Biopolymer based intervention for *Salmonella* in water

Moushumi Ghosh and Santosh Pathak
Department of Biotechnology and Environmental Sciences, Thapar University, Patiala 147 004, India

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This study evaluated the potential of a bacterial polymer for binding and removal of *Salmonella* spp. currently deemed as a biowarfare agent in water. The biopolymer previously characterized as a polysaccharide with flocculating activity is produced extracellularly by the bacterium, *Klebsiella terrigena*. The biopolymer could effectively remove 3 log cfu/mL of *Salmonella typhimurium* ATCC 23564, within a period of 30 min from amongst other indicator (*Escherichia coli* DH5α and *Enterococcus faecalis* ATCC 35550) and enteropathogens of moderate concern (*Staphylococcus aureus* ATCC 9144) spiked in water samples at ambient temperature. The optimum dose of biopolymeric flocculant for removal of *Salmonella* was 2 mg/L. FISH and CLSM demonstrated that the removed *S. typhimurium* was selectively bound to the biopolymer matrix. The results indicate a significant possibility of using this biopolymer for rapid detection and removal of key biowarfare agents (for instance *Salmonella*) transmitted through water.

**Keywords:** Biopolymer, biowarfare, flocculation, polysaccharide, *Klebsiella terrigena*, *Salmonella typhimurium*

**Introduction**

Enteric *Salmonella* infection is a global problem both in humans and animals, and has been attributed to be the most important bacterial etiology for enteric infections worldwide\(^1\). The natural habitat of *Salmonella* is the gastrointestinal tract of animals, including birds and human beings\(^2\). This organism finds its way into the river water, coastland estuarine sediments through fecal contamination. Aquatic environments are the major reservoirs of *Salmonella* and aid its transmission between the hosts\(^2\). The survival rate of *Salmonella* in such aquatic environments is very high, outliving even *Vibrio cholerae* in highly eutrophic river water\(^3\). Besides, *Salmonella* spp. are moderately easily disseminated, causing moderate morbidity and low mortality, and require enhancements of diagnostic and surveillance capability. For these reasons, this pathogen has been categorized currently as category B biowarfare agent by CDC (Centers for Disease Control, Atlanta, USA). Since bioterrorism is difficult to predict or prevent, reliable detection as well as highly specific platforms are especially important to minimize dissemination of biothreat agents and to protect the public health.

Although inorganic and organic synthetic high MW polymers are being used extensively\(^5\) as economical options for potable water treatment, these polymers have been implicated in health problems. Besides, the polymers are difficult to biodegrade and do not have selectivity required for removal of biowarfare agents. Therefore, a biodegradable, safe polymer with flocculating ability preferably with specificity towards one or more biowarfare agents is required to be developed as an alternative to existing synthetic flocculants, for countering threats to potable water. Microbiologically produced flocculants are generally expected to be readily biodegradable and harmless to the environment and humans, indicating their potential to replace the existing synthetic flocculants\(^6\). Several types of bioflocculants have been previously reported, and some of them exhibit efficient flocculating activities comparable to synthetic flocculants for not only inorganic but also for organic suspended particles\(^7\). However, the possibility of selective removal of high risk pathogens (deemed as biowarfare agents) from potable water or sources of fresh water used frequently has not been explored. In view of these facts the application of biopolymer as a possible sustainable intervention measure was anticipated. We, therefore, evaluated the potential of a biopolymer previously characterized from *Klebsiella terrigena* in our laboratory; the biopolymer possesses high flocculating ability against a wide range of colloidal particles. Visualization of biopolymer (using FISH) binding, effective dose of this biopolymeric

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\(^*\)Author for correspondence: Tel: 91-175-2393421; Fax: 91-175-2393738 E-mail: mghosh@thapar.edu
floculant and the influence of other possible enteropathogens and indicator microorganisms in water affecting specific removal of *Salmonella* spp. were also determined.

**Materials and Methods**

**Strain and Culture Conditions**

An industrial wastewater isolate, previously isolated and identified by our group as *Klebsiella terrigena* was used in this study. The strain has been deposited (Accession number MTCC 7805) at the Microbial Type culture collection and Gene bank, Institute of Microbial Technology, Chandigarh. The strain was preserved in glycerol stock solution at −80°C.

