CHAPTER 2

LITERATURE REVIEW

2.1 Chitin

Various reviews of chitin have appeared from the turn of the century, one in 1909 reviewing knowledge on the material from 1811. In the late 1930s and early 1940s, there was a considerable interest in chitin and its derivatives, but the production costs and shortage of waste raw materials probably caused loss of this early interest. Substantially increased in the raw material supply and improved technology aroused the interest again in the 1970s when commercial and experimental quantities were made available (Lower, 1984).

The name of chitin comes from the Greek word "chiton", meaning a coat of mail and was apparently first used by Herry Brandconnot in 1811. Chitin is a component of by products from the fishing industry and it is found naturally in the shells of crustaceans, insect exoskeletons, fungal cell walls, microfauna and plankton. The material in fact was first isolated from the skeleton structure of King crabs (Lower, 1984). It is found in association with proteins and minerals, such as calcium carbonate. Different species of crustaceans, shrimp and crab shell wastes have been widely used for the isolation of chitin. Since the biodegradation of chitin is very slow in the crustacean shell waste, accumulation of large quantities of the waste cause a major concern in the seafood processing industry. Disposal of shellfish processing discards, thus, has been a challenge for most of the shellfish-producing countries. Therefore, a production of value added product, such as chitin, chitosan and their derivatives and an application of these products in different fields are of major interest (Shahidi *et al.*,1999).

2.1.1 Molecular structure of chitin

Chitin is a white, hard and inelastic substance. It is a linear polymer of 2-acetyl-2-amino-2-deoxy-β-D-glucose having 1000-3000 basic residues and is also known as N-acetyl-D-glucosamine. It is one of the most abundant polysaccharides in nature and has an analogue of cellulose. The chitin is structurally similar to cellulose, except in the C-2 position the hydroxyl group of cellulose is replaced by an acetamide groups (Kurita, 1997). The structure details of chitin is shown in Figure 1.

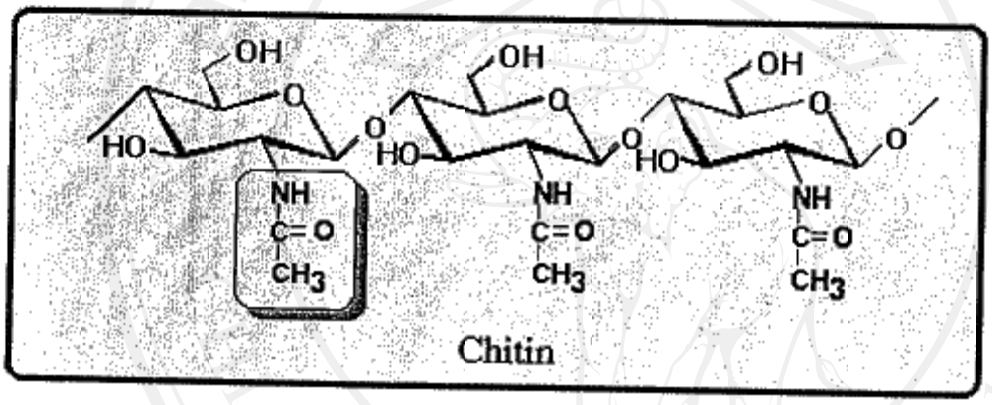

Figure 1 Structure of chitin
(Source: www.user.chollian.net/~chitin/chitikimchi.html)

Muzzarelli (1998) reported details of the chitin structure. Upon crystallization, native chitin becomes a highly ordered biopolymer. Chitin has a strong crystal structure and a high degree of crystallinity. Three crystallographic models of chitin were performed, including α-chitin, β-chitin and γ-chitin. Each of crystallographic models differs in a formation of crystal system and factors effecting a crystal lattice of unit cells within a crystal conformation. These differences are due to the arrangement pattern of molecules in the crystal lattice. The long chain of chitin is arranged in a pleated sheet in the lattice crystal of a unit cell, that can be categorized into two patterns which are parallel and anti-parallel patterns. α-chitin shows an anti-parallel arrangement that involves two anti-parallel chitin chains per unit cell and one 2-

acetamido-2-deoxy-β-D-glucose as an independent residue. This form is found in shrimp and crab shells. While, β-chitin from a squid pen has a parallel arrangement of polymer chains. The chain arrangement of γ-chitin is a mixed pattern of parallel and anti-parallel patterns. In nature, the α-chitin form is found more than the other forms because of its highly intermolecular and intramolecular hydrogen bond formation making the chain very stiff and stable. The crystallographic models of α-chitin and β-chitin are shown in Figure 2.

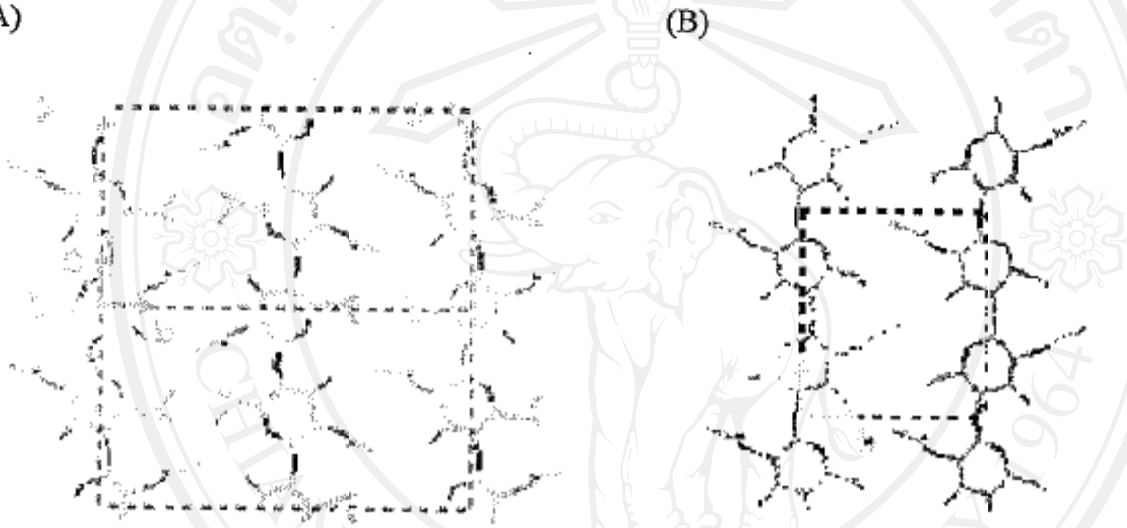

Figure 2 The crystallographic models of α-chitin (A) and β-chitin (B) (Source: www.cermav.cnrs.fr/poster_virtuel/putaux/chitine/introduction1.html)

2.1.2 General properties of chitin

Chitin is a biodegradable and non-toxic compound. It is insoluble in water, dilute aqueous salt solution and most organic solvents. Some chitins are dissolved in strong mineral acids; formic acid, methanesulphonic acid, dichloroacetic acid and trichloroacetic acid. It is partially soluble in the mixtures of *N,N*-Dimethylacetamide (DMA) plus 5% lithium chloride (LiCl) and *N*-methyl-2-pyrrolidinone plus LiCl. In these systems, chitin can be dissolved for up to 5%. The chitin in the DMA plus LiCl solution will become viscous and when the solution is exposed to moisture, the chitin

will be precipitate like a rubber-like solid, which swells enormously in water. Films and filaments may be produced from the DMA plus LiCl solutions, and some reactions can be carried out in this medium in order to modify chitin chemically; for instance, chitin is chlorinated, brominated, sulfated or reacted with acid chloride or with isocyanates. These solvent systems are suitable for the analytical characterization of chitins in terms of polydispersity (Ornum, 1992 and Muzzarelli, 1998).

Chitin isolates are differed from each other in many respects, including: the degree of acetylation, typically close to 0.90 and element analysis, with N content typically close to 7% and the ratio of nitrogen to carbon (N/C) is 0.146 for fully acetylated chitin or 0.153 for the acetylation degree of 0.8. Molecular size and monodispersity of the average molecular weight of chitin *in vivo* are probably in the order of MDa, but chitin isolates have low values due to partial random depolymerization occurring during the isolation and depigmentation steps. The average molecular weight of commercial chitins is 0.5-1.0 MDa (Muzzarelli, 1998).

2.1.3 Chitin production

Shrimp outer skeleton and crab shell are the most suitable biological sources for the production of chitin. Table 1 showed the yields of chitin/chitosan from the shellfish waste. Shrimps from tropical areas are reported to contain chitin 30-60% higher than the shrimps from the ocean in regions with a moderate or cold climate. In South East Asia, however, most of the shrimps are produced by pond cultures by both nature and intensive systems with highly popular breeding techniques that are rapidly expanding. The shrimp is usually processed manually in medium sized farms. This process results in massive amounts of biowaste.

In Thailand, being the largest exporter of frozen shrimps, the business has led to a production of more than 200,000 metric tons of biowaste. For Asia as a whole, the total amount of shrimp biowaste is estimated to be at least ten times more. The sharp increase in the production of shrimp in the South East Asia is not accompanied by a sharp increase in the production of chitin. The majority of the biowaste is discarded or

grounded for animal's feed. In some rural fisherman's villages, the biowaste is left on land, polluting the environment. A small portion of the waste is transported to Japan for further processing. A variable but small portion is processed in the South East Asia itself (Steven, 1996). The worldwide market for chitin derivative products in 2000 is shown in Table 2.

Table 1 Chitin and chitosan yields from shellfish in 2000.

Waste	Shell Dry		Chitin		Chitosan	
	Tons	% Yields	Tons	% Yields	Tons	% Yields
Shrimps Shell 8 tons (wet)	~2	25	~1.25	63	~1	80
Crab Shell 7 tons (wet)	~3.8	54	~ 1.25	33	~ 1	80

Based on data from Sandford (2003).

Table 2 Worldwide market for chitin derivative products in 2000.

Derivatives	Production (Tons)	Yield from chitin	Chitin (Tons)	Chitin for Nutraceuticals (Tons)
Glucosamine	4,000	60%	6,667	5,500
Chitosan	2,000	75%	2,667	1,300
Oligosaccharide	500	50%	1,000	1,000
TOTAL	6,500	-----	10,334	7,800

Based on data from Sandford (2003).

