COMPARATIVE STUDY ON THE BASIS OF ANTIBACTERIAL ACTIVITY OF COMMERCIAL GRADE AND LOCALLY PRODUCED CHITOSAN FROM SHRIMP (*PENAEUS MONODON*) SHELL WASTE

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A Thesis Submitted
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SEPTEMBER, 2013

DEDICATED TO MY

BELOVED PARENTS

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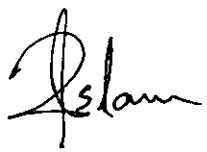
DECLARATION

This thesis description is the original research work of the authors, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the written concurrence of the concerned supervisor. Legal action would be taken in any case of violation regarding the declaration.



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The author

ABSTRACT

The antibacterial activity of chemically deacetylated chitosan, produced from shrimp (*Penaeus monodon*) shell was tested against gram negative (*Salmonella typhi, Escherichia coli, Shigella dysenteriae*) and gram positive (*staphylococcus aureus*) bacteria. An analytical grade of commercial chitosan was included for comparison. The commercial grade chitosan possesses higher antibacterial activity against both types of bacteria, while chitosan prepared in the present study possess moderate activity. For commercial grade chitosan solution, the zones of inhibition were recorded 15.21±0.707mm for *S. typhi,* 16.21±0.707mm for *E. coli,* 15±0mm for *S. dysenteriae,* and 12.42±1.414mm for *S. aureus.* On the other hand it was 13.01±0.71, 13.06±0.35, 14.21±0.71, and 10.10±0.35mm for *S. typhi, E. coli, S. dysenteriae,* and *S. aureus* respectively for produced chitosan. The IC_{50} and MICs value of produced chitosan was quite higher than the commercial grade chitosan. For IC_{50} value, commercial grade had shown prominent results over produced chitosan. The lowest IC_{50} value of 0.433mg/ml was seen against gram negative (*E. coli)* bacteria for commercial grade whereas it was relatively higher, 1.12mg/ml for chitosan produced in lab. For gram positive (*S. aurius)* bacteria, IC_{50} value is quite higher (0.73 mg/ml for commertial grade and 1.2 mg/ml for laboratory grade chitosan) than other gram negative bacteria.The MIC was 5mg/ml and 3mg/ml against *E. coli* and *S. dysenteriae* respectively and MBC was 5mg/ml and 4mg/ml against *E. coli* and *S. dysenteriae* respectively. Whereas for gram positive *S. aureus* the MIC was 3mg/ml and MBC was 4mg/ml for commercial grade and MIC 4mg/ml and MBC 5mg/ml for lab grade chitosan.

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ABBREVIATION

Ch-	Chitosan
Cfu-	Colony forming unit
DD-	Degree of Deacetylation
E. coli-	*Escherichia coli*
IC_{50} -	Inhibitory Concentration 50
kDa-	Kilo Dalton
LD50-	Lethal Dosage 50
MBC -	Minimum Bactericidal Concentration
MIC -	Minimum Inhibitory Concentration
MW-	Molecular Weight
NA -	Nutrient agar
NB -	Nutrient broth
OD-	Optical Density
SD -	Standard Deviation
S. typhi-	*Salmonella typhi*
S. dysecteriae-	*Shigella dysenteriae*
S. aureus-	*Staphylococcus aureus*
Tet. -	Tetracycline

CHAPTER ONE

INTRODUCTION

INTRODUCTION

Chitin is a polysaccharide of animal origin found abundantly in nature and characterized by a fibrous structure. It forms the basis of the main constituent of the outer skeleton of insects and crustaceans like shrimp, crabs and lobster (Kumar *et al.*, 2005). According to Chen (1998) the chitin structure can be modified by removing the acetyl groups, which are bond to amine radicals in the C2 position on the glucan ring, by means of a chemical hydrolysis in concentrated alkaline solution at elevated temperature to produce a deacetylated form known as chitosan.

No *et al.*, (2002) stated that antibacterial activity of chitosan is effective in inhibiting growth of bacteria. The antimicrobial properties of chitosan depend on its molecular weight and the type of bacterium. For gram-positive bacteria, chitosan with 470 KDa was the most effective, except for *Lactbacillus sp.*, whereas for gram negative bacteria, chitosan with 1,106 KDa was effective. Chitosan generally showed stronger bactericidal effects for gram positive bacteria (*Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, and L. bulgaris*) than for gram-negative bacteria (*E.coli, Pseudomonas fluorescens, Salmonella typhymurium, and Vibrio parahaemolyticus*) in the presence of 0.1% chitosan. The antimicrobial activity of chitosan is described to be associated with molecular weight, degree of acetylation, concentration of chitosan and bacterial inoculum size was showed by Chen *et al.*, (1996) and Fernandes *et al.*, (2008). It is reported by Peter *et al.* (2008) that lower molecular weight chitosan is more effective against Gram-negative bacteria, whereas high molecular weight chitosan is effective against Gram-positive bacteria in atomic force microscopy of cell wall structure and nano indentation study.

In this research work it is tried to show the activity of commercial grade and chitosan produced from local shrimp against four pathogenic bacteria, one gram positive strain *staphylococcus aureus* and three gram negative strain *Escherichia coli, Shigella dysenteriae, Salmonella typhi.* These pathogenic bacteria are all around us contaminating our food, water readily, causing spoilage of perishable food items.

The bacterial effectiveness on gram-positive or gram-negative bacteria is however, somewhat controversial. It is stated by Coma *et al.*, Jeon *et al.*, No *et al.* (2001-03) and Dutta (2009) that chitosan generally showed stronger effects for gram-positive bacteria (e.g. *Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, L. bulgaris*, etc.) than for gram-negative bacteria (e.g. *E. coli, Pseudomonas fluorescens, Salmonella typhymurium, Vibrio parahaemolyticus,* etc.). Conversely, it has been demonstrated that hydrophilicity in gram-negative bacteria is significantly higher than in gram-positive bacteria, making them most sensitive to chitosan Chung *et al.*, (2004). The charge density on the cell surface is a determinant factor to establish the amount of adsorbed chitosan. More adsorbed chitosan would evidently result in greater changes in the structure and

in the permeability of the cell membrane. This would suggest that the antibacterial mode of action is dependent upon the host microorganism Masson *et al.* (2008).

Chitosan production can be for environment as shrimp waste is utilize to produce chitosan instead disposing the waste into the environment. As Bangladesh produce shrimp in large quantity, we can produce large amount of chitosan and can use that for various purposes. It can be used as food preservative, coating material, fertilizer, water purification agent etc. However in our locality we are lacking the analysis of various prospects of chitosan. In this context my research work is intended to fulfil the following objectives:

1. To study the antibacterial effect of chitosan.
2. To investigate the anti bacterial activities of chitosan produced from local shrimp shell waste.
3. To compare the antibacterial activity of produced chitosan with commercial chitosan.

CHAPTER TWO

LITERATURE REVIEW

LITERATURE REVIEW

2.1. Natural Antimicrobials

Traditional antimicrobials such as sorbate, benzoate, sulfite, etc. have been used as reliable preservatives to control microbial hazards in the food industry for decades (Raybaudi-Massilia *et al.*, 2009). However, these compounds do not satisfy the concept of "natural" or "healthy" foods that consumers are increasingly demanding. With consumers growing awareness and concerns regarding chemically synthesized preservatives (traditional antimicrobials), novel and safe natural antimicrobials targeting food pathogens with minimum adverse effects have attracted much more attention (Richard & Patel, 2005). On the other hand, natural antimicrobials can be used as a promising alternative of traditional antimicrobials for preserving foods such as in fresh cut fruit and fruit juices (Raybaudi-Massilia *et al.*, 2009).

Natural antimicrobials are derived from many sources, ranging from animal (chitosan, lysozyme, and lactoperoxidase) to plant (essential oils, aldehydes, esters, herbs and spices) and to microbial origin (nisin) (Tiwari *et al.*, 2009). Table 1 summarizes representative natural antimicrobials from different origins and their antimicrobial activities.

Regarding the development of natural antimicrobials from the animal origin, many researchers focused on the potential use of chitosan in food preservation. Currently, chitosan has been approved as functional food in some Asian countries, such as Japan and Korea in the last decade (Aranaz *et al.*, 2009). However, it has not been officially proclaimed as GRAS (Generally Recognized as Safe) substances by the U.S. Food and Drug Administration (Raafat & Sahl, 2009).

2.2. Chitin

The name chitin was first used by Bradconnot in 1811, which was derived from the Greek word chiton, meaning a coat of mail (Lower, 1984; Skaugrud & Sargent, 1990). Chitin, as the second most abundant natural polymers on Earth after cellulose (Shahidi *et al.*, 1999; Singla & Chawla, 2001; No *et al.*, 2007), distributes widely in nature and is mainly present as the structural component of crustacean shells (Raafat & Sahl, 2009). The principal sources of chitin are summarized in Table 2 (Felt *et al.*, 1998). Chitin is an insoluble linear mucopolysaccharide (Raafat & Sahl, 2009) (Fig.1) composed of 2-acetamido-2-deoxy- β-D-glucose (N-acetyl glucosamine) linked by a β (1→4) bonds (Kumar, 2000). It can be regarded as cellulose with hydroxyl group at C-2 position replaced by acetamino group (Suzuki, 2000).

Table 1. Representative Natural Antimicrobials and their Antimicrobial Activities

Origin	Antimicrobials	Spectrum of activity	Application	Reference
Animal	Chitosan	Bacteria, yeast and mold	Fruits, vegetables, meat, milk, and seafood	(No *et al.*, 2007)
	Lactoperoxidase	*S.* Enteritidis, *E. coli* O157:H7, *Shigella* spp.	Fruit and vegetable juices	(Touch *et al.*, 2004; Van Opstal *et al.*, 2006)
	Lysozyme	*L. monocytogenes, C. jejuni, B. cereus* and *S.* Typhimurium	Orange juice	(Liang *et al.*, 2002; Raybaudi-Massilia *et al.*, 2009)
Plant	Essential oils	*E. coli* O157:H7, *S. enterica*	Apple juice, Apple cider	(Friedman *et al.*, 2004; Liang *et al.*, 2006)
Microbial	Nisin	Only gram-positive bacteria, i. e. *L. monocytogenes, B. cereus, C.botulinum*	Fresh-cut water melon, orange juice	(Raybaudi-Massilia *et al.*, 2009)

Table 2. Principal Sources of Chitin

Organism		Chitin content (%)
Crustacean	Crab[a]	72.1
	Shrimp[a]	69.1
	Lobster[a]	69.8
Insects	True fly[a]	54.8
	Sulphur butterfly[a]	64.0
Fungi	*Aspergillus niger*[b]	42.0
	Mucor rouxii	44.5

[a]Organic weight of cuticle ; [b]Dry weight of the cell wall.

Chitin's immunogenicity is exceptionally low, in spite of the presence of nitrogen. Chitin can be degraded by chitinase. Because of its high insolubility in ordinary solvents, chitin cannot be isolated by the solvent extraction method. However, chitin is fairly stable in mild acidic and

basic conditions, and thus may be obtained as the residue after decomposition of other components by acid and alkali (Kurita, 2006).

2.3. Chitosan

Chitosan is a biomaterial, primarily produced from the alkaline deacetylation (40-50% NaOH) of chitin. Since this N-deacetylation is almost never complete, chitosan is considered as a partially N-deacetylated derivative of chitin (Kumar, 2000).

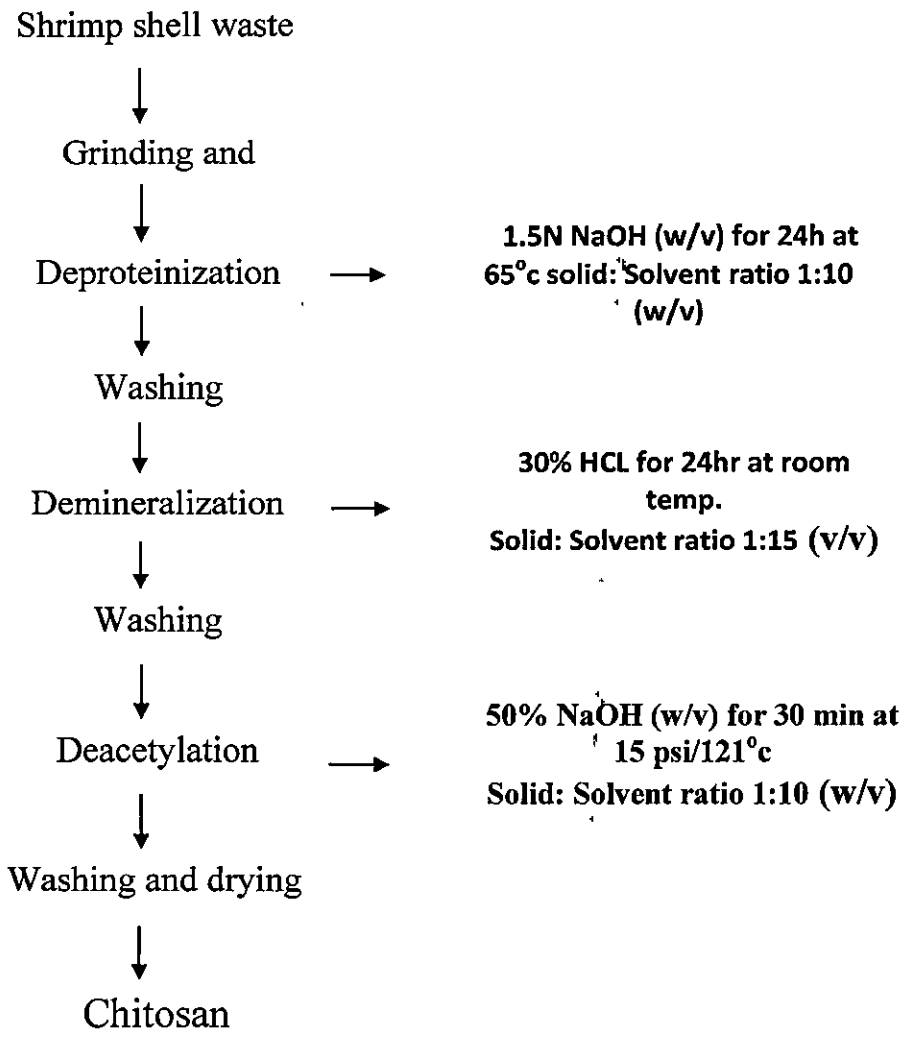

Figure 1: Shrimp shell Chitosan production (No and Meyers, 1995; Sultana. J, 2004)

Advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan (Rabea *et al.*, 2003). Structurally, chitosan is a high-molecular-weight linear heteropolysaccharide composed of a β (1→4) linked two monosaccharides, N-acetyl-D-glucosamine and D-glucosamine (Rãafat & Sahl, 2009) (Fig. 1). Chtiosan has three types of reactive functional groups, an amino group as well of both primary and secondary hydroxyl groups at the C-2, C-3, and C-6 positions, respectively (Furusaki *et al.*, 1996). The varied proportion of the two monosaccharides in chitosan contributes to different properties, such as degrees of deacetylation (DD; 75-95%), molecular weights (Mw; 50-2,000 kDa), viscosities, pKa values and so on (Singla & Chawla, 2001).

