DEVELOPMENT OF QUATERNIZED BIOPOLYMERIC FLOCCULANTS FOR WATER TREATMENT

A thesis
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for the award of the degree of

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IN
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Certificate

Certified that the thesis **"Development of quaternized biopolymeric flocculants for water treatment"** which is submitted by **Ms. Gurpreet Kaur Khaira**, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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Candidate Declaration

I hereby declare that the work which is being presented in this thesis "**Development of quaternized biopolymeric flocculants for water treatment**" submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Moushumi Ghosh**, Associate Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

Gurpreet kaur khaira
18/7/2014

Gurpreet Kaur Khaira

Human subtlety will never devise an invention more beautiful, more simple, or more direct than does Nature—because in her inventions, nothing is lacking—and nothing is superfluous. . .

Leonardo da Vinci

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Abstract

Providing safe drinking water is an important public health issue and recent studies have recognized commonly used flocculants and disinfectants responsible for unintended health hazards. An increased resistance of waterborne pathogens to synthetic biocides and environmental considerations underpin an urgency to develop strong, economically viable and ecofriendly replacements of conventional synthetic flocculants and disinfectants. Distinctive nature of microbial extracellular polymers containing amino groups can be explored in hope to obtain effective and safe substitute to chemical biocides. Although, many natural and synthetic water treatment agents are available in the market, but natural products are not as efficient as the semi-synthetic or synthetic compounds in terms of efficacy. Thus in the present study, an attempt was made to design a semi-synthetic flocculant-disinfectant replete with sustainability, cost effectiveness, biocompatibility, non-toxic polymers with high inactivation efficacy of waterborne pathogens.

In an attempt to develop antibacterial polymer(s) with dual flocculant-disinfectant property, we chemically altered the surface properties of an amino sugar rich biopolymeric flocculant produced by *Klebsiella terrigena.* Chitosan, most extensively modified polymer, was used as reference compound due to its structural similarity to the bioflocculant. Alteration were brought about in the native biopolymers through quaternization, and the trimethyl biopolymeric derivatives (N,N,N trimethyl biopolymer and N,N,N trimethyl chitosan, abbreviated as TMB and TMC, respectively) were analyzed physically, chemically and for flocculating properties. TMB did not differ significantly ($p<0.05$) in either chemical, physical properties or flocculating ability when compared to its native counterpart. The antibacterial activity of water-soluble derivatives was investigated against six prevalent water borne-pathogens, viz. *Salmonella typhimurium* ATCC

25315, *Shigella flexneri* 2a, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* O157:H7 ATCC 32150. Results revealed an inactivation of 4 log CFU/ml of all water borne pathogens with TMB as compared to TMC which resulted in inactivation of 3 log CFU/ml, over a short contact time (30-60 minutes) and low dosage (30-90µg/ml) at ambient temperature. The biochemical and physiological insights on mode of action of quaternized derivatives suggested membrane damage by TMB to be the principal mechanism for inactivation against all the bacteria.

In the *Salmonella* Ames assay, TMB failed to induce His^{+} revertants in a significant manner ($p<0.05$) indicating that TMB lacked mutagenic or carcinogenic potential. Further, study on mortality, gross toxic effects, behavioral changes and the hematological, histopathological and general examinations indicated no adverse effects in acute toxicity studies in Swiss albino mice fed with the quaternized biopolymer (140mg/Kg/bodyweight/day) over a period of 30 days. Overall the study 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 water borne pathogens.

Keywords: Bioflocculant, antibacterial polymer, water borne pathogens, quaternization, cell permeability, toxicity.

CONTENTS

List of Abbreviations

MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NFA	N-2-fluorenylacetamide
2-AA	2-aminoanthracene
DADMAC	poly (diallyl dimethyl ammonium chloride)
DBPs	Disinfection Byproducts
NOM	Natural Organic Matter
THMs	Trihalomethanes
HAAs	Haloacetic acids
BDCM	Bromodichloromethane
CPC	Cetylpyridinium chloride
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetra acetic acid
EPS	Extracellular polymeric substances
FIB	Flocculant isolation broth
BHI	Brain heart infusion
FTIR	Fourier transform infrared spectroscopy
GPC	Gas permeation chromatography
H_2O_2	Hydrogen peroxide
BF	*Klebsiella* biopolymeric flocculant
QBF	Quaternized Biopolymeric flocculant
TMC	N,N,N trimethyl chitosan derivative
TMB	N,N,N trimethyl biopolymeric derivative
LB	Luria Bertani
NTU	Nephelometric Turbidity Units
OD	Optical Density
ppm	Parts per million
rpm	Revolution per minute
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TAL	Thin Agar Layer
UV	Ultraviolet

List of Symbols

%	Percentage
µ	Micron
C	Carbon
Ca	Calcium
Da	Dalton
g	Gram
H	Hydrogen
hr	Hours
Hz	**Hertz**
kb	Kilo base
KDa	Kilo Dalton
kV	Kilo volt
L	Litre
M	Molar
mA	Milli ampere
mg	Milligram
Mg	Magnesium
Min	Minutes
mL	Milliliter
N	Nitrogen
NCC	Nebauer cell counter
ng	Nanogram
OD	Optical Density
Pa	Pascal
Sec	Seconds
U	Unit
V	Volt
v/v	volume by volume
w/v	Weight by volume
β	Beta
µg	Microgram
µl	Microlitre

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LIST OF PUBLICATIONS

Publications in peer reviewed journals

1. **Khaira, G.K.**, Ganguli, A. and **Ghosh, M.** (**2014).** Antimicrobial efficacy and *in vivo* toxicity studies of a quaternized biopolymeric flocculant. Journal of Water and Health. doi:10.2166/wh.2014.186.
2. **Khaira, G.K**., Ganguli, A. and **Ghosh, M**. **(2013)** Synthesis and evaluation of antibacterial activity of quaternized biopolymer from *K. terrigena.* Journal of Applied Microbiology, 116, 3: 511-518.
3. **Khaira, G.K.,** Ganguli, A. and **Ghosh, M**. **(2013)** Rapid inactivation of Salmonella by a quaternized biopolymeric flocculant. Water Science & Technology: Water Supply, 14, 1:31-37.
4. **Khaira, G.K.,** Kumarya, R., Chhibber, M. and **Ghosh M**. **(2013)** Development of a quaternized chitosan with enhanced antibacterial efficacy. Journal of water & health, 11, 3: 410–418.

Conferences proceedings

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2. **Khaira, G.K.,** Chhibber, M. and **Ghosh, M.** Development of quaternized biopolymer flocculant for effective removal and inactivation of *Salmonella* in potable water. 13th International Symposium on Biopolymers, ISBP2012, Cairns, Queensland, Australia. (Oct. 7-10, **2012**).
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CHAPTER 1

Introduction

1. INTRODUCTION

Natural water is a complex colloid system containing organic and inorganic substances as well as thin-dispersed components. They are too small to be settled by gravity or filtered through the common filtration media. In addition, colloidal suspensions are stable in water due to their electrical charge. During the past decade, substantial alterations in the composition of water colloid systems have occurred due to the presence of diverse chemical and biological contaminants arising mainly from sewage, industrial wastes and agricultural chemicals (Tripathy and Ranjan 2006). This has led to deterioration in the quality of natural water in terms of chemical and biological constituents. Since majority of waterborne contaminants exist as dispersed solid particles or colloids, separation of such colloidal particles from suspension stabilizing forces that prevent aggregation is essential. Flocculants cause solid–liquid separation by an aggregation process of colloidal particles, termed as flocculation resulting in flocs which can be removed (Brostow et al. 2009) easily. Therefore a combination of Coagulation/Flocculation, sedimentation and filtration is the most widely accepted water treatment technology (Tripathy and Ranjan 2006).

Currently used organic and inorganic chemical flocculants, possess (Bolto and Gregory 2007) potential disadvantages due to their recalcitrant, neurotoxic and carcinogenic nature (Yokoi et al. 1997). Therefore, biomaterials, mimicking the functions of synthetic flocculants have been sought after as possible alternatives for ensuring safety of environment and human health during water treatment practices (Salehizadeh and Shojaosadati 2001). Flocculation has been shown to be mediated by the presence of a variety of biomaterials, for instance, microorganisms or bioflocculants-which are biodegradable macromolecular flocculant secreted by microorganisms (Gao et al. 2006). A wide gamut of studies have testified the efficacy of bioflocculants in comparison to traditional flocculants (Ma et al. 2008; Ghosh et al. 2009a,b). These includes starch, alginates, guar gum, polyglutamic acid, bacterial exopolysaccharides, products based on chitin, glue and gelatin which are mainly polymers derived from animal, plants or microbial sources. Key features of bioflocculants that have been ascribed to their efficiency are high molecular weights, shear stability, low dosage requirement, inertness to pH changes, formation of large cohesive flocs (Li et al. 1999; Ghosh et al. 2009a,b). Besides, biopolymeric flocculants can be tailored from the knowledge of molecular weight, molecular weight distribution, chemical structure, nature and ratio of functional groups on the polymeric backbone.

Microbial pathogens which lie in the ambit of colloidal particles present a formidable challenge to the separation process from water since they require inactivation prior to human consumption. Chemical disinfectants used to achieve this have singular disadvantages owing to their toxicity, carcinogenicity and induction of resistance to pathogens. An obvious resolution to this problem lies in application of effective disinfectants of biological origin. Biomaterials, especially biopolymers owing to their attributes as flocculants and amenability of functional group modification are desirable candidates for this purpose. The cationic nature rendered upon biopolymers through modifications such as quaternization is deemed to be highly effective on account of hydrophobicity and potential disruptive effects on the cell membrane of target pathogens. The biopolymer chitosan, a copolymer of glucosamine and N-acetyglucosamine units linked by 1,4-glucosidic bonds is obtained through the alkaline hydrolysis of chitin (Tan et al. 2013) and represents an interesting example of modified biopolymer. The high biodegradability and non-toxicity to mammals have enabled chitosan to be widely used as antimicrobial agent, either alone or blended with other natural polymers (El Hadrami et al. 2010; Badawy and Rabea 2011).

The antimicrobial activity of chitosan against a variety of bacteria and fungi has been well reported (Muzzarelli et al. 2001; Badawy and Rabea 2011; Xia et al. 2011). Unfortunately, this activity is limited to acidic conditions because of its poor solubility above pH 6.5, since chitosan starts to lose its cationic nature and become poorly soluble under these conditions (Badawy and Rabea, 2011). Therefore, subsequent efforts have focused on the preparation of derivatives soluble in water over a wide pH range as water solubility is an important factor in applications of chitosan as an antimicrobial agent (Lim and Hudson 2004), moreover, its bactericidal application on Gram positive bacteria are less pronounced restricting its usage in terms of a broad spectrum biocidal (Takemono et al. 1989; Kim and Choi 2002).

Though the above deliberations clearly indicate the feasibility of biopolymers to be functionalized as novel disinfectant agents apt for water treatment, it also poses a challenge in terms of designing superior biopolymeric agents lacking the inherent drawbacks of solubility and wide functionality. Biopolymeric flocculants especially those elaborated by bacteria have been explored as a judicious alternative to water treatment and may be viewed upon as interesting agents for this purpose. Systematic studies therefore must adequately characterize microbial exopolymers with potential flocculating properties prior to structural modification to bestow upon a biocidal functionality for a dual purpose ‘water treatment agent”. Importantly, the biopolymeric flocculants should qualify the criterion of non toxicity /carcinogenicity through *in vitro* and *in vivo* tests. In fact, extensive safety assessment have been carried out for several commercially valuable microbial exopolysaccharides, especially dextran, beta-glucan and the differences in molecular weight and route of administration have been shown to significantly affect their distribution and excretion (Honarkar and Barikani 2009; Delaney et al. 2003; Jonker et al. 2010). For biopolymeric flocculant, human exposure is expected through an oral route. Therefore mutagenicity/carcinogenicity through initial *in vitro* screens may offer leads for further *in vivo* oral toxicity based assays.

In this study, a pre-characterized bioflocculant with unique properties of robustness and high flocculating ability for waterborne pathogens (Ghosh et al. 2009a,b) was chosen for development of disinfectant. Chitosan was used as reference compound, since the bioflocculant bears structural similarity with chitosan, a natural coagulant (Ghosh et al. 2009a; Badawy and Rabea 2011). An attempt was also made to design a semi-synthetic flocculant-disinfectant with high inactivation efficacy of waterborne pathogens. It was anticipated that an efficient and robust flocculant–disinfectant would be beneficial in formulating a novel approach for disinfecting water.

Accordingly the specific Objectives of this thesis are

1. Development of quaternized derivatives of bioflocculant and evaluation of the efficacy of developed variant
2. Studies on physiological, biochemical and molecular responses of microbial inactivation
3. Toxicological studies on the synthesized derivative of bioflocculant

CHAPTER 2

Review of Literature

2. REVIEW OF LITERATURE

Over the turn of the century, quality of water has been strongly recognized as an indicator of socio-economic development. This is because the quality of water, whether used for drinking or domestic purposes, food production, or recreational purposes has an important impact on health. In fact, water of poor quality can cause disease outbreaks and can contribute to background rates of disease manifesting themselves on different time scales.

2.1 Colloids in water environment

Colloidal particles are present in virtually all raw waters. These particles constitutes of minerals, organic and biological matter and they fall into a general category of very fine material, normally less than one micron in size, having electrostatic surface charges. The larger size particles are relatively easy to settle and can be easily removed by filtration. The fine and smaller size particles present the real challenge as their settling time is intolerably slow and they easily escape the process of flocculation. Most of the colloidal particles are negatively charged, hence experience repulsion due to this like charge present on the adjacent particles, which in turn prevents the formation of agglomerates and process of flocculation. Bacteria range in size from 0.2 to 5µm and viruses from 0.005 to 0.1µm which is within the size range considered colloidal.

The theory of colloid stability is based on the recognition of two forces in any stabilized solution: (i) the electrostatic repulsion which opposes aggregation, and (ii) a universal attractive Vander Waals force which acts to bind particles together (Sharma et al. 2006, Tripathy and Ranjan 2006). The energy of Brownian motion is sufficient to prevent the particles from settling under gravity and particles remain suspended for long periods of time. In general, most colloidal materials have negative charge. Initially, attraction from the negative colloid causes some of the positive ions to form a firmly attached layer around the surface of the colloid, the layer of counter-ions i.e., Stern layer (Ivanov et al. 2007; Gogtas 2012). Additional positive ions are still attracted by the negative colloid but now they are repelled by the positive stern layer as well as by other nearby positive ions that are also trying to approach the colloid (Ivanov et al. 2007). A dynamic equilibrium result forming a diffuse layer of counter-ions and the negative colloid and its positively charged atmosphere produce an electrical potential across the diffuse layer. This is highest at the surface and drops off progressively with distance, approaching zero at the outside of the diffuse layer (Koohestanian et al. 2008). The potential curve is useful because it indicates the strength of the repulsive force between colloids and the distance at which these forces come into play. A particular point of interest on the curve is the potential at the junction of the Stern layer and the diffuse layer, called as zeta potential. The magnitude of zeta potential is usually used as indication of colloidal particle stability (Crocker and Grier 1998; Koohestanian et al. 2008).

2.2 Water treatment system overview

Colloidal particles are the major causes of objectionable turbidity, color, taste and odor in drinking water. Conversion of stable state dispersion to the unstable state is termed destabilisation and the processes of destabilisation are coagulation and flocculation (Tripathy and Ranjan 2006). The enormous efforts to improve the quality and safety of water have been documented; to date the most common strategy for potable water treatment remains to be coagulation, a chemical technique directed toward destabilization of particle suspension (Tripathy and Ranjan 2006). Coagulation is usually followed by flocculation, which is a slow mixing technique promoting the aggregation of the destabilized (coagulated) particles followed by filtration (slow sand). Other treatment options include the use of activated alumina, granulated activated carbon as well as ion exchange and reverse osmosis (APHA 1975, Chaiket et al. 2002, Tripathy and Ranjan 2006).

2.2.1 Flocculation

Flocculants are generally used as sedimentation aids for the solid liquid separations. Coagulation is the first stage of destabilization, and basically its action is to neutralize or reduce electric charges and promote the agglomeration of these particles. Inorganic coagulants such as ferric chloride and aluminium sulphate have been used to clarify water for years (Tripathy and Ranjan 2006; Norton et al. 2009). After colloidal suspensions have been destabilized by coagulants, flocculant polymers are frequently used to enhance the performance of clarification processes. Because of their very high molecular weights (very long chain of monomers), and of their ionic charge, flocculants will bridge destabilized particles together (Koohestanian et al. 2008). The forces involved in this bridging between particle and polymer are mainly ionic and hydrogen bonding. Flocculants are generally classified into three major groups: 1) inorganic flocculants such as aluminium sulphate and poly aluminium chloride, 2) Organic synthetic polymers such as polyacrylic acid and polyacrylamide derivatives, 3) bioflocculants such as chitosan, sodium alginate, gelatin and microbial polymers (Kurane et al. 1986). The synthetic flocculants are available in three ionic forms, cationic, anionic and non-ionic. Polyacrylamide, polyacrylic acid, poly (styrene sulphonic acid), poly (diallyl dimethyl ammonium chloride) (DADMAC), etc are some of the most important synthetic flocculants (Tripathy and Ranjan 2006).

However, several synthetic flocculants have been implicated as neurotoxic and strongly carcinogenic to human (Yokoi et al. 1997). The role of aluminium, a major component of poly (aluminium chloride) in induction of Alzheimer’s disease was reported by Sanchez-Martin et al. (2012). Accumulation of sludge produced following enormous use of inorganic synthetic flocculants intensify the issue of toxicity and environmental concern (Brostow et al. 2009; Salehizadeh and Shojaosadati 2001).

2.2.2 Disinfection practice

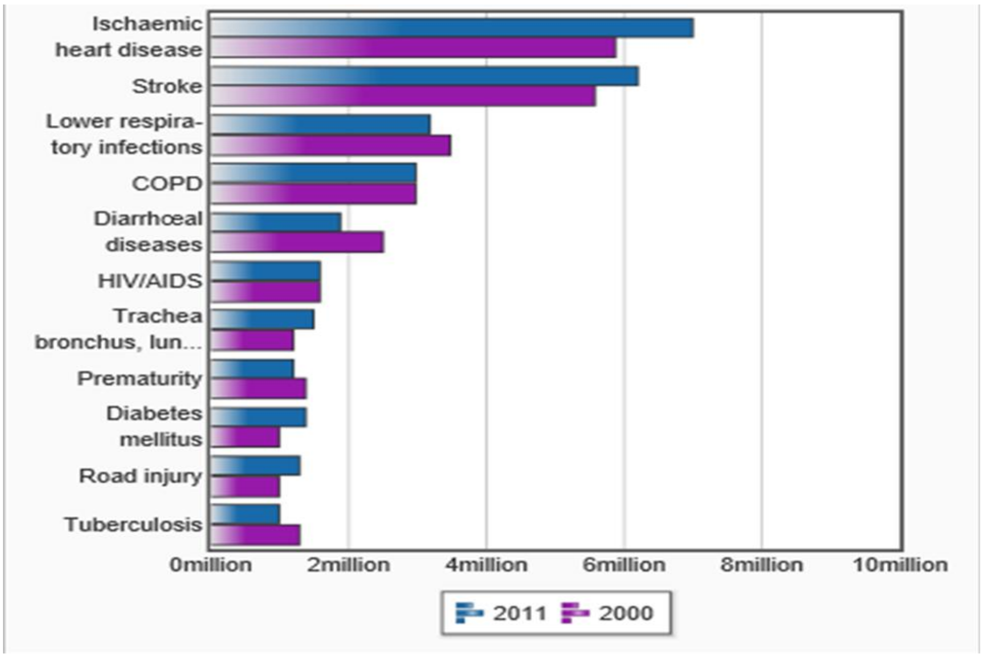
Disinfection technologies kill or screen biological contaminants present in a water supply. Currently, chlorination, microfiltration, ozone, and ultraviolet light are the four major technologies used in water disinfection (Tripathy and Ranjan 2006; Lantagne 2010). Chlorination is the most widely used method of disinfection of stored potable water. However, the concern over disinfection by-products (DBPs) produced by chlorine led to a series of studies to investigate alternative disinfectants (Chaiket et al. 2002; Lantagne 2010). DBPs are formed when chemical disinfectant reacts with the DBP precursors (natural organic compounds) present in the water to form potentially harmful chemical by-products trihalomethanes (THMs) and haloacetic acids (HAAs), both of which are carcinogenic in large quantities and results in itching of eyes and skin irritation on contact (Adamowicz et al. 2011). In fact, over 250 different types of DBPs have been identified till date, and the behavioral profile of twenty have been elucidated (Sadiq and Rodriguez 2004). Ozonization and UV irradiation are other effective methods, which are expensive and more suitable for point of use water rather than supply and distribution system (Krasner 2009).

2.4.2.1 Disinfectant by-products in water

Most widely used disinfectants viz chlorine gas, chloramine and chlorine dioxide are known to be strong respiratory irritants (Adamowicz et al. 2011). Evidence from few animal and human studies suggested that chlorine, hypochlorite solutions, chloramine and chlorine dioxide themselves probably do not contribute to the development of cancer or any toxic effects. However, the wide variety of disinfection by-products (DBPs) that result from reactions of chlorine and other disinfectants with Natural organic matter (NOM) which is found in virtually all water sources pose major health risk. Trihalomethanes (THMs), haloacetic acids (HAAs) and bromodichloromethane (BDCM) are known DBPs which have been reported to induce cytotoxicity in the liver and kidneys, also reduced the sperm motility in rats (WHO 2000). Among the haloaldehydes and haloketone, chloral hydrate induces hepatic necrosis in rats at doses equal to or greater than 120mg/kg of body weight per day (WHO 2000). Limited toxicity data are available for the other halogenated aldehydes and ketones, however, liver tumours were noted in a lifetime drinking-water study with chloroacetaldehyde (WHO 2000).

The toxic action of chlorite is primarily in the form of oxidative damage to red blood cells at doses as low as 10mg/kg of body weight. There are indications of mild neuro-behavioural effects in rat pups at 5.6mg/kg of body weight per day. There are conflicting data on the genotoxicity of chlorite (Couri et al. 1982). The toxicity of chlorate is similar to that of chlorite, but chlorate is less effective at inducing oxidative damage. It does not appear to be teratogenic or genotoxic *in vivo* (Couri et al. 1982).

2.3 Leading Infectious diseases: water borne diseases as global threat

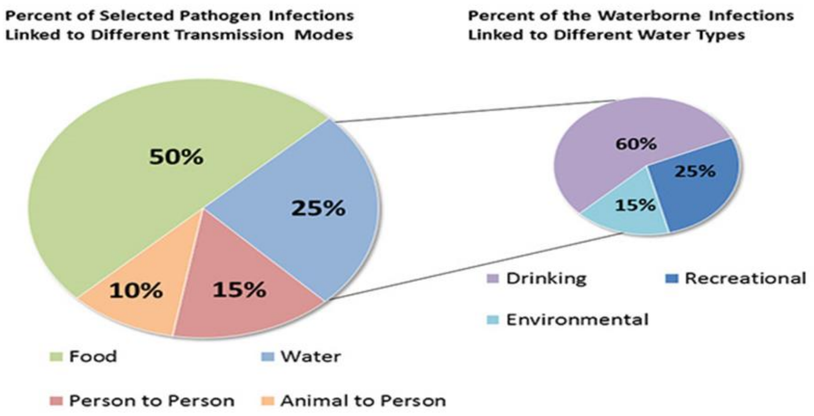


Source: http://www.who.int/mediacentre/factsheets/fs310/en/

Figure 2.1: Comparison of top ten leading causes of death in world over the past decade, 2000 and 2011

WHO estimates that in 2008, diarrhea occupied a leading position among diseases as a cause of death and illness, killing 2.5 million people and causing approximately 4 billion cases of illness annually (WHO 2008). Burden of water borne disease estimates are constantly updated. WHO is currently reassessing these estimates including the global burden of disease attributable to water, sanitation and hygiene. Based on the work conducted to date, the number of deaths due to diarrhoea will be substantially lower and likely less than 2 million. While the preliminary estimate shows a significant decline in diarrhoeal relate deaths compared to prior WHO estimates, diarrhoea remains a leading cause of mortality (WHO 2011a,b; Liu et al. 2012).

Figure 2.1 shows the comparison of top ten diseases leading to death over the last decade (WHO 2013). Mostly, the sufferers of diarrhea are children, setting back growth and development of a country. According to WHO (2007), 80% of the illness in developing countries are water related e.g. cholera, dysentery etc., and is a vicious killer. Moreover, millions of people are exposed to dangerous levels of biological contaminants and chemical pollutants in their drinking water due to inadequate water treatment systems. Therefore, if the present situation is not dealt seriously, it will persist and continue to cause substantial loss of human lives.



Source: CDC, http://www.cdc.gov/healthywater/burden/goals.html

Figure 2.2: Attribution water borne illnesses to sources

Figure 2.2 indicates the percentage of selected pathogen infections linked to contaminated water and the percentage of water borne pathogens linked to different water types. About 25% of overall pathogen infections are linked to water as transmission mode and drinking water contamination with pathogenic microorganisms leads to 60% of infectious diseases**.**

2.4 Water borne pathogens

Table 2.1: Water borne bacterial diseases

Causative agent	Disease
Escherichia coli, particularly serotypes such as O148, O157 and O124	Acute diarrhea and gastroenteritis
Vibrio cholerae, serovarieties O1 and O139, *Vibrio parahaemolyticus*	Cholera, Gastroenteritis
Salmonella enterica subsp. *enterica* serovar Paratyphi *Salmonella enterica* subsp. *enterica* serovar Typhi *Salmonella enterica* subsp. *enterica* serovar Typhimurium	Typhoid fever and serious salmonellosis
Shigella dysenteriae *Shigella flexneri* *Shigella boydii* *Shigella sonnei*	Bacillary dysentery or shigellosis, Gastroenteritis
Pasteurella tularensis	Tularemia
Brucella melitensis	Brucellosis
Pseudomonas pseudomallei	Melioidosis
Leptospira icterohaemorrhagiae (spirochaetales)	Leptospirosis (Well’s disease)

Sources: Leclerc et al. 2002; Cabral 2010

It is clear from the foregoing deliberation that waterborne pathogens present a formidable threat to water; these pathogens include various types of bacteria, protozoan parasites, viruses and other microorganisms (Cabral 2010). Surface and groundwater, both are contaminated by bacteria and viruses, whereas parasitic protozoa appear predominantly in surface water. Their entry into drinking water occurs upon contamination of the water source by sewage and animal waste, or when wells are not properly sealed or constructed.

2.4.1 Salmonella

A Gram-negative, facultative rod-shaped (Figure 2.3), non-spore forming, and predominantly motile bacteria, all strains of *Salmonella* are pathogenic for humans, causing enteric fevers such as typhoid and paratyphoid fevers, gastroenteritis, and septicaemia. *Salmonella typhimurium* is known to cause typhus abdominalis in humans and is commonly found in contaminated and infected food, water or fly excrement (Baudart et al. 2000).

Figure 2.3: Electron photomicrograph of *Salmonella typhimurium* cells.

The principal habitat of *Salmonella* is the intestinal tract of humans and animals. Serovars of *Salmonella* can be found predominantly in one particular host, or can be ubiquitous, or can have an unknown habitat (Ashbolt 2004, Baudart et al. 2000). Salmonellosis caused by human serovars, Typhi and Paratyphi A, is transmitted through fecal contamination of water and may cause grave diseases often associated with invasion of the bloodstream (Baudart et al. 2000; Ashbolt 2004; Haley et al. 2009). *Salmonella* finds its way into the river water, estuarine, coastland, sediments through fecal contamination (Ghosh et al. 2009a; Haley et al. 2009; Wery et al. 2008). It has been reported that the survival rate of *Salmonella* is higher in aquatic environments even outliving *Vibrio cholera* (Chao et al. 1987; DiRita 2001).

A combination of characteristics which makes *Salmonella* an effective pathogen includes the presence of an endotoxin typical of Gram-negative organisms, as well as the ‘Vi’ antigen which is thought to increase the virulence (Levantesi et al. 2012). Invasin, a protein is also produced and excreted by *Salmonella* that allows non-phagocytic cells to take up the bacterium, where it is able to live intracellularly. The emergence of antibiotic resistant strains of *Salmonella* is of great concern worldwide, and is thought to be mainly due to the excessive use of antimicrobial agents in humans and animals. Resistance to major antibiotics such as streptomycin, ampicillin, kanamycin, tetracycline, chloramphenicol and sulfonamides is commonly observed (Briggs and Fratamico 1999). Detected *Salmonella* outbreaks were mostly linked to contaminated drinking waters sourced from municipal water system, well and tank water (Bhatta et al. 2007; Lienemann et al. 2010).

2.4.2 Shigella

A Gram-negative, non-motile, non-spore forming, rod-shaped bacteria which is known to be closely related to *Escherichia coli* and *Salmonella* (Ahmed and Shimamoto 2014). Shigellosis is a disease that causes dysentery that result in the destruction of the epithelial cells of the intestinal mucosa in the cecum and rectum. *Shigella* bacteria multiply within colonic epithelial cells and cause inflammation, mucosal ulceration, and bleeding. This disease is mainly transmitted by fecal-oral contact.

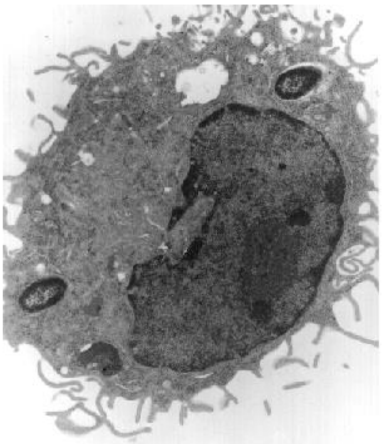


Figure 2.4: Macrophage infected with *Shigella*

Global burden of shigellosis is estimated to be 164.7 million cases per year, of which 163.2 million cases are reported in developing countries, where 1.1 million deaths occurred (WHO 2005). More than half of all these deaths attributable to shigellosis involved children younger than five years. Several reports mentioned that the incidence in developing countries could be up to twenty times greater than those in developed countries; however incidence varies within the same region (ECDOC 2010) or even in each country (CDC 2009;

Woodward and Rodgers 2000). Four species of *Shigella* viz. *S. flexneri, S. sonnei, S. dysenteriae*, and *S. boydii* are known to be pathogenic. *Shigella sonnei* is most common in developing countries and is responsible for 60% to 80% of cases reported worldwide. In South America, however, the most common species is *Shigella flexneri* (serotype 2) followed by *S. sonnei* (CDC 2009; Woodward and Rodgers 2000; Kotloff 1999). Paraguay and Chile reported *Shigella flexneri* to be more prevalent than *Shigella sonnei* (Rolfo et al. 2012).

Antibiotic treatment is necessary in cases of bacteremia and severe diarrhoea in shigellosis. However, early antibiotic resistance in *Shigella* was detected in Japan, where dysentery caused by *Shigella* infection was a large problem (Davies 2007). 80%-90% of *Shigella* isolates were reported to be resistant to sulfonamides by 1950 and by 1965, resistance to chloramphenicol, tetracycline and streptomycin had been reported (Pazhani et al. 2004). Since then, resistance continues to increase (McDonnell and Russell 1999).

2.4.3 Escherichia coli O157:H7

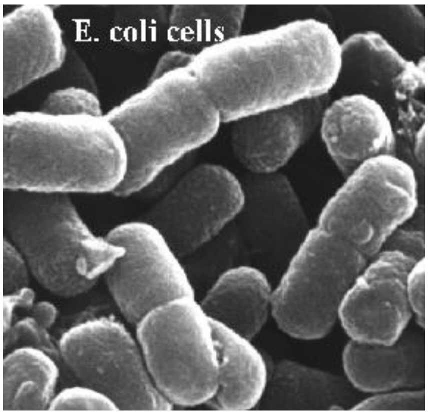


Figure 2.5: *Escherichia coli* cells

A Gram-negative facultative anaerobe, it is a rod-shaped bacteria, approximately 0.5-2µm in size (Drasar and Barrow 1985). Although most of the *E. coli* strains are non-pathogenic, strains possessing virulence traits cause serious diarrheal infections in humans. These pathogenic *E. coli* strains are divided into groups based on the mechanisms through which they interact with the human intestinal tract and symptoms (e.g., some produce specific types of toxin, whereas others invade, bind to or cause structural alterations of intestinal cells) (Nataro and Kaper 1998). Six groups are enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC), enteropathogenic (EPEC) and diffuse adherent (DAEC) *Escherichia coli* (Nataro and Kaper 1998). A member of EHEC group, *E. coli* O157:H7 can cause diarrhoea that ranges in severity from mild and self-limiting to severe and life threatening, up to 10% of *E. coli* O157:H7 infections can progress to haemolytic uraemic syndrome (Sack 1975; Nataro and Kaper 1998). Children and the elderly are most susceptible to the complications that arise from EHEC infections.

E. coli O157:H7 has been most commonly associated with waterborne outbreaks worldwide (Michino et al. 1999; Dundas et al. 2001; Salvadori et al. 2009). The first outbreak of *E. coli* O157:H7 infection was documented in Canada. It was associated with a municipal water supply and known as the Walkerton outbreak of 2000. This outbreak has been reported as largest bacterial waterborne outbreak in the country till date (Salvadori et al. 2009). In North America, the outbreaks of *E. coli* O157:H7 involving consumption of drinking water contaminated with human sewage or cattle faeces have been documented (Olsen et al. 2002). Although Shigatoxigenic group of *Escherichia coli* (STEC) are generally considered to be a problem in the developed nations, they have even been isolated from developing countries (Sehgal et al. 2008). The first major outbreak of bloody diarrhea in the developing world associated with O157 occurred in Swaziland in 1992 (Effler et al. 2001). In India, Hamner et al. (2006) reported seriously polluted nature of the Ganges in Varanasi due to release of raw sewage into the river. Also, *E. coli* O157:H7 was found to be a significant cause of high incidence of waterborne diseases, including dysentery and cholera in the region (Hamner et al. 2006).