**Purification and Determination of Flocculating Potential**

For the production of flocculating material, the strain was grown in 50 mL of FIB medium on a rotary shaker (120 rpm/min) at 30°C for 48 h. Cells were removed from the culture medium by centrifugation at 12,000 g for 30 min at 4°C. The polysaccharide was separated from the supernatant by the addition of two volumes of ethanol and precipitation at 4°C for 24 h. The precipitated polysaccharide was collected by filtration (Whatman GF Filter), dissolved in deionized water, dialyzed extensively against deionized water and lyophilized. Protein concentration was determined by Lowry method. Crude polysaccharide was dissolved in deionized water and re-precipitated by adding a 10% solution of cetylpyridinium chloride (CPC). The precipitated polysaccharide complex was collected by centrifugation at 10,000 g for 20 min at 4°C and re-dissolved in a 10% NaCl solution. The precipitated polysaccharide was recovered by addition of three volumes of ethanol. The extracted polysaccharide was dissolved in deionized water, dialyzed twice against deionized water and lyophilized. Flocculating activity of the purified polymeric suspension in water measured against distilled water as control by the method of Kurane.

Activity (%) was defined and calculated as 

\[
\frac{[B - A]}{B} \times 100.
\]

**Salmonella Removal Assays**

Cultures of *Salmonella typhimurium* ATCC 23564, *Staphylococcus aureus* ATCC 9144, *Escherichia coli* DH5α and *Enterococcus faecalis* ATCC 35550 were grown overnight in BHI broth (HiMedia, Mumbai), harvested by centrifugation, cell pellets of all the cultures were washed thrice 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 (3 replicates). Purified biopolymeric material in different doses was then added to each replicate, mixed thoroughly by hand rotation and allowed to stand at ambient temperature for 1 h. Aliquots of each sample were carefully removed by a vacuum pump, diluted in maximum recovery diluent (MRD), aliquots from each combination were plated onto the following respective selective media, viz XLD (Xylose Lysine Desoxycholate Agar), BPA (Baird Parker Agar), VRBA (Violet Red Bile Agar) and BEA (Bile Esculin Azide Agar). All the plates were incubated at 37°C for 18-24 h; counts were expressed as log cfu/mL. Control experiments comprised of similar combinations but lacked biopolymers. The agglomerated biopolymer in sediment was used for Fluorescent *in situ* hybridization (FISH).

**Salmonella spp. Removal Demonstrated by FISH**

The first step of the FISH technique is the fixation of the biopolymer flocculated sample. Two equal proportions (from 10 mL), 5 mL each were disposed into 50 mL sterile Falcon tubes. The first aliquot was fixed by mixing with 300 μL of 4% paraformaldehyde in PBS, and incubated at 4°C for 16 h. Fixed samples were washed twice with PBS, resuspended in 50% ethanol in PBS, and stored at −20°C. The remaining 5 mL of the samples was centrifuged (8000 rpm, 20 min) and pellets were resuspended in 5 mL of a 96% ethanol/PBS solution (1:1, v/v) and stored at −20°C until used for FISH experiments; 1 μL of a solution of DNA interacting dye (DAPI) (200 ng μL⁻¹) at 42°C for 2 h; 2 μL of the fixed cell solution was added to a teflon poly-lysin coated slide (Merck, Darmstadt, Germany). The sample was allowed to dry on the slide at 42°C for 20 min and subsequently dehydrated by passing through successive washes of 50, 80, and 96% ethanol for 3 min each. Hybridizations were performed in 9 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) in the presence of 10% Sal 3, 25% ENF191, 30% Eub338 formamide, 1 μL of Sal3-Cy3 (indocarbocyanine) labeled oligonucleotide probe or EUB338 labeled with indodicarbocyanine(Cy5). ENF191 labeled with FLUOS [5(6)-carboxyfluorescein-N-hydroxysuccinimide ester]. These oligonucleotide probes were 5'-labeled and unlabeled competitor oligonucleotides were obtained from MWG (Ebersberg, Germany). The sequences of
oligonucleotide probes used in this study (Table 1) bound to nearly all members of the domain bacteria, and were used to verify that all the cells present were permeable for probes and thus accessible for in situ hybridization with more specific probes\textsuperscript{11-16}.

After hybridization, the slides were gently washed with distilled water and immersed in buffer containing 20 mM Tris-HCl, pH 7.2, 10 mM EDTA, 0.01% SDS and 440 mM NaCl for 15 min at 43°C. Slides were removed from buffer, rinsed with distilled water and air-dried\textsuperscript{10}. Fixed cells of \textit{Salmonella typhimurium} ATCC 23564 and \textit{Escherichia coli} DH5α were always used as positive and negative controls, respectively.