The production of chitin and their derivatives are mainly done by chemical processes at present. The recovery of chitin, as a bulk commodity, depends upon acid and hot alkali treatments to remove minerals and proteins respectively. The process is energy consuming, produces large volumes of aggressive wastes and does not allow recovery of by-products such as proteins, minerals and pigments. Almost as important is the non-specific nature of the chemical processes which gives a product varying in degree of acetylation and molecular weights (Simpson *et al.*, 1994).

a) Chemical method

The process for chitin manufacturing can be varied to some degree, depending upon the local situation. The process of chitin production shows a relationship between individual steps in the chitin production manufactured from shellfish by-products. The first step is done by grinding the shells to a uniform particle size, followed by an extraction of protein. The shell waste consists of three major components: protein, mineral matter and chitin. The percentages of these components are varied depend on species. The first step, however, is a removal of the remaining protein after the meat extraction in the seafood processor. Following the protein removal, the mineral matter is reacted out using a hydrochloric acid. The product of the hydrochloric acid reaction is calcium chloride, which is soluble and is rinsed out from the residual solid that is dissolved during the process. Once the chitin stage is achieved, the residual solid is rinsed and then dried. The reactions of acid and alkali can be seen in Figure 3. The dried chitin can be stored indefinitely until it is converted to chitosan or for some other use (Ornum, 1992 and Fan *et al.*, 2002). The details of general steps are summarized as followed:

1) Deproteinization

This step has been used to deproteinize a shellfish waste for a chitin production by either a digestion with sodium hydroxide (NaOH), refluxing of shells with dimethylformamide or an extraction with a mild sulfurous acid (H_2SO_3) solution at 25°C for 3 hr. By far, the most widely used method for deproteinization involves the

digestion with alkali, or a dilute aqueous solution of NaOH for protein removal, with constant stirring for prolonged hours in an inert atmosphere.

2) Demineralization

The demineralization process with hydrochloric acid (HCl) involves extraction of the shells with the acid at ambient temperature for 3 hr. In this extraction, calcium carbonate ($CaCO_3$) is converted into a soluble calcium chloride ($CaCl_2$) with an evolution of CO_2.

3) Decoloration

The exoskeletons of crustacea contain coloring matters, mainly carotenoids, which do not appear to be complexed with either inorganic materials or proteins, since previous treatments have removed these components. The decoloration of chitin may be achieved by applying of organic solvents, such as ethanol. Other ways of removing these pigments include the use of cold formic acid on the carapace, or a mixture of ammonium sulfate $(NH_4)_2SO_4$ and sulfuric acid (H_2SO_4). The treatment that gives the most acceptable commercial products is extraction of the pigments by refluxing with absolute acetone, followed by a treatment with 75% acetone and then bleaching with 0.315% cold sodium hypochlorite (Simpson *et al.*, 1994).

Shell waste

Grind to small pieces

Deproteinisation

Demineralisation

Decoloration

Chitin

Figure 3 Chitin manufacturing process (Ornum, 1992).

b) Biological method

Hence the recovery of chitin and chitosan could be achieved by an action of fermentation systems, extracted enzymes or enzymes transposed into organisms suited for industrial processes as is shown in Figure 4.

Most biotechnology routes apply an enzymatic deproteination either by fermentation of waste with proteolytic organisms, or the use of added proteases from various sources or a combination of both. Hall (1996) reviewed that the addition of proteases to hydrolyse fish waste proteins is well known and has been applied to crustacean wastes. A range of protease from plant, animal and marine source has been applied, optimum conditions determined and the effectiveness of deproteination assessed. For example, Gogne (1993), used chymotrypsin, papain, and a bacterial protease and chymotrypsin at pH 8 for 72 hr at 40°C with an enzyme : substrate ratio of 7:1000 (w/w). The results showed similar to those of the chemical deproteination. Some specific protease associated with the turnover of chitin in crustacea might be best suited to this approach, but their expense will probably preclude them compared with the cheaper general protease available.

The fermentation requires an organism producing an extracellular protease. Shimahara *et al.* (1982) pioneered this work with *Pseudomonas maltophilia* LC 102 and claimed 95% deproteination of Kuruma prawn *(Peneaus japonicus)* carapace. Bustos and Healy (1994) applied the same organism to scampi *(Nethrops norvegicus)* waste and achieved promising results of 70% deproteination in 40 hr without a complete optimisation. In both routes, the applied enzymes must not lead to depolymerisation or deacetylation.

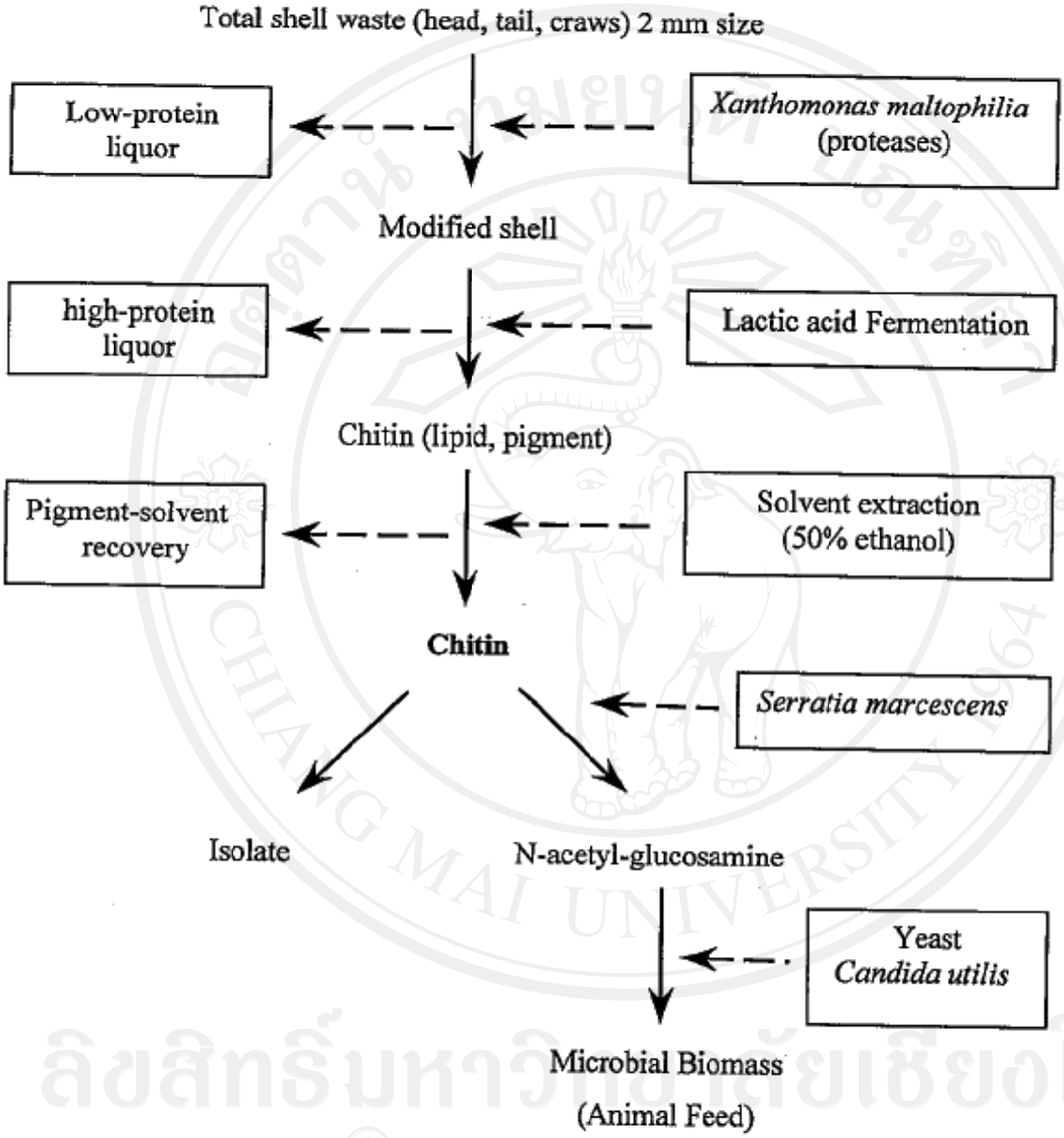

Figure 4 Total bioconversion process (Hall, 1996).

2.2 Chitosan

Chitosan is the name used for low acetyl substituted forms of chitin. It is sticky, colorless and shows a non-newtonian behaviour in solution. In general, chitosan has a N content higher than 7% and a degree of acetylation lower than 0.04. Commercial chitosan may contain insoluble highly acetylated fractions that come from the core of the granules submitted to heterogeneous deacetylation.

2.2.1 Structure of chitosan

Chitosan is composed primarily of glucosamine, 2-amino-2-deoxy-β-D-glucose, known as (1→4), 2-amino-2-deoxy-D-glucose. Up to now, only a few studies on the molecular conformation of chitosan have been reported. The structure detail of chitosan is shown in Figure 5. There is still not one simple model can describe chitosan, in spite of the fact that several models have been published for chitin, α-chitin and β-chitin (Muzzarelli and Muzzarelli, 1998). Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively. Chemical modifications of these groups have provided numerous useful materials in different fields of application (Shahidi *et al.*, 1999).

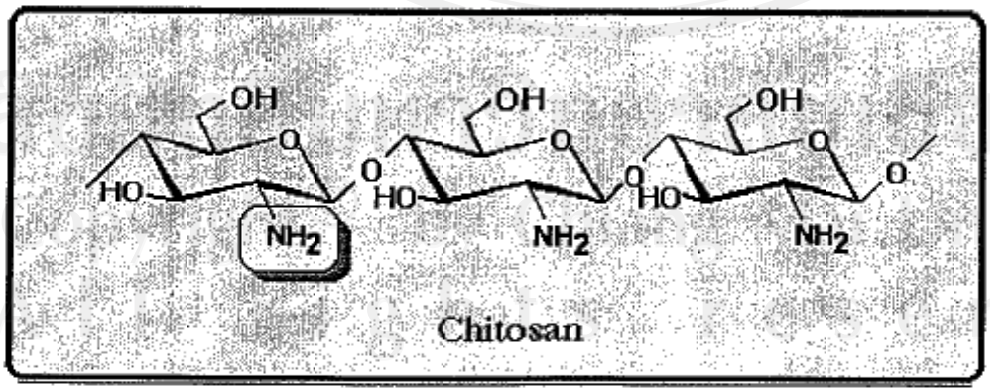

Figure 5 Structure of chitosan

(Source: www.user.chollian.net/~chitin/chitikimchi.html)

2.2.2 Physicochemical characteristic of chitosan

The difference between chitin and chitosan lies in the degree of deacetylation. Generally, the reaction of deacetylating chitin in an alkaline solution cannot reach completion even under a harsh treatment. Commercial chitosan is mainly produced by deacetylating chitin obtained from seashell materials. The quality and properties of chitosan products, such as purity, viscosity, deacetylation, molecular weight, and polymorphous structure, may vary widely because many factors in the manufacturing process can influence the characteristics of the final product (Li *et al.*, 1997).

1) Degree of deacetylation

The degree of deacetylation is one of the most important chemical characteristics of chitosan. A chitosan products have degree of deacetylation between 79-91%. This determines the content of free amino groups in the polysaccharide. Methods for checking the removal of acetyl groups in chitosan include infrared spectroscopy, titration, gas chromatography, and dye adsorption. A review from Li *et al.* (1997), it was suggested that first derivative ultraviolet spectrophotometry at 199 nm was probably the best method for nondestructively and accurately determining the degree of acetylation in chitosan samples. With this technique, the *N*-acetylglucosamine absorbance readings were linearly dependent on concentration and were not influenced by the presence of acetic acid. Another method for analyzing the degree of deacetylation in chitosan was observed under acid conditions. There was a 1:1 stoichiometry for the interaction of amino groups in chitosan with sulfonic acid groups in dye ions. The dyeing agent of C.I. Acid Orange 7 was found to be the most rapid method for determining the deacetylation value in chitosan.

