

Review Article

A Biopolymer Chitosan and Its Derivatives as Promising Antimicrobial Agents against Plant Pathogens and Their Applications in Crop Protection

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Received 22 January 2011; Revised 8 March 2011; Accepted 22 March 2011

Academic Editor: Bruno Sarmento

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Recently, much attention has been paid to chitosan as a potential polysaccharide resource. Although several efforts have been reported to prepare functional derivatives of chitosan by chemical modifications, few attained their antimicrobial activity against plant pathogens. The present paper aims to present an overview of the antimicrobial effects, mechanisms, and applications of a biopolymer chitosan and its derivatives in crop protection. In addition, this paper takes a closer look at the physiochemical properties and chemical modifications of chitosan molecule. The recent growth in this field and the latest research papers published will be introduced and discussed.

1. Introduction

Plant pathogens are considered economically important agricultural micro-organisms around the world. They induce decay on a large number of agricultural crops during the growing season and postharvest. Chemical pesticides provide the primary means for controlling the plant pathogens. However, continuous use of such compounds has faced two major obstacles: increasing public concern regarding contamination of perishable agricultural products with pesticide residues and proliferation of resistance in pest populations [1–3]. Hence, there is growing emphasis on environmentally friendly technologies in pest control, and evaluation of various alternatives to reduce dependency on harmful synthetic pesticides [4–6]. Consequently, several nonchemical treatments have been proposed for pest control. Although these approaches have been shown to reduce pests, each has limitations that can affect their commercial applicability. When used as stand-alone treatments, none of the nonchemical control methods has been clearly shown to offer a consistently economic level of disease control that warrants acceptance as an alternative to synthetic pesticides.

Among these strategies, some satisfactory results have been reported using natural compounds such as chitosan as safe alternative to hazardous pesticides with negligible risk to human health and the environment [7]. Chitosan, as the most abundant naturally occurring amino-polysaccharide, possesses many of these attributes and has attracted attention because of its unique physiochemical characteristics and biological activities [6, 8, 9]. From a biological standpoint, chitosan and its derivatives are very attractive for agriculture applications, which are closely related to human safety and fitness. For example, these compounds can function as seed soaking, root applying, and spray agents; all of these play an important role on plant disease control and stress resistance [6, 10].

The origin of chitosan (pronounced *Kite-O-San*) can be traced back to 1811 when “chitin”, from which it is derived, was first discovered by Henri Braconnot, a professor of the natural history in France. According to some researches, while Braconnot was conducting research on mushrooms, he isolated what was later to be called chitin [11, 12]. Chitin was the first polysaccharide identified by man, preceding cellulose by about 30 years. In the 1830s, there was a man

who authored an article on insects in which he noted that similar substance was present in the structure of insects as well as the structure of plants. He then called this amazing substance as “chitin”. Basically, the name chitin is derived from Greek, meaning “tunic” or “envelope”. The concept was further known in 1843 when Lassaigne demonstrated the presence of nitrogen in chitin. In 1859, Professor C. Rouget subjected chitin to alkali treatment, which resulted in a substance that could, unlike chitin itself, be dissolved in acids. The term “chitosan” was given to deacetylated chitin by Hoppe-Seiler [13]. While chitin remained an unused natural resource for a long time, interest in this polymer and its derivatives such as chitosan and chitooligosaccharides has increased in recent years due to their unique properties. Intense interest applications grew in the 1930s; however, the lack of adequate manufacturing facilities and competition from synthetic polymers hampered the commercial development in this period. Renewed interest in the 1970s was encouraged by the need to better utilize shellfish shells and the scientists worldwide began to chronicle the more distinct properties of chitin and chitosan to understand the potential of these natural polymers. In the early 1960s, chitosan was investigated for its ability to bind with the red blood cells. That time also, it was considered as a hemostatic agent. Then, for the past three decades, chitosan has been used in water purification. Since then, numerous research studies have been undertaken to find ways to use these materials. Today, it is known as a dietary supplement that is good for weight loss. In fact, it has been marketed for such purpose for about 20 years in Japan as well as in Europe. Many people even call it as the “fat blocker” [14–18].

Chitosan is a linear aminopolysaccharide of glucosamine and *N*-acetylglucosamine units and is obtained by alkaline deacetylation of chitin extracted from the exoskeleton of crustaceans such as shrimps and crabs, as well from the cell walls of some fungi [19]. The following major characteristics of chitosan make this polymer advantageous for numerous applications: (1) it has a defined chemical structure; (2) it can be chemically and enzymatically modified; (3) it is physically and biologically functional; (4) it is biodegradable and biocompatible with many organs, tissues, and cells; (5) it can be processed into several products including flakes, fine powders, beads, membranes, sponges, cottons, fibers, and gels. Consequently, chitosan has found considerable application in various industrial areas [6, 20–22].

Owing to its high biodegradability, nontoxicity, and antimicrobial properties, chitosan is widely-used as an antimicrobial agent either alone or blended with other natural polymers. To broaden chitosan’s antimicrobial applicability, comprehensive knowledge of its activity is necessary. The paper reviews the current trend of investigation on antimicrobial activities of chitosan and its derivatives against plant pathogens. The antimicrobial activity depends on several factors such as molecular weight, degree of deacetylation, solubility, positive charge density, chemical modification, pH, concentration, hydrophilic/hydrophobic characteristic, chelating capacity, and type of microorganism. Mode of antimicrobial action is discussed in parts of the active compound and the target microorganisms collectively and

independently in same complex. It has immense structural possibilities for chemical modifications to generate novel properties, functions, and applications especially in agricultural area. Therefore, different physicochemical properties and chemical modifications of chitosan molecule are also comparatively discussed. Finally, the general antimicrobial applications of chitosan and perspectives about future studies in this field are considered.

2. Chitosan Structure and Natural Origin

Chitin, occurring as a structural polysaccharide in the outer skeleton of animals belonging to the phylum Arthropoda (animals with an outer skeleton) and a component of the cell walls of certain fungi and algae, is quite abundant. It is also produced by a number of other living organisms in the lower plant and animal kingdoms, serving in many functions where reinforcement and strength are required. In contrast, chitosan is much less abundant in nature than chitin and has so far been found only in the cell walls of certain fungi [23]. Chitin is the raw material for all commercial production of chitosan and glucosamine, with estimated annual production of 2000 and 4000 tons, respectively [24]. Most commonly, chitin forms the skeletal structure of invertebrates. At least 10 Gtons (1×10^{13} Kg) of chitin are constantly present in the biosphere [25]. Chitin is a linear polymer of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit), which is insoluble in aqueous solvents. It also has many structural similarities with cellulose such as conformation of the monomers and diequatorial glycosidic linkages. Chitosan may be considered as a family of linear binary copolymers of (1 → 4)-linked A-units and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit). The term chitosan does not refer to a uniquely defined compound; it merely refers to polysaccharides having different composition of A and D units, which is a white, hard, inelastic, and nitrogenous polysaccharide. It has been proposed to define chitin and chitosan based on their solubility in aqueous acetic acid, that is, chitosan as soluble and chitin as insoluble [26].

3. Production of Chitosan

A variety of procedures have been developed and proposed over the years for preparation of pure chitosan. Several of these form the basis of chemical processes for industrial production of chitosan from crustacean shell waste. For chitin production, the raw materials most abundantly available are the shells of crab, shrimp, and prawn (69–70%) [16, 19, 27]. Because chitin is associated with other constituents, harsh treatments are required to remove them from chitinous material to prepare chitin and then chitosan on a large scale. Proteins are removed from ground shells by treating them with either sodium hydroxide or by digestion with proteolytic enzymes such as papain, pepsin, trypsin, and pronase [28]. Minerals such as calcium carbonate and calcium phosphate are extracted with hydrochloric acid. Pigments such as melanin and carotenoids are eliminated with 0.02% potassium permanganate at 60°C or hydrogen

peroxide or sodium hypochlorite. Conversion of chitin to chitosan generally is achieved by hydrolysis of acetamide groups of chitin. This is normally conducted by severe alkaline hydrolysis treatment due to the resistance of such groups imposed by the *trans*-arrangement of the C2-C3 substituents in the sugar ring [29]. Thermal treatments of chitin under strong aqueous alkali are usually needed to give partially deacetylated chitin (degree of acetylation, DA < 30%), regarded as chitosan. Usually, this process is achieved by treatment with concentrated sodium or potassium hydroxide solution (40–50%) at 100°C or higher to remove some or all the acetyl groups from the polymer [19, 30, 31]. This process, called deacetylation, releases amine groups (NH₂) and gives the chitosan a cationic characteristic. This is especially interesting in an acid environment where the majority of polysaccharides are usually neutral or negatively charged. The deacetylation process is carried out either at room temperature (homogeneous deacetylation) or at elevated temperature (heterogeneous deacetylation), depending on the nature of the final product desired. However, the latter is preferred for industrial purposes. In some cases, the deacetylation reaction is carried out in the presence of thiophenol as a scavenger of oxygen or under N₂ atmosphere to prevent chain degradation that invariably occurs due to peeling reaction under strong alkaline conditions [32]. One other method of preparing chitosan of improved purity is to dissolve the materials in an acid (e.g., acetic acid) and filter to remove extraneous materials. The clarified product is then lyophilized to give a water-soluble chitosonium acid salt or precipitated with NaOH, washed, and dried to give a product in the free amine form [19].

Recent advances in fermentation technologies suggest that the cultivation of selected fungi can provide an alternative source of chitin and chitosan. The amount of these polysaccharides depends on the fungi species and culture conditions. Fungal mycelia are relatively consistent in composition and are not associated with inorganic materials; therefore, no demineralization treatment is required to recover fungal chitosan. Usually, the Zygomycetes class has higher amounts of chitin and chitosan in their cell walls when compared to other classes of fungi [33–42]. The use of biomass from fungi has demonstrated great advantages, such as: independence of seasonal factor, wide-scale production, simultaneous extraction of chitin and chitosan, extraction process is simple and cheap resulting in reduction in time and cost required for production, and also absence of proteins contamination, mainly the proteins that could cause allergy reactions in individuals with shellfish allergies [33, 43–47]. However, to optimize the production of chitin and chitosan from fungi, complex or synthetic media, which are expensive are usually used. It has become necessary to obtain economic culture media that promote the growth of fungi and stimulate the production of the polymers. Recently, microbiological processes were used for chitin and chitosan production by *Cunninghamella elegans* grown by submerge fermentation in economic culture medium, yam bean (*Pachyrhizus erosus* L. Urban), as substrate [48]. The main characteristic of yam bean is the simple manipulation and low nutrition

requirements when compared with other similar cultures, and tuberous roots yields are up to 60 ton/ha. The extraction of chitin and chitosan from different species of mushrooms (i.e., *Agaricus bisporus*, *Auricularia auriculajudae*, *Lentinula edodes*, *Trametes versicolor*, *Armillaria mellea*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Pleurotus eryngii*) has been also illustrated [39, 49]. The mushroom, *P. sajor-caju*, showed highest yield of biomass and *L. edodes* was the lowest when compared with others under submerged fermentation. The processes and conditions for the extraction of chitin and chitosan from mushroom were nearly same in the methods of Crestini et al. [50] and Pochanavanich and Suntornsuk [39], and were different in Mario et al. [49]. More over, the chitin composition and structure have been studied in insects, terrestrial crustaceans, and nematodes. However, their demineralization studies were carried out using HCl (1–2 N) for 0.3–96 h at 25–100°C, which is stronger than the demineralization process of aquatic crustacean materials [38, 51–55].

4. Physiochemical Properties of Chitosan

4.1. Crystalline Structure. Since chitosan is a heterogeneous polymer consisting of GlcN and GlcNAc units, its properties depend on the structure and composition. Ogawa and Yui [56] studied the crystalline structure of different chitin/chitosan samples prepared by two different procedures: (a) the partial deacetylation of chitin, and (b) the partial reacylation of a fully deacetylated chitin (pure chitosan). It was observed that the partially reacylated material was less crystalline than pure chitosan. They also showed that, for the preparation of a less crystalline chitosan sample, it is preferable to proceed via reacylation of fully deacetylated chitosan rather than direct solid-state deacetylation of chitin. This preferred treatment also resulted in less anhydrous crystals. Generally, the step of dissolving the polymer results in a decrease in the crystallinity of the material. However, it also depends on the secondary treatment (reprecipitation, drying, and freeze-drying). In addition, the origin may affect the residual crystallinity of chitosan, which in turn controls the accessibility to internal sorption sites and the diffusion properties (rehydration and solute transport) and also deacetylation procedure may affect the solid state structure of chitosan [57–59].

4.2. Degree of N-Acetylation. An important parameter to examine closely is the DA in chitin, that is, the ratio of GlcNAc to GlcN structural units. In chitin, the acetylated units prevail (DA ≥ 90%), where as chitosan is fully or partially *N*-deacetylated derivative with a DA of less than 30%. This ratio has a striking effect on chitin and chitosan solubility and solution properties. To define this ratio, attempts have been made with several analytical methods [18, 60–65], which include IR, UV, and NMR spectroscopies, pyrolysis gas, gel permeation chromatography (GPC), thermal analysis, various titration schemes, and acid hydrolysis. Furthermore, in case of chitin, the DA determines the suitable conditions of deacetylation, a complex process still requiring investigation in order to assess the feasibility of quicker preparation of

chitosan. Thus, the search for quick, user-friendly, low cost, and accurate method to determine the DA has been one of the major concerns over many decades. However, the inherent complexity of this particular system turns this apparently simple analytical problem into a very difficult one, which is well illustrated by the extensive number of different techniques, either destructive or nondestructive, yielding direct or nondirect and frequently non reproducible values [26, 66–69]. It is worth noting that nondestructive methods offer the advantage of avoiding manipulations of the polymers such as hydrolysis, pyrolysis, or derivatization, the consequences of which are not always well known. Especially, FTIR spectroscopy is considered to be a very attractive technique, as it is nondestructive, fast, sensitive, user-friendly, low priced, and suitable for both soluble and nonsoluble samples [62]. Proton NMR spectroscopy is a convenient and accurate method for determining the chemical structure of chitosan and its derivatives [61, 70–73]. NMR measurements of chitosan compounds are, however, limited to samples that are soluble in the solvent, which limits the analysis of chitosan with DA values lower than 0.3 in aqueous solutions. A typical proton NMR spectrum of chitosan is shown in Figure 1. The signal at δ 3.20 ppm was attributed to H-2 of GlcN residue. The intense band at 4.8–5.30 ppm is related to OH groups and HDO (solvent). In this region, as observed more clearly from an extended spectrum, some different anomeric protons (H-1 of GlcN and GlcNAc units) are appeared at 4.88–5.00 ppm. The DA is calculated from the integral ratio between protons of acetyl group and the GlcN protons. The degree of deacetylation (DDA) is calculated from the integral ratio between the proton on C-2 and the glucose unit protons [71, 74, 75]. The $^1\text{H-NMR}$ spectra also allowed us to propose a method to determine the degree of substituent (DS) value and the determination is based on the ratio between the protons of the substituent and the protons of the pyranose unit [71, 76–79].

The role of the DA on the chain stiffness has essentially been assessed by viscometry considering the Mark-Houwink-Kuhn-Sakurada (MHKS) relationship [80, 81], $[\eta] = KM^a$, and by static light scattering through the relation $R_G = KM^\nu$, where $[\eta]$ and R_G correspond to the intrinsic viscosity and the radius of gyration, respectively. Experiments have been performed either with parent samples having different degrees of polymerization [82] or with samples fractionated by size exclusion chromatography [83]. Many authors found an increase of the a coefficient with DA and concluded that acetyl groups induce some stiffness to the chains [82, 84, 85], whereas the dependence of ν on DA is less significant [83, 85, 86].

4.3. Molecular Weight. As polysaccharides in general, chitosans are polydisperse with respect to molecular weight (MW). The MW difficulty encountered in this respect concerns the solubility of the samples and dissociation of aggregates often present in polysaccharide solution. As to choosing a solvent for chitosan characterization, various systems have been proposed, including an acid at a given concentration for protonation together with a salt to screen the electrostatic interaction because of the MW chitosan is

an average over the whole distribution of Mws. The MW heterogeneity of polysaccharides can be described by several types of average MW. The two most common methods in use for averaging are the number-average, M_n (which weighs the polymer molecules according to the number of molecules having a specific MW) and the weight-average, M_w (which weighs the polymer molecules according to the weight of molecules having a specific MW). The MW of chitosan depends on its source and deacetylation conditions (time, temperature, and concentration of alkali). Chitosan obtained from deacetylation of crustacean chitin may have a MW over 100,000 Da. Consequently, it is necessary to reduce the MW by chemical or enzymatic methods to much lower MW for easy application and high biological activity.

In order to evaluate the MW of polymeric chain, various methods can be used extensively. Viscometric [87] and GPC [88] techniques are easy to perform and low time consuming. On the other hand, they are empirically related to the MW, because the measurement depends upon the hydrodynamic volume of the macromolecule, which is a function of the MW conformational properties, and polymer-solvent interaction. As a consequence, a calibration curve is required. The viscosity of chitosan solutions is measured by using Ubbelohde Viscometer. The running times of the solution and solvent are used to calculate the relative viscosity (η_{rel}), specific viscosity (η_{sp}), and reduced viscosity (η_{red}) as follows: $\eta_{\text{rel}} = t_{\text{ch}}/t_{\text{sol}}$; $\eta_{\text{rel}} = t_{\text{ch}}/t_{\text{sol}}$; $\eta_{\text{red}} = \eta_{\text{sp}}/c$, where t_{ch} and t_{sol} are the running times of the chitosan solution and solvent, respectively and c is the chitosan concentration in g/dL. The intrinsic viscosity, defined as $[\eta] = C(\eta_{\text{red}})_{c=0}$, is obtained by extrapolating the η_{red} versus concentration data to zero concentration and the intercept on the ordinate is the intrinsic viscosity [82, 89–91]. Finally the average molecular weight (M) is calculated based on the MHKS equation ($[\eta] = KM^a$), where K and a are viscometric parameters depending on the solvent [92, 93]. For example, a chitosan dissolved in 0.5 M $\text{CH}_3\text{COOH}/0.2$ M CH_3COONa , K and a were found to be 3.5×10^{-4} and 0.76, respectively according to Terbojevich et al. [94] and Wang et al. [82]. On the contrary, the light scattering (LS) method [95–97] gives absolute values for MW, but the technique is more difficult and sometimes the data are not easy to interpret.

4.4. Solubility and Charge Density. Chitin and chitosan degrade before melting, which are typical for polysaccharides with extensive hydrogen bonding. This makes it necessary to dissolve them in an appropriate solvent system to impart functionality. For each solvent system, polymer concentration, pH, counter ion concentration, and temperature effects on the solution viscosity must be known. When the DDA of chitin reaches about 50% (depending on the origin of the polymer), it becomes soluble in aqueous acidic media and is called chitosan. Comparative data from solvent to solvent are not available. As a rule, the maximum amount of polymer is dissolved in a given solvent towards a homogeneous solution. Subsequently, the polymer is regenerated in the required form. A coagulant is required for polymer regeneration or solidification. The nature of the coagulant is also highly dependent on the solvent and

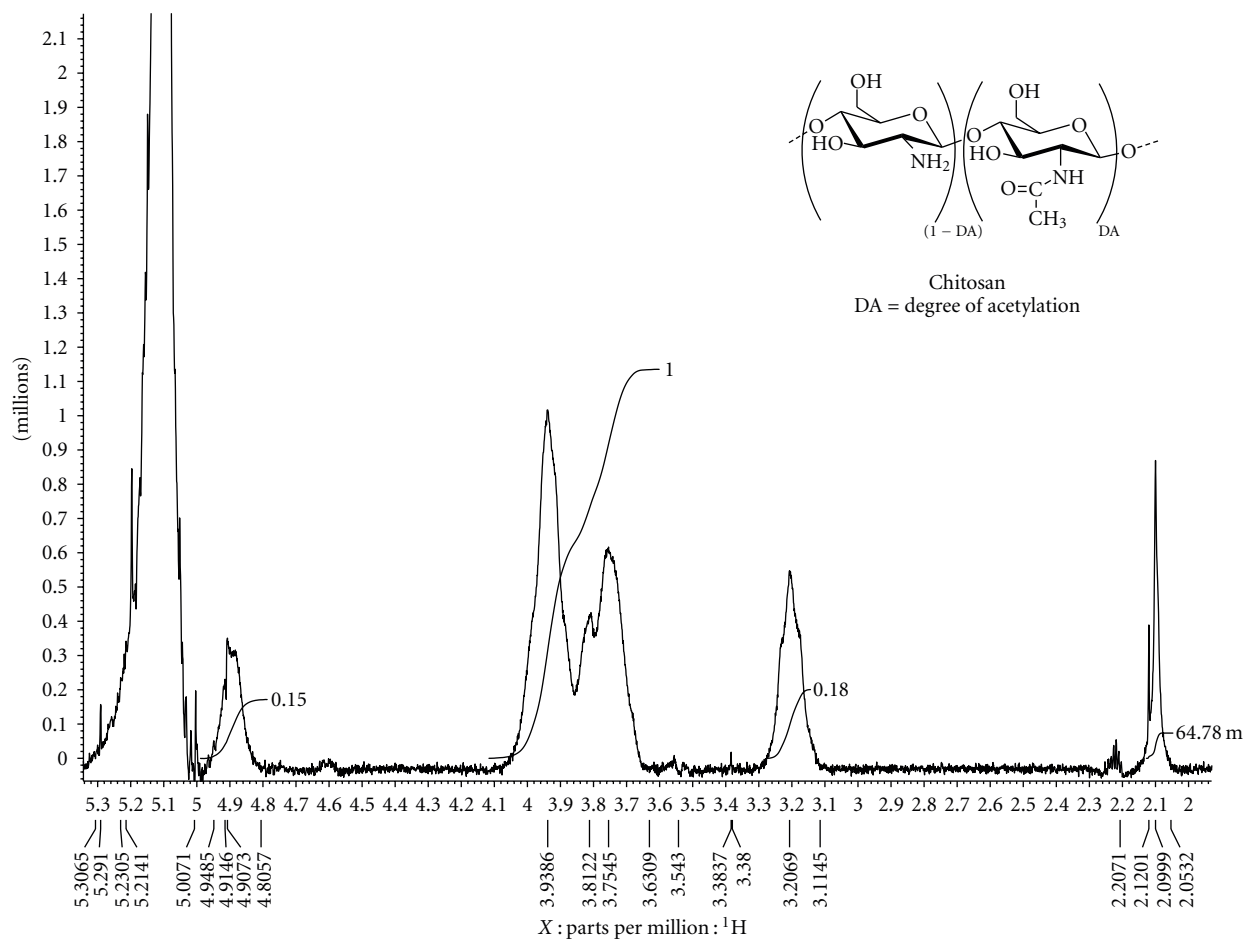


FIGURE 1: ^1H -NMR spectrum (300 MHz) of chitosan in 0.5% $\text{CD}_3\text{COOD}/\text{D}_2\text{O}$ at 25°C .

solution properties as well as the polymer used [63, 98]. Chitosan, being a cationic polysaccharide in neutral or basic pH conditions, contains free amino groups on C-2 of GlcN unit and hence, is insoluble in water. In acidic pH, amino groups can undergo protonation thus, making it soluble in water. Therefore, solubility of chitosan depends upon the distribution of free amino and *N*-acetyl groups [99]. Usually 1–3% aqueous acetic acid solutions are used to solubilize chitosan [100]. The macromolecule chains are stretched caused by electrostatic repulsion of the NH_3^+ groups. The stretched chains will tend to be coiled with addition of salt because of the charge screening effect of added salt. The extent of solubility depends on the concentration and type of acid, where as the solubility decreases with increasing concentration of acid and aqueous solutions of some acids such as phosphoric, sulfuric, and citric acids are not good solvents [101]. The charge density of chitosan, that is, the degree of protonation of amino groups, is determined by the chemical composition, MW, and external variables such as pH and ionic strength. Dissociation constants (pK_a) for chitosan range from 6.2 to 7, depending on the type of chitosan and conditions of measurement [61, 91, 102, 103]. A number of solvents for chitin and chitosan can be found in the literature. Generally, the solubility decreases with an

increase in MW [63, 104]. Moreover, few attempts have been made to enhance chitosan's solubility in organic solvents [105–108]. However, many other attempts have been made to enhance its solubility in water. One major reason is because most biological applications for chemical substances require the material to be processible and functional at neutral pH. Thus, obtaining a water soluble derivative of chitosan is an important step towards the further application as a biofunctional material [70, 109, 110].

4.5. Viscosity. Viscosity is an important factor in the conventional determination of chitosan MW and in determining its commercial applications. Higher MW chitosan often render highly viscous solutions, which may not be desirable for industrial handling. However, lower viscosity chitosans may facilitate easy handling. The solution viscosity of chitosan depends on its molecular size, cationic character, and concentration as well as the pH and ionic strength of the solvent [111]. The determination of the intrinsic viscosity of polyelectrolyte is an effective method to study the sensibility of polyelectrolyte to the addition of salt. Therefore, the dilute solution viscosity of three chitosan samples (MW = 2.6×10^5 , 5.6×10^5 , and 1.06×10^6 Da) was measured both in solutions of NaCl with different ionic strengths

(0.01, 0.03, 0.06, and 0.10 mol/L) and in those of NaCl, KCl, CaCl₂, and BaCl₂ with the same ionic strength (0.06 mol/L), respectively [112]. It was found that the response of the intrinsic viscosity of chitosan to the added salts proceeds in the order of anion Na⁺ > K⁺ > Ba²⁺ > Ca²⁺. According to the comparison of the ionic radius and hydrate number among the four anions, it is clear that the influence of intrinsic viscosity of chitosan on the added salt was due to both the anion radius and anion solvent power. Meanwhile the parameter of the MHKS equation was estimated to be 0.78, 0.70, 0.67, and 0.62 in corresponding added salt ionic strength (0.01, 0.03, 0.06, and 0.10 mol/L) [113].

4.6. Chemical Reactivity. Chitosan has three reactive groups, that is, primary (C-6) and secondary (C-3) hydroxyl groups on each repeat unit and the amino (C-2) group on each deacetylated unit. These reactive groups are readily subject to chemical modifications to alter mechanical and physical properties of chitosan. The typical reactions involving the hydroxyl groups are etherification and esterification. Selective *O*-substitution can be achieved by protecting the amino groups during the reaction [71]. The presence of a nucleophilic amino group allows for selective *N*-substitution, such as *N*-alkylation and *N*-acylation by reacting chitosan with alkyl halides and acid chlorides, respectively [71, 75, 114–117]. The alternative method for the *N*-alkylation is a reductive alkylation, where the amino group is converted to an imine with an aldehyde or ketone, and subsequently reduced to an *N*-alkylated derivative [26, 70, 76, 118–120].

4.7. Film-Forming Properties. In recent years, increasing interest in edible films has developed mainly due to concern over the disposal of conventional synthetic plastic materials derived from petroleum. Degradation of plastics requires a long time and most of them end up overburdening on landfill. Conversely, edible films from renewable agriculture products not only are degraded readily after their disposal, but also can extend the food shelf life, thus improving the quality of food. Among various available edible film materials, considerable attention has been given to chitosan because of its unique properties. It has been extensively studied for applications as films or membranes. These films can be described as biofilms with a homogeneous matrix, stable structure, good water barrier, and mechanical properties [121–124]. The functional properties of chitosan films are improved when chitosan is combined with other film-forming materials. Hoagland and Parris [125] prepared chitosan-pectin laminated films by interaction between the cationic groups on chitosan with the anionic groups on pectin. Hosokawa et al. [126] reported that when biodegradable films were made from chitosan and homogenized cellulose oxidized with ozone the number of carbonyl and carboxyl groups on the cellulose interacting with the amino groups on the chitosan increased. The water resistