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THOMSON REUTERS
International Journal of Medical Research and Pharmaceutical Sciences
Volume 10 (Issue 4): April 2022 ISSN: 2394-9414
Impact Factor- 4.174

# CHITOSAN AND ITS OLIGOMERS – CHARACTERISTICS AND PRODUCTION METHODS AT INDUSTRIAL SCALE

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***Keywords:***
*Polysaccharides, Chitosan, Biopolymer, Extraction, Production methods.*

***Abstract***
Chitosan has received considerable attention as a functional biopolymer with a wide range of applications in food, agriculture, medicine, pharmaceutics and cosmetics, due to its valuable physicochemical and biological properties. It is a cationic polymer obtained by deacetylation of chitin, found abundantly in crustacean, insect, arthropod exoskeletons, and molluscs. This review focuses on the up-to-date methods for industrial production of chitosan and some of its basic characteristics such as chemical structure, degree of deacetylation, molecular weight and crystallinity. The mechanisms of the most common chemical production methods are summarized. The main technological steps of demineralization, deproteinization and deacetylation are discussed, as well as the impact of the different chemical agents and reaction parameters used during the production process. Chitosan can also be obtained through biotechnological extraction involving enzymes or microorganisms. Enzymatic and fermentation-based approaches are also reviewed and compared with the chemical techniques, emphasizing on their pros and cons from industrial perspective.

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## Introduction

Chitosan is a chitin-derived biopolymer. Chitin is a widespread polysaccharide in nature and can be obtained from various species of crustaceans insects, algae and fungi (Figure 1).[1,2] Due to its low solubility in water, chitin is usually processed into chitosan, whose structure is soluble in aqueous acidic medium.[3,4] Shrimps, crabs, lobsters and krill are the most common sources for the production of chitosan. The average content of chitin in the shells of crustaceans is about 20-30 %, while the shells of some lobster species such as *Nephrops sp.* and *Homarus sp.* comprise up to 60-75 % chitin.[5] To ensure high quality of the final chitosan, the use of shells of the same species is recommended. The shells of different marine sources have different compositions, which implies a wide variety of parameters of the production process. For example, the wall of shrimp shells is thin and chitin extraction is an easier process compared to other crustaceans. Before isolating chitin, the selected starting material must be cleaned, dried and ground into small pieces. In the shells of crustaceans, chitin forms a complex network with proteins and calcium carbonate deposits. A demineralization process is required in order to remove the proteins. The polysaccharide must be purified also from inorganic calcium carbonate.[6] About half of the total mass of the crustaceans used in the food sector is disposed of as waste, which can provide tons of chitin a year. Therefore, sources of chitosan are available and inexpensive.[7]

Historically, chitin was first isolated from fungi and later from beetles cuticles. More recently, chitosan derived from fungi (*Mucor rouxii*, *Aspergillus niger*, *Penicillum crysogenum*, *Lactarius vellereus*) and insect cuticles (lady bug, silk worm, wax worm, butterfly) has been gaining interest.[7] Such chitosan can be better controlled in terms of low viscosity and exhibits a very high deacetylation degree. Moreover, fungi do not require demineralization because of their very low inorganic content. However, the production of chitosan from such materials is less profitable and the industrial waste from crustacean shells remains the main source for the production of these polymers.[8]

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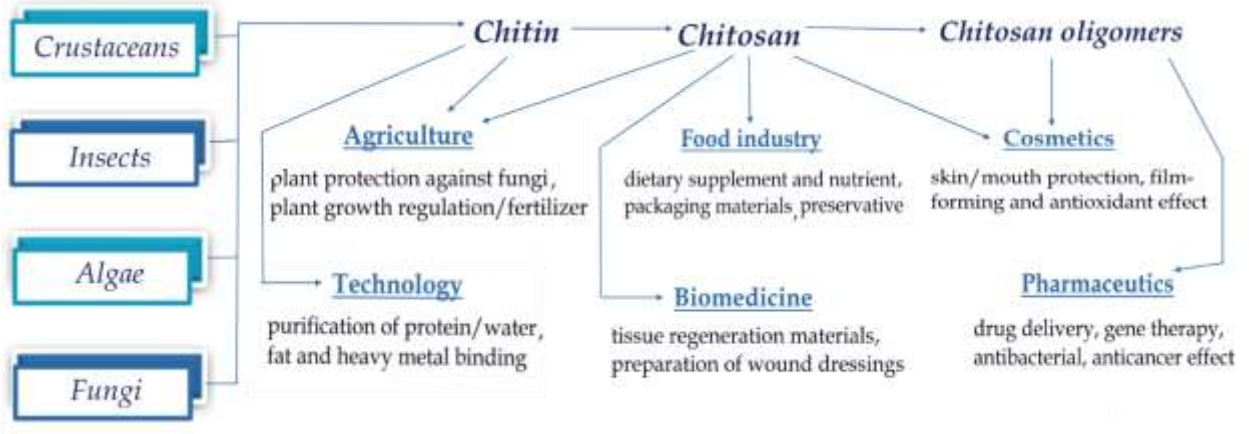


