CELLULAR EXPRESSION OF TWO KEY WATER BORNE PATHOGENS TOWARDS ANTIMICROBIAL QUATERNIZED CHITOSAN

A Thesis

submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Under the guidance of:

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JULY 2012

CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled "**Cellular expression of two key water borne pathogens towards antimicrobial quaternized chitosan**" in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology and Environmental Sciences (DBTES), Thapar university, Patiala, is an authentic record of my work during the period of six months from January, 2012 to June 2012, under the guidance of Dr. Moushumi Ghosh, Assoc. Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled "**Cellular expression of two key water borne pathogens towards antimicrobial quaternized chitosan**" submitted by Amit Kumar in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by him. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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To my parents, for all the support and motivation they have given me throughout my life.....

ACKNOWLEDGEMENT

I thank the Almighty for showering his blessings throughout the preparation of my thesis. I take this opportunity to acknowledge the warmth, guidance and co - operation of my teachers, colleagues, friends and family members in this study. I am pleased to have the opportunity to express my gratitude to those precious people who have contributed to this thesis.

First and foremost, I would like to express my profound gratitude and great indebtedness to my supervisor, Assoc. Prof**. Dr. Moushumi Ghosh**, for her benevolent supervision of my M Sc. Thesis. I am very grateful to her for giving me the opportunity to conduct this research project within her fascinating research group, for her continuous support and constructive criticism, and for encouraging and challenging me throughout the progress of the study; never accepting less than my best efforts.

I express my sincere thanks to **Dr. M.S Reddy**, Professor and Head, Department of Biotechnology and Environmental sciences (DBTES), Thapar University, Patiala for providing the best laboratory facilities.

Special thanks to Ms. ***Gurpreet Kaur Khaira***, a research scholar, DBTES, for her help, support and valuable suggestions throughout my project work. I also wish to express my thanks to Ms. ***Taranpreet Kaur*** and Ms**.** ***Seema Bhanwar***, research scholars, DBTES for their constant encouragement and help. I am thankful to Ms. ***Richu Singla***, **Mr.** ***Mukesh Singh*** **and Ms.** ***Gaatha Sharma*****,** research scholars, DBTES for their valuable companionship and suggestions.

I was fortunate enough to avail the ungrudging help and assistance from all our laboratory staff, Mr. Babban, Mr. Mohinder, Mr. Surinder and Mrs. Lalita.

Last but not least I am very thankful to my wonderful family and friends for their encouragement. I thank my friends ***Tania*** and ***Charu***, for their timely help and support. Thanks to my dear friends, ***Abhishek***, ***Rabia, Khushi, Gagandeep*** and all the other **classmates**. I am forever indebted to my dear sister ***Shweta*****,** who encouraged me in attaining my goal. Special thanks I owe to my **parents** for their support and love.

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CONTENTS

ABSTRACT

The antibacterial activity of chitosan and its quarternized derivative i.e., TMC against *Salmonella typhimurium* and *Shigella flexneri* was evaluated and an attempt to understand the mechanism of action was attempted in this study. The two key gram-negative water borne pathogens was evaluated for viability at different incubation times. Further, the integrity of the cell membranes of both bacterial cells were investigated by determining the release from cells of materials that absorb at 260 nm, electric conductivity measurements and leakage release of cytoplasmic β-galactosidase activity. Results revealed that after treatment with TMC, the electric conductivity of bacterial suspensions increased, followed by increasing of the units of average release for alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenase (G6PDH). SDS PAGE and occurrence of protein in cell free supernatant indicated that the soluble proteins decreased or disappeared in the treated *Salmonella* cells, demonstrating that TMC performed its antibacterial function via increasing the permeability of cell membranes. The results obtained with two water borne pathogens demonstrate the complexity of the mode of action of Quaternized biopolymer. An explanation of the antibacterial mechanism is proposed involving the cell wall disruption due to interaction between positive amino groups present in the TMC with the negatively charged bacterial cell surface. Several lines of evidence suggest that its site of action is the outer membrane, but a probability of more than one target site would explain quaternized chitosan's antimicrobial action.

Keywords: Water borne pathogens, Quaternized chitosan, Antibacterial activity, Membrane integrity.

List of Abbreviations

APS	Ammonium Per Sulphate
BHI	Brain Heart Infusion broth
CFU	Colony Forming Unit
DNA	Deoxyribonucleic acid
EDTA	Ehylenediamine-tetraacetic acid
et al.	Et alteri / et alii (and others)
KDa	Kilo Dalton
MIC	Minimum Inhibitory Concentration
OD	Optical Density
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEMED	N,N,N´,N´-tetramethylethylendiamine
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet

List of Symbols

°C	degree(s) Celsius
g	gram
h	hour
mL	Millilitre
mg/ml	Milligram per millilitre
mg/L	Milligram per litre
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
µM	Micromolar
Min	Minute
Mm	Millimetre
mV	Milivolt
MW	Molecular weight

N	Normality
Na	Sodium
Nm	Nanometer
%	Percentage
rpm	Revolutions per minute
Sec	Second
spp.	Species (plural)
w/v	Weight by volume
U	Unit
V	Volt
wt	Weight

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INTRODUCTION

Water-borne diseases have now become a global burden. According to WHO (2010), 80% of the illness in developing countries are water related. In waterborne infections, pathogens are usually spread by contaminated water with untreated or poorly treated sewage. Examples of such pathogens are *Salmonella* spp., *Shigella* spp., *Vibrio cholerae* and *E. coli*. Although, chlorine is considered as the primary disinfectant of choice for removal of various water borne pathogens because of its potential oxidizing capacity. However, in the recent years, many waterborne pathogens are reported to be resistant to chlorine.

Salmonella and *Shigella* are key gram-negative water borne pathogens, as the infections caused by them, represents a major health problem worldwide. These concerns have been further reinforced in recent years by the emergence of antimicrobial resistance among the major groups of the enteric pathogens. Studies with *Salmonella* and *Shigella* are of particular relevance because these species can occupy multiple niches, including human and animal hosts.

Billions of dollars are spent annually on synthetic products designed for removal of water-borne pathogens. However, the potential market for products made from natural polysaccharides is growing rapidly. Carbohydrate based polymers provide the broad spectrum of raw materials that exhibit biodegradability, biocompatibility and versatility. Another very interesting inherent property of natural polysaccharides is their biological functionality. A very well known naturally occurring copolymer with known antimicrobial activity is chitosan, composed of monosaccharides that have amino groups in the nonanomeric position. Biocides such as quaternary ammonium salts of chitosan (QACs) can play an important role in the control of bacteria in a variety of applications and are thus a precious resource that must be managed so as to be protected from loss of activity over time.

SCOPE OF THE STUDY

The antimicrobial efficacy of chitosan is generally well documented; however, the precise mechanism of its mode of action is still unknown. Chitosan acts on both Gram-positive and Gram-negative bacteria. The non-solubility of chitosan in neutral and alkaline aqueous solutions limits its application as potential antibacterial polymer. Water-soluble chitosan derivatives can be obtained by the introduction of permanent positive charges on the polymer chain resulting in cationic polymer, independent of the pH of aqueous medium. The antimicrobial action of QACs involves perturbation of cytoplasmic and outer membrane lipid bilayers through association of the positively charged amino group with the negatively charged bacterial cell surface.

Antibacterial derivatives of chitosan have been far less studied than antibiotics in terms of efficacy, mechanism of action and epidemiology of bacterial resistance in water borne pathogens. Also, no systematic study has been carried out till date on development of tolerance and resistance of pathogens against antimicrobial chitosan or its derivatives. Now understanding the bacterial defence strategies will help in developing concepts for reducing the development and spread of resistant bacterial strains. Elucidation of metabolic responses of these survivor cells may provide important insights about the target sites and the mode of action of such compounds. To assess the potential of chitosan and its quaternary ammonium salt as antibacterial biopolymer through examination of the tolerance and resistance mechanism of *Salmonella typhimurium* and *Shigella flexneri* 2a.

REVIEW OF LITERATURE

2. REVIEW OF LITERATUR

2.1WATER BORNE PATHOGENS

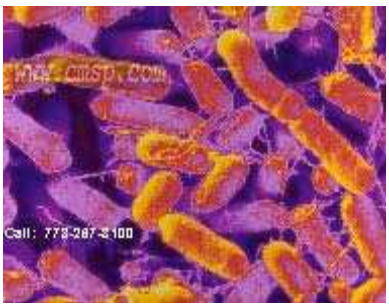
Diarrhoea occupies a leading position among diseases as a cause of death and illness, killing 1.8 million people and causing approximately 4 billion cases of illness annually (Water Dome, 2002). Mostly, the children suffer from diarrhoea, setting back growth and development. Water-borne diseases have now become as a global burden. According to WHO (2007), 80% of the illness in developing countries are water related e.g. cholera, dysentery etc and they are acting as vicious killer. It is indeed disheartening that after more than four decades of independence, our country has not succeeded in providing water, especially safe drinking water in rural and many urban areas, though we often hear claims of progress on all fronts. Inadequately treated water may contain disease-causing organisms, or pathogens. Pathogens include various types of bacteria, viruses, protozoan parasites, and other organisms. Bacteria and viruses contaminate both surface and groundwater, whereas parasitic protozoa appear predominantly in surface water. They get into drinking water when the water source is contaminated by sewage and animal waste, or when wells are improperly sealed and constructed. They can cause gastroenteritis, *Salmonella* infection, dysentery, shigellosis, hepatitis, and giardiasis (a gastrointestinal infection causing diarrhea, abdominal cramps, and gas (Santos *et al*., 2003). The presence of coliform bacteria, which is generally a harmless bacteria, may indicate other contamination to the drinking water system.

Shigella and *Salmonella* are the major water borne pathogens that cause abdominal distress. Salmonellosis and shigellosis are significant and persistent causes of diarrheal diseases among humans in developing countries (Wray *et al.,*1978). While many of the symptoms are same, several differences exist in the method of contamination, treatment and duration. *Salmonella* and *Shigella* infections represent a major health problem worldwide, particularly in

the developing countries where they are recognized as the most frequent cause of morbidity and mortality (Santos *et al*., 2003).The impact of lives lost, together with the high costs to local public health care systems, makes prevention and control a priority (Gleick, 2002).

2.2 *Salmonella*

Salmonella is a gram-negative, facultative rod-shaped, non-spore forming, predominantly motile bacteria. All strains of *Salmonella* are pathogenic for humans, causing enteric fevers such as typhoid and paratyphoid fevers, gastroenteritis, and septicaemia. Over 2,000 antigenic types of *Salmonella* occur. *Salmonella typhimurium* is known to cause typhus abdominalis in humans and is a bacterium commonly found in contaminated and infected food, water or fly excrement (Wray et al.,1978)



Salmonella is nearly as well-studied as *E. coli* from a structural, biochemical and molecular point of view, and as poorly understood as *E. coli* from an ecological point of view. *Salmonella* DNA base composition is 50-52 mol% G+C. The genera with DNA most closely related to *Salmonella* are *Escherichia, Shigella,* and *Citrobacter* (Akbarmehr, 2011).