2.4.4 Aeromonas

A Gram-negative, rod-shaped bacteria, it is found in virtually all surface water types (freshwater, marine and estuarine) in all but the most extreme conditions of pH, salinity and temperature (Cabral 2010). *A. hydrophila, A. veronii* biovar *sobria* (syn. *A. sobria*) and *A. caviae*--account for roughly 85% of human infections and are therefore considered to be the species of most importance for drinking water systems (Janda and Abbott 2010). The organisms have been isolated from the gastrointestinal tracts and infected tissues of cold-blooded and warm-blooded animals such as fish, birds, reptiles and domestic livestock (Janda and Abbott 2010). Its occurrence in retail food items, such as poultry, meat and dairy products has been well reported (Janda and Abbott 2010). Aeromonas have even been detected in the distribution systems of chlorinated drinking water supplies worldwide (Sharma et al. 2003; Janda and Abbott 2010; Kivanc et al. 2011).

Diarrhoea associated to *Aeromonas* infection has been encountered worldwide, in almost persons of all age groups (Sharma et al. 2003; Janda and Abbott 2010). *Aeromonas* species have been associated with gastroenteritis, the most common symptom is watery diarrhoea, accompanied by fever and abdominal pain, dysenteric type of illness with bloody stools to chronic or subacute watery diarrhoea (Kaper et al. 1981; Janda and Abbott 2010). Although, factors responsible for the pathogenicity and virulence of *Aeromonas* species or strains are poorly understood, a number of potential virulence components have been identified that would appear to enable the organisms to behave as human pathogens. These include components such as pili, fimbriae and flagella for attachment and colonization; external lipopolysaccharides, capsules or surface layers to assist in evading host defences; and toxins, haemolysins, proteases and other enzymes for causing damage to host cells (Janda and Abbott 2010). Research has identified that a known diarrhoea-causing strain of *A. hydrophila* possesses four prospective virulence factors: two haemolysins (Act and HlyA), a heat-stable enterotoxin (Ast) and a heat-labile enterotoxin (Alt) (Erova et al. 2008; Janda and Abbott 2010). Despite such findings, the role and relative significance of each remain uncertain, as studies have also found these factors distributed among numerous clinical and environmental strains in different combinations (Erova et al. 2008; Janda and Abbott 2010).

Although, the transmission of *Aeromonas* through drinking water is still debatable as there is no direct evidence of acquiring *Aeromonas* infection through drinking water, the presence of *Aeromonas* in finished drinking water supplies and distribution samples has been well documented, suggesting a possible route of transmission. Hence, for effective removal of *Aeromonas* from drinking water, maintaining an adequate disinfectant residual in distribution system is required as the potential exists for *Aeromonas* to regrow in the distribution system (WHO/UNICEF 2010).

2.4.5 Yersinia

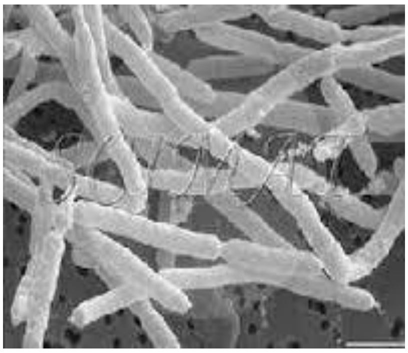
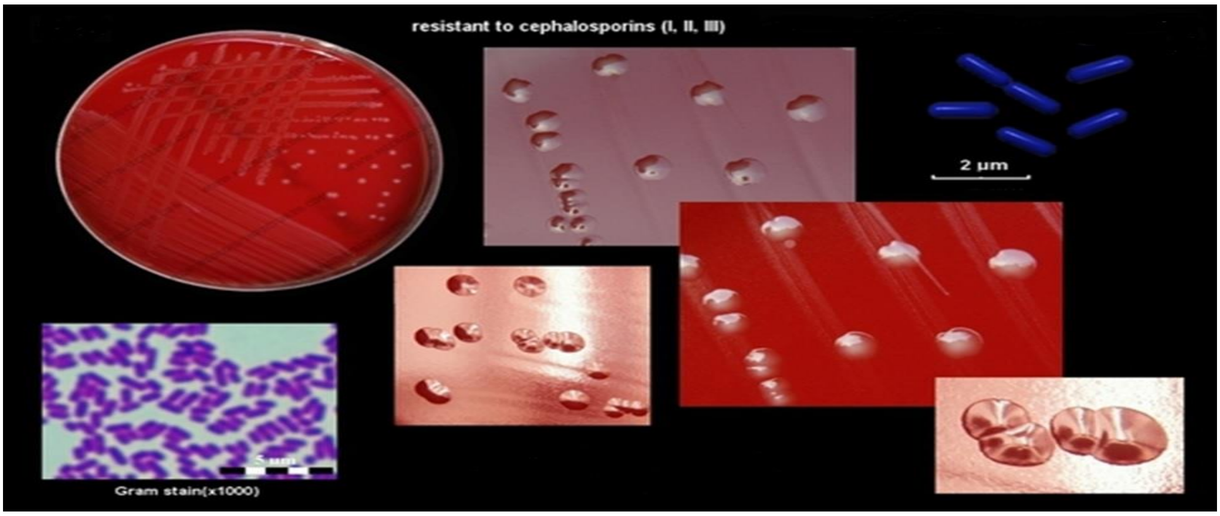


Figure 2.6: Electron micrograph of *Yersinia enterocolitica* cells

Yersinia is a Gram-negative facultative anaerobe, non-sporulating rods of 0.5-0.8µm in diameter and 1-3µm in length and belongs to the family Enterobacteriaceae (Percival et al. 2004). *Yersinia enterocolitica* is most frequently associated with enterocolitis, acute diarrhea, terminal ileitis, mesenteric lymphadenitis and pseudoappendicitis (Bercovier et al. 1980; Abdel-Haq et al. 2000) with the spectrum of disease ranging from asymptomatic to life-threatening sepsis, especially in infants. Schleifstein and Coleman (1939) provided the first recognized description of five human isolates of *Y. enterocolitica.* A 103-kd protein, known as invasin and determined by the *INV* gene, mediates bacterial invasion. The best-defined pathway is through the action of invasin (Young et al. 1992). The enterotoxin produced by *Y. enterocolitica* is similar to that produced by the heat-stable *Escherichia coli*; however, it likely plays a minor role in causing disease, as diarrheal syndromes have been observed in the absence of enterotoxin production. In addition, the toxin does not appear to be produced at temperatures above 30°C. The plasmid-mediated outer membrane antigens are associated with bacterial resistance to opsonization and neutrophil phagocytosis (Janda and Abott 2010).

Waterborne outbreaks of gastroenteritis involving *Yersinia enterocolitica* have been recorded on numerous occasions, with improper treatment, post-treatment contamination or consumption of untreated water supplies being the most frequent causes (Sharma et al. 2003). Isolation of *Yersinia enterocolitica* in developing countries is uncommon. However, in India, it has been isolated from wastewater, river, ground water and from well water (Sinha et al. 2000; Singh et al. 2003; Sengupta and Sharma 2013). World-wide surveillance data on *Y. enterocolitica* show great changes over the past two decades and bring forth its emerging nature (Sharma et al. 2003). The strains present in the aquatic environment are extremely heterogeneous, belonging to biotype 1A. It has been shown that biotype 1A strains of *Y. enterocolitica* may be pathogenic by some novel mechanisms. Thus, the importance of *Y. enterocolitica* as an emerging waterborne pathogen needs to be assessed further.

2.4.6 Listeria



Source: microbiologyinpictures.co

Figure 2.7: *Listeria monocytogenes* cultivated on columbia sheep blood agar at 37˚C for 24 h

A Gram-positive facultative anaerobe, rod-shaped coccobacillus, typically measuring 0.5µm in diameter and 0.5 to 2µm long **(Figure 2.7),** *Listeria monocytogenes* has the ability to grow at low temperature, a range of pH values between 4.3 and 9.6, and can reproduce at temperatures between 1 and 45°C (Low and Donachie 1997; Acha and Szyfres 2003; Roberts and Wiedmann 2003). *L. monocytogenes* is divided into 11 serovars; however, most human and animal cases are caused by serovars 4b, 1/2b, and 1/2 a (Low and Donachie 1997; Acha and Szyfres 2003).

Listeriosis occurs worldwide, but is seen mostly in industrialized countries, mainly found in soil, plants and water (Roberts and Wiedmann 2003). Animals, including cattle, sheep and goats, can also carry the bacteria. In infected people, symptoms vary, ranging from mild flu-like symptoms such as nausea, vomiting and diarrhea to more serious infections such as meningitis and other potentially life-threatening complications (Percival et al. 2004). People who are more susceptible to *Listeria* infections are the elderly, pregnant women, newborn infants and people with weak immune systems.

2.5 Resistance in bacteria against conventional disinfectants

The definition of bacteria as resistant or susceptible against an antimicrobial agent is critical. A bacterium is protected from its environment by a membrane, the integrity of which is essential to survival of the bacterium. This membrane consists of basic compounds such as phospholipids and lipopolysaccharides, and is stabilised by Mg^{++} and Ca^{++} cations. Thus, if ionised disinfecting molecules are absorbed or repelled by electrical charges at the initial contact and absorption stage, the following means of action will theoretically be possible: - non-polar molecules may dissolve and enter the lipid phase - specific carrying systems will lead other molecules through the membrane- other molecules will be able to disturb the organisation of the membrane by remaining bound to certain sites.

The mechanisms by which the bacteria develops resistance to antimicrobials are rather complex. Transport across the cell membrane, enzymatic inactivation and target alterations might be involved (Rice 1999). Understanding the bacterial defense strategies will help in developing concepts for reducing the development and spread of resistant bacterial strains. Disinfectants act on microorganisms in two different ways: growth inhibition (bacteriostasis, fungistasis) or lethal action (bactericidal, fungicidal or virucidal effects) (McDonnell and Russell 1999). Only the lethal effects are of interest in disinfection, and as the objects of treatment have no inherent means of defence, lethality is the desired objective. Although microbiologists have been working for more than a century on the problems associated with disinfection, understanding of the mode of action of active molecules remains vague: numerous hypotheses exist but few certainties. Many authors have long maintained that disinfectants and antiseptics act in a non-specific manner, in contrast to antibiotics which have distinct cellular targets within the microorganism (McDonnell and Russell 1999; Bloomfield 2002; Moore et al. 2008). Although studies still need to be performed in this field, it is clear that this distinction cannot be made for some molecules. It should also be noted that there may be considerable variation (in terms of pH, hardness, salinity, etc.) in the media surrounding the target microorganisms, and the state in which the latter is present (e.g. bacterium isolated or included in complex biofilm). The cumulative factors govern killing efficacy of the biocidal.

Biocides mostly interact with microorganisms initially at the cell surface (McDonnell and Russell 1999; Foged and Nielsen 2008; Siedenbiedel and Tiller 2012). Resistance is thus significantly influenced by cell wall composition and components of the outer surface which determine this interaction and subsequent uptake by the cell. Gram-negative bacteria possess a complex structure, with a much thinner peptidoglycan layer closer to the cytoplasmic membrane. Moreover, the periplasmic space is situated outermost and delimited externally by an asymmetric outer membrane with the outer surface constituted in essence by lipopolysaccharide (LPS). The resultant layer provides a formidable barrier that can restrict the uptake of biocides (McDonnell and Russell 1999). Moreover, the outer membrane of

Gram-negative bacteria acts as a permeability barrier because the narrow porin channels limit the penetration of hydrophilic molecules (Delcour 2009).

2.6 Biopolymeric flocculants: potential alternatives to water treatment chemicals

Drinking water is mostly contaminated due to inadequacies of distribution system as well as operational limitations of the conventional treatment process leading to regrowth of pathogenic microbes. Treatment options for stored potable water must therefore selectively remove both microbes and components resulting in such conditions.

Given the inadequacies of currently used synthetic biocidals for water treatment, natural products and technologies offer a logical choice over chemical counterparts for the treatment process. Polymers of natural origin are readily biodegradable (Salehizadeh and Shojaosadati 2001; Yasaman et al. 2010), non-toxic nature, and do not cause secondary pollution, these aspects have designated them as interesting candidates for further research (Li et al. 2010).

Over a decade, several studies have reported high molecular weight microbial polymers with versatile structure having high flocculating activity and selective binding to contaminants (Salehizadeh and Shojaosadati 2001; Deng et al. 2005; Ghosh et al. 2009a,b; Nwodo et al. 2014). These unique features of biopolymers can be directly exploited for rapid and efficient treatment of stored potable water. Although biopolymers like chitosan are known to be endowed with native flocculant properties as well as natural biocidal activity, their applications are limited to the area of food and pharmaceuticals only (Tan et al. 2013). Attempts have also been made to chemically modify cellulose and chitosan to enhance their biocidal properties but no research have emphasized on biopolymers which may replace or minimize the usage of water treatment chemicals especially for disinfection. Moreover, few, if any reports are available on chemical modification of polymers of microbial origin to achieve the former.

To harness biopolymers as flocculants it is important to understand their mechanism of action. However, in comparison with conventional chemical and synthetic flocculants, the flocculating mechanisms of bioflocculants are less well understood. According to the proposed mechanisms by some authors, bioflocculants cause aggregation of particles and cells by bridging and charge neutralization (Salehizadeh and Shojaosadati 2001). Bridging mechanisms occur after the particles are adsorbed onto the chains of bioflocculant. Many particles could adsorb to a long molecular chain, and the particles adsorbed on the chain could be adsorbed simultaneously by other flocculant chains, leading to the formation of three-dimensional flocs that are capable of settling fast (Deng et al. 2005). Since most bioflocculants and particles are negatively charged, charge neutralization seldom occurs in the bioflocculation process. Cations such as Ca^{2+}, Fe^{2+}, Al^{3+} are known to stimulate flocculating activity by neutralizing and stabilizing the residual negative charge of functional groups and by forming bridges between particles.

Apart from their strong binding ability, as noted before, the special advantages of bioflocculants such as efficiency, biodegradable nature and safety to humans and environment make them desirable to be applied for drinking and wastewater treatment, downstream processing, and fermentation processes (Salehizadeh and Shojaosadati 2001; Survase et al. 2007).

2.6.1 Flocculants from natural sources

Chitosan, cellulose and low-cost starch are the most abundant natural polymers and they have been reported as alternatives to synthetic flocculants (Tripathy and Ranjan 2006). Chitosan, a deacetylated product of chitin, has many interesting properties, such as antimicrobial activity and nontoxicity and it has been used as coagulant that aid in conjuction with alum. Chitosan has its applications in several fields such as the food, medical, cosmetic, and textile industries (Binsu et al. 2006: Badawy and Rabea 2013; Tan et al. 2013). Cellulose is the most abundant natural polysaccharide in which the anhydroglucose units alternate up and down and are joined together by β-1,4-glycosidic bonds. It has been used in a variety of ways in everyday life such as textiles, papermaking, food processing, packaging, building materials, and pharmaceuticals. Guar gum is another naturally occurring cheaper and non-toxic polymer which has been applied as a flocculant aid in potable water treatment due to its non-ionic nature (Salehizadeh and Shojaosadati 2001).

The traditional use of the *Moringa Oleifera* seeds for domestic household water treatment has been well known in certain rural areas in the Sudan (NRC 2006). In West Asia, one of the best known uses for *Moringa* is the use of powdered seeds to flocculate contaminants and purify drinking water (Berger et al. 1984). The active ingredient in the *M.* o*leifera* seed has also been identified as a polyelectrolyte (Ndabigengesere and Narasiah 1998). Its use for coagulation, co-coagulation, or coagulant aid has been a subject of investigation in many parts of the world (Jahn 1986, 1988).

2.6.1.1 Microbially derived flocculants

Different algae, bacteria and fungi are reported to produce extracellular polymeric substances which function as bioflocculants with various properties (Kurane et al. 1986; Sutherland 1998; Jang et al. 2001; Gao et al. 2006; Wu and Ye 2007; Ghosh et al. 2009a,b; Kavita et al. 2013). In recent years, the study of bioflocculants has attracted wide attention because of their special attributes and properties, due to which they can potentially be applied in drinking and wastewater treatment, downstream processing, and fermentation processes (Salehizadeh and Shojaosadati 2001). Several types of bioflocculants have been previously reported, and some of them have shown efficient flocculating activities comparable to those of the synthetic flocculants for not only inorganic but also organic suspended particles (Yokoi et al. 1997, Sutherland 1998). Bioflocculant produced from *Rhodococcus erythropolis* (Kurane et al. 1986) has been reported to be effective for various colloidal suspensions and pigments. A bioflocculant, MBFA9 was produced from a strain of bacteria isolated from a soil sample, identified as *Bacillus mucilaginosus* and its application in starch waste water treatment has been reported (Deng et al. 2005).

A highly efficient bioflocculant from *Vagococcus* sp. has been produced repoerted not only in the area of wastewater treatment, but also in drinking water processing, food and fermentation industry (Gao et al. 2006). Many microorganisms such as *Klebsiella sp.* (Dermlim et al. 1999; Ghosh et al. 2009a), *Corynebacterium glutamicum* (Wu et al. 2007), and *Citrobacter* (Jang et al. 2001) have been found to produce bioflocculants. Removal of water pathogen, *Cryptosporidium* oocysts and biowarfare agents such as *Salmonella* has been successfully reported by the use of bioflocculants isolated from *Klebsiella terrigena* (Ghosh et al. 2009a,b).

2.7 Biopolymer modifications

Biopolymers are the suitable primers for alteration in order to develop new molecular designs (Salehizadeh and Shojaosadati 2001). They have different functional groups present on the polymeric backbone which can be modified by grafting and cationization. The biopolymers are natural in origin, fairly shear stable, biodegradable and can be easily altered. The components and structures of biopolymers are complex and different bioflocculants produced by different bacteria can have different properties (Deng et al. 2005). Infrared spectroscopic studies of various bioflocculants show the presence of carboxyl, hydroxyl, methoxyl, amino and phosphate groups in the structure (Gao et al. 2006).

The structure of chitosan is useful to the synthetic organic chemist interested in site-selective modifications. Such modifications have resulted in several derivatives of chitosan with distinct properties and applications. The presence of multiple nucleophilic groups within the chitosan backbone requires following a suitable synthetic protocol in order to obtain the desired selectivity. The synthetic transformation steps performed are often relatively simple, exploiting the differences in the nucleophilicities of primary amino group (at C-2) versus the two hydroxy groups (at C-3 and C-6). However, the degree of substitution varies greatly upon the reaction conditions. Broadly speaking, the derivatives reported in literature mainly fall under one of the three categories; *N*-alkylation, *N* and/or *O* acylation and grafting of chitosan on a growing or preexisting polymer chain (Sajomsang et al. 2008, 2009a,b; Tan et al. 2013; Badawy and Rabea 2011).

Among the various methods of modification, graft polymerization has been the most used method. Grafting of polysaccharides such as chitosan and cellulose has led to the formation of functional derivatives by covalent binding of a molecule, the graft, onto the polymer backbone (Badawy and Rabea 2013). Chitosan has two types of reactive groups, amine and hydroxyl groups, that have been grafted. Grafting of biopolymers lead to improved properties such as increased chelating and complexion properties enhanced adsorption properties. Graft polymerization of maleic acid onto carboxymethyl chitosan and hydroxypropyl chitosan using ammonium persulfate as initiator has been reported (Avadi et al. 2004; Sajomsang et al. 2008, 2009a,b; Xu et al. 2010). Cationic polysaccharides have been prepared by the reaction of a polysachcharide with various reagents possessing positively charged groups such as amino, sulphonium and phosphonium groups.

2.7.1 Quaternized derivatives of biopolymers

Positive charges attached to surfaces, particles, polymers, peptides or bilayers have been used as antimicrobial agents by themselves or in sophisticated formulations. Various modifications have been performed on polymers such as cellulose and chitosan to produce new properties; for example, methylation improved binding, thickening and stabilizing properties of polymers

(Hiatt and Rebel 1971), conversion to the phosphate ester introduced flame-resistant properties and ion-exchange capability in polymers (Isogai et al. 1986) and quaternization (introduction of quaternary ammonium groups) conferred adhesive properties (Ebringerova et al. 1994; Belalia et al. 2008; Tan et al. 2013).

Among the modifications mentioned above, quaternization often efficiently confers properties of commercial value on the existing polysaccharides (Tan et al. 2013). This modification changes the functionality while maintaining most of the other properties of the polysaccharide intact. Quaternary ammonium compounds formed after methylation of amino sugar group of polymers, impart them with positive charge and enhanced antibacterial activity (deBritto et al. 2011). Though enormous scope of quaternized polymers for human use has been highlighted, the possibility of surface modification of known or unknown effective bioflocculants to impart biocidal properties for water treatment remains to be explored.

Quaternary ammonium salts of amino sugar rich biopolymers might be a good candidate as polycationic biocide. In polycationic biocides, generally, it is assumed that the charge density of the polyelectrolyte increases with an increase in the molecular weight which leads to enhanced adsorption of polycations onto the negatively charged cell surfaces (Kim et al. 2003; Hansen et al. 2011). This is favourable for binding of the polycations to the cytoplasmic membrane of bacterial cells and other particles in suspension. Quaternary ammonium compounds are widely used as disinfectants, antiseptics, pharmaceutical products and cosmetics. These compounds penetrate into the bacterial cell wall, react with the cytoplasmic membrane and subsequently induce cell wall lysis caused by autolytic enzymes (McDonnell and Russell 1999). Several attempts have been made to modify chitosan through quaternization for its use in food, medical and textile industries (Kim et al. 2003; Avadi et al. 2004; deBritto et al. 2011; Badawy and Rabea 2011)). N-Alkyl chitosan derivatives were prepared by introducing alkyl groups into the amine groups of chitosan via a Schiff's base intermediate (Jia et al. 2001). Quaternization of the N-alkyl chitosan derivatives with methyl iodide produced water-soluble cationic polyelectrolytes. The antimicrobial activities of the chitosan quaternary ammonium salts increased with an increase in the chain length of the alkyl substituent; this increased activity was ascribed to the contribution of the increased lipophilic properties of the derivatives (Belalia et al. 2008).

There is a considerable interest in biologically active derivatives of chitosaccharides and N,N,N-trimethylchitosan is one of the most promising chitosan derivatives that is currently being investigated (Sajomsang et al. 2009a,b; Runarsson et al. 2010; Badawy and Rabea 2011; Tan et al. 2013). The intensive methylation of chitosan generates the N,N,N-trimethyl derivative, characterized by possessing permanent positive charges in chains as a consequence of the quaternization of the amino groups in the C-2 position. Trimethyl chitosan (deBritto et al. 2011) is reported to be water-soluble polyelectrolyte with good intestinal absorption enhancing properties for hydrophilic and macromolecular drugs. Likewise, triethyl chitosan and diethylmethyl chitosan were synthesized and characterized with different degrees of quaternization (Avadi et al. 2004) for pharmacological and pharmaceutical experiments. It was shown that unlike chitosan but like trimethyl chitosan, the solubility of triethyl chitosan is not pH dependent and is soluble in water at room temperature.

Various derivatives of chitosan have been achieved but because of the strong molecular forces and hence low affinity for ordinary organic solvents appropriate for carrying out chemical reactions, chitin and chitosan are less accessible to potential reactants than cellulose. There are difficulties in performing reactions with chitosan due to lack of stability, multi-functionality and low reactivity. Hence chemical modifications of chitosan are complicated and not easy to control in general (Kurita et al. 1982; Badawy and Rabea 2011).

2.8 Quaternary ammonium compounds (QACs) as disinfectants

Quaternary ammonium compounds, also known as QACs, is synthesized by an irreversible reaction between alkyl halides and tertiary amines (Garcia et al. 1999; Steichen 2001). QACs can be classified in three major groups depending on type of the functional groups: monoalkonium, dialkonium and benzalkonium halides (Garcia et a. 1999; Zachvieja 2001; Zhang et al. 2011). QACs are large molecules having molecular weights typically between 300 and 400g/mole and are composed of two distinctly different moieties: hydrophobic alkyl groups, and a hydrophilic positively charged central N atom which retains its cationic character at all pH values (Zhang et al. 2011).

Historically, the applications of QACs was initiated in the 20th century, and over time developed rapidly. They exhibit a broad spectrum of antimicrobial activity over a wide range of pH and are used in domestic, industrial, agricultural and medical applications, pesticides, fungicides, sanitizers/disinfectants (McDonnell and Russell 1999; Richards and Cavill 1976). The most commonly used QACs are dialkonium and benzalkonium chlorides (Tiedink 2001). QACs irreversibly bind to the phospholipids and proteins of the bacterial membrane, thereby impairing permeability (Russell 1983; Mcbain et al. 2004).

2.9 Toxicity assessment of water treatment chemicals

Toxicities of chemical flocculants have been tested primarily for human consumption, warm-water species of fish, and for various invertebrates. Aquatic organisms respond to their total environment (physical and chemical components) and in this way integrate the effects of all the various chemical and physical parameters. Two types of assays are used in general, static bioassay with fish in a tank of standing test water and the continuous flow bioassay in which solution is renewed continually. Various fish species are used for the toxicity assays, for example, rainbow trout *(Salmo gairdneri* Richardson), fathead minnow (*Pimephales* *promelas* Rafinisque), bluegills (*Lepomis macrochirus* Rafinesque) and goldfish (*Carassius auratus*) are some of the standard fishes (Arthur and Eaton 1971; Anderson and DeFoe 1980; Biesinger et al. 1982; Bradbury et al. 1989; Ankley et al. 1992; Ankley and Johnson 2004). Few tests have been conducted on flocculant toxicity to salmonid or other cold-water fish. Results of most toxicity tests have shown that purity of the chemical, ionic form, and concentration of flocculant are the critical factors in determining toxicity. Some of the water treatment chemicals are added in excess of the amounts required to bind with sediment particles which remain dissolved in the water and are potentially available to the aquatic biota. The polymeric flocculants can be obtained in higher grades containing fewer impurities and are hence the higher grade chemicals are used for potable water or where toxicity to aquatic organisms is of particular concern.

Biesinger et al. (1976) tested the three poly-electrolyte flocculants on rainbow trout, lake trout (*Alvelinus namaycush*), and three crustaceans, *viz*. a mysid (*Mysis relicta*), a copepod (*Limnocalanus macrurus*), and a cladoceran (*Daphnia magna*). They concluded that some of the cationic polyelectrolytes tested are particularly toxic at concentrations which could be released into aquatic environments. Two cationic polyelectrolytes were also shown to impair reproduction of Daphnia at 0.1mg/l and 1.0mg/l. Biesinger and Stokes (1986) tested the toxicities of 28 different polymers to daphnids, fathead minnows, midges (Insecta: Chironomidae) and gamrnarids (Crusteacea: Amphipoda) and found that for the three nonionic chemicals tested, concentrations in excess of 100mg/l were required to produce 50% mortality (LC) in the populations.

Another important factor is the route of exposure to a toxic substance. There is a possibility of ingested chemicals completely getting neutralized by the liver, or the compound may enter the blood stream directly. Hence there can be different target organs depending upon the route of exposure. Selective toxicity is the type where the level of toxicity of one compound differs among different species. Another major factor in determining the toxicity of a compound is the formation of metabolites. During biotransformation, detoxification can occur where the compound gets transformed into a less toxic form, or biotoxification can occur to make it more active or toxic.

Among the bioassays carried out to assess the toxicity or mutagenicity of a compounds, Ames test is worth mentioning. The *Salmonella typhimurium*/microsome assay (*Salmonella* test; Ames test) is a widely accepted short-term bacterial mutagenesis assay for identifying substances that can produce genetic damage that leads to gene mutations (Mortelmans and Zeiger 2000). The test uses a number of *Salmonella* strains with preexisting mutation. Since DNA is chemically the same in all organisms, any living organism can be used to test for mutagens. Thus, bacteria can be used as a first step in identifying potential human carcinogens without waiting for long-lived mammals to develop cancer. The *Salmonella* mutagenicity test was specifically designed to detect chemically induced mutagenesis (Ames et al. 1975; Maron and Ames 1983). Over the years, its value has been recognized by the scientific community, and by government agencies and corporations. The test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs because there is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained. International guidelines have also been developed (e.g., Organisation for Economic Co-operation and Development (OECD); International Commission on Harmonization (ICH)) for use by corporations and testing laboratories to ensure uniformity of testing procedures prior to submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides (Mortelmans and Zeiger 2000).

International regulations regarding human health require that all new products should be tested for their safety prior to their use. Toxic effects are divided into some general categories such as acute, chronic, lethal, cumulative, delayed, long term and short term effect. Among these, acute toxicity which is usually lethal, and chronic which may be lethal or sublethal are mostly considered for toxicological evaluation. The analysis of toxic properties of the flocculants and disinfectants using *in vivo* mammalian system (mice or rats) are of enormous value which reflects indirect toxic effects on humans. Various drugs, chemicals, antibacterial agents have been tested for their acute toxicity using Swiss albino mice and their lethal doses are reported. The aim of this research was to develop quaternized biopolymeric flocculants and investigate the mechanism of their antimicrobial activity toward the creation of non-toxic antimicrobials. Although there are a number of reports regarding the toxicity evaluation of polysaccharide polymers, knowledge on biodegradable microbially produced flocculants, which have always been considered safe, is still limited.

CHAPTER 3

Materials & Methods

3. MATERIALS AND METHODS

3.1 Chemicals and media

Chitosan (85% deacetylated) was procured from Sigma Aldrich (St. Louis, MO, USA). All chemicals and reagents used were of the highest analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (St. Louis, MO, USA) and Hi-Media (Mumbai, India). Chemicals of molecular biology grade were procured from New England Bio labs, UK. Media solutions were sterilized by autoclaving at 121°C and 15psi for 20mins and were allowed to cool below 50°C before use. Luria-Bertani (LB) agar (10.0g tryptone, 5.0g yeast extract, 10.0g NaCl, 15g agar per liter, pH 7.0±0.2) were made by the addition of 17g/l of agar prior to autoclave sterilization. FIB (flocculant isolation broth) media was used for bioflocculant production (Appendix 1).

3.2 Bacterial strains

Biopolymer producing strain: An industrial wastewater isolate, *Klebsiella terrigena* (Accession number EU082029), was used in this study (Ghosh et al. 2009). The amino rich biopolymer producing strain was preserved in glycerol stock solutions at -80°C. The strain was revived in LB agar plate and incubated overnight at 37ºC and a single colony was picked and transferred in FIB broth and was grown to mid log phase at 37ºC.

Indicator strains: For testing the antibacterial activity of antibacterial polymer(s), selected bacterial surrogate strains, *Salmonella typhimurium* ATCC 25315, *Shigella flexneri* 2a, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria* *monocytogenes* ATCC 19111 and *Escherichia coli* O157:H7 ATCC 32150 were maintained in Tryptone Soy Broth (TSB) with 2% glycerol and stored at 4ºC. The cultures were revived prior to every test in Brain Heart Infusion (BHI) agar plate and incubated overnight at 37ºC and a single colony was picked and transferred in BHI broth and incubated for 18-24h at 37ºC.

3.3 Extraction and purification of biopolymeric flocculants

For extraction and purification of biopolymeric flocculants, method of Ghosh et al. (2009) was followed. 1l cultures of *Klebsiella terrigena* in FIB media, pH 7±0.2 was incubated in a rotary shaker (120rpm/min) at 30°C (Labcon, 5081U, USA) for 48hours and were centrifuged at 12,000g for 30min at 4°C (Sigma 2-16KC, Germany). The supernatant fraction was then separated from the cellular debris by the addition of two volumes of ethanol to 100ml of concentrated supernatant and allowed to precipitate at 4°C for 24h. The precipitated polymer was collected by filtration (Whatman GF Filter) and dialyzed extensively using a dialysis tubing (Cellulose, MWCO 12000 Da), against deionized water. The bioflocculant was re-precipitated by addition of a 10% solution of cetylpyridinium chloride. The precipitated polymer complex was collected by centrifugation at 10,000g for 20min at 4ºC and re-dissolved in 10% NaCl solution. Fractionation and purification were achieved using gel chromatography on a Sepharose 4B column followed by elution with a 0.4M NaCl buffer. Three volumes of ethanol were added to recover the purified bioflocculant, which was further dialyzed and lyophilized to powder. Weight of the bioflocculant was measured using a weighing balance (Sartorius CPA 225D, Germany).

3.4 Determination of hexosamine content for screening of bioflocculants with amino rich sugar

To determine the hexosamine content in purified biopolymers, Elson-Morgan method (1934) was followed. 250µl of standards (0-1mg/ml, galactosamine) and samples were added to 50µl of reagent A. Then each mixture was heated to 100ºC for 3min. After cooling rapidly to room temperature 1.5ml of reagent B was added, washing down any condensate formed. The mixture was incubated 37ºC for 20min. After cooling to room temperature, the absorbance was measured at 585nm. The hexosamine content present in the sample was calculated from standard curve (Galactosamine, 0-1mg/ml). A standard curve for hexosamine and preparation of reagent A and B is shown in Figure 1 in Appendix II.

