Rapid inactivation of *Salmonella* by a quaternized biopolymeric flocculant

Gurpreet Kaur Khaira, Abhijit Ganguli and Moushumi Ghosh

ABSTRACT

We report the characteristics and biocidal properties of a biopolymeric flocculant produced by *Klebsiella terrigena* capable of flocculating *Salmonella* efficiently from water. In order to impart an antimicrobial function, the native biopolymer was quaternized and the trimethyl biopolymeric derivative (TMB) was analysed physically, chemically and for flocculating properties. The quaternized biopolymer was evaluated for antimicrobial effects against four strains of *Salmonella* namely, *S. typhimurium* ATCC 23564, MTCC 1251, MTCC 98 and *S. typhi* MTCC 733. TMB did not differ significantly ($p < 0.05$) in either chemical and physical properties or flocculating ability when compared to its native counterpart. TMB completely inactivated *Salmonella* (60 μg/mL, at ambient temperature) within 60 min of exposure. Cell injury and death were evidenced by rapid increase in electrical conductivity in the media and release of intracellular alkaline phosphatase and glucose-6-phosphate dehydrogenases indicating permeation of cells. Electron micrographs revealed grossly altered morphology suggesting damage to the cell membranes as a possible reason for inactivation. The results of this study suggest a potential application of the developed biocidal bioflocculant for water treatment.

Key words | biocidal, bioflocculant, cell membrane, *Salmonella*, quaternization

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INTRODUCTION

Waterborne diseases pose a great burden on global public health (WHO 2008). *Salmonella* spp. (*Salmonella paratyphi* A, B and C) are among the prevalent water-borne pathogenic bacteria in developing nations (Sharan *et al.* 2011). They are of considerable significance as they result in approximately 5.5 million cases (both waterborne and foodborne diseases) of enteric fever each year (Sharan *et al.* 2011). Transmission through water becomes evident considering that individuals infected with *Salmonella* shed the organisms in their faeces, which can enter domestic sewage which, if insufficiently treated or inadequately disinfected, can consequently contaminate drinking water sources (Maskey *et al.* 2006). Environmental considerations demand the development of effective, economically viable and ecofriendly replacements of conventional synthetic flocculants and disinfectants for water treatment, based upon renewable organic materials which degrade naturally.

In recent years, cationic polymers with modified quaternary ammonium groups with antimicrobial properties have been developed. Some of these polymers, for example chitosan, have shown promising results as antimicrobials (Siedenbiedel & Tiller 2012). The biocidal activity of these polymers is attributed to their ability to interact with the bacterial cell wall, which results in disruption of the underlying cytoplasmic membrane (Choi *et al.* 2001; Mcbain *et al.* 2004). No studies to date have attempted (to the best of our knowledge) to chemically modify microbial biopolymeric flocculants, to endow biocidal properties against waterborne pathogens.

An extracellular biopolymeric flocculant from an environmental isolate of *Klebsiella terrigena* has been thoroughly investigated in our earlier studies (Ghosh *et al.* 2009a, b); the bioflocculant possesses unique attributes in terms of robustness and high flocculating ability for waterborne pathogens. We envisaged that imparting an

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antimicrobial function to this flocculant may enable inactivation and simultaneous removal of these pathogens during water treatment. Therefore, in this study we chemically modified the bioflocculant by quaternization, compared its intrinsic characteristics with its native structure and investigated its biocidal properties with a possible mechanism of inactivation against *Salmonella*.

MATERIALS AND METHODS

All the chemicals were purchased from the Sigma chemical company (Sigma, MO, USA).

Bacterial strains and culture conditions

An industrial wastewater isolate, previously isolated and identified in our laboratory as *Klebsiella terrigena* (Accession number MTCC 7805), was used in this study. The strain was preserved in glycerol stock solutions at −80 °C. For production of biopolymer, the strain was grown in 1 L of medium (polypeptone 5 g/L, diammonium sulphate 2 g/L, yeast extract 1 g/L, $CaCl_2$ 0.7 g/L, NaCl 0.1 g/L, $MgSO_4$ 2 g/L, K_2HPO_4 1 g/L, glucose 1 g/L, agar 3 g/L) on a rotary shaker (120 rpm/min) at 30 °C for 48 hr. Cultures of *S. typhimurium* (ATCC 23564, MTCC 1251, MTCC 98) and *S. typhi* MTCC 733 were grown in brain heart infusion (BHI) broth by incubating at 37 °C, for 6–8 hr with agitation (120 rpm).

