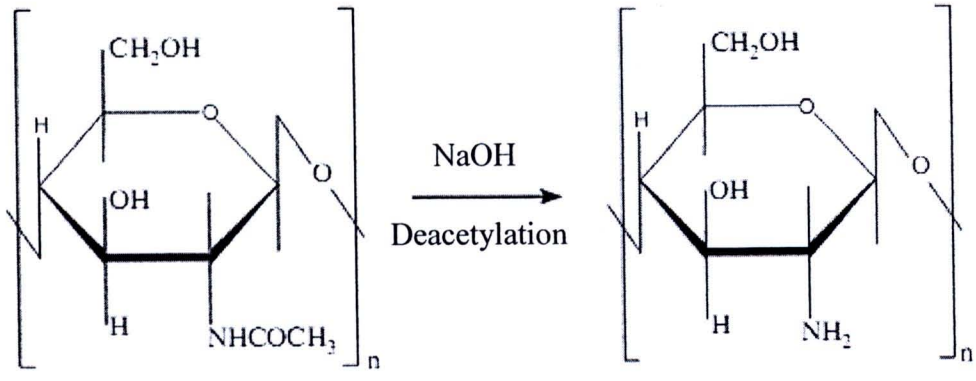
CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Chitosan

Chitosan is a cationic polymer comprising of copolymers of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine as shown in Figure 2.1. Chitosan is obtained from chitin, which is a natural polysaccharide found particularly in the shell of crustacean, cuticles of insects and cell walls of fungi. Chitin is the second most abundant polymerized carbon found in nature.



Chitin Chitosan

Figure 2.1 Chemical structure of chitin and chitosan.

Hirano (1996) presented about the production and consumption of chitin and chitosan, as well as their practical applications in biotechnology. The applications include the use as: 1) cationic agents for polluted waste-water treatment, 2) agricultural materials, 3) food and feed additives, 4) hypocholesteolemic agents, 5) biomedical and pharmaceutical materials, 6) wound-healing materials, 7) blood anticoagulant, antithrombogenic and hemostatic materials, 8) cosmetic ingredients, 9) textile, paper, film and sponge sheet materials, 10) chromatographic and immobilizing media, and

11) analytical reagents. It has been proved to be biologically renewable, biodegradable, biocompatible, non-antigenic, non-toxic and biofunctional. The term chitosan has been used to describe a series of polymers of different degrees of deacetylation defined in terms of the percentage of primary amino groups in the polymer backbone. The degree of deacetylation of typical commercial chitosan is usually between 70% and 95%, with molecular weight between 10 and 1000 kDa. The properties, biodegradability and biological role of chitosan are dependent on the relative proportion between of N-acetyl-amine and D-glucos amine residues. In preparing chitosan, ground shells are deproteinated and demineralized by sequential treatments with alkali and acid, after which the extracted chitin is deacetylated to chitosan by alkaline hydrolysis at high temperature. Production of chitosan via this process is inexpensive, easy and can provide additional control over chitosan final properties. In addition, chitosan molecule has amino and hydroxyl groups which can be modified chemically providing high chemical versatility. Morever, it can be metabolized by certain human enzymes, especially lysozyme. Therefore, it is considered biodegradable. Chitosan is also a bioadhesive material. The adhesive property of chitosan in a swollen state has shown to persist well during repeated contact between chitosan and the substrate, impling that, in addition to the adhesion by hydration, many other mechanisms, such as hydrogen bonding and ionic interactions might also have involved.

Khor et al. (2003) reviewed the extraction of chitin from shellfish sources. More than 40 years have lapsed since this biopolymer had aroused the interest of the scientific community around the world for its potential biomedical applications. Chitin, together with its variants, especially its deacetylated counterpart chitosan, has been shown to be useful as a wound dressing material, drug delivery vehicle and a candidate for tissue engineering. The promise for this biomaterial is vast and will continue to increase as the chemistry to extend its capabilities and new biomedical applications are investigated. It is interesting to note that a majority of these works has come from Asia. Japan has been the undisputed leader, but other Asian nations, namely Korea, Singapore, Taiwan and Thailand have also made notable contributions.