Muzzarelli and Muzzarelli (1998) reviewed that an important parameter to examine chitosan closely is the degree of deacetylation in chitin, i.e. the ratio of *N*-acetyl-D-glucosamine to D-glucosamine units. This ratio has a striking effect on chitin solubility and solution properties. Chitosan is the universally accepted non-toxic *N*-deacetylated derivative of chitin, where chitin is *N*-deacetylated to such an

extent that it becomes soluble in dilute aqueous acetic acid and formic acid. In chitin, the acetylated units prevail (degree of acetylation typically 0.90). Chitosan is the fully or partially *N*-deacetylated derivative of chitin with a typical degree of acetylation of less than 0.35. To define this ratio, attempts have been made with many analytical tools, which include IR spectroscopy (Figure 6), pyrolysis gas chromatography, gel permeation chromatography and UV spectrophotometry, first derivative of UV spectrophotometry, ^{1}H-NMR spectroscopy, ^{13}C solid state NMR (Figure 7), thermal analysis, various titration schemes, acid hydrolysis and HPLC, separation spectrometry method and more recently, near-infrared spectroscopy.

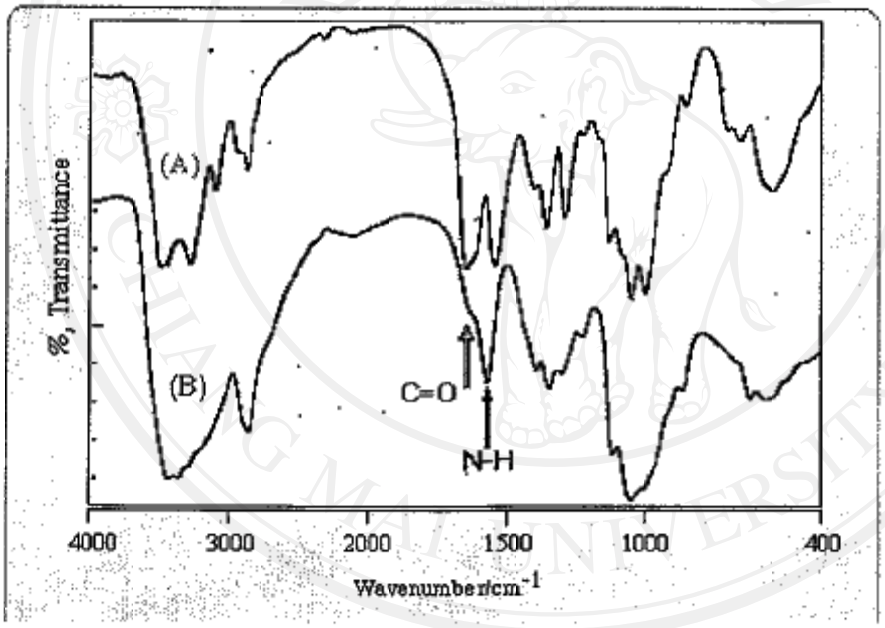

Figure 6 IR spectra of Chitin (A) and Chitosan (B)
(Source: www.user.chollian.net)

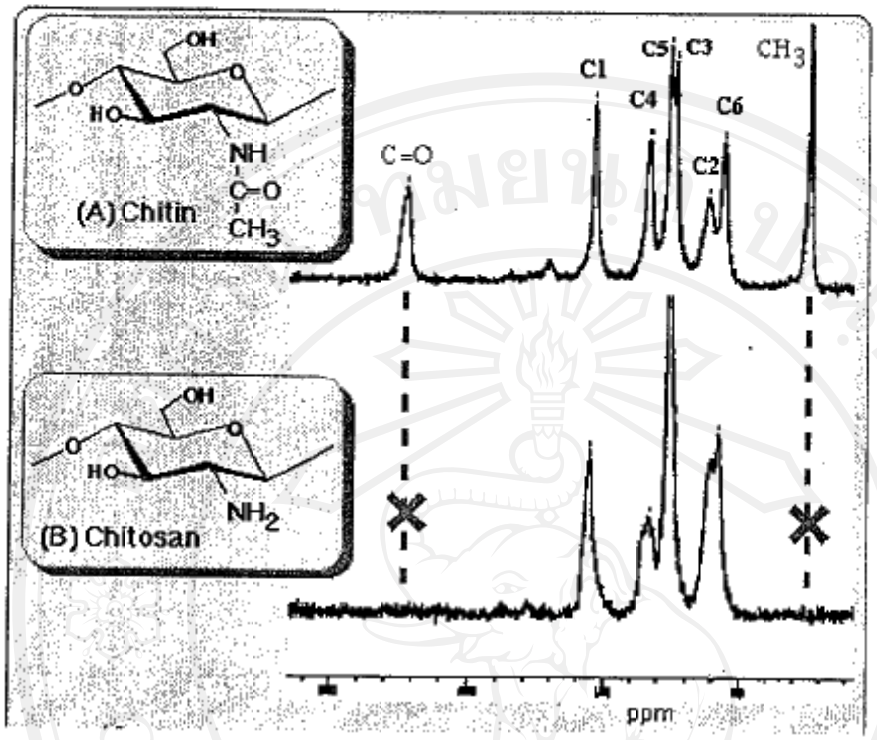

Figure 7 ^{13}C NMR spectra of chitin (A) and chitosan (B) by CP-MA (Source: www.user.chollian.net)

2) Molecular weight

Li *et al.* (1997) reviewed that the molecular weight of native chitin is usually larger than one million dalton while commercial chitosan products fall between 100,000 and 1,200,000 Da. During the manufacturing process of chitosan, harsh conditions can lead to degradation of the chitosan product. For example, with the Horowitz method, after a 30 minutes treatment at 180°C, a chitosan sample with a chain length of only twenty units was obtained. In general, factors such as dissolved oxygen, high temperature, and shear stress, can cause a further degradation of chitosan products. Dissolved oxygen can slowly degrade chitosan. At temperatures over 280°C, thermal degradation of chitosan takes place and the polymer chains

rapidly break down. On the other hand, shear degradation caused by hydrodynamic forces favors breakage of long-chain chitosan molecules to a critical length. Below this point, shearing does not have any further effect on the molecular weight distribution. This process can provide a relatively narrow molecular weight distribution. Several procedures for preparing low molecular weight chitosan have been reported. For example, Takeda *et al.* (1989) obtained a colorless sample with an average molecular weight of only 60,000 Da by treating chitosan solution with 0.05% chlorine dioxide (ClO_2). In contrast, Kushino and Orihara (1988) treated a chitosan solution with enzymes such as papain, cellulase, and acid protease and obtained a product with an average molecular weight of 36,000 Da.

The molecular weight of chitosan can be determined by methods such as chromatography, light scattering, and viscometry. Among these method, viscometry is the most simple and rapid method for the determination of molecular weight. Although in 1978, Bough *et al.* indicated that the molecular weight of chitosan was not always directly related to its viscosity because of the presence of colloidal particles, the viscometry method is still widely employed for determining the relative molecular weight of chitosan. In 1988, Maghami and Roberts tested a series of chitosan samples having relatively the same molecular mass distribution but different extents of *N*-acetylation by using a Mark-Houwink equation. It was shown that the equation was applicable to chitosan with the *N*-acetylation range from 0% to 40%.

Chitosan molecular weight distributions have been obtained using a High Performance Liquid Chromatography (HPLC). The weight-average molecular weight (Mw) of chitin and chitosan has also been determined by light scattering. A laser light-scattering spectrometry provides absolute measurements of Mw of chitosan in an acetate buffer. Viscometric constants are available for computing the Mw from the intrinsic viscosity of an aqueous chitosan and from an organic chitin solution.

In 1992, Audy and Asselin have described a gel electrophoresis procedure for the determination of the hydrolysis products of chitosans. Electrophoresis is performed on a polyacrylamide slab gel in the presence of 7 M urea and 5.5% acetic acid; chitosan

migrates as a polycation. Chitosan was stained with Calcofluor White M2R or with Coomassie Brilliant Blue R-250. Furthermore, converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution, which in turn influences the agglomeration. The natural chitin has a molecular weight of more than $1x10^6$ Da, while chitosan has a range of molecular weight between $1x10^5$ to $1.2x10^6$ Da depending on the steps in the chitosan manufacturing (Kumar, 2000).

3) Viscosity

The viscosity of chitosan in solution is influenced by many factors, such as the degree of polymer deacetylation, molecular weight, concentration, ionic strength, pH and temperature. Li *et al.* (1997) reviewed that in general, as the temperature rises, the viscosity of the polymer solution decreases. However, a pH change in the polymer solution may give different results depending on the type of acids employed. With acetic acid, the viscosity of chitosan tends to increase with decreasing pH, whereas with hydrochloric acid the viscosity decreases when the pH is lowered. In 1984, Kienzle–Sterzer *et al.* studied the persistence length of chitosan molecules in dilute solution. They indicated that the intrinsic viscosity of chitosan was a function of the degree of ionization as well as ionic strength. An increase in either chitosan ionization or ionic strength would decrease the polymer solution's intrinsic viscosity. Based on their study, they proposed that chitosan in dilute solution behaves as a non-draining worm-like molecule, its molecular configuration being dictated by electrostatic interaction between polyion and counterious.

Kubota and Kikuchi (1998) reviewed that if the pH of a solution is increased, the intermolecular and intramolecular electrostatic repulsions between cationic charges are reduced because chitosan has an amino group. This allows the chitosan chains to come closer and thus lowers the hydrodynamic volume of chitosan molecules. This effect may enhance the interchain and intrachain of the hydrogen-bonding. Similarly as the ionic strength increases, the intrinsic viscosity decreases due to the shielding effect of the counterions.

4) Solubility

Chitosan is insoluble in water, alkali, and organic solvents, but it is soluble in most solutions of organic acids when the pH of the solution is less than 6. Acetic and formic acids are two of the most widely used acids for dissolving chitosan. Some dilute inorganic acids, such as nitric acid, hydrochloric acid, perchloric acid and phosphoric acid, can also be used to prepare a chitosan solution but only after prolonged stirring and warming. Sometimes, however, a white gel-like precipitate is formed in a nitric solution after polymer dissolution. Chitosan is soluble in hydrochloric acid and nitric acid in the range of 0.15-1.1%, but it is insoluble in 10% acid solution (Li *et al.*, 1997). Chitosan is insoluble in any common organic solvents (e.g. dimetylformamide and dimetylsulfoside) and at hydrogen ion concentrations above pH 6.5. Its amino group will ionize by the range of pK_a of 6.2-6.8, the coefficiency of ionization depends upon the density of polymer ion (Ornum, 1992). In addition to dissolve chitosan in acid solutions, mixtures such as dimethylformamide with dinitrogen tetroxide at a ratio of 3:1 also appear to be good solvents for chitosan.