2.2.1 Prevalence

The principal habitat of the *Salmonella* is the intestinal tract of humans and animals. *Salmonella* serovars can be found predominantly in one particular host, can be ubiquitous, or can

have an unknown habitat. Typhi and Paratyphi A are strictly human serovars that may cause grave diseases often associated with invasion of the bloodstream. Salmonellosis in these cases is transmitted through faecal contamination of water. This organism finds its way into the river water, coastland, estuarine, sediments through fecal contamination. Aquatic environment are the major survivors of *Salmonella* and aid its transmission between the hosts (Ghosh *et al.,* 2009) . The survival rate of *Salmonella* is higher in aquatic environment, even outliving *Vibrio cholera*. Reports are also available on the prevalence of salmonellosis in livestock and poultry in various regions (Ghosh *et al.,* 2009). Although many would argue that the preferred environment for *Salmonella* is the gut of animals, the entire lifecycle of this organism must be considered and there is clear evidence that much of the survival and persistence of this so-called pathogen is associated with environments outside of the host animal (Harthy *et al*., 2012).

2.2.2 Pathogenesis of *Salmonella*

Typhoid is strictly a human disease. The incidence of human disease decreases when the level of development of a country increases, where these hygienic conditions are missing and the probability of fecal contamination of water remains high and so is the incidence of typhoid. *Salmonella* infections in humans vary with the serovar, the strain, the infectious dose, the nature of the contaminated water, and the host status. (Wray *et al*.,1978) An oral dose of at least 10^5 *Salmonella* Typhi cells are needed to cause typhoid in 50% of human volunteers, whereas at least 10^9 *S. Typhimurium* cells (oral dose) are needed to cause symptoms of a toxic infection (Santos *et al*., 2003). In the pathogenesis of typhoid the bacteria enter the human digestive tract, penetrate the intestinal mucosa (causing no lesion), and are stopped in the mesenteric lymph nodes. There, bacterial multiplication occurs, and part of the bacterial population lyses. From the mesenteric lymph nodes, viable bacteria and LPS (endotoxin) may be released into the

bloodstream resulting in septicemia Release of endotoxin is responsible for cardiovascular "collapsus and tuphos" (a stuporous state—origin of the name typhoid) due to action on the ventriculus neurovegetative centers (Santos *et al*., 2003)

2.2.2.1 Virulence Factors

A combination of characteristics makes *S*.typhi an effective pathogen. This species contains an endotoxin typical of gram-negative organisms, as well as the Vi antigen which is thought to increase the virulence. It also produces and excretes a protein "invasion" that allows non-phagocytic cells to take up the bacterium, where it is able to live intracellularly. It is also able to inhibit the oxidative burst of leukocytes, making innate immune response ineffective (Santos et al., 2003)

2.2.3 Resistance in *Salmonella*

During the last decade, antibiotic resistance and multiresistance of *Salmonella* spp. have increased a great deal. Plasmid-borne antibiotic resistance is very frequent among *Salmonella* strains involved in pediatric epidemics. Resistance to ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline, and sulfonamides is commonly observed(Wray *et al*.,1978). Multiple drug resistance in non-typhoidal *Salmonella* infections was common by the end of the 1960s and has increased drastically since then there have been several outbreaks of multiply resistant *S. typhimurium* associated with cattle and poultry . In 1988, in England, a multiple resistant *S. typhimurium* strain, DT104, was first isolated. During the following decade, 90% of human DT104 isolates were found to be multiply resistant, i.e. ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline resistant (Cookson, 2005). This strain has since spread through Europe, the U.S. and many other parts of the world. From 1997 to the present,

resistance to trimethoprim and fluoroquinolones has appeared in DT104 isolates . Since 1986, there have been an increasing number of reports of multiple resistant *S. typhi* throughout the world. In Mexico, chloramphenicol resistance first appeared, followed by resistance to ampicillin, streptomycin, sulfonamides, and tetracycline .Fluoroquinolone resistance has also been reported in *S. typhi* in developed countries. (Akbarmehr, 2011)

2.3 *Shigella*

Shigella is a gram-negative, nonmotile, nonspore forming, rod-shaped bacteria closely related to *Escherichia coli* and *Salmonella* (Ghosh *et al*., 2011) All strains of *Shigella* are pathogenic, causing bacillary dysentery in humans that result in the destruction of the epithelial cells of the intestinal mucosa. *Shigella* infection is typically via ingestion (fecal–oral contamination). Some strains of *Shigella*, are known to produce enterotoxin and Shiga toxin, which are associated with causing haemolytic uremic syndrome. Humans and the higher primates are the reservoir for this organism. The organism can be found in the faeces for weeks after symptoms have ceased. It can survive in human faeces for days if the samples remain moist.

2.3.1 Prevalence/Habitat

Members of the genus *Shigella* normally inhabit the intestinal tract of humans but do not infect animals. Their presence in water is, therefore, an indication of human faecal contamination. Infection is commonly by person-to-person contact, or by the consumption of contaminated food or water. Gastro-intestinal disease is commonly a symptom of infection of which dysentery is the most severe. The disease is typical of conditions of poor hygiene and sanitation (Ghosh *et al*., 1998).

2.3.2 Pathogenesis

Shigella infection is typically via ingestion (fecal–oral contamination); depending on age and condition of the host, as few as 100 bacterial cells can be enough to cause an infection. *Shigella* causes dysentery that result in the destruction of the epithelial cells of the intestinal mucosa in the cecum and rectum. Some strains produce enterotoxin and shiga toxin, similar to the verotoxin of E. coli O157:H7 and other verotoxin-producing Escherichia coli. Both shiga toxin and verotoxin are associated with causing hemolytic uremic syndrome (Ghosh *et al*., 1998).

Four species of *Shigella*, *S. flexneri, S. sonnei, S. dysenteriae*, and *S. boydii*, are pathogenic. In the U.S., most shigellosis is caused by *S. sonnei*, but around the world, *S. flexneri* is the most common. *Shigellae* are capable of surviving the acidity of the stomach, and consequently, the infectious dose is low (Ghosh *et al*., 2011). Invasion and tissue destruction in the intestinal epithelial layer leads to watery diarrhea, then bloody, mucoid diarrhea, as well as cramps and fever. Usually, shigellosis is limited to gastrointestinal illness, but it can progress to bacteremia. *S. dysenteriae* is often the culprit in serious cases and can have a mortality rate of 20%. Throughout the world, 165 million cases of shigellosis occur each year, almost all of which are in developing countries (WHO 2010). However, 20-30 thousand cases occur in the U.S. annually.

2.3.3 Resistance

Antibiotics are useful in treatment of shigellosis since they reduce the length of time of the symptoms, as well as bacterial shedding, but antibiotic treatment is necessary in cases of bacteremia and severe diarrhea. Early antibiotic resistance was detected in Japan, where dysentery caused by *Shigella* infection was a large problem. By 1950, 80%-90% of *Shigella* isolates were resistant to sulfonamides, and by 1965, resistance to tetracycline, chloramphenicol,

and streptomycin had been reported. Since then, resistance continues to increase (McDonnell *et al.,* 1999).

2.4CONVENTIONAL METHODS FOR REMOVAL OF WATER-BORNE PATHOGENS

Throughout history, mankind has been striving to improve the safety of water. The purpose of disinfection is to kill or inactivate microorganisms, so that they cannot reproduce and infect human hosts. The processes involved in treating water for drinking purpose involves screening, prechlorination, aeration, coagulation and flocculation, sedimentation, filtration and prechlorination (Wolfe *et al.*1990).The most important part of the water treatment process is the disinfection of the water, which is most commonly done using chlorination. Instead of chlorination, other methods of disinfection are also available but they carry many drawbacks with them. Though chlorination also possesses some drawbacks, instead it is a method of choice in water disinfection.

2.5STRUCTURE OF GRAM-NEGATIVE BACTERIA

Many gram-negative bacterial cells have complex cell walls. Wide-range of studies on the structure of gram-negative cells has been concentrated on *E. coli* in particular. The outermost regions of the cell wall consist of various components that form a structure called the outer-membrane. This membrane is a lipid bilayer which contains phospholipids (phosphatidylethanolamine and phosphatidyl-glycerol) on its inner surface and

lipopolysaccharide (3-deoxy-D-*manno*-octulosonic acid, hexoses, heptoses, ethanolamine, and phosphoric acid) exclusively on its outer surface. The core lipopolysaccharide is linked to an antigenic side chain. The side chain comprises sugar units, which form the outmost layer of the cell and is the main source of its antigenic characteristics (Maillard, 2002). The arrangement of the lipoprotein (made of amino and fatty acids) anchors the outer-membrane to the peptidoglycan layer. Peptidoglycan is the component of the cell which contributes to wall strength and constitutes about 10% of the wall mass of gram-negative cells. Parallel to the peptidoglycan is the cytoplasmic membrane, which likes the outer-membrane, is a lipid bilayer (Makino, 1989). It consists of a double layer of phospholipids and has proteins embedded in it. The membrane surrounds the cytoplasm of the cell, separating it from the environment.

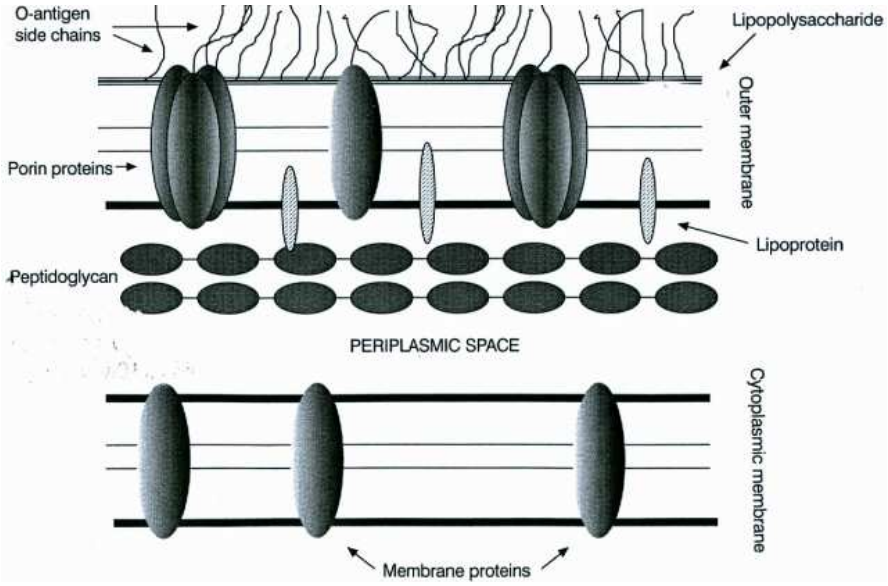


Fig. 2.1 Strucure of Gram-negative bacterial cell wall

2.6MODE OF ACTION OF ANTIBACTERIAL COMPOUNDS

2.6.1 Physical and chemical agents

There are four main targets for antibacterial drugs: the cytoplasm itself, cytoplasmic enzymes, nucleic acids and ribosomes. Gram-negative bacteria were found to be more resistant to chlorine (Maillard, 2002). Membrane permeability loss was the first lethal effect of chlorine on *E. coli,* due to damage to the external membrane, producing an imbalance in the transmembrane ionic gradient. When sublethal chlorine levels were present, respiration inhibition was revealed as the first effect.