Dutta et al. (2008) summarized all the known methods of formation of chitosan based films with antimicrobial properties and discussed their subsequent applicability in the area of food preservation. Active biomolecules such as chitosan and its derivatives have a significant role in food application area. Chitosan-based films have proven to be very effective in food preservation. The presence of amino group in C2 position of chitosan provides major functionality towards biotechnological needs, particularly, in food applications. Chitosan-based polymeric materials can be formed into fibers, films, gels, sponges, beads or even nanoparticles. Chitosan films have shown potential to be used as a packaging material for the quality preservation of a variety of food. Chitosan has exhibited microbial activity in a wide variety of pathogenic and spoilage microorganisms, including fungi, and Gram-positive and Gram-negative bacteria.

2.2 Electrospinning Process

2.2.1 General description of electrospinning process

The process of electrospinning, namely utilizing electrostatic forces to generate polymer fibers, can be traced back to the process of electrospraying, in which solid polymer droplets are formed rather than fibers. In fact, a number of processing parameters must be optimized in order to generate fibers as opposed to droplets. A typical electrospinning apparatus can be used to form fibers, droplets, or a beaded structure depending on the various processing parameters, such as distance between source and collector or applied potential. In recent works, a greater understanding of processing parameters has led to the formation of fibers with diameters in the range of 100-500 nm, typically referred to as nanofibers. A typical electrospinning set up is consisted of a capillary through which the liquid to be electrospun is forced; a high voltage source with positive or negative polarity, which provides electrical charges to the liquid; and a grounded collector (Figure 2.2). A syringe pump, gravitational force, or pressurized gas are typically employed as a mean to force the liquid through a small-diameter capillary forming a pendant drop at

the tip of the capillary. An electrode from the high-voltage source is then immersed in the liquid or can be directly attached to the capillary if a metal needle is used.

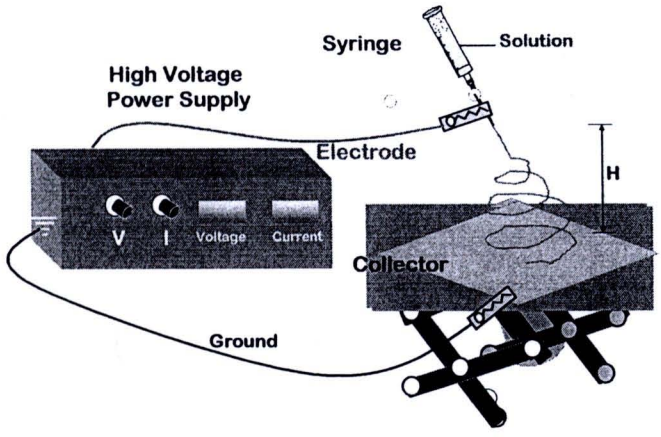


Figure 2.2 Schematic of a typical electrospinning system.

Upon the application of the potential from the high-voltage source, charges are injected into the liquid. Increasing the electric field strength causes the repulsive interactions between like charges in the liquid and causes the attractive forces between the oppositely charged liquid and at the collector to begin to exert tensile forces on the liquid, elongating the pendant drop at the tip of the capillary. As the electric field strength is increased further, a point will be reached at which the electrostatic forces balance out the surface tension of the liquid. If the applied voltage is increased beyond this point a fiber jet will be ejected from the apex of the cone and accelerates toward the grounded collector. Huang et al. (2003) recognized an electrospinning technique for the fabrication of polymer nanofibers. Various polymers have been successfully electrospun into ultrafine fibers in recent years, mostly from polymer solution in solvent and some in melt form. Potential applications based on such fibers, including their uses as reinforcement in nanocomposite, have been realized. Comprehensive reviews have been presented on researches and developments related to electrospun polymer nanofibers including processing, structure and property characterization, applications, and modeling and simulations.

Nowadays, the development of the nanofibers has led to resurgence in interest regarding the electrospinning process due to potential applications in filtration, protective clothing, and biological applications such as tissue engineering scaffolds and drug delivery devices as schematically shown in Figure 2.3.