A reviewed from Li *et al.* (1997) repeated that water-soluble chitosans, dissolved in the absence of acid, are frequently required when acids are undesirable substances in products such as cosmetics, medicines, and food. It has been shown that chitosan with 50% deacetylation from a homogeneous processing is water-soluble. The reason for this effect was found to be the presence of a small proportion of scarcely deacetylated macromolecules contained in the chitosan sample prepared by heterogeneous processes. These molecule, which came from the more crystalline regions of the polysaccharide, tended to remain aggregate in solution and were more resistant to chemical reactions, whereas, under a homogeneous condition, an uniform distribution of *N*-acetyl groups decreased the polymer crystallinity and increased the solubility. Other methods for improving the water solubility of chitosan have been investigated. In 1988, Kushino and Asano reported a procedure for preparing a water-soluble chitosan salt. Chitosan was dissolved in an aqueous solution, concentrated (to 10%) by evaporation, and finally spray-dried at 175°C to provide a water-soluble salt.

Chemical modification of chitosan, on the other hand, provides an alternative way to improve the biopolymer's water solubility.

5) Coagulating ability

Chitosan is a good coagulating and flocculating agent. It has many amino groups that can ionize to be positively charge and bind to substances which negatively charge such as protein, solids, dyes and other polymers. However, chitosan behaves quite differently with respect to transition metal ions. In the review of Li *et al.* (1997), they indicated that the nitrogen in the amino group of the chitosan molecule acts as an electron donor and is presumably responsible for selective chelating with metal ions. The complexation of the chitosan nitrogen with metal ions was confirmed by Ogawa and Oka in 1984. They proposed that a metal ion (i.e. cupric ion) could coordinate with four amino groups in the D-glucosamine dimer residue of the chitosan chain. The free amino group in chitosan was considered to be much more effective for binding metal ions than the acetyl groups in chitin. This leads us to consider that the higher free anion group content of chitosan should give higher metal ion adsorption rates. However, Kurita *et al.* (1979) indicated that the adsorption ability of chitosan is dependent on many other factors, such as crystallinity, deacetylation, and affinity for water. In their studies, they found that samples with 55% deacetylation, prepared by a homogeneous hydrolysis process, showed the highest adsorption ability. These sample were amorphous and had the best solubility in water. The effect of structure on the adsorption ability was also revealed. The ability of metal adsorption, for example, could be enhanced by cross-linking, controlled *N*-acetylation, and complexation with other polymers like glucan.

2.2.3 Chitosan production

The industrial production and use of chitosan have been steadily increased since the 1970s. The production of chitosan increased 37% each year from 1978 to 1983. In 1994, the total world consumption of chitin and chitosan was estimated to be more than 1,000 tons with 800 tons being used (Hirano, 1996). By 1997, two US companies produced chitosan from crab waste (Leffler, 1997). According to the most recent OPD Chemical Buyers Directory, chitosan is now manufactured or distributed by 45 companies (Tilton, 1998). The cost price for technical chitosan is about US$ 15-20/kg. For medical purposes, a much better quality of chitosan can be obtained but the price is around US$ 200/kg.

Chitin prepared from shellfish waste may be processed further into chitosan by deacetylation. Deacetylation may also be achieved by either chemical or biological method. The chitosan manufacturing process is shown in Figure 8.

a) Chemical method

Some of the limitations with the chemical approach for preparing chitosan from chitin include the following:

(1) It requires high temperature and therefore entails high energy cost.

(2) It involves the use of high volumes of concentrated NaOH, which is caustic.

(3) There is a simultaneous production of liquid waste containing protein and other non-protein nitrogenous compounds.

It has been suggested that deacetylation proceeds to about 70% with the first hour of treatment with 50% NaOH solution at 100°C, but it progresses only gradually after this, reaching about 80% in 5 hr. Further, deacetylation can rarely be extended beyond 80% unless the alkali fusion procedure is applied along with fractionation. Extended treatments with hot concentrated NaOH solution could result in an almost completely N-deacetylated product (i.e. about 90% deacetylation), however, such a treatment is

also accompanied by degradation of the molecular chain. Since a treatment with alkali leads to depolymerization of the material, deacetylation may be carried out in an inert atmosphere to alleviate the depolymerization problem and thereby recover chitosan of higher viscosity. A method for preparing chitosan having a degree of deacetylation of up to 100% by alkali treatment from chitin could be done with repeated washing of the intermediate products with water (Simpson *et al.*, 1994).

b) Biological method

The conversion of chitin to chitosan is being tried using a fungal enzyme chitin deacetylase. A number of microorganisms have been shown to produce a chitin deacetylase including *Mucor* spp., *Absidia* spp., *Aspergillus* spp., *Rhizopus* spp., *Saccharomyces* spp. and *Colletotrichum* spp. In spite of intensive research at different laboratories in the world, an efficient system for enzymatic deacetylation has not become available. It is expected that bioprocess technology for the isolation of chitin and chitosan will not only lead to a better quality product but also to a sustainable and more economical production system (Steven, 1996). The conversion of chitin to chitosan by chitin deacetylases (CD) has been extensively studied and several organisms with this ability have been screened by Shimahara *et al.* (1989). In addition, chitin deacetylases have been extracted from several sources for direct application to chitosan production. Generally, the fermentation or enzymatic approaches give low yields but highly deacetylated forms of chitosan. Low yields may be overcome by process optimisation techniques. Chitosan may also be prepared from chitin using deacetylating enzymes from microbial sources. The presence of these enzymes was probably inferred from the occurrence of chitosan in the cell walls of *Phycomyces blakesleeanus* and *Mucor rouxii*. Thus, preparation of chitosan from chitin by biological methods maybe accomplished by fermentation, whereby cultures of chitin deacetylase producing microorganism are inoculated in a media containing chitinous substrate, as demonstrated with *Aspergillus niger* mycelia (Simpson *et al.*, 1994).

N-acetyl-glucosamine (NAG) is the basic monomer of chitin and chitosan and its production by the action of a chitinase has been proposed culminating in a full process design. The chitinase produced by *Serratia marcescens* and yeast *Pichia kudriawzeii* was able to utilize the NAG. In theory, this route in conjugation with a biotechnology-based recovery of chitin, as proposed above, leads to full conversion of a waste to a usefull biomass product. Since shellfish wastes are rich in various other useful nutrients and biochemicals e.g. protein, carotenoid pigments and minerals apart from chitin and chitosan, a recovery scheme based primarily on enzymatic methods and mild chemical treatments has been proposed to ensure maximum economic use of this abundant waste material or by a direct treatment of chitin polymers with extracts of microbial chitin deacetylase enzymes. These enzymes were first recovered by Araki and Ito in 1974 from *Mucor rouxii* (Simpson *et al.*, 1994).

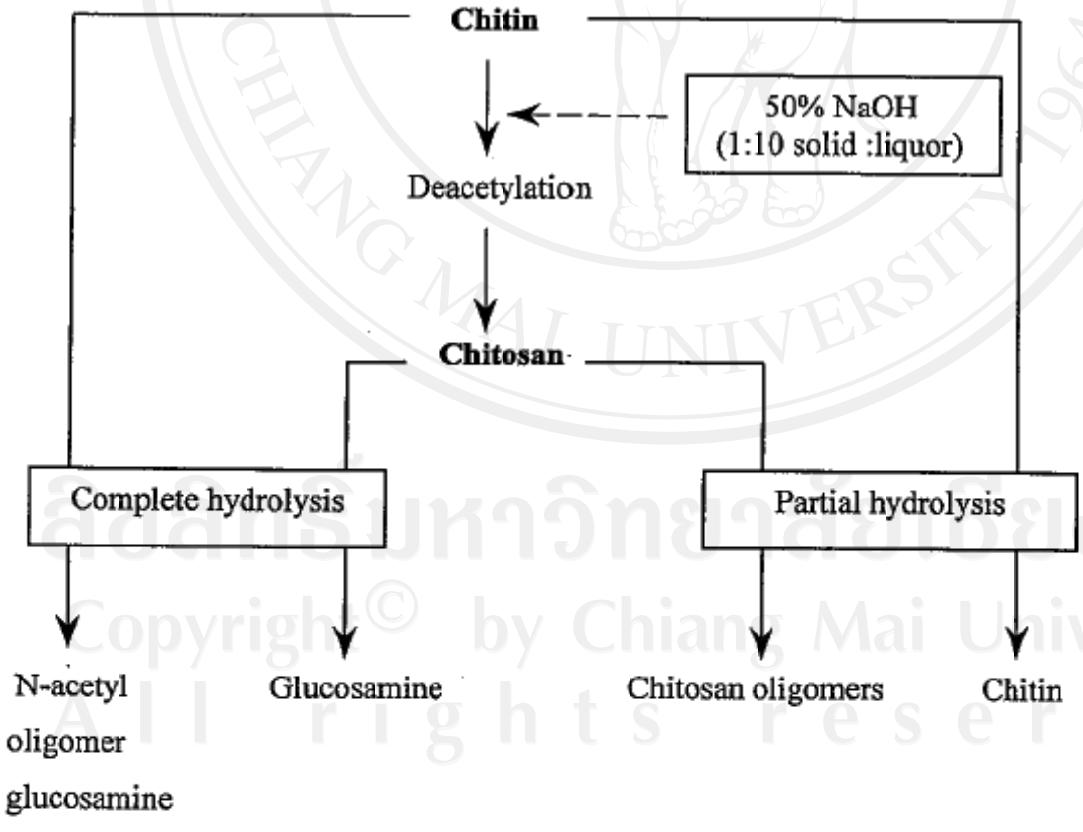

Figure 8 Chitosan manufacturing process (Shahidi *et al.*, 1999).

2.2.4 Application of chitosan

In 1970s, the major applications of chitosan were centered on sludge dewatering and metal ion chelation. Many potential products using chitosan have been developed. The total estimated consumption of chitosan in 1994 was 800 tons/year, as shown in Table 3 (Steven, 1996). For the present trend, commercial applications of the natural polymers, chitosan are increasing. The functional use of chitosan, and its worldwide markets will be discussed. Major application areas include: fat reducing dietary supplement, wound dressings, tissue engineering, immunopotentiation, anti-bacterial, anti-odor, oral and nasal drug delivery, lotions, creams and shampoos, functional foods, water purification, agricultural biocide, fibers and textiles, paper, oilfield drilling/recovery additive, etc. The difference in value between the applied products and the low-cost polymer is one of the main driving force pushing studies on new application of chitosan (Li *et al.*, 1997).

Table 3 The consumption of chitosan in the worldwide market.

Application	Tons/year
Flocculating and dewatering agents	350
Living waste-water treatment (200)	
Food manufacturing waste-water treatment (100)	
Sugar manufacturing etc. (50)	
Food and feed additives	185
Health foods (80)	
Animal feed (60)	
Food processing (45)	
Agricultural materials	120
Textiles and fabrics	50
Cosmetic ingredients	40
Medical and veterinary materials	20
D-Glucosamine and oligosaccharides	13
Paint and dyeing additives	10
Natural thickners	10
Beads, films, gel, sponges etc.	1
Chromatographic media and chemical reagents	1
TOTAL	800

Based on data from Steven (1996).