**Microscopy and Digital Image Analysis**

The slides were then mounted with Citifluor AF1 immersion oil solution (Citifluor Ltd., London, UK) and examined with a Nikon microscope (Nikon, Lewisville, TX) fitted for epifluorescence microscopy with a high-pressure metal halide lamp and filter sets F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, D460/50, for DAPI detection). The probe positive signals were visualized and pictures of fluorescent cells were recorded using a Confocal laser scanning microscope (CLSM 510, Zeiss, Oberkochen, Germany). For the detection of Cy5 (Helium –Neon 633 nm) Fluos-labeled cells, an argon laser (450 to 514 nm) was used and for Cy3, helium-neon laser (543 nm) was used to visualize probe positive signals. For each microscope field, fluorescence conferred by the different probes was recorded in separate images. For each hybridization experiment, 30 microscope fields at random positions and in random focal planes were recorded using a Zeiss Plan-Neofluar 40×/1.3 oil objective. This procedure (30 images at low magnification) allowed us to record a high number of probe-target cells and thus to accurately determine the relative abundance of heterogeneously distributed probe-target cells. All the acquired pictures corresponded to optical sections obtained by adjusting the pinhole diameter of the CLSM were accordingly recorded as 8-bit images of 512/512 pixels with a resolution of 1.6/1.6 pixels per μm.

**Results and Discussion**

**Salmonella Removal Assays**

The initial counts of spiked enteropathogens (\textit{S. aureus} ATCC 9144, \textit{E. faecalis} ATCC 35550) and \textit{E. coli} DH5α in tap water samples are presented in Table 2. Following addition of biopolymer and subsequent to the treatment process, aliquots of tap water samples were diluted in 0.1% MRD and suitable dilutions plated onto respective selective media as well as on TAL (Thin Agar Layer plates). Significant reduction (P<0.5) in numbers of \textit{Salmonella} (3 log reduction) were observed in both selective and TAL plates. Counts of \textit{S. aureus}, \textit{E. faecalis} and \textit{E. coli} DH5α remained unaltered. Biopolymer dose optimization trials carried out indicated that the optimum dosage of the \textit{K. terrigena} bioflocculant was 2 mg/L for achieving the removal of 3 log cfu/mL spiked \textit{S. typhimurium} (Table 3). A higher removal efficiency of \textit{Salmonella} spp. by the biopolymer at doses exceeding that of 2 mg/L was not observed.

**FISH for Visualization of Selective Agglomerated \textit{Salmonella} by the Biopolymer**

After the tap water was treated with the biopolymer, visualization and simultaneous detection

Table 2—Selective removal of \textit{S. typhimurium} ATCC 23564 from mixed cultures in tap water by purified \textit{Klebsiella terrigena} biopolymer

<table>
<thead>
<tr>
<th>Microorganisms used</th>
<th>Initial counts (log cfu/mL)</th>
<th>Final counts (log cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. typhimurium} ATCC 23564</td>
<td>3.0</td>
<td>a</td>
</tr>
<tr>
<td>\textit{S. aureus} ATCC 9144</td>
<td>3.0</td>
<td>2.91</td>
</tr>
<tr>
<td>\textit{E. coli} DH5α</td>
<td>3.0</td>
<td>2.93</td>
</tr>
<tr>
<td>\textit{E. faecalis} ATCC 35550</td>
<td>3.0</td>
<td>2.98</td>
</tr>
</tbody>
</table>

Results are average of three independent trials. Each trial comprised of three replicates

\textsuperscript{a}Biopolymer for removal studies was dosed at 200μg /L

Table 1—Sequences and target organisms of oligonucleotide probes used in this study

<table>
<thead>
<tr>
<th>Probes</th>
<th>Probe target</th>
<th>Sequence</th>
<th>Position</th>
<th>Target organism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB</td>
<td>16S rRNA</td>
<td>GCCTGCTCCCCTGGAGGAGT</td>
<td>\textit{E. coli} 338-355</td>
<td>Bacterial kingdom</td>
<td>11</td>
</tr>
<tr>
<td>Non-EUB</td>
<td></td>
<td>ACTCTTTACGGGGAGGACG</td>
<td></td>
<td>Negative control</td>
<td>12</td>
</tr>
<tr>
<td>GAM42a</td>
<td>23S rRNA</td>
<td>GCCTTCCCACATGTTT</td>
<td>\textit{E. coli} 1027-1043</td>
<td>γ proteobacteria</td>
<td>13</td>
</tr>
<tr>
<td>Sal3</td>
<td>23S rRNA</td>
<td>AACATCTCCACCTTG</td>
<td>\textit{S. enterica} 1713-1730</td>
<td>\textit{Salmonella enterica}</td>
<td>14, 15</td>
</tr>
<tr>
<td>ENF 191</td>
<td>16S rRNA</td>
<td>GAA AGC GCC TT TAC TTATGCG</td>
<td>\textit{E. faecalis} 191-233</td>
<td>Enterococcus faecalis</td>
<td>16</td>
</tr>
</tbody>
</table>