***Figure 1. Sources and some applications of chitin, chitosan and chitosan oligomers***

## Chemical structure

Chitin is a polymer of N-acetyl-D-glucosamine (Figure 2). Its chemical structure is similar to cellulose; the hydroxyl groups are substituted by acetyl amine groups. Unlike other abundant polysaccharides containing carbon, hydrogen and oxygen, chitin also contains nitrogen (6.89 %) in its structure.[8]

When chitin is deacetylated and the repeating units in the polymer are predominantly free of acetyl functional groups such as β-1,4-D-glucosamine, the polymer is known as chitosan. The process of deacetylation is carried out to varying degrees depending on the target applications, so that numerous products with different degrees of deacetylation can be obtained. The mole fraction of the N-acetylated repeating units is defined as degree of acetylation (DA), while the percentage of the repeating units of β-1,4-D-glucosamine in the polysaccharides is defined as degree of deacetylation (DD). When the polysaccharide structure has a DD of more than 50 %, the resulting polymer is chitosan. Most commercial chitosans have DD values between 70-90 %.[5]

The functional groups of chitosan molecules include free reactive amino groups at C-2, the primary hydroxyl group at C-3 and the secondary hydroxyl group at C-6. The glycosidic bonds and the acetamide groups can also be considered functional groups. Various chitosan derivatives can be obtained by chemical modification of its reactive units. The polymer can be functionalized by a variety of mechanisms using the free amino and hydroxyl groups of the molecule that are prone to chemical reactions. Some of the most common modification techniques involve phosphorylation, thiolation, N-phthaloylation and cross-linking. Chemical modifications can not only improve the physical and chemical properties of chitosan, but can also expand the application range of chitosan and its derivatives.[4]


***Figure 2. Chemical structure of chitin and chitosan***

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**Degree of deacetylation**

The degree of deacetylation is one of the most important characteristics of chitosan, which determines many of its properties and applications. The polymer can be classified on the basis of its DD value as chitosan with a high degree of deacetylation (70-99 %) or with a low degree of deacetylation (50-70 %).[4]

Studies report that chitosan with a higher DD has stronger biological activity. This may be due to the higher concentration of amino groups in the molecule of the polymer. The protonation of these functional groups is vital for the manifestation of the biological effects of chitosan.[5]

Deacetylation of chitin to produce chitosan is performed to obtain a soluble polysaccharide. Therefore, solubility is directly associated with DD. When the polymer DD reaches about 50 %, the polysaccharide becomes soluble in aqueous acidic media. Dissolution occurs by protonation of the $–NH_2$ functional group on the C-2 position of the D-glucosamine units, whereby the polymer is converted to a polyelectrolyte in the acidic media. The concentration of required protons is at least equal to the concentration of $-NH_2$ units involved.[9]

The biodegradability of chitosan in living organisms is also related to the polymer DD. In the human body, chitosan is degraded mainly due to the activity of lysozyme and bacterial enzymes present in the colon. The enzymatic degradation is dictated by physical (dissolution) and chemical (cleavage of glycoside bonds) processes. The enzymes first diffuse from the surrounding media to the surface of the polymer chain. Subsequent adsorption leads to the formation of an enzyme-polymer complex. Specific interactions between chitosan and lysozyme must be provided to cleave the glycoside bond of chitosan. The acetyl groups present in the chitosan structure are responsible for the lysozomal binding. However, the low quantity of acetyl groups is not necessarily associated with low degradability. Chitosan biodegradation is a complex process that depends on several other factors, such as polymer composition, crystallinity, molecular weight, pH of the environment and others.[10]

**Molecular weight**

The properties of chitin and chitosan also depend on the degree of polymerization (DP), which represents the length of the polymer chain and correlates with the molecular weight (MW). The degree of polymerization is the number of monomer units in the polymer or oligomer structure. The chain lenght of most chitosans cannot be determined accurately and the average MW in kDa is often used for its characterization.[3] The MW of chitosan usually varies in the range 50-1000 kDa depending on the starting material used for its production. The polymer can be classified according to its MW as low molecular weight chitosan (LMW) (<300 kDa) or high molecular weight chitosan (HMW) (>300 kDa).[4] MW affects some vital polymer properties such as solubility, viscosity, adsorption on solids, strength, elasticity and biofunctionality.