However, these properties can greatly influence its physicochemical characteristics and directly affect its application (Raafat & Sahl, 2009). In contrast to chitin's insolubility, the presence of free amine groups along the chitosan chain allows it to dissolve in diluted acids such as acetic acid, lactic acid, and formic acid due to the protonation of these groups, rendering the corresponding chitosan salt soluble. Therefore, there are important experimental variables that should be taken into account when working with chitosan solutions such as the nature of the salt counter-ion, DD, Mw, pH, ionic strength, and the addition of non-aqueous solvents (Aranaz *et al.*, 2009).

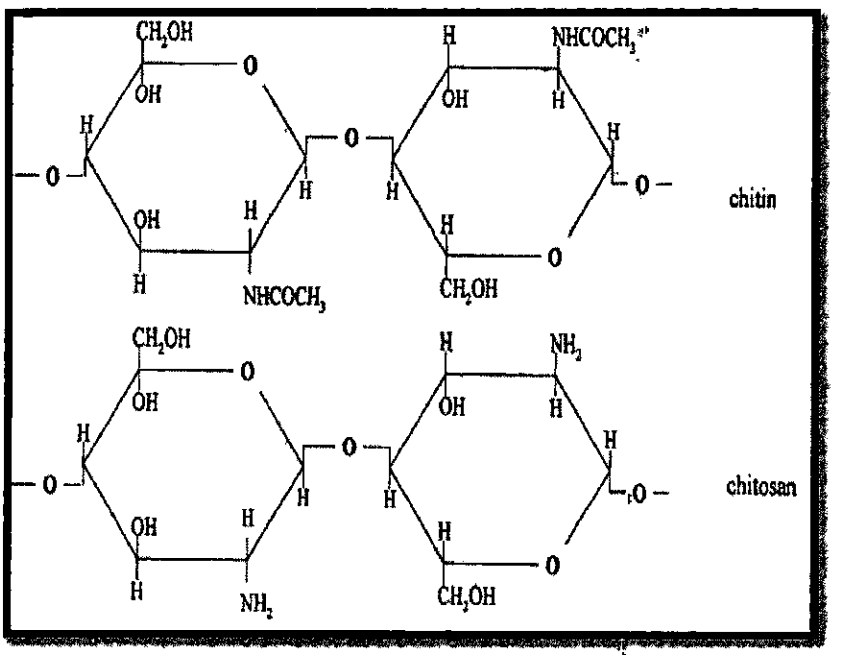

Figure 2: Structures of Chitin and Chitosan (Raafat & Sahl, 2009)

2.4. Biological Properties of Chitosan

Chitosan and its derivatives have attracted much commercial interest with regards to medical, pharmaceutical, and industrial applications due to the possession of several interesting properties: biodegradability, biocompatibility, and low toxicity (No *et al.*, 2007; Raafat & Sahl, 2009). Additionally, other properties such as analgesic, antitumor, hemostatic, hypocholesterolemic, antimicrobial, and antioxidant properties of chitosan have also been reported (Koide, 1998; Kumar, 2000; Kumar *et al.*, 2004).

2.4.1. Biodegradability

Traditionally, several methods can be used to produce chitosan oligomers, such as physical, chemical, and enzymatic methods. Physical methods including ozone treatment and ultraviolet radiation is preferred due to their accelerated degradation of chitosan (Yue *et al.*, 2009). Chitosan is absent from mammals but it can be degraded *in vitro* by several non-specific enzymes from a variety of sources, such as lysozymes, pepsin, papain, cellulase, pectinase, proteases, and lipases (Pantaleone *et al.*, 1992; Darmadji & Izumimoto, 1994; Yalpani & Pantaleon, 1994; Kumar *et al.*, 2005). Moreover, it also can be catalyzed by chitosanases (chitosan *N*-acetyl-glucosamino-hydrolases). The biodegradation of chitosan leads to the release of non-toxic oligosaccharides of variable lengths which can be subsequently incorporated into glycosaminoglycans and glycoproteins, to metabolic pathways or be excreted (Pangburn *et al.*, 1982).

Chitosan degradation kinetics seems to be inversely related to the degree of crystallinity that is controlled mainly by the DD value. Moreover, the distribution of acetyl groups also affects biodegradability since the absence of acetyl groups or their homogeneous distribution (random rather than block) results in very low rates of enzymatic degradation (Aiba, 1992; Francis *et al.*, 2000).

Finally, several studies investigated the relationship between degradation and the DD value. It seems that degradation rate increases while the DD value was decreased. (Hirano *et al.*, 1989; Sashiwa *et al.*, 1991; Kurita *et al.*, 2000). For example, Kofuji *et al.* (2005) investigated the enzymatic behaviors of various chitosans by observing changes in the viscosity of chitosan solution in the presence of lysozyme, and found chitosan with a low DD tended to be degraded more rapidly. However, other authors reported that differences in degradation are due to variations in the distribution of acetamide groups in the chitosan molecule (Sashiwa *et al.*, 1991; Aiba, 1992; Shigemasa *et al.*, 1994). This occurs due to differences in deacetylation conditions, which influences viscosity of the chitosan solution by changing the inter- or intra-molecular repulsion forces (Sashiwa *et al.*, 1991).Therefore, the biodegradation rate of chitosan cannot be estimated from the DD value alone.

2.4.2. Biocompatibility

Another attractive biological property of chitosan is its biocompatibility. For example, the function of chitosan is not affected by the host and it does not elicit any undesirable local or systemic effects. Chitosan is well tolerated by live tissues, including the skin, ocular membranes, as well as the nasal epithelium (Shigemasa & Minami, 1995). However, this property also depends on the characteristics of the sample (natural source, method of preparation, Mw, and DD, etc.).

2.4.3. Low Toxicity

Low toxicity is another attractive feature of chitosan compared with other natural polysaccharides. Chitosan has an LD50 of around 16 g/kg, very similar to that for salt and glucose by *in vivo* toxicity assays carried out on mice (Singla & Chawla, 2001). Nevertheless, people with shellfish allergy should be contraindicated. It is reported that toxicity of chitosan is dependent on the DD value. In a previous study, chitosans with DD values higher than 35% showed low toxicity, while a DD value under 35% caused dose-dependent toxicity (Aiba, 1992).

2.5. Economic Aspects and Regulatory Status

2.5.1. Economic Aspects

The commercial production of chitin and chitosan is mostly obtained from crustacean shells waste, such as shrimps, crabs, lobster, crawfish, etc. which is economically feasible and ecologically desirable because large amounts of shell wastes are available as a by-product of the seafood industry. Chitosan has been commercially produced in North America, India, Japan, Poland, Norway and Australia (Kumar, 2000; Raafat & Sahl, 2009).

Bangladesh is one of the major shrimp producing countries in the world. The annual production of shrimp is 134715 metric ton. Most of the shrimp (104517.23 metric ton) has been exported from Khulna division of Bangladesh. *Peneus monodon* (Bagda/ Black tiger) is widely cultivated shrimp species in Khulna. Around 49% *Peneus monodon is* cultivated in this region (Fishery statistical year book, 2007-2008). In Khulna there are 35 fish processing plants among 124 fish plants in Bangladesh (Quassem, *et al.,* 2003). Shrimp is normally export headless and often peeled off the outer shell, thus the waste generated comprises mainly of the shrimps head, shell & tail (Knorr, 1991) which ultimately dump in the open place as well as in the river and cause environment pollution.

It is necessary to ensure the total utilization of shrimp waste on a global scale. Now a days, waste from fish processing industries is only being used in animal and fish feed (Cira *et al,* 2002) in

our country. But there is a huge potential for chitan/chitosan production from shrimp waste; especially in the South West (Khulna, Bagerhat, Sathkhira) and South East (Cox's Bazar, chittagong) region.

2.5.2. Regulatory Status

Until now, chitosan has been approved as food additive or supplement in Japan, Korea, England, Italy, Portugal, and the United States (Novack et al., 2003; No et al., 2007). However, it has not been officially proclaimed as GRAS substances by the U.S. FDA (Raafat & Sahl, 2009).

2.6. Antimicrobial Activity of Chitosan

2.6.1. Overview

With the consumers increasing demand for more natural and safer food without chemical preservatives, the applications of novel natural antimicrobials has attracted much more attention in recent years. In that regards, much attention has been focused on the safety and efficiency of chitosans from animal origin as the natural antimicrobials. The antimicrobial activity of chitosan has been observed in a wide variety of microorganisms, including bacteria, yeast, and fungi (Rabea *et al.*, 2003). Moreover, chitosan has several advantages over other types of natural antimicrobials, such as higher antimicrobial activity, broader spectrum of activity, higher killing rate and lower toxicity toward mammalian cells (Rabea *et al.*, 2003). For example, one study suggested that chitosan could be used as an antimicrobial preservative in emulsion formulations for mucosal as well as for parenteral applications (Jumaa *et al.*, 2002). Similarly, another study proposed that chitosan be used as an adjunct in the potentiation of antimicrobial effect of benzonates and others (Sagoo *et al.*, 2002). In addition, chitosan was found capable of potentiating the antimicrobial activity of a number of preservatives, such as phenethyl alcohol, benzoic acid, and phenylmercuric acetate against numerous bacteria strains (Raafat & Sahl, 2009).

The reported minimum inhibitory concentrations (MICs) of chitosan vary widely with the bacteria, ranging from 0.005% to 1.5% (w/v) (Shahidi *et al.*, 1999; Jeon & Kim, 2000; No *et al.*, 2002). Chitosan has been shown to inhibit both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae, Vibrio* spp., and *Salmonella* Typhimurium. Wang *et al.* (1992) reported that a much higher concentration of chitosan (1-1.5%) is required for complete inhibition of *S. aureus* after two days of incubation. Another study found that chitosan concentrations (>0.005%) were sufficient to complete inactivation of *S. aureus* (Shahidi *et al.*, 1999). Simpson *et al.* (1997) found that 0.02% of chitosan was required to inhibit *B. cereus*

growth, while it also reported that these bacteria can be inactivated by chitosan in another study (Shahidi *et al.*, 1999).

Numerous studies have shown the effect of chitosan on *E.* coli inhibition. Darmadji and Izmimoto *et al.* (1994) reported that chitosan with concentration of 0.1% was required to inhibit *E. coli* growth in meat preservation. But another showed that lower concentration (0.0075%) of chitosan was enough to inhibit the *E. coli* growth (Simpson *et al.*, 1997). Moreover, concentrations of 0.5 or 1% of chitosan were capable of completely inactivatiton the *E. coli* growth at pH 5.5 (Wang, 1992). Besides, No *et al.* (2002) found that *V. parahaemolyticus* growth was effectively inhibited by 1-3 log cycles at a 0.1% concentration of chitosan with molecular weight of 470 kDa while *L. monocytogenes* was completely inhibited at 0.1% concentration by chitosan of Mw=746 kDa.

2.6.2. Factors Affecting the Antimicrobial Activity

There are numerous studies exploring the antimicrobial activity of different chitosans from various sources using diverse testing conditions. Discrepancies in the results obtained in those studies were observed. No *et al.* (2002) reported that chitosan (0.1%) was more effective in inhibiting Gram-positive than Gram-negative bacteria. In another study, chitosan had stronger antimicrobial activity against *S. aureus* than *S. enterica* and *V. vulnificus*, suggesting that chitosan is more effective at inhibiting Gram-positive than Gram-negative bacteria (Chhabra *et al.*, 2006). In direct contrast, Helander *et al.* (2001) demonstrated that chitosan presented a higher antimicrobial activity against Gram-negative than Gram-positive bacteria.

On the one hand, chitosan's *in vitro* antimicrobial activity is dependent upon various intrinsic and extrinsic factors, such as Mw, DD, viscosity, solvent, pH, test strains, temperature, and metal ions etc. (Raafat & Sahl, 2009). On the other hand, methodologies applied in varied studies will be another factor contributing to different results of antimicrobial activity of chitosan.

2.6.2.1. Intrinsic Factors

Although many studies have explored the intrinsic and extrinsic factors affecting the antimicrobial activity of chitosan, it is still difficult to pinpoint the influence of Mw or the DD value on the antimicrobial activity of chitosan. For example, it was reported that chitosan possessed a higher antimicrobial activities with decreasing Mw for gram-negative bacteria, not for gram-positive bacteria (No *et al.*, 2002). In the same study, it found that the minimal inhibitory concentrations (MICs) of chitosans ranged from 0.05% to above 0.1% for different bacteria tested and Mw of chitosan used.

It was suggested that chitosan with Mw of 470 kDa was more effective for inhibiting the growth of Gram-negative bacteria, whereas that with 1,106 kDa was less effective. For Gram-positive bacteria, chitosan with Mw of 470 kDa was less effective. Another study (Jeon *et al.*, 2001)

demonstrated that low Mw chitosans (5-10 KDa) showed the strongest antimicrobial activity against pathogenic bacteria. Zheng et al. (2003) reported that among 5 chitosans with Mw less than 300 kDa, the antimicrobial activity against *S. aureus* was strengthened as the Mw increased, while the effect on *E. coli* was weakened which was in agreement with Chen *et al.* (1998). 2006 study that the antimicrobial activity of low Mw chitosan is higher than the high Mw samples against *E. coli*. Another study (Shin *et al.*, 1997) showed that chitosan with Mw of 40 kDa could inhibit the growth of 90% of *S. aureus* and *E. coli* at a concentration of 0.5% and chitosan with Mw of 180 kDa could almost completely inhibit the growth of *S. aureus* and *E. coli* at a concentration of 0.05%. It has been suggested by Jeon et al. (2001) that an Mw of more than 10 kDa is required for proper inhibition of microorganisms by chitosans. It was also reported that *Campylobacter* spp. were the most sensitive microorganisms to chitosan, and the MIC of chitosan for *Campylobacter* ranged from 0.005 to 0.05% (Raybaudi-Massilia *et al.*, 2009). Moreover, according to studies by Shigemasa et al. (1995) and Liu *et al.* (2001), chitosans with a high DD were more effective than those with a low DD value in inhibiting the growth of microorganisms, which probably was due to the higher percentage of protonated amine groups.

2.6.2.2. Extrinsic Factors

The antimicrobial activity of chitosan is inversely influenced by pH, i.e., stronger antimicrobial activity was observed at lower pH. Based on the studies of Liu *et al.* (2006), No *et al.* (2002), and Rabea *et al.*(2003), chitosan showed its antimicrobial activity only in an acidic medium, which was caused by the poor solubility of chitosan at pH above 6.5. The reason that requiring a pH at least 6.5 for chitosan to maintain its antimicrobial activity may be due to the presence of predominantly positive-uncharged amino groups as well as poor solubility of chitosan (Papineau *et al.*, 1991; Sudharshan *et al.*, 1992).