Biopolymer production

Cells were removed from the culture medium by centrifugation (12,000 *g*) and the biopolymer was separated by the addition of two volumes of ethanol (99.5%) to 500 mL of concentrated supernatant, and allowed to precipitate at 40 °C for 24 hr. The precipitated polymer was collected by filtration (Whatman GF Filter) and dialysed extensively, against deionized water. Crude biopolymer was purified by addition of a 10% solution of cetylpyridinium chloride. The precipitated polymer complex was collected by centrifugation at 10,000 rpm for 20 min at 4 °C and redissolved in 10% NaCl solution. Three volumes of ethanol were added to recover the purified biopolymer, which was further dialysed and lyophilized (Ghosh *et al.* 2009a, b).

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

Purified biopolymer (150 mg) was dissolved in dimethylsulphate (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, followed by stirring the solution at ambient temperature for 6 hr. The product was precipitated using acetone, filtered and vacuum dried. White precipitates obtained were redissolved in deionized water (20 mL), subjected to dialysis using a dialysis tubing (Cellulose, MWCO 12000,) for one day; and lyophilized to obtain powder (4.5 mg) (Belalia *et al.* 2008).

NMR analysis was also carried out: ^1H NMR spectra of biopolymer and *N*, *N*, *N*-trimethyl derivative (TMB) were recorded using a Bruker Avance II (400 MHz) spectrometer. For this analysis, samples were dissolved in D_2O (supplementary data; available online at http://www.iwaponline.com/ws/014/169.pdf).

Physico-chemical analysis of TMB

The total sugars, neutral sugar, uronic acids, amino sugar content and pyruvic acid of the biopolymer and TMB were performed as described by Yokoi *et al.* (1997). Elemental analysis was carried out with a 2400 II elemental analyser (Perkin Elmer Company, USA). Fractionation and purification were achieved using gel chromatography on a Sepharose 4B column followed by elution with a 0.4 M NaCl buffer. The ultrastructure of purified TMB was observed by scanning electron microscopy (SEM) (JSM 541-V, JEOL, Japan).

Measurement of flocculating activity

TMB and biopolymer were evaluated for their ability to flocculate suspended solids of varied size. A standard solution of suspended solids of 100, 1,000 and 2,000 NTU was made by suspending active carbon, silica, magnesium hydroxide, cellulose and yeast in 100 mL water. A bacterial suspension (gram-negative bacteria including *Salmonella*) was also used for this study. A suspension was prepared by diluting the suspension from stock (0.4–0.7%) to desired turbidity with a turbidimeter (Cyber Scan TBDIR1000 Meter, The

Netherlands). An assay was performed by adding 10 mL $CaCl_2$ (5 mM), 0.5 mL biopolymer (2 mg/L) and 9.5 mL of distilled water to 80 mL of these solutions. The pH was adjusted to 7 ± 0.2 and the solutions were allowed to stand at room temperature for 5 min. Twenty millilitre aliquots were withdrawn from the upper phase and the turbidity was measured. Flocculating activity was calculated by recording optical density with a spectrophotometer at 550 nm. Flocculating activity of purified biopolymer and TMB in water was measured against distilled water as a control according to the method of Kurane *et al.* (1986). Activity (%) was defined and calculated as:

$$(B - A)/B \times 100$$

where A is optical density of sample at 550 nm and B is optical density of reference at 550 nm.

The activity was expressed as the mean value from triplicate determinations.

Determination of antibacterial activity

The antibacterial spectrum of the biopolymeric derivative was determined against *S. typhimurium* ATCC 23564, MTCC 1251, 98 and *S. typhi* MTCC 733. The cultures were revived in BHI broth by incubating at 37 °C for 6 hr. For a preliminary screening, an agar disc diffusion assay (supplementary data; available online at http://www.iwaponline.com/ws/014/169.pdf) was performed and diameters of inhibition zones were observed (data not shown). The experiments were performed in triplicates.