*Previously published probes used in this study are referred to.
of the biopolymer matrix was carried out directly, using FISH in combination with CLSM. The probes used in this study are given in Table 1. Hybridization with probe EUB338, which is complementary to a portion of the 16S rRNA gene conserved in the domain bacteria, was used to visualize the entire bacterial population in the specimens. Also all detected cells stained with the DNA intercalating dye DAPI demonstrated that they were permeable to probes and therefore good candidates for in situ hybridization. The results obtained with the non-EUB probe indicated the absence of non-specific interaction between the probe and constituents of the cellular matrix. Hybridizations with probe EUB338 and the γ-proteobacterial probe GAM42a resulted in strongly positive fluorescence hybridization signals for E. faecalis and S. typhimurium (data not shown) (Fig 1A). The more specific probe Sal3, which binds to 23S rRNA of all Salmonella enterica subspecies tested so far (excepting only subspecies IIIa), was then used to verify the presence of Salmonella in the biopolymeric matrix. Sensitivity testing showed that the probes were able to detect as low as 10^4 cfu/mL. Fig. 1 B and C demonstrate that with the universal eubacterial probe EUB 338-Cy5, bacteria could be detected by FISH, the observed bacteria were located within the biofloculant matrix; FISH positive signals obtained using simultaneous EUB338-Cy5 and Sal3-Cy3 oligonucleotide probe signals revealed distinct morphotype of small bacterial rods, detected as Salmonella. Phase contrast microscopic analysis allowed 3-dimensional reconstruction of the biopolymer matrix and confirmed that the cells observed were bacteria; the spatial distribution of the bacteria was also evident. The organisms were located enmeshed on the surface and were also visible within layers of the biopolymer. We could also successfully carry out FISH of E. faecalis ATCC 35550 when ENF191–Fluos probe was also used with EUB338-(Cy5). On hybridization with probe EUB338, no hybridization signals of the active bacterial population were observed in the fixed biopolymer specimen. Before fixation, the sample was subjected to flocculate E. fecalis (10^7 CFU/mL) in tap water. Although hybridization with probe ENF191 was successful in the tap water sample without biopolymer addition as shown in Fig. 1. FISH positive signals for the test organism were visualized as light blue in the unflocculated control water sample. This clearly

Table 3—Optimization dose of purified Klebsiella terrigena biopolymer for flocculating S. typhimurium ATCC 23564 spiked in tap water

<table>
<thead>
<tr>
<th>Biopolymer dose (mg/L)</th>
<th>Initial</th>
<th>Final counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>3.0</td>
<td>2.65</td>
</tr>
<tr>
<td>0.08</td>
<td>3.0</td>
<td>2.41</td>
</tr>
<tr>
<td>0.1</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.2</td>
<td>3.0</td>
<td>b</td>
</tr>
<tr>
<td>0.4</td>
<td>3.0</td>
<td>2.98</td>
</tr>
<tr>
<td>0.6</td>
<td>3.0</td>
<td>2.97</td>
</tr>
<tr>
<td>1.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Salmonella typhimurium counts (log cfu/mL)
Results are average of three independent trials. Each trial comprised of three replicates

Fig. 1—(A), Confocal laser scanning micrograph of FISH of the unflocculated control water sample. Hybridization with oligonucleotide probes ENF191–FLUOS, Sal3-Cy3 and Eub338-Cy5. Excitation wavelengths: 488 nm (FLUOS), 543 nm (Cy3), and 633 nm (Cy5) for green, red and infrared fluorescence, respectively. Signals displayed as an rgb image. E. faecalis appear in light blue, S. typhimurium in pink; (B) & (C), Phase contrast and Confocal laser scanning micrographs, respectively of FISH of the sample showing selective binding of Salmonella to biopolymer. Phase contrast and CSLM analysis of agglomerated biopolymer matrix after FISH with S. typhimurium have aggregated on the biopolymer matrix. Hybridization with oligonucleotide probes Eub338-Cy5 and Sal3-Cy3 excitation wavelengths were 543 nm (Cy3), and 633 nm (Cy5), red and infrared fluorescence, respectively. Signals displayed as an rgb image by overlay of infrared fluorescence in both channels, blue and red. The signals of the S. typhimurium appear pink. Measure bars=5µm.
indicated that the biopolymer possessed specific binding affinity for the cells of Salmonella spp., although taxonomically both the organisms belong to same family and subclass.