The alternation of ionic strength in a medium may affect the antimicrobial activity of chitosan (Raafat & Sahl, 2009). However, results varied from different studies. For example, one study reported that the presence of divalent cations reduces the antimicrobial activity of shrimp chitosan against *E. coli* (Tsai & Su, 1999), likely because the increase of metal ions, especially divalent ions, could decrease the chelating capacity of chitosan (Kong *et al.*, 2010). In contrast, Chung et al. (2003) suggested that the higher ionic strength could enhance the solubility of chitosan and therefore increase its antimicrobial activity. It is probably due to existing cations in medium may interact with the negative-charged components mainly on the cell wall of bacteria besides polycationic chitosan, consequently weakening the antimicrobial activity.

2.6.3. Antimicrobial Mode of Action

The exact mechanism of the antimicrobial action of chitosan remains to be elucidated, but several factors influence the antimicrobial activities of chitosan. The mode of antimicrobial action of chitosan is discussed below.

The polycationic nature of chitosan (pKa = 6.3) is prerequisite for antimicrobial activity. As pH is below the pKa of chitosan, electrostatic interaction between the polycationic structure (NH3+ groups of glucosamine) and the predominantly anionic components of the microorganisms surface (such as Gram-negative lipopolysaccharides and cell surface proteins) plays a very important role in the antimicrobial activity of chitosan. Eventually, the interaction between the positively charged NH_3^+ groups and the negatively charged microbial cell surface contribute to the leakage of protein and other intracellular components of the microbial cells, ultimately resulting in the impairment of vital bacteria activities (Muzzarelli *et al.*, 1990; Helander *et al.*, 2001; Je & Kim, 2006; Raafat *et al.*, 2008; Kong *et al.*, 2010). The number of amino groups linking to C-2 on chitosan backbones is important in electrostatic interaction, which indicate that large amount of amino groups are capable of enhancing the antimicrobial activity. Therefore, chitosan with higher DD shows a stronger inhibitory effect than that of a lower DD chitosan (Kong *et al.*, 2010).

The different MWs of chitosan and its physical states render distinctive modes of antimicrobial action. LMw water-soluble chitosan was able to penetrate cell wall of bacteria and then interact with DNA and inhibit synthesis of mRNA and DNA transcription (Sudharshan *et al.*, 1992). For HMw water-soluble chitosan and solid chitosan could only interact with cell surface without penetrating into the cell wall and lead to altering cell permeability or form an impermeable layer around the cell, thus blocking the transport of essential solutes into the cell (Kong *et al.*, 2010).

CHAPTER THREE

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

3.1. Instruments used in the experiments

1. Laminar air flow (SAARC)
2. Incubator (Sanyo, Japan)
3. Autoclave (Local)
4. Spectrophotometer (Hitachi, U-2910)
5. pH meter (pH Tester H196107, Hanna Instruments, USA)
6. Digital Balance (OHAUS, PA104 Corp. USA)
7. Water Distiller (Jinan Mao, China)
8. Refrigerator(Walton)
9. Micropipette (Gilson, France)
10. Beaker
11. Test tubes
12. Petri dish
13. Inoculating loop (Nicrome, 2mm), etc.

3.2. Chemical used in the experiment

1. Nutrient Broth (HIMEDIA, M002)
2. Agar powder (HIMEDIA, M002)
3. Glacial Acetic Acid (Merck, Germany)

3.3. Chitosan Sample

Chitosan samples were collected from Sigma Aldrich, Germany and Bioprocess Technology lab, Khulna University.

3.4. Bacteria Strains and Culture Conditions

The four bacterial strains (Table 3) used in this study were selected from our lab which were collected from ICDDR'B as clinical isolates and were maintained in nutrient broth (NB) media, which included 3 Gram-negative bacteria (*Escherichia coli, Vibrio cholera, Shigella dysentery*) and 1 Gram-positive bacteria (*Staphylococcus aureus*). All strains were grown on tryptic nutrient agar (TSA; BD Diagnostic Systems, Sparks, MD) supplemented with 2% NaCl. Cultures were incubated at 37°C for 24 h.

Table 3. Representative Bacterial Strains

Strain group	Strain ID and serotype	Source and reference
Gram-negative		
Escherichia coli	ATCC 25922	ICDDR,B isolate
Salmonella typhi	ATCC 14035	ICDDR,B isolate
Shigella dysentery		ICDDR,B isolate
Gram-positive		
Staphylococcus aureus	ATCC 29213	ICDDR,B isolate

Table 4. List of bacteria with diseases caused by them

Gram positive/negative	Bacteria	Disease
Negative	*Escherichia coli*	Gastroenteritis, urinary tract infections, and neonatal meningitis
	Shigella dysenteriae	Bacillary dysentery
	Salmonella typhi	Typhoid
Positive	*Staphylococcus aureus*	Scaled skin syndrome

Source: Pharmaceutical Microbiology, Hugo and Russell, 6th ed. (1998)

Methodology

3.5. Methods to Detect Antimicrobial Activity of Chitosan

The antimicrobial activities of chitosan may be determined using four main methods, agar dilution, broth microdilution, well plate and disk diffusion which are standard methods recommended by Clinical and Laboratories Standards Institute (CLSI) for measuring *in vitro* susceptibility of bacteria to antimicrobial agents used in clinical settings (CLSI, 2009; CLSI, 2009). Since these methods apply different principles, the results obtained may differ.

Besides methods, antimicrobial susceptibility testing results can also be affected by many other factors, such as the microorganisms tested and the degree of solubility of each test-compound (Valgas *et al.*, 2007). Among the four methods, well plate has been the most popular one used to examine the antimicrobial activity of natural antimicrobials including chitosan (Kim & Kim, 2007; Mayachiew *et al.*, 2010). In this research work this method is used also.

3.6. Preparation of inoculum

Stock cultures were maintained at 4° C on nutrient agar slant. Active cultures for experiments were prepared by transferring a loop-full of cells from the stock cultures to test tubes of Nutrient Broth (pH 7 ± 0.2) that were incubated without agitation for 24 hrs at 37° C. In this exercise we used turbidity estimations to determine the growth characteristics of a bacterial culture.

3.7. Preparation of Chitosan

Chitosan compounds prepared from crab shell waste were purchased from Sigma Aldrich (Germany), and the other chitosan sample was made in Bioprocess Technology Lab (Khulna University). All compounds were acid-soluble. The compounds were placed in separate vials and dried in an oven under 60°C before the experiment.

Chitosans were dissolved in acetic acid (1%, v/v) to obtain a stock solution (10mg/ml). The pH values for all chitosan solutions were adjusted to 5.9 (No *et al.*, 2002) with 1 N HCl (Sigma-Aldrich, St. Louis, MO) and 1 N NaOH. The stock solutions were autoclaved, kept in the refrigerator, and diluted in water and acid before use.

3.8. Well plate Method for antibacterial activity

Well plate method was used to screen the antibacterial activity. From the overnight culture nutrient broth, small portion of bacterial culture was transferred into vial containing nutrient broth and incubated at 37° C until the growth reached the log phase (4-6 hrs; 5×10^6 cfu/ml). We adjusted the pH of the medium. The pH of medium was 6.5. The NA plates were prepared by pouring 20 ml of molten media into sterile petriplates and were allowed to solidify. Then plates were seeded properly by pouring the culture broth with a Pasteur pipette to make bacterial lawn.

The excess culture broth was withdrawn from the plates and allowed to dry. Wells were made with a sterilized pasture pipette which diameter was 6mm. Then the wells were poured with chitosan solution and tetracycline for comparing the zone of inhibition. All these were done in laminar air flow cabinet. The plates were incubated overnight at 37° C and inhibition zones were measured in millimeter (mm) with a transparent scale and noted down.

3.9. Determination of Inhibitory Concentration 50 (IC_{50})

In this method an increasing in concentration of the sample (dissolved in acetic acid) were added to different test tubes containing particular culture. Calculated amount of chitosan was added from the stock solution (10 mg/ml) to the sterilized screw-capped one drum vials each containing 1 ml of NB to obtain seven different concentrations (0.005, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2 µg/ml respectively). Then 10 µl of freshly grown inoculum (5×10^6 cfu/ml) was added to each vial. Additional vials were also included as control; media with inoculums; media with sample (0.01, 0.05 and 0.25 mg/ml) and media with of solvent and bacteria. The first control tubes were included to check the turbidity of media due to bacterial growth, the second was to check turbidity caused by sample and the third control tubes were to check whether the solvent itself caused any inhibition. All the tubes were incubated at 37° C. After incubation, ODs were taken by spectrophotometer (Hitachi, U-2910) at 700 nm, noted down and then calculated to find out the percentage of inhibition and finally IC_{50} value for each extract.

3.10. Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of chitosan sample was determined by the tube dilution method. In this method, an increasing in concentration of extract was added to different screw-capped vials.

Sterilized screw-capped one drum vials each containing 2 ml of nutrient broth were taken. Then calculated amount of sample was added from the stock solution (10 mg/ml) to the vials to obtain a range of different concentrations (0.005, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2 mg/ml) of the sample. Then 10 µL of freshly grown inoculums (5×10^6 cfu/ml) was added to each vial. Additional vials were also included as control, designated as media with inoculums; media with sample and media and 50 µL of solvent and bacteria. All the tubes were incubated at 37° C. After incubation, vials having lowest concentrations of extract showing no turbidity were primarily selected as MIC of the specific sample. However, in case of excessively stained extract subculture from the tubes were made to determine the MIC. The whole process was replicated for testing each sample.

3.11. Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) of chitosan sample was determined by measuring the viability loss of bacterial culture with an increasing in concentration of chitosan. Vials containing different concentration of sample were plated out on NA plate to test the viability. Null colony formation indicated the MBC concentration(s).

3.12. Data Analysis

All experiments were conducted in triplicate, and mean values and standard deviations of the diameter of inhibition zone in disk diffusion assay were calculated from the experimental data obtained.

CHAPTER FOUR

RESULTS

RESULTS

Chitosan solutions of different concentration were used to find out the results and they were calculated in terms of zone of inhibition of selected bacteria by well plate test, Inhibitory Concentration (IC_{50}) from optical density, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

4.1. Well plate test

Well plate method was used to find out the antibacterial activity of chitosan solution. The diameters of zone of inhibition (Figure 3 and 10) were measured in mm as average of two results (mm ± SD) and are presented in Table 4 and Table 5. The tested solution showed as promising.

4.2. Inhibitory Concentration (IC_{50}) value

To obtain the strength of the chitosan samples, percentages of inhibition were calculated (formula: Appendix 1) using the ODs at different concentrations for each sample and IC_{50} values were computed for each solution. For commercial grade chitosan the lowest value of IC_{50} 0.433 mg/mL against *E. coli* was seen. Whereas for local grade chitosan it was seen 0.88mg/ml against *Shigella* (Appendix: Table 7; Figures 8, and 19). An increase in the percentage of inhibition was eminent with the headway of concentrations. Commercial grade chitosan was on the apex showing the lowest IC_{50} value of only 0.433 mg/mL against *E. coli* followed by 0.463 mg/mL against *S. typhi,* 0.55mg/ml against *Shigella* and 0.73mg/ml against *S. aurius* (Appendix: Table 7; Figures 4-7). On the other hand it was quite high for local grade chitosan. The lowest IC_{50} value was 0.88mg/ml against *Shigella,* followed by 0.99mg/ml against *E. coli* and 1.33mg/ml for both *S. typhi* and *S. aurius.* (Appendix: Table 7; Figures 15-18).

4.3. Minimum Inhibitory Concentration (MIC)

After obtaining the percentages of inhibition, concentrations with higher values were taken into account to find out MIC of the fractions. Vials were prepared with appropriate concentrations for selected fractions and bacteria. An escalating transparency was observed down with an increase in concentration (Figures 10, 11, 12, 13, 21, 22, 23 and 24). Commercial grade chitosan raised same result 3 mg/mL against *S. typhi* and *S. aureus,* whereas it was 2 mg/mL for both *Shigella* and *E. coli.* For local grade chitosan the MIC value was 3 mg/mL against *E. coli* and 4mg/ml against *S. aureus,* whereas it was 3mg/mL for both *Shigella* and *S. typhi.*

4.4. Minimum Bactericidal Concentration (MBC)

MBC values were determined by plating suspensions from culture vials of MIC assay. The vials that showed no visual growth were streaked onto NA plate to check the viability and at a certain concentration no visible growth of bacteria was found onto the plate. Thus, MBC values of extracts were recorded (Appendix: Table 7) and streak plates are presented in figure 25 and 26.

At 4 mg/mL, *S. typhi* and *S. aureus* were completely inhibited the bacterial growth by commercial grade chitosan solution, while *Shigella* and *E. coli* was absolutely inhibited at 3 mg/mL. For local grade chitosan solution the concentration was 4mg/ml for *S. typhi* and *Shigella* whereas and *E. coli* and *S. aureus* was absolutely inhibited the bacterial growth at 5mg/mL.

Table 5: Mean diameter of zone of inhibition (mm ± SD) for commercial grade chitosan

Bacteria	Ch	Tet.
Salmonella typhi	15.21±0.707	20.5 ± 0.71
Escherichia coli	16.21±0.707	21.5 ± 0.71
Shigella dysenteriae	15±0	21±1.42
Staphylococcus aureus	12.42±1.414	23 ± 0.00

Well diameter: 6 mm, Concentration: 30 µg/disc; **Ch**- Chitosan; **Tet**- Tetracycline.

Table 6: Mean diameter of zone of inhibition (mm ± SD) for local grade chitosan

Bacteria	Ch	Tet.
Salmonella typhi	15.00±0.71	20.5 ± 0.71
Escherichia coli	13.06±0.35	21.5 ± 0.71
Shigella dysenteriae	14.21±0.71	21±1.42
Staphylococcus aureus	10.10±0.35	23 ± 0.00

Well diameter: 6 mm, Concentration: 30 µg/disc; **Ch**- Chitosan; **Tet**- Tetracycline.