LMW chitosans are associated with lower viscosity and lower density. They have a higher capacity for cell penetration and improved aqueous solubility. Some of them have inhibitory activity against various pathogens and can be used as food preservatives.[4] LMW chitosans demonstrate more significant biological effects than those with HMW. For example, chitosan with MW lower than 20 kDa has stronger bioactivity than chitosan with MW higher than 120 kDa. The relationship between the length of the polysaccharide chain and the activity exhibited can be explained with the solubility of the polymer. Chitosan with MW below 22 kDa is soluble in water without acidification. When the MW is above 30 kDa, protonation of the amino group by acid is required to dissolve in water. Polymer chains with MW below 9 kDa demonstrate significantly better aqueous solubility.[5]

HMW chitosans, on the other hand, exhibit higher viscosity conductivity, and higher adsorption to lipophobic molecules. They are commonly used as dietary ingredients, emulsifiers in food formulations or as food packaging materials.[4]

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**Crystallinity**

Crystallinity is another key characteristic of chitin and chitosan, relevant to their properties and specific applications. In solid state, polymer chains are assembled through a network of H-bonds which controls their solubility, swelling and reactivity. The intermolecular and intramolecular hydrogen bonds in the chitin chain are highly crystalline, which interferes with the solubility of the polymer in water. The water-binding capacity of chitin and chitosan varies depending on their origin and differences in crystallinity.[11]

Depending on its source, chitin occurs as two allomorphs, namely α- and β- forms. α-Chitin is more stable compared to β-chitin. In nature, it forms fibrils with a high index of crystallinity (80 %). The polysaccharide chains are arranged in an anti-parallel orientation, which allows the formation of a great number of bonds between them.[7] α-Chitin is more abundant and can be found in the cell walls of yeast and fungi, shrimp shells, insect cuticles, as well as in the tendons and shells of crabs and lobsters. It can also be obtained by in vitro biosynthesis or recrystallization from solutions.[6]

The polymer chains of β-chitin are arranged in parallel orientation and the crystallinity index of its fibrils is around 70 %.[7] β-Chitin is commonly found in squid pens and in tubes synthesized by vestimentiferan worms and pogonophorans. *β*-Chitin is more reactive and readily soluble than the *α*-form due to its hydrated structure and weaker intermolecular hydrogen bonds.[4]

Chemical modifications are often used to improve the water solubility of chitin and chitosan by introducing hydrophilic groups on the amino group or the hydroxyl group of their molecules. Thus, the original hydrogen bonds in the polymer structure are destroyed and its crystallinity decreases.[11]

## Chemical Production Methods

Chitosan is obtained mainly from marine sources by N-deacetylation of chitin. Chitin can be converted to chitosan by chemical or biotechnological processing.

Chemical methods are preferred for industrial scale production of chitosan due to the short duration of the process, relatively simple technology, low cost and high productivity.[5] The process of chitosan production can be divided into three main steps – demineralization, deproteinization and deacetylation (Figure 3).

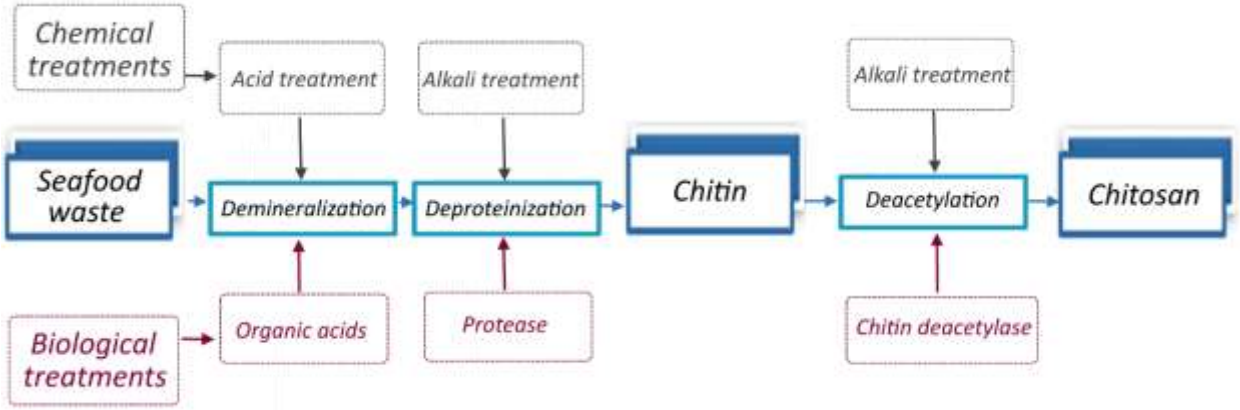


***Figure 3. Chitin and chitosan production by chemical and biological treatments***

The source materials are first treated with an acidic solution to remove calcium carbonate. The efficiency of the process depends on the type and concentration of acid used and the duration of the reaction. Although various acids can be used for demineralization such as $HNO_3$, $H_2SO_4$, HCOOH and $CH_3COOH$, it is concluded that HCl at a

concentration of up to 10 % removes calcium most effectively. The optimal duration of the reaction is 2-3 hours, as studies reported that duration beyond this time leads to a slight decrease in calcium content.[5]