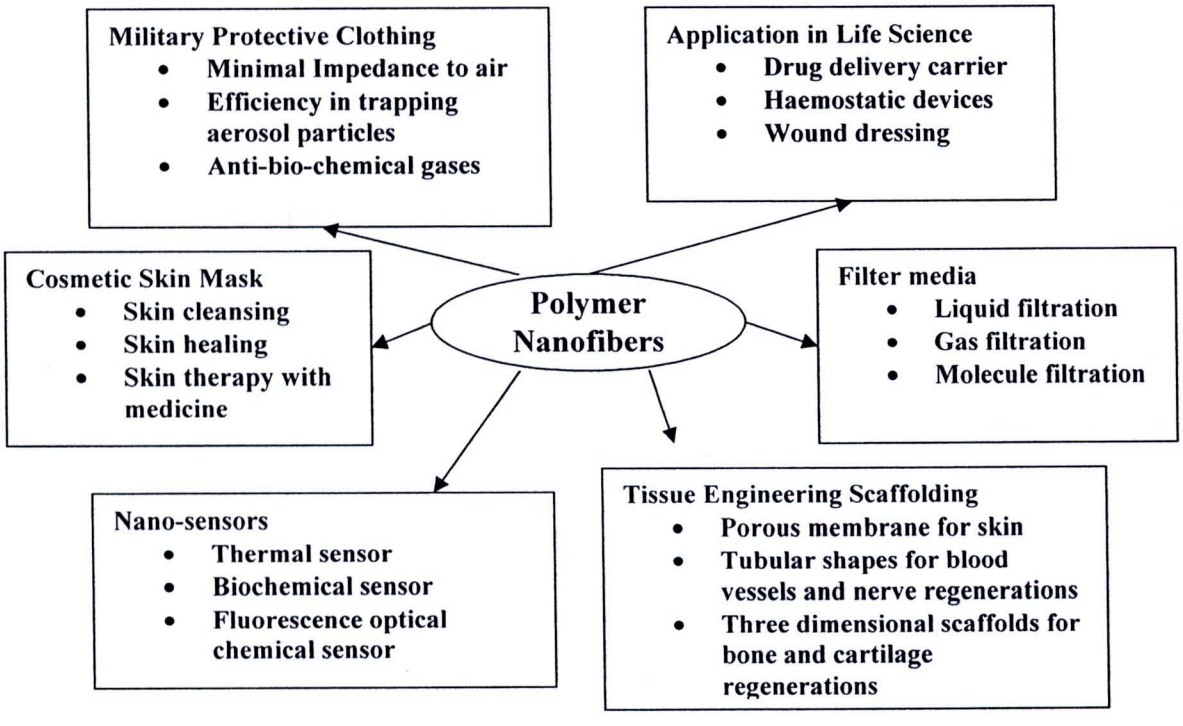


Figure 2.3 Potential applications of electrospun polymer nanofibers (Huang et al., 2003).

Ma et al. (2005) prepared cellulose nanofibers membrane by electrospinning as affinity membrane. Cellulose acetate (CA) solution (0.16 g/ml) in a mixed solvent of acetone/DMF/trifluoroethylene (3:1:1) was electrospun into nonwoven fiber mesh with the fiber diameter ranging from 200 nm to 1 μm. The CA nanofiber mesh was heat-treated at 208 °C for 1 h to improve structural integrity and mechanical strength, and then treated in 0.1 M NaOH solution in H_2O/ethanol (4:1) for 24 h to obtain regenerated cellulose (RC) nanofiber mesh, which was used as a novel filtration membrane.

2.2.2 Electrospinning of chitosan

Geng et al. (2005) produced electrospun chitosan nanofibers from aqueous chitosan solution using concentrated acetic acid solution as a solvent. A uniform nanofibrous mat with average fiber diameter of 130 nm was obtained. The concentration of the aqueous acetic acid higher than 30% was prerequisite for chitosan nanofiber formation, because more concentrated acetic acid in water progressively decreased surface tension of the chitosan solution and concomitantly increased charge density of jet without significant effect on solution viscosity. However, acetic acid solution higher than 90% did not dissolve enough chitosan to make spinnable viscous concentration. Only chitosan of a molecular weight of 106,000 g/mol produced bead-free chitosan nanofibers, while low- or high-molecular-weight chitosans of 30,000 and 398,000 g/mol did not. Average fiber diameters and size distribution decreased with increasing electric field and more bead defects appeared at 5 kV/cm or more.