Many authors reported that the first sites attacked by ozone were the cell wall and membrane, either through glycoproteins, glycolipids or certain amino acids as tryptophane. Some studies suggested that ozone alters proteins and unsaturated bonds of fatty acids in the membrane structures, leading to cell lysis. Chlorhexidine is another important antibacterial agent, that affects the outer membrane of Gram-negative bacteria with the release of periplasmic enzymes and disturbs the functionality of the inner membrane (Wolfe *et al.*1990).

In the case of chlorination it has been found that it affects the cell respiration, transport and DNA activity. Water chlorination caused an immediate decrease in oxygen utilization in both bacteria *(Escherichia coli, Mycobacterium fortuitum)* and fungi *(Candida parapsilopsis).* Among the disinfectants used in water treatment, chlorine dioxide has been mentioned as disrupting the permeability of the outer bacterial membrane (Gleick, 2002). These results were supported by previous findings, which found that the outer membrane proteins and lipids were sufficiently altered by chlorine dioxide to increase permeability, allowing it to reach the internal structures. Besides chemical agents, the physical agent most used for disinfection in water treatment is UV radiation, which is efficient at inactivating vegetative and resistant forms of

bacteria, viruses and vegetative and resistant forms of bacteria, viruses and pathogenic protozoa. For UV radiation the target is always nucleic acid (Wolfe *et al.*1990).

2.6.2 Mechanisms of antibiotic action

2.6.2.1 Inhibitors of Cell Wall Synthesis:

There are two major groups of cell wall synthesis inhibitors, the β-lactams and the glycopeptides. The β-lactams include the penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. In gram negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured. In gram positive cells, autolysis is triggered by the release of lipoteichoic acid (Rodgers *et al.,* 1989).

The glycopeptides are a group of antibiotics that include vancomycin, avoparcin, and others that bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall. These drugs are large molecules that are excluded from gram negative cells by the outer membrane, thus limiting their action to gram positive organisms (Makino, 1989).

2.6.2.2 Inhibitors of protein synthesis:

Antibiotics take advantage of structural differences between bacterial ribosomes and eukaryotic ribosomes. The aminoglycoside antibiotics are a group whose mechanism of action is not completely understood. The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins. These drugs enter bacterial cells by an active transport that involves quinones that are absent in anaerobes and streptococci, thus excluding these organisms from the spectrum of action. Streptomycins act by binding to the 30S ribosomal subunit. Kanamycins and

neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin (Rodgers *et al.,* 1989).

2.6.2.3 Inhibitors of nucleic acid synthesis:

The sulfonamides and the diaminopyrimidines indirectly inhibit nucleic acid synthesis by inhibiting folate synthesis. Folate is a coenzyme necessary for the synthesis of purines and pyrimidines. Although both types of drugs are useful on their own, they exhibit a synergistic effect when combined (Makino, 1989).

2.7NATURAL POLYSACCHARIDES AS FUTURE BIOCIDAL AGENTS

The potential market for products made from natural polysaccharides (polymers) has expanded rapidly. These polysaccharides can form many other derivatives which will pose no threat to the environment after their use. An unique feature of natural polysaccharides is their biological functionality, therefore these polysaccharides are envisaged as ideal furturistic biocides. Some attributes of these polymers are as follows:

a) They are easy and inexpensive to synthesize.
b) They are easy to store for longer duration and are less dependend on surrounding temperature.
c) They don't decompose and form harmful byproducts.
d) They are biocidal to a broad spectrum of pathogenic microorganisms with brief contact of time.

2.8 ANTIMICROBIAL CHITOSAN DERIVATIVES

Many attempts have been made to enhance chitosan's solubility in neutral water, since it is desirable in chitosan's many applications e.g., cosmetics, food, and biomedicines. This has been achieved by synthesizing *N*-substituted derivatives. Some other *N*-substituted reactions include *N*-alkylation, *N*-acylation, and *N*-hydroxyacylation. Remarkable change in water solubility of chitosan due to *N*-substitution is due to reduced regularity of intermolecular hydrogen bonding, which creates space for water molecules to fill in and solvate the hydrophilic groups of the polymer backbone (Britto *et al.,* 2011).

One of the most unique biological properties of chitosan is its antibacterial activity. Chitosan inhibits the growth of a wide variety of bacteria (Chung *et al*., 2004). However, chitosan exhibits its antibacterial effect at pH less than 6.5 (acidic range) due to its poor solubility in neutral water. To analyze chitosan's antibacterial activity at pH above 6.5, chitosan derivatives are usually prepared. The most popular derivatives include those that comprise acidic (anionic) or quaternary ammonium (cationic) moieties on the polymer backbone. The preparation of water-soluble methylated chitosan and chitooligomer derivatives and its antibacterial activity was investigated. The antibacterial activity against a variety of gram positive and gram negative bacteria was assessed by a quantitative assay based on conventional agar dilution tests (Chung *et al*., 2004).

The antimicrobial efficacy of chitosan is generally well documented; however, the precise mechanism of its mode of action is still unknown. Electron microscopical examinations of various chitosan-treated cells microorganisms suggest that its site of action is at the microbial cell surface (Muzzarelli *et al*., 1990). In Gram-negative microorganisms, an abnormally expanded periplasmic space was observed, while intracellular materials appeared to be more tightly packed and lacking any organization.

2.8.1 Mode of action

2.8.1.1 Outer Membrane Disruption

Among the various models proposed the most acceptable being the interaction between positively charged chitosan/chitosan derivative molecules and negatively charged microbial cell membranes. In this model the interaction is mediated by the electrostatic forces between the protonated NH^{+}_{3} groups and the negative residues (Tsai et al., 1999), presumably by competing with Ca^{2+} for electronegative sites on the membrane surface.

This electrostatic interaction results in two fold interference: (i) by promoting changes in the properties of membrane wall permeability, thus provoke internal osmotic imbalances and consequently inhibit the growth of microorganisms (Shahidi *et al.,* 1999). (ii) by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase (Sudarshan *et al*., 1999. Chen *et al*., 2000)

Interaction with negatively-charged surface components of many fungi and bacteria, causes extensive cell surface alterations, leakage of intracellular substances and ultimately resulting death of cells (Tsai *et al.,* 1999). As early as 1982, Young *et al.* demonstrated in their study that chitosan increases the membrane permeability of plant cells, presumably by binding to polygalacturonate, a component of plant cell walls, inducing the leakage of electrolytes, UV-absorbing materials, and proteins into the medium. Helander *et al.2001,* studied the effects of chitosan treatment on the cell membranes of Gram-negative bacteria and found evidence for extensive cell surface alterations, marked by thickening and formation of vesicular structures on

the outer membranes of both *Escherichia coli* and *Salmonella typhimurium*. They reasoned that chitosan binds to the outer membrane of Gram-negative bacteria, thereby affecting its barrier properties, probably through complex formation with various lipopolysaccharides (Davydova *et al.,* 2000).

Liu *et al*., (2004), hypothesize that this electrostatic interaction takes place between chitosan and negatively-charged cell membrane components (i.e. phospholipids or proteins), affecting membrane integrity and permeability, which causes leakage of intracellular substances, and finally the death of the cell.

Raafat *et al., (*2008), observed under transmission electron microscope the ultrastructural changes of *S. simulans 22* cells upon exposure to positively charged chitosan. It was possible to observe and identify chitosan molecules attached on bacteria cell surfaces. In the interacting sites the cell membrane became locally detached from the cell wall, giving rise to "vacuole-like" structures underneath the wall. The detachment generates ions and water efflux, provoking decreases on the internal bacteria pressure. Visual confirmation of an effective membrane lysis been also reported on gram-negative and gram-positive bacteria.

Chung *et al.*, (2008) proposed that the inactivation of *E. coli* by chitosan occurs via a two-step sequential mechanism: an initial separation of the cell wall from the cell membrane, followed by destruction of the cell membrane. Their conclusion was based on similarities between the antibacterial pattern of chitosan and those of polymyxin and EDTA.

Concerning the bacteria surface polarity, the outer membrane of gram-negative bacteria consists essentially of lipopolysaccharides containing phosphate and pyrophosphate groups which render to the surface a density of negative charges superior to that observed for gram-positive ones (membrane composed by peptidoglycan associated to polysaccharides and teichoic

acids). This supports the evidence that the leakage of intracellular material observed by chitosan in gram-negative is superior to that reported in gram-positive bacteria (Helander *et al* 2001, Chung *et al* 2008). Several *in vitro* experiments in which gram-negative bacteria appear to be very sensitive to QAC, exhibiting increased morphological changes on treatment when compared to gram-positive (Helander *et al.,* 2001).

Liu *et al*., (2006).,reported that as a polymeric macromolecule, chitosan is unable to traverse the outer membrane of gram-negative bacteria because the membrane functions as an outer permeability barrier against macromolecules. Therefore, the possibility of chitosan's direct access to the intracellular parts of the cell (e.g. cytoplasmic membrane) is not possible.

The function of the outer membrane of Gram-negative bacteria is to act as a protective permeability barrier. The outer membrane is impermeable to large molecules and hydrophobic compounds from the environment. LPS is essential to the function of the outer membrane. It establishes a permeability barrier that is permeable only to low molecular weight, hydrophilic molecules. In the *E. coli* the ompF and ompC porins exclude passage of all hydrophobic molecules and any hydrophilic molecules greater than a molecular weight of about 700 daltons (Maillard, 2002).

2.8.1.2 Protein Synthesis Inhibition

Another proposed mechanism is the binding of chitosan with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms (Sebti *et al.,* 2005). In this the chitosan molecules is assumed to be able to pass through the bacterial cell wall, composed of multilayers of cross-linked murein, and reach the plasma membrane. Observation by confocal laser scanning microscopy confirmed the presence of chitosan oligomers (a chain with few number of monomer units) inside *E. coli*

exposed to chitosan under different conditions. Raafat *et al.,* (2008), stated that in spite of been accepted as a possible mechanism, the probability of it occurring is rather low. The prevailing contention is that chitosan acts essentially as an outer membrane disruptor rather than as a penetrating material (Helander *et al.,* 2001).

2.8.1.3 Chelation of Metals

The third mechanism is the chelation of metals, suppression of spore elements and binding to essential nutrients to microbial growth. It is well known that chitosan has excellent metal-binding capacities where the amine groups in the chitosan molecules are responsible for the uptake of metal cations by chelation (Helander *et al* 2001). In a recent model proposed by Wang et al., (2005), the metal is arranged as an electron acceptor connected to one or more chitosan chains via -NH_2 and by forming bridges to hydroxyl groups. It is unquestionable that chitosan molecules in bacteria surrounds might complex metals and blockage some essential nutrients to flow, contributing to cell death (Sudarshan *et al*., 1999). Nevertheless, this is, evidently, not a determinant antimicrobial action since the sites available for interaction are limited and the complexation reach saturation in function of metal concentration.

2.9RESISTANCE AGAINST BIOCIDES

The definition of bacteria as resistant or susceptible against an antimicrobial agent is critical. It is also very important to note the difference between intrinsic and acquired resistance to an antimicrobial compound. Intrinsic resistance can best be described as resistance of an entire species to an antimicrobial compound, based on inherent (and inherited) characteristics requiring no genetic alteration. This is usually due to the absence of a target for the action of a given compound or the inability of a specific drug to reach its target. For example, *Pseudomonas*

aeruginosa exhibits high intrinsic resistance to many antibiotics due to its drug efflux pumps and restricted outer membrane permeability (Akbarmehr., 2011) .