Chitin is then purified from proteins and other organic components using bases. The main parameters affecting the process are the alkali concentration and the reaction temperature. Aqueous solution of NaOH at a concentration of 1-10 % is preferred for deproteinization of chitin. The reaction is carried out at a temperature of 65-100 °C for 0.5-12 hours.[12]

Deacetylation of chitin is the final step for obtaining chitosan. In conventional deacetylation, chitin is soaked in aqueous alkali solvents and chitosan is produced as an insoluble precipitate deacetylated up to 85-99 %. A hot solution of NaOH at a concentration of 30-50 % is usually used.[6] Although acid hydrolysis is also possible for deacetylation of chitin, this method is not preferred because it leads to depolymerization of the chitin backbone.[3] The deacetylation process can be time-consuming, and the reaction time depends on the temperature. For example, at room temperature chitosan with 10 % DA can be obtained after 580 hours.[6] At higher temperatures (80-120 ºC) deacetylation can be performed within 12-24 hours. Prolonged high-temperature alkali treatment is not recommended, as it may cause depolymerization. Although atmospheric conditions have negligible effect on the deacetylation process, it has been observed that chitosan with higher molecular weight is obtained under atmospheric nitrogen and with the addition of sodium borohydride which prevents polymer degradation.[6] Nitrogen is commonly used in the production of chitosan to protect the system from oxygen and avoid depolymerization. Sodium borohydride is usually added to NaOH solution as a reducing agent. The main parameters affecting the resultant DA of chitosan are the concentration and type of the alkali reagent, temperature and reaction time. Investigations have shown that HMW (300-1000 kDa) chitosan can be produced with a well-controlled DA using fixed reaction conditions. High NaOH concentration, high temperatures and prolonged incubation time significantly increase the reaction rate, but at the same time lead to significant reduction in DP, resulting in LMW chitosan.[3] Therefore, optimum conditions for efficient deacetylation should be determined while avoiding severe depolymerization.

Excess NaOH used in chitosan production is considered a serious economic and environmental issue. Sodium cations can contaminate soil and water systems. An alternative approach to reduce NaOH is to mix chitin with NaOH powder (ratio 1:5) by extrusion at 180 °C instead of using a solution. Thus, highly deacetylated and soluble chitosan can be obtained with only half of the NaOH needed for the aqueous system.[13] KOH could also be used to deproteinize and deacetylate chitin as a more environmentally friendly alternative to NaOH. Potassium is an essential element for plant growth and a common component in fertilizers.[5]

## Biotechnological Production Methods

Biotechnological methods are another possible technique for chitosan production. They use enzymes to deproteinize and deacetylate chitin. Various proteinases and deacetylases, mainly of microbial, fungal or marine origin, have been used for these two reactions.[7] Although enzymatic deacetylation is carried out at a lower temperature (25-60 °C) and does not require alkaline treatment, this method is not widely used in industry due to its higher cost compared to chemical processing. Enzymes are considerably more expensive than the generic bases used in chemical methods. Moreover, different types of enzymes are required for deproteinization and for deacetylation purposes.

However, expensive enzymes are not the only limitation for biotechnological techniques. Enzymatic methods are less efficient than chemical techniques. They cannot provide complete purification of the polysaccharide and about 5-10 % of the protein content remains bound to the isolated chitin. Furthermore, the resulting chitosan is usually less deacetylated. This requires enzymatic techniques to be applied in combination with chemical ones.[7]

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The economic aspect of the biotechnological method of production can be partly solved by applying a fermentation process. It involves microorganisms that constantly multiply in the reaction medium and generate the desired enzymes. Commonly used bacterial species include *Lactobacillus sps*. such as *L. paracasei*, *L. plantarum*, and *L. helveticus*, which secrete lactic acid.[4] The acid serves to demineralize the starting material, forming calcium lactate. Disadvantages of the fermentation method include country-specific microorganisms, a risk of contamination, requiring specialized equipment, long duration of the process (usually seven days) and the need for post-fermentation separation and purification.