Bhattarai et al. (2005) presented controllable chitosan/PEO nanofibers with an average diameter from a few microns down to 40 nm that were fabricated by electrospinning. The nanofibers can be deposited as a nonwoven membrane or as a highly aligned bundle. Rheological study combined with SEM characterization revealed that the spinnability of chitosan solution was substantially improved when the solution viscosity was reduced. Introducton of Triton X-100™ as a surfactant and DMSO as a cosolvent into chitosan solution allowed the solution to be spinnable at high chitosan/PEO ratios, and substantially improved the spinnability of the solution and the fibrous structure of as-spun nanofibers. The nanofibrous membrane of chitosan/PEO with a ratio of 9 to 1 retained good structural integrity in water and exhibited better adhesion of chondrocytes than its cast film counterpart. SEM images further confirmed that chitosan/PEO nanofibers promoted the adhesion of chondrocyte (HTB-94) and osteoblast (MG-63) cells
and maintained characteristic cell morphology and thus cell phenotype, and may serve as a potential candidate for bone tissue engineering.

Lei et al. (2006) prepared nanofibers with average diameters between 20 and 100 nm by electrospinning of 82.5% deacetylated chitosan (M_v = 1600 kDa) mixed with poly(vinyl alcohol) (PVA, M_w = 124–186 kDa) in 2% (v/v) aqueous acetic acid. The formation of bicomponent fibers was feasible with 3% (v/v) acetic acid containing up to an equal mass of chitosan. Finer fibers, fewer beaded structures and more efficient fiber formation were observed with increasing PVA content. Nanoporous fibers could be generated by removing the PVA component in the 17/83 chitosan/PVA bicomponent fibers with 1 M NaOH (12 h). Fiber formation efficiency and composition uniformity improved significantly when the molecular weight of chitosan was halved by alkaline hydrolysis (50 wt % aqueous NaOH, 95 °C, 48 h). The improved uniform distribution of chitosan and PVA in the bicomponent fibers was attributed to better mixing mostly due to the reduced molecular weight and to the increased deacetylation of the chitosan.

Homayoni et al. (2009) studied the problem of chitosan with the electrospinning technique. Because of chitosan high viscosity, which limits its spinability, the problem could be resolved through the application of an alkali treatment which hydrolyzes chitosan chains and so decreased its molecular weight. Solution of the treated chitosan in 70–90% acetic acid aqueous solution produced nanofibers with appropriate quality and processing stability. Decreasing the concentration of acetic acid in the solvent increased the mean diameter of the nanofibers. Optimum nanofibers are achieved with chitosan which was hydrolyzed for 48 h. The diameter of these nanofibers (140 nm) was strongly affected by the electrospinning conditions as well as by the concentration of the solvent. FTIR investigations proved that neither the alkali treatment nor the electrospinning process change the chemical nature of the polymer.

2.3 Cell Immobilization

Attachment of cells onto a solid surface is probably the mildest among cell immobilization techniques. In its simplest form, it is also one of the processes that does not result in a high-value added product. The success of the technique depends,

in the first instance, upon the properties of the cells themselves. The natural evolution of species has produced many organisms that are capable of adhering to surface. Some techniques in waste water treatment, e.g. the trickling filter system, have made use of this property for decades. In adsorption, there is generally an initial weak attachment of the cells, which can be easily reversed. This is followed by development of stronger (multiple attachment) binding. Extracellular material produced by the cells is often an important factor in fixing the cells to the adsorption substrate. This step may be followed by a natural entrapment of the cells in a biopolymer matrix.

For industrial processes, the selection of the appropriate support material has been largely fortuitous and has relied mainly upon the organism's own ability to attach to the surface. The more recent active interest in the adsorption process has led to study and development of all kinds of support materials, ranging from simple crude mineral substances to complex ion-exchange derivatives of organic polymers. In addition, the physical form of the support has received considerable attention, particularly with regard to porosity and shape of the material. Techniques for the immobilization of cells can be classified into two methods, i.e., attachment and entrapment.