3.5 Determination of flocculating activity

The flocculating activity of the purified bioflocculant was determined by the method described by Kurane et al. (1986). Kaolin clay was used as suspension material for estimating the flocculating activity in all the experiments, unless otherwise noted. A volume of 0.05ml of bioflocculant (BF) dissolved in water (concentration as specified in different experiments) and 0.95ml of distilled water was added to 8ml of kaolin suspension (5g/l) with 1ml $CaCl_2$ (10mM) in a tube. The pH was adjusted to 7 with 1N NaOH and 1N HCl and solution mixed gently; 0.05ml of distilled water was added in place of BF to the reference tube. The solution was thoroughly vortexed for 30sec and allowed to stand at room temperature for 5min. 2ml of aliquots were withdrawn from the upper phase and O.D. was recorded at 550nm with a spectrophotometer (U-2800, Hitachi, Japan).

The Flocculating activity was calculated in percent as follows:

Flocculating activity (%) = (B-A) *100 / B

Where A – Optical density of sample at 550 nm

B – Optical density of reference at 550 nm

3.6 Synthesis of quaternized biopolymeric derivatives

Methyl iodide method (Belalia et al. 2008): BF (500mg) was dissolved in deionized water (50ml) and 0.5ml of formaldehyde was added to the solution. The solution was further kept for stirring at room temperature for 30 min and sodium borohydride (41.25mg) was added. After one hour of stirring, the pH of the solution was adjusted to 10 by using NaOH (1M). White precipitate formed was filtered and washed till it reached pH 7. Trapped moisture was removed by refluxing it with dry benzene through azeotropic distillation. Evaporation of benzene resulted in N-methyl BF (yield 90%; 450mg). N-methyl BF (450mg) was dispersed in N-methyl-2-pyrrolidone (12ml) with sodium iodide (0.5gm). The mixture was vigorously agitated at 60°C for 1h after which 15% w/v NaOH (2.2ml) and methyl iodide (1ml) were added. The product was precipitated using acetone (36ml), filtered and vacuum dried after the continued stirring for 6h at 60°C. White precipitates obtained were re-dissolved in deionized water (20ml) and purified through dialysis method by using a dialysis tubing (Cellulose, MWCO 12000 Da) for one day. The dialysate was lyophilized (yield 80%; 360 mg) and the resultant derivative was named as TMB1. The same method was repeated with chitosan and the derivative obtained was named as TMC1 (yield 70%; 280 mg).

Dimethyl Sulphate (DMS) method (deBritto 2011): Purified BF (500mg) was dissolved in dimethyl sulfate (8ml) and deionized water (2ml). The solution was then filtered to eliminate the impurities.

Sodium hydroxide (600mg) and sodium chloride (440mg) were added to the resulting suspension, followed by stirring the solution at ambient temperature for 6h. The product was precipitated using acetone, filtered and vaccum dried. White precipitates obtained were re-dissolved in deionized water (20ml) and subjected to dialysis method by using a dialysis tubing (Cellulose, MWCO 12000 Da) for one day. Dialyzed solutions were then lyophilized and the resultant derivative of BF was named as TMB2 (yield 70%; 350mg). The same method was repeated with chitosan and the derivative obtained was named as TMC2 (yield 60%; 300mg).

3.7 Characterization of quaternized biopolymeric derivatives

Determination of degree of quaternization (DQ %): To measure the degree of quaternization (DQ) of quaternized polymers, the amount of chloride on the quaternized biopolymeric flocculant was titrated with aqueous silver nitrate solution at room temperature. 1% (w/v) silver nitrate ($AgNO_3$) solution was poured into a 25ml burette. Then, 5.0ml of 1% (w/v) quaternized biopolymeric flocculant solution and 4.0ml of 1% (w/v) potassium chromate (K_2CrO_4) solution were transferred to a 125ml Erlenmeyer flask. The silver nitrate solution was added to the 1% (w/v) quaternized biopolymeric flocculant solution slowly drop by drop with swirling the flask until the entire solution in the flask turned a very light orange-red color, indicating the endpoint had been reached. The total volume of the titrant (silver nitrate solution) was recorded and the corresponding amount of chloride on the quaternized biopolymeric flocculants was calculated. Each sample was titrated three times to obtain an average DQ value.

NMR Analysis: ^{1}H-Nuclear magnetic resonance (NMR) spectra of the BF, Chitosan, TMC1 and TMB2 polymers were obtained with an NMR spectrometer (Bruker Avance II (400 MHz), Switzerland) by dissolving samples of the polymers in D_2O at 80°C with suppression of the water peak.

Molecular weight determination: Molecular weight determination of BF and TMB was performed with a Bruker Daltonics MALDI-TOF mass spectrometer. The MALDI matrix used was 2, 5-dihydroxybenzoic acid (DHB). The number-average molecular weights, Mn, and average weight, M_w of the polymer sample were determined in the linear mode.

Particle size and Polydispersity Index (PDI) measurement: The particle size and size distribution (polydispersity index (PDI)) of polymers were measured by Zetasizer (Malvern Instruments, UK), based on the dynamic light scattering (DLS) technique.

3.7.1 Compositional analysis

3.7.1.1 Determination of total sugar

The total carbohydrate content of the BF and TMB was determined by phenol sulphuric acid method as described by Dubois et al. (1956). The reagent was prepared by dissolving phenol in water (5% w/v). 200µl of samples, standards and controls (containing up to 100µg carbohydrates) were mixed with 200µl of phenol reagent (Appendix I). 1.0ml of concentrated sulphuric acid was rapidly and directly added to the solution surface without allowing it to touch the sides of the tube. The solutions were left undisturbed for 10min before shaking vigorously. The absorbance was determined at 490nm after a further 30min. All experiments were conducted in triplicate. The amount of total carbohydrates present in the sample solution was calculated from standard curve (prepared using glucose, 0-1mg/ml) (Figure 2 in Appendix II).

3.7.1.2 Determination of protein

Folin-Lowry method was used to determine the total protein in the native and quaternized biopolymeric flocculants, Lowry et al. (1951). Bovine serum albumin (BSA) was used as standard in different concentrations (0.1-0.5mg/ml) and, biopolymer stock was 1mg/ml. 50ml of reagent A and 50ml of reagent B were mixed to make the complex-forming reagent. 1ml of freshly mixed complex-forming reagent was added to 0.2ml of the sample or standard. The solution was left undisturbed for 10min at room temperature. The absorbance was measured at 750nm. The amount of total protein present in the sample was calculated from the standard curve prepared by using pure BSA as standard (Figure 3 in Appendix II).

3.7.1.3 Determination of uronic acid

The uronic acid content of the BF and TMB was determined by method described by Haug and Larsen (1962). 250µl of sample or standards (0-1mg/ml, D-glucuronic acid) and controls were cooled in an ice bath. 1.5ml of ice cold reagent A (Appendix I) was carefully added with mixing and cooling in ice bath. The mixture was rapidly cooled in the ice-bath. 50µl of reagent B (Appendix I) was added and mixed well. Reheating was done at 100ºC for 15min. The mixture was cooled rapidly to room temperature and the absorbance was measured at 525nm. The uronic acid content present in the sample was calculated from standard curve (D-glucuronic acid, 0-1mg/ml) (Figure 4 in Appendix II).

3.7.1.4 Determination of pyruvic acid

The pyruvic acid content of the BF and TMB was determined by method described by Friedman and Haugen (1943). For the determination of the pyruvic acid content of the biopolymer samples (5mg dissolved in 5ml distilled water) were firstly treated with perchloric acid (50%) for de-proteinization and were kept at 30ºC for 30min. Then 1ml of DNP reagent (Appendix I) was added to the extract. Further, 4ml of water and 10ml 2.2N NaOH were added. The tubes were shaken and absorbance was measured at 416nm. A standard curve was made by using pyruvic acid (0-3mg/ml).

3.7.1.5 Elemental analysis

The powdered BF and TMB were analysed for C, H, N on a 2400 II elemental analyser (Perkin Elmer Company, Bedford, Massachusetts, USA).

3.8 Effect of temperature, pH and time on degree of quaternization of TMC and TMB

To study the effect of temperature, pH and time on degree of quaternization of TMB and TMC, reactions were set at range of temperatures, 20 to 40 ºC, at pH in range of 4 to 9. The samples were withdrawn after 3 hours and degree of quaternization at regular intervals up to 12 hours was measured by titration method.

3.9 Evaluation flocculating efficiency

3.9.1 Comparison of efficiency of TMB with other synthetic and natural flocculants

The comparative study of the TMB was done with chemical flocculants like polyacrylamide (6.00 x 10^7 Da) and CHEMFLOC 985W (2.5 x 10^6) (Chemical Systems, New Delhi, India) and the natural flocculants xanthan (2.5 x 10^5), chitosan (2 x 10^4) and polyglutamic acid (5 x 10^5). The flocculant activity of the various flocculants was checked by flocculation of kaolin suspension and their flocculating activity was calculated as described earlier. A stock solution of these flocculants was prepared (10mg/l). Flocculation of kaolin clay was carried at a pH range of 4-9 to determine their optimum pH. The effect of various cations was studied by replacing Ca^{2+} with other cations (Cu^{2+}, Fe^{2+}, Al^{3+}, Mg^{2+} and Co^{2+}, Fe^{3+}) in the kaolin suspension.

3.9.2 Flocculation of colloidal solution

Experiments were performed with TMB and TMC to examine the flocculation of suspended solids of varied size at varied concentrations. A standard solution of suspended solids of 400NTU was made by suspending powders of active carbon, silica, magnesium hydroxide, cellulose and cells of yeast in 100ml water. A bacterial suspension (Gram-negative and Gram-positive bacteria including *Salmonella, Yersinia*, *Aeromonas, Listeria* and *Escherichia*) was also used in 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, Netherlands). An assay was performed by adding 10ml $CaCl_2$ (5mM), 0.5ml TMB (0-60µg/ml) and 9.5ml of distilled water to 80ml of these solutions. The pH was adjusted to 7±0.2 and the solutions were allowed to stand at room temperature for 5min. 20ml of aliquots were withdrawn from the upper phase and its turbidity was measured.

3.9.3 Statistical Optimization of TMB dosage by Response Surface Methodology (RSM)

RSM used the common form of center composite design (CCD) which is called center composite face design (CCFD) that consists of 2k factorial points (k means factors = 2), 2k axial points and two replicated at the center point to provide estimation of the experimental error variance. The range of and levels of experimental variables investigated in this study are presented in Table 3.1. The actual values of the coded levels for these factors were selected and based on preliminary experiments. Twenty runs were required for a complete set of the experiments. The second order polynomial equation was used to prove the relationship between the factors and the investigated response. CCD developed by the Design Expert software, version 8.0.7.1 (Stat Ease Inc. Minneapolis, USA, trial version) was adopted to optimize dosage of TMB for maximum flocculating activity and turbidity removal. The flocculating activity and turbidity removal were selected as the dependent variables, while the TMB concentration for dose optimization and turbidity level were selected as independent variables.

Table 3.1: Actual values in terms of coded factors for low, medium and large size particles

	-alpha	-1	0	+1	+alpha
Dosage (ppm)	**5.8**	10	20	30	34
Turbidity (NTU)	**8.5**	50	150	250	291.4

In order to further validate and confirm the suitability of the model, verification experiments with numerically optimized levels of tested variables were performed.

3.10 Evaluation of Antibacterial activity

Preparation of 0.5 McFarland turbidity standards: To standardize the inoculum density for a susceptibility test, Barium Sulphate ($BaSO_{4)}$ turbidity standard, equivalent to a 0.5 McFarland standards was used. A 0.5 McFarland standard was prepared as described in NCCLS (1993). 1% (v/v) solution of sulfuric acid was prepared by adding 1ml of concentrated sulfuric acid to 99ml of water and mixed well. A 1.175% (w/v) solution of barium chloride was prepared by dissolving 2.35g of dehydrated barium chloride ($BaCl_2.H_2O$) in 200ml of distilled water. To make the turbidity standard, 0.5ml of the barium chloride solution was added to 1% of 99.5ml sulfuric acid solution and mixed well. A small volume of those turbid solutions was transferred to a screw-capped tube of the same type as used for preparing the control inocula and stored in the dark at room temperature.

3.10.1 Agar disc diffusion assay

Agar disc diffusion assay was used to evaluate the antibacterial activity of quaternized biopolymeric flocculants against six selected water borne pathogens. The disc test was performed according to a procedure developed by Pulusani et al. (1979) with some modifications: 10ml of the melted BHI agar medium was seeded with 100µl broth culture of the test organism in a 100mm sterile petri dish. When the soft agar had hardened, uniform sized discs (diameter 6mm) prepared from whatmann filter paper no. 1 soaked in sterile solution of 1mg/ml of each native biopolymers (BF and chitosan) and quaternized biopolymeric flocculants (TMB1, TMB2, TMC1 and TMC2) were placed on the agar surface. After incubation for 20±4h at the appropriate temperature for each organism tested, the diameter of the inhibition zone around the disc was measured. All the tests were performed in triplicates.

3.10.2 MIC determination

Inoculum were obtained from an overnight agar culture of the test organism. Inoculum for the MIC test was prepared by taking at least three to five well-isolated colonies of the same morphology from an agar plate culture. The top of each colony was touched with a sterile loop and the growth was transferred into a tube containing 4 to 5ml of normal saline. The broth culture was incubated at 37ºC until it achieved the turbidity of the 0.5 McFarland standards (usually 2 to 6h), a suspension containing approximately 1 to 2×10^{8} CFU/ml was obtained. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity comparable to that of the 0.5 McFarland standards.

Antibacterial activity of chitosan, BF, TMC and TMB was assayed by microdilution method, using a sterile 96 well-microtiter plate reader (Bioscreen C, Thermo labsystems, Helsinki, Finland) (Raafat et al. 2008). Briefly, the growth of indicator organisms in broth (300µl) containing antibacterial derivatives was studied in micro titre plates (100 wells). Each well was inoculated with 10µl broth culture (grown overnight) of the test organism diluted to about 10^{7}CFU/ml adjusted as McFarland standard. The optical density at 600nm was measured automatically at 10min interval, using a wideband filter (405-600nm), and the plates were shaken at 3min interval for 20s. The MIC was read as the least concentration of the antibacterial agents that was sufficient to completely inhibit visible bacterial growth. The MIC determinations were carried out in triplicates, with two independent experiments performed.

3.10.3 Time-kill assay

Initial inocula of 10^7CFU/ml was adjusted using McFarland standard. Cultures of the indicator strains were incubated separately in the absence (control) and presence of different concentrations (relative to MIC) of native polymers and their quaternized derivatives, for a period of 24h at 37°C. The absorbance (600nm) was measured at regular interval of 30min, aliquots of 0.5ml were withdrawn at the appropriate time and were plated on BHI agar plates to determine the viable counts by pour plate technique. Data was analyzed by expressing growth as CFU/ml. The surviving $\log_{10}$ CFU/ml was plotted against time for each of the different quaternized biopolymer concentrations. All the experiments were performed at least three times.

3.11 Determination of antibacterial activity in real time conditions

3.11.1 Water quality analysis

Collection of water samples: Water samples (stored in distribution tanks, hand pump, piped, canal surface water) were collected from Patiala and nearby regions of Punjab including rural & semi urban, urban areas where degree of contamination is diverse and of high magnitude. For the collection of river water samples, the sterile containers were first rinsed with water from the source before collecting the water sample by holding the bottle at the bottom and plunging it below the water surface. The mouth of the bottle was placed opposite the water current. If there was no current, it was created artificially by pushing the bottle forward. The bottle was filled leaving about

30mm of empty space to allow mixing during laboratory analysis (Buckalew et al. 2006). The samples were transported to the laboratory on ice and were stored at 4^{o}C until their use.

The water samples were analyzed for parameters such as turbidity, electrical conductivity, pH, alkalinity, hardness, total dissolved solids, chlorides, sulphate, nitrate, fluorides, sodium, potassium, calcium, magnesium, bicarbonate, and bacteriological parameter such as coliform bacteria as described in "Standard methods for the examination of water and wastewater American Public Health Association (APHA) (APHA et al. 1998) **(Table 3.2)**.

Table 3.2: Analysis of water quality parameters

Parameter	Analysis method
Turbidity (NTU)	Turbiditimetric
pH	Potentiometric
Total dissolved solids (TDS) (mg/l)	Gravimetric
Total hardness (mg/l)	EDTA titrimetric method
Calcium (mg/l)	EDTA titrimetric method
Magnesium (mg/l)	Titrimetric method
Chloride (mg/l)	Argentometric Titration Method
Sulphates (mg/l)	Spectrophotometric method
Fluorides (mg/l)	Spectrophotometric method
Nitrates (mg/l)	Phenol disulphonic acid (PDA) method
Coliform bacteria (CFU/ml)	MPN method

3.11.2 Preparation of the synthetic water and removal of pathogenic bacteria

Synthetic water was formulated as recommended in APHA (1998). It was used in all experiments in order to standardize the tests with microorganisms. **Table 3.3** shows the concentrations of the reagents added to deionized water in order to prepare the synthetic water. The deionized water used for the preparation of the synthetic water must have an electrical conductivity of 1µS/cm at 25ºC. Color values of the synthetic water were about 10 color units (cu), which was obtained using 0.1% solution humic substance. Turbidity values of the synthetic water were about 5.0 nephelometric turbidity units (NTU), which was obtained using a 1.0% clay solution (sodium montmorillonite clay). These parameters were monitored with a spectrophotometer (DR4000, HACH). Samples were prepared at the beginning of the experiments and the turbidity measurement was carried out immediately after sample preparation, without altering the original sample qualities, such as temperature or pH. Water pH was measured using an Orion pHmeter (model no. 370).

Table 3.3: Composition of synthetic water

Reagent	Concentration (mg/l)
$NaHCO_3$	12
$CaSO_4$	6
$MgSO_4$	7.5
KCl	0.5
NaOH	200
KH_2PO_4	4000

All the standard indicators water borne pathogens (mentioned previously) were pre-grown in BHI broth, harvested by centrifugation. Cell pellets of all the cultures were washed thrice with sterile water, mixed, re-suspended in synthetic water, mixed in equal proportions and spiked in 1L of synthetic water held in Erlenmeyer flasks (3 replicates). TMB and TMC at different concentration was then added to each replicate, mixed thoroughly by hand rotation and allowed to stand at ambient temperature for 10, 30 and 60min. Control experiments comprised of similar combinations but lacked TMB and TMC. In parallel experiments, chlorine was added at different concentrations prepared from 10 mg/l of stock solution. Free chlorine solutions were prepared from reagent grade chemicals (10% sodium hypochlorite from Panreac, Barcelona, Spain). The bacterial cell count was done using the colony count method. The efficiency of the TMB, TMC and chlorine in destroying bacteria was evaluated by comparing the initial and final bacterial count after treatment.

3.12 Cellular leakage measurement

Preparation of washed cell suspensions: Pregrown bacterial cells were separated by centrifugation at 11,000×g for 10min from the culture medium (BHI) after 24h of growth at 37°C. Cell pellets were washed in 10mM PBS (pH 7.0) and diluted with the same buffer to approximately 10^7CFU/ml at an absorbance (OD 600nm) of 1.

3.12.1 Electrical conductivity measurement

Cellular leakage of the treated bacterial cells was determined by measuring electrolyte leakage into the incubation medium by conductivity meter (DDS-307, Precision and Scientific Instrument Co. Ltd., Shanghai, China). TMB and TMC were then added to bacterial cells suspensions. The treated cell suspension was centrifuged at 8,000×g for 15min, and the supernatant was analysed for electrical conductance with 10 min interval. All the experiments were performed in triplicates.

3.12.2 Determination of potassium ion leakage

TMB and TMC were then added to the prepared bacterial cells suspensions. The treated cell suspension was centrifuged at 8,000 × g for 15min, and the supernatant was analysed for potassium using an atomic absorption spectrophotometer (AAS 5EA, Analytic Gena, Germany) with an airacetylene flame and a wavelength of 324.8 nm. Three replicates per sample were analysed. The intracellular level of the K^+ ion was expressed in percentage released and plotted against time.

3.12.3 Determination of the leakage of 260-nm-absorbing material

TMB and TMC were added to bacterial cells suspensions. The cell suspension was centrifuged at 8,000 × g for 15 min, and the supernatant was then decanted into a plastic UV cuvette (1cm path length). The absorbance was read against a buffer control at 260 nm and graph was plotted against time.

3.12.4 Intracellular ATP measurements

Cells were harvested and washed as described above and-incubated for 10 min at 25˚C in 50mM HEPES (pH 7.0 or 8.0) buffer containing 0.2% (wt/vol) glucose. Total ATP and extracellular ATP in target pathogens after treatment with TMB were determined with a luminometer (Lumac/3M Biocounter M2010A) as described by Guihard et al. (1993). Intracellular ATP concentrations were calculated by using the intracellular volumes of 3 and 2ml/mg of protein for indicator strains. ATP leakage kinetics was determined on a bacterial suspension prepared as above. Aliquots were withdrawn at intervals of 10min for one hour and viable counts determined.

3.12.5 Leakage of glucose, lactate dehydrogenase (LDH) and protein from treated bacterial cells

To examine the effect of quaternized derivative on cell leakage and the viability of the indicator strain, inoculum of the test organism (1ml) was inoculated into sterile deionized water (10ml) with or without quaternized derivative in a culture tube. The mixture, containing water-soluble biopolymeric derivative (TMB and TMC) and indicator cultures were incubated at 37°C with shaking (120rpm) for 12h. At regular interval of 1h, aliquots were withdrawn for determination of protein and glucose contents and lactate dehydrogenase (LDH) activity. The cell suspension was centrifuged at 8,000×g for 15min, and the supernatant was measured for LDH activity, protein and glucose contents. The glucose content was analyzed by a glucose assay kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). A sample or glucose standard (0.0–3.0mg/dl, 200µl) was added to reagent (1ml) containing glucose dehydrogenase. After incubation at 25°C for 15min, the absorbance at 334 nm was recorded. The LDH activity was analyzed by an LDH assay kit (Clontech, USA). A 200µl sample was added to 1ml reagent containing NADH and incubated at 25°C. LDH activity was then determined by measuring the rate of decrease of the NADH concentration which was monitored by recording the change of absorbance at 334nm. The protein concentration was measured by absorbance at 280nm.

3.12.6 Extraction and FT-IR analysis of Lipopolysaccharides (LPS) of target pathogens

Lipopolysaccharide from the pathogens treated with quaternized derivatives and control cells was extracted by hot phenol-water method as described by Westphal and Jann (1965) with some modifications. In brief, bacterial suspensions (10^8 CFU/ml) were centrifuged at 10,000 × g for 5min. The pellets were washed twice in PBS (pH=7.2) (0.15M) containing 0.15mM $CaCl_2$ and 0.5mM $MgCl_2$. Pellets were then re-suspended in 10ml PBS and sonicated for 10min on ice. In order to eliminate contaminating protein and nucleic acids, treatment with proteinase K, DNase and RNase was performed prior to extraction step. For this purpose, proteinase K (100µg/ml) (Roche, Mannheim, Germany) was added to the cell mixture and the tubes were kept at 65°C for an additional hour. Mixture was subsequently treated with RNase (40µg/ml) (Roche, Mannheim, Germany) and DNase (20µg/ml) (Roche, Mannheim, Germany) in the presence of 1µl/ml 20% MgSO4 and 4µl/ml chloroform and incubation was continued at 37°C overnight. At the next step, an equal volume of hot (65–70°C) 90% phenol was added to the mixtures followed by vigorous shaking at 65-70°C for 15min. Suspensions were then cooled on ice, transferred to 1.5ml polypropylene tubes and centrifuged at 8500 × g for 15min. Supernatants were transferred to 15ml conical centrifuge tubes and phenol phases were re-extracted by 300µl distilled water. Sodium acetate at 0.5M final concentration and 10 volumes of 95% ethanol were added to the extracts and samples were stored at –20°C overnight in order to precipitate LPS. Tubes were then centrifuged at 2000×g, 4°C for 10min and the pellets were re-suspended in 1ml distilled water. Extensive dialysis against double distilled water at 4°C was carried out at the next step until the residual phenol in the aqueous phases was totally eliminated. Final purified LPS product was lyophilized and stored at 4°C.

LPS were analyzed by a Nicolet magma, FTIR spectrometer equipped with a Maillard-I-Alanine doped deuterated triglycine sulphate (DLa TGS) detector using KBr pellets. To prevent absorption from ambient, the system was purged with dry nitrogen. For each spectrum, 64 scans were co-added at spectral resolution of 4cm^{-1}. The spectra covered the wave number ranging from 4000-400cm^{-1} frequencies for all sharp bands were determined accurately from the original baseline corrected spectra of corresponding group using a software OPUS (version 6.5). The different FT-IR spectra graphs were drawn by origin software (version 6.0).

3.12.7 Electron microscopic examination

Cultures of the indicator bacterial strains were grown to the early exponential phase and then split into two portions: one was treated with TMB (MIC) and incubated at 37°C, while the other served as an untreated control. After 30-min incubation, the cells were washed twice with PBS and fixed in a fixative (2% glutaraldehyde, 2% paraformaldehyde, and 0.5% $CaCl_2$ in 0.1 M cacodylate buffer).

For Transmission Electron Microscopic (TEM) analysis, the samples were post fixed in 1.33% osmium tetroxide, dehydrated in graded ethanol and infiltrated with a 1:1 mixture of propylene oxide. The samples were then embedded in Spurr low viscosity embedding media. Thin sections of samples were then viewed with a Philips CM 120 transmission electron microscope. Bacteria treated with MIC and sub-MIC values of TMB were used for the TEM analysis.

For Scanning Electron Microscopic ***(***SEM) analysis, samples were fixed using 3.7% paraformaldehyde followed by rinsing in sterile water and ethanol. Subsequently, samples were air-dried by lyophilization, sputter-coated with gold and imaged using Analytical Scanning Electron Microscope (Variable Pressure Scanning Electron Microscope, Hitachi S3400N, NIPER, Chandigarh) to explore changes in morphology, resulted from modification process on the bacterial cell surfaces.

3.13 Agglutination assay

The effect of TMB on agglutination of *S. typhimurium* by *Salmonella* H-antigen type specific antibody was performed using *Salmonella* typing serum obtained from BBL Microbiology Systems as described in the package insert. 20µl of antiserum was added to glass slide and mixed with the *S. typhimurium* culture treated (with MIC and sub-MIC concentration of TMB and untreated) (Herikstad et al. 2002). Slides were tilted for 5-10sec and then observed for agglutination. Positive reaction was read as visible agglutination whereas negative reaction was seen as homogenous milky turbidity.

3.14 Toxicological evaluation of synthesized antimicrobial compound

3.14.1 Determination of antimutagenecity by Salmonella (Ames) assay

The mutagenicity of TMB was assessed by the Ames test using *Salmonella typhimurium* strains TA98 and TA100. The assay was performed according to Maron and Ames (1983), and conducted under both absence and presence of metabolic activation mixture (S9 mix). Standard mutagens sodium azide (10µg per plate), N-2-fluorenylacetamide (NFA) (10µg per plate) and 2-aminoanthracene (2-AA) (2.5µg per plate) were used in the test. TMB (50,100,150µg/ml) were applied to *S. typhimurium* cells. The number of sodium azide-, NFA- and MNNG- induced His+ revertant were counted. Antimutagenic potency (%) on *Salmonella* was calculated as

$$AP= (M-M_{TMB}/M) *100$$

Where, AP is the percent antimutagenecity potency, 'M' refers to mutagen induced His+ revertants in absence of TMB and M_{TMB} refers to mutagen induced His+ revertants in presence of TMB.

3.14.2 Acute toxicity studies in Swiss albino mice

The description of the test item used in the study is presented in **Table 3.4.** Test item Quaternized Biopolymer (TMB) was prepared freshly as and when required for animal administration. Calculated amount of test item was weighed aseptically in sterile eppendorf and suspended in calculated volume of PFS. The mixture was vortexed for 5min followed by sonication for 1min. A slightly viscous solution was obtained which was injected immediately to animals.

Female Swiss albino mice weighing 20 to 25g and age 8-10 weeks were taken for the experiment. Animals were acclimatized in standard animal house environmental conditions **(Table 3.5)** for 7 days before the start of experiment.

Table 3.4: Description of Test item

Species	Mus *musculus*
Strain	Swiss Albino
Source	Animal Facility – VMRC
Sex	Female
Age	8-10 weeks
Number of animals	22
No. of animals / group	**Sighting Study-** 2 animals / group **Main Study-** 4 animals / Group
Acclimatization	7 Days
Identification of animals	**Pre-randomization-** By tail marking with temporary marker; **Post-randomization-** Body marking with picric acid (H- Head, B- Body, T- Tail, UM- unmarked); **Cage Labeling-** Labeling with complete study details
Randomization	Animals were randomly selected based upon their body weight and allotted to different groups.

3.14.2.1 Description of the Test Procedure

Table 3.5: Animal husbandry

Target Room Temperature	22 ± 3 °C
Target Relative humidity	30 to 70%
Light/dark cycle	12-hourly
No. of animals per cage	**Sighting Study-** 2 animals / cage **Main Study-** 4 animals / cage
Feed	Conventional feed purchased from a commercial supplier would be provided *ad libitum* to the animals.
Water	Filtered drinking water *ad libitum*.

Animals were randomly selected and grouped on the basis of their body weight into total of 10 groups *viz.* G1, G2, G3, G4 & G5 consisting of 2 animals each for sighting study **(Table 3.6)** and G6, G7, G8, G9 & G10 consisting of 4 animals each for main study **(Table 3.7).** Starting with the Sighting Study to decide the dose levels for final study G1, G2, G3, G4 & G5 were aseptically injected 20, 60, 180, 140 & 100 mg/kg i.p. of TMB suspended in Pyrogen Free Saline (PFS).

Table 3.6: Sighting Study

Group	**Treatment**	**Dose (mg/kg bw)***	**Route of administration**	**No. of animals / group**
G1	TMB	60	i.p.	2
G2		180	i.p.	2
G3		220	i.p.	2
* Dose levels were selected on the basis of clinical signs and mortality observed				

Table 3.7: Main Study

Group	**Treatment**	**Dose (mg/kg bw)***	**Route of administration**	**No. of animals / group**
G4	TMB	180	i.p.	4
G5		200	i.p.	4
G6		220	i.p.	4
G7		240	i.p.	4
* Dose Levels were selected on the basis of mortality observed in Sighting Study. Total 4 Dose Levels were selected representing 0 % to 100 % Mortality.				

To avoid unwanted mortality and animal use in the experiment, all the animal groups were not subjected to “Quaternized Biopolymer” treatment simultaneously. Next higher or lower dose level was administered only after making some decision from the clinical outcome of previous dose level injected i.e. any mortality or clinical toxicity observed in previously dosed animal group.

The objective of Sighting Study was to obtain LD_0 & LD_{100} (optimum dose levels that would cause 0 % & 100 % lethality respectively). From the mortality outcome of Sighting Study, Starting dose level was selected for main study and G4, G5, G6, & G7 were aseptically injected gradually increasing dose levels of 180, 200, 220 & 240 mg/kg bw i.p. of " Quaternized Biopolymer" in a similar stepwise manner as the Sighting Study in order to get 0 % to 100 % mortality pattern. Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24h, with special attention given during the first 4 hours, and daily two times thereafter, for a total of seven days. Clinical signs of toxicity, Body weight change (observed for thirty days) and mortality were recorded daily in controlled data recording sheets (DRS).

3.14.2.2 Observations

Body weight of all experimental animals was recorded daily during the study period. Body temperature of all experimental animals was recorded at 0min (pre-dosing) and 30, 60, 90, 120, 180, 240 & 480 min post-dosing with a precise digital clinical thermometer. All the animals were observed for any clinical signs of toxicity and data was recorded in corresponding DRS. Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4hours, and daily two times thereafter, for a total of seven days for any toxicity related mortality.

3.14.2.3 Hematology

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

3.14.2.4 Histology

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

3.15 Statistical analysis

All the experiments were performed in triplicate and the data were analyzed by statistics software package SPSS (version 11.5). The aim of the statistical treatment was to establish that a result is robust to repetition (or replication) of the study. For every experiment, the average and standard deviation (SD) were calculated as measures of central tendency and dispersion of the values acquired with the experiment. However, the screening and rejection of atypical values (outliers) was always done. The paired samples *t*-test was used for confirmatory statistical analysis in order to ascertain the significance of a difference between two means. The model underlying a *t*-test assumes that the data have been derived from normal distributions with equal variance, being considered a parametric test. The homogeneity of variance was assessed by the Levene´s test for equality of variance. The output of the statistical test is the *P*-value. When the *P* - value of a statistic was less than the significance level, the assumption of the statistic was said to be significant. If the *P*-value was larger than the significance level, the assumption was accepted. The statistical calculations were based on a significance level equal or higher than 95%.

The quantitative characters were statistically analyzed. The mean, standard deviation (SD) and standard error (SE) were calculated for each of the characters using the following formula.

$$\text{Mean, } x = \_x/N$$

$$\text{Standard error (SE)} = SD/\sqrt{N}$$

Where x= measured characters, N= number of observations and SD = Standard deviation

Each bar in the graph represents mean ± SD of triplicate data

CHAPTER 4

Results

Microbially synthesized extracellular polymers represent a prospective class of alternative water treatment chemical agents. Their unique ability to flocculate colloidal particles rapidly in water and structural plasticity enabling 'add on function' have begun to be exploited. Since pathogenic microorganisms are important colloidal particles in water, 'add on function' in biopolymeric flocculants that impart effective biocidal property can offer novel technological solution for water treatment. A systematic evaluation of newly identified biopolymers including their antimicrobial efficacy, robustness, chemical structure and non-toxic nature are pre-requisites for technological purposes. Thus, this study attempted to develop quaternized antimicrobial derivative of bioflocculant against water-borne bacterial pathogens. Further, efficacy of the developed variants were compared with chitosan, a polymeric counterpart with similar structure. Finally, the toxicological profile of the developed variant was evaluated.