Acquired resistance can arise either through mutation or horizontal gene transfer. The major mechanisms of acquired resistance are the ability of the microorganisms to destroy or modify the drug, alter the drug target, reduce uptake or increase efflux of the drug, and replace the metabolic step targeted by the drug.

Biocides interact with microorganisms initially at the cell surface. 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 .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. On the other hand, investigations into the potential mechanism(s) of chitosan resistance are lacking, So, there is a need to conduct a detailed investigation of the determinants of chitosan resistance in water borne pathogens (Morat *et al*.2005).

Understanding the bacterial defense strategies will help in developing concepts for reducing the development and spread of resistant bacterial strains. 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, 1999). This is particularly true for organisms such as *Pseudomonas aeruginosa,* where the high Mg^{2+} content of the outer membrane aids in producing strong LPS-LPS links. On the other hand, in *Proteus spp.,* the presence of a less acidic type of lipopolysaccharide is a

contributory factor to its resistance to chlorhexidine and other cationic biocides. Moreover, the outer membrane of Gramnegative bacteria acts as a permeability barrier because the narrow porin channels limit the penetration of hydrophilic molecules (Rodgers *et al.,*1989).

2.9.1 Resistant Small Colony Variants

In vitro studies highlighted important changes in the physiology of bacteria that have acquired resistance to certain biocides such as cationic compounds and oxidising agents .It has been well reported that *in vitro* sub-lethal (often sub-MIC) concentration of a biocide decreases the susceptibility of bacteria to that biocide and modifies the antibiotic susceptibility profile (Cookson, 2005).

A recent study evaluated the selection potency of cetylpyridinium chloride, a quaternary ammonium compound widely used worldwide for disinfection in hospitals, on *Serratia marcescen* (McDonnell, 1999). The authors demonstrated that a resistant strain was selected exhibiting noticeable resistance stability (over 60 generations) and a large MDR phenotype including resistance against cetylpyridinium chloride, quinolones, tetracycline and chloramphenicol. This resistance level is associatedwith the overproduction of the SdeAB efflux pump that expels the toxic compounds, i.e.biocides and antibiotics, from the bacterial cells. Recently, Kastbjerg, *et al.* 2010 observed that while a sub-lethal concentration of quaternary ammonium compounds (QAC) and triclosan increased virulence gene expression in *Listeria monocytogenes*, other disinfectants based on chlorine and peroxides decreased such expression.

MATERIALS AND METHODS

3. MATERIALS AND

3.1 Bacterial Strains and Culture Conditions

Cultures of *Salmonella typhimurium* ATCC 25315, and *Shigella flexneri* 2a were revived twice in brain heart infusion broth by incubating at 37°C for 12 hrs. Both strains used in the current study were stored as glycerol cultures (40% [vol/vol]) at -70°C.

3.2 Agar disc diffusion

Discs of uniform size were prepared from the Whatmann filter paper. Overnight grown cultures, 100ul was spread onto BHI (Brain Heart Infusion) agar plates with a sterile cotton-tipped swab to form an even lawn. For the dose response study, paper discs diluted with different chitosan (100-1000 µg/mL) and quaternized chitosan concentrations (50-500 µg/mL) were placed on the surface of each BHI plate using a sterile pair of forceps. Then the plates were incubated aerobically at 37°C for 24 hours and the diameter of zone inhibition was measured.

3.3 MIC determination

The minimum inhibitory concentration (MIC) is a measure of the susceptibility of a bacterial strain towards a specific antimicrobial substance. Values of the MIC of the different antimicrobials were determined by a standard broth microdilution assay. Briefly, different concentrations of antibacterial agent (chitosan and quaternized chitosan) were added in culture medium (selected bacterial strains) in a sterile 96 well microtiter plates in High Throughput Growth Analyser (BIOSCREEN C, Helsinki, Finland). The inoculated microtiter plates were then incubated at 37°C, absorbance was measured at 600 nm.

Susceptibility tests were repeated at least three separate times to check the reproducibility of the results, and the mean of these values were taken.

3.4 Killing Kinetics

Samples of the bacterial cultures were removed at regular intervals to record survival counts, expressed as CFU/ml. The surviving $\log 10^7$ CFU/ml was plotted against time for each of the chitosan and TMC. The number of viable cells in a bacterial suspension was estimated through 10-fold serial dilutions in physiological saline (0.9% [wt/vol] NaCl). 50 µl-aliquots of the appropriate dilutions were plated onto the surface of agar plates, and incubated for 24 –48 h at 37°C. The developed colonies were then counted, and the number of colony forming Units (CFU)/ml in the bacterial suspension was calculated based on the respective dilution factor.

3.5 Development of Variants of *Salmonella* and *Shigella*

3.5.1 Estimation of sublethal concentration of *Salmonella* and *Shigella*

Sub lethal concentration was determined by using Quaternized chitosan (TMC), slightly less than its calculated MIC value. An initial inoculum of around 10^7 CFU/ml was taken and then incubated at 37°C. The range of Quaternized chitosan concentrations tested were (360 -390 µg/mL) for *Shigella* and (270-290 µg/mL) for *Salmonella* to encompass sublethal-to-lethal TMC levels. After 20 min of contact, samples were removed and the surviving count was determined. Curves were constructed, comparing mean surviving $\log 10^7$ CFU/ml versus TMC concentrations. The colonies were further sub cultured and again its sub lethal concentration was calculated.

3.6Study On Mode Of Action

3.6.1 Cellular leakage study

Leakage is best considered as a measure of the disruption of the cell permeability and it might reflect a bacteriostatic effect rather than cell death (Maillard, 2002).

3.6.1.1 Electric conductivity assay

Bacterial samples (wild type and variants) were incubated at 37 ◦C (at log phase) and then harvested by centrifugation at 11,000 × g for 10 min. The pellets were washed and resuspended in 0.1 M phosphate buffer (pH 7.4). The final cell suspensions were adjusted to 10^7 CFU mL−1 and mixed with TMC (MIC and sublethal concentrations). The mixtures were then incubated at room temperature and measured for their electric conductivity every 10 min for 2 h on 4510 conductivity meter (Jenway, UK). The experiment without compound was used as blank control.

3.6.1.2 Leakage of UV absorbing materials

Bacterial cell membrane integrity was examined by determination of the release of material absorbing at 260 nm (A260, Chen and Cooper, 2002). Bacterial cultures (wild type and variants) were grown as above, harvested, washed and resuspended in 0.5% NaCl solution. Ratio of 260/280 was calculated to detect the presence of protein in the supernatant of treated cells.

3.6.1.3 Zeta Potential Measurements

The cell surface charge was determined by particle micro electrophoresis using a Zetasizer (Malvern Instruments) in order to measure the zeta potential. Bacterial cells were

grown in BHI and suspended in 1mM KCl at a concentration of approximately 1×10^7 cells/ml . Experiments were repeated three times.

3.6.2 Leakage of Enzymes

3.6.2.1 β-galactosidase activity assay

Inner membrane permeabilization was determined by measuring the release of cytoplasmic beta-galactosidase activity from *Salmonella* and *Shigella* into the culture medium using ONPG as the substrate (Ibrahim *et al,* 2000). Logarithmic-phase bacteria (Wild type and variants), grown in BHI broth were harvested by centrifugation at 11,000 × g for 10 min, washed and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance of 1.2 at 420 nm. A 1.6-mL aliquot was mixed with TMC (at sublethal concentration) of 1.6 mL and 30 mM ONPG. The production of o-nitrophenol over time was determined by monitoring the increase in absorbance at 420 nm spectrophotometrically.

3.6.2.2 Alkaline phosphatase (ALP) and Glucose-6-phosphate dehydrogenase assay (G6PDH)

ALP activity was determined using the method described by Malamy and Horecker (1964). The reaction mixture (total volume: 1 mL) contained 0.1 mg p-nitrophenylphosphate in 0.5 M Tris–HCl buffer (pH 8). The reaction was followed at 28ºC by measuring the optical density of the suspension at 420 nm. A unit of released ALP activity was defined as the amount of enzyme that produced 1 µM of p-nitrophenol-equivalent in 1 min at 28ºC.

G6PDH activity was determined using the method described by Malamy and Horecker (1964). A 0.2-mL aliquot of the cell suspension was added to the reaction mixture. G6PDH

activity was determined in a solution (total volume: 1 mL) containing 0.05 M Tris-HCl (pH 8), 0.01 M CaCl2, 1.0 µM glucose-6-phosphate and 0.4 µM triphosphopyridine nucleotide (TPN). The reaction was followed at 28 ◦C by measuring the optical density of the suspension at 340 nm. A unit of released G6PDH activity was defined as the amount of enzyme that reduced 1 µM of TPN-equivalent in 1 min at 28ºC.

3.7Protein Profiling of Water Borne Pathogens

Leakage of Proteins in bacterial cell free supernatant was determined by the method of Lowry et al (1951). In addition,an electrophoretic separation of the protein samples was conducted using common sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli , with a 4% stacking gel and 12% resolving gels. The SDS-PAGE was conducted in a vertical uigel apparatus (Amersham Biosciences) at a voltage of 90 V for 120 - 140 min. Later the proteins were visualized with coomassie brilliant blue R-250.

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSION

4.1 Preliminary screening of antibacterial properties in chitosan and quaternized chitosan

4.1.1 Agar disc diffusion assay:

The inhibitory effect of chitosan and quaternized chitosan against *Shigella flexneri 2a* and *Salmonella typhimurium* is shown in table 4.1. Results revealed that higher concentration of chitosan was required to have inhibitory effect against the selected water borne pathogens. However, in the case of quaternized chitosan with permanent positive charge, it was found that lower concentration i.e. 50 to 200 µg/mL also had pronounced effect against both the bacterial strains.

Table 4.1 Antibacterial activity and Zone of inhibition of chitosan and TMC (mm)

Test organism	Zone of inhibition diameter (mm)											
	TMC concentration (µg/mL)						Chitosan concentration (µg/mL)					
	50	100	200	300	400	500	100	300	500	700	900	1000
Salmonella	11	14	16	19	22	24	N.D	N.D	14	17	20	22
Shigella	N.D	10	13	15	17	20	N.D	N.D	12	16	18	19

The larger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial. The major disadvantages of this method is that it is unable to generate the

MIC value (i.e., not quantitative). Therefore this method is not always reliable for determining the antimicrobial activity of natural antimicrobials since the polarity of the natural compounds can affect the diffusion of compounds onto the culture medium. Compounds with less polarity diffused slower than more polar ones (Moreno *et al.*, 2006). Due to these concerns, disk diffusion may not be a suitable one to determine the antimicrobial activity of natural compounds. The method was considered here in order to screen the antibacterial activity of chitosan and TMC against the two major water borne pathogens as in many previous studies, disk diffusion was used to determine the antimicrobial activities of chitosan (Kulkarni *et al.*, 2005; Coma *et al.*, 2006; Kim & Kim, 2007); also chitosan was reported to be effective against bacteria, yeast, and fungi without mentioning MIC values. For these reasons, the MIC determination was carried out through broth dilution assay.