**Production of chitosan oligomers**

Many studies have demonstrated that in addition to chitosan, its oligomers also show significant biological activity and have potential applications in medicine and food industry. Chitosan oligomers can act as probiotics, which positively change the balance of the intestinal microflora, inhibit the growth of harmful bacteria, promote good digestion and boost immune function. Some of them exhibit antibacterial, antifungal, antioxidant, and immunomodulatory effect.[3] Chitosan oligomers are chitosan hydrolysates, composed mainly of 1,4-linked D-glucosamine and partially of 1,4-linked N-acetyl-D-glucosamine. Various degradation reactions can be applied to shorten the chitosan structure to oligomer units. By reducing the size of the polymer chain, important physicochemical properties such as solubility and viscosity, as well as a number of biological and technological characteristics can be altered. The process of polymer degradation must be precisely controlled to obtain chitosan oligomers with desired molecular weight and properties. Oligomer structures are obtained by breaking the chemical bonds between N-acetylglucosamine (A) and glucosamine (D) monomer units in a chitosan polymer chain. Depending on the degradation method used, some of the four types of glycosidic bonds -D-D-, -A-A-, -A-D- and -D-A- are destroyed with priority, which allows the formation of different products. Among the most used methods for preparing chitosan oligomers are acid hydrolysis, ultrasonic processing, redox depolymerization and enzymatic processing.[14] Studies report that hydrochloric acid mainly affect A-A and A-D glycosidic bonds, compared to D-D and D-A, which is why the reducing ends are dominated by acetylated units. Along with the process of degradation and hydrolysis of O-glycosidic bonds between N-acetylglucosamine and glucosamine monomers, deacetylation and hydrolysis of N-acetyl linkage are performed to a lesser extent.[15] Nitrous acid is another reagent that can be used to obtain chitosan oligomers. It has a specific action on the primary amine in the glucosamine unit of the polymer structure. The disadvantage of this method is that it produces mainly monomers and the yield of oligomers is low. Another undesirable result is the possible formation of 2,5-anhydro-D-mannose after breaking the glycosidic bond.[14] Chitosan oligomers can be formulated by sonication. Ultrasonic exposure results in a moderate reduction in the chitosan polymer chain, regardless of its molecular weight. The main factor influencing the degradation rate is chitosan DA.[16]

Redox depolymerization using hydrogen peroxide as a reagent, is a faster method for reducing the polymer chain compared to sonication. Chitosan oligosaccharides of different composition can be produced by this technique. The molecular weight of the products obtained depends mainly on the concentration of the hydrogen peroxide used and the temperature at which the reaction takes place.[17]

The enzymatic method is the most selective due to enzyme-specific recognition. It allows more precise control of the degradation process and obtaining oligomers with the desired molecular weight. The most common enzymes used are glycosyl hydrolases, which are specific for chitosan (chitosanases) or chitin (chitinases). These enzyme families catalyze the hydrolysis of β-1,4-glycosidic bonds in the polymer chain and produce mainly oligosaccharides and only a limited amount of monomers.[14]

Non-specific enzymes from different families - proteases, hemicellulases, lipases and others – can be used to degrade chitosan.[3] Lysozyme, pepsin, papain and pronase are some of the proteolytic enzymes that can be used for chitosan depolymerization. However, the enzymatic reaction mainly leads to the production of LMW polymer and a low amount of oligosaccharides. Studies report that enzymes showed lower affinity for HMW chitosan, leading to a

slower degradation rate. This could be attributed to the limited flexibility of the longer polymer chain in the reaction medium. The enzyme hemicellulase can also cause depolymerization of chitosan. The rate of the process depends mainly on the polymer DD. Oligomers with different chain length can be observed within 4 hours of the reaction time. Lipases have also proved to hydrolyze chitosan. The observed degradation rate is much slower compared to other enzymatic reactions mentioned and is influenced by the reaction temperature.[14]

## Conclusion

According to literature, there are two main groups of preparation approaches used in industry to produce the polysaccharide chitosan from seafood waste material - chemical and biotechnological methods. The comparison between these techniques shows that the chemical treatments are simpler and quicker, producing biologically active chitosan with lower molecular weight and higher degree of deacetylation. On the other hand, the biological methods use milder reaction conditions, but involve the use of microorganisms, which creates a risk for contamination of the final polysaccharide product. Furthermore, the fermentation techniques require specialized equipment, long process duration and the need for post-fermentation separation and purification, which does not make them preferred for industrial production. Although the chemical techniques have more advantages over the biological ones, further development and optimization is required in order to make these technological processes more efficient and safe.

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