4.1 Screening of amino sugar containing polymeric flocculants from bacteria

A library of fifty exopolymers were made after screening their culture supernatants that were endowed with an ability to flocculate kaolin suspension. It consisted of exopolymers showing >65% flocculating activity. Amongst them, five biopolymeric flocculants were found to possess high amino sugar content (>15%). A high yielding polymer with maximum amino sugar content (38.6%) which exhibited >80% of flocculating activity **(Table 4.1),** was selected for development of antibacterial derivative. This polymer was obtained from a non-pathogenic environmental isolate; the organism was identified as *Klebsiella terrigena*. The polymer under study was already well characterized for its physico-chemical structure and its mechanical properties by previous investigator in our laboratory (Ghosh et al. 2009a,b, Pathak, 2011).

Table 4.1: Bioflocculant activity, yields and amino sugar content profile of five selected biopolymeric flocculants

Biopolymeric flocculant	Flocculating activity (%)	Yield (mg/l or g/l)	Amino sugar content (%)
RD1	74 ±0.6	200 (0.2g/l)	15.1 ±0.35
RD2	70 ±0.2	150 (0.15g/l)	18.2 ±0.06
RD3	78 ±0.8	189 (0.189g/l)	20.3 ±0.04
BF	**83 ±0.7**	**250 (0.25g/l)**	**38.6 ±0.06**
RD5	76 ±0.4	190 (0.19g/l)	20.0 ±0.02

4.2 Synthesis and characterization of quaternized biopolymeric derivative(s)

Amino group present in the polysaccharide backbone of the bioflocculant were transformed into quaternary ammonium compounds synthetically so that the polymer that could effectively bind, would also be able to inactivate the water borne pathogens (WBPs) simultaneously. Methylation is an effective approach in quaternization of polymers. Synthesis of trimethyl biopolymeric derivatives leads to methylation of the amino groups in the C-2 position of biopolymer to form quaternary amino groups with fixed positive charges on the repeating units of the quaternized polymer chain. Also the advantage of introducing methyl groups at different levels on the polymeric backbone can be controlled externally with ease. Quaternization process using either dimethyl sulphate or methyl iodide have been well documented for obtaining quaternary ammonium compounds of chitosan (deBritto et al. 2007; Belalia et al. 2008; Tan et al. 2013). Thus, the suitability and applicability of the same was attempted in case of the selected biopolymeric flocculant in the present study.

Table 4.2: DQ (%) of quaternized derivatives

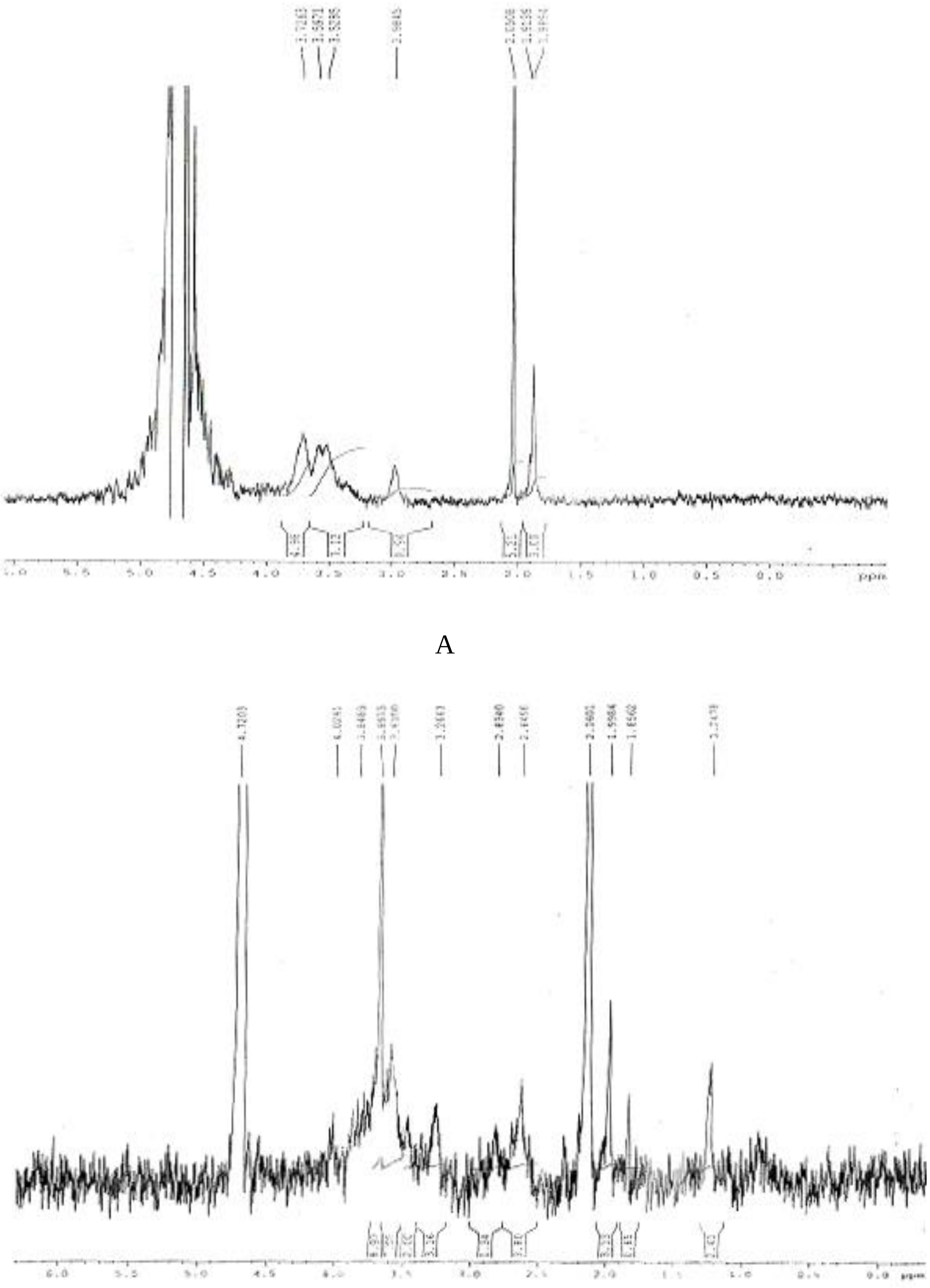
Sample	Degree of Quaternization (%)
BF	-
TMB1	30±0.4
TMB2	**73±0.7**
Chitosan	-
TMC1	67±0.4
TMC2	**69±0.75**

Two quaternized derivatives of each chitosan (TMC1 and TMC2) and BF (TMB1 and TMB2) were synthesized. Out of the two afore mentioned methylating agents, DMS resulted in high DQ in both, chitosan and BF derivatives **(Table 4.2).** Therefore it was considered appropriate to use the quaternized derivatives obtained using DMS for the further studies.

Table 4.3: Effect of reaction time on the DQ of TMC and TMB

Time	Degree of Quaternization (%)	
	TMC	TMB
1 h	13±0.70	17±0.40
3 h	31±0.90	50±0.50
6 h	**52±0.75**	**73±0.55**
9 h	52±0.70	73±0.21
12 h	53±0.85	73±0.15

During the reaction a heterogeneous medium was generated that comprised of different proportion of mono-, di- and tri- methylated species as resulted from potentiometric analysis. However, a homogenous medium that consisted of purely tri- methylated species was achieved by the optimization of time. It was observed that the completion of quaternization reaction occurs within 6h, where a maximum DQ of 73% was achieved **(Table 4.3).**



A

B

Figure 4.1 ^{1}H NMR spectra of the chitosan dissolved in D_2O/ CF_3COOH mixture (A) and N,N,N trimethyl chitosan derivative, TMC dissolved in D_2O (B).

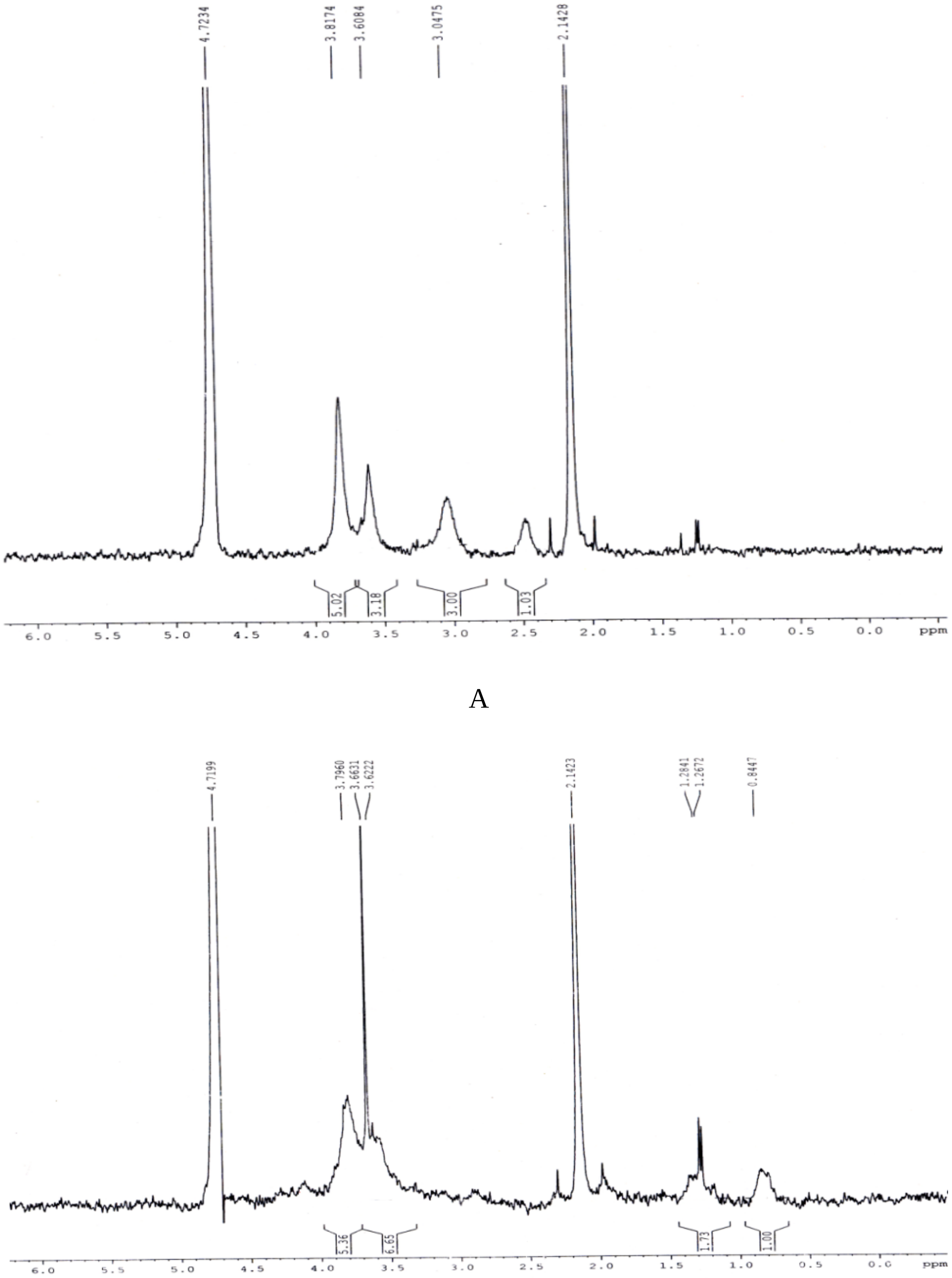


Figure 4.2 ^{1}H NMR spectrum of BF (A) and N, N,N trimethyl biopolymeric derivative, TMB (B) dissolved in D_2O

The singlet peak at 3.66ppm of ^{1}H NMR spectra shown in **Figure 4.1** and **4.2** represents the presence of tri-methylated product of chitosan (TMC) and bioflocculant (TMB). The results were derived on the basis of ^{1}H NMR spectral analysis of quaternized chitosan reported by Sieval et al. (1998) in quaternized chitosan where a peak at 3.1 ppm was assigned to dimethyl amino group and peak between 4.7 and 5.7 ppm were assigned to ^{1}H protons.

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 was 66.8% and 2.45% (w/w), respectively, indicating a primarily polysaccharide structure of TMB similar to the native biopolymer. The acidic polysaccharides including uronic acid (2.83%) and pyruvic acid (7.4%) did not differ significantly than native biopolymer. However, the amino sugar content was reduced to 0.34% in TMB from 5.8% in BF.

The molecular weights (MW) of BF and its quaternized derivative is very important for industrial and scientific uses since MW affects the functionalities. To estimate the molecular weight distribution of each BF and TMB, its weight-average molecular weight (M_w) and number-average molecular weight (M_n) were measured by Matrix Assisted Laser Desorption and Ionization--Time of Flight Mass Spectroscopy (MALDI-TOF). An increment in the molecular weight after quaternization was clearly understood by MALDI-TOF spectra of TMB in comparison to BF **(Figure 4.3)**. These results were also in supported by ^{1}H NMR spectra previously.

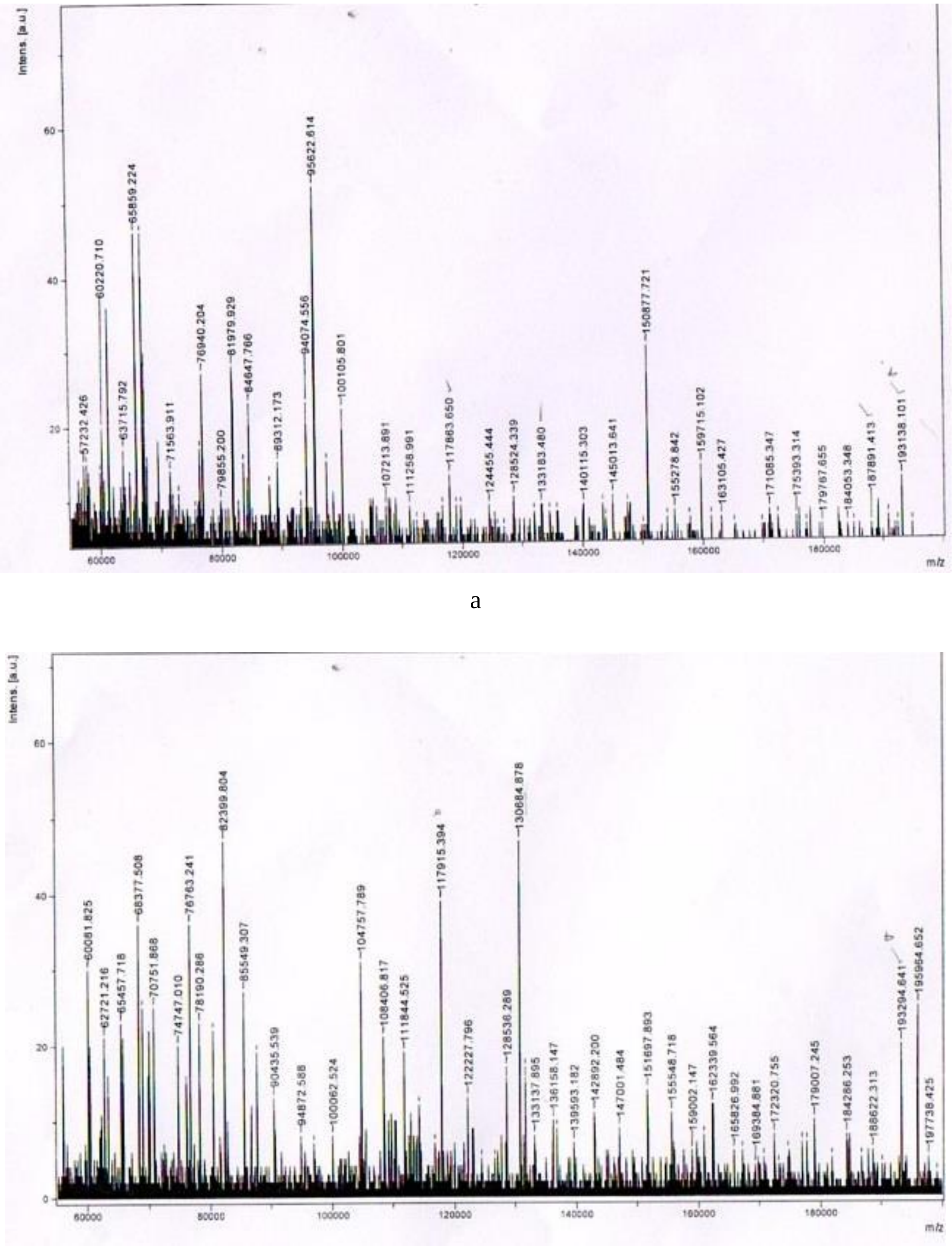
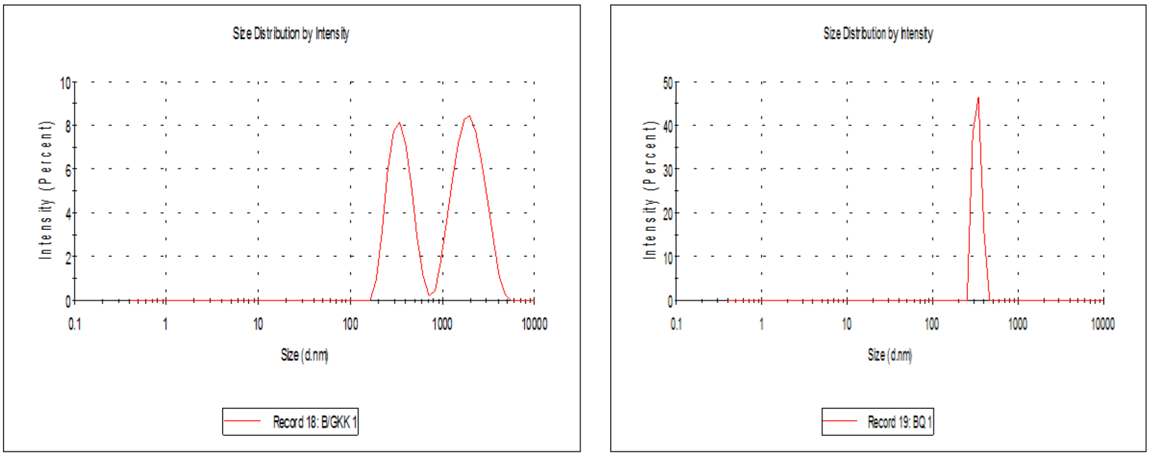


Figure 4.3: MALDI-TOF spectra of a) BF and b) TMB



A **b**

Figure 4.4: Size distribution intensity of BF (a) and TMB (b)

Dynamic Light Scattering (DLS) analysis demonstrated the particle size of the native BF as 729.893nm whereas its quaternized counterpart was of 1453nm in size. **Figure 4.4** shows the size distribution intensity of TMB and BF. The Polydispersity index (PDI) was determined from the ratio of the weight average molecular weight to the number average molecular weight. Interestingly, the native BF because of low PDI value of 0.306, perhaps is monodispersible in nature, whereas TMB with PDI of 0.997 reflects its polydispersible nature. Therefore, it was inferred that disperisbility of TMB improved due to introduction of methyl groups to the biopolymeric flocculant chain. This enhanced functionality imparted due to quaternization can allow TMB to be more effective in the process of flocculation and dispersion.

TMB and TMC were readily soluble in aqueous solutions at pH 7 **(Table 4.4).** As expected, chitosan became insoluble at pH 7 due to deprotonation of amines at pH higher than 6.3 (Badawy and Rabea 2011). The yields of the quaternized products were reported as percentage weight fractions, which means the w/w percentage of the product, where the product refers to the quaternized derivative and the reactant is the polymer used for the quaternization reaction **(Table 4.4)**.

Table 4.4: Recovery and solubility of quaternized derivatives

Polymer	**Yield (%)**	**Solubility in water at pH 7**
BF	-	+
TMB	**74**	+
Chitosan	-	-
TMC	**67**	+

Further the effect of temperature and pH on degree of quaternization of TMC and TMB were also examined, although no significant effect was observed. The conditions for the quaternization reaction were carried out at 37°C and neutral pH so that the flocculating activity of the polymer does not get affected. If however, after every quaternization reaction, the flocculating activity was checked to ensure the efficacy of the polymer.

4.3 Flocculating efficiency of quaternized derivatives

4.3.1 Comparative study with synthetic and natural flocculants

The activity of the quaternized biopolymeric derivatives, TMC and TMB was compared of its efficacy with other natural (Chitosan, Xanthan, *Moringa olifera*; Biopolymeric flocculant obtained from *Klebsiella terrigena* (BF)) and synthetic flocculants (CHEM FLOC985W and Polyacrylamide) under their most optimal conditions of dose, pH, temperature and cations. TMB and BF both showed better flocculating activity than other synthetic and natural flocculants like xanthan (87.6±4.4), chitosan (77.9±2.6) and polyglutamic acid (73.5±3.4).

Table 4.5: Comparison of flocculating efficiency of TMB with other flocculants

Flocculants	Dosage	Cations	Temperature	pH	Flocculating Activity (%)
TMB	5mg/l	Not required	RT	7±0.02	94±2.8
TMC	10mg/l	Not required	RT	7±0.02	79±1.6
BF	20mg/l	Not required	RT	7±0.02	93.4±3.4
Xanthan	10mg/l	Ca^{2+}	40˚C	7±0.02	87.6±4.4
Γ-PGA	8 mg/l	Ca^{2+}	30˚C	7.5±0.2	73.5±3.4
Chitosan	25mg/l	Not required	40˚C	7.5±0.2	77.9±2.6
Polyacrylamide	30mg/ml	Not required	35˚C	8±0.02	92.6±4.2
CHEMFLOC985W	8mg/ml	Fe^{3+}	RT	8±0.02	93.8±3.9

The flocculating activity of BF was approximately similar to that of polyacrylamide (92.6±4.2) and of TMB was approximately similar to that of a commercially available flocculant CHEMFLOC985W **(Table 4.5)**. In addition, it did not require Fe^{3+} for flocculation.

4.3.2 Statistical optimization of TMB dose

In order to predict and develop a clear understanding of flocculation performance of the quaternized product under real time conditions, two sets of simulation studies were carried out. In preliminary experiments, flocculant activity of TMB was assessed against colloidal particles of different sizes in range of 0.5 to 100µm. The effective concentration of quaternized derivative in removing suspensions that are composed of colloids of known size was screened **(Figure 4.5)**. The varied size of colloidal particles used (~0.5-0.8µm (bacterial cell suspension), ~4-7µm (Kaolin, yeast cell suspension, cellulose), ~15-20µm (silica), ~100µm (Active carbon)) were broadly differentiated into low, medium and large sizes respectively. Further the dose required for maximum flocculation of low, medium and large size particles at various turbidity levels was optimized by response surface methodology (RSM).

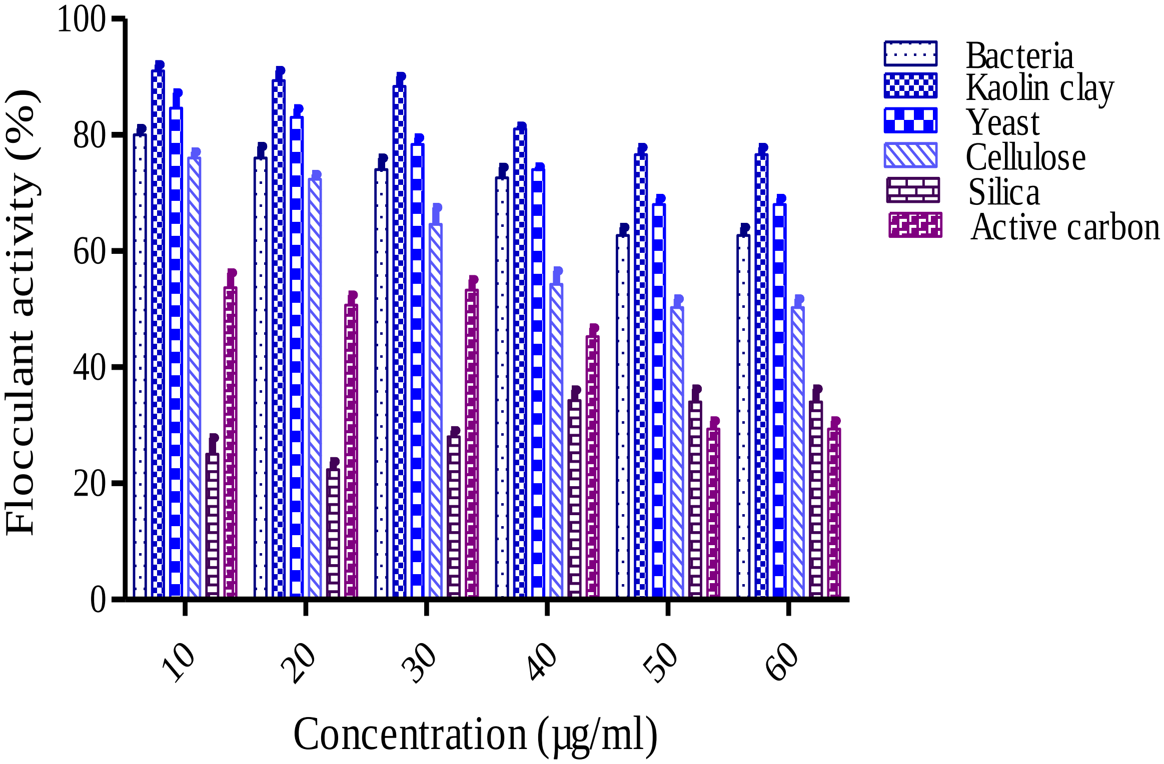


Figure 4.5 Flocculating activity (%) against varied size colloidal particles in solution applied with different doses of TMB ranging from 10-60µg/ml. The corresponding size of the particles are following [~0.5-0.8µm (bacterial cell suspension), ~4-7µm (Kaolin, yeast cell suspension, cellulose), ~15-20µm (silica), ~100µm (Active carbon)]

Results revealed that TMB aggregated a wide range of colloidal particles, over concentrations of 10-60µg/ml. As illustrated in **Figure 4.5**, the most effective flocculation was achieved in suspensions comprising low sized particles at even low concentration of the polymer at a fixed turbidity of 400NTU. The flocculation efficiency against the suspension of medium sized particles, ranged from 30-40% flocculant activity (that too at relatively higher concentration of polymers). The suspensions of large sized particles showed 50-65% flocculant activity for TMB at the same concentration range that flocculated in the range of 70-90% activity in case of low sized particle suspension. This might be due to non-uniform distribution of pore size on the biopolymer surface; also the number of sites available for binding low sized particles might be more than that of binding medium and large sized particles.

On the basis of preliminary experimental data, in the second set of experiments, the solutions of each of the colloidal particles were made that was differentiated into three types of turbidity levels low (10-100NTU), medium (100-200NTU) and high (200-400NTU) comprising of low (bacteria ~0.5-0.8µm), medium (silica ~15-20µm) and large size (active carbon~100µm) colloidal particles for optimization studies.

Response surface methodology was employed to determine the relationship between the flocculation process responses (flocculating activity and turbidity removal) with the most important variables (TMB dosage and turbidity of solution comprising of low, medium and large sized particles). There are several response models that can be derived, such as linear, interactive, quadratic and cubic models. These models may be correlated with the experimental data, but significant selection of the best model is required because the selected model correlates with the experimental data depending on the adequacy of that selected model.

Table 4.6: Experimental variables and results of flocculation experiments

a. Low size particles

Run	A:Dose (ppm)	B:Turbidity (NTU)	Flocculating activity (%)	Turbidity removal (%)
1	-1	1	20	32
2	-1.41421	0	40	44
3	0	0	78	70
4	1	-1	65	58
5	1.414214	0	55	50
6	0	1.414214	45	56
7	0	0	85	89
8	-1	-1	92	89
9	0	0	85.6	89.7
10	0	-1.41421	84	83
11	1	1	56	59
12	0	0	84.9	88.9
13	0	0	85.3	89

b. Medium size particles

Run	A:Dose (ppm)	B:Turbidity (NTU)	Flocculating activity (%)	Turbidity removal (%)
1	0	0	93	88.5
2	1	-1	50	47
3	-1	1	49	53
4	0	0	93	88.9
5	1.414214	0	30	32
6	0	1.414214	80	75
7	-1.41421	0	20	23
8	0	0	93	88
9	0	-1.41421	49	53
10	1	1	45	51
11	-1	-1	25	28
12	0	0	93	88
13	0	0	93	88.4

c. Large size particles

Run	A:Dose (ppm)	B:Turbidity (NTU)	Flocculating activity (%)	Turbidity removal (%)
1	-1.41421	0	63	65
2	1	-1	96	93
3	0	0	85.4	85
4	1.414214	0	92	88
5	0	0	85.8	85
6	0	0	85.5	84
7	-1	-1	70	74
8	0	-1.41421	85	86
9	-1	1	68	70
10	0	0	85.6	85
11	0	0	85.6	85
12	0	1.414214	75	82
13	1	1	80	78

Thus, according to the experimental data, the quadratic model was suggested to represent the correlation between experimental data and all responses, because it has the lowest standard deviation and p value, as well as the highest coefficient of determination (R^2), adjusted R^2 and predicted R^2 values. However, the cubic model was not recommended in this study because it had insufficient points to estimate the coefficients of the model. **Table 4.6**a, b and c shows the experimental variables and the results of the flocculation experiments for low, medium and large size particles.

Analysis of variance (ANOVA) was employed to determine the statistical significance of all analyses. Based on the optimization of parameters and the experimental results (obtained from CCD), the relationship between the flocculating activity and the significant parameters (dose of TMB and turbidity level of solution) was established in the form of a quadratic polynomial equation. The equation for the model in terms of coded factors is present in **Table 4.7.**

Table 4.7: Quadratic model equation for flocculating activity and turbidity removal

Low size particles $Y_{\text{Flocculating activity (R1)}} =+83.76+3.78*A-17.02*B+15.75*A*B-17.57*A^2-9.07* B^2$ $Y_{\text{Turbidity removal (R2)}} =+85.32+0.56* A-11.77* B+14.50\ AB-18.85* A^2-7.60* B^2$
Medium size particles $Y_{\text{Flocculating activity (R1)}} =+93.00 +4.39 * A +7.86 * B -7.25 * AB -34.62 * A^2 -14.88 * B^2$ $Y_{\text{Turbidity removal (R2)}} =+88.36+3.72* A+7.51* B-5.25* AB-30.68* A^2-12.43* B^2$
Large size particles $Y_{\text{Flocculating activity (R1)}}= +85.58 +9.88* A-4.02* B-3.50* AB-4.10* A^2-2.85* B^2$ $Y_{\text{Turbidity removal (R2)}} =+84.80+7.44* A-3.08* B-2.75* AB-4.52* A^2-0.78* B^2$
Where, ‘Y’ is the response value for flocculating activity (%) and turbidity removal (%), ‘A’-Dose of TMB, ‘B’-Turbidity of solution

Table 4.8. Analysis of variance (ANOVA) results showing the terms in each response quadratic model.

a. Low size particles

Response	Source	Sum of Squares	Df	Mean Square	F Value	p-value	Remark
	Model	5895.45	5	1179.09	53.58	< 0.0001	Significant
Flocculating activity	A-Dose	114.10	1	114.10	5.19	0.0569	
	B-Turbidity	2317.25	1	2317.25	105.30	< 0.0001	
	AB	992.25	1	992.25	45.09	0.0003	
	A^2	2146.90	1	2146.90	97.56	< 0.0001	
	B^2	571.96	1	571.96	25.99	0.0014	
Turbidity removal	Model	4610.40	5	922.08	18.13	0.0007	Significant
	A-Dose	2.51	1	2.51	0.049	0.8304	
	B-Turbidity	1108.82	1	1108.82	21.80	0.0023	
	AB	841.00	1	841.00	16.53	0.0048	
	A^2	2471.15	1	2471.15	48.58	0.0002	
	B^2	401.54	1	401.54	7.89	0.0262	

b. Medium size particles

Response	Source	Sum of Squares	Df	Mean Square	F Value	p-value	Remark
	Model	9957.72	5	1991.54	145.96	< 0.0001	Significant
Flocculating activity	A-Dose	154.37	1	154.37	11.31	0.0120	
	B-Turbidity	493.62	1	493.62	36.18	0.0005	
	AB	210.25	1	210.25	15.41	0.0057	
	A^2	8340.11	1	8340.11	611.25	< 0.0001	
	B^2	1539.24	1	1539.24	112.81	< 0.0001	
Turbidity removal	Model	7723.04	5	1544.61	1998.10	< 0.0001	Significant
	A-Dose	110.47	1	110.47	142.90	< 0.0001	
	B-Turbidity	451.69	1	451.69	584.31	< 0.0001	
	AB	110.25	1	110.25	142.62	< 0.0001	
	A^2	6547.91	1	6547.91	8470.36	< 0.0001	
	B^2	1074.82	1	1074.82	1390.38	< 0.0001	

c. Large size particles

Response	Source	Sum of Squares	Df	Mean Square	F Value	p-value	Remark
	Model	1113.59	5	222.72	486.05	< 0.0001	Significant
Flocculating activity	A-Dose	780.37	1	780.37	1703.03	< 0.0001	
	B-Turbidity	129.14	1	129.14	281.83	< 0.0001	
	AB	49.00	1	49.00	106.94	< 0.0001	
	A^2	117.08	1	117.08	255.51	< 0.0001	
	B^2	56.60	1	56.60	123.53	< 0.0001	
Turbidity removal	Model	691.86	5	138.37	30.87	0.0001	Significant
	A-Dose	442.93	1	442.93	98.83	< 0.0001	
	B-Turbidity	76.00	1	76.00	16.96	0.0045	
	AB	30.25	1	30.25	6.75	0.0355	
	A^2	142.44	1	142.44	31.78	0.0008	
	B^2	4.18	1	4.18	0.93	0.3664	

Table 4.8 shows the probability (*p* value) of the quadratic model for flocculating activity and turbidity removal that implies that the model is significant. To evaluate the quality of the model developed, the coefficient of determination (R^2) was used which gave the proportion of total variance in the response predicted by the model. The closer R^2 is to 1, the better the model predicts the response (Trinh and Kang 2011). The determination coefficients (R^2 values) between the observed and predicted values for in case of low, medium and large size particles for flocculating activity and turbidity removal, indicated a good agreement between the observed and predicted values **(Table 4.9).** Thus the predication of experimental data is considered to be satisfactory.