1) Water Treatment and Papermaking

As environmental protection is becoming an important global ploblem, the relevant industries pay attention to the development of technology which does not cause environmental problems. One of the earliest applications of chitosan was for chelating harmful metal ions, such as copper, lead, mercury and uranium, from waste water. Chitosan is considered to be safe for human use. Studies on the chelating ability of chitosan began about twenty years ago. In 1973, Muzzarelli documented the effectiveness of chitin, chitosan and other chelating polymers for their ability to chelate transition metal ions. He indicated that chitosan was a powerful chelating agent and exhibited the best collection ability of all the tested polymers because of its high amino group content. In another interesting study dealing with the recovery of uranium from water, Hirano *et al.* (1984) showed that 40-74% of the available uranium could be recovered from river and lake water, and 3% from seawater. The extremely low recovery rate from seawater might have been caused by the presence of large quantities of other metal ions, which would have competed with uranium for complexing with chitosan. Recent studies showed that the chelating ability of chitosan could be further improved by homogeneous hydrolysis, cross linking, controlled *N*-acetylation, and complexation with other polymer like glucan. Examples of adsorption of metal ions by chitosan from different crustacean sources are presented in Table 4.

From the reviewed of Li *et al.* (1997), chitosan is known to be an excellent coagulating and flocculant agent due to the high density of amino groups on the polymer chain that can interact with negatively charged substances, such as proteins, solids and dyes. In 1978, Wu *et al.* investigated the effectiveness of different chitosans for removing proteins from whey cheeses. They found that the effectiveness of chitosan in coagulating solids and proteins was inversely proportional to the molecular weight of the polymer. The optimal concentrations for the chitosans tested ranged from 70-150 mg/ml, with a resultant reduction to more than 90% in the turbidity. In another area, adsorbants (prepared by mixing chitosan with cellulose fibers) were employed for sorption of dyes from wastewater. The list of dyes that could be adsorbed by chitosan is shown in Table 5. The chitosan-cellulose fibers were

able to adsorb 264 mg/g reactive dyes and 421 mg/g acid dyes compared with an adsorption capacity of less than 80 mg/g dye for coconut charcoal. Furthermore, in 1989, No and Meyers successfully used crawfish chitosan for recovery of organic compounds and coagulation of suspended solids from seafood-processing streams. In one study, they used two amino-Cu crawfish chitosan columns to recover amino acids from waste water. The two columns were operated in turn to absorb the amino acids and elute Cu. Recoveries were reported to be 15% for glycine, 21% for proline, and 47-93% for the other amino acids.

Table 4 Rate of adsorption of metal ions by chitosan from different sources.

Metal ions	mg of metal adsorbed per g of chitosan [a]			
	Crab	Prawn	Squid	Squilla
Fe^{3+}	17.6/23.4/23.4	11.7/15.7/23.4	17.6/20.5/23.4	14.6/17.6/29.3
Co^{2+}	4.1/4.7/5.9	5.3/7.1/7.1	4.7/4.7/7.4	4.7/4.7/4.7
Ni^{2+}	35.2/55.2/64.6	47.0/64.6/82.1	29.3/64.6/82.1	29.3/52.8/76.3
Hg^{2+}	241/281/321	311/331/341	321/346/366	351/381/411
Cu^{2+}	21.1/36.2/39/3	30.2/42.3/66.4	27.2/45.3/51.3	42.3/60.4/60.4

[a] First number = 30 min, second = 60 min, third = 120 min of treatment. 1 g of chitosan powder was added to 100 ml of 0.1 M solution of the metal ions.
Based on data from Knorr (1991).

Table 5 Dyes that could be adsorbed by chitosan.

C.I. Number	Commercial Name	Chemical Class
Acid Blue 193	Lanasyn Navy S-DNL (S)	1:2 Chromium complex of monoaza
Acid Blue 40	Nylosan Blue E-2GL (S)	Anthraquinone
Direct Yellow 44	Solophenyl Yellow 5G (CGY)	Diazo
Direct Blue 78	Solophenyl Blue 4GL (CGY)	Triazo

Based on data from Kim *et al.* (1997).

Several early applications of chitosan in the pulp and paper industries have been reviewed by Muzzarelli in 1983 (Li *et al.*, 1997). These included a surface treatment of paper with a 1% chitosan solution to increase its bursting strength and folding endurance, without any decrease in the paper's brightness. With a development of new technology, such as color photocopying, different types and higher quality paper and fibers are required. In the fiber industry, for example, a treatment with a 0.5% chitosan solution improved the fiber's color fastness (Tokunaga, 1988). In the paper making area, Aizawa and Noda (1988) placed a chitosan layer on a photographic paper to increase its antistatic properties since an electrostatic discharge can cause a serious decrease in picture quality. The surface resistance to electrostatic charging was reported to have increased more than ten thousand times after the treatment with the chitosan solution.

2) Medical and Pharmaceutics

Although chitosan by itself is hemostatic (stops bleeding). By utilizing the hemostatic effect, chitosan bandages and sponges were prepared for surgical treatment and wound protection (Kibune *et al.*, 1988 and Motosugi *et al.*, 1988). In contrast, Tokura *et al.* (1988) immobilized partially sulfated chitosan oligomers on the surface of molded chitosan materials, such as artificial blood vessels and fibers, to produce antithrombogenic medical products. There are other medical applications that have also been investigated. Some derivatives, such as sulfated chitosan, are blood anticoagulants, as shown in Table 6. Li *et al.* (1997) reviewed that anticoagulant membranes for blood ultrafiltration can be prepared by immobilizing bioactive molecules like PGE1, hirudin, heparin, or tithrombin on chitosan In 1988, Uragami and Mori formed membranes by complexing quaternized chitosan with sodium heparin and found that the ultrafiltration rate of the membranes could be decreased by increasing the degree of heparinization. The *in vivo* tests showed that no thrombus formed on the membrane.

Chitosan has also been found to be effective against cholesterol. Various hypolipemic formulations containing chitosan, including particles, powders, solutions and injections, were prepared for oral administration (Suzuki *et al.*, 1988). In oral tests on mice, these medicines effectively decreased the blood cholesterol level up to 66%. The hypocholesterolemic activity of chitosan was probably due to an inhibition of micelle formation (Muzzarelli, 1985). Variously called "the fat magnet", "the fat trapper", or "a sponge", chitosan is thought to inhibit fat digestion by dissolving in the stomach, emulsifying fat in the stomach contents, and forming a gel in the intestine which entraps the fat and prevents intestinal absorption (Kanauchi *et al.*, 1995). Some sources of chitosan diet aid that are available for consumers via internet are listed in Table 7.

Table 6 Blood anti-coagulant and LPL (Lipoprotein Lipase)-releasing activities of some sulfated derivatives of chitosan and chitin

Sulfated derivative	Mw ($x10^3$)	D.S. for sulfate	Anticoagulant activity[a] (units mg^{-1})	LPL-releasing activitiy[b] (mol eq/l of plasma)
Chitin	26	2.0	355 (2.0)	100 (0.1)
Chitosan	12-22	1.7	239 (1.4)	3200 (3.4)
N-Desulfated chitosan	22	0.7	n.d.[c]	Inactive
N-Hexanoyl Chitosan	27	1.8	n.d.[c]	Inactive
CM-chitosan	245-540	0.6	26 (0.10)	700 (0.7)
Heparin	21		174 (1.0)	950 (1.0)

[a] The dosage was 0.1mg/kg of body weight, and the maximum LPL activity is shown in mole equivalents of free acids per liter of plasma after incubation at 37°C for 30 min, and the activity relative to heparin is shown in parentheses.

[b] With respect to the activated thromboplastin time (ATPP); the activity relative to heparin is shown in parentheses.

[c] Not determined

Based on data from Hirano (1996)

Li *et al.* (1997) reviewed that chitosan could selectively aggregate leukemia tumor cells *in vitro*, producing a dense aggregate and inhibiting cell growth. In 1984, Hadwiger *et al.* confirmed the interaction of chitosan with DNA and built a model to explain the resistance response of host cells to the pathogen. In 1989, Bronner *et al.* observed that chitosan, which has a strong affinity for DNA, was transmitted from the mite to the nuclei of the punctured cells within one hour. The special affinity of chitosan for biomolecules has been utilized to reduce side effects of drugs. For example, in 1989, Ouchi *et al.* covalently linked an antineoplastic prodrug, 5-fluorouracil, to chitosan with hexamethylene spacers via carbamoyl bonds. In mice tests, the chitosan bound prodrug exhibited an enhanced inhibition effect against tumor cells without displaying any apparent toxicity. The new drug was reported to be effective for treating leukemia by decreasing the tumor weight. It also showed a fivefold growth inhibition ratio for Meth-A fibrosarcoma over the prodrug itself.

Chitosan, as a polymer, is a good additive in a preparation of biomaterials. Allan *et al.* (1984) indicated that it maybe possible to employ chitosan in creating soft contact lenses either by casting or molding procedures. Contact lenses are required to be optically clear, safe, wettable and permeable. Among these properties, gas permeability is particularly important because oxygen must be transported from the tear fluid to the eye surface, whereas carbon dioxide needs to diffuse back from the eye surface to the fluid. Several techniques for preparing contact lenses have been reported resulting in chitin *n*-butyrate lenses, chitosan lenses and blue chitosan lenses.

Chitosan was also employed for making artificial skin. The artificial chitosan dermis was tested by inserting it into a cut on the back of rats. A normal inflammatory reaction was observed after 2 days, followed by a cell colonization after 7 days. Finally, a low molecular weight chitosan (Mw = 1,500 Da) was found to be useful for treating a bone disease. The low molecular weight polysaccharide effectively increased the alkaline phosphatase activity, thereby accelerating bone formation (Namita *et al.*, 1988).

Table 7 Some chitosan products sold to consumers over the internet.

Name	Suppliers
1. Chito-Max	Body Wise
2. ChitoPro	Utimate Nutrition
3. Mega Chitosan Plus	Utraceutic
4. Chitosan (250 mg capsules)	Body Systems Technology
5. Chitosan Plus	Nature's RX
6. Diet Fuel	Twinlab
7. Fat Zapper	Head Start
8. Souther Blue Chitosan	McFarlan
9. Chitosan (500 mg capsule)	Adbiotk

Based on data from Adbiotk (1999); Body Systems Technology (1999); Bodywise (1999); Headstart (1999); Natures RX (1999); Netrition (1999); Southern Blue (1999) and Utranutrition (1999).