2.3.1 Entrapment method

Entrapment method employs a variety of matrices that have been used for cell immobilization such as natural polymeric gels (e.g. agar, carrageenan, alginate, chitosan and cellulose derivatives) and synthetic polymers (e.g. polyacrylamide, polyurethane, polyvinyl) Entrapment in natural polymeric gels has become a preferred technique for cell immobilization because of the toxicity problems associated with synthetic polymeric materials. The use of natural gels is, however, limited by their mechanical strength and the lack of open spaces to accommodate active cell growth resulting in their rupture and the release of cells into the growth medium. The advantages accruable from such bio-structures are reusability, non-toxicity,

mechanical strength for necessary support and open spaces within the matrix for growing cells, thus, avoiding rupture and diffusion problems.

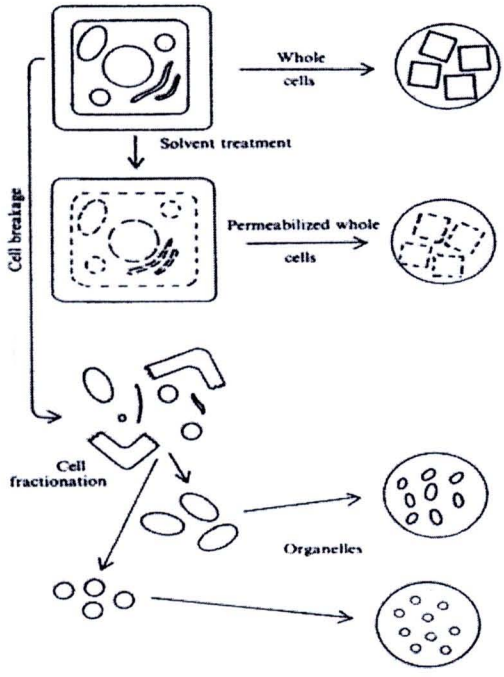


Figure 2.4 The immobilization of the biocatlytic activity of cells by entrapment.

2.3.2 Attachment method

Attachment method has utilized synthetic foams such as polyurethane foams and nylon sponge as a substrate for immobilization. Recently, stainless steel sponges have also been described as a suitable synthetic material for fungi immobilization (Ignacio et al., 2006). One of the advantages found for the use of stainless steel sponges is that dyes do not adsorb onto it. Also, natural supports (i.e., organic materials) can be used to immobilize fungi by the attachment method. Neverthless, the use of natural supports can cause problems in a bioreactor. For example, degradation and loss of the supporting material may lead to blockages in the waste stream and a constant flow of wastewater may lead to permanent losses of enzymes from the fungal extracellular matrix.

2.4 Background for Bacteria Used in This Research

For individual bacterial cell, Gram was a scientist who invented a technique called Gram staining by which bacteria can be divided into two groups based on the chemical and physical properties of their cell walls. The difference in Gram-reaction of these two groups of bacteria is thought to be due to a difference in the structure of their cell walls. Gram-positive cell walls consist of many layers or peptidoglycan and do not posses a lipid outer membrane. Gram-negative cell walls on the other hand have only one or a few layers of peptidoglycan but posses an outer membrane consisting of various lipid complexes. The term Gram-negative or Gram-positive refers to the staining procedure used to determine the cell wall composition of unknown bacteria, which helps determining the appropriate antimicrobial treatment by physicians. It does not refer to the electrical charge of the bacteria. Both Gram-negative and Gram-positive bacteria are negatively charged. The characteristics listed in Table 2.1 are generally presented for the between differences of Gram-positive and Gram-negative bacterial. Schematic cross sections of these structures are shown in Figure 2.5.

Table 2.1 Characteristics of Gram-positive and Gram-negative bacteria.