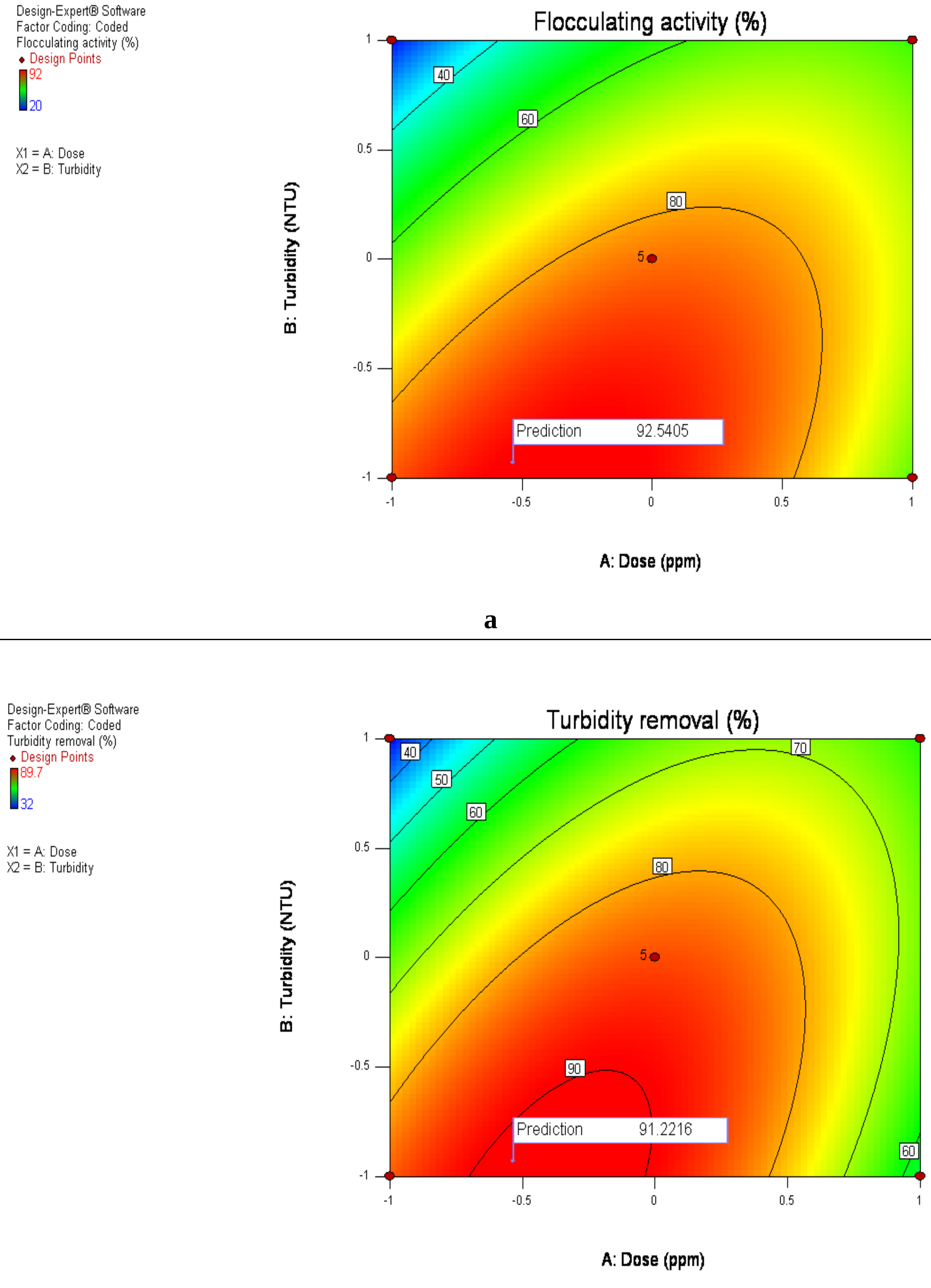
Table 4.9: Model statistics

Response	**Probability**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**Adequate precision**	**C.V %**
Low size particles						
Flocculating activity	<0.0001	0.9745	0.9563	0.8572	21.687	6.96
Turbidity removal	0.0007	0.9283	0.8771	0.8184	11.296	10.33
Medium size particles						
Flocculating activity	<0.0001	0.9905	0.9837	0.9324	30.071	5.91
Turbidity removal	<0.0001	0.9993	0.9988	0.9954	111.524	1.42
Large size particles						
Flocculating activity	<0.0001	0.9971	0.9951	0.9800	70.914	0.83
Turbidity removal	0.001	0.9566	0.9256	0.6977	19.152	2.60

Notes: R^2: coefficient of determination; Adj. R^2: adjusted R^2; Pred. R^2 : predicted R^2 ; Adeq. Prec.: Adequate precision; CV: coefficient variation.

The measures of the adequate precision for the response models were 21.687 and 11.296 (low size particles), 30.071 and 111.524 (medium size particles) and 70.914 and 19.515 (large size particles) for the flocculating activity and turbidity removal models. The signal to noise ratio is represented by these adequate precision values and a ratio greater than four is desirable. Hence, in this study the adequate precision values for both the models in all particle sizes were more than four which indicated that the quadratic model equation can be used within the range of factors in the design space. The coefficient of variance (CV), represents the ratio of the standard error of estimate to the mean value of the observed model (represented as %). The model can be normally considered reproducible when its CV value is less than 10% (Trinh and Kang 2011). The low CV values of the models in the case of low, medium and large size particles indicated the good precision and reliability of the experiments.

Figures 4.6, 4.7 and 4.8 shows the contour plot for the flocculating activity at various dosages model, revealing the maximum flocculating activity and turbidity removal of more than 90% occurs at polymer dosage of 14.5ppm in the case of low size particles at low turbidity of solution. This maximum flocculating activity at a lower dose at lower turbidity level may occurs due to the fact that the polymer becomes ionized, can easily neutralize the residual charge on particles and expand the chain on the bridge. The maximum flocculating activity of 93.997% of medium size particles occurred at TMB dosage of 21.5ppm accompanied by 89.3851% of turbidity removal at the medium turbidity of the solution. The large size particles were flocculated at maximum value of 94.7134% in a low turbidity solution at a polymer dosage of 27ppm which also reduced the turbidity of the solution by 92.289%. The common turbidity removal mechanism by forming flocs composed accompanied or followed by sweep flocculation of colloidal particles (Tripathy and Ranjan 2006).



a

b

Figure 4.6. 2D contour plot for (a) flocculating activity; and (b) for reduction in turbidity in case of low size particles.

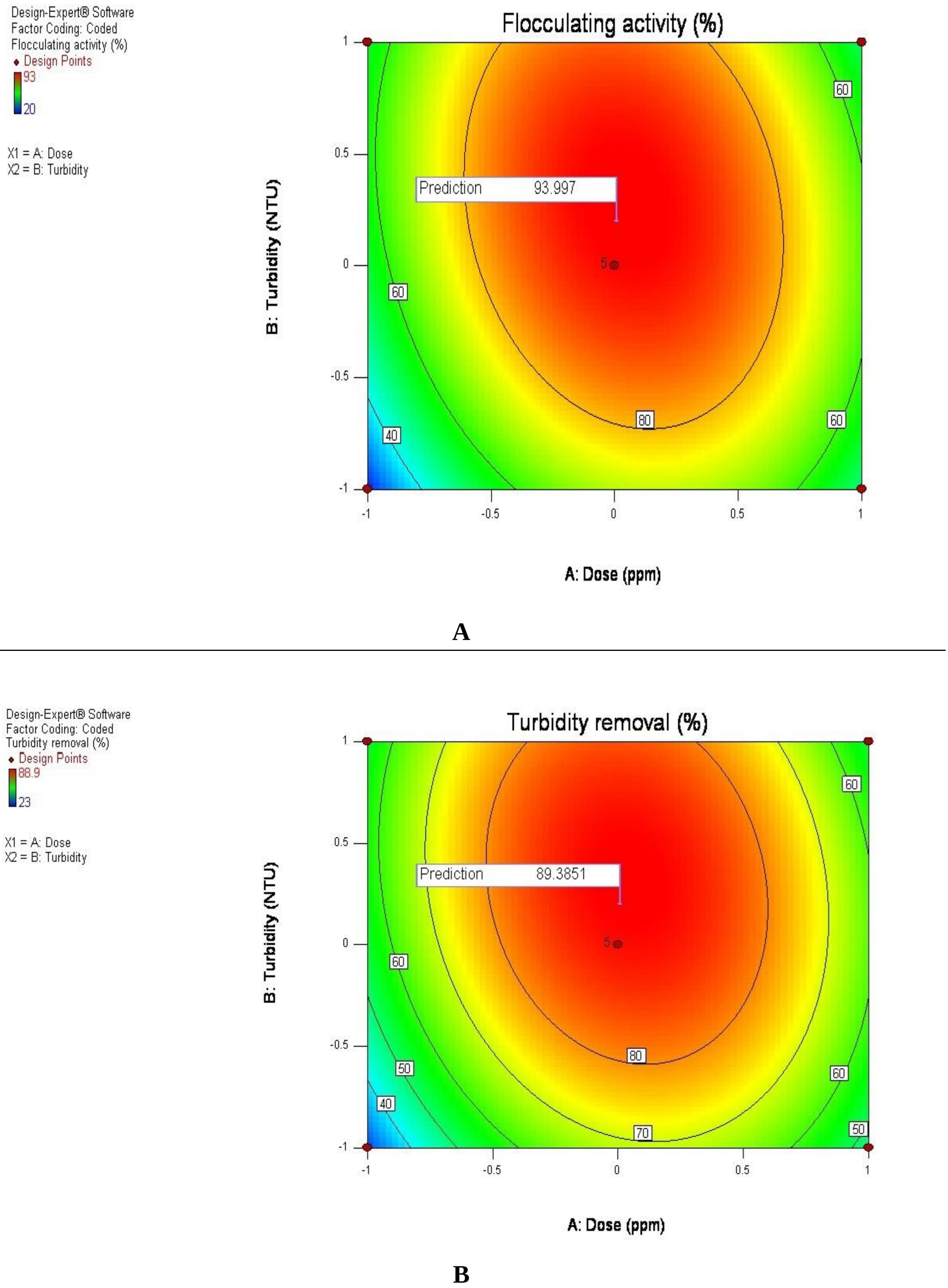
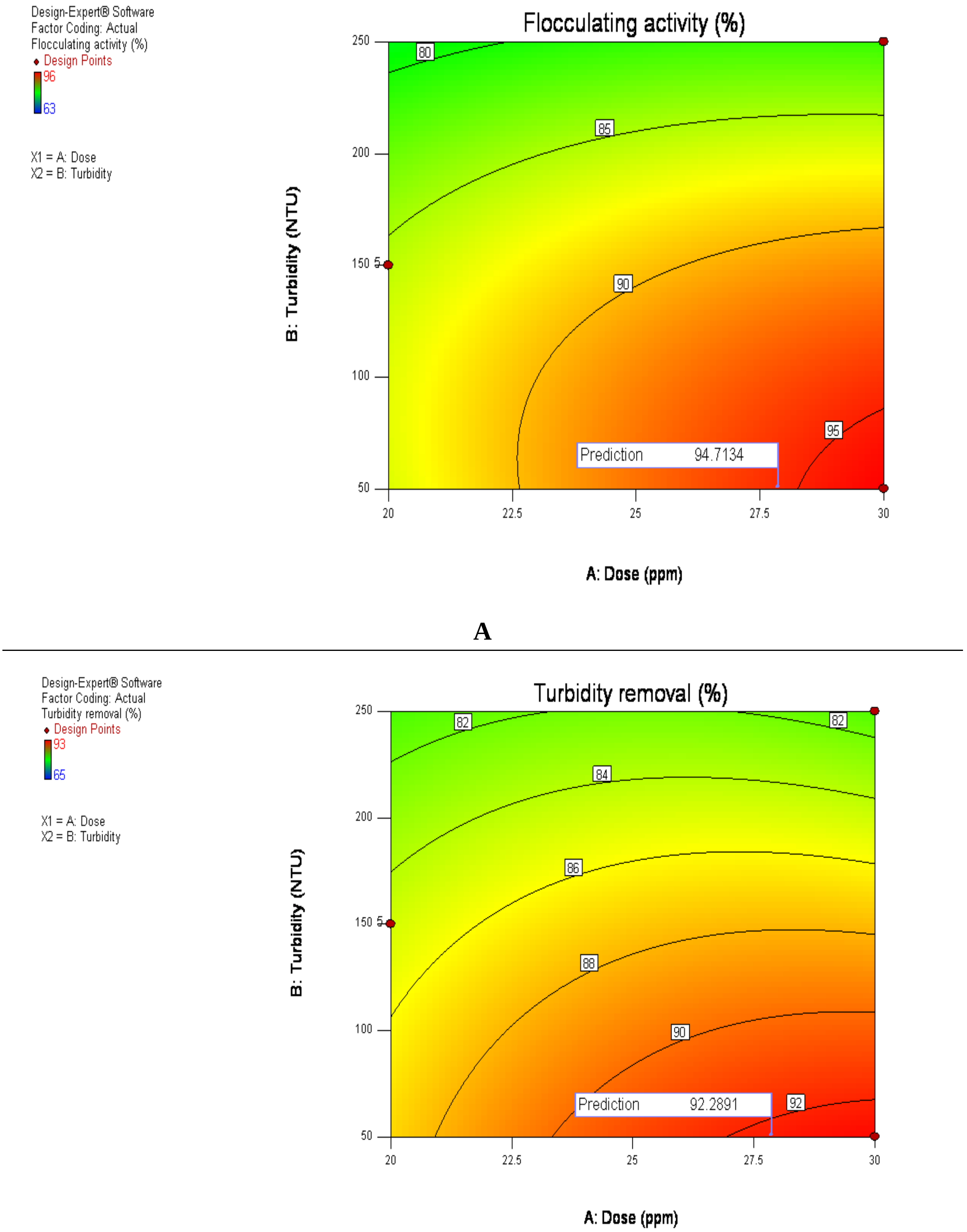


Figure 4.7 2D contour plot for (a) flocculating activity; and (b) for reduction in turbidity in medium size particles.



A

B

Figure 4.8: 2D contour plot for (a) flocculating activity; and (b) for reduction in turbidity in large size particles.

In order to have a practical understanding of the biopolymeric flocculants efficiency, an evaluation was carried out against mixed suspension containing all different colloidal size particles together at three levels of turbidity. The optimized doses from the RSM model were used in this study and the turbid solutions ranging from 10 to 400NTU were considered. Overall results revealed that the degree of flocculation achieved with various sized of particle suspension was found to be in agreement with results obtained in the optimization studies against individual particle sized at different turbidity of solution.

As is shown in the **Figure 4.9**, high dosage of the polymer does not contribute to a noticeable increase in the flocculating activity which corresponds to the turbidity removal. As the size of colloidal particles is variable in the mixed suspension, at low turbidity of solution the dose which was earlier found suitable for large particle size is now effective for medium size particles at low turbidity levels. 20ppm of TMB dose was equally effective at lowest turbidity but still somewhat lesser than the optimized dose for highest particle size. At the highest turbidity of the mixed suspension the dose was nearly equal to that of the dose with lowest turbidity of highest particle size. Overall results revealed that 15 to 20ppm of TMB was efficient in removal of different size particles in mixed suspension at all the levels of turbidity.

This phenomenon might be due to the increase of polymer and particle complexes that start to form and the maximum removal of turbidity is at the dosage value when almost all polymer sites are occupied by the particles. Beyond that optimum dosage value, the turbidity removal decreases probably due to lack of polymer sites for interaction with the colloidal particles. The flocculant dosage leading to the maximum flocculation efficiency value is considered as optimal dosage value. The mechanism of the flocculation with TMB may be explained by a combination of charge neutralization and polymer bridging.

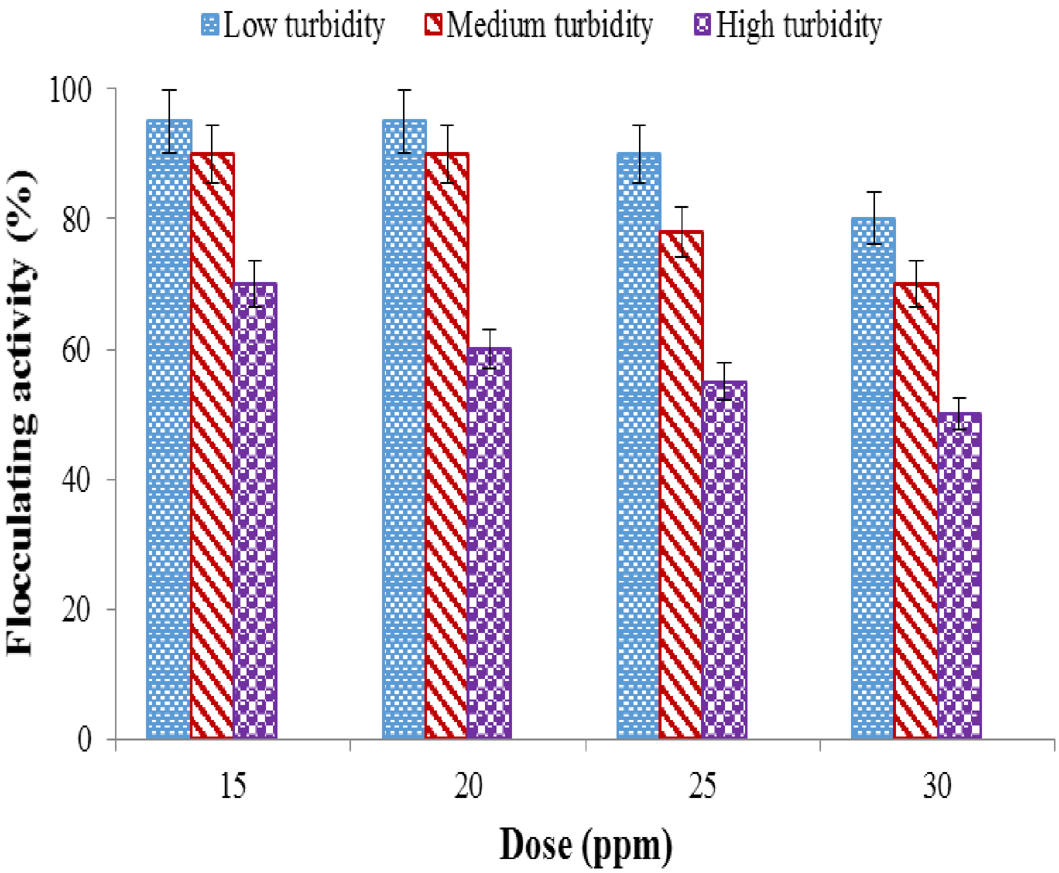


Figure 4.9 Effect of various doses of TMB on mixed suspension of colloidal particles

4.4 Inactivation of indicator pathogenic bacteria by quaternized polymeric flocculant derivatives

In twenty water samples (collected from tanks, hand pumps, piped, canal surface water of different locations in Punjab and Haryana including rural and semi urban, urban areas where degree of contamination is diverse and of high magnitude, the pathogenic bacteria detected in the impacted samples are given in **Table 4.10.**

Table 4.10: Prevalence of water borne pathogenic bacteria in random potable water samples

Pathogen or indicator group	**Counts in sampled water**	**Impacted samples (%)**
Heterotrophic count	$3X10^6$ - $4.2X10^8$CFU/mL	48 %
Coliforms, Fecal coliforms, *E.coli*	>240/100mL MPN	30 %
Salmonella spp	2.2 $X10^4$CFU/mL	12 %
Yersinia spp	$1.67X10^3$CFU/mL	10 %
Aeromonas spp	$1.06X10^4$CFU/mL	13 %
Listeria spp	3.0 $X10^3$ CFU/mL	9 %

The human pathogens that present serious risk of disease whenever present in drinking water include *Salmonella* species, *Shigella* species, pathogenic *Escherichia coli*, *Vibrio cholerae*, *Yersinia*, *Listeria*, various viruses such as Hepatitis A, Hepatitis E, Rota virus and parasites such as *Entamoeba histolytica* and *Giardia* species and so on (Maskey et al. 2006; Jyoti et al. 2010; Sharan et al. 2011). On the basis of literature cited, *Salmonella typhimurium* ATCC 25315, *Shigella flexneri* 2a, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* O157:H7 ATCC 3215 were selected to further evaluate antibacterial activity of quaternized polymers.

Antibacterial activity of chitosan, BF and their derivatives were analyzed against the six water borne pathogens by agar disc diffusion assay. Both TMB and TMC demonstrated antibacterial activity against all the bacteria (*Salmonella typhimurium* ATCC 25315, *Shigella flexneri* 2a, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* O157:H7 ATCC 3215) at neutral pH indicated by diameters of inhibition zones measured in mm scale.

Table 4.11 Zone of inhibitions shown by the pathogens against bioflocculant (BF), chitosan and their derivatives, TMB and TMC (Values represent the diameter (mm) of zone produced around each disc and are average of 3 separate experiments)

Bacteria	**Concentration of compounds (µg/ml)** **Diameter of zone of inhibition (mm)**									
	BF		Chitosan		TMB			TMC		
	25	100	25	100	25	75	100	25	75	100
***Salmonella typhimurium* ATCC 23564**	NZ	14±0.6	NZ	10±0.4	14±0.5	22±0.4	25±0.5	NZ	12±0.4	15±0.7
Shigella flexneri 2a	NZ	12±0.2	NZ	8±0.6	15±0.3	24±0.6	25±0.9	NZ	10±0.3	12±0.6
***Aeromonas hydrophila* ATCC 35654**	NZ	13±0.7	NZ	11±0.3	13±0.1	20±0.3	23±0.5	9±0.3	14±0.7	18±0.4
***Yersinia enterocolitica* ATCC 9610**	NZ	13±0.5	NZ	12±0.2	10±0.6	19±0.6	21±0.3	10±0.4	17±0.9	19±0.5
***Escherichia coli O157:H7* ATCC 32150**	NZ	14±0.2	NZ	11±0.8	10±0.7	16±0.8	16±0.5	9±0.1	16±0.4	18±0.6
***Listeria monocytogenes* ATCC 19111**	NZ	13±0.1	NZ	9±0.7	NZ	14±0.5	16±0.2	8±0.2	11±0.4	11±0.5

ND: Not determined; NZ: No zone observed; Diameters includes the diameter of disc (6 mm)

With quaternized biopolymeric flocculants, TMC and TMB, significant ($p<0.05$) inhibitory effect than that of BF and chitosan was observed **(Table 4.11)**. However, TMB showed higher inhibitory effect against all the Gram-negative bacteria in comparison to TMC. These observed effects may be explained on the basis of structural resemblance in possession of trimethyl ammonium moieties at amino groups of polymer backbone. The orders of zone of inhibition with TMB were found to be *Salmonella> Shigella> Aeromonas> Yersinia> E.coli 0157:H7> Listeria* whereas the orders of zone of inhibition with TMC were found to be *E.coli 0157:H7> Yersinia> Aeromonas> Salmonella> Shigella> Listeria.* The Gram-positive *Listeria* was inhibited the least by both the quaternized derivatives.

The aim of the present study was to systematically investigate the extent of antibacterial activity of quaternized chitosan and newly reported quaternized bioflocculant, TMB. A key aspect of this study is the examination of minimum inhibitory concentration (MIC) of quaternized bioflocculants, then, monitoring their bactericidal effect.

4.5 MIC determination

A semi-quantitative measurement of in-vitro activity of antibacterial agent (TMB and TMC) was carried out to estimate the lowest concentration of TMB and TMC that could inhibit the visible growth of pathogenic microorganisms after overnight incubation.

The results of the treatment outcomes of native polymers and their quaternized derivatives against all the six selected pathogens in BHI media at ambient temperature are shown in **Table 4.12.**

Table 4.12 Minimum inhibitory concentration (MIC) of BF, Chitosan and quaternized derivatives, TMC and TMB against six bacterial pathogens (in BHI media)

Bacteria	MIC (µg/ml)	
	TMC	TMB
S. typhimurium	74	60
A. hydrophilla	71	62
Y. enterocolitica	67	65
E. coli O157:H7	70	68
L. monocytogenes	80	72
S. flexneri 2a	78	62

The MIC values of TMB and TMC were 60µg/ml and 74µg/ml, respectively for *Salmonella*. The MIC of TMB against *Shigella flexneri* Type 2a was 62 whereas 78µg/ml of TMC was required to inhibit the bacteria. Inhibition of the growth of *E. coli* O157:H7 occurred at 68 and 70µg/ml of TMB and TMC respectively. In earlier studies with quaternized derivatives of chitosan, *E.coli* and *S. aureus* have been adopted as model of Gram-negative and Gram-positive bacteria, respectively, and MIC values were reported in the range of 32-168µg/ml for both the bacteria (Sajomsang et al. 2008). Although, the antibacterial activity depends upon the degree of quaternization and molecular weight of the starting material, trimethyl chitosan synthesized in present work seems promising as compared to earlier derivatives (Sajomsang et al. 2009b). In the case of *Aeromonas*, the MIC of TMB and TMC were 62 and 71µg/ml, respectively. The inhibition of *Yersinia* in BHI was achieved in 60min with MIC value of 65 and 67µg/ml of TMB and TMC, respectively. To inhibit the growth of *Listeria monocytogenes,* 72µg/ml and 80µg/ml of TMB and TMC were needed, respectively. However, other bacteria as *Enterococcus faecalis and Pseudomonas aeruginosa* have shown susceptible to trimethyl chitosan, with MIC of 128 and 256g/ml respectively (Runarsson et al. 2010; Xu et al. 2010). *Streptococcus mutans*, responsible for dental caries in humans, was also susceptible to chitooligosaccharide quaternized via covalent addition of glycidyl trimethylammonium chloride (Kim et al. 2003). The observed MIC values of all the tested Gram-negative bacteria were in the range of 50-90µg/ml with TMB (Table 4.12). The optimization of time in order to kill the bacteria in BHI media and water was further carried out in killing kinetics study.

4.6 Killing kinetics

Figure 4.10 (a-f) reveals a comparative analysis of antibacterial activity of quaternized biopolymeric flocculants and their native counterparts against six water borne pathogens. 4 log reductions were evident in all the pathogens treated with TMB and TMC at different concentrations and exposure time. Results revealed 99.9% inactivation of all TMB treated Gram negative pathogens and 90% inactivation of *Listeria*, which is the only gram positive bacterium used in the study. Treatment with TMC at respective time resulted in 99% inactivation of all the pathogens and 90% inactivation of *Listeria*.

Finer analysis of **Figure 4.10a** indicated that TMB at 60µg/ml exerted a marked inhibitory effect against *Salmonella*. The onset effect of this was rapid and resulted in a reduction of 4 log cycles within 60min. In a similar fashion, **Figure 4.10b** demonstrates that when *Aeromonas hydrophila* was exposed to TMB (62µg/ml), a reduction of cells by 3 log CFU/ml occurred within the first hour. Following this a rapid decrease was observed within next 15-20min and finally cell numbers were reduced by 4 log CFU/ml within 75-80min of treatment.

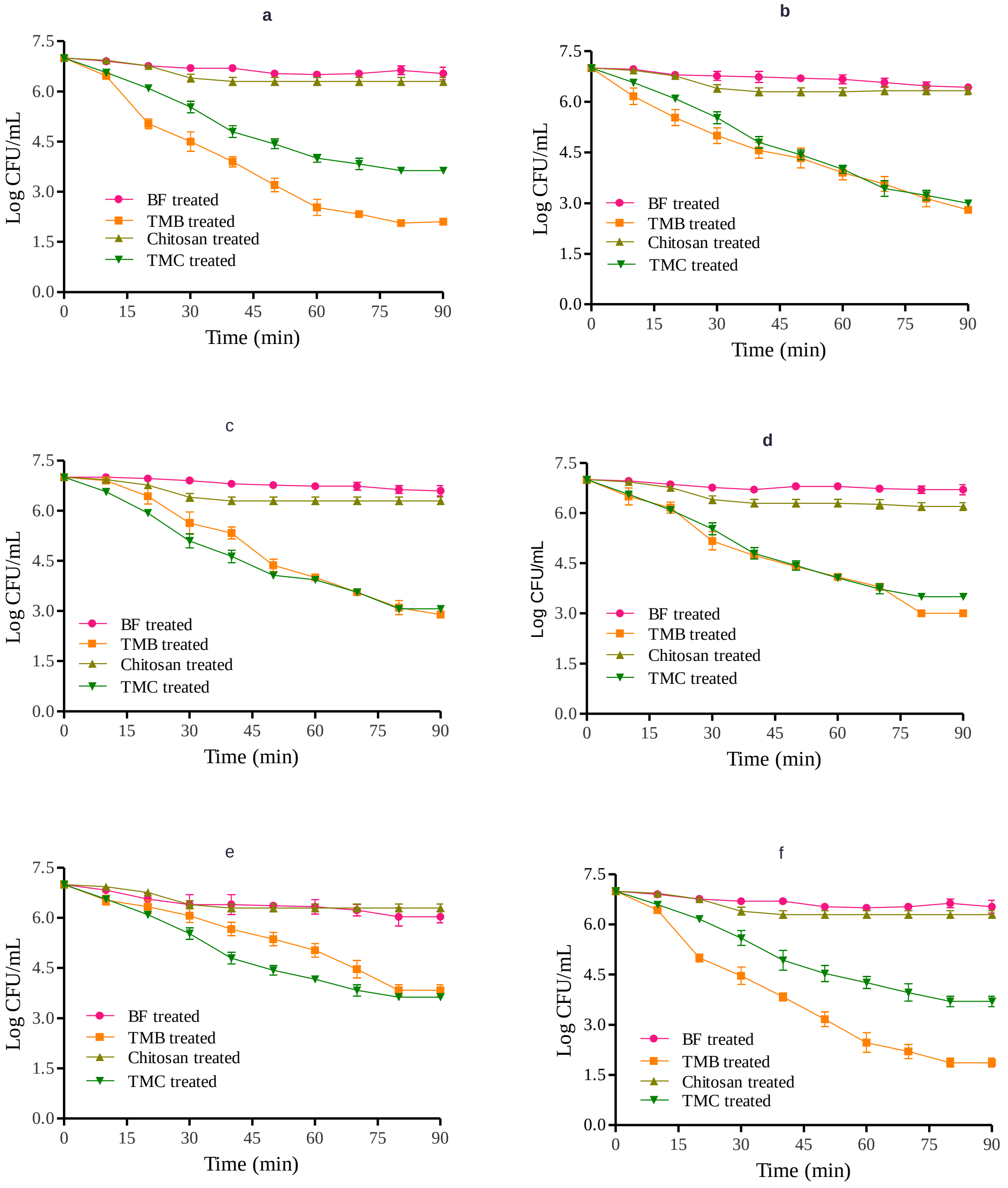


Figure 4.10 Killing kinetics for BF (●), N,N,N- trimethyl biopolymer, TMB (■), chitosan (▲) and N,N,N-trimethyl chitosan, TMC (▼) against, a) *Salmonella typhimurium* ATCC 23564, b) *Aeromonas hydrophila* ATCC 35654, c) *Yersinia enterocolitica* ATCC 9610, d) *Escherichia coli* O157:H7 ATCC 35150, e) *Listeria monocytogenes* ATCC 1911 f) *Shigella flexneri 2a*. Each value is expressed as mean ± SD (n = 3)

Figure 4.10c indicates that when *Yersinia* cells were treated with TMB at 65µg/ml for 75min, a reduction of 4 log cycles was observed. **Figure 4.10d** demonstrates that when *E. coli* O157:H7 was exposed to TMB at 68µg/ml, it exerted a 4log CFU/ml reduction in 80min. For *Listeria monocytogenes,* however, cell numbers declined slowly over 70-90 minutes (**Figure 4.10e**) when exposed to 72µg/ml of TMB. The effect of TMB at 62µg/ml on the growth of *Shigella flexneri* Type 2a is shown in **Figure 4.10f**, where a reduction of 4 log CFU/ml was evident after 60-70min of treatment. However, treatment of indicator pathogenic bacteria with TMC at concentration range of 60-80µg/ml for 60-80 minutes resulted in approximately 3log reduction.

A appropriate assays were used to investigate the rate at which TMB and TMC kill the target bacteria in nutrient rich media as well as in simulated conditions. The permanent loss of reproductive activity being a definite sign of bactericidal activity, an antimicrobial agent which enables a reduction of target organisms by 4 log cycles is considered effective (McDonnell and Russell 1999).

4.7 Effect of quaternized biopolymers on synthetic water spiked with indicator strains

In order to obtain information on the flocculant-disinfectant activity of newly designed biopolymeric flocculant in real time applications, 216 water samples from various sources near Patiala city, Punjab, INDIA were collected and analysed for water quality parameters (**Table 4.13)**.