To investigate the killing kinetics, the antibacterial activity of TMB was assayed by the microdilution method, using a sterile 96-well microtiter plate reader (Bioscreen C, Thermo labsystems, Helsinki, Finland). Briefly, serial two-fold dilutions of TMB solutions were prepared in the appropriate culture medium in sterile 96-well round bottom polystyrene microtiter plates (Raafat *et al.* 2008). Final TMB concentrations used were 1–100 μg/mL. The four strains of *Salmonella* 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. The solutions of biopolymer and TMB were added, and the minimal inhibitory concentration (MIC) was determined (Andrews 2001) for 3 hr.

Electrical conductivity measurement

The mid-log phase cells of *Salmonella* were harvested by centrifugation at $11{,}000 \times g$ for 10 min. The pellets were washed and resuspended in 0.1 M phosphate buffer (pH 7.4). The final cell suspensions were adjusted to 10^7 CFU/mL and mixed with TMB and biopolymer (40 and 60 μg/mL, 100 and 150 μg/mL, pH 7.0, respectively), and then the mixtures were incubated at room temperature. Measurements of their electrical conductivity were taken every 10 min for 2 hr.

Permeation of the cytoplasmic membrane

The biopolymer and TMB (100 μg/mL) were added to a 0.85% (w/w) NaCl solution containing *Salmonella* strains each at 10^7 CFU/mL. Following incubation at 37 °C for 8 hr, a 0.2 mL aliquot of the cell suspension was withdrawn and added to the reaction mixture. Alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenases (G6PDH) activity was determined using the method of Malamy & Horecker (1964) (supplementary data; available online at http://www.iwaponline.com/ws/014/169.pdf).

Electron microscopy

Salmonella cells treated with TMB as described above were used to visualize structural damage, following 20, 40 and 60 min of incubation (supplementary data; available online at http://www.iwaponline.com/ws/014/169.pdf) by SEM.

RESULTS AND DISCUSSION

Synthesis and characterization of *N, N, N* trimethyl biopolymeric derivative

Figure 1 depicts the ^{1}H-NMR spectra of the native biopolymer and quaternized biopolymer (TMB), respectively. The spectra revealed an intense signal at 3.66 ppm corresponding to the trimethylammonium group. Sieval *et al.* (1998) indicated that the peak at 3.6 ppm is assigned to the trimethyl amino group, the