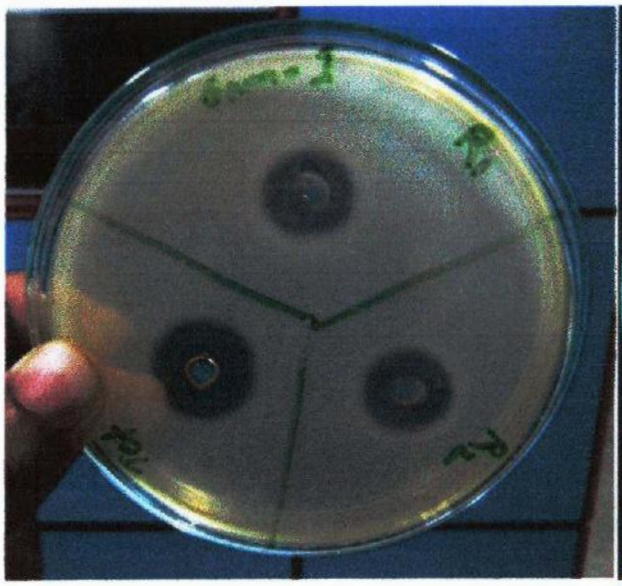

Fig: *Salmonella typhi*

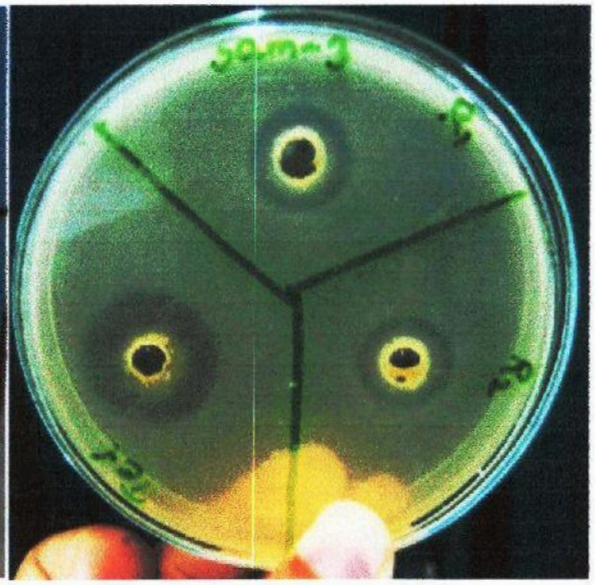

Fig: *Escherichia coli*

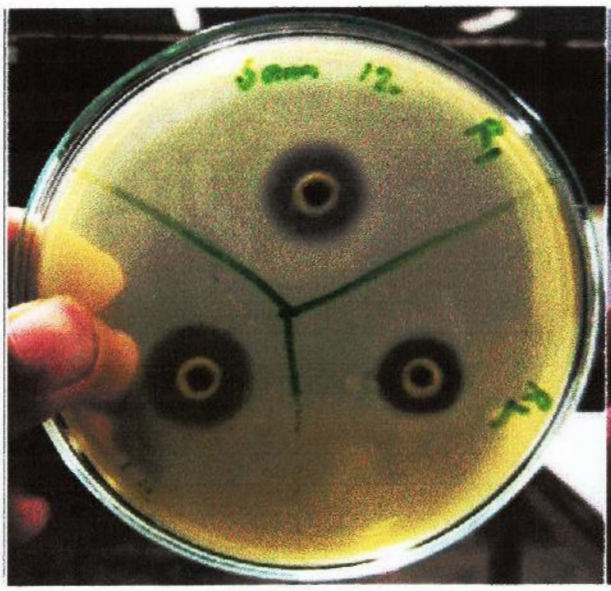

Fig: *Shigella dysenteriae*

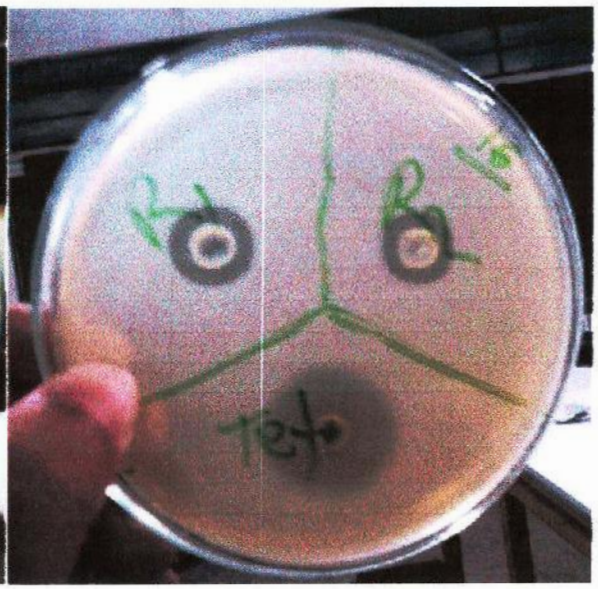

Fig: *staphylococcus aureus*

Figure 3: Zone of inhibition of commercial grade chitosan against test bacteria

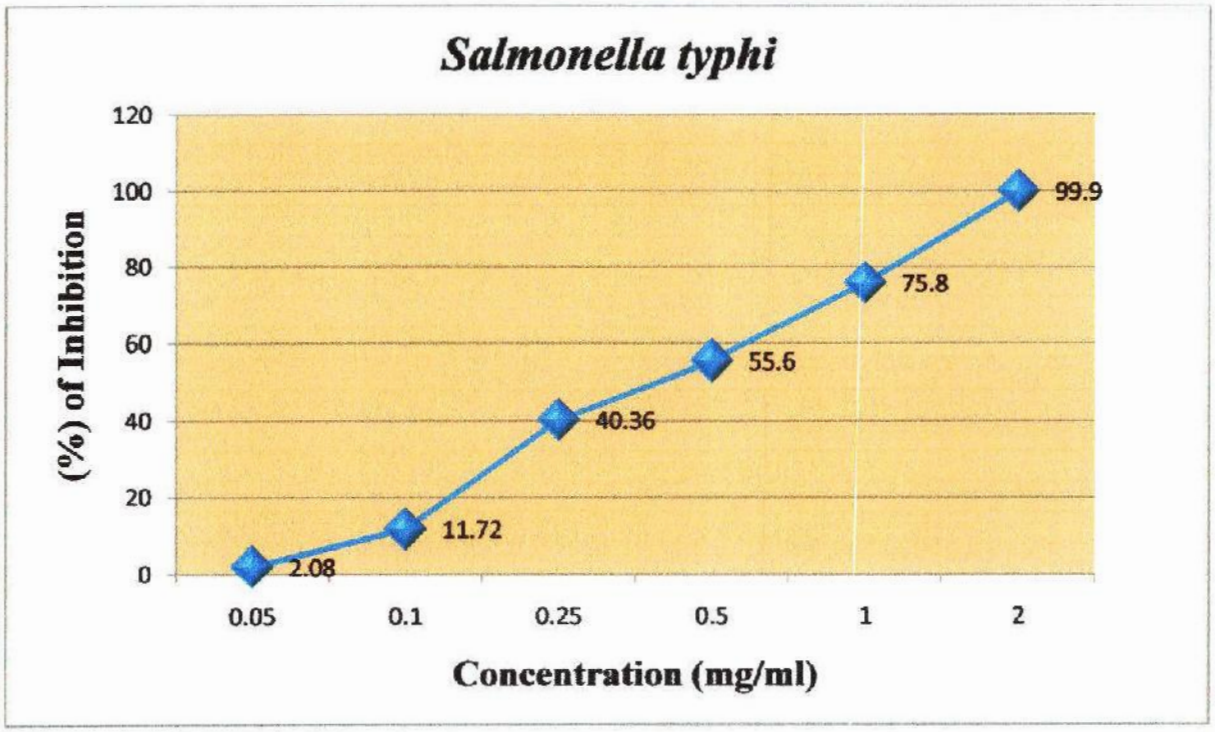

Figure 4: Inhibition (%) of *S. typhi* by commercial grade chitosan sample

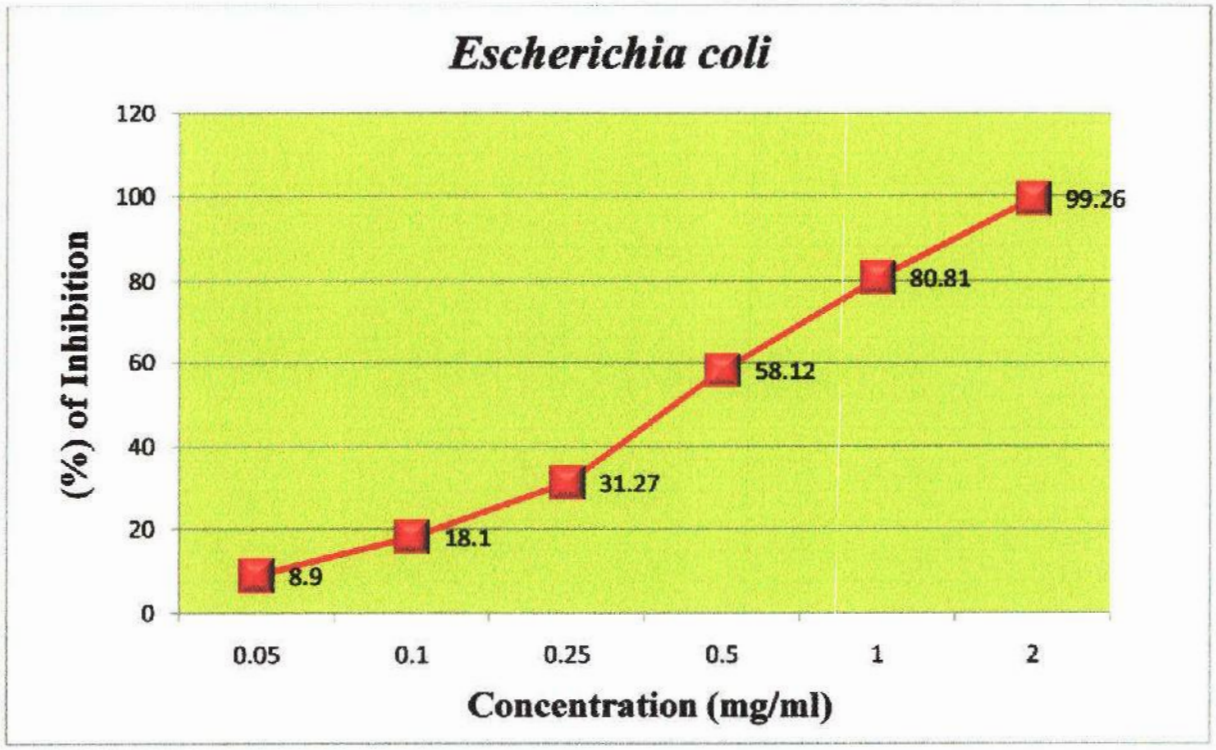

Figure 5: Inhibition (%) of *E. coli* by commercial grade chitosan sample

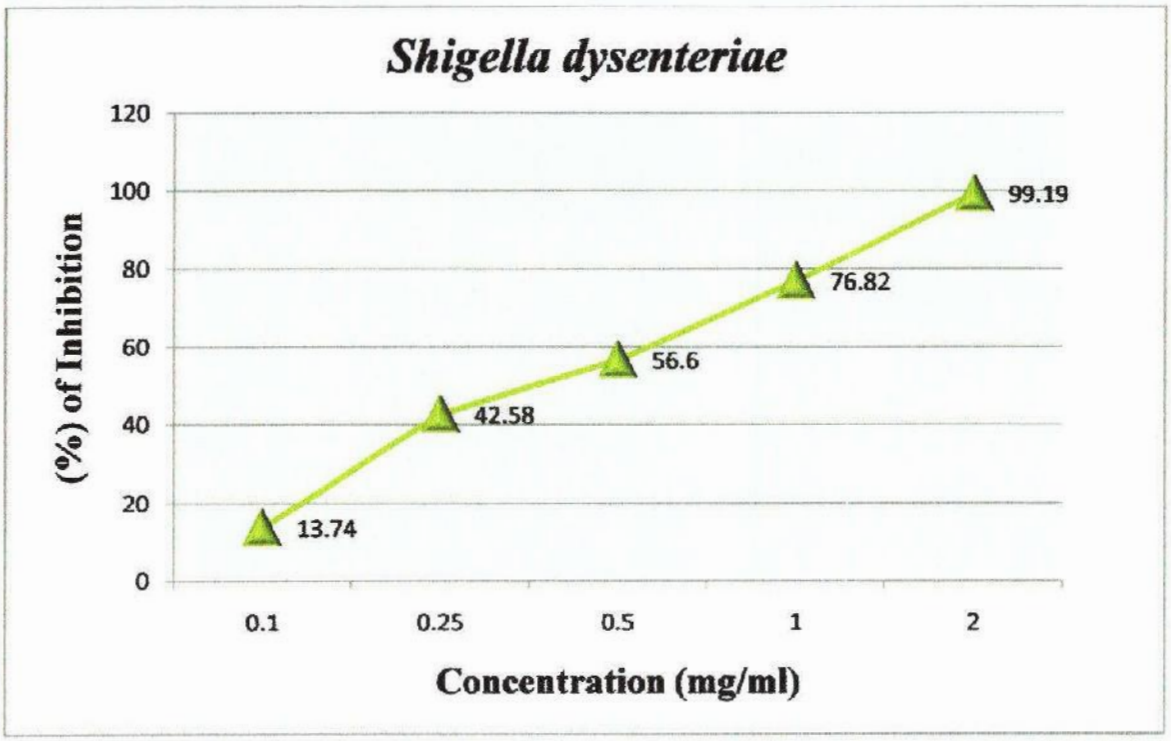

Figure 6: Inhibition (%) of *S. dysecteriae* by commercial grade chitosan sample