Table 4.13: Water quality analysis

Characteristics	Acceptable limits	Limit of rejection	No. of water samples	Samples within acceptable limits	Samples in range of rejection
Turbidity (NTU)	2.5	10	216	107	108
pH	7.0 to 8.5	<6.5 or >9.2	216	209	7
TDS (mg/l)	500	1500	216	98	118
Total hardness (mg/l)	200	600	216	105	111
Calcium (mg/l)	75	200	216	114	102
Magnesium (mg/l)	<30	150	216	124	92
Chlorides (mg/l)	200	1000	216	111	105
Sulphates (mg/l)	200	400	216	109	107
Fluorides (mg/l)	1.0	1.5	216	169	47
Nitrates (mg/l)	45.0	45.0	216	134	82
Iron (mg/l)	0.1	1.0	216	153	73

On the basis of information acquired from these samples, synthetic water was formulated and effect of TMB and TMC on spiked water borne pathogens was recorded. The results are shown in **Table 4.14.**

The initial count of spiked water borne pathogens in synthetic water samples was 6 log CFU/ml. Significant reduction ($P<0.5$) in numbers of all the pathogens (3-5 log reduction) were observed in both selective media and Thin Agar Layer (TAL) plates. The TAL plate method was adopted for enumerating injured pathogens following treatment with TMB; this method resuscitates injured cells simultaneously selecting them and was therefore used in conjunction with respective pathogen selective medium (Wu et al. 2001). The TMB dose optimization trials carried out indicated that optimum dosage was in range of 5-10mg/l for achieving the removal of 6-7 log CFU/ml of water borne pathogens spiked in synthetic water **(Table 4.14)**. On the other hand, chlorine used at the same concentration as of TMB, resulted in 99.99% inactivation of *Salmonella typhimurium*, *Shigella flexneri* and *Aeromonas hydrophilla*. Chlorination required less time as compared to TMB for inactivating *Yersinia enterocolitica*, *E.coli* and *Listeria monocytogenes*.

Table 4.14 Comparative efficiency of quaternized biopolymer (TMB and TMC) and synthetic disinfectant (chlorine) in synthetic water spiked with selected microorganisms at initial inoculums of 6 $\log_{10}$ CFU, (pH-6-7) at 25°C

Bacteria	**Dose (mg/l)**	**TMB**			**TMC**			**Chlorine**		
		Cell count after treatment at exposure time (min)			Cell count after treatment at exposure time (min)			Cell count after treatment at exposure time (min)		
		10	30	60	10	30	60	10	30	60
Listeria monocytogenes	6	5.5	4	1	6	3	1	4.6	1.2	0
E. coli O157:H7	5	6	1.6	0	6	3	1	2.2	0	0
Salmonella typhimurium	4	4.6	1	0	5	3	1	5	3	0
Shigella flexneri 2a	4	4.5	1.2	0	5	3.5	1	5	2	0
Yersinia enterocolitica	6	5.4	2	0	6	4	1	2.2	1.1	0
Aeromonas hydrophila	5	5.3	3	0	6	3	1	4	2	0

Currently, chlorination is the most common method of disinfecting public drinking water worldwide. Therefore, any novel biocidal agent designed for water disinfection must necessarily be compared with chlorine. The pathogen removal efficiency of chlorine in comparison to TMB in water against selected water borne pathogens was thus compared. We observed some differences during this study in terms of bacterial inactivation, which may be attributed to variability between strains, slight pH or temperature differences. Simulation trials in synthetic water indicated inactivation of Gram-negative microorganisms during the first 30-60 min of treatment at low dosage of TMB and TMC (5-10mg/l).

Table 4.15 shows the effect of TMB and TMC on the major parameters of pH, total hardness, total dissolved solids, turbidity and coliform count in synthetic water. The quaternized biopolymeric flocculants investigated in this study were able to reduce both the turbidity and bacterial load from the synthetic water to a varying degree, with higher bacterial load removal rate observed with increasing concentrations of the polymers. TMB and TMC at dose of 10ppm and 15ppm, respectively, could effectively reduce the total hardness and total dissolved solids from synthetic water. The initial turbidity of synthetic water was set as 50NTU which was reduced to 96% by addition of TMB and TMC lead to 70% reduction in the turbidity of water. A lower turbidity removal rate was observed with increase in polymer concentrations in most cases. This is because the optimum amount of flocculants in the suspension causes the microorganisms and the fine particles to aggregate and settle. However, when the optimum concentration is exceeded, the aggregated particles can re-disperse and this disturbs particle-settling. This has been attributed to an increase in the repulsive energy between the flocculants and the microorganisms, which causes hindrance in floc formation (Mishra and Bajpayee 2002; Kim et al. 2013).

Table 4.15 Data of synthetic water samples before and after treatment with different doses of TMB and TMC

Parameter	**Before treatment**	**After treatment with different concentrations (mg/l) of TMB and TMC for 30minutes**								**WHO stds.**
		1		**5**		**10**		**15**		
		TMB	**TMC**	**TMB**	**TMC**	**TMB**	**TMC**	**TMB**	**TMC**	
pH measurement	7.5±0.05	7±0.07	7.2±0.09	7±0.04	7±0.08	7±0.01	7±0.04	7±0.01	7±0.02	6.5-8.5
Total hardness (mg/l)	280±0.88	100±0.68	260±0.82	50±0.12	200±0.9	24±0.85	120±0.65	24±0.57	100±0.4	500
TDS	350±0.60	150±0.5	300±0.8	80±0.01	240±0.5	30±0.08	200±0.5	30±0.06	180±0.4	500
Turbidity (NTU)	50±0.02	5±0.6	35±0.75	2±0.008	30±0.24	2±0.005	10±0.1	1±0.002	7±0.5	10
MPN/100 ml	240±0.88	50±0.04	140±0.08	2±0.04	80±0.69	0.0±0.0	20±0.44	0.0±0.0	10±0.56	Nil

The total coliform bacteria count reduced significantly on addition of TMB at 100ppm. However, 10MPN/100ml of bacterial count was still measured on the addition of 15ppm of TMC. It is worth noting that there was no significant reduction in pH of the synthetic water (pH 7.5) after the addition of TMB and TMC as the final pH ranged between 6.55 - 6.92. This makes quaternized biopolymeric flocculant derivatives preferable in practical terms as no further chemical addition is necessary to correct the pH of the finished water.

4.8 Biochemical responses of water borne pathogens

Insights on mode of action of antibacterial agents are crucial both for structure refinement/design modification of antibacterial agents and fate of target pathogens. A battery of assay is usually carried out for evaluating the overall biochemical response of target bacterial cells. In order to elucidate the possible mechanism of the inhibitory action of TMB and TMC against water borne pathogens, target pathogens were further assessed for their membrane potential, permeability intactness, cellular leakage estimation (intracellular K^+ content), intracellular ATP concentration, measurement of glucose and/or lactate dehydrogenase activity. Cellular damage and death was also studied by electron microscopy and FT-IR spectroscopy.

4.8.1 Electric conductivity of treated bacterial cell suspensions

To evaluate the relationship between antibacterial activity of quaternized polymers and membrane permeability of pathogenic bacteria, electrical conductivities of samples were determined as shown in **Figure 4.11.** The electric conductivity of the cell suspensions for indicator strains *Salmonella* treated with TMC (50 and 70µg/ml) and TMB (40 and 60µg/ml) showed a time-dependent increase. In TMC treated bacterial suspension, electrical conductivity increased for 10min beyond which no further increase was observed, either by increasing the duration of exposure or TMC dose. In case of TMB treated bacterial suspensions, the electric conductivity increased by 6.0% and 9.5% for 40 and 60µg/ml, respectively, compared with the controls**.** These results indicated that 60µg/ml TMB was more effective than 40µg/ml of TMB. Differences in the changes of the electrical conductivity between the treatments also suggested that the effect of TMB on the membrane permeability of *Salmonella* and *Shigella* was greater than the other four bacterial pathogens.

4.8.2 Potassium leakage

K^+ specific ion electrode was used to detect membrane impairment and leakage of cellular contents from all the bacterial cells after exposure to different concentrations of TMB and TMC (5-100µg/ml). **Figures 4.12** represents the effects of 10 and 60 µg/ml of TMB upon the membranes of target bacteria as indicated by potassium (K+) leakage. TMB initiated a gradual, dose-dependent flux of K^+ ions from *Salmonella typhimurium* and *Shigella flexneri*, which was rather incomplete. Exposure to TMB at 20µg/ml led to marked leakage effects upon all organisms with a rapid onset. Moreover, treatment of bacterial cells with TMC at 70µg/ml resulted in a gradual leakage of potassium ions from the target cells. The initial K+ ion concentration of the bathing solution for all microorganisms in all experiments was 118 mM. The release of potassium ions in all the pathogens is strongly related to exposure time, as with further increase in time and dose, leakage became stagnant suggesting a complete exhaustion of intracellular K^+ pool on account of extensive membrane damage.

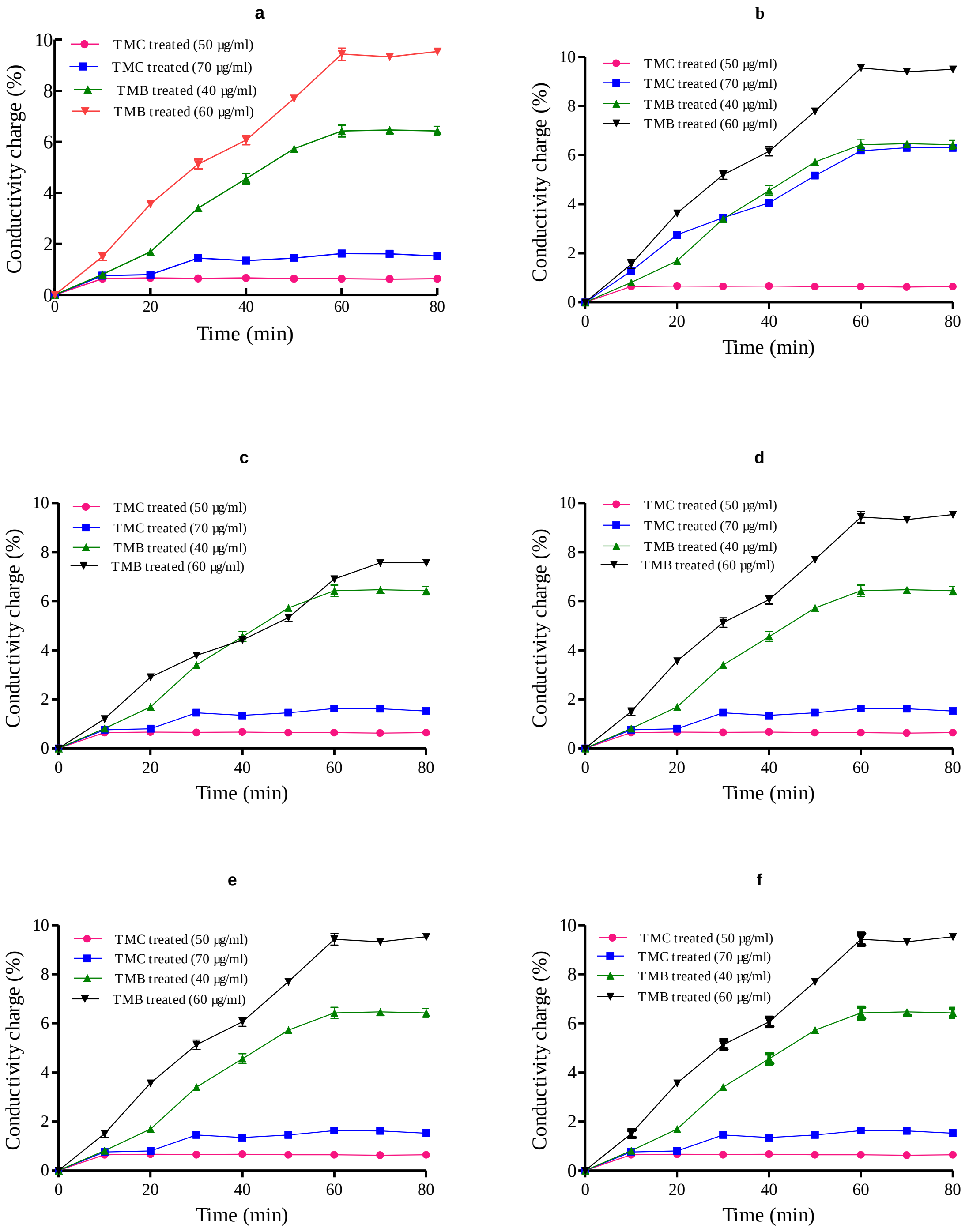


Figure 4.11 Electric conductivity of suspensions of 10^7 CFU/ml of a) *Salmonella typhimurium ATCC 23564*, b) *Shigella flexneri 2a*, c) *Aeromonas hydrophila* ATCC 35654, d) *Listeria monocytogenes* ATCC 1911, e) *Yersinia enterocolitica* ATCC 9610, and f) *Escherichia coli* O157:H7 ATCC35150. Each value is expressed as mean ± SD (n = 3).

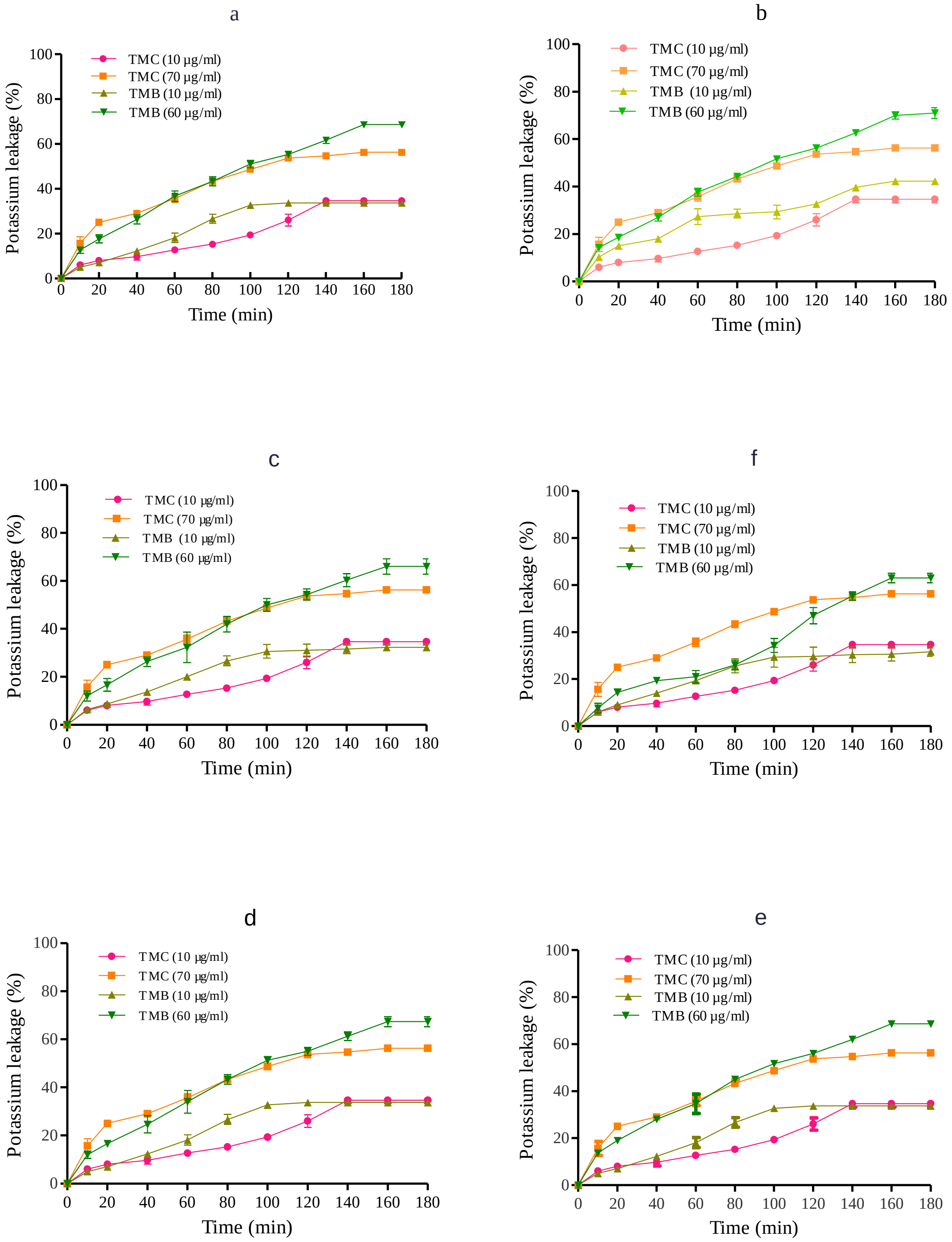


Figure 4.12 Potassium release from a) *Salmonella typhimurium* ATCC 23564, b) *Shigella flexneri 2a*, c) *Aeromonas hydrophila* ATCC 35654 d) *Yersinia enterocolitica* ATCC 9610, e) *Escherichia coli* O157:H7 ATCC 35150 and f) *Listeria monocytogenes* ATCC 1911 after treatment with TMB and TMC. Each value is expressed as mean ± SD (n = 3).

4.8.3 Leakage of UV-absorbing materials

The leaked UV-absorbing materials from the bacterial cells treated with quaternized polymers were measured, as an indicator of permeability alteration. If bacterial membrane is damaged to a certain extent, the release of cytoplasmic constituents of the cell (large molecules such as DNA, RNA, and other materials) can be monitored. Since these nucleotides have strong UV absorption at 260 and 280 nm, membrane integrity can be determined through the detection of absorbance at these wavelengths.

The treatment of Gram-negative bacterial cells with TMB (10-70µg/ml) resulted in a gradual leakage of UV-absorbing substances (likely representing nucleotide and coenzyme pools) from bacterial cells, followed by a plateau for up to 2h **(Fig 4.13).** This leakage was also found to be concentration and time dependent. Optical density measurements of the treated culture revealed around 50% reduction in culture density after 2h, which was attributed to aggregation and flocculation of the cells in the presence of TMB. However, treatment of *Salmonella* with TMC at a higher concentration of 120µg/ml for 130minutes led to release of UV-absorbing substances from the bacterial cells. Increasing the concentrations of TMB and TMC resulted in increased levels of UV-absorbing substances for all the other microorganisms, *Shigella* (TMB-80µg/ml; 65min and TMC-130µg/ml; 130min), *E.coli* (TMB-100µg/ml; 145min and TMC-90µg/ml; 120min), *Aeromonas (*TMB-80µg/ml; 90minutes and TMC-120µg/ml; 110min), *Yersinia (*TMB-100µg/ml; 70min and TMC-140µg/ml; 160min), *and Listeria (*TMB-150µg/ml; 90min and TMC-186µg/ml; 190min).

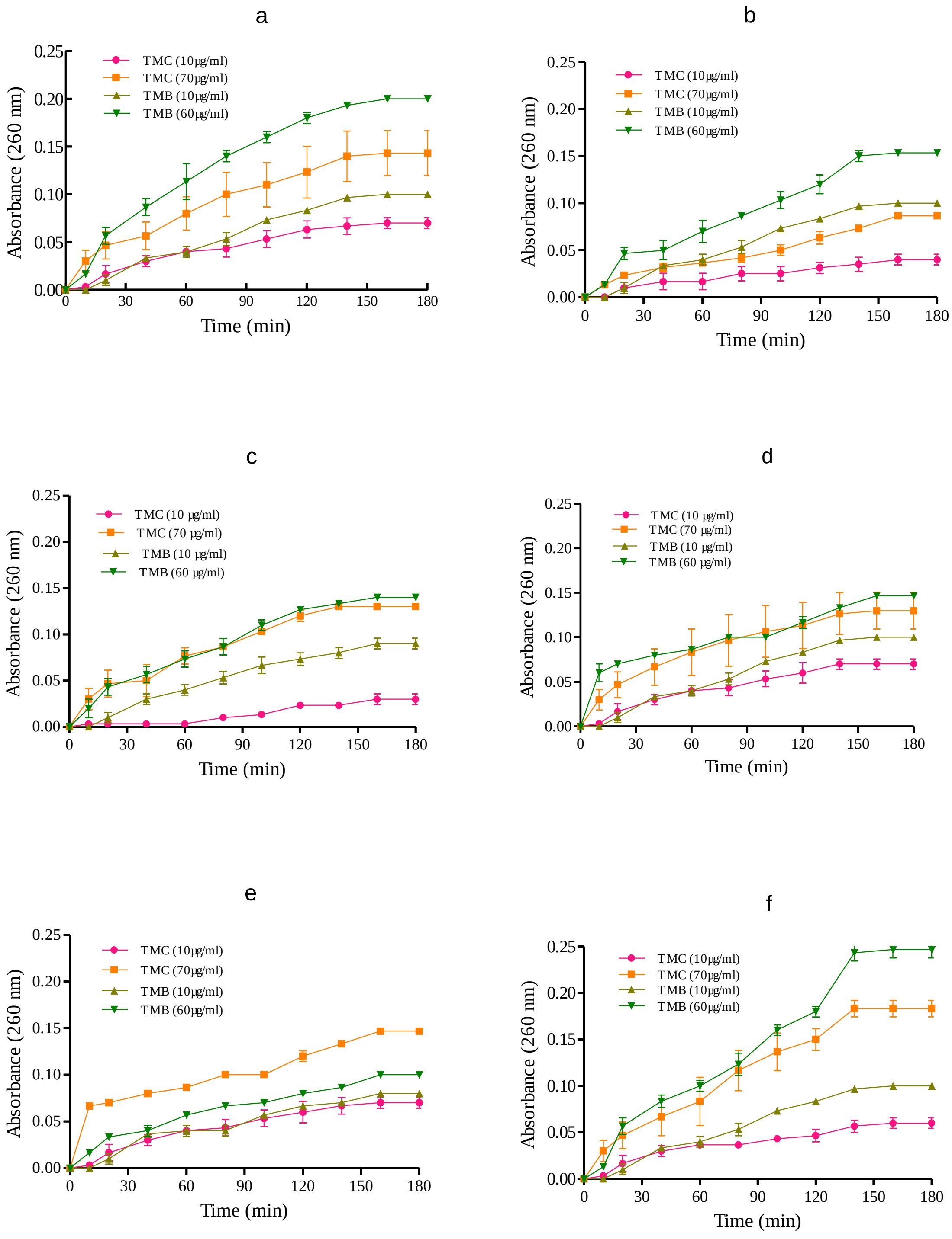


Figure 4.13 Leakage of UV-absorbing substances from a) *Salmonella typhimurium* ATCC 23564, b) *Shigella flexneri 2a*, c) *Aeromonas hydrophila* ATCC 35654 *d) Yersinia enterocolitica* ATCC 9610, *e) Escherichia coli* O157:H7 ATCC 35150 and *f) Listeria monocytogenes* ATCC 1911 after treatment with TMB and TMC. Each value is expressed as mean ± SD (n = 3).

Table 4.16 Effect of TMB (76µg/ml) and TMC (80µg/ml) on the leakage of protein in the extracellular media of target pathogens after 3 hours

Bacterial strains	**Absorbance (280nm)**	
	TMB	**TMC**
Salmonella typhimurium	0.616 ± 0.04	0.36± 0.08
Aeromonas hydrophilla	0.520 ± 0.06	0.50 ± 0.07
Yersinia enterocolitica	0.44 ± 0.06	0.53 ± 0.02
***E. coli* O157:H7**	0.46 ± 0.08	0.49 ± 0.09
Listeria monocytogenes	0.42 ± 0.02	0.43 ± 0.03

Each value is expressed as mean ± SD (n = 3)

Table 4.16 summarizes the effect of TMB and TMC on leakage of protein from target pathogens in the extracellular media. Treatment of pathogens with a concentration of 76µg/ml of TMB and 80µg/ml of TMB and TMC led to rapid leakage of protein from all the bacterial cells within a period of 3h. The amount of protein detected in post treatment increased gradually over time for all organisms. A stronger correlation between leakage and duration of exposure (3h) were revealed in Gram-negative than in Gram-positive organisms.

4.8.4 Leakage of Glucose and Lactate dehydrogenase (LDH)

In order to elucidate the possible mechanism of the bactericidal action of quaternized biopolymers, leakage of glucose and Lactate dehydrogenase (LDH) enzyme from treated pathogens were studied. In the present study, treatment of pathogens with a concentration of 76µg/ml of TMB and 80µg/ml of TMC led to rapid leakage of glucose and LDH within a period of 3h (**Table 4.17 and 4.18**), though a minor increase in glucose and LDH levels in the extracellular media was observed after 6h, no further increase in the levels of glucose, protein or LDH occurred thereafter.

Table 4.17 Effect of TMC (80µg/ml) and TMB (76µg/ml) on leakage of lactate dehydrogenase in the extracellular media of target pathogens after 3 hours

Bacterial strains	LDH activity (U/L)	
	TMB	**TMC**
Salmonella typhimurium	18.28 ± 0.06	14.36 ± 0.08
Shigella flexneri	17.44 ± 0.08	13.97 ± 0.05
Aeromonas hydrophilla	16.37 ± 0.07	15.37 ± 0.06
Yersinia enterocolitica	15.82 ± 0.08	16.0 ± 0.80
***E. coli* O157:H7**	15.63 ± 0.06	14.63 ± 0.08
Listeria monocytogenes	14.0 ± 0.70	13.0 ± 0.60

Each value is expressed as mean ± SD (n = 3)

Table 4.18 Effect of TMC (80µg/ml) and TMB (76µg/ml) on leakage of glucose concentration in the extracellular media of target pathogens after 3 hours

Bacterial strains	Glucose (mg/dl)	
	TMB	**TMC**
Salmonella typhimurium	0.48 ± 0.04	0.34 ± 0.02
Shigella flexneri	0.42 ± 0.06	0.30 ± 0.04
Aeromonas hydrophilla	0.40 ± 0.05	0.39 ± 0.06
Yersinia enterocolitica	0.35 ± 0.05	0.49 ± 0.09
***E. coli* O157:H7**	0.36 ± 0.08	0.35 ± 0.07
Listeria monocytogenes	0.34 ± 0.04	0.33 ± 0.02

Each value is expressed as mean ± SD (n = 3)

Several authors have proposed that the antimicrobial action of chitosan could be explained by a more direct disturbance of membrane functions. The reactive amino groups in chitosan could interact with a multitude of anionic groups on the surface of the cell to alter its permeability. This causes the leakage of intracellular components such as glucose and LDH, resulting in a destabilized cell membrane beyond repair and subsequent cell death.

4.8.5 ATP leakage

Killing kinetics often reflect the mode of action, and it was hypothesized that differences between different water borne pathogens regarding their sensitivity and time-kill might be due to different modes of interaction with the quaternized derivative. Therefore, an ATP bioluminescence assay was employed to determine whether cell envelope perturbation was involved in the antibacterial effect, and if so, whether the organisms differed in the degree of ATP leakage.

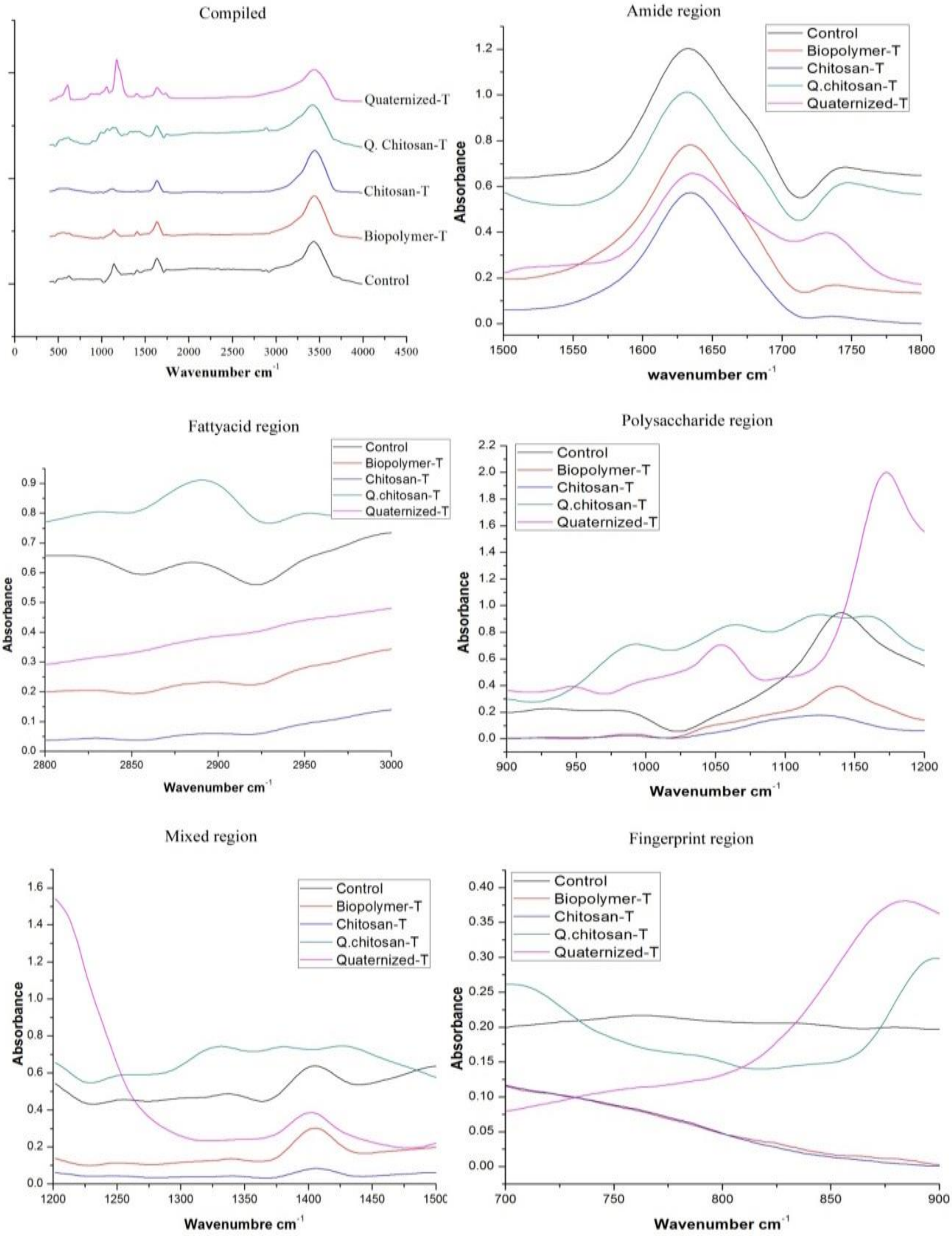
Table 4.19 ATP content of pathogens treated with quaternized biopolymeric flocculants

Time (min)	**Bacteria**	**Untreated**	**Treated (TMC)**	**Treated (TMB)**
0	*Salmonella typhimurium*	8.80±0.01	8.78±0.7	8.08±0.2
	Shigella flexneri	8.70±0.04	8.67±0.3	8.06±0.1
	Aeromonas hydrophilla	8.66±0.03	8.59±0.1	8.11±0.5
	Yersinia enterocolitica	8.76±0.05	8.70±0.2	8.15±0.4
	E. coli O157:H7	8.79±0.02	8.75±0.4	8.09±0.2
	Listeria monocytogenes	8.65±0.01	8.60±0.6	8.13±0.2
45	*Salmonella typhimurium*	8.79±0.03	8.20±0.4	8.05±0.2
	Shigella flexneri	8.68±0.01	8.65±0.3	8.04±0.6
	Aeromonas hydrophilla	8.63±0.04	8.60±0.5	8.03±0.7
	Yersinia enterocolitica	8.70±0.01	8.65±0.3	8.04±0.2
	E. coli O157:H7	8.75±0.02	8.73±0.1	8.07±0.5
	Listeria monocytogenes	8.60±0.02	8.54±0.09	8.10±0.6
90	*Salmonella typhimurium*	8.65±0.04	8.17±0.3	8.04±0.4
	Shigella flexneri	8.65±0.03	8.60±0.2	8.10±0.8
	Aeromonas hydrophilla	8.59±0.04	8.54±0.3	8.08±0.4
	Yersinia enterocolitica	8.65±0.03	8.60±0.7	8.06±0.08
	E. coli O157:H7	8.71±0.02	8.69±0.1	8.05±0.06
	Listeria monocytogenes	8.55±0.01	8.50±0.5	8.04±0.2

The impact of TMB and TMC on the energetic condition of sensitive cells was determined by measuring the cellular ATP levels **(Table 4.19).** Upon addition of quaternized polymers and within 45-90 minutes of incubation, a non-significant increase in extracellular ATP content was observed for all the indicator strains. ATP is a marker for cell viability; it is present in all metabolically active cells and the cell concentration declines very rapidly upon cells undergoing cell death (Kangas et al. 1984; Lundin et al. 1986). The sensitivity, linearity, and signal stability of the ATP-based assay supports its reliability for assessing cell proliferation or death in addition, its simplicity makes it convenient method for assessing efficacy of antimicrobial compounds.

4.8.6 Analysis of Lipopolysaccharides (LPS) isolated from treated pathogens

Fourier transform infrared (FT-IR) absorbance spectroscopy (in the mid-IR range, usually defined as 4000-400cm^{-1} or 2500-25000nm) has sufficient resolution to distinguish intact microbial cells at the strain level. The resulting spectra for microbial cells represent the “total” biochemical composition of those cells. FTIR spectroscopy measures vibrations of functional groups and highly polar bonds. Therefore spectral features of many important biochemical constituents create a “fingerprint” for a bacterial strain. The importance of FT-IR spectroscopy as a physicochemical method to determine the global chemical features of cells suggests its adequacy to study the molecular changes after treatment with antibacterial agents (Ashtiani et al. 2013). In fact, FT-IR methods have been used to study sonication-injured *L. monocytogenes* (Lin et al. 2004), heat injured Gram-negative and Gram-positive bacteria (Al-Qadiri et al. 2008) and heat killed *E. coli* in ground beef (Davis et al. 2010) in the recent past.


