers of all the pathogens (3–5 log reduction) were observed in both selective and TAL plates. TMB dose optimization trials carried out indicated that the optimum dosage was in range of 5–10 mg l^{-1} for achieving the removal of 6–7 log CFU ml^{-1} of waterborne pathogens spiked in sterile tap water (Table 2). On the other hand, chlorine used at the same concentration as TMB resulted in 99.99% inactivation of all the pathogens at higher exposure time in comparison to TMB.

Oral toxicity studies

Mortality of animals was not observed over the period of experiment. Body weight gains (Table 3), rectal temperature profile (results not shown) and feed consumption in all TMB concentrations tested was comparable to control group values.

Table 2 | Comparative efficiency of quaternized biopolymer (TMB) and synthetic disinfectant chlorine in water spiked with selected microorganisms at initial inoculums of 6 $\log_{10}$ CFU

		TMB (pH 6–7) at 25 °C			Chlorine (pH 6–7) at 25 °C		
		Viable cells after exposure time (min)			Viable cells after exposure time (min)		
Microorganisms	Dose (mg l^{-1})	10	30	60	10	30	60
L. monocytogenes	6	5.5	4	1	4.6	1.2	0
E. coli	5	6	1.6	0	2.2	0	0
S. typhimurium	4	4.6	1	0.0	5	3	0
Y. enterocolitica	6	6	2	0	2.2	1.1	0
A. hydrophila	5	5.6	3	0	4	2	0

Behavioural changes or changes in body weight of TMB exposed mice were not observed. Gross and microscopic examination revealed no changes attributable to the administration of either native biopolymer or its quaternized derivative (TMB). Haematology showed no significant ($p > 0.05$) treatment-related changes that were evident during the experimental period (Tables 4 and 5) upon comparison to the control group. Liver sections from mice fed with TMB revealed a similar profile as the blank groups in liver tissues, suggesting the absence of any untoward response.

DISCUSSION

Few synthetic polymers quaternized for biocidal properties have been adequate for biological processes. For instance, in spite of the high bactericidal efficacy of the quaternized poly (vinylpyridine)s, the minimal biocompatibility has limited their use for biological purposes. The incorporation of hydrophilic and biocompatible polymers by copolymerization has been proposed to improve both the antimicrobial efficiency and the biocompatibility (Munoz-Bonilla *et al.* 2012). In contrast, biopolymers are generally considered as environmental friendly materials in terms of biodegradability, and inherent biocompatibility, and for these reasons, biopolymers have been successfully used in various industries and for water treatment (Honarkar & Barikani 2009; Nwodo *et al.* 2012). The structural diversity and plasticity of biopolymers as well as their safety qualifies them for

Table 3 | Mean body weight changes following administration of TMB in mice; body weight was recorded every fifth day (five animals per dose)

	Days							
Dose (mg kg^{-1} bw)	0	5	7	10	15	20	25	30
0	29.5 ± 0.7	29.0 ± 1.4	28.5 ± 0.7	30.0 ± 1.4	27.5 ± 0.7	28.0 ± 0.0	29.0 ± 0.0	28.0 ± 0.0
25	34.0 ± 2.8	33.0 ± 2.8	33.5 ± 2.1	33.5 ± 3.5	32.0 ± 2.8	32.5 ± 3.5	31.5 ± 3.5	32.0 ± 2.8
80	36.0 ± 0.0	34± 2.1	33.6± 2.1	32.5± 2.1	33.3± 2.1	34± 2.1	34± 2.1	34.5± 2.1
120	34.7 ± 3.5	32.5 ± 3.5	31.0 ± 3.0	30.5 ± 2.5	32.5 ± 3.5	32.5 ± 2.5	33.5 ± 3.5	31.5 ± 3.5
140	33.5 ± 2.1	31.0 ± 2.1	30.8 ± 2.4	32.0 ± 2.1	33.0 ± 2.8	31.0 ± 3.1	33.0 ± 3.2	32.0 ± 3.1

Table 4 | Means of red blood cell parameters in mice administered TMB by gavage for 30 days

	Dose				
Parameter	0	25	80	120	140
Red blood cells (millions/mm^3)	9.0 ± 0.2	7.4 ± 0.2	6.5 ± 0.9	7.0 ± 1.5	8.3 ± 0.2
Haemoglobin (g dL^{-1})	13.1 ± 2.0	13.3 ± 1.2	13.4 ± 1.2	15.4 ± 2.4	18.5 ± 3.5
Haematocrit (%)	49.3 ± 5.3	53 ± 3.8	51 ± 3.4	50 ± 5.1	48.7 ± 2.0
Mean corpuscular volume (μm^3)	50.8 ± 2.2	60 ± 10.5	64.6 ± 7.3	63.2 ± 10.5	50 ± 2.3
Mean corpuscular haemoglobin (pg)	17.2 ± 0.7	18.2 ± 1.0	21.1 ± 3.0	20.5 ± 2.5	21.9 ± 4.0
Mean corpuscular haemoglobin concentration (%)	28.2 ± 4.3	25 ± 4.8	26 ± 4.2	30 ± 2.5	39 ± 9.3