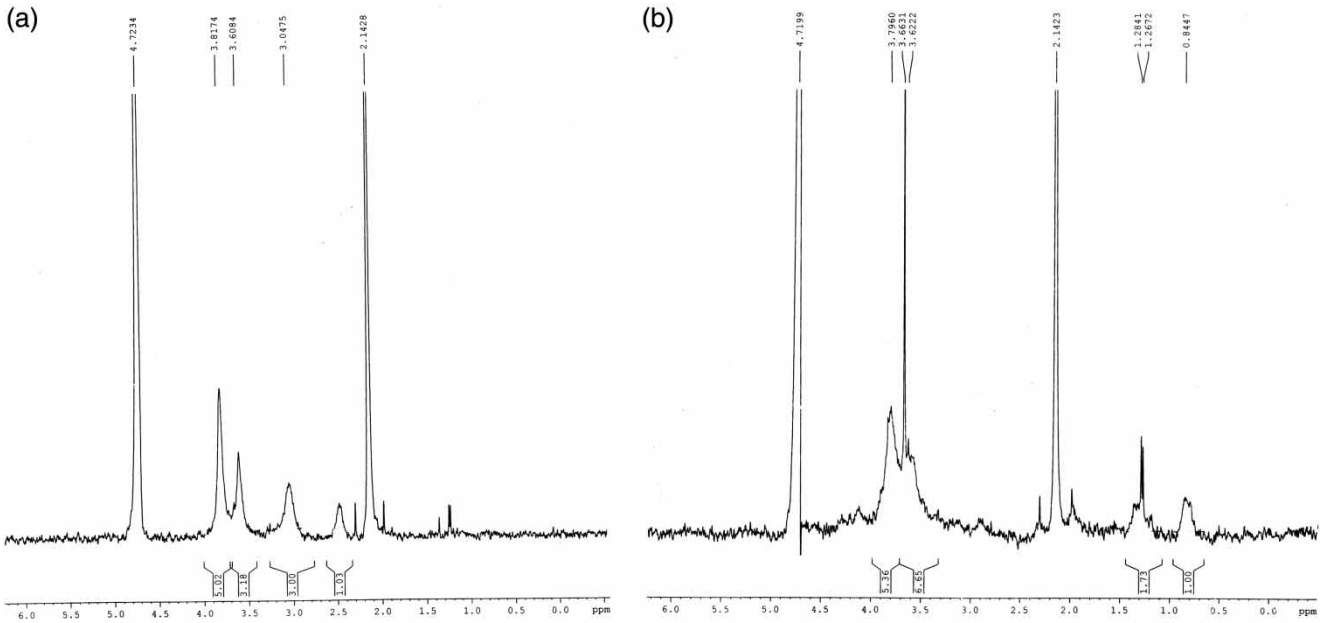

Figure 1 | ^{1}H NMR spectra of biopolymer (a) and TMB (b) dissolved in D_2O.

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.

Methylation of the amino groups in the C-2 position of the biopolymer to form quaternary amino groups with fixed positive charges on the repeating units of the TMB polymer chain may be inferred from the obtained results.

Physico-chemical characteristics of TMB

Both physical and chemical characteristics of TMB were compared with its native counterpart (Table 1).

Table 1 | Compositional analysis of biopolymer and TMB

S.no.	Components	Biopolymer composition (%)	TMB composition (%)
1.	Total sugar	69.8	66.8
2.	Amino sugar	6.87	5.8
3.	Protein	6.73	2.45
4.	Pyruvic acid	0.6	7.4
5.	Uronic acid	1.12	2.83
6.	Carbon	14.73	12.73
7.	Hydrogen	1.23	1.01
8.	Nitrogen	0.64	0.44

The SEM observations indicated a similar porous surface morphology of TMB to that of the native structure. The small pores were non-uniform in size and distribution, and appeared as interconnected channels (supplementary data; available online at http://www.iwaponline.com/ws/014/169.pdf).

Flocculating efficiency of the quaternized biopolymer

TMB and the biopolymer, at the optimum doses of 0.2 and 2 ppm, respectively, showed similar flocculating activity at low, medium and high levels of turbidity.

The biopolymer and TMB aggregated a wide range of colloidal particles, over concentrations of 0.1–10 ppm. As illustrated in Figure 2, the most effective flocculation was achieved in suspensions comprising very small and small sized particles even at low concentrations of the polymer. The flocculation efficiency in the suspension of medium sized particles ranged from 30 to 40% (that too at relatively higher concentrations of polymer). The suspensions of large sized particles showed 50–65% flocculant activity at the same concentration range that flocculated in the range of 70–90% in the case of small and very small sized particle suspensions.