Compiled

Amide region

Fattyacid region

Polysaccharide region

Mixed region

Fingerprint region

Figure 4.14 FT-IR spectra of LPS isolated from *Salmonella typhimurium* (control), biopolymer treated bacterial cells (Biopolymer-T) and quaternized biopolymer treated bacterial cells (Quaternized-T).

The Lipopolysaccharide (LPS) region of inactivated *Salmonella typhimurium* strains following biopolymer, chitosan, TMC and TMB treatment was examined by mid-infrared spectroscopy (4000-400cm^{-1}) (**Figure 4.14**). The spectrum of non-treated (control) cells was visually similar to the spectra in previous studies for *Salmonella* sp. (Al-Qadiri et al. 2008). The structure of fatty acid, polysaccharide and fingerprint region of the isolated LPS was completely altered as observed from the symmetrical stretching of CH_2 groups (which belongs to lipids), following the TMB treatment. TMC treatment resulted in complete alteration of the polysaccharide, mixed and the fingerprint region of the LPS, with no effect observed on the fatty acid region of the LPS. Figure 4.13 clearly shows that no changes on the amide region of LPS isolated from *Salmonella typhimurium* were observed after treatment with any of the polymers or their quaternized derivatives.

Several antibacterial molecules resulted in the permeabilization of Gram-negative membranes by release of LPS. For instance, the release of LPS from *S. enterica* serovar Typhimurium has been reported after exposing the bacteria to polycations, protamine, and a 20-residue lysine polymer (lysine20) (Vaara and Vaara 1983). The antimicrobial activity of lactoferrin and lactoferricin is accompanied by the release of LPS from the membrane of *Salmonella* (Yamauchi et al. 1993). Results obtained in the present study clearly depicts that quaternized biopolymeric flocculants have a major effect on the LPS regions of *Salmonella typhimurium*.

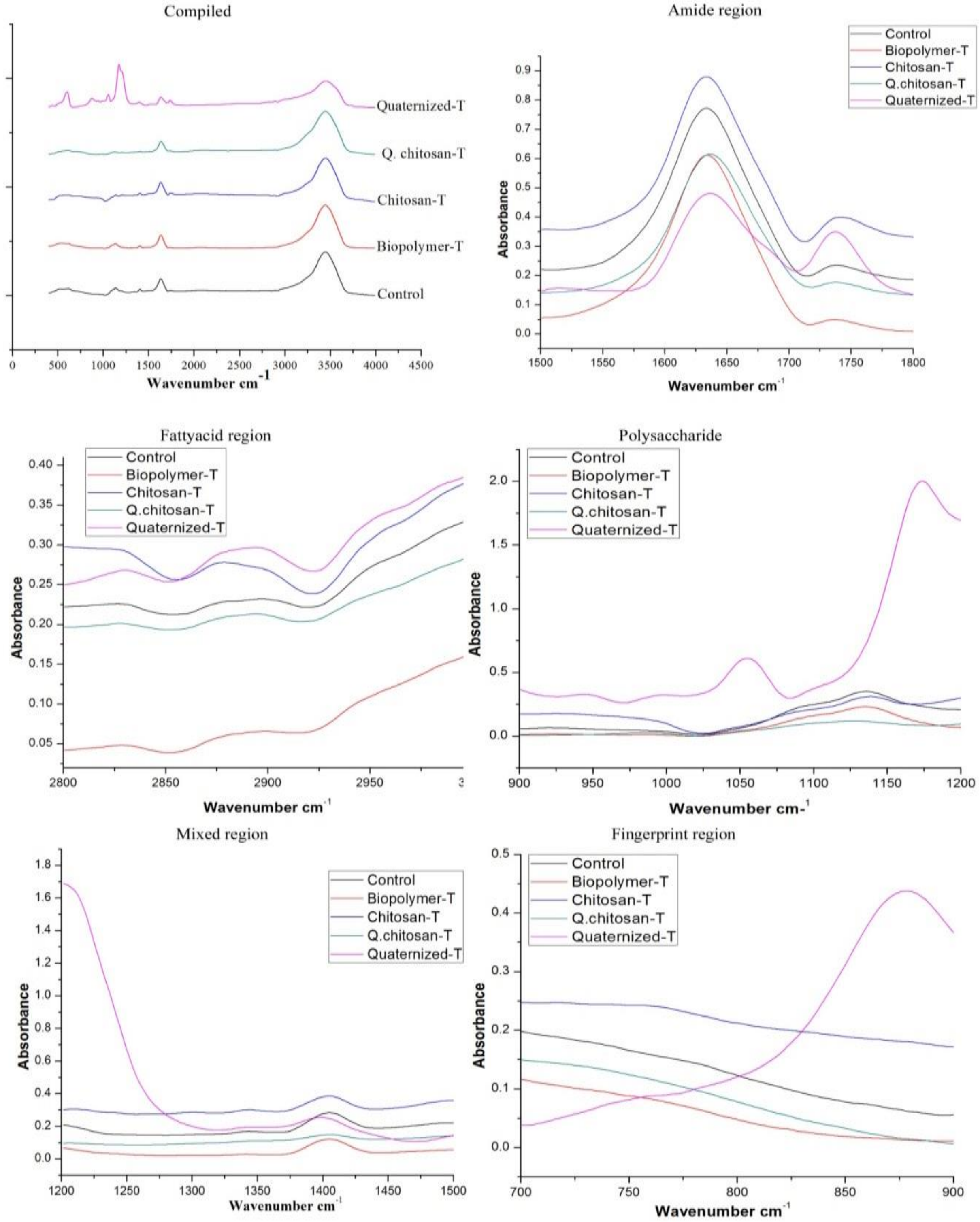


Figure 4.15 FT-IR spectra of LPS isolated from *Shigella flexneri 2a* (control), biopolymer treated bacterial cells (Biopolymer-T) and quaternized biopolymer treated bacterial cells (Quaternized-T).

The LPS of inactivated *Shigella flexneri 2a* strains following biopolymer, chitosan, TMB and TMC treatment was examined by mid-infrared spectroscopy (4000-400cm^{-1}) (**Figure 4.15**). Treatment with biopolymer, chitosan and TMC resulted in no changes on the LPS region of *Shigella flexneri,* whereas the structure of amide, polysaccharide and fingerprint region were completely altered on treatment with TMB. Hence, results reveal significant effect of TMB on the structure of LPS region of *Shigella flexneri.*

Figure 4.16 shows the FTIR spectra of LPS of inactivated *Aeromonas hydrophila* strains following biopolymer, chitosan, TMC and TMB treatment. Results revealed that among the native polymers, chitosan showed no changes on the structure of LPS region, whereas, BF completely altered the amide and polysaccharide region of LPS. Also some effect on the fingerprint region of LPS structure after biopolymeric flocculant treatment can be clearly seen by a peak at 1400cm^{-1}, due to the symmetric stretching of vibration of COO^{-} group of fatty acid and amino acid. TMB treatment of *Aeromonas hydrophilla* completely altered the LPS structure at fatty acid, polysaccharide and fingerprint region. Quaternized derivative of chitosan, TMC also affected the amide, polysaccharide and mixed regions of LPS.

In the case of mid-infrared spectroscopy (4000-400cm^{-1}) of the LPS region of *Yersinia enterocolitica* strains following biopolymer, chitosan, TMC and TMB treatment, inferred the complete alteration of polysaccharide region of the LPS structure whereas TMC had no significant effect on the LPS structure of bacteria (Figure 4.17). In the case of biopolymeric flocculant treated bacterial LPS region, no changes were observed. The native chitosan, however, affected the amide (1550 cm^{-1}-60 cm^{-1} at Amide II due to N-H bending vibrations coupled to C-N stretching) and polysaccharide (1061 cm^{-1} due to stretching of C-O, C-C groups and in-plane bending of O-CH) regions of LPS structure of bacteria.

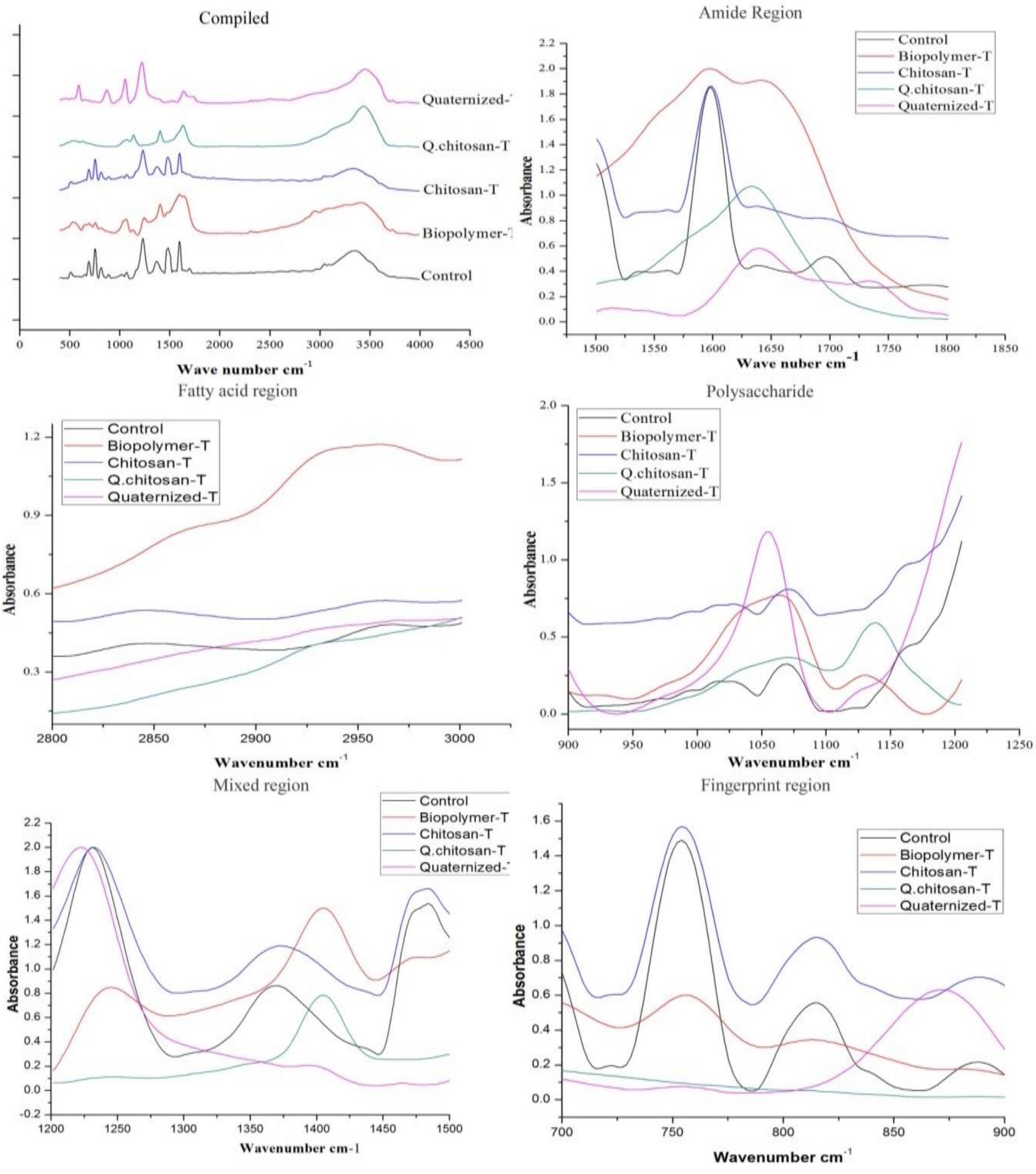


Figure 4.16 FT-IR spectra of LPS isolated from *Aeromonas hydrophila* (control), biopolymer treated bacterial cells (Biopolymer-T) and quaternized biopolymer treated bacterial cells (Quaternized-T).

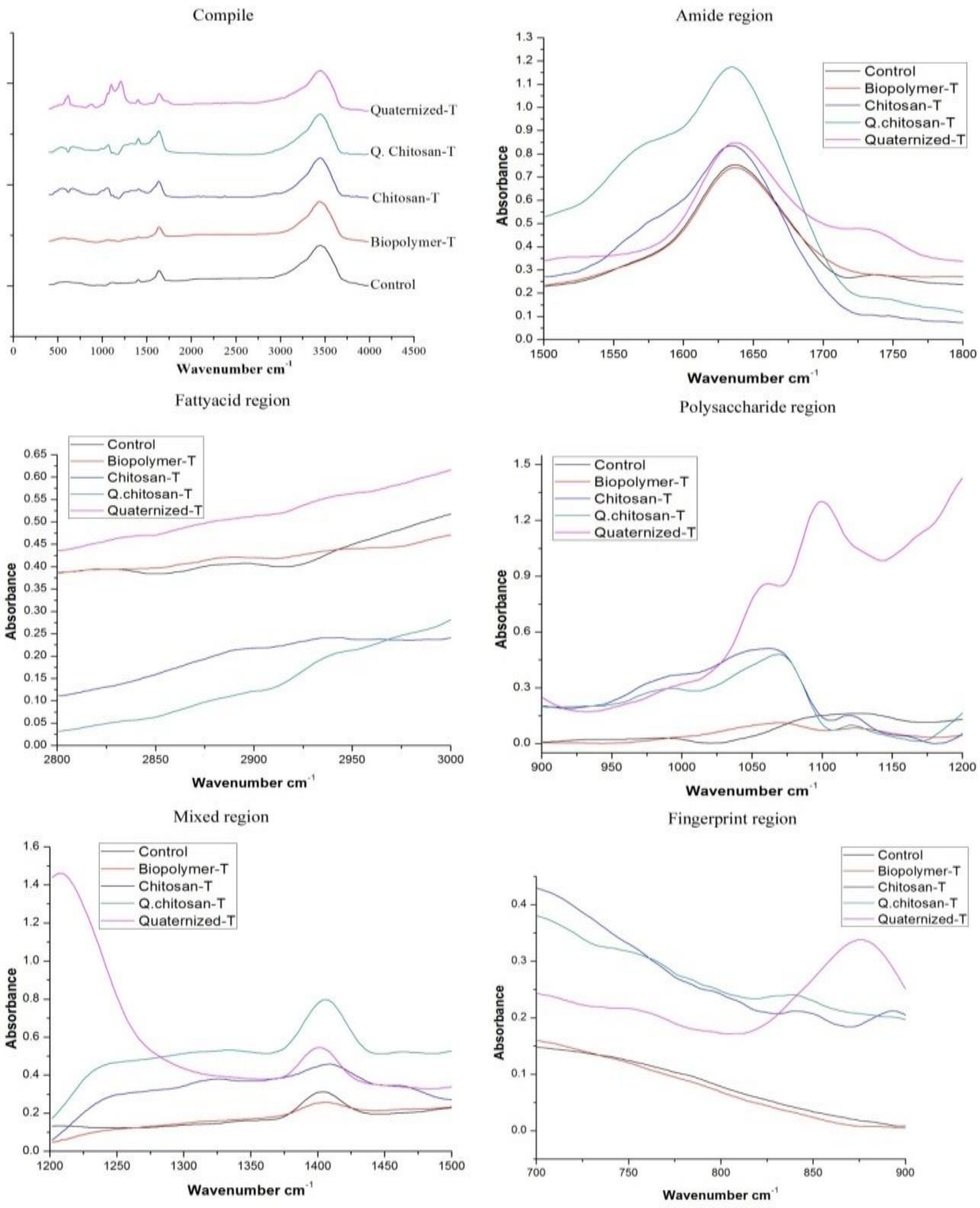


Figure 4.17 FT-IR spectra of LPS isolated from *Yersinia enterocolitica* (control), biopolymer treated bacterial cells (Biopolymer-T) and quaternized biopolymer treated bacterial cells (Quaternized-T).

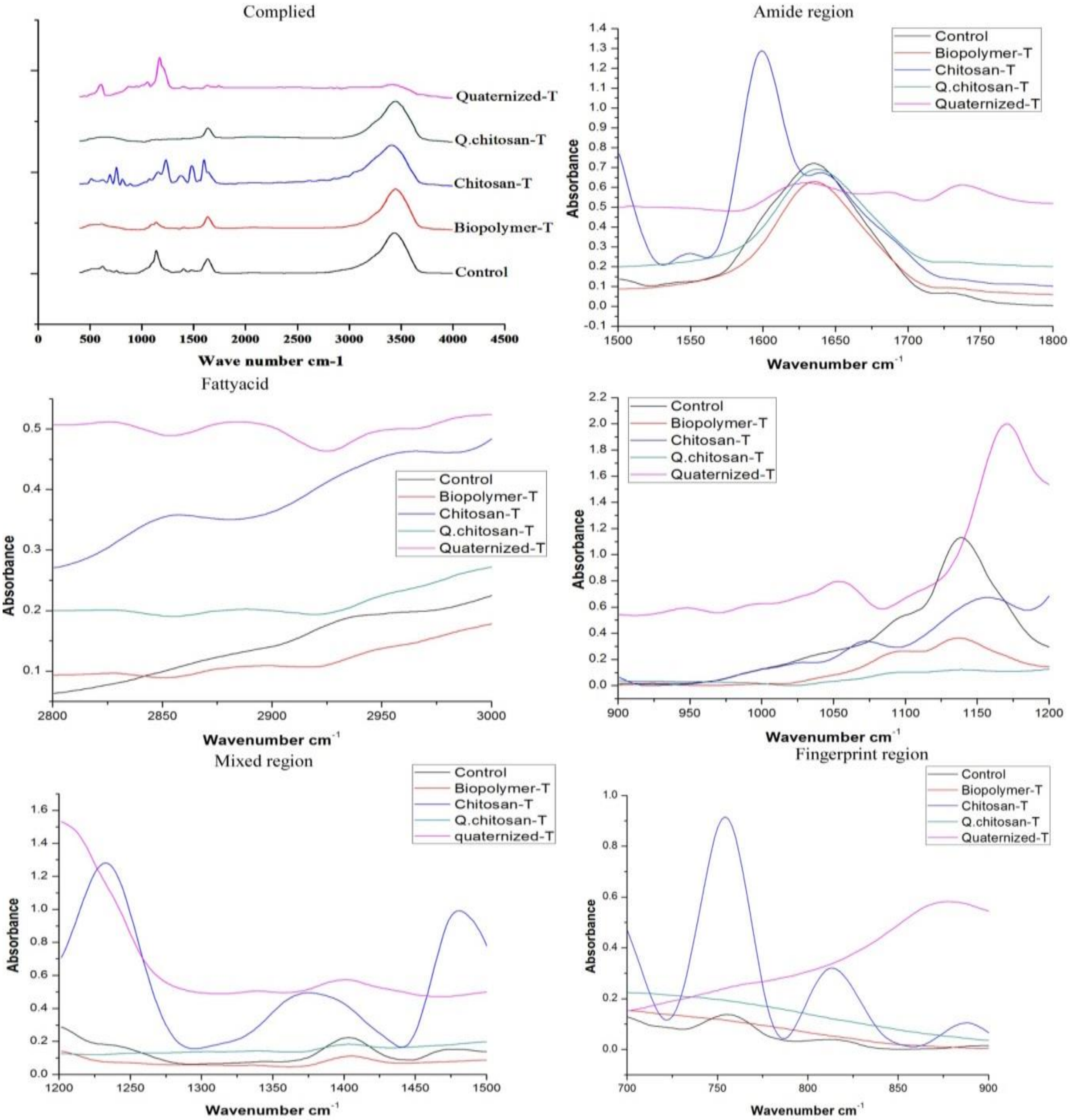


Figure 4.18 FT-IR spectra of LPS isolated from *Escherichia coli O157:H7* (control), biopolymer treated bacterial cells (Biopolymer-T) and quaternized biopolymer treated bacterial cells (Quaternized-T).

Figure 4.18 shows the alteration on structure of LPS region isolated from *Escherichia coli* O157:H7, following the treatment with native polymers and their quaternized derivatives. Native as well as quaternized polymeric flocculants showed a significant effect on various regions of the LPS structure of *E. coli* O157:H7. Biopolymeric flocculant completely altered the polysaccharide and the fingerprint region. Treatment with chitosan resulted in a peak at 1553cm^{-1} predominantly α-sheet of amide II (amide II band mainly stems from the C-N stretching and C-N-H bending vibration weakly coupled to the C=O stretching mode), and a peak in fatty acid region at 2848cm^{-1} due to stretching vibration of CH_2, CH_3 of phospholipids, cholesterol and creatine, also it has completely altered the fingerprint region. TMB treatment of bacteria completely altered the amide, polysaccharide, mixed and fingerprint region of LPS structure whereas TMC had no effect on amide region and completely altering the polysaccharide, mixed and fingerprint regions.

Although, LPS are the major toxins of Gram-negative bacteria (endotoxin) but an early study suggested *L. monocytogenes* is unique among Gram-positive bacteria in that it might possess LPS which serves as an endotoxin (Wexler and Oppenheim 1979). While the polysaccharide is non-toxic, the lipid A part is responsible for the toxic activities of these bacteria which result in septic shock. Later it was found to not be a true endotoxin. *Listeria* cell walls consistently contain lipoteichoic acids, in which a glycolipid moiety, such as a galactosyl-glucosyl-diglyceride, is covalently linked to the terminal phosphomonoester of the teichoic acid. This lipid region anchors the polymer chain to the cytoplasmic membrane. These lipoteichoic acids resemble the lipopolysaccharides of Gram-negative bacteria in both structure and function, being the only amphipathic polymers at the cell surface.

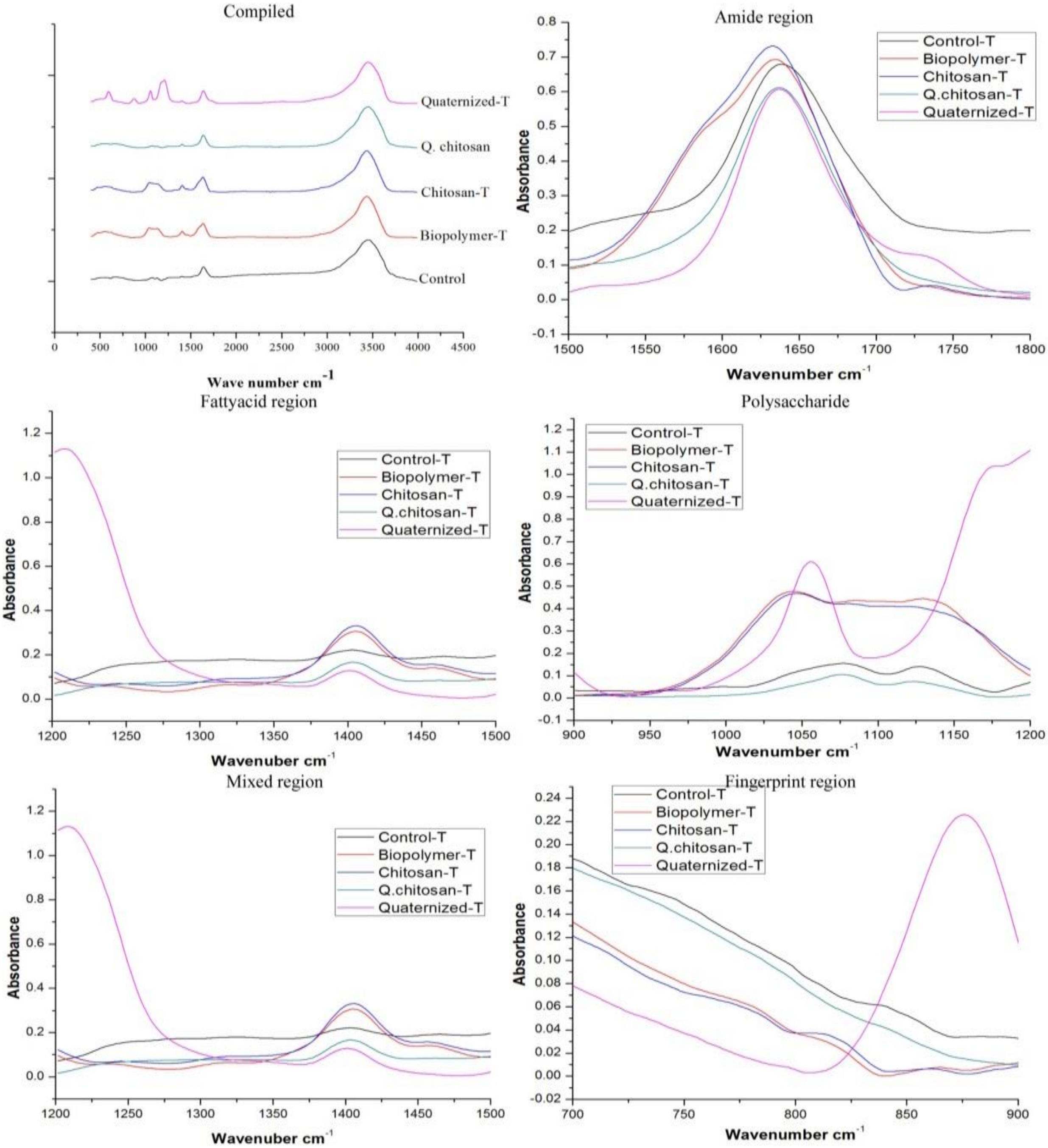


Figure 4.19 FT-IR spectra of LPS isolated from *Listeria monocytogenes* (control), biopolymer treated bacterial cells (Biopolymer-T) and quaternized biopolymer treated bacterial cells (Quaternized-T).

The LPS of inactivated *Listeria monocytogenes* strains following biopolymer, chitosan, TMB and TMC treatment was examined by mid-infrared spectroscopy (4000-400cm^{-1}) (**Figure 4.19**). No changes were observed in the fingerprint region, a peak at 1717 cm^{-1} in amide region raises from C=O stretching vibrations in the case of TMB treated bacterial LPS structure. Also, after TMB treatment, peak at 1050 cm^{-1} due to symmetric stretching CO-O-C and C-O stretching coupled with C-O bending of the C-OH of carbohydrates, 1107cm^{-1} and 1110 cm^{-1} due to stretching vibration of CO and CC and ring of polysaccharide and pectin and cellulose was observed in the polysaccharide region and in the mixed region, peak at 1208 cm^{-1} due to PO_2^- asymmetric was recorded. On treatment with TMC, changes in only mixed region was observed with a peak at 11400-500 cm^{-1} due to ring stretching vibrations mixed strongly with CH in plane bending and symmetric CH_3 bending modes of the methyl group of proteins. In both the native polymers, chitosan and biopolymer, similar effects were observed with changes observed in amide, fatty acid and polysaccharide region of LPS structure of *Listeria.*

Gram positive bacteria are generally known to lack LPS. However, *Listeria* is an exception (Wexler and Openheim, 1979). Though bactericidal activity was observed for *Listeria,* further studies are warranted to define the exact mechanism of cellular damage.

4.8.7 Electron microscopic examination

To correlate spectroscopic data with ultra-structural changes caused by quaternized biopolymeric flocculant, TMB, both treated and untreated indicator bacteria was subjected to scanning electron microscopic examination at various time intervals (Figure 4.20). Extensive intracellular changes were observed in TMB treated Gram-negative when compared to the non-treated cells including cytoplasmic membrane disruption and blebbing.

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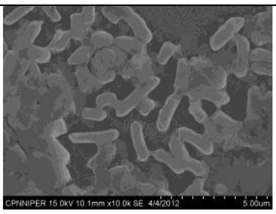
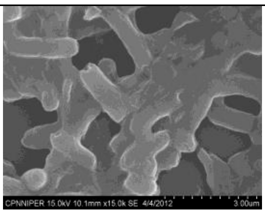
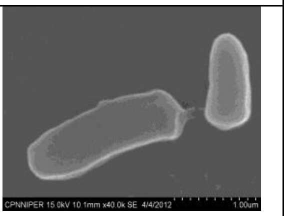
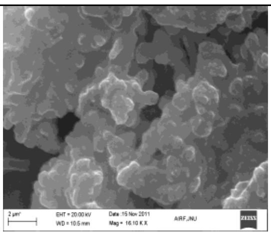
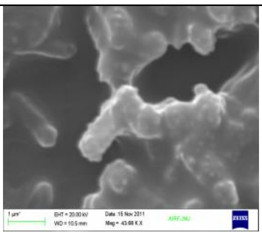
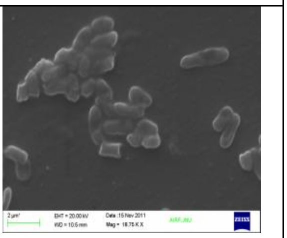
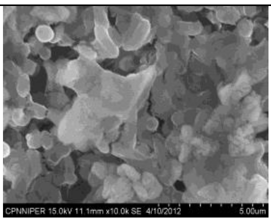
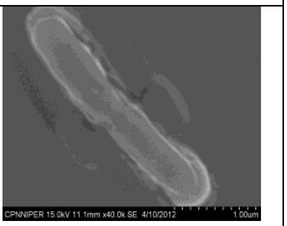
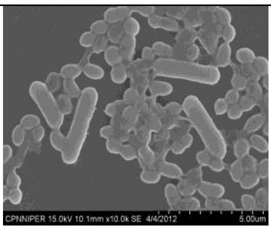
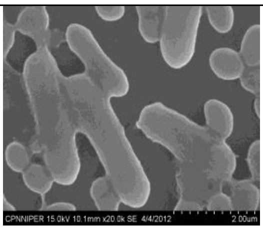
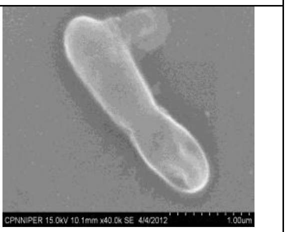
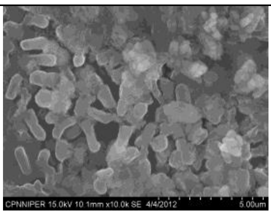
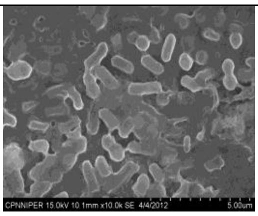
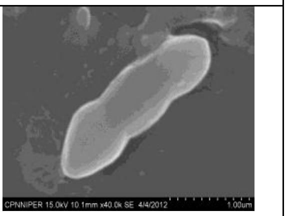
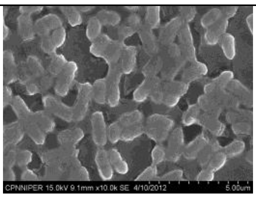
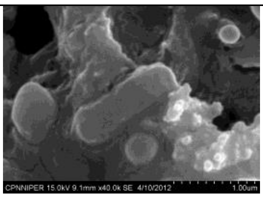
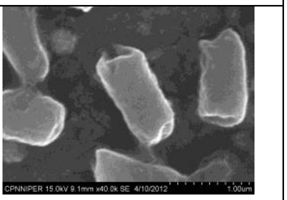
Bacteria	0 min	30min	60min
Salmonella typhimurium			
Shigella flexneri			
Aeromonas hydrophilla			
Yersinia enterocolitica			
Escherichia coli			
Listeria monocytogenes			

Figure 4.20 Scanning electron micrographs of indicator bacteria treated with TMB in mid-logarithmic growth at 10^7CFU/ml at various time intervals.

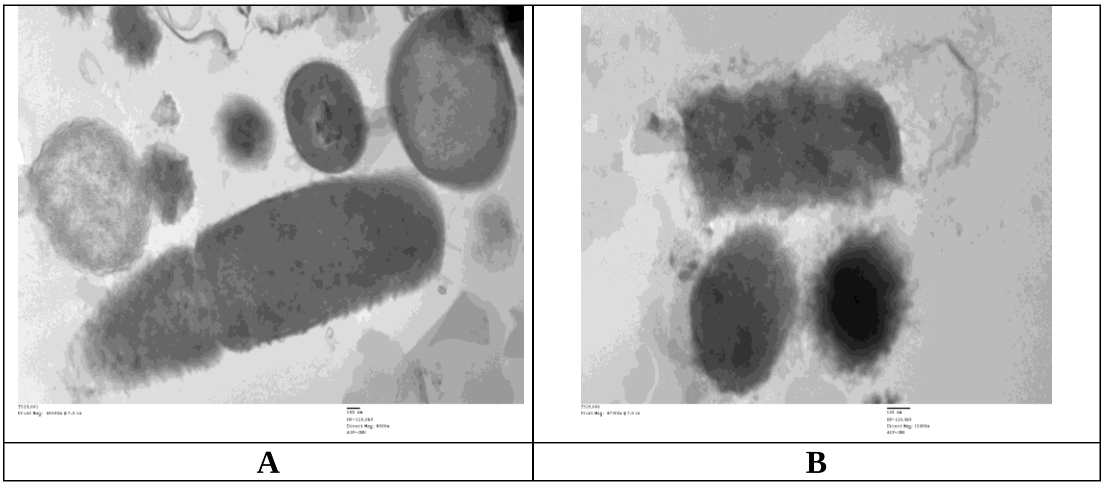
Besides, remarkable modifications of cell membrane and disruption of cell membranes of Gram-negative bacteria occurred at low TMB concentration. The electron micrographs for *Listeria monocytogenes* obtained from scanning microscopy observations showed important morphological damages. Cells treated with TMB at 80µg/ml for 60 minutes revealed deformed and interconnected cells with shrinkage of cell surface and lysis of cytoplasmic material.

A summary of the electron microscopic studies, FT-IR spectroscopy, 260nm absorbing material, extracellular K^+ leakage and glucose leakage assays revealed that membranous structures and cell wall to be the target sites of quaternized biopolymer.

4.9 Effect of TMB on outer cell wall of *Salmonella typhimurium*

The purpose of this study was to investigate the occurrence of sublethal injury and the relationship between membrane damage and loss of viability in *Salmonella* after exposure to TMB. TMB affected the ultrastructure of the cell wall of *Salmonella* and altered the LPS structure of the bacteria. Further the effect on the cell wall of *Salmonella typhimurium* exposed to a sub-MIC levels of TMB was studied using, electron microscopic examination and agglutination that involves the outer cell wall constituents.