Components	Gram- positive	Gram- negative
Peptidoglycan	60-100%	5-20%
Lipid content	0.2%	10-20%
Polysaccharide	35-60%	15-20%
Thickness	20-80 nm.	10 nm.
Teichoic acid	Accessory polymers covalently linked to Peptidoglycan	No
Cell wall	1 membrane layer	2 layers

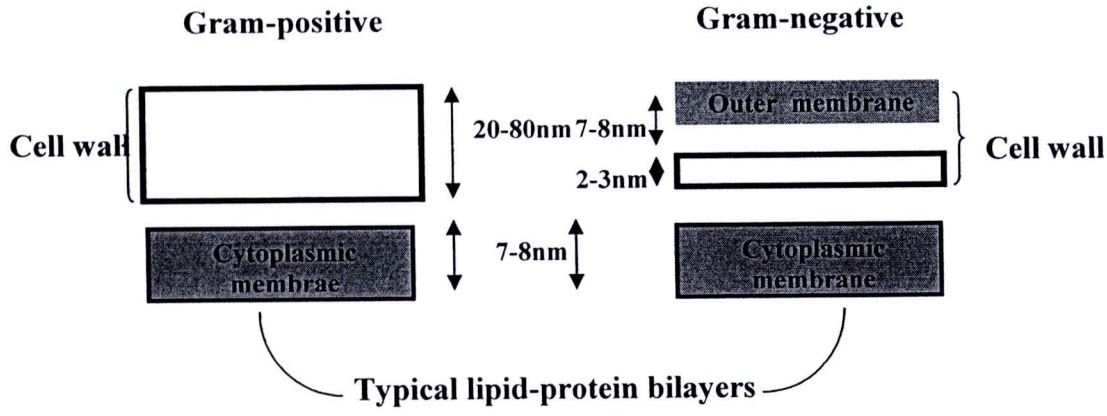


Figure 2.5 Schematic cross sections of cell structure.

2.4.1 Acinetobacter baylyi

Acinetobacter is a Gram-negative bacterium that is readily found throughout the environment including drinking and surface water, soil, sewage and various types of foods. *Acinetobacter* is also commonly found as a harmless coloniser on the skin of healthy people and usually poses very few risks. In this study, *Acinetobacter baylyi* strain GFJ2 was isolated from fruit peel. *Acinetobacter* cells are short, measuring 1.0-1.5 by 1.5-2.5 microns elliptoid during growth. They often become more coccoid during the stationary phase. Cells are found in pairs or small clusters. The groups form smooth, pale colonies on solid media. *Acinetobacter* is strictly aerobic, catalase positive, and oxidase negative. It is the last properly that can be used to distinguish *Acinetobacter* from other infective bacteria. These bacteria can use various selection of organic materials as source for carbon.

2.4.2 Brevibacillus agri

Brevibacillus is a Gram-positive bacteria related to *Bacillus*. *Bacillus* is a genus of rod-shaped bacteria and a member of the division *Firmicutes*. *Bacillus* species are obligate aerobes, and test positive for the enzyme catalase. *Bacillus*

includes both free-living and pathogenic species. Under stressful environmental conditions, the cells produce oval endospores that can stay dormant for extended period. The cell wall of *Bacillus* is a structure on the outside of the cell that forms the second barrier between the bacterium and the environment, and at the same time maintains triangle shape and withstands the pressure generated by the cell's turgor. The cell wall is composed of teichoic and teichuronic acids. The role of the cytoskeleton in shape generation and maintenance is important.

Kongpol et al. (2009) presented about a *Brevibacillus agri* strain 13, which was isolated and characterized as a Gram-positive organic-solvent-tolerant bacterium able to grow at 45 °C. It can tolerate high concentration (5% and 20%, v/v) of various organic solvents with a broad range of log P_{ow} when the organic solvent was provided as a nonaqueous layer. Although it can tolerate a number of aromatic solvents, it cannot utilize them as a sole carbon source. The surface characteristics of cells exposed to organic solvent were investigated using the bacterial adhesion to hydrocarbon test, a contact angle measurement, ζ potential determination, and fluorescence microscopy analysis and compared with that of non-exposed cells. The results showed that although it has a hydrophilic cell surface, it has a unique indigenous cell surface characteristic in which the cells can stabilize solvent-in-water emulsion by adhering to the solvent—water interface of the solvent droplets. The tolerance and predilection of *B. agri* strain 13 toward organic solvents may suggest its potential application as a whole-cell biocatalyst for the biotransformation process of water-immiscible substrate(s).