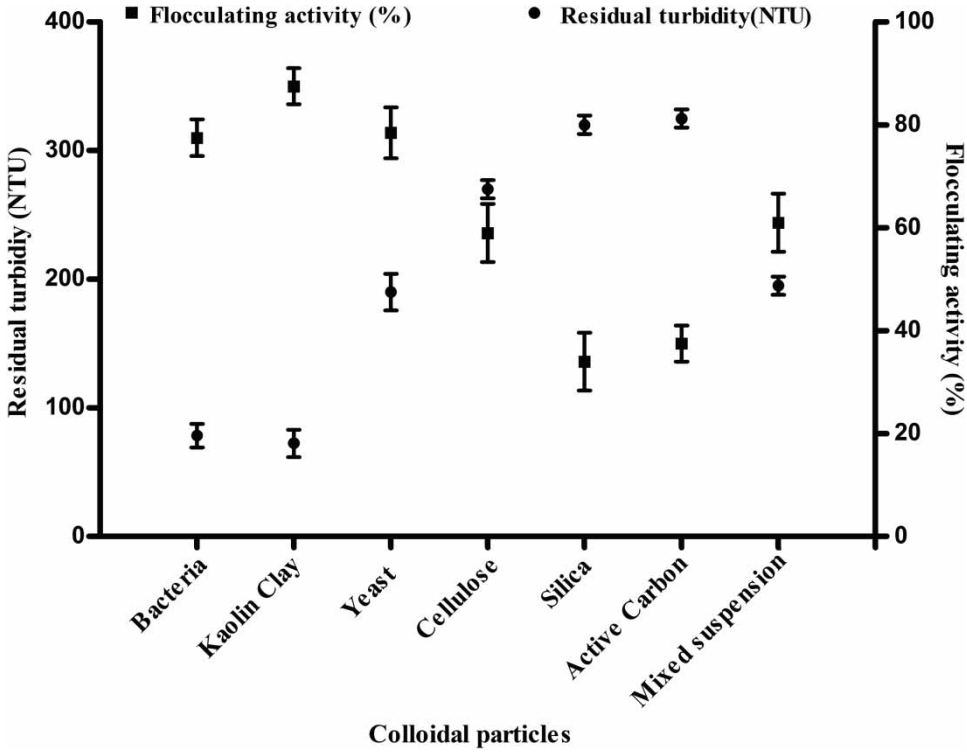

Figure 2 | Efficiency of removal of varied size colloidal particles in solution (at 100 NTU) dosed with TMB (0.2 ppm). The corresponding sizes of the particles are the following: ~0.5–0.8 μm (bacterial cell suspension), ~4–7 μm (kaolin, yeast cell suspension, cellulose), ~15–20 μm (silica), ~100 μm (active carbon).

This might be due to non uniform distribution of pore size on the biopolymer surface; also the number of sites available for binding small and very small sized particles might be more than that for binding medium and large sized particles.

Biocidal activity of quaternized biopolymeric flocculant against *Salmonella*

The antibacterial activity of the biopolymer and TMB was assessed thereafter against *Salmonella* by observing the zones of inhibition and determining MICs (Table 2). Results obtained by the agar disc diffusion assay indicated a strong inhibition by TMB, the native biopolymer failed to inhibit *Salmonella* (data not shown). Moreover, TMB significantly inhibited the growth of *Salmonella* in 60–90 min at concentrations ranging from 50 to 100 μg/mL.

Table 2 | Minimum inhibitory concentration (MIC) of quaternized biopolymer (TMB)

S.no.	Cultures	MIC (μg/mL) of TMB
1.	*S. typhimurium* ATCC 23564	63
2.	*S. typhimurium* MTCC1251	62
3.	*S. typhimurium* MTCC 98	62
4.	*S. typhi* MTCC 733	60

The analysis of bacterial growth in the presence of various concentrations of quaternized biopolymer over time indicated no significant change ($P > 0.05$) in growth behaviour at a concentration of 10 μg/mL, with respect to the control (cells without TMB). For concentrations of 10 and 35 μg/mL, the growth profile was different from the control ($P < 0.05$) whereas at concentrations of ≥50 μg/mL, the quaternized biopolymer inhibited cell growth. The killing efficacy of 60 μg/mL of TMB when challenged upon approximately 10^7 cells/mL of pathogen is illustrated in Figure 3, 3.5 log reduction was achieved by 60 min at ambient temperature.

The higher antibacterial activity of TMB could be due to the interaction between the positively charged amino groups of the biopolymer and negatively charged cell surface of gram negative bacteria.