3) Cosmetics and Enzyme technology

An application of chitosan for cosmetics was reviewed several years ago. The use of chitosan could help to remove the leftover starch contained in shampoos. Its use would also have an effect of conferring shine and strength to hair due to interactions between the polysaccharide and hair proteins. Recently, more reports have been published on the use of chitosan and its derivatives in cosmetic products (Li *et al.*, 1997). For example, Yabe *et al.* (1988) prepared a series of colored cosmetic powders for makeup by spray-drying a mixture of pigments and chitosan granules. The powders showed high fastness to sunlight and sweat. Other reports on the use of chitosan for cosmetics include nail polishes (Lang *et al.*, 1989), moisturizers (Matsumura *et al.*, 1989), hair and skin fixatives (Lang and Wendel, 1988) and hair

conditioners (Lang and Wendel, 1988; Mizushima, 1988 and Iwao, 1989). Cleansers can also contain chitosan. One of these, a bath lotion containing chitosan lactate, chitosan sucinate, and chitosan alkyl phosphates, was prepared by Bandai *et al.* (1988). He claimed that this lotion resulted increased skin softness.

In the review from Li *et al.* (1997), they indicated that amino and hydroxy groups on the chitosan polymer provide places for physical or chemical linkages and modifications. Chitosan, therefore, has been widely used as a carrier for enzyme immobilization. Enzymes immobilized on chitosan gels include α-chymotrypsin, α-galactosidase, invertase, β-galactosidase, α-amylase, lactase and cyclodextrin glucanotransferase. The immobilization usually involved a cross-linking step using glutaraldehyde. In addition, in 1989, Braun and co-worker immobilized penicillin G acylase on chitosan powder and beads by a covalent linkage. They found that a covalently linked enzyme was active longer than an absorbed one. Another interesting application was reported by Katayama (1988), who prepared a glucose electrode by forming an immobilized enzyme-chitosan membrane on a nylon net that was covered with a hydrogen peroxide permeable membrane.

4) Chitosan Membrane and Cell Encapsulation

Li *et al.* (1997) reviewed that chitosan membranes may be prepared in various ways: evaporation of chitosan solvents, cross-linking with bifunctional reagents, chelating with anionic counterions or complexing with polymers and proteins. The direct evaporation of chitosan spreaded on a glass plate is the most simple technique for the preparation of chitosan films and generally produces a water-soluble film. This type of film was found to be amorphous and to have a comparable water vapor permeability to cellophane. In 1977, Muzzarelli reported a procedure for the preparation of reverse osmosis membranes. These membranes were prepared by dissolving chitosan in a diluted acidic solution mixed with organic solvents, such as methanol, ethanol and acetone, in a 6:4 ratio of water to solvent. The solution was spreaded on a glass plate followed by air drying. By this method a 3-μm-thick membrane was obtained which had a very high water permeability while rejecting

81% of the calcium chloride. In 1989, Kanke *et al.* compared two different types of chitosan films-monolayer and double-layer films. They found that the permeability of the double-layer films could be controlled by changing operation variables.

A cross-linked chitosan membrane can be prepared by an addition of bifunctional reagents, such as aldehydes, carboxylic anhydrides and glutaraldehyde, to the chitosan solution. In 1978, Hirano, for example, published a procedure for the preparation of *N*-arylidene-chitosan and *N*-acyl-chitosan membranes. A chitosan-hydroacetate solution was first poured into a glass petri dish to give a thin liquid layer. After the addition of either an aldehyde or carboxylic anhydride, films formed within a few hours. These membrane were reported to neither solubilized nor swollen by soaking for one week in water, 2 N NaOH, dimethyl sulfoxide or formamide. The mechanism of cross-liking gelation has been studied. In 1989, Roberts and Taylor's study of the gelation behavior of the chitosan-glutaraldehyde system, and the effect of variables, including the activity coefficients of electrolytes and concentrations of chitosan, glutaraldehyde and electrolytes were examined. They suggested that the cross-linking mechanism involves formation of a Schiff's base structure.

A cell encapsulation technology is important in the development of new cell transplantation techniques for hormone delivery in medicine. One of the most important advantages of microencapsulation is the ability to provide a sheltered surrounding the cells. The semipermeable capsule membrane allows small molecules to diffuse through but prevents the passage of large molecules and cells. Encapsulation may provide potentially new forms of treatment for a number of metabolic diseases of various organs. The transplantation of insulin-producing islets of Langerhans immobilized in alginate-polylysine microcapsules, for example, may be an effective new way of treating diabetes in animals. Since the earliest industrial applications of encapsulation, the technique has the potential for providing higher cell densities and product concentrations. Chitosan-copolymer capsules have been successfully used to culture *Bacillus*, plant cells and hybridoma cells (Li *et al.*, 1997).

The capsule membrane permeability and molecular weight cutoff can be controlled by modulating the viscosity, average molecular weight and concentration of the membrane-forming polymers, pH and ionic strength, as well as the reaction time. The authors found that the durability of chitosan-alginate capsule membranes depended on the chitosan molecular weight- the lower the chitosan molecular weight, the stronger and thicker the membranes. They postulated that this effect could be due to the limitation of the molecular size with respect to mass diffusion of chitosan through the pores in the alginate gel matrix (Goosen, 1987; Daly *et al.*, 1989; Shioya and Rha, 1989 and Kim and Rha, 1989).

5) Agriculture

Chitosan has many potential applications in agriculture because the polymer is naturally occurring and biodegradable. Therefore, it should not cause pollution problems. One application that is known to favor is the termination of certain seeds by protecting them from a fungal attack during storing and by influencing favorably the microflora in the microenvironment during germination. Aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* found in preharvest crops such as maize and peanuts are secondary metabolites that induces toxic responses in human and animals. Because chelating agents that bind zinc were reported to inhibit aflatoxin production, it was speculated that *N*-carboxymethylchitosan reduced an aflatoxin yield for more than 90%, whereas the fungal growth was reduced to less than one-life. Toxigenic fungal cells showed that the *N*-carboxymethylchitosan presence inhibited spore germination and sporulation of mycelia (Cuero *et al.*, 1991).

Rice (*Oryza sativa*), soybean (*Glycine hyspida*), pine (*Pinus thumbergii*) and other seeds showed an increase in chitinase activity during germination and seedling growth, when the seeds were contacted with *N*-carboxymethylchitosan (1.7 mg/g dry weight) and with chitosan oligomer (Hirano *et al.*, 1990). Similarly, *N*-carboxymethylchitosan was able to double the protein contents in maize seeds, which also had a higher total cellular RNA content (20.6 mg/kg) than controls (11.0 mg/kg) (Osuji and Cuero, 1992).

6) Food industry

Chitosans have been of interest in the past few decades due to their potential broad range of industrial applications. However, only limited attention has been paid to food application of these versatile biopolymers. Conversion of processing discards into valuable by-products and alternative materials has been identified as a timely challenge for food research and development associated with numerous applications of chitinous polymers. In that sense, these biopolymers offer a wide range of unique applications including bioconversion for the production of value-added food products, preservation of foods from microbial deterioration, formation of biodegradable films, recovery of waste material from food processing discards, purification of water and clarification and deacidification of fruit juices (Table 8) (Shahidi *et al.*, 1999).

Owing to the high chelating and coagulation ability of chitosan, the polymer has been utilized widely in the food industry. In the case of beverages, for example, chitosan was used to remove dyes from orange juice and to remove solids, β-carotene, and acid substances from apple and carrot juice. In addition to the above, chitosan was also used to extend the shelf-life due to its antibiotic properties (Li *et al.*, 1997). Chitosan is a good clarifying agent for grapefruit juice either with or without pectinase treatment (Chen and Li, 1996) and a highly effective fining agent for apple juice which can afford zero turbidity products with 0.8 kg/m^3 of chitosan (Soto-Perlata *et al.*, 1989). The use of edible films and coatings to extend the shelf-life and improve the quality of fresh, frozen and fabricated food has been examined during the past few year due to their ecofriendly and biodegradable nature (Kester and Fennema, 1986 and Kittur *et al.*, 1998). Chitosan and chitosan-laminated film containing antimicrobial agent provide a type of active package such that the preservatives released from the film deposit on the food surface and inhibit the microbial growth (Labuza and Breene, 1989 and Chen *et al.*, 1996). The application of a chitosan coated film for control of enzymatic browning in litchi fruit was studied by Zhang and Quantick (1997). They reported that the chitosan film coating delayed changes in the contents of anthocyanins, flavonoids and total phenolics.

Now, consumers demand for food products with fewer synthetic additives but increased safety and shelf-life. These demands have increased the importance of natural antimicrobial agents which prevent the growth of pathogenic and spoilage micro-organisms. There are a lot of reports on antimicrobials such as nisin and chitosan, applications in areas such as postharvest storage of fruits and vegetables, and ways of combining antimicrobials with other preservation techniques to enhance the safety and quality of foods (Shahidi *et al.*, 1999).

Table 8 Food applications of chitin, chitosan and their derivatives in the food industry

Area of application	Examples
1. Antimicrobial agent	- Bactericidal
	- Fungicidal
	- Measure of mold contamination in agriculture commodities
2. Edible film industry	- Controlled moisture transfer between food and surrounding environment
	- Controlled release of antimicrobial substances
	- Controlled release of antioxidants
	- Controlled release of nutrients, flavors and drugs
	- Reduction of oxygen partial pressure
	- Controlled rate of respiration
	- Controlled enzymatic browning in fruits

Table 8 (continued)

Area of application	Examples
3. Additive	- Clarification and deacidification of fruits and beverages
	- Natural flavor extender
	- Texture controlling agent
	- Emulsifying agent
	- Food mimetic
	- Thickening and stabilizing agent
4. Nutritional quality	- Dietary fibre
	- Hypocholesterolemic effect
	- Livestock and fish feed additive
	- Reduction of lipid absorption
	- Production of single cell protein
	- Antigastritis agent
	- Infant feed ingredient
5. Recovery of solid materials from food processing waste	- Affinity flocculation
	- Fractionation of agar

Based on data from Shahidi *et al.* (1999).

Antimicrobial action of chitosan

Chitosans show an antimicrobial activity along with the ability to resist environmental conditions. They are also source of nutrients. The antimicrobial activity of chitosan has been reported by several authors. In this context, the unusual antimicrobial activity of chitosan against different groups of microorganisms, such as bacteria, yeast, fungi and viral has received considerable attention in recent years. Chitosan has been used as an antimicrobial compound by its external application (exogenous) to the host, to the substrate or media and to a physical surface containing microbial population. Because of the positive charge on the C-2 of the glucosamine monomer at pH below 6.0, chitosan is more soluble and has a better antimicrobial activity than chitin (Shahidi *et al.*, 1999 and Yalpani *et al.*, 1992).

The exact mechanism of the antimicrobial action of chitosan is still unknown, but different mechanisms have been proposed, including an interaction between positively charged chitosan molecules and negatively charged microbial cell membrane that leads to the leakage of proteinaceous and other intracellular constituents (Sudharshan *et al.*, 1992; Fang *et al.*, 1994 and Chen *et al.*, 1998). Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth. It also activates several defense processes in the host tissue, acts as a water binding agent and inhibits various enzymes. Binding of chitosan with DNA and an inhibition of mRNA synthesis occur via penetration of chitosan to the nuclei of microorganisms and interfering with the synthesis of mRNA and proteins (Shahidi *et al.*, 1999).