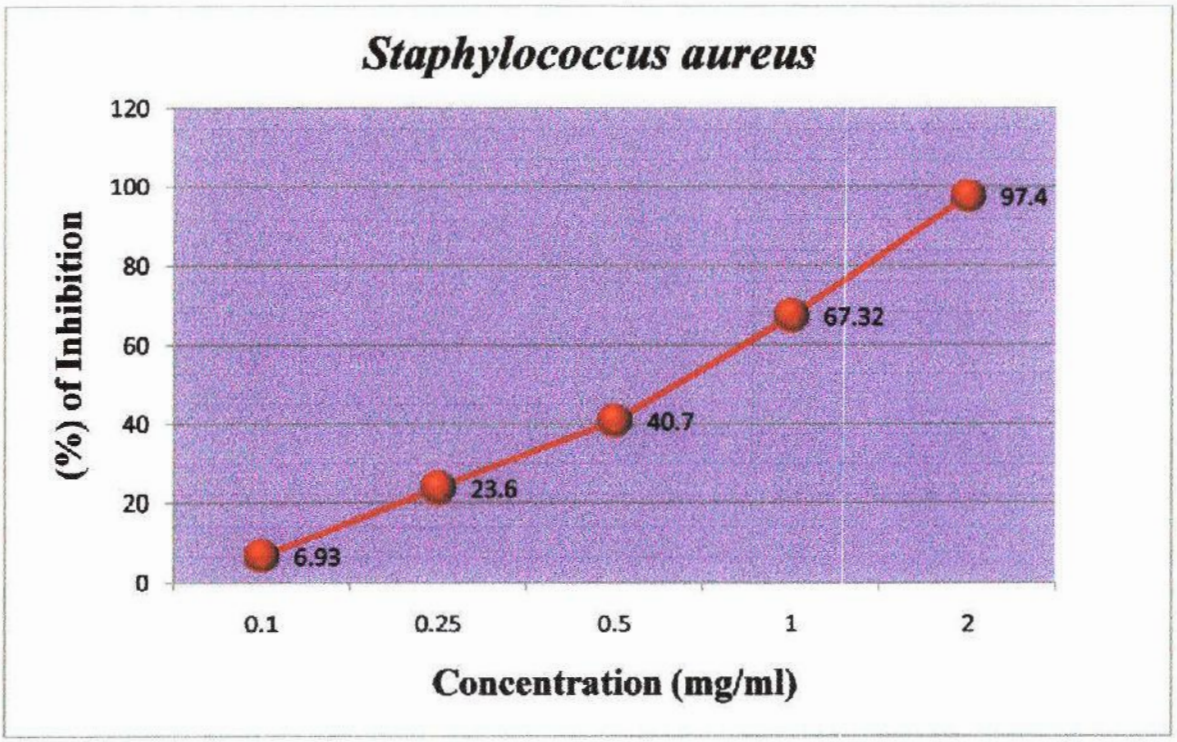

Figure 7: Inhibition (%) of *S. aureus* by commercial grade chitosan sample

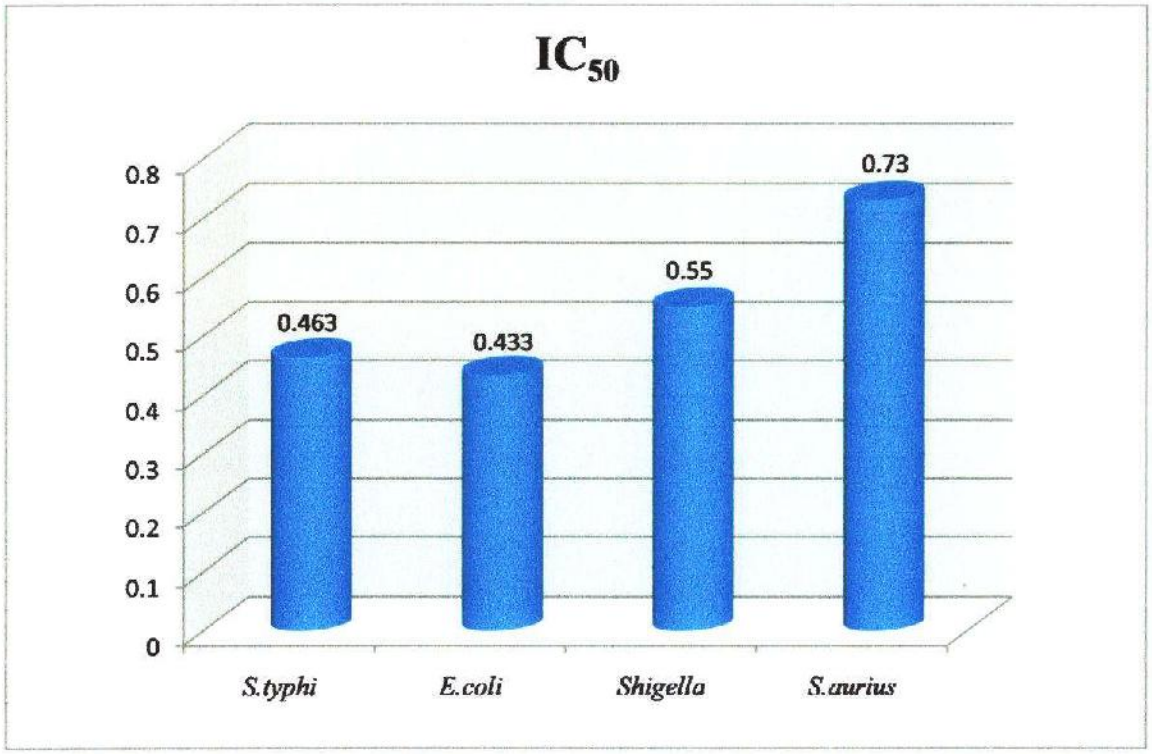

Figure 8: IC_{50} values of commercial grade chitosan solution against four test bacteria

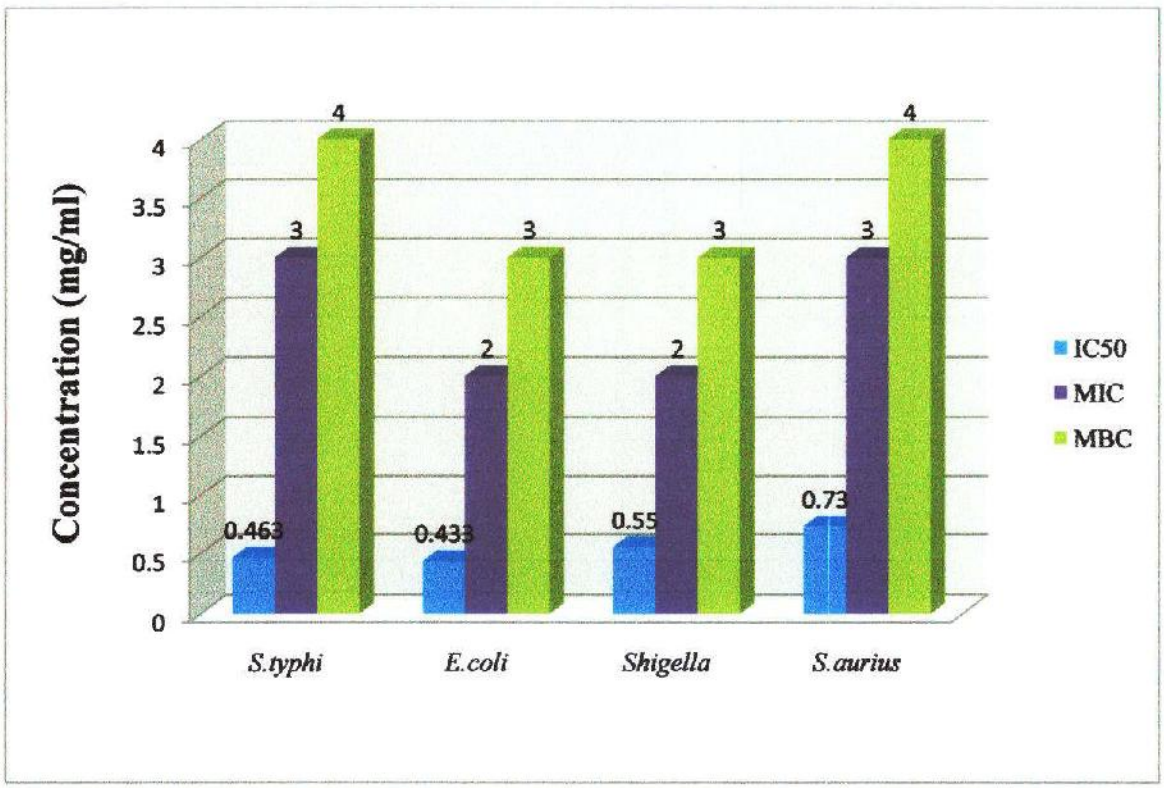

Figure 9: IC_{50}, MIC and MBC values for test bacteria by commercial grade chitosan sample.

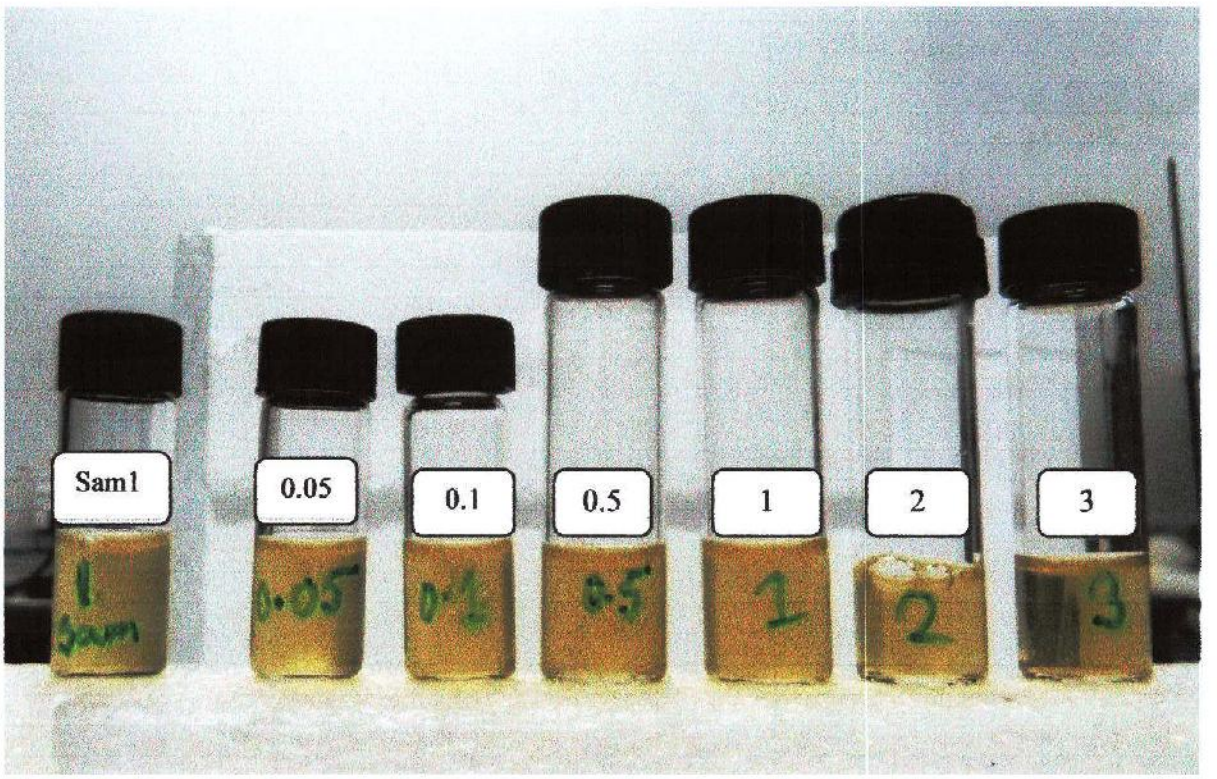

Figure 10: Vials with increase conc. of commercial grade chitosan against *S. typhi*

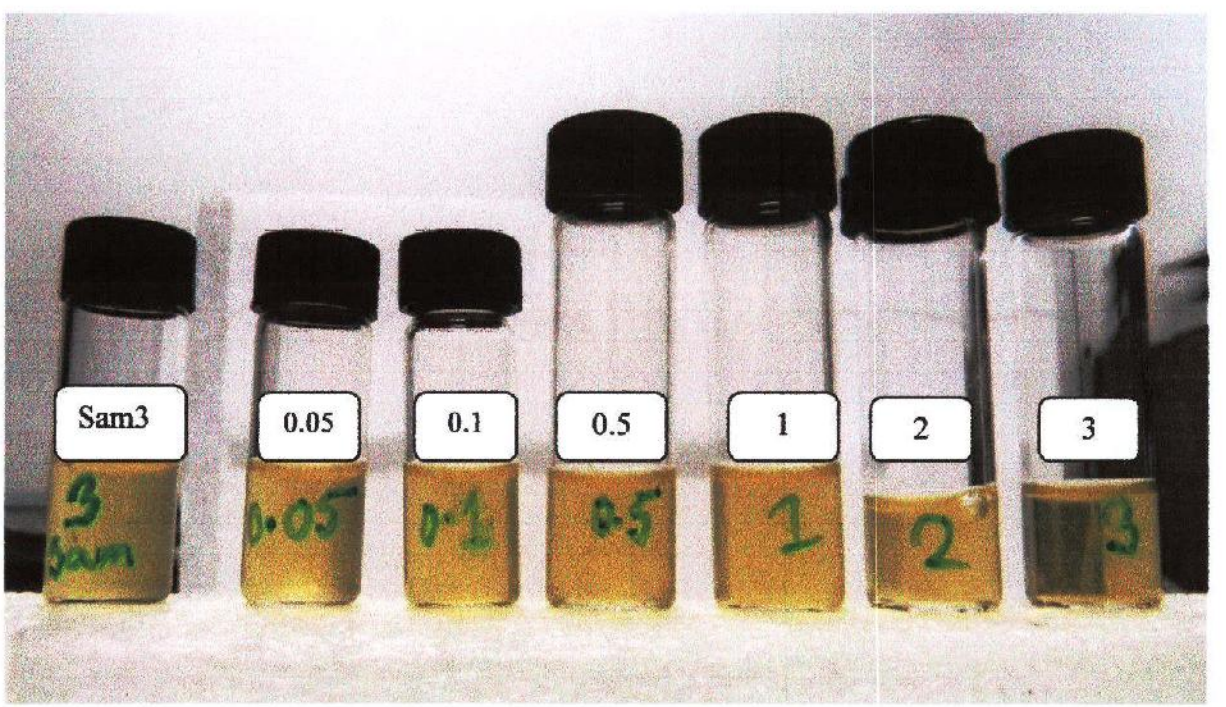

Figure 11: Vials with increase conc. of commercial grade chitosan against *E. coli.*