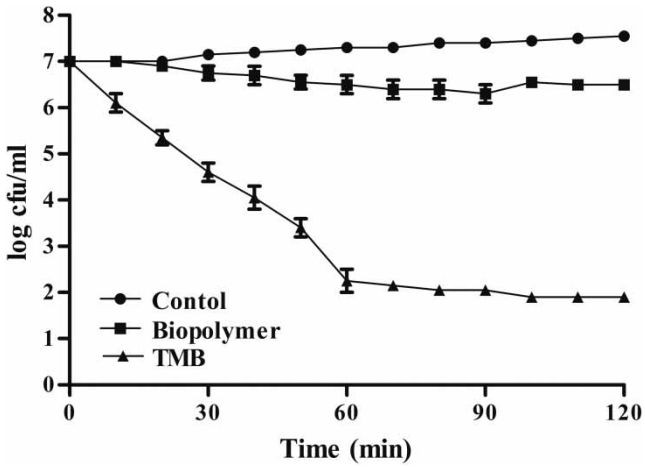

Figure 3 | Time-dependent killing efficacy of TMB (60 μg/mL) and biopolymer (146 μg/mL) against *Salmonella*.

Electrical conductivity of cell suspensions of TMB treated bacteria

The electrical conductivity of the cell suspensions for *Salmonella* treated with biopolymer (100 and 150 μg/mL) and TMB (40 and 60 μg/mL) showed a time-dependent increase (Figure S-2, supplementary data; available online at http://www.iwaponline.com/ws/014/169.pdf). Differences in the changes of the electrical conductivity between the treatments also suggested that the effect of TMB on the membrane permeability of *Salmonella* was greater than the biopolymer.

Effect of TMB on leakage of enzymes

The effects of biopolymer and TMB on the leakage of ALP and G6PDH have been expressed as units of enzyme released (data not shown). In the supernatant of *Salmonella* strain treated with native biopolymer, a complete absence of ALP and G6PDH was observed as expected, in contrast to the TMB. In the supernatant of TMB treated *Salmonella*, the release of enzymes reached a plateau in 3 hr for ALP and in 6 hr for G6PDH. These results agreed with those of Malamy & Horecker (1964) who reported that ALP was an extracellular enzyme, while G6PDH was found in the cell membrane, suggesting the cell membrane to be a likely target of TMB.

Scanning electron microscopy

To observe the ultra structural change caused by TMB treatment, SEM data were collected for treated bacterial cells at various time intervals. The intracellular changes were observed in TMB treated *Salmonella* when compared to the non-treated cells. Remarkable modifications of cell membrane and disruption of cell membranes occurred after only a short period of exposure (Figure 4).

Overall, the observations of the present study indicated the efficacy of TMB in inactivating *Salmonella* by directly damaging cell membranes; whether flocculation of *Salmonella* is followed by inactivation remains to be examined. Based on the results of this study, a potential application of the TMB for inactivating *Salmonella* in water may be envisaged, given the efficacy, flocculating ability and safety demonstrated by oral toxicity studies in mice (unpublished observations). Thus the newly developed bioflocculant may have important applications in removing and inactivating *Salmonella* in water.

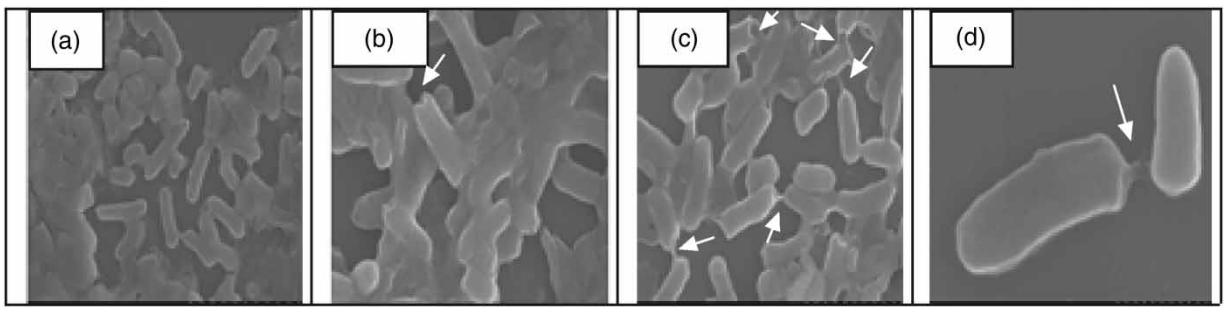

Figure 4 | SEM of *S. typhimurium* treated with TMB. *S. typhimurium*, in mid-logarithmic growth at 10^7 CFU/mL, was incubated with TMB (60 μg/mL): (a) 0 min; (b) 30 min; (c) 60 min; (d) view of a single cell.