The results were found to be consistent with previous studies, which reported higher bactericidal effect of chitosan for Gram-positive bacteria. The tested Gram-negative bacteria (*Salmonella* and *Shigella*) were insensitive to the antimicrobial activity of chitosan, with concentrations values of <300 μg/ml; on the other hand, strains were much more sensitive, with values as low as 50-100 μg/ml of Quaternized derivative. These results were found to be in good agreement with the previous studies done on the antibacterial activity of chitosan and it derivatives which indicated that the derivatives of chitosan with cationic charge, especially ammonium salt, exhibit particularly high antibacterial activity than that of chitosan (Belalia *et al*., 2008). They have also indicated that the hydrophobicity, cationic charge of the substituent, and the flexible movement of the alkyl chain introduced to chitosan strongly affected the antibacterial activity of the chitosan derivatives against many bacterial strains specifically *S. aureus* and *E. coli* (Kim *et al*., 2002). After the quaternization, the chitosan became water-

soluble poly-electrolyte with a high charge density. Jia et. al (2001) suggested that the target site of such quaternized chitosan is the negatively charged cell wall of bacteria.

4.1.2 MIC Determination

Figure 4.2 shows the minimum inhibitory concentration of TMC required to kill selected water-borne pathogens effectively. *Salmonella typhimurium* ATCC 25315 was found to be much sensitive to the TMC as it requires the minimum dose of it (300 μg/mL), followed by *Shigella flexneri* 2a, which have the MIC value of 400 μg/mL.

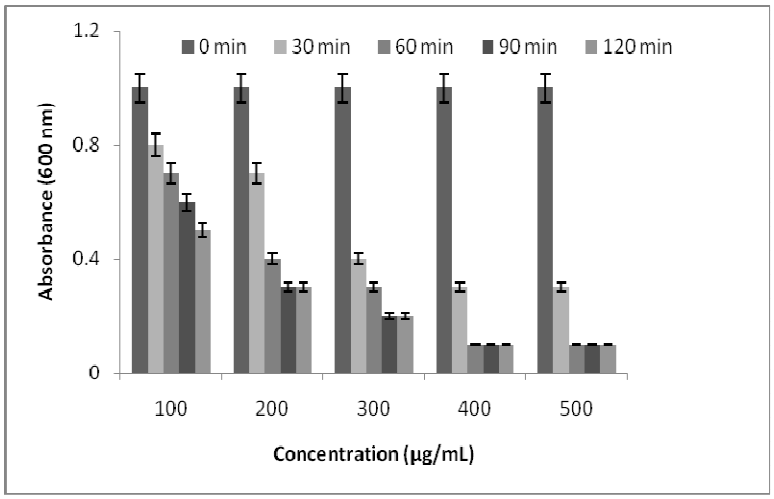


Fig. 4.1 (A) Optical density of *Salmonella* cells at different concentration exposed at various time period

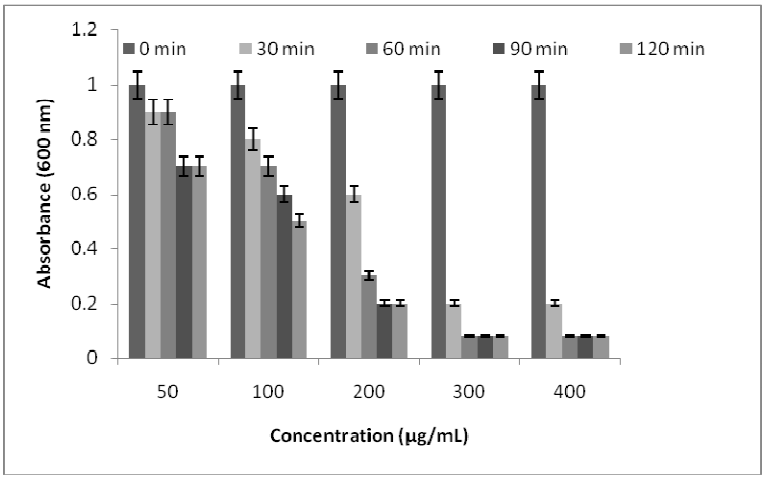
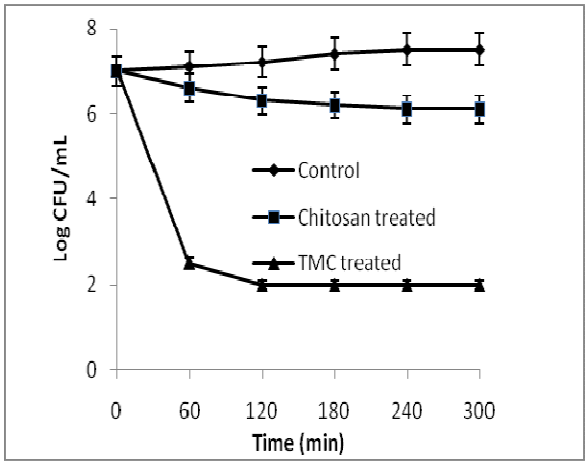


Fig. No. 4.1 (B) Optical density of *Shigella* cells at different concentration exposed at various time period

The antimicrobial activity of chitosan and TMC was also investigated by means of killing assays, conducted as described earlier. The kinetics of chitosan's and TMCs antimicrobial activity against the indicator strains, *Salmonella and Shigella* (Figure 4.3, A and B, respectively) was carried out.

A



B

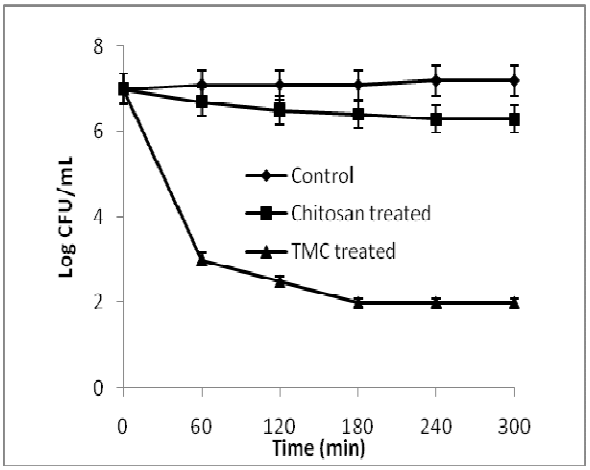


Fig 4.2 Killing kinetics of indicator strains in presence of chitosan and quaternized chitosan

Approximately 10^7 CFU/ml of *Salmonella typhimurium* (A) and *Shigella flexneri 2a* (B), were incubated with chitosan concentrations, equivalent to MIC and TMC concentrations, equivalent to MIC, incubated with shaking at 37°C. Surviving counts were estimated and plotted as a growth curve.

The results revealed the dose dependent inhibitory effect of quaternized chitosan against both the bacterial strains. In contrast to the bioactivity of chitosan, the inhibitory activity of TMC was maintained during the incubation time. After quaternization, the chitosan became a water-soluble polyelectrolyte, with a permanent cationic charge density. Jia *et al*., 2001 also found that the antibacterial activity of quaternized chitosan against *E. coli* was stronger than that of chitosan. This different behaviour could also be due to the lower polymerization degree of N,N,N-trimethylchitosan compared to the starting polymer. As already mentioned, the quaternization led to a reduction in chitosan molecular weight due to temperature and alkaline synthesis conditions (Runarsson *et al*., 2007). N,N,N-trimethylchitosan could then penetrate through the cellular membrane of bacteria and act on the intercellular material, leading to an improvement of its antibacterial action. Jia *et al*. (2001) reported the effect of equally trimethylated N,N,N-trimethylchitosan derivatives with different molecular weights on *Escherichia coli*, where the low molecular weight derivatives were found to be most effective. Tokura *et al*. (1997) suggested that the small oligomers of chitosan serve as nutrients for bacteria, whereas the 32 higher oligomers are toxic by virtue of their charge-mediated adhesion to the cell membrane, which in turn prevents the uptake of nutrients through the cell wall.

It has been reported that the target site of cationic disinfectants is the cytoplasmic membranes of the microbes (Franklin *et al*., 1981). The main constituents of the cytoplasmic membrane are membrane proteins and phospholipids. The phospholipids of bacteria are phosphoglycerides that have both a hydrophilic and a hydrophobic end (Costerton *et al*., 1975). Thus, quaternized chitosan derivatives with a alkyl group would be expected to strongly interact with cytoplasmic membranes due to the hydrophobic affinity between the introduced alkyl group and the phospholipids, leading to a higher antibacterial activity. Therefore, it was reported that the hydrophobic property due to the introduced alkyl group was an important factor in enhancing the antibacterial activity against *S. aureus* (Kim *et al*., 2002).

The inhibitory activity of TMC towards Gram-negative bacteria can be considered in terms of its chemical and structural properties. As a polymeric macromolecule, TMC is unable to pass the outer membrane of Gram-negative bacteria, since this membrane functions

as an efficient outer permeability barrier against macromolecules (Nikaido, 1996). Therefore, direct access to the intracellular parts of the cells by TMC is unlikely. A key feature of TMC is its positive charge of the amino group at C-2. This creates a polycationic structure, which can be expected to interact with the predominantly anionic components lipopolysaccharides, proteins of the Gram-negative surface (Je *et al*., 2006).

4.2 Development of Variants

At sublethal dose of biopolymer there was emergence of colonies of slow-growing subpopulations of *Shigella* and *Salmonella* strains that are generally more resistant to the action of antimicrobials than their parent strain. There must be certain intrinsic metabolic characteristics of these survivor colonies which contribute to the increased resistance against biopolymers

resulting in decreased metabolism, leading to slow growth and their reduced uptake of Quaternized biopolymer.

Results obtained revealed that at the MIC values of TMC, the number of surviving cells was drastically reduced within 3 h. The sublethal dose of TMC measured against both the bacterial strains was found as 270 µg/mL and 380 µg/mL for *Salmonella* and *Shigella*, respectively. These survivors are considered as variants here, and they constitute the slow-growing subpopulations of *Salmonella* and Shigella *strains.* These variants are generally more resistant to the action of antimicrobials than their parent strain (Rafat *et al* 2008).

The effect of TMC (at MIC value) on the growth of *Salmonella* and *Shigella* is depicted in Fig. 4.3.

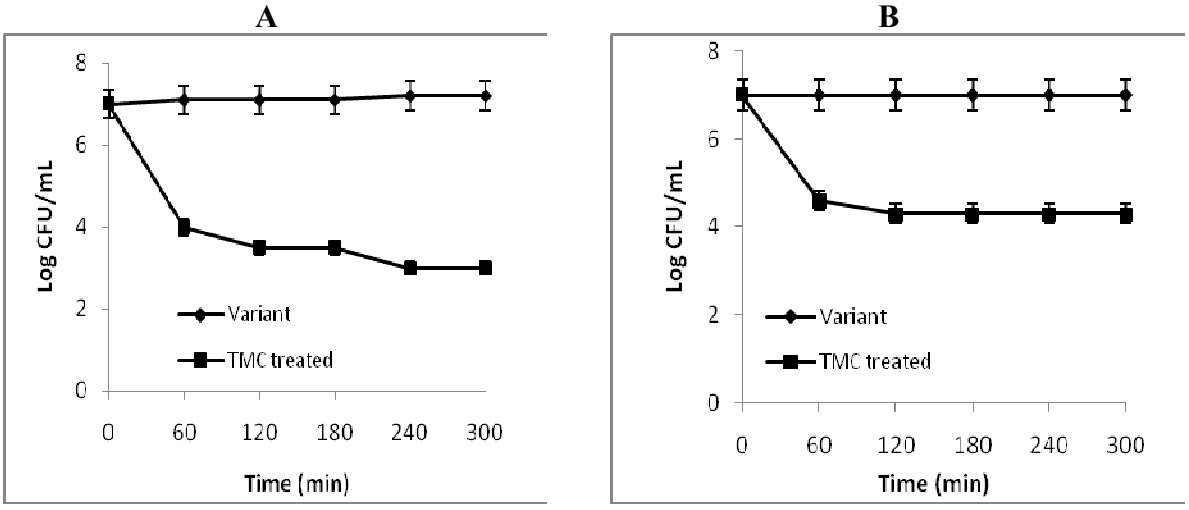


Figure 4.3 Killing kinetics of Variant types of *Salmonella* (A) and *Shigella* (B)

In the earlier studies with other gram-negative bacterial strains, it was found that their intrinsic metabolic characteristics contributed to the increased resistance to cell wall-active antibiotics and aminoglycosides, as well as cationic peptides. The reason behind the slow growth of variants may be the decreased metabolism, and their reduced uptake of TMC. The metabolic alterations taking place in the bacterial cells can be explained by considering the mode of action of the antibacterial chitosan on wild type as well as variant types of *Salmonella* and *Shigella* strains.

4.3 Effect of Antimicrobial chitosan on Bacterial Pathogens

4.3.1 Bacterial cell surface charge determination

The cell surface charge was determined by particle micro electrophoresis using a Zetamaster Particle Electrophoresis Analyser (UK) in order to measure the zeta potential. The surface charge of cells is often determined as its zeta potential. Table 4.2 shows the values of zeta potential of bacterial cells. In both the cases, there was a notable change in the charge between the treated and control strains. Cell surface charge can be accessed on the basis of zeta potential, which is the potential of the interfacial region between the bacterial surface and the aqueous environment. One of the primary existing methods to characterize cell surface charge involves microelectrophoresis. This method requires measurement of the rate of migration of suspended cells within an applied electrical field at a fixed temperature pH and ionic strength of media.

Cell surface charge was found to be high in both the bacterial strains suggesting the interaction between the cationic polymer and gram-negative cell wall.

Table 4.2 Zeta potential values after and before treatment with TMC

Bacteria	Before Treatment	After Treatment
Salmonella	-12.5	-9.99
Shigella	-16.1	-7.59

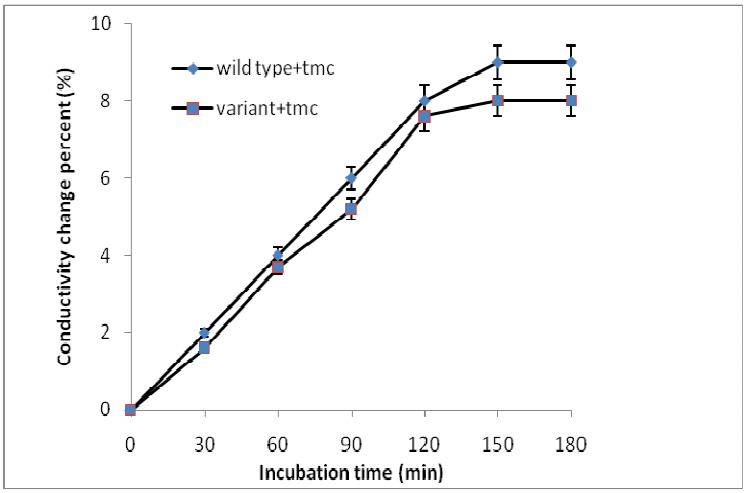
4.3.2 Leakage studies

In terms of susceptibility to antimicrobial agents, the cytoplasmic membrane, which regulates the transfer of solutes and metabolites in and out of the cell cytoplasm, is often considered as one of the major target sites (Helander *et al*.2001). Damage to the membrane can take several forms: (i) physical disruption of the membrane; (ii) dissipation of the proton motive force (PMF) and (iii) inhibition of membrane-associated enzyme activities (Je *et al*. 2006). Leakage is best considered as a measure of the disruption of the cell permeability barrier, therefore particular attention was paid in this study to assess the effect of chitosan on membrane integrity, using several assays which help detect the leakage of intracellular components from whole cells.

4.3.2.1 Electric conductivity assay

Bacterial suspensions (wild and variant type) were treated with TMC in their log phase. Fig. 4.4 depicted the increase in conductivity of bacterial cells treated with quaternized biopolymer, which suggested the increase in permeability of cell membrane.

A



B

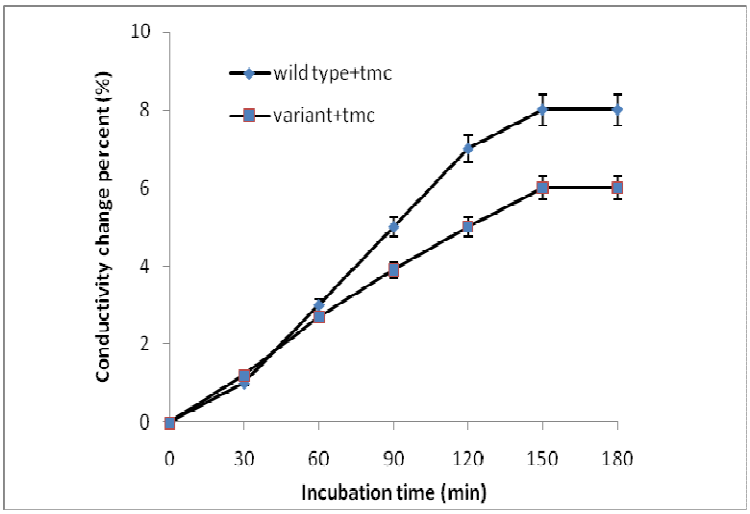


Fig. 4.4 Electric conductivity of cell suspensions for Quaternized chitosan treated (A) *Salmonella typhimurium* and (B) *Shigella flexneri* (wild and variant)

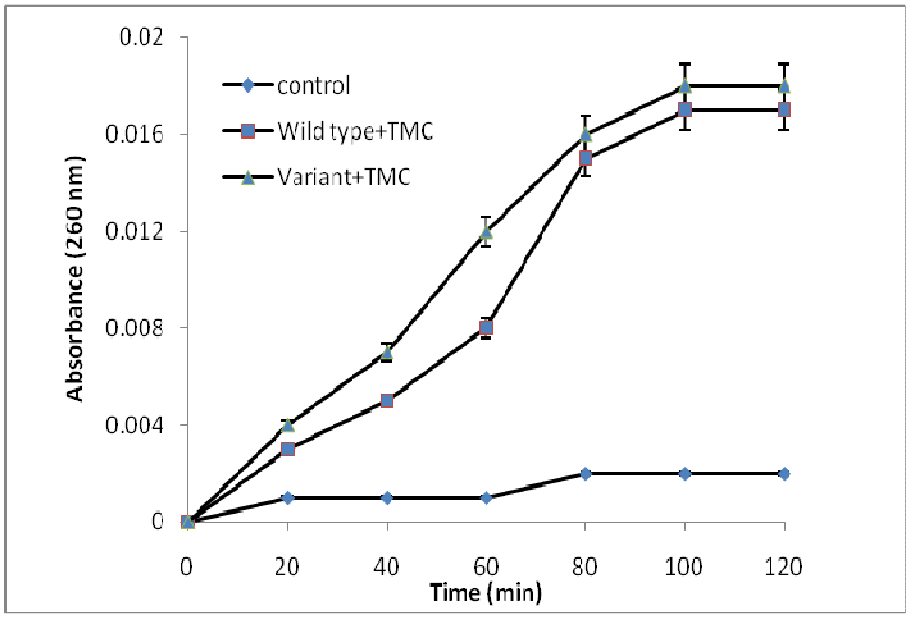
The electric conductivity of the cell suspensions for *Salmonella* and *Shigella* cells showed a time-dependent increase. It was found that the electric conductivity of suspensions for wild type *Salmonella* cells treated with TMC began to increase for the first 30 minutes, increased till 60 minutes and after that, and stabilized after this with no significant increase. In the case of variant type cells, same pattern was observed in the beginning, but the conductance remains to

increase within 2 hours. The effect of TMC on the membrane permeability of *Salmonella* was greater on the survivors than the normal bacterial cells treated with the MIC value of TMC.

In the case of *Shigella* cells treated with TMC (at MIC value) and survivor cells, it was found that increasing pattern of electric conductivity was similar.

4.3.2.2 Leakage of UV-absorbing materials

To test whether other larger molecules would be able to escape from cells treated with TMC, we measured the absorbance, at 260 nm, of cell-free supernatants of bacterial suspensions treated with TMC, in order to detect the leakage of UVabsorbing substances, likely representing nucleotide and coenzyme pools. Treatment of *Salmonella* with TMC resulted in a time-dependent, gradual leakage of UV-absorbing substances from bacterial cells, followed by a plateau up to 2 hours (Figure 4.5). Chemical and electrophoretic analyses of cell-free supernatants of chitosan-treated cell suspensions, using both conventional protein determination and SDS-PAGE, showed that interaction of chitosan with *Salmonella* either involved no release of proteins, or their amount was too small to be detected.



B

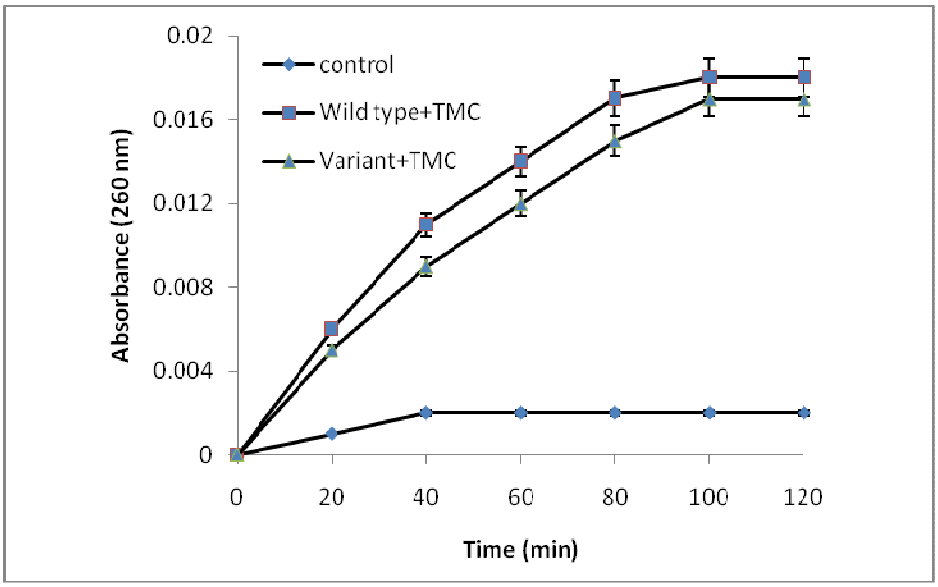


Fig 4.5 Time course for leakage of cellular components from *Salmonella* (A) and *Shigella* (B) (wild and variant type) upon exposure to TMC.

Leakage of UVabsorbing cellular components from *Salmonella* cells as a function of time. 260 nm materials were measured in the supernatant of cultures growing in BHI at 37°C and treated with TMC.