Choi *et al.* (2001) showed the morphological changes of chitooligosaccharide-treated cells by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). When *Actinobacillus actinomycetemcomitans* cells were treated with 0.1% chitooligosaccharides for 30 min, the cells were markedly degraded from spherical shape to irregularly condensed masses with bleb-like structures (Figure 9). The ultrastructure picture observed by TEM confirmed this finding that the microorganism cell had extra- and intracellular changes compared with the non-

treated cells (Figure 10). The changes include a separation of the cytoplasmic membrane from the cell envelope, coagulation of the cytosolic components, and disruption of the outer membrane structure, in which the membrane became sloughing, breaching and blebbing. Helender *et al.* (2001) demostrated the mode of antimicrobial action of chitosan on Gram-negative bacteria with special emphasis on its ability to bind and to weaken the barrier function of the outer membrane (OM). They found from chemical and electrophoretic analyses of cell-free supernatants of chitosan-treated cell suspensions that interaction of chitosan with *E. coli* and the salmonellae involved no release of lipopolysaccharide or other membrane lipids. However, chitosan rendered *E. coli* to be more sensitive to the inhibitory action of dyes and bile acids using selective media. An electron microscopy showed that chitosan caused extensive cell surface alterations and covered the OM with vesicular structures. Chitosan thus appeared to bind to the OM, explaining the loss of the barrier function.

Some mechanisms have been suggested to understand this antibacterial action (Chen *et al.*, 2001; Fang *et al.*, 1994 and Sudharshan *et al.*, 1992).

1) Reaction with bacterial teichoic acid, polelectrolyte complexes
2) Chelation of metals present in metalloenzymes
3) Alteration of the bacterial adhesion
4) Inhibition of the enzymes that link glucans to chitin
5) Prevention of nutrients permeation

Some mechanisms for antifungal activity of exogenous chitosan have been suggested (Sudharshan *et al.*, 1992; Shahidi *et al.*, 1999 and Seo *et al.*, 1992).

1) Introduction of phenylpropanoid and octadecanoid pathways
2) Introduction of chitosanase
3) Introduction of chitosanase with many polypeptides
4) Effect of chitosans on the plant enzymes in relation to plant resistance against fungal pathogens
5) Introduction of phenolic compounds and/or phytoalexins
6) Introduction of morphological and/or physiological changes

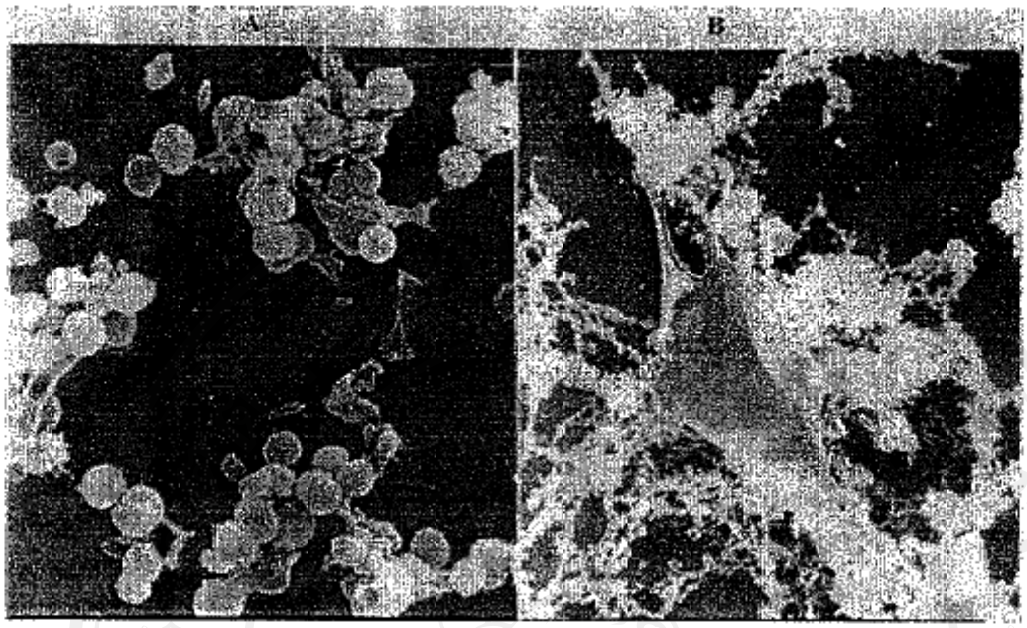

Figure 9 Scanning electron photomicrograph of *A. actinomycetemcomitans* cell after treatment with the chitooligosaccharides for 30 min. Non-treated cells (A) and treated cells (B) (Choi *et al.*, 2001).

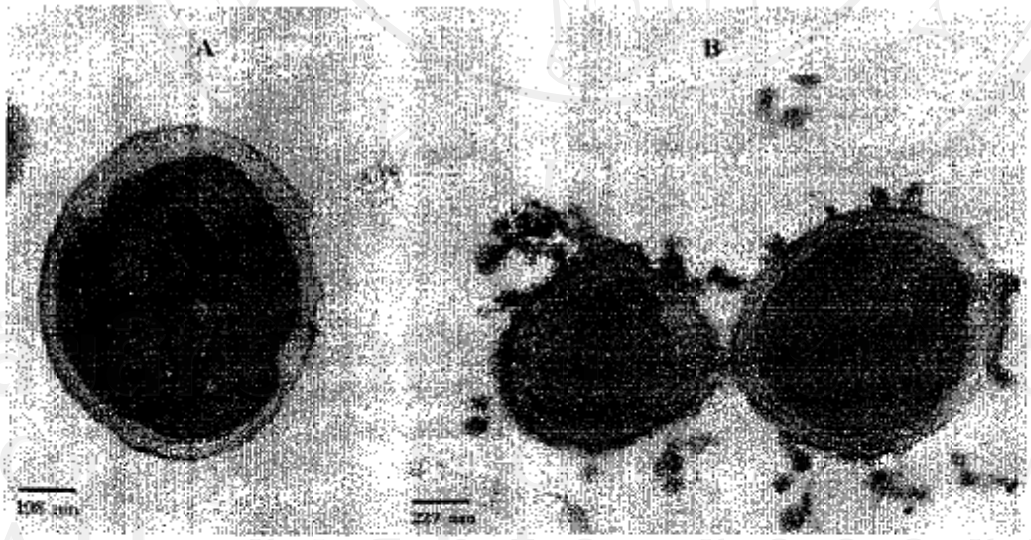

Figure 10 Transmission electron photomicrograph of *A. actinomycetemcomitans* cell after treatment with the chitooligosaccharides for 30 min. Non-treated cell (A) and treated cells (B) (Choi *et al.*, 2001).

2.2.5 Nutritional effect of chitosan in foods

Multiple actions of chitosan in food systems relate to their effects as a dietary fibre and as functional ingredients. The United States Food and Drug Administration (USFDA) approved chitosan as a feed additive in 1983 (Knorr, 1986). Chitosan is also used in the food industry as a food quality enhancer in certain countries (Shahidi *et al.*, 1999). Recently, Hirano *et al.* (1990) demonstrated the nutritional significance of chitosan in animals and indicated the effectiveness of chitosan as a feed additive.

Chitosan is not only very useful, it is also very safe. It has been used extensively in numerous industrial, health and food application. To determine the relative safety of various foods, scientists run experiments to determine the food's toxic level or LD50. The relative LD50 in humans then would be 1.33 g/day/kg body weight. Given that an average person weights 150 pounds or 70 kg, this means that the toxic amount for person would be greater than 90 g/day (Hennen, 1996). Chitosans like a dietary fibre that is non digestibility in the upper gastrointestinal tract, high viscosity, polymeric nature and high water binding in the lower gastrointestinal tract, which are all responsible for the effective hypocholesterolemic potential of dietary fibres (Muzzarelli, 1997). Chitosan shows most of these criteria and has a highly characteristic property in relation to other dietary plant fibres. Due to the ability of forming ionic bonds at low pH, it can bind *in vitro* to different types of anions such as bile acids or free fatty acids (Muzzarelli, 1996). Large properties of these bound lipids are thus excreted. Bound triacylglycerols would escape hydrolysis by lipase, promoting the excretion of fatty materials including cholesterol, sterols and triacylglycerols.

Razdan and Petterson (1994) observed increase high density lipoprotein (HDL) concentrations after feeding chitosan containing diet to broiler chicken. This could be attributed to enhance a reverse cholesterol transport in response to intestinal losses of dietary fats. Chitosan appears to reduce the absorption of bile acids or cholesterol; either of these effects may cause a lowering of blood cholesterol. This effect has been repeatedly demonstrated in animals, and a preliminary human study showed that 3–6

grams chitosan per day of taken for two weeks resulted in a 6% drop in cholesterol and a 10% increase in HDL (the "good") cholesterol (Healthnote, 2003).

Chitosans have remarkable spectrum of physiological activities. Some of these activities have been well documented, for other only suggestion is available. The lists of a number of significant physiological actions are followed (Koide, 1998):

1) Hemostasis
2) Hypocholesterolemia
3) Immune cell migration and Cell proliferation
4) Wound healing and bone repair
5) Tissue reconstruction
6) Carcinogenesis
7) Inflammation

2.3 Food Spoilage

2.3.1 Food spoilage definition

A spoiled food is simply a food that is unacceptable to a consumer for a reason that the food is not of quality expected and not safe due to an undesirable change in the color, flavor, odor and texture (Schuler *et al.*, 2002 and Garbutt, 1997). The terms of "spoiled" and "unspoiled" are subjective since acceptance is dependent upon consumer expectation and is not related to food safety (Forsythe, 2000). Types of food spoilage fall into categories, according to the cause of spoilage which are (Garbutt, 1997 and Forsythe, 2000):

1) Non-microbial spoilage can be caused by foreign materials or enzymes that occur in the foodstuff naturally. It may be due to a number of causes including insect damages; physical injuries due to bruising, pressure, freezing, drying and radiation; the activity of indigenous enzymes in animal and plant tissues and chemical changes that are not induced by microbial or naturally occuring enzymes.

2) Microbial spoilage is caused by microorganisms and their products. Microorganisms grow and metabolism resulting in possible pH changes and formation of toxic compounds, off odors, gas and slime formation. For example, an organoleptic change brought about by the growth of micro-organisms. This is by far the most important and common cause of food spoilage.

2.3.2 Food spoilage microorganisms

During harvesting, processing and handling operation, food may become contaminated with a wide range of microorganisms. Subsequently, during distribution and storage the conditions will be favorable for certain organisms to multiply and cause spoilage. Growth of microorganisms in food can cause spoilage by producing an unacceptable change in taste, odor, appearance, texture and a combination of these factors. Which microorganisms that will develop or what biochemical and chemical reactions that occur is dependent upon the food composition and its intrinsic and extrinsic parameters. Spoilage is not only due to the visible growth of microorganisms but also to the production of end metabolites which result in off odors, gas and slime. Although there is much progress in the characteristic of the total microbial flora and metabolites developing during spoilage, not much is known about the identification of specific microorganisms in relation to food composition. Despite the fact that food spoilage is a huge economical problem worldwide, the mechanisms and interaction leading to food spoilage are very poorly understood. Although the total microbial flora may increase during storage, some specific spoilage organisms are the actual cause of chemical changes and production of off odors. The shelf life of food is dependent upon the growth of spoilage flora and this microbial growth can be reduced through cold storage and packaging. The higher the initial microbial load the shorter the shelf life of food due to an increase in microbial activities. A wide variety of microorganisms may initially be present in food and grow if favorable conditions are present. On the basis of susceptibility to spoilage, food may be classed as nonperishable (or stable), semi-perishable and perishable. This classification depends

on intrinsic factors including water activity, pH, presence of natural antimicrobial agents, etc (Forsythe, 2000).

Microbial spoilage in food is the beginning of a complex natural process of decaying that under natural circumstances, leads to recycling of the elements present in the animal or plant tissue in the natural environment. A spoiled food thrown onto a compost heap and then used in soil cultivation will undergo microbial degradation that will eventually lead to the release of carbon as carbon dioxide, nitrogen as nitrate and mineral elements that used by green plants as nutrient sources. Spoilage organisms are often the primary invaders of dead plant and animal tissues growing rapidly at the expense of low molecular weight compounds present in the food.

Microorganisms present in a food product are originated from either the raw materials and ingredients or from contamination. The means by which such microorganisms cause spoilage are varied and depended on the organisms present and type of food products in which they are growing. The ability of these organisms to grow and cause spoilage in a product is dependent on factors affecting microbial activity according to:

1) Extrinsic factors applied to food
   - Temperature
   - Relative humidity
   - Oxygen concentration
   - Physical state

2) Intrinsic factors of food
   - Moisture content or water activity
   - pH
   - Oxidation-reduction potential
   - Nutrient availability
   - Inhibitory substances

2.3.3 Meat and Meat products

Meat and meat products are excellent sources of protein. One hundred grams of lean meat can provide up to half our daily protein requirement. It is also a good source of vitamin B-complex and minerals, especially iron, which is of good bioavailability in meat. However, bacteria, yeasts and molds also find meat as a suitable substrate for growth, making meat highly perishable. Fresh meat is a good culture medium allowing a broad range of microorganisms to develop because nutrients and free water are present in sufficient amount to support microbial growth, pH is close to neutral and the redox potential at the surface is higher than at the core. Glucose, glycogen, amino acids and variety of low molecular weight compounds are readily available and can sustain the growth of indigenous flora up to 10^9 cell per gram (Saucier, 1999).

At chill temperatures (refrigeration at 5°C and below), the microflora in meat is quickly being dominated by psychrotrophic organisms that grow at the expense of water soluble nutrients present in the meat. *Pseudomonas* spp., particularly *Ps. fluorescens* and *Ps. fragii* are the organisms associated with the spoilage of good quality, low pH meat. The spoilage symptoms of putrid or fruity off odors and flavors are produced as a result of amino acid breakdown. As long as glucose is available as a carbon source for energy production, amino acids are utilized for protein production and growth. However, when the numbers of bacteria at the meat surface reach a critical level (in a good quality meat this is about 10^7 organisms/cm^2 of meat surface or per g of comminuted meat), glucose as an energy source for growth becomes exhausted and cannot be replenished by diffusion at a rate fast enough to supply the requirements of the organisms. In this situation, spoilage bacteria switch to amino acids as a carbon source producing metabolic by-products that are responsible for the characteristic of off odors and flavors. *Ps. fragii* produces fruity off odor and others, e.g. *Ps. fluorescens*, produce putrid odors associated with the breakdown of the sulphur-containing amino acids cysteine and methionine. The human olfactory sense can detect the breakdown products of the sulphur-containing amino acids, e.g. dimethyl sulphide, methanethiol and hydrogen sulphide, at extremely low concentrations. Later, when other amino acid breakdown products, such as ammonia,

amines and indole can be noticed, the pH of the meat rises rapidly, accelerating the spoilage rate and eventually reaching pH 8.0. When the level of spoilage bacteria reaches $10^8/cm^2$ or per g and above, visible slime becomes evident. Only when the meat is grossly spoiled and well beyond a condition acceptable to consumers, the break down of muscle fibre structure associated with the proteolytic activity of Psudomonad*s* can be detected.

The high pH of dark cutting meat coupled with a lower glucose content compared with a good quality meat mean that not only do spoilage bacteria grow more quickly, but the threshold level of organisms at the surface when amino acids become utilized as carbon sources is lower (about 10^6 organisms/cm^2 or per g). This gives a faster onset of spoilage symptoms and therefore shorter storage life than good quality meat held at the same temperature (Garbutt, 1997). Carcass meat at temperatures above 20°C will readily be spoiled by bacteria originating from the animal's intestines which have contaminated the meat during slaughtering. The spoilage flora is dominated by mesophilic organisms such as *E. coli, Aeromonas* spp., *Proteus* spp. and *Micrococcus* spp. (Forsythe, 2000).

Meat safety and Microbiological guideline

Meat and meat products are part of the daily diet of many individuals and represent an important economic component of the agricultural activities of several countries. Meat safety can be challenged in various ways including chemical residues (e.g. pesticides antibiotics), disease in animals (e.g. transmissible spongiform encephalopathy) but, most importantly, by microbial contamination with pathogenic microbes or their toxin. The microbial world is in constant evolution, with basically infinite adaptive capacities towards conditions that we will use to control it, all making meat safety as a perpetual issue. On the verge of the new millennium, with the world population still rising and with the increasing market globalization where meats will travel greater distances and for longer time periods, a concerted effort among regulatory agencies, scientist and industry is necessary in order to develop novel

alternatives for food preservation and to identify new methods for investigating and detecting causative agents of meat-borne illnesses (Saucier, 1999).

The number and types of microorganisms present in a food product may be used to judge the microbiological safety and quality of that product. Safety is determined by the presence or absence of pathogenic microorganisms or their toxins, the number of pathogens and the expected control or destruction of these agents. Tests for indicator organisms may be used to assess either microbiological quality or safety when a relationship between the occurrence of an indicator organism and the likely presence of a pathogen or toxin has been established. The level of spoilage microorganisms reflects the microbiological quality or wholesomeness of a food product as well as the effectiveness of measure used to control or destroy such microorganisms. Currently, microbiological criteria are used to assess the safety of food, adherence to Good Manufacturing Practices (GMPs), the keeping quality (shelf-life) of certain perishable foods and the suitability of a food or ingredient for particular purpose. When appropriately applied, a microbiological guideline can be a useful means for ensuring the safety and quality of food, which in turn elevates consumer confidence. Different scientific organizations have been involved in developing general principles for application of microbiological guidelines by regulatory agencies and the food industry (Smoot and Pierson, 1997). For example, the microbiological guidelines for some ready-to-eat meat products were established by the Public Health Laboratories (PHLS) of the United Kingdom (UK) (Table 9). Another guideline is provided by the Alberta Provincial Public Health Laboratory, Canada (Table 10).

There are four grades of microbiological quality- related to the actual aerobic colony count, number of indicator organisms and the presence/number of pathogens determined by the microbiological examination of the food (Gilbert *et al.*, 2000). The terms used to express the microbiological quality of the meat products are:

1) Satisfactory – test results indicating good microbiological quality.
2) Acceptable – an index reflecting a borderline limit of microbiological quality.
3) Unsatisfactory – test results indicating that further sampling may be necessary and that environmental health officers may wish to undertaken a further inspection of the concerned premise to determine whether hygiene practices for food production or handling are adequate or not.
3) Unacceptable / potentially hazardous – test results indicating that urgent attention is needed to locate the source of the problem; a detailed risk assessment is recommended. Such results may also form a basis for prosecution by environmental health departments, especially if they occur in more than one sample. Food examiners will wish to draw on their own experience and expertise in determining the advice and comments they wish to give and they will be required to do this if invited to give an expert opinion during legal proceeding.

Table 9 Guideline for the microbiological quality of ready–to eat meat products.

Criterion	Microbiological quality (CFU/g unless stated)			
	Satisfactory	Acceptable	Unsatisfactory	Unacceptable /potentially hazardous
Aerobic colony count (30°C/48 hr)	$< 10^3$	10^3 - $< 10^4$	$\geq 10^4$	N/A
Indicator organism				
Enterobacteriaceae	< 100	100 - $<10^4$	$\geq 10^4$	N/A
E. coli (total)	< 20	20 - < 100	≥ 100	N/A
Listeria spp. (total)	< 20	20 - < 100	≥ 100	N/A
Pathogens				
Salmonella sp.	ND in 25g			Detect in 25g
Campylobacter spp.	ND in 25g			Detect in 25g
E. coli O157 & other VTEC	ND in 25g			Detect in 25g
V. cholerae	ND in 25g			Detect in 25g
V. parahaemolyticus	< 20	20 - < 100	100 - $<10^3$	$\geq 10^3$
L. monocytogenes	< 20**	20 - < 100	N/A	≥ 100
S. aureus	< 20	20 - < 100	100 - $<10^4$	$\geq 10^4$
C. perfringens	< 20	20 - < 100	100 - $<10^4$	$\geq 10^4$
B. cereus and other Pathogenic *Bacillus* spp.#	$< 10^3$	10^3 - $<10^4$	10^4 - $<10^5$	$\geq 10^5$

N/A not application ND not detection

** Not detected in 25 g for certain long shelf-life product under refrigeration.

If the *Bacillus* count exceed 10^4 CFU/g, the organism should be identified.

Based on data from Gilbert *et al*. (2000).

Table 10 Alberta Health and Food Microbiological guideline.

Analysis	Food "to be cooked" (excluding ground meat)	Ground Meat "to be cooked"	Food "ready-to-eat" (including Steak Tartar but excluding culture of fermented products)
Standard Plate Count* (Aerobic)	< 1,000,000 CFU/g	< 5,000,000 CFU/g	< 100,000 CFU/g
Total Coliforms	< 1,000 CFU/g	< 1,000 CFU/g	< 100 CFU/g
C. perfringens	< 1,000 CFU/g	< 1,000 CFU/g	< 100 CFU/g
Yeast	< 1,000 CFU/g	< 1,000 CFU/g	< 100 CFU/g
Molds	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
E. coli	< 100 CFU/g	< 100 CFU/g	< 10 CFU/g
S. aureus (coagulase +Ve)	< 100 CFU/g	< 100 CFU/g	< 100 CFU/g
Salmonella *E. coli 0157:H7,* *Y. enterocolitica* *and Campylobacter*			0 CFU/g (zero tolerance)

* Food which are vacuum packaged, modified atmospheric, or controlled atmospheric packaged, the standard plate count may exceed 1,000,000 CFU/g due to the desired presence of lactic acid bacteria which can serve to inhibit the growth of pathogenic and aerobic spoilage bacteria.

Based on data from http://www.norwestlabs.com/pdf/FoodAppendices.pdf.