2.5 Interaction between Cell and Chitosan

Hong et al. (2001) presented the bacterial activities of six chitosans and six chitosan oligomers with different molecular weights (Mws) against four Gram-negative (*Escherichia coli, Pseudomonas fluorescens, Salmonella typhimurium, and Vibrio parahaemolyticus*) and seven Gram-positive bacteria (*Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, and L. bulgaricus*). Chitosan generally showed stronger bactericidal effects

with gram-positive bacteria than gram-negative bacteria. The minimum inhibitory concentration (MIC) of chitosan ranged from 0.05% to > 0.1% depending on the bacteria and Mws of chitosan.

Fierro et al. (2007) showed the effect of chitosan immobilization of *Scenedesmus* spp. cells on its viability, growth and nitrate and phosphate uptake. *Scenedesmus* sp. (strains 1 and 2) and *Scenedesmus obliquus* immobilized in chitosan beads showed high viability after the immobilization process. Immobilized *Scenedesmus* sp. strain 1 had higher growth rate than its free living counterpart. The immobilized cells accomplished 70% nitrate and 94% phosphate removal within 12 h of incubation while the free-living cells removed 20% nitrate and 30% phosphate within 36 h of treatment. Blank chitosan beads were responsible for up to 20% nitrate and 60% phosphate uptake at the end of the experiment. Chitosan is a suitable matrix for immobilization of microalgae, particularly *Scenedesmus* sp., but this system should be improved before being need for water quality control.

Hui et al. (2004) studied about the bactericidal activity of chitosan acetate solution against *Escherichia coli* and *Staphylococcus aureus* was by evaluating the enumeration of viable organisms at different incubation times. Morphologies of bacteria treated with chitosan were observed by transmission electron microscopy (TEM). The interaction of chitosan with synthetic phospholipid membranes was studied. Results showed that chitosan increased the permeability of the outer membrane, inner membrane and ultimately disrupted bacterial cell membranes, with the release of cellular contents. This damage was likely caused by the electrostatic interaction between NH_3^+ groups of chitosan acetate and phosphoryl groups of phospholipid components of cell membranes.

Chung and Chen (2007) investigated the bacterial activity of chitosan by assessing the mortality rates of *Escherichia coli* and *Staphylococcus aureus* based on the extent of damaged or missing cell walls and the degree of leakage of enzymes and nucleotides from different cellular locations. Chitosan was found to react with both the cell wall and the cell membrane, but not simultaneously, indicating that the

inactivation of *E. coli* by chitosan occurs via a two-step sequential mechanism: an initial separation of the cell wall from its cell membrane, followed by destruction of the cell membrane. The similarity between the bacterial profiles and patterns of chitosan and those of two control substances verified this mechanism. The bacterial activity of chitosan could be altered by blocking the amino functionality through coupling of the chitosan to active agarose derivatives. These results verify the status of chitosan as a natural bactericide.

Desai et al. (2009) fabricated nanofibrous filter media by electrospinning of chitosan/PEO blend solutions onto a spunbonded non-woven polypropylenesubstrate. They demonstrated the usage of chitosan-based nanofibrous filter media to effectively filter out heavy metal ions, pathogenic microorganisms, and contaminant particulate media from both air and water media. Heavy metal binding, anti-microbial and physical filtrations efficiencies of these chitosan-based filter media were studied and correlated with the surface chemistry and physical characteristics of these nanofibrous filter media. Filtration efficiency of the nanofiber mats was strongly related to the size of the fibers and its surface chitosan content. Hexavalent chromium binding capacity up to 35 mg chromium/g chitosan was exhibited by chitosan-based nanofibrous filter media along with a 2–3 log reduction in *E. coli* bacteria. After 6 h of contact time, the chitosan blend fibers did show 2–3 log reduction in *E. coli*. Air and water filtration efficiencies of the nanofibrous filter media were measured using aerosol and PS beads suspended in water respectively. It was shown that the nano fibrous filter had high efficiencies which correlated with the fibrous media size and shape. These results indicated the advantage of chitosan nanofibers in filters and its commercial applicability.