Table 5 | Means of white blood cell parameters of mice administered TMB by gavage for 30 days

	Dose				
Parameter (%)	**0**	**5**	**15**	**50**	**150**
Eosinophils	0.8 ± 0.9	1.3 ± 0.7	1.6 ± 0.6	1.4 ± 1.0	1.34 ± 0.6
Monocytes	4.4 ± 1.0	4.8 ± 1.5	4.8 ± 1.2	4.2 ± 1.5	4.2 ± 1.6
Lymphocytes	83.07 ± 2.5	83 ± 6.4	82.8 ± 3.0	80.3 ± 7.2	80.1 ± 8.5
Neutrophils	11.2 ± 2.0	13 ± 4.2	11.4 ± 4.1	12.3 ± 8.2	13.02 ± 4.2

furthering desirable chemical changes to enhance or endow with 'customized' functionalities.

The biopolymer produced by *K. terrigena* possesses excellent flocculating properties suitable for water treatment, and physical and chemical characteristics of this biopolymer have been well documented (Ghosh *et al.* 2009a, b). Methylation of the amino groups in the C-2 position of biopolymer to form quaternary groups with fixed positive charges on the repeating units of the TMB polymer chain may be inferred from the obtained results. In addition, some degree of mono- and di-methylation of the biopolymer structure and methylation on the 3 and 6 hydroxyl groups of the biopolymer chain may be expected.

MIC is the main microbiological parameter to predict the efficacy of antimicrobials. However, it is well known that MIC may vary according to the inoculum size used (inoculum effect) (Mizunaga *et al.* 2005). In our study, MIC of the tested quaternized biopolymer against all the selected pathogens was not affected by an increase in the inoculum size (data not shown).The antibacterial activity of quaternized biopolymeric derivative could be due to the positive charges on the flocculant chain, as a consequence of the quaternization of the amino groups. The activity resulted from the interaction between the positively charged amino groups of the biopolymer and negatively charged cell surface of Gram-negative bacteria and also due to the flocculant activity of the biopolymer.

Currently, chlorination is the most common method of disinfecting drinking water worldwide. The present study determined removal efficiency of chlorine in comparison to TMB in water against selected waterborne pathogens. Differences between the earlier findings and the results of the present study in inactivation of bacteria by chlorine may be attributed to variability between strains, slight pH or temperature differences. In sterile tap water most inactivation of Gram-negative microorganisms occurred during the first 30–60 min of treatment and the dosage of TMB required was low.

A few studies have elaborately documented the safety of polysaccharide biopolymers, for instance, chitin from crustacean shells (Honarkar & Barikani 2009) beta-glucan from barley and yeast (Delaney *et al.* 2003; Jonker *et al.* 2010). A general interpretation from these studies suggests that differences in the molecular structures of polysaccharide chains (e.g., chain length, type of linkage) in biopolymers influence physico-chemical properties and, consequently, biological activity as well as a differential response in mice under *in vivo* conditions (intestinal behaviour); however, these biopolymers were intended as dietary supplements. Such detailed examination, including caecum, urine and faeces, was not deemed important since the present study was designed to judge the toxicity of the antimicrobial polymer designed for treating water.

It may be argued that a predominantly polysaccharide structure of the *K. terrigena* biopolymer, and the absence of associated lipid eliminated the possibility of a cytotoxic effect as indicated in preliminary toxicity studies with cell lines (unpublished observations). However, in view of the chemical change brought about by quaternization, it was desirable to evaluate the toxicity of the quaternized biopolymer. A critical comparison of the results obtained from our studies was not possible due to the lack of similar studies, where microbially or plant-derived biopolymers have been chemically modified in part for rendering antimicrobial property. The absence of negative effects of TMB on behaviour, body weight, haematology and organ histology at concentrations representing 1,000-fold more than the anticipated TMB dosage required to inactivate the pathogens in water, suggest the safety, and therefore, a potential applicability of the *K. terrigena* biopolymer as a flocculant disinfectant for water treatment.

CONCLUSIONS

This study reported for the first time the biocidal activity of a biopolymeric flocculant on waterborne pathogenic bacteria. The biocidal function of the native bacterial exopolymer was achieved by quaternization. The newly developed biocidal biopolymeric flocculant was found to be safe at a 'no observed adverse effect level' (NOAEL) dose of 140 mg kg^{-1} day^{-1} in mice in oral toxicity studies, suggesting a potential application of the quaternized biopolymer for water treatment.

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