CONCLUSIONS

This study effectively demonstrated the biocidal effects of a polymeric flocculant produced by *K. terrigena*. Quaternization was effective in imparting biocidal properties to the native biopolymer, the quaternized biopolymeric flocculant led to rapid inactivation of *Salmonella* strains. Cell death occurred presumably by a rapid release of cytoplasmic enzymes and changes in electrical conductivity. Gross morphological changes in cell membranes of bacteria were observed from electron micrographs of exposed cells. The newly developed biocidal biopolymeric flocculant may have important applications in removing and inactivating *Salmonella* in water.

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REFERENCES

Andrews, J. M. 2001 Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy* **48** (1), 5–16.

Belalia, R., Grelier, S., Benaissa, M. & Coma, V. 2008 New bioactive biomaterials based on quaternized chitosan. *Journal of Agricultural and Food Chemistry* **56** (5), 1582–1588.

Choi, B. K., Kim, K. Y. & Yoo, Y. Z. 2001 *In vitro* antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *International Journal of Antimicrobial Agents* **18** (6), 553–557.

Ghosh, M., Pathak, S. & Ganguli, A. 2009a Effective removal of cryptosporidium by a novel bioflocculant. *Water Environment Research* **81** (2), 160–164.

Ghosh, M., Pathak, S. & Ganguli, A. 2009b Application of a novel biopolymer for removal of *Salmonella* from poultry wastewater. *Environmental Technology* **30** (4), 337–344.

Kurane, R., Takeda, K. & Suzuki, T. 1986 Screening and characteristics of microbial flocculants. *Agricultural and Biological Chemistry* **50** (9), 2301–2307.

Malamy, M. H. & Horecker, B. L. 1964 Release of alkaline phosphatase from cells of *Escherichia coli* upon lysozyme spheroplast formation. *Biochemistry* **3** (12), 1889–1893.

Maskey, A. P., Day, J. N., Phung, Q. T., Thwaites, G. E., Campbell, J. I., Zimmerman, M., Farrar, J. J. & Basnyat, B. 2006 *Salmonella enterica* serovar Paratyphi A and *S. enterica* serovar Typhi cause indistinguishable clinical syndromes in Kathmandu, Nepal. *Clinical Infectious Diseases* **42** (9), 1247–1253.

Mcbain, A. J., Ledder, R. G., Moore, L. E., Catrenich, C. E. & Gilbert, P. 2004 Effects of quaternary-ammonium-based formulations on bacterial community dynamics and antimicrobial susceptibility. *Applied and Environmental Microbiology* **70** (6), 3449–3456.

Raafat, D., Bargen, K., Haas, A. & Sahl, H. 2008 Insights into the mode of action of chitosan as an antibacterial compound. *Applied and Environmental Microbiology* **74** (12), 3764–3773.

Sharan, R., Chhibber, S. & Reed, R. H. 2011 Inactivation and sublethal injury of *Salmonella typhi*, *Salmonella typhimurium* and *Vibrio cholera* in copper water storage vessels. *BMC Infectious Diseases* **11**, 204.

Siedenbiedel, F. & Tiller, J. C. 2012 Antimicrobial polymers in solution and on surfaces: overview and functional principles. *Polymers* **4** (1), 46–71.

Sieval, A. B., Thanou, M., Kotze, A. F., Vehoef, J. C., Brusse, J. & Junginger, H. E. 1998 Preparation and NMR characterization of highly substituted *N*-trimethyl chitosan chloride. *Carbohydrate Polymers* **36** (2), 157–165.

WHO 2008 Guidelines for drinking-water quality. http://www.who.int/water_sanitation_health/dwq/fulltext.pdf (accessed 9 April 2013).

Yokoi, H., Yoshida, T., Mori, S., Hirose, J., Hayashi, S. & Takasaki, Y. 1997 Biopolymer flocculant produced by an *Enterobacter* sp. *Biotechnology Letters* **19** (6), 569–573.

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