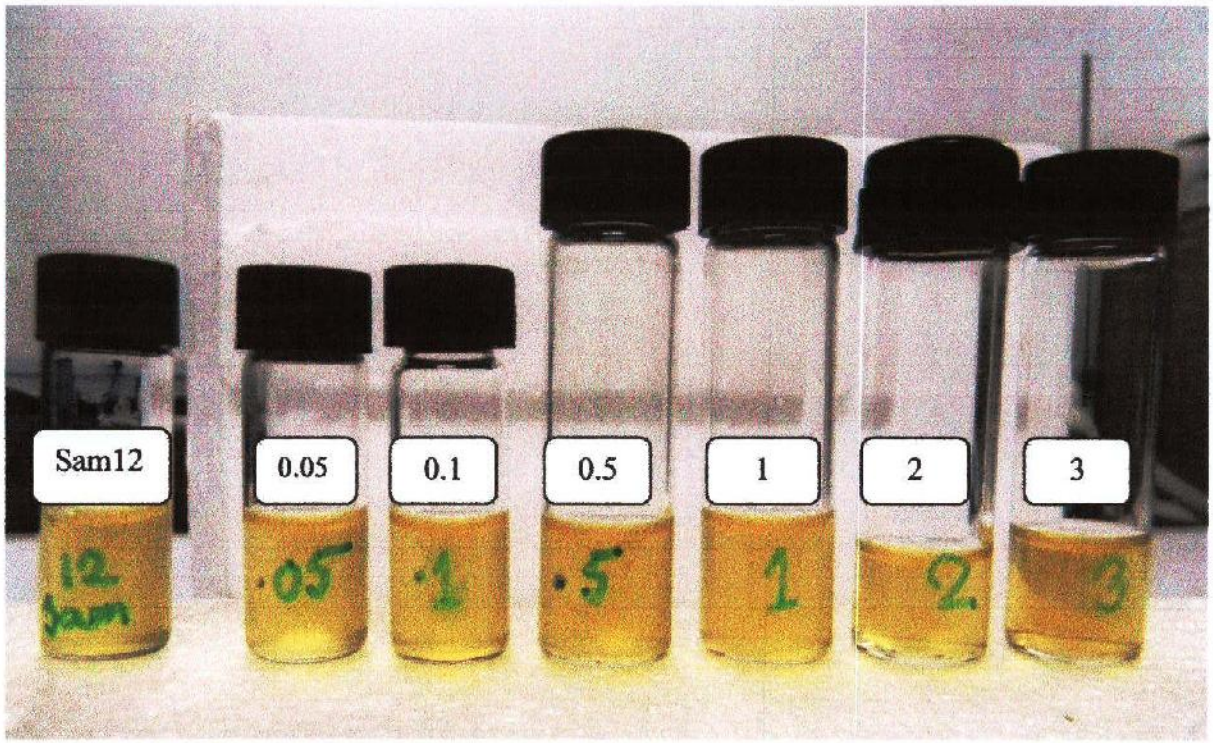

Figure 12: Vials with increase conc. of commercial grade chitosan against *S. dysenteriae*

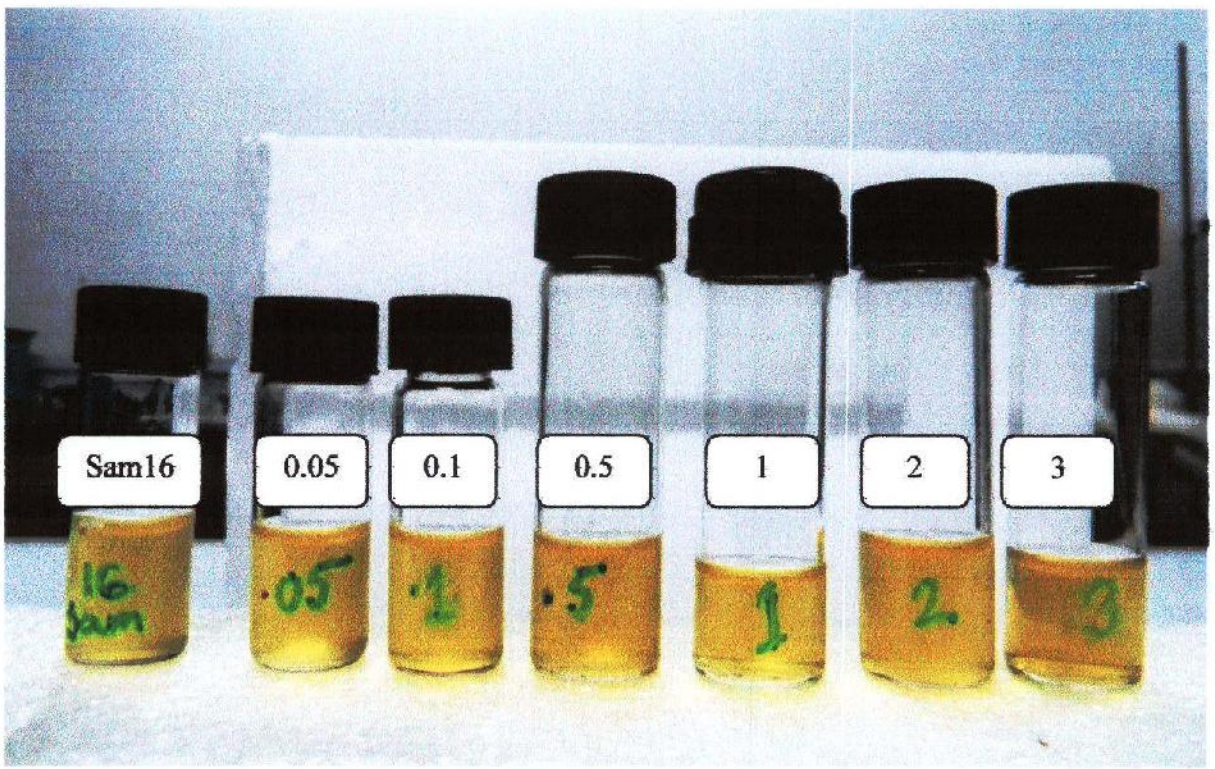

Figure 13: Vials with increase conc. of commercial grade chitosan against *S. aureus.*