Floculation by polymers may be viewed upon as a process where bacteria adhere to each other through the adsorbed flocculant on their surfaces. The attachment or aggregation is controlled by a complex interplay of interaction forces between the bacterium in a bulk phase and a substratum and other bacterium. The most relevant interactions are van der Waals forces (VDW), electrostatic interactions, hydrophobic interactions and polymer interactions. Since microbes as well as naturally occurring surfaces usually carry negative charge, the electrostatic interactions between them are repulsive and the bacterial adhesion must occur as a result of attraction due to other interactions, such as VDW, hydrophobic or polymer interactions. The Klebsiella terrigena biopolymer, previously characterized as a polysaccharide, possesses excellent flocculating activity against colloidal particles. The flocculating activity is stable over a wide pH range. It was therefore anticipated that this biopolymeric flocculant may be useful for removal of bacterial cells as well from waterbodies. The results of Salmonella removal through direct plating on selective media were confirmed by the TAL method, which can resuscitate injured microorganisms exposed to various environmental matrices, by simultaneously enriching them as well as providing selectivity, thus allowing adequate enumeration of cells. Absence of Salmonella in both cases confirmed complete removal of the cells.

To further elucidate the observations, FISH and CLSM of fixed biopolymer samples were carried out. Hybridization with probe EUB338, which is complementary to a portion of the 16S rRNA gene conserved in the domain bacteria, was used to visualize the entire bacterial population in the fixed biopolymer sample. All the cells stained with DAPI demonstrated that they were permeable to probes and, therefore, good candidates for in situ hybridization. Also by using the control probe non EUB338 and DAPI staining, non-specific binding could be excluded, which gave confidence to the results achieved; in addition, autofluorescence was excluded by viewing the sections prior to the FISH procedure. Repeated microscopic evaluation confirmed the unambiguity of the interpretation of the images obtained. The bright signal intensities of the bacteria indicated a high amount of rRNA, which is an evidence for physiological activity of the cells at the time of sampling. CLSM, which was carried out in conjunction with FISH allowed optical sectioning and 3-dimensional reconstruction for localization and observation of the spatial distribution of bacteria in different layers of the bioflocculant material. The more specific probe Sal3, which binds to 23S rRNA of all S. enterica subspecies tested so far (excepting only subspecies IIIa), was then used to verify the presence of Salmonella in the biopolymeric matrix after the removal by the bioflocculant. Specificity testing of the Sal3 oligonucleotide probe demonstrated that it was highly specific and hybridized only with target organisms. Sensitivity testing showed that the probes were able to detect as low as 10^3 cfu/mL. FISH technique may also have a potential value in elucidating the specificity and thus the mechanism of binding of biopolymeric material to Salmonella. It is difficult to explain why hybridization signals of the active bacterial population of E. faecalis were not observed in the fixed biopolymer matrix, when the sample was allowed to flocculate E. faecalis (10^7 cfu/mL) in tap water before fixation. Nevertheless, explanation of the observed selective binding cannot be made considering purely electrostatic interactions as key players. There is also increasing evidence that many of the surface appendages, especially of pathogens, carry specific adhesins involved in specific interactions with receptors on the host cell. It is possible that attachment of Salmonella to the biopolymer occurred through a specific binding mechanism. Absence of such mechanisms in S. aureus, E. faecalis or E. coli might be the possible explanation for this observation. However, these propositions require detailed information of the biopolymer structure.

**Conclusion**

The present investigation demonstrated for the first time the use of biopolymers for selectively targeting and removing Salmonella, an extremely important pathogen, which can be transmitted through water. The importance of pathogens (as well as toxins) has gained enormous significance currently, for global security reasons, following several reports of intentional use of these microorganisms (and toxins) as potential bio-weapons. Consequently, methods for rapid, accurate detection, concentration and possible destruction of pathogens in either food or water have been advocated as key prerequisites for developing efficient counter measures to these biowarfare agents.
Biopolymers such as the one described in this study, may be used at multiple level platforms by polymer engineering approaches for instance, covalent attachment of fluorescent or suitable chromophoric groups (which will signal upon attachment of the target pathogen). We anticipate that incorporation of suitable antimicrobial agents in the biopolymer may lead to inactivation of select biowarfare agents; therefore, engineering pre-characterized biopolymers for antimicrobial activity is under investigation.

Acknowledgement
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References