The structure of the cell was markedly altered by TMB in a concentration and time dependent manner at MIC value of 60µg/ml treated for 60min **(Figure 4.21a)** and at sub MIC value of 30µg/ml treated for 180min **(Figure 4.21b).** Complete alterations were noted for cells exposed to 60µg/ml of TMB for 24h; concentrations as low as 30µg/ml yielded similar changes in cultures exposed in excess of 12h.



A **B**

Figure 4.21 Transmission electron micrographs of *Salmonella* treated with 60µg/ml for 60min(A) and 30µg/ml for 180min(B) of TMB

The effect of TMB on the agglutination of control and at different concentrations of TMB exposed *Salmonella* is summarized by **Table 4.20.** *Salmonella* cells exposed to 20µg/ml of TMB for 1 min were not agglutinated by anti-H antibody regardless of whether the cells were in BHI or saline. *Salmonella* grown in the presence of MIC levels of TMB (60µg/ml) for 24h, washed, and then incubated with TMB at different concentrations were not agglutinated by anti-H antibody as were the unexposed control. The organism did not agglutinate with anti-H antibody when incubated with TMB and, when washed once with saline, did not show agglutination on re-incubation with anti-H antibody. These results suggested an inactivation of the H antigen and loss of infectivity.

Table 4.20: Effect of TMB on the agglutination of *Salmonella typhimurium* by anti-H antibody

Bacteria	**TMB concentration (µg/ml)**						
	0	**10**	**20**	**30**	**40**	**50**	**60**
***S. typhimurium* cells (control)**	+	+	–	–	–	–	–
***S. typhimurium* cells treated with TMB**	–	–	–	–	–	–	

4.10 Mutagenicity studies

Mutagenicity and toxicity are important criteria and required to be addressed as the newly designed quaternized biopolymeric flocculant, TMB is intended primarily for applications in water. A simple and convenient *in vitro* study was designed to examine the mutagenicity of TMB using the *Salmonella* Ames assay.

The Ames *Salmonella*/microsome mutagenicity assay, a short term bacterial reverse mutation assay is specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations (Mortelmans and Zeiger 2000). The test uses a number of *Salmonella* strains with pre-existing mutations in histidine operon that incapacitate the bacteria to synthesize the required amino acid, histidine, thus rendering non viability in its absence. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate, containing a trace of histidine, only those bacteria that revert to histidine independence (his+) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, upon challenging mutagen to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner. New mutations at the site of these pre-existing mutations, or nearby in the genes, can restore the gene's function and allow the cells to synthesize histidine. These newly mutated cells can grow in the absence of histidine and form colonies (Mortelmans and Zeiger, 2000). In the current past, various studies have used this assay as a rapid, safe indicator of carcinogenicity/mutagenicity of molecules intended for human applications.

Table 4.21: Mean number of revertants per plate after incubation with different concentrations of TMB with (+S9) and without (-S9) metabolic activation

Sample		Number of revertants per plate (Mean±SD)			
		TA98		TA100	
		(-) S9 mix	(+) S9 mix	(-) S9 mix	(+) S9 mix
Control		20±4	22±3	111±7	98±5
sodium azide (10µg/plate)^{PC}		-	-	1500±40	-
2-NFA (10µg/plate)^{PC}		902±150	-	-	-
2-AA (2.5µg/plate)^{PC}		-	882±40	-	4000±356
TMB	(50µg/ml)	20±2	21±4	43±6	48±2
	(100µg/ml)	18±4	27±8	39±2	41±2
	(150µg/ml)	20±6	21±2	34±4	38±3

PC- Positive control

TMB at 50ug/ml, 100ug/ml and 150ug/ml did not exert any mutagenic or carcinogenic effect as evidenced by insignificant number of revertants on plates used with or without metabolic activation system when compared with negative control **(Table 4.21).** Whereas positive controls produced a considerable increase in mutant colony frequencies showing the sensitivity of the tester strains. TMB was found to be a non-mutagen/carcinogen in bacterial reverse mutation tests.

4.11 Acute toxicity studies of Quaternized biopolymeric flocculant

In order to establish human safety of the quaternized biopolymeric floculant and conform to its regulatory clearance, toxicity assessment was carried out at Venus remedies Ltd, Baddi, INDIA using rat models (Swiss Albino Mice). Different concentrations of “Quaternized BF” were administered intraperitoneally to groups of animals anticipating that the toxic/virulent dose levels would result in clinical toxicity and some death/s in a dose dependent manner (Marra and Girard, 2006).

4.11.1 Effect on Body weight

Preliminary study- seven days

Sighting Study: Mean body weight change of all the survival treatment groups of Sighting Study has been mentioned in **Table 4.22**. Among the three "Quaternized Biopolymer" treatment groups, G-1 (60mg/kg) & G-2 (180mg/kg) did not show any significant change in body weights. Body weights of G-3 (220mg/kg) could not be recorded because of dose related mortality of all the animals in these groups within 24h after dosing.

Table 4.22 Sighting study- Mean body weight change

Group	Dose (mg/kg bw)	0	1	2	3	4	5	6	7
G-1	60	23.5 ±0.7	23.0 ±0.0	24.0 ±0.0	24.0 ±0.0	23.5 ±0.7	23.0 ±0.0	23.5 ±0.7	23.0 ±0.0
G-2	180	23.0 ±1.4	23.0 ±2.8	22.0 ±2.8	22.5 ±2.1	23.5 ±2.1	23.0 ±2.8	23.0 ±1.4	22.5 ±2.1
G-3	220	23.0 ±1.4	D	D	D	D	D	D	D

D: All animals died; NA: Not applicable as only single animal was left

Main study: Mean body weight change in body weight of all the survival treatment groups of Main Study has been mentioned in **Table 4.23.** Treatment groups, G-4 (180 mg/kg) did not show any significant change in body weights. A statistically significant decrease in body weights of survival animals of group G-5 (200 mg/kg) & G-6 (220 mg/kg) was observed over the observation period of 7 days (15.0 % & 22.6 % respectively at day-7 as compared to day-0). Body weights of G-7 (240 mg/kg) could not be recorded because of dose related mortality of all the animals in these groups within 24h after dosing.

Table 4.23: Main Study- Mean Body Weight change

Group	Dose (mg/kg bw)	0	1	2	3	4	5	6	7
G-4	180	23.0± 0.8	22.3± 1.0	22.3 ± 1.3	22.0± 1.2	22.3± 0.5	21.8± 0.5	22.8± 0.5	22.3± 0.5
G-5	200	22.8± 0.5	22.7± 0.6	22.0 ± 1.0	21.3± 0.6	21.0± 1.0	20.3± 0.6	19.7± 0.6	19.3± 0.6
G-6	220	23.3± 0.5	22.0± NA	21.0 ± NA	20.0± NA	20.0± NA	19.0± NA	19.0± NA	18.0± NA
G-7	240	23.0± 0.8	D	D	D	D	D	D	D
D- All animals died; NA- Not applicable as only single animal was left									

Final study-thirty days: The body weight gain profile in the TMB concentrations screened from preliminary study of seven days, comparable to control group values are presented for the final study of thirty days in **Table 4.24.** No Changes in body weight of TMB exposed mice were observed.

Table 4.24: Mean body weight changes following administration of TMB in mice; body weight was recorded every 5th day (five animals per dose).

Dose (mg/kg/ bw)	**Days**						
	0	**5**	**10**	**15**	**20**	**25**	**30**
0	29.5±0.7	29.0±1.4	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±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	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	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	32.0±2.1	33.0±2.8	31.0±3.1	33.0±3.2	32.0± 3.1

4.11.2 Rectal temperature (seven day study)

Sighting Study: Rectal temperature of all the treatment groups of Sighting Study has been mentioned in **Table 4.25**. Treatment groups G-1 (60mg/kg) & G-2 (180mg/kg) did not show any significant change in rectal temperature over the observation period of 24h. However, a statistically significant decrease (hypothermia) was observed in G-3 (220mg/kg) within 1h of “Quaternized Biopolymer” administration lasting upto 8h followed by the death of animals within 24h.

Main Study: G-4 (180mg/kg) did not show any significant change in rectal temperature over the observation period of 24h. 2 out of 4 animals in G-5 (200mg/kg) showed hypothermia followed by death of 1 animal within 24h post “Quaternized Biopolymer” administration but other hypothermic animal recovered and survived. All the 4 animals in G-6 (220mg/kg) showed hypothermia and 3 out of 4 died following hypothermia. All the 4 animals of group G-7 (240mg/kg) showed hypothermia and death within 24h post “Quaternized Biopolymer” administration. Rectal temperature of all the survival treatment groups of Main Study has been mentioned in **Table 4.26.**

4.11.3 Clinical Signs of Toxicity

Gross and microscopic examination revealed no changes attributable to the administration of quaternized derivative (TMB).

Hematology showed no treatment-related changes were evident from over the experimental period (**Table 4.27** and **4.28**) 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.

Table 4.25: Sighting Study- Individual animal body temperature data

Sighting Study- Rectal Temperature Record										
		Rectal Temperature (Degree Celcius) →								
Group / Dose	**↓Animal No. Time points→**	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**180 min**	**240 min**	**480 min**	**24 hrs**
Group-1 (60 mg/kg)	H	35.3	35.2	35.4	34.9	35.0	35.2	35.2	35.1	L
	B	35.1	34.9	35.0	35.0	35.2	35.0	35.0	35.1	L
Group-2 (180 mg/kg)	T	36.2	35.6	35.4	35.7	35.9	35.2	35.7	36.0	L
	UM	36.7	36.3	36.0	35.7	36.2	36.2	36.1	36.3	L
Group-3 (220 mg/kg)	H	37.3	33.3	NR	NR	NR	NR	NR	NR	D
	B	37.6	NR	NR	NR	NR	NR	NR	NR	D

L-Animals live, D- All animals dead; NR- Not readable i.e lower than the measurable limit of thermometer

Table 4.26: Main Study- Individual animal body temperature data

Main Study- Rectal Temperature Record										
		Rectal Temperature (Degree Celcius) →								
Group / Dose	**↓Animal No. Time points→**	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**180 min**	**240 min**	**480 min**	**24 hrs**
Group-4 (180 mg/kg)	H	36.6	36.2	36.0	36.1	35.9	36.2	36.3	36.3	L
	B	35.8	35.3	35.4	35.6	35.2	35.4	35.4	35.6	L
	T	35.2	34.7	34.3	34.3	34.6	34.4	34.9	34.8	L
	UM	36.3	35.9	35.4	35.6	35.8	36.1	36.0	36.2	L
Group-5 (200 mg/kg)	H	36.4	34.4	33.3	NR	NR	NR	NR	34.3	L
	B	36.0	33.2	NR	NR	NR	NR	NR	NR	D
	T	35.7	35.2	34.6	33.7	34.6	35.0	35.0	35.2	L
	UM	36.2	34.6	34.2	34.4	35.3	35.4	35.4	35.7	L
Group-6 (220 mg/kg)	H	35.6	NR	NR	NR	NR	NR	NR	NR	D
	B	35.7	33.2	33.0	33.4	33.3	34.2	34.0	34.4	L
	T	36.0	34.0	NR	NR	NR	NR	NR	NR	D
	UM	36.5	NR	NR	NR	NR	NR	NR	NR	D
Group-7 (240 mg/kg)	H	36.2	NR	NR	NR	NR	NR	NR	NR	D
	B	35.9	NR	NR	NR	NR	NR	NR	NR	D
	T	36.6	NR	NR	NR	NR	NR	NR	NR	D
	UM	35.3	32.6	NR	NR	NR	NR	NR	NR	D

L	**Animal Live**
D	**Animal Dead**
NR	**Not Readable (lower than the measurable limit of Clinical thermometer**

Table 4.27: Means of red blood cell parameters in mice administered TMB by gavage for 30 days

Parameter	Dose				
	0	25	80	140	**200**
Red blood cells (millions/ mm3)	9.0±0.2	7.4±0.2	6.5±0.9	7.0±1.5	**8.3±0.2**
Hemoglobin (g/dL)	13.1±2.0	13.3±1.2	13.4±1.2	15.4±2.4	**18.5±3.5**
Hematocrit (%)	49.3±5.3	53±3.8	51±3.4	50±5.1	**48.7±2.0**
Mean corpuscular volume (µ^3)	50.8±2.2	60±10.5	64.6±7.3	63.2±10.5	**50±2.3**
Mean corpuscular hemoglobin (pg)	17.2±0.7	18.2±1.0	21.1±3.0	20.5±2.5	**21.9±4.0**
Mean corpuscular Hemoglobin concentration (%)	**28.2± 4.3**	**25±4.8**	**26±4.2**	**30±2.5**	**39±9.3**

Table 4.28: Means of white blood cell parameters of mice administered TMB by gavage for 30days

Parameter	Dose			
	0	50	140	200
Eosinophils (%)	0.8±0.9	1.6±0.6	1.4±1.0	1.34±0.6
Monocytes (%)	4.4±1.0	4.8±1.2	4.2±1.5	4.2±1.6
Lymphocytes (%)	83.07±2.5	82.8±3.0	80.3±7.2	80.1±8.5
Neutrophils (%)	11.2±2.0	11.4±4.1	12.3±8.2	13.02±4.2

Gross necropsy was performed for the animals died during the experiment (after the administration of test item). Pin point to ecchymotic hemorrhages on heart, kidney and liver was observed in animals died within 48h post-test item administration. No gross pathological change was observed in survival animals necropsied on termination day**.**

It was found, however, that the minimum inhibitory concentrations of all bacteria tested are in a concentration range of TMB (between 40 and 70µg/ml) in which there was no observed significant toxic activity compared to the control. This fact is more significant in respect to Gram-negative bacteria, for which the bactericidal concentration values are also described in the same range. It must be noted that the MIC concentrations are much below the toxicity level. It has been reported earlier in an *in vivo* study, that chitosan's have no evidence of toxicity and the LD50 of chitosan in mice is 16g/kg of body weight (Kean and Thanou 2010). Finally TMB, if remaining in water in case of improper filtration raises no safety considerations as aluminium and chlorine does with Alzheimer's disease and disinfection by products.

CHAPTER 5

Discussion

Bacteria synthesize vast array of polymeric substances extracellularly and each biopolymer has its own material specific properties. This diverse structural variability, composition and sequence of monomers, degree of polymerization, substituents of monomeric units, altogether not only serve as an indicator of its possible applications but also make them suitable primers for chemical modifications to achieve desirable function. An amino sugar rich extracellular polymer obtained from an environmental isolate *Klebsiella terrigena*, displayed high flocculating ability for water borne pathogens (Ghosh et al. 2009a). We anticipated that rendering an antimicrobial function to the same may enable inactivation and simultaneous removal of these pathogens from water and would lead to development of an effective and safe substitute to water treatment chemicals.

Microbial exopolymer with antimicrobial properties, in particular, has gathered considerable interest due to their enormous scope of modification and wide gamut of application. In the present study, the amino group present in the polysaccharide backbone of the bioflocculant were transformed into quaternary ammonium compounds synthetically by using methylating agents which resulted in the formation of trimethyl biopolymeric flocculant (TMB) (Khaira et al. 2013b). Quaternary ammonium compounds (QACs) are membrane active agents with a target site, predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane (Tan et al. 2013). The possible explanation for the mechanism is that the amino group of biopolymers behaves as a nucleophile that bonds to and yields products with a variety of electrophiles, so the methylation reaction proceeds via electrophilic substitution of nitrogen (Badawy and Rabea, 2011; Tan et al. 2013). The main parameter that characterizes a quaternized derivative is the average degree of quaternization (DQ). The DQ is usually determined by NMR spectroscopy (Sieval et al. 1998), although potentiometric and conductimetric methods have also been widely used (Belalia et al. 2008). However, since the reaction is conducted in a heterogeneous medium, mono-methylated, di-methylated, and tri-methylated species are generated randomly and may lead to misinterpretation of the ^{1}H NMR spectrum. Resulting derivatives of chitosan and BF i.e. TMC and TMB, respectively, with higher DQ were considered for evaluating their flocculating and antibacterial activity against water borne pathogens.

Dosage is crucial in any water treatment plant as over-dosing often leads to insufficient removal of turbidity as well as public health concerns with organic polymers and high operating cost, whereas under-dosing can also have a similar issue of insufficient removal of turbidity (Bouyer et al. 2005). Understanding the interaction between the flocculant dose with its flocculating activity as well as turbidity removal efficiency is important for optimization of flocculation process. The correlation between turbidity removal and flocculant performance as reported by Kim et al. (2013) who depicted that a decrease in the organic matter content or turbidity of water reduced the electrostatic repulsion between dispersed particles and flocculants, thus promoting the flocculation performance. For the optimization of dosage for effective flocculation of varied colloidal particles at different turbidity levels, a design of experiment (DOE) was employed to study the effect of variables and their responses using a minimum number of experiments. Response surface methodology (RSM) with central composite rotatable design (CCRD) signified the ability of quaternized biopolymer to effectively flocculate varied size particles in mixed suspension at various levels of turbidity. The findings indicated that application of RSM to the maximum flocculation and turbidity removal had R^2 value closer to 1.

The lower value of coefficient of variation (CV), the "Adequate Precision", denotes a higher reliability of the experiments and a very high significance of the model (Venil et al. 2009).

Upon optimization of the significant variables for flocculation process, the surface plots helped a better understanding of the interactions of the significant variables and thus enabled the determination of the optimal level of each variable for maximizing flocculating activity and turbidity removal. TMB formed stronger flocs that settle more easily than with simple flocculation electrolytes. An explanation to this effect lies in the high molecular weight of a flocculant, which in turn is related to the chain length of the polymer and act as an important factor in the flocculation reaction. A large molecular weight polymeric flocculants have long chain lengths and sufficient number of free functional groups which can act as bridge to bring many suspended particles together, and hence cause a larger floc size in the flocculation reaction (Kurane et al. 1986). Long chain polymers when added in small dosage to a suspension of colloidal particles, adsorb onto them in such a manner that an individual chain can become attached to two or more particles thus “bridging” them together (Salehizadeh and Shojaosadati 2001). The essential requirements for polymer bridging are that there should be sufficient unoccupied particle surface for attachment of polymer segments from chains attached to other particles and that the polymer bridges should be of such an extent that they span the distance over which inter-particle repulsion prevails (Salehizadeh and Shojaosadati 2001; Tripathy et al. 2006). Thus, at lower dosages, there is insufficient polymer to form adequate bridging links between particles. With excess polymer, particle surface available for attachment of segments are insufficient leading to destabilization of particles, this may involve some steric repulsion. The dispersity of TMB was improved by introduction of methyl groups to the biopolymeric flocculant chain as indicated by the Polydispersity index of TMB and BF. This enhanced functionality imparted due to quaternization allows TMB to be more effective in the process of flocculation and dispersion. The results obtained in our study therefore provide a proof-in-concept demonstration of quaternized biopolymeric flocculants for water purification. A general implication of these findings may be envisioned in terms of practical utility, since application may in part reduce operational costs in flocculation treatment, as well and effectively reduce the concentration of residual metallic elements (e.g. aluminium) in water (Ma et al. 2008). Important related characteristics of these polymers include robust operability over wide pH range, thermal stability, excellent rheological properties as well as easy disperisbility in water.

The grim current scenario makes the assessment of microbial safety of water a prime consideration to human health. *Yersinia enterocolitica*, *Aeromonas, Salmonella, Listeria*, *Shigella and E. Coli* O157:H7 are among the prevalent water borne pathogens (Dev et al. 1991; Nath et al. 1999: Shane 2000; Maskey et al. 2006). It was therefore pertinent to examine the biocidal efficacy of developed quaternized biopolymeric flocculants, TMB and TMC on these pathogen types. The biocidal activity of these polymers is attributed to their ability to interact with bacterial cell wall, which results in disruption of the underlying cytoplasmic membrane (Mcbain et al. 2004; Raafat et al. 2008). Overall, all the six pathogens were inactivated by TMB at low dosage (60-70µg/ml) within short contact time (30-70min) (Khaira et al. 2013b,c). The Minimum inhibitory concentration (MIC) of TMB used to inhibit *Salmonella* was found to be 60µg/ml which led to 4 log reduction whereas a higher concentration of 74µg/ml of TMC that is required for same exposure time of 60min led to 3 log reduction of bacterial cells. In this study, antibacterial activity of the synthesized biopolymeric derivative was examined only at low concentrations and after short-exposure times with the intention of investigating its potential practical use as an antibacterial agent against bacterial pathogens. The antimicrobial activity of chitosan against a variety of bacteria and fungi originates from its polycationic nature (Muzzarelli et al. 2001; Badawy and Rabea, 2011; Xia et al. 2011). Unfortunately, this activity is limited to acidic conditions because of its poor solubility above pH 6.5, since chitosan starts to lose its cationic nature and become poorly soluble (Badawy and Rabea, 2011). As water solubility is an important factor in applications of chitosan as an antimicrobial agent, the chemical modification of chitosan and several derivatives with high solubility in water have been attempted. Moreover chitosan derivatives containing carboxyl groups such as N,O-(carboxyalkyl and aryl) chitosans, N,N-(dicarboxyethyl) chitosan and N-(carboxyacyl) chitosans have been reported as water soluble compounds (Kurita et al. 1982; Shigemasa et al. 1995).

Discrepancies exists among different reports on the antibacterial activity of chitosan and its quaternary derivatives. Trimethyl chitosan exhibits enhanced antibacterial activity against *E. coli* in comparison to chitosan and a decrease in pH results in stronger activity (Avadi et al. 2004). However, TMC synthesized in the present study, was found effective against the water borne pathogens, but required higher dose and longer exposure time for the inactivation of target pathogens in comparison to TMB. In another report, modification of the free amino group of the chitosan backbone decreased its antibacterial activity (Xu et al. 2010). These reports showed that quaternization does not always enhance the antibacterial activity of chitosan and that the effect of pH on the antibacterial activity of quaternary ammonium chitosan is uncertain. The discrepancies among different reports on the antibacterial activity of chitosan and its quaternary derivatives are most likely caused by various intrinsic and extrinsic factors that are related to the chitosan itself (e.g., type, molecular weight, degree of deacetylation, viscosity, solvent and concentration).

The battery of assays performed to estimate the leakage from bacterial cells on treatment with TMB revealed prominent cellular leakage in all the studies as well as cellular damage and death clearly discernable by electron microscopy and FT-IR spectroscopy (Khaira et al. 2013a,b,c). Nevertheless, explanation of the observed selective binding and killing cannot be arrived at by considering purely electrostatic interactions as key players. There is also increasing evidence that surface appendages, especially of pathogens, carry specific adhesins involved in interactions with receptors on the host cell (Wilson et al. 2002). TMB completely altered the lipopolysaccharide structure of *Salmonella* which affects virulence by the amount produced the O side chain length and composition, and the degree of glycosylation (Murray et al. 2006; Patel et al. 2010). Flagella are antigenic surface structures of *Salmonella* and flagellin is the major structural protein of flagella and carries the serotype-specific H- antigenic determinants (Levantesi et al. 2012). It is possible that TMB binds to the outer cell wall in a specific manner blocking the recognition of the H antigen by H-specific antibody within minutes of exposure of the organism to TMB.

It has been accepted that the antimicrobial activity of the quaternized biopolymer is affected by microbial species. Similar observations were noted in our study with *Listeria*. The explanation for TMB being not as effective against *Listeria* as against *Salmonella, Shigella, Aeromonas, Yersinia and E.coli* lies in the fact that overcoming the outer membrane is a prerequisite for any material to exert bactericidal activity towards Gram-negative bacteria (McDonnell and Russell 1999). The negative charge on the cell surface of the tested Gram-negative bacteria was higher than on the tested Gram-positive bacteria, leading to more polycationic quaternized polymer being adsorbed and higher inhibitory effects against the Gram-negative bacteria (McDonnell and Russell 1999; Tan et al. 2013).

Despite the distinction between Gram-negative and Gram-positive bacterial cell walls, antibacterial modes both begin with interactions at the cell surface and compromise the cell wall or outer membrane first. For Gram-positive bacteria, lipoteichoic acids may provide a molecular linkage for quaternized derivatives at the cell surface, allowing it to disturb membrane functions (Tan et al. 2013). LPS and proteins in the outer membrane of Gram-negative bacteria are held together by electrostatic interactions with divalent cations that are required to stabilize the outer membrane. Polycations may compete with divalent metals such as Mg^{2+} and Ca^{2+} ions present in the cell wall which could disrupt the integrity of the cell wall or influence the activity of degradative enzymes (Kong et al. 2010). As a polymeric macromolecule, TMB was unable to traverse the outer membrane of bacteria, since this membrane functions as an efficient outer permeability barrier against macromolecules. The increased membrane permeability leads to destabilization of the cell membrane, leakage of intracellular substances and, ultimately, the death of cells (Raafat and Sahl 2009). The highly reactive amino groups in biopolymers have the ability to interact with anionic groups on the cell surface and to form polyelectrolyte complexes with bacterial surface compounds, thereby forming an impermeable layer around the cell, which prevents the transport of essential solutes into the cell (Tan et al. 2013). Overall the results obtained in this study are similar to those reported in most instances suggesting membrane damage by TMB to be the principal mechanism for inactivation. Bacterial LPS are unique and complex glycolipids that provide characteristic components within the outer membranes of Gram-negative bacteria. Certain external agents that either release LPS and other components from the outer membrane or intercalate in the membrane can abolish the integrity of the outer membrane, a more extensive action however also results in release of LPS leading to cell lysis (Alakomi et al. 2000). Therefore structural changes in LPS are reminiscent of the extent of damage incurred. The FTIR profiles indicated marked deviation from LPS of untreated pathogens in comparison to treated counterparts suggesting the severity of damage especially in case of Gram negative pathogens

To ensure safety of TMB in terms of carcinogenicity or mutagenicity, the Ames test was used. The *Salmonella*/microsome assay has been used to analyze the mutagenicity of molecules *in vitro*, with sufficient accuracy based on his+ revertants. None of the three different concentrations of TMB tested exhibited significant revertant frequency of the *Salmonella* tester strains indicating TMB to lack the ability to act as a carcinogen/mutagen. It has been argued based on numerous evidences that Ames test alone may not guarantee a complete toxicological profile of a molecule intended for human applications. Therefore additional toxicological studies on polysaccharide biopolymers have elaborately documented their safety. For instance, Honarkar and Barikani (2009) carried out toxicity studies on chitin from crustacean shells, Delaney et al. (2003) and Jonker et al. (2010) reported studies on beta-glucan from barley and yeast in mice models. A general interpretation from these studies suggest 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 differential response in mice under *in vivo* conditions (intestinal behavior). However these biopolymers were intended as dietary supplements.

In Swiss albino mice fed with the quaternized biopolymer (140 mg kg/ body weight/ day) over a period of 30 days, mortality of animals was not observed during the period of experiment. Body weight gains, rectal temperature profile and feed consumption at all TMB concentrations tested was comparable to control group (Khaira et al. 2014). 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 upon comparison to the control group. Moreover, liver sections from mice fed with TMB revealed a similar profile as that of the control groups, suggesting the absence of any untoward response. These results were sufficient to indicate the safety of the quarternized derivatives when delivered through oral route.

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, hematology, and organ histology at concentrations representing 1000 folds more than the anticipated TMB dosage required to inactivate the pathogens in water suggest the safety and therefore a potential applicability of the TMB as a disinfectant cum flocculant in water treatment.

Additionally, whether this compound induces bacterial resistance or its mechanisms merits further detailed investigation as it was beyond the scope of the present study. Future work should also aim at demonstrating the molecular details of the underlying mechanisms and their relevance to the antimicrobial activity of quaternized biopolymers.

In summary, the work aimed at examining the following objectives:

1. Development of quaternized derivatives of bioflocculant and evaluation of the efficacy of developed variant
2. Studies on physiological, biochemical and molecular responses of microbial inactivation
3. Toxicological studies on the synthesized derivative of bioflocculant

Overall, aim of this study was to develop a safe semi-synthetic candidate which can be possibly used as a replacement of chemical flocculants and disinfectants in water treatment.

The salient findings in the study are as follows:

- From a library of fifty purified bioflocculants, a polymer obtained from an environmental isolate *Klebsiella terrigena*, which possessed high flocculating activity (>80% activity against kaolin suspension) and 38.6% of amino sugar content was selected for rendering antibacterial activity through quaternization. The bioflocculant has been pre-characterized in terms of compositional analysis and molecular mass and known to aggregate a wide range of colloidal particles.
- Chitosan and the biopolymeric flocculant (BF) were quaternized through two different methods, where methyl iodide and dimethyl sulphate were used as methylating agents, respectively, resulting in two quaternized derivatives of each chitosan (TMC1 and TMC2) and BF (TMB1 and TMB2). The ^{1}H-NMR spectra the derivatives TMB (73%) and TMC (67%), with higher DQ, confirmed the quaternization by the singlet peak at 3.66 ppm, equivalent to trimethyl ammonium group. Quaternized derivatives with higher DQ were

obtained during six hours of reaction time, further changes in pH and temperature conditions of the reaction failed to significantly change the DQ values.

- The optimum dose of TMB for effective flocculation and turbidity removal of different particle sizes were statistically optimized with Response Surface Methodology and Central Composite Design. The optimum dose was further used against a mixed suspension comprising of low, medium and large size colloidal particles and results revealed that 15 to 20ppm of TMB is efficient to remove the different size particles in mixed suspension at all the levels of turbidity. High molecular weight TMB with Polydispersity index of 1 was found to be more effective in flocculating different sizes of colloidal particles (ranging from 0.5µm to 100µm) as compared to TMC over concentrations of 10-40ppm and mechanism of the flocculation with TMB may be explained by a combination of charge neutralization and polymer bridging.
- An inactivation of 4 log CFU/ml of *Salmonella typhimurium* ATCC 25315, *Shigella flexneri* 2a, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* O157:H7 ATCC 32150 was noted for TMB as compared to TMC which resulted in inactivation of 3 log CFU/ml, over a short contact time (30-60 minutes) and low dosage (60-90µg/ml) at ambient temperature. The inhibitory effect of TMB on pathogens was observed in the order as *Salmonella typhimurium*> *Shigella flexneri*> *Aeromonas hydrophila*> *Yersinia enterocolitica*> *E.coli* 0157:H7> *Listeria monocytogenes.* On comparing the data of inhibitory effect of TMC on different indicator strains, the order of effect was observed as *Aeromonas hydrophila*> *Salmonella typhimurium*> *E.coli* 0157:H7> *Shigella flexneri*> *Yersinia enterocolitica*> *Listeria monocytogenes.*

- In order to elucidate the mode of action of quaternized biopolymers, a battery of assays was carried out for evaluating the overall biochemical response of target bacterial cells. The electric conductivity measurements, leakage of potassium ions, intracellular metabolites and enzymes (proteins, glucose and Lactate dehydrogenase (LDH)) UV-absorbing substances from bacterial cells treated with TMB (40 to 70µg/ml) and TMC (60-100µg/ml) resulted in a destabilized cell membrane beyond repair and subsequent cell death. The impact of TMB on the energetic condition of sensitive cells was determined by measuring the cellular ATP levels. Upon addition of TMB (2,800AU/ml) and 10min of incubation increase in extracellular ATP was observed. For *Salmonella*, ATP release was highest suggesting a potential damage of cytoplasmic membrane of target pathogen.
- The Lipopolysaccharide (LPS) of inactivated pathogenic strains post TMB treatment was scrutinized by mid-infrared spectroscopy (4000-400cm^{-1}). The structure of fatty acid, polysaccharide and fingerprint region of the isolated LPS was completely altered as observed from the symmetrical stretching of CH_2 groups (which belongs to lipids), following treatment with quaternized biopolymer. A summary of the above findings suggest membrane damage by TMB to be the principal mechanism for inactivation against all the bacteria. To correlate spectroscopic data with ultra-structural changes caused by TMB treatment, scanning electron micrograph studies were carried out for treated bacterial cells at various time intervals. Profound intracellular changes were observed in TMB treated bacterial cells upon comparison to their non-treated counterparts.
- Investigation on the occurrence of sublethal injury and the relationship between membrane damage and loss of viability in Gram negative bacteria *Salmonella* after exposure to TMB was carried out. Results of transmission electron microscopic examination and agglutination

of target pathogens after treatment at MIC and sub-MIC levels revealed complete damage of the cell wall of the bacteria. TMB probably bound to the outer cell wall in a specific manner and blocked the recognition of the H antigen by H-specific antibody within minutes of exposure of the organism to TMB.

- Simulation trials in synthetic water indicated that quaternized biopolymeric flocculants investigated in this study were able to reduce both the turbidity and bacterial load from the synthetic water to a varying degree, with higher bacterial load removal rate observed with increasing concentrations of the polymers.
- For assuring safety during application, the *in vitro Salmonella* Ames test was applied; results revealed that TMB lacked any mutagenic or carcinogenic potential. Further, in acute toxicity studies using Swiss albino mice model, mortality, gross toxic effects and behavioral changes were not observed. Moreover, histopathology, lipid profile and blood chemistry of biopolymer fed rats indicated no significant alterations when compared to control rats. The newly developed biocidal biopolymeric flocculant was found to be safe at a "no observed adverse effect level" (NOEAL) dose of 140 mg/Kg/day in mice in oral toxicity studies.

Overall results suggested that quaternization of amino rich biopolymer isolated from a bacterium led to a water-soluble bioactive agent with enhanced inhibitive capability against all the selected bacterial pathogens. Also it was tolerated well without any signs of toxicity and may have potential application as a safe, antimicrobial bioflocculant for both removing and inactivating water borne pathogens.

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