Attempts to detect protein leakage upon TMC treatment failed in the case of *Shigella flexneri,* indicating that either no proteins leaked out of the cells or their amount was too small to be detected. The cytoplasmic cell membrane undoubtedly is the target for many antimicrobial agents. When antimicrobial agents interact with bacterial membranes, it causes frequently fundamental changes in bacterial membrane and function (Je *et al*. 2006). If bacterial membrane became compromised, low molecular weight species, leach out first, and followed by nucleotides such as DNA, RNA and other materials. The release of these intracellular components is evidenced by strong UV absorption at 260 nm - an indication of membrane damage (Raafat *et al* 2008).

In similar experiments, Young *et al*., 1982, found that chitosan treatment greatly increased the membrane permeability of suspension-cultured plant cells, reflected by leakage of electrolytes and UV-absorbing materials from *Phaseolus vulgaris* cells. The low amount of soluble protein found in the medium after treatment with a chitosan concentration as high as 500 µg/ml was explained by binding of proteins to chitosan molecules attached to insoluble cell material or their precipitation by chitosan. In summary, the leakage experiments uniformly indicated that chitosan efficiently permeabilized the plasma membranes of staphylococci for small cellular constituents. These data were in fair agreement with previous reports that described chitosan as a membrane-perturbing compound (Helander *et al* 2001).

4.3.3 Leakage of Enzymes:

a. β-galactosidase activity

The inner layer of Gram-negative bacteria consists of phosphatidyl glycerol and cardiolipin .The cytoplasmic β-galactosidase is released as a consequence of change in inner membrane permeability ((Ibrahim et al, 2000). As depicted in Fig. 4.6, when wild and variant

type *Shigella* cells were treated with TMC at MIC concentration, a lag time of about 10 min was followed by a progressive release of the cytoplasmic β-galactosidase for up to 120 min to reach a steady state.

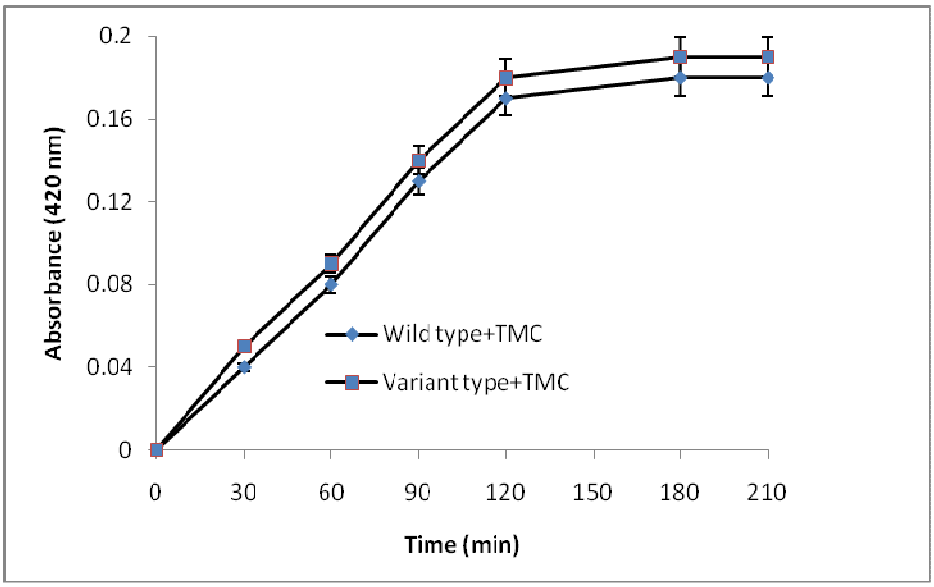


Fig. 4.6 Release of β-galactosidase enzyme withe time in *Shigella flexneri* (wild and variant) cells treated with TMC

These results indicated that the release of cytoplasmic β-galactosidase caused by TMC was time- and dose-dependent, which was coincident with the results reported by Liu, Du, Wang, and Sun (2004) and Xing et al. (2009).

The TMC interacts with the highly negatively charged O specific oligosaccharide units of the Outer membrane (OM) consisting of the anionic LPS. Destabilization of the OM is necessary to gain access to the IM. The Inner membrane (IM) of Gram-negative bacteria is composed of anionic lipids like phosphatidyl glycerol (PG) and cardiolipin which would favour the

association of cationic molecules. Therefore, TMC bind the anionic inner membrane through electrostatic interactions.

Salmonella typhimurium does not produce alkaline phosphatase, nor β-galactosidase enzyme (Schlesinger et al., 1968). Hence, the positive results were not obtained in the case of *Salmonella*, neither in wild nor in variant type.

b. ALP AND G-6-PDH enzyme assay

Figure 4.7 shows the alkaline phosphatase enzyme released from the bacterial membrane of *Shigella* (wild and variant type) with time.

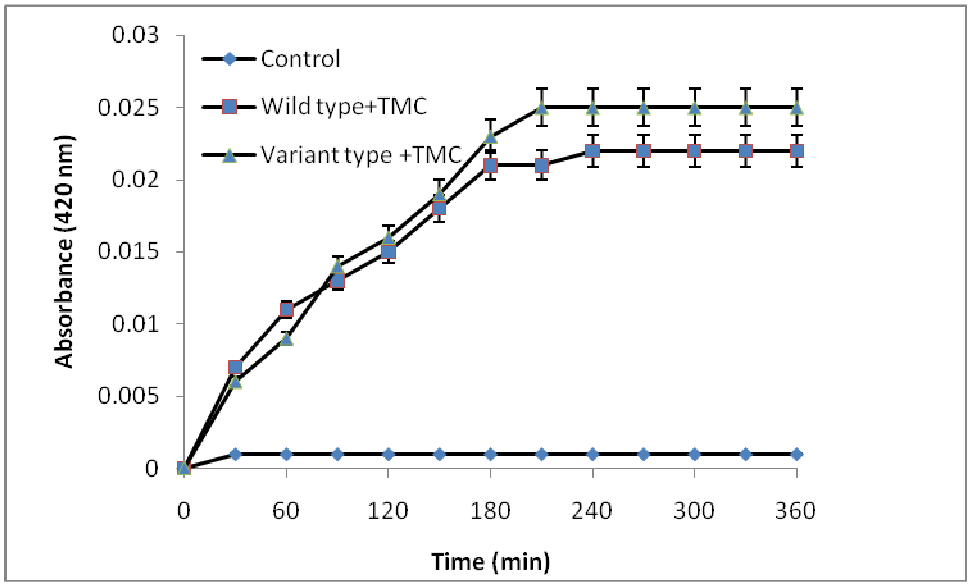


Fig. 4.7 Alkaline phosphatase activity in *Shigella* (wild and variant) cells with respect to time

Salmonella does not make alkaline phosphatase and thus would not be expected to have a regulatory gene that specifically controls the synthesis of alkaline phosphatase enzyme (Yagil and Harmoni, 1976). Results obtained in the case of *Shigella flexneri* cells treated with quaternized chitosan, revealed that the units of average release for ALP was higher in variant type cells, as compared to the wild type. The control, which consisted of bacteria solution, showed zero units of the enzyme released. Further, the release of enzyme reached a plateau in 3 h in both the cell types.

Alkaline phosphatase has been shown to be associated with cell walls of a number of gram-negative bacteria (Schlesinger *et al*.1968). It has been established that alkaline phosphatase is associated with a structural cell wall component which is present both inside and outside the double-track layer of the cell wall, and further studies have indicated that this component is lipopolysaccharide. Cheng et al.()have concluded that this enzyme is evenly distributed in the periplasmic space or on the cell surface of *Pseudomonas aeruginosa*, and an even distribution of alkaline phosphatase and of 5'-nucleotidase has also been noted in *Escherichia coli*.

Figure 4.8 (A) depicts the increase in Glucose-6-phophate dehydrogenase activity within 6 hours. Results obtained showed release of enzyme reached a plateau in 5 h in both, wild as well as variant type of *Salmonella* cells treated with quaternized chitosan.

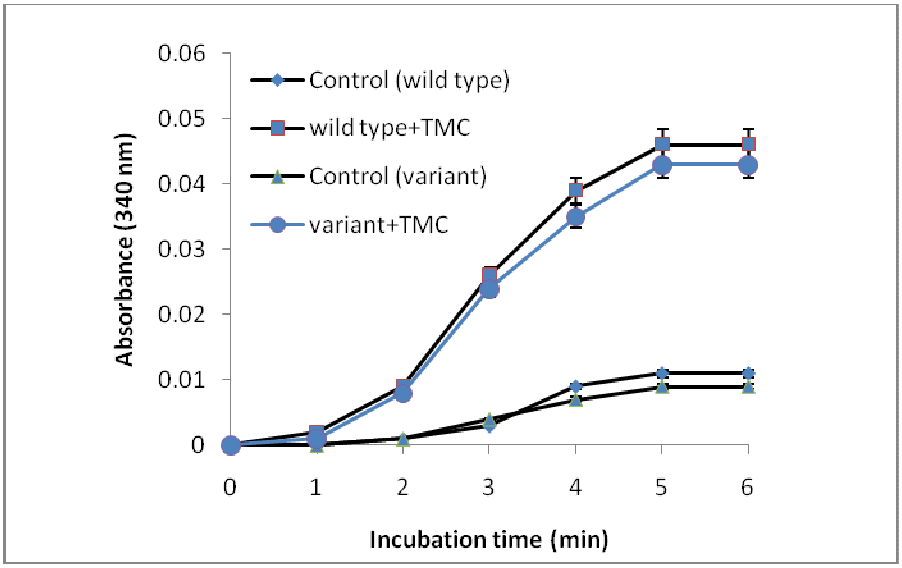


Fig. 4.8 (A) Glucose-6-phosphate dehydrogenase activity in *Salmonella typhimurium* (wild and variant) cells with respect to time

Glucose 6-phosphate dehydrogenase is an intracellular enzyme that catalyzes the NAD^+- or $NADP^+$-dependent conversion of glucose-6-phosphate to 6-phosphogluconate. Lundberg et. al., (1999) examined the function of G6PD in *Salmonella typhimurium*, a pathogenic bacterium specifically adapted to survival within phagocytic cells, and represented the first demonstration of an essential role of G6PD in microbial pathogenesis. Both reactive oxygen and nitrogen intermediates produced by phagocytic cells appear to contribute to host defence against *Salmonella* (Lundberg *et al*.,2001).

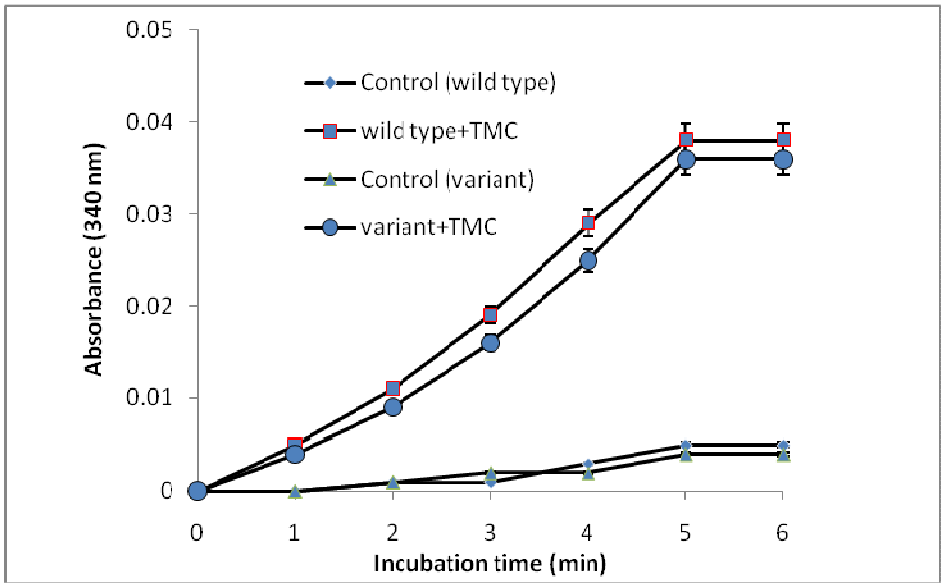


Fig. 4.8 (B) Glucose-6-phosphate dehydrogenase activity in *Shigella flexneri* (wild and variant) cells with respect to time

G6PDH has been used previously as cytoplasmic marker in Escherichia coli (Calcott & Macleod 1975), and its leakage will indicate disruption of membrane integrity. Cell disruption by Tris apparently exposes G6PDH to the substrate, and the appearance of G6PDH activity should therefore indicate cell disruption. As shown in Figure 4.8 (B), significant release of glucose-6 phosphate enzyme has been observed within 5 hours.

Malamy and Horecker (1964) reported that ALP was an extracellular enzyme, while G6PDH was found in the cell membrane. The locations of these two enzymes clearly provided the answer as to why ALP was the first molecule to be released into the medium, followed by G6PDH.

4.4 Protein profiling of Antibacterial chitosan treated bacterial cells by SDS-PAGE

Determination of Protein content

Concentration of protein was estimated by method of Lowry *et al.,* 1951, with Bouvine Serum Albumin (BSA) as standard. The protein concentration was found to be more in wild type in comparison to its variant in *Salmonella typhimurium*. However, there was no significant difference in case of *Shigella flexneri*.

Table 4.3 Protein content (mg/mL) of both the wild type strains and their respective variants

Bacterial strains	Wild type	Variant type
Salmonella typhimurium	0.8	0.9
Shigella flexneri	0.6	0.58

As shown in Fig. 4.9, the normal cell protein electrophoresis bands of *Salmonella typhimurium* (wild type as well as variant type) appeared strong and clear. After the wild type cells of *Salmonella typhimurium* were treated with TMC for 60 minutes, the bands of all-molecular weight proteins for *Salmonella* appeared obviously shallow, even disappearing altogether. The protein content reduced after treatment with TMC. On the other hand, protein contents in the cell-free supernatant increased compared with control culture supernatants. These results suggested that TMC decreased the content of cellular soluble proteins by permeating and disrupting the cell membrane.

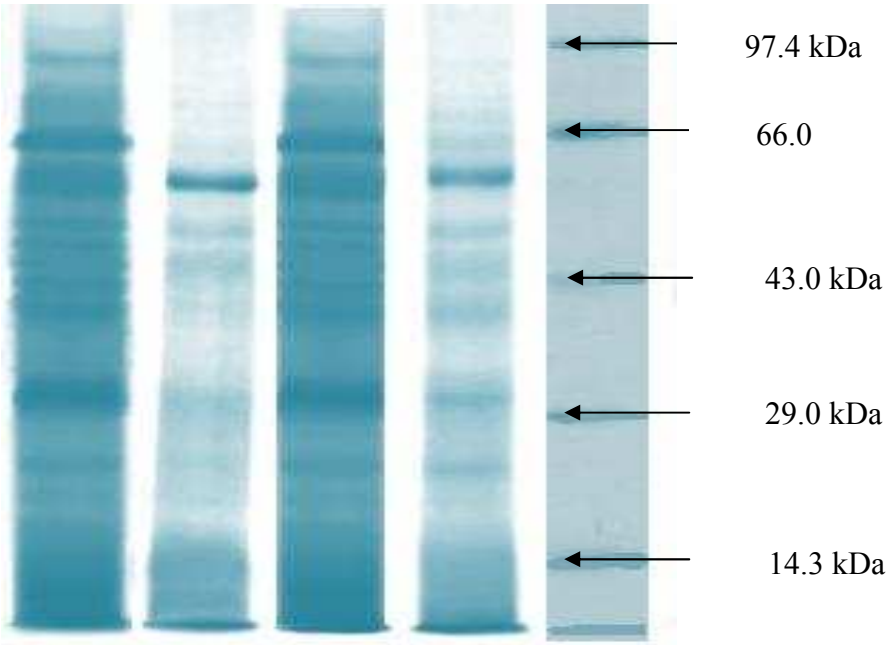


Figure 4.9 SDS PAGE of total cellular protein of *Salmonella typhimurium* (wild and variant type) after treatment with TMC

Lane 1- Total cell protein of *Salmonella typhimurium* (wild type)

Lane 2- Total cell protein of *Salmonella typhimurium* (wild type) treated with TMC

Lane 3- Total cell protein of *Salmonella typhimurium* (variant type)

Lane 4- Total cell protein of *Salmonella typhimurium* (variant type) treated with TMC

Lane 5- A 97.4 kDa protein marker

In the case of *Shigella flexneri* 2a, the protein contents of TMC treated wild as well as variant type cells remained same even after increasing the incubation time (30 to 180 minutes). However, in the *Shigella* cell*s*, treated with QAC, the ratio 260/280 in the cell free supernatant comes

out to be less than 1.5. This shows either no leakage of proteins from the cells or their amount was too small to be detected. The difference in the protein profiling of *Shigella* and *Salmonella,* may be due to their differences in the structures of cell membranes. Cui et al. (2009) proposed that chitosan derivatives probably inhibited protein synthesis or control gene expression, but sufficient data to support the statement has not been presented. However, significant effect of antibacterial biopolymer on membrane integrity of Gram-negative bacteria has been observed, but further studies on mechanism of action are required.

CONCLUSION

CONCLUSION

The results reported here demonstrated that Quaternized chitosan (TMC) is endowed with potential antibacterial activity against two major gram-negative water borne pathogens, *viz*. *Salmonella typhimurium* and *Shigella flexneri*. The experimental data presented in this study provide evidence that TMC disrupts the barrier properties of the outer membrane of Gram-negative bacteria, due to the interactions of this polycation with cell membranes, consequently

increasing the membrane permeability. It displayed a dose-dependent growth-inhibitory activity, sub-lethal concentration was determined and variants of both the bacterial strains were developed.

In the current study, a systematic evaluation of TMC's mode of action was carried out in an attempt to understand the underlying molecular mechanisms of this activity, bearing in mind that such mechanisms most probably differ among various species. The results obtained indicated that TMC had a stronger effect on the cell membrane of *Salmonella typhimurium* than on *Shigella flexneri* 2a. Significant leakage of UV absorbing materials, intracellular and extracellular enzyme has been depicted. The significant effect of quaternized biopolymer on the protein content of wild as well as variants of *Salmonella typhimurium* was observed. In general the complex mechanisms by which these processes are coupled or interrelated have not been successfully ascertained. Further studies should aim at clarifying the molecular details of the underlying mechanisms and their relevance to the antibacterial activity of quaternized biopolymer, particular with regard to the bacterial resistance mechanism against this antibacterial polymer.

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ANNEXURE

Culture medium	Source / Composition
Brain-heart infusion medium (BHI broth)	Calf brain infusion solids (12.5), beef heart, solids (5.0), proteose, peptone(10.0), glucose (2.0), sodium

chloride (5.0), di-sodium phosphate (2.5)

Bacterial strains	**Media**	**Culture conditions**
Salmonella typhimurium ATCC 25315	BHI broth	37°C with aeration
Shigella flexneri 2a	BHI broth	37°C with aeration

β-Galactosidase Activity Assay

i) Z buffer, per 50 mL:

- 0.80g $Na_2HPO_4.7H_2O$ (0.06M)
- 0.28g $NaH_2PO_4.H_2O$ (0.04M)
- 0.5 mL 1M KCl (0.01M)
- 0.05 mL 1M $MgSO_4$ (0.001M)
- 0.135 mL b -mercaptoethanol (BME) (0.05M)
- bring to approximately 40 mL with H_2O, dissolve all the salts

The pH was adjusted to 7.0 and solution was stored 4^0C

ii) ONPG should be dissolved fresh each day. Dissolve the ONPG to a final concentration of 4mg/ml in 0.1M Phoshphate buffer pH 7.0

iii) Phoshphate buffer (pH 7.0) per 100ml:

1.61 g $Na_2HPO_4.\ 7H_2O$(0.06M)

0.55 g $Na_2HPO_4.\ .H_2O$ (0.04M)

Glucose-6-Phosphate Dehydrogenase assay

i) 250 mM Glycylglycine Buffer, pH 7.4 at 25°C Prepared 100 ml in deionized water using Glycineglycine

ii) 60 mM D-Glucose 6-Phosphate Solution (G 6-P) -Prepared 2 ml in deionized water using D- Glucose 6-Phosphate

iii) 20 mM Nicotinamide Adenine Dinucleotide Phosphate Solution (NADP) -Prepared 2 ml in deionized water using Nicotinamide Adenine Dinucleotide phosphate).

iv) 300 mM Magnesium Chloride Solution ($MgCl_2$) Prepared 2 ml in deionized water using Magnesium Chloride

v) Glucose-6-Phosphate Dehydrogenase Enzyme Solution (Immediately before use, prepared a solution containing . 0.3 - 0.6 unit/ml of Glucose-6-Phosphate Dehydrogenase in cold Reagent (i).

Alkaline Phosphatase assay

The assay mixture (1 ml)

i) 1 **M** Tris buffer, pH 8

ii) 1 µmole of p-nitrophenylphosphate was dissolved in Reagent (i)

Protein determination

Solutions	Composition
Solution A	3% Na_2CO_3 in 0.1 M NaOH
Solution B	2% $CuSO_4 \cdot 5H_2O$
Solution C	4% K-Na-Tartarate
Alkaline copper-reagent	2% solution B, 2% solution C, 96% solution A
Bovine serum albumin (BSA)	1 mg/ml phosphate buffer

SDS-PAGE

Solutions	Compositions
Acrylamide	30% [wt/vol] Acrylamide-Bisacrylamide-solution (19

:1)

Solution A	3 M Tris-HCl; pH 8.5
Solution D	0.96 M Tris, 0.8 % SDS [wt/vol]
Ammonium persulfate (APS)	10% [wt/vol] (freshly prepared)
SDS	10% [wt/vol] (freshly prepared)
sample buffer	2.5 ml 0.5 M Tris-HCl (pH 6.8), 2 ml 50% glycerol [wt/vol], 0.8 ml β-mercaptoethanol ,0.3g bromophenol blue [wt/vol].
Electrophoresis buffer	25 mM Tris, 192 mM glycine,10% [wt/vol] SDS; pH 8.5
Coomassie-staining solution	0.3g coomassie brilliant blue R-250, 450 ml methanol, 20ml glacial acetic acid, add100 ml d. water
Destaining solution	300 ml methanol, 100ml glacial acetic acid, add 1 l d. water