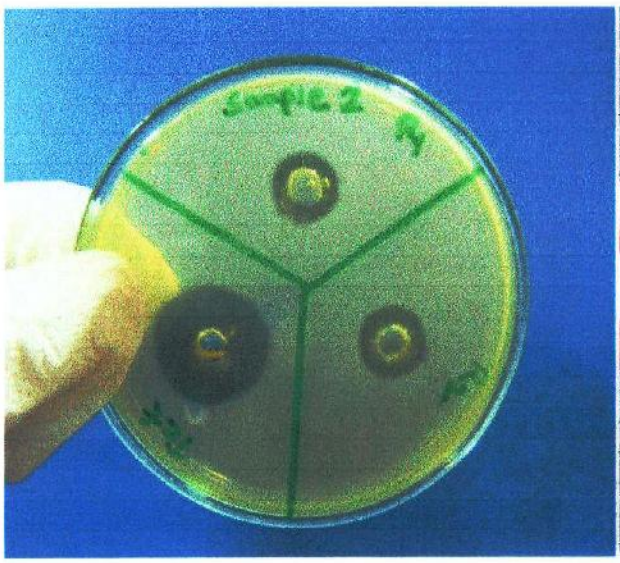

Fig: *Salmonella typhi*

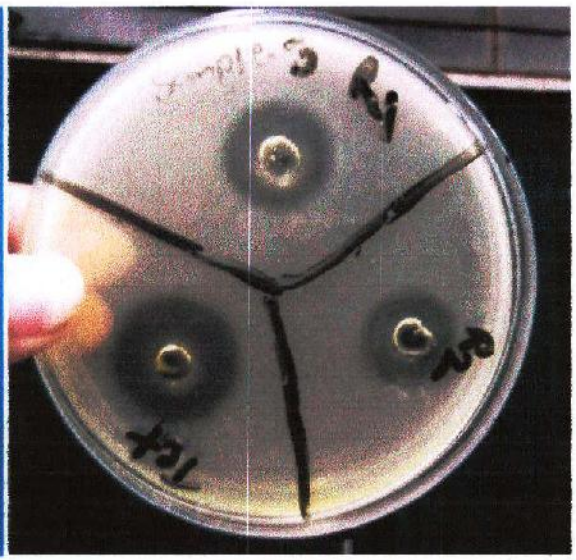

Fig: *Escherichia coli*

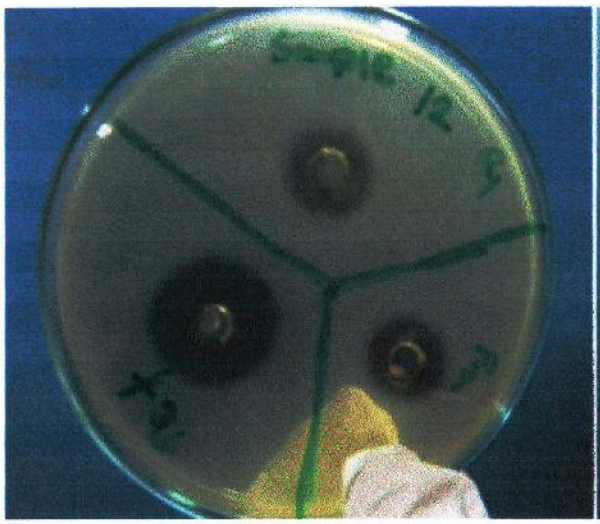

Fig: *Shigella dysenteriae*

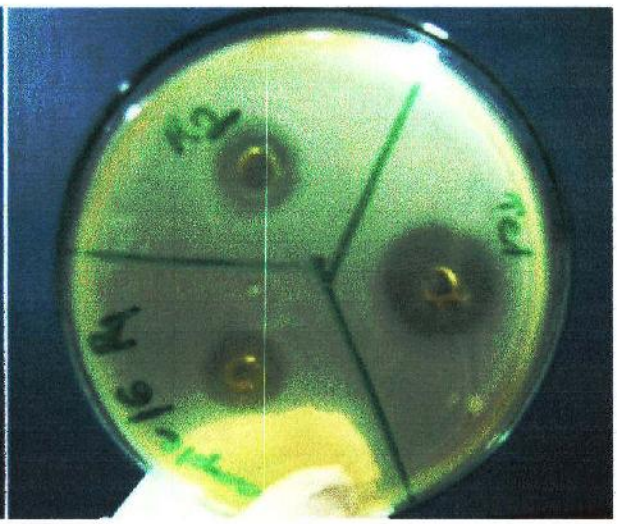

Fig: *staphylococcus aureus*

Figure 14: Zone of inhibition of local grade chitosan against test bacteria

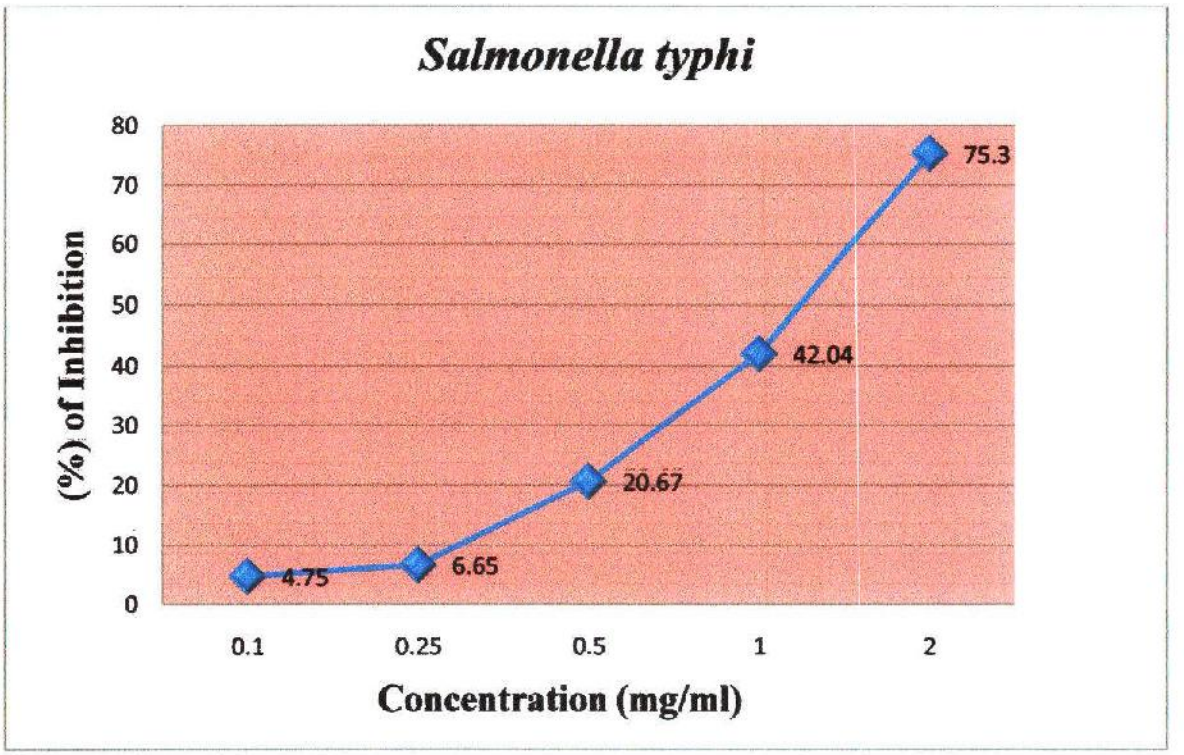

Figure 15: Inhibition (%) of *S. typhi* by local grade chitosan

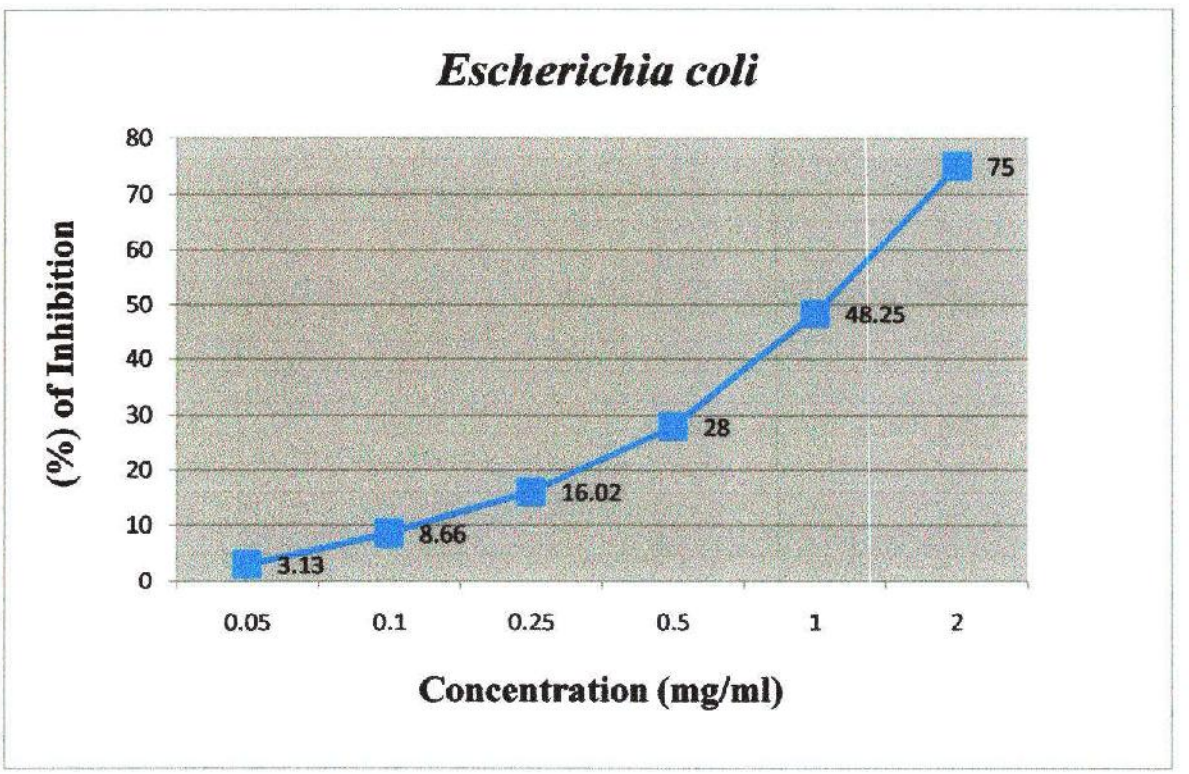

Figure 16: Inhibition (%) of *E. coli* local grade chitosan

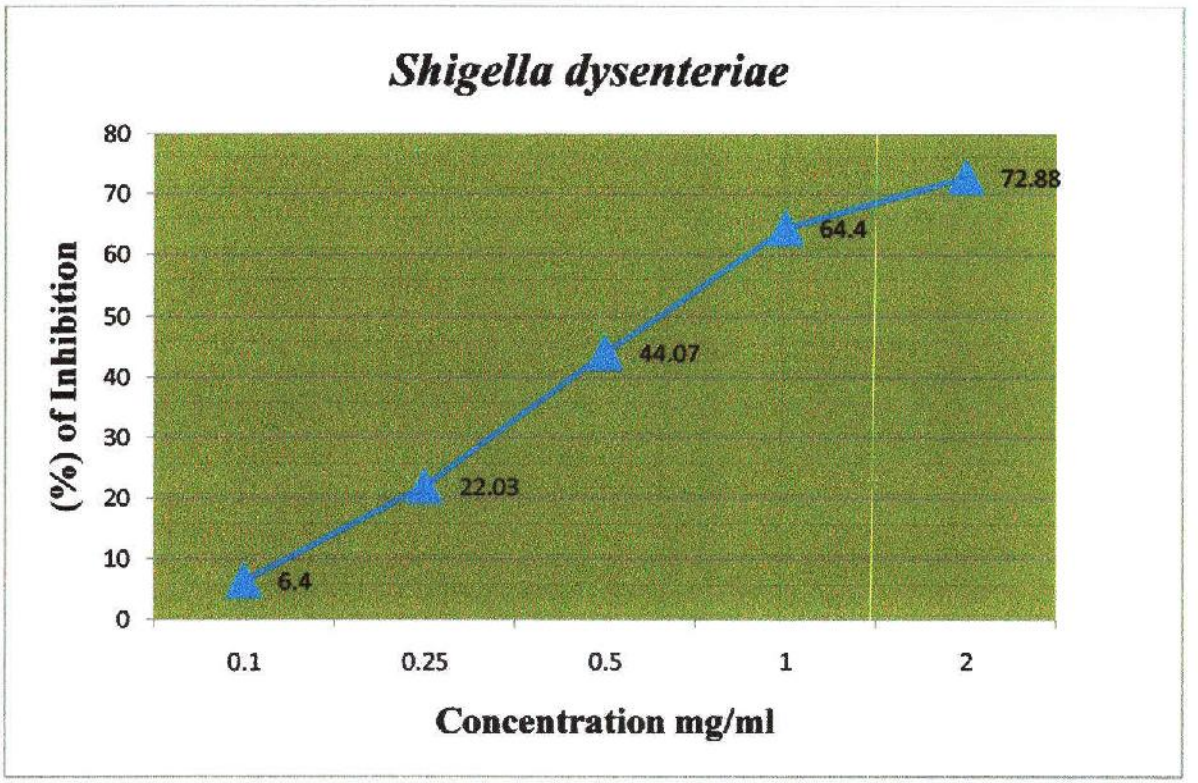

Figure 17: Inhibition (%) of *S. dysenteriae by* local grade chitosan

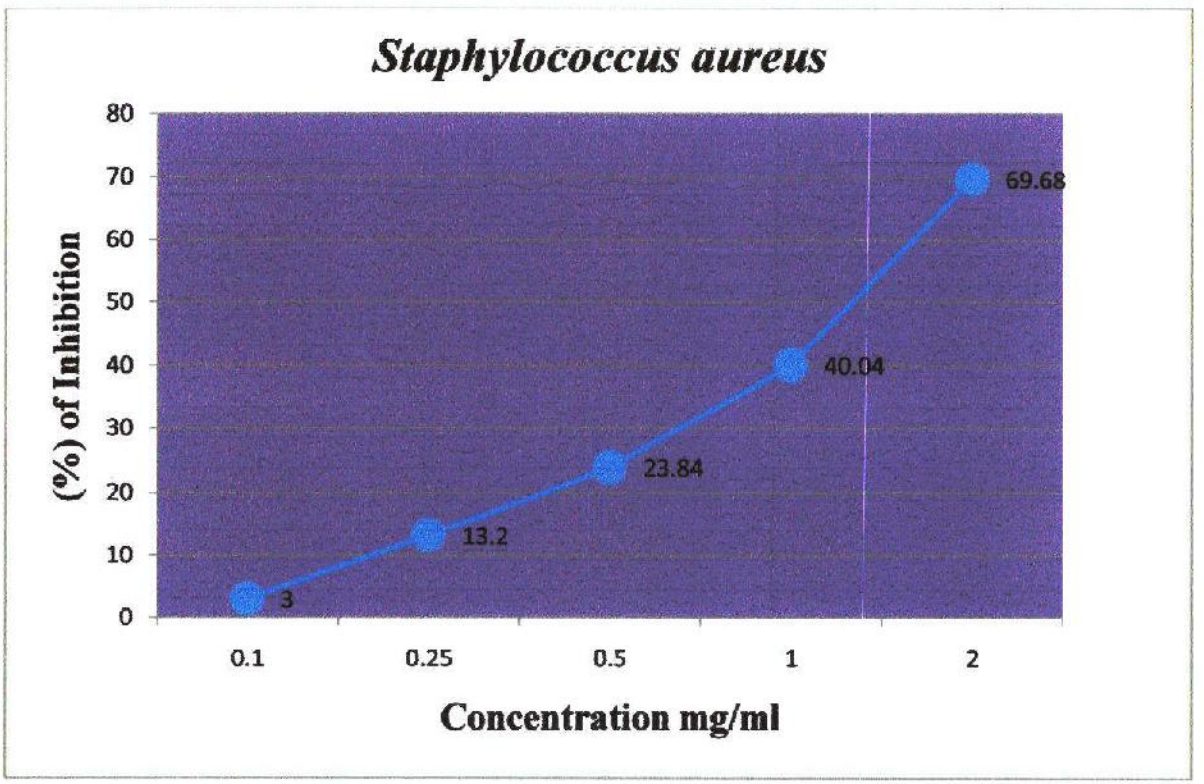

Figure 18: Inhibition (%) of *S. aureus by* local grade chitosan

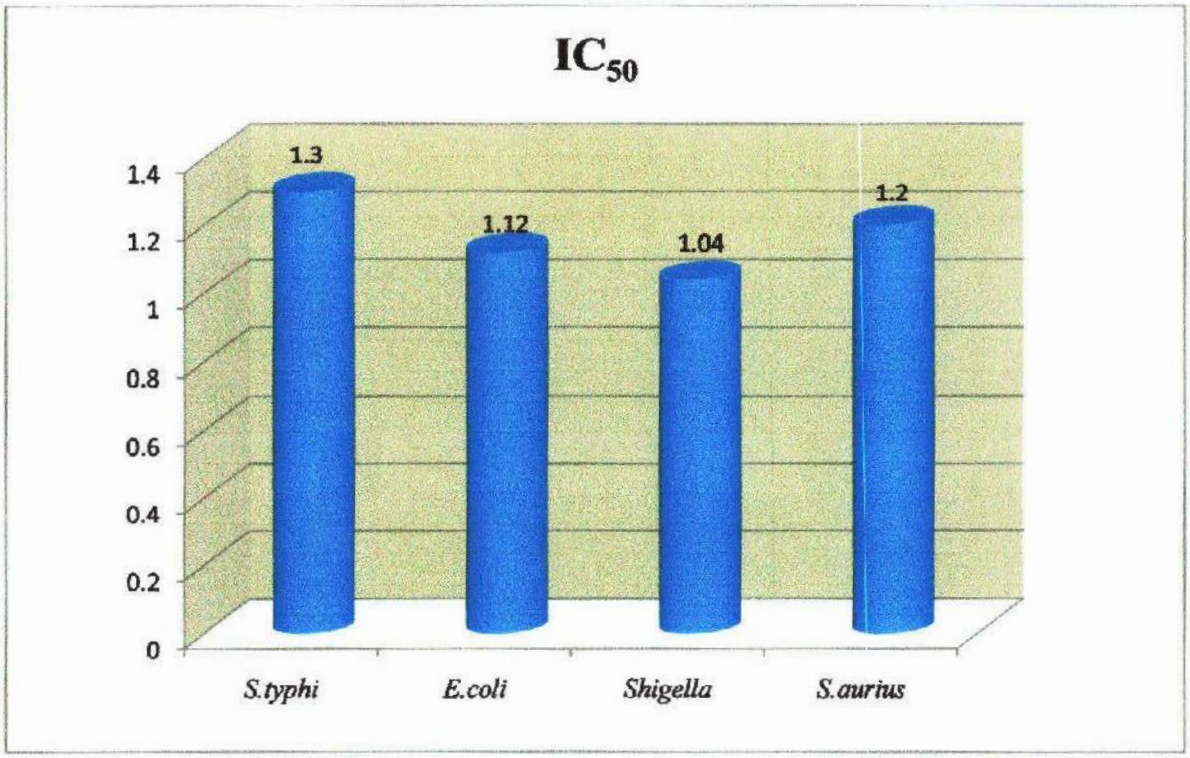

Figure 19: IC_{50} values of local grade chitosan solution against four test bacteria

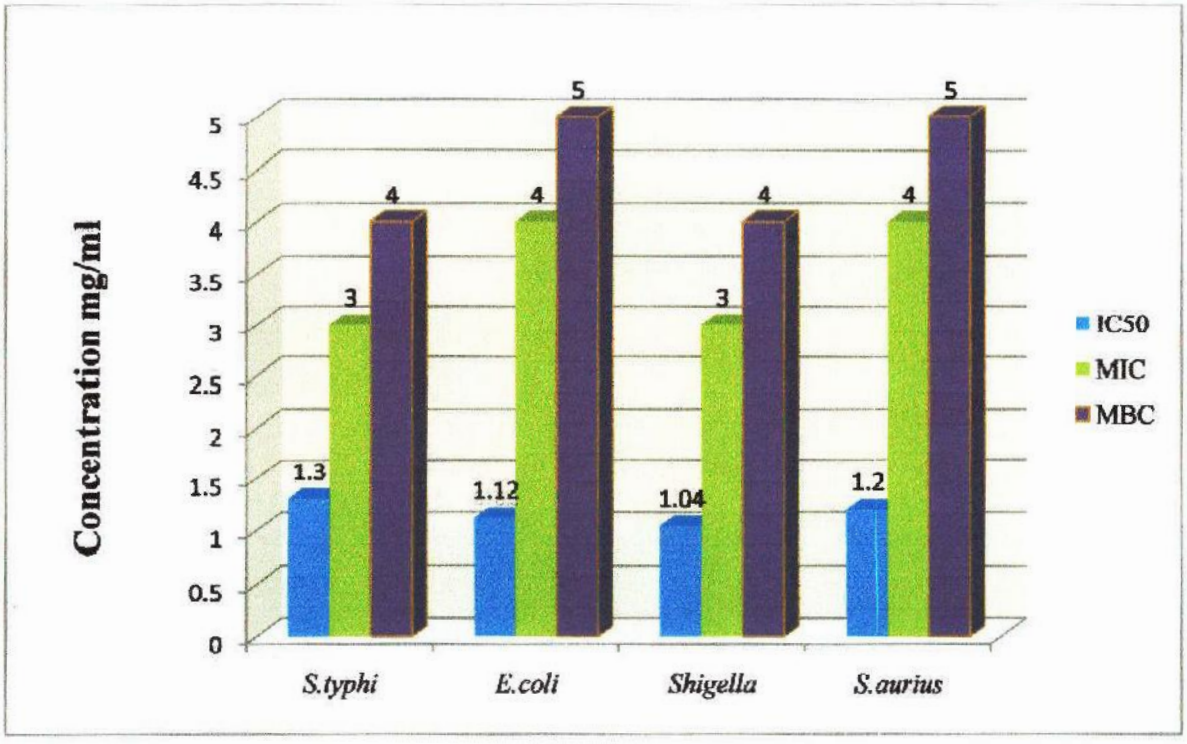

Figure 20: IC_{50}, MIC and MBC values for test bacteria by local grade chitosan sample.

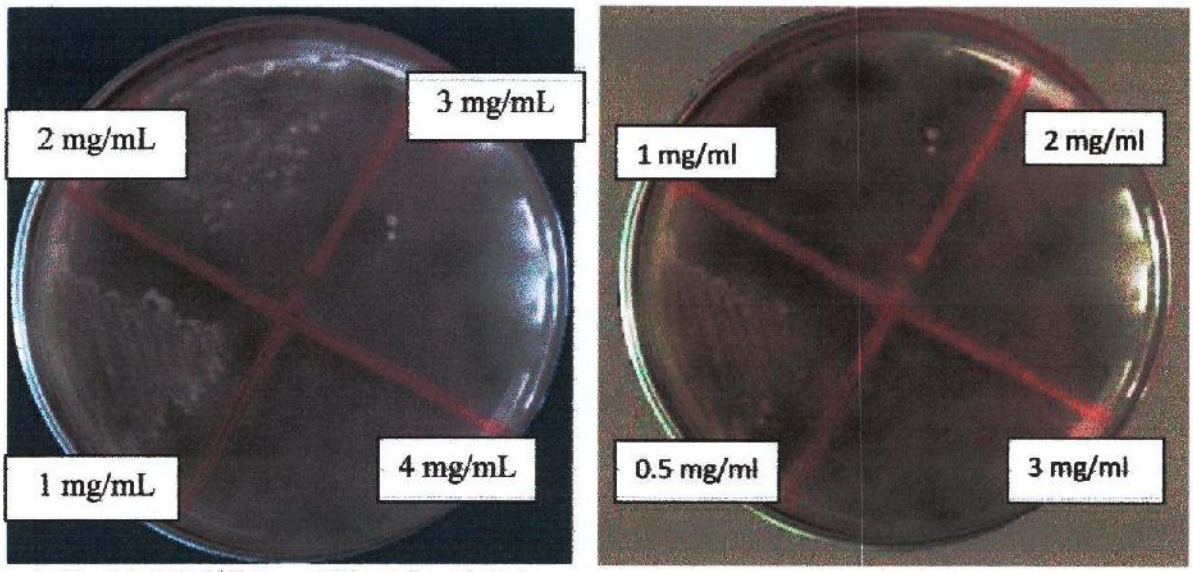

Figure 25: Plate for screening MBC for *S. typhi* and *E. coli* by commercial grade chitosan

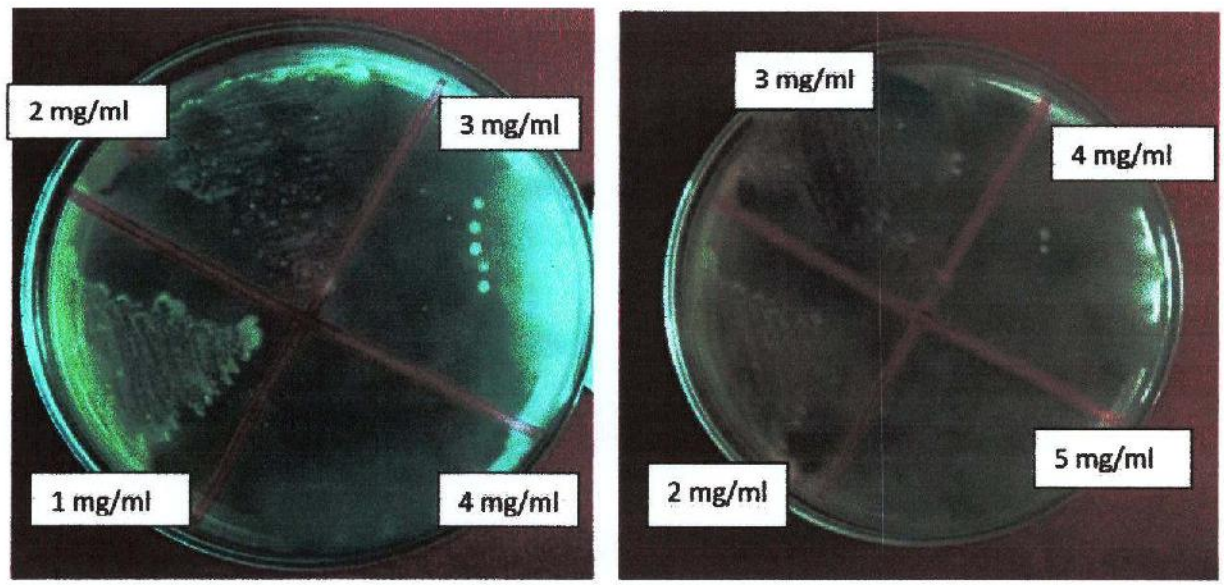

Figure 26: Plate for screening MBC for *S. typhi* and *E. coli* by local grade chitosan

4.5 Comparative Study between Commercial and Local Grade chitosan

Commercial grade Chitosan possess strong antibacterial activity against both G (+ve) (*Staphylococcus aureus)* and G (-ve) (*Salmonella typhi, Escherichia coli, Shigella dysenteriae)* bacteria, while local grade possess lower level of activity against these four types of bacteria.

4.5.1 Comparative Study of Antibacterial Activity against G (+ve) and G (-ve) of two Chitosan samples

Antimicrobial activity of chitosans was evaluated based on the diameters of clear inhibition zone surrounding the well. If there is no inhibition zone, it is assumed that there is no antimicrobial activity. Comparing commercial grade with lab grade chitosan it was found that commercial grade chitosan gave increased zone of inhibition than lab grade chitosan. The result is numerically presented (Table 7) below to determine which types of bacteria are mostly affected by these chitosan samples:

Table 7: Comparative Study of Zone of inhibition (mm ± SD) of two Chitosan samples

Bacteria	Zone of inhibition (mm ± SD)	
	Commercial grade	Local grade
Salmonella typhi	15.21±0.707	13.01±0.71
Escherichia coli	16.21±0.707	13.06±0.35
Shigella dysenteriae	15±0	14.21±0.71
Staphylococcus aureus	12.42±1.414	10.10±0.35

4.5.2 Comparative Study of Commercial grade Chitosan and local grade chitosan against G (-ve) and G (+ve) Bacteria:

Considering the data of OD values, now we will compare numerically the activity of two chitosan samples against G (-ve) and G (-ve) bacteria. The results for IC_{50}, MIC and MBC are numerically presented (Table 8) below:

Table 8: Comparative Study of IC_{50}, MIC and MBC of two Chitosan samples

Bacteria	IC_{50} (mg/mL)		MIC (mg/mL)		MBC (mg/mL)	
	Commercial grade	Local grade	Commercial grade	Local grade	Commercial grade	Local grade
Salmonella typhi (-Ve)	0.463	1.3	3	3	4	4
Escherichia coli (-Ve)	0.433	1.12	2	4	3	5
Shigella dysenteriae (-Ve)	0.55	1.04	2	3	3	4
Staphylococcus aureus(+Ve)	0.73	1.2	3	4	4	5

Two different chitosan samples were investigated to seek out the IC_{50}, MIC and MBC values against respective bacteria. Commercial grade chitosan showed prominent over local grade chitosan.

CHAPTER FIVE

DISCUSSION

DISCUSSION

There are several studies that have explored the antimicrobial activity of different chitosan compounds from various sources by employing diverse testing conditions. However, the discrepancies in the results obtained in many instances were observed, partially because chitosan's in *vitro* antimicrobial activity is relied on various intrinsic and extrinsic factors, such as molecular weight (Mw), degree of deacetylation (DD), pH, and test strains (Raafat & Sahl, 2009). Many researches are mainly focused on exploring the intrinsic and extrinsic factors effect on the antimicrobial activity of chitosan. For example, No et al. (2002) revealed that chitosan showed stronger inhibition effects for Gram-positive bacteria than Gram-negative bacteria and chitosan showed a higher antimicrobial activity than chitosan oligomers. Another study reported that the antimicrobial activity of chitosan with Mw of less than 300 kDa was strengthened as the Mw increased, while the effect on *E. coli* strains was weakened (Zheng & Zhu, 2003).

On the other hand, the different methodologies applied in the in *vitro* susceptibility testing are contributing to these discrepancies also. Disk diffusion, agar dilution, well plate and broth micro dilution methods currently are all available to examine the antimicrobial activity of chitosan in different studies (Liu *et al.*, 2004; Kim & Kim, 2007; Raafat *et al.*, 2008). However, the lack of standardized and reliable *in vitro* susceptibility method makes direct comparison of the results obtained among the numerous studies impossible. There is few data on comparison and evaluation the different methods to determine the antimicrobial activity of chitosan in one study. Therefore, this study appears to be the first study where well plate method was used to determine the antimicrobial activity of chitosan of different sources against total 4 gram-positive and gram-negative bacteria. Also comparing the MICs obtained by well plate method and evaluating the results generated in order to compare whether commercial grade chitosan is more active than local grade chitosan and vice-versa and suggest chitosan's antimicrobial activity.

The antimicrobial activity of chitosan tested in this study differed with the molecular weight and type of bacteria species. According to the data, a higher inhibition activity was observed for commercial and lab grade chitosan against gram negative bacteria while a lower inhibition activity was observed for commercial and local grade chitosan against gram positive bacteria. Differences in molecular weight of chitosan could be the reason resulting in these variations in antimicrobial activity of chitosan and its derivatives. Some study reported that the antimicrobial activity of chitosan with low Mw is higher than the high Mw chitosan against *E. coil* (Liu *et al.*, 2006), while the antimicrobial activity was strengthened with lower Mw chitosan found in present study. Another study demonstrated that low molecular weight chitosan (5-10 KDa) showed the highest antimicrobial activity against pathogenic bacteria (Jeon *et al.*, 2001). That is probably because small molecule with lower Mw is easier to penetrate the cell membrane of bacteria than large Mw of chitosan, which contribute to the leakage of protein and other intracellular components of the microbial cells, ultimately resulting in the impairment of vital bacteria activities. In this study, the antimicrobial activity of chitosan varied from their Mw and

the bacteria tested, except for local grade chitosan with higher Mw showed relatively lower antimicrobial activity against *Salmonella typhi.*

Numerous studies have shown the effect of chitosan on *E. coli* inhibition. In this study also found that commercial grade chitosan with low Mw was able to inhibit the growth of *E. coli* strains at 1% concentration which is in accordance with findings of Wang. He found that concentrations of 0.5% or 1% of chitosan was capable to complete inactivate the *E. coli* growth at pH 5.5 (Wang, 1992). But another showed that lower concentration (0.0075%) of chitosan was enough to inhibit the *E. coli* growth (Simpson *et al.*, 1997). Moreover, local grade chitosan with higher Mw also able to inhibit the growth of *E. coli* but the concentration need to be higher than the commercial grade chitosan.

In addition, according to the MIC values in the current study, commercial grade chitosans showed a higher or similar antimicrobial activity against gram-negative bacteria, including *Escherichia coli, Shigella dysenteriae, and Salmonella typhi.* Similarly, No et al. (2002) found that chitosan generally showed stronger antimicrobial activity with gram-positive bacteria than gram-negative bacteria.

Comparing commercial grade with local grade chitosan it was found that commercial grade chitosan gave increased zone of inhibition than local grade chitosan. If we follow the data of two different grade chitosan then we can see the difference. For commercial grade chitosan solution, the zone of inhibition were recorded 15.21±0.707mm for *S. typhi,* 16.21±0.707mm for *E. coli,* 15±0mm for *Shigella dysenteriae,* and 12.42±1.414mm for *Staphylococcus aureus* (Table 5). On the the other hand it was 13.01±0.71, 13.06±0.35, 14.21±0.71, and 10.10±0.35mm for *S. typhi, E. coli, Shigella dysenteriae,* and *Staphylococcus aureus* respectively (Table 6) for local grade chitosan. After this preliminary screening, solutions were subjected to evaluate IC_{50} values. A range of concentrations were applied to estimate the percentage of inhibition by computing the ODs. Considering the data, it was found that the IC_{50} and MICs value of local grade chitosan is quite higher than the commercial grade chitosan. For IC_{50} value, commercial grade had shown prominent results over local grade. The lowest IC_{50} value 0.433mg/ml was seen against gram negative (*E. coli)* bacteria for commercial grade whereas it was relatively higher, 1.12mg/ml for local grade. For gram positive (*S.aurius)* bacteria, IC_{50} value is quite higher than other gram negative bacteria. It was seen 0.73mg/ml for commercial grade and 1.2mg/ml for local grade.

However two different chitosan samples were investigated to seek out the MIC and MBC values against respective bacteria. Commercial grade chitosan showed lowest MIC (2mg/ml) and MBC (3mg/ml) against gram negative *E. coli* and *Shigella dysenteriae.* These results were much lower than the MIC and MBC of local grade chitosan. The MIC was 4mg/ml and 3mg/ml against *E. coli* and *Shigella dysenteriae* respectively and MBC was 5mg/ml and 4mg/ml against *E. coli* and *Shigella dysenteriae* respectively. Whereas for gram positive *Staphylococcus aureus* the MIC

was 3mg/ml and MBC was 4mg/ml for commercial grade and MIC 4mg/ml and MBC 5mg/ml for local grade chitosan.

The results of this investigation have revealed that Chitosan that was produced in our laboratory is not equivalent to the commercial grade but it poses the quality that has in commercial grade. It has now proved that locally produced chitosan has moderate antibacterial activity. But higher concentration is needed for inhibit total bacterial growth and that is due to higher molecular weight and low Degree of Deacetylation (DD). The variation in the values of DD between the two chitosan samples used in this study was anticipated as their manufacturing process and condition differ from one to another. The dependence of DD on the source and method of purification has been reported the dependency of DD values on the type of analytical methods used (Baxter *et al.*, 1992 Li *et al.*, 1997,). The varied values of DD for the same of chitosan could be ascribed to different sample preparation, experimental condition as well as type of instrument used and its sensitivity (Sabnis *et al.*, 1997, Blair *et al.*, 1987). Also the chemical basis of this method is based on the reaction with the amine group, the presence of protein contaminants remained in the sample during the extraction process could adversely interfere the results.

However, it is possible to maintain the same quality as commercial grade chitosan by carefully handling the production process in the laboratory and indicating that continuation of this study is needed for future work.

CHAPTER SIX

CONCLUSION

CONCLUSION

The production of chitin and chitosan has gained much attention over the last few years. Now a day's, chitosan is being used in highly purified and diversified products which suits different needs in agriculture, medical fields, cosmetic field etc. The main objective of this study was to investigate chitosan (produced by chemical deacetylation) produced locally exclusively from shrimp of our country (as region or source may affect activity) whether possess antibacterial activity and to compare this chitosan's quality with commercial grade chitosan. Qualities of resulted products were evaluated by antibacterial activity test in terms of zone of inhibition, IC_{50}, MIC and MBC.

The results showed that for commercial grade chitosan solution, the zone of inhibition were much bigger in diameter than locally made chitosan. Considering the data of OD values, it was found that the IC_{50} and MICs value of locally made chitosan is quite higher than the commercial grade chitosan. For IC_{50} value, commercial grade had shown prominent results over local grade. It was also seen in case of MIC and MBC also. There was a contradiction among researcher that chitosan is more active against gram positive or gram negative bacteria. The present research demonstrates chitosan was more active against gram negative bacteria than gram positive bacteria that support the findings of Chen YM, Chung YC, Wang LW, Chen KT, and Li SY. They cited that chitosan or its derivatives is more effective for gram-negative bacteria than gram-positive bacteria. The result reveals that chitosan produced by chemical treatment from shrimp (*Penaeus monodon*) waste was an active antibacterial agent, inhibits both gram negative and gram positive bacteria. So it is a broad spectrum antibacterial agent. However, the recovery process itself could still be improved.

In conclusion, this study appears to be the first one where we find a comparative representation of commercial grade chitosan with local one and conformation of the antibacterial activity of locally made chitosan. Also in future this study will help to establish commercially feasible chitosan industry in Khulna region by maintaining the quality of international standard.

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APPENDIX

APPENDIX

1. $\%\ \text{of inhibition} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100\%$

Table 7: Inhibition (%), IC_{50}, MIC an MBC result of fractionated sample of commercial grade chitosan

Conc.(mg/mL)	Inhibition (%)	IC_{50} (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
S. typhi				
0.05	2.08 ± 0.0021	**0.463**	**3**	**4**
0.1	11.72 ± 0.012			
0.25	40.36±0.0007			
0.5	55.6±0.0057			
1	75.8±0.0028			
2	99±0.0028			
3	100			
E. coli				
0.05	8.9 ± 0.002	**0.433**	**2**	**3**
0.1	18.1 ± 0.004			
0.25	31.27 ± 0.005			
0.5	58.12 ± 0.002			
1	80.81±0.005			
2	99.26±0.0028			
3	100			
S. dysenteriae				
0.1	13.74 ± 0.004	**0.55**	**2**	**3**
0.25	42.58 ± 0.003			
0.5	56.97 ± 0.006			
1	78.82 ± 0.0007			
2	99.19±0.003			
3	100			
4	100			
S. aureus				
0.1	6.93±0.02	**0.73**	**3**	**4**
0.25	23.6±0.003			
0.5	40.70±0.007			
1	67.32±0.011			
2	97.4±0.002			
3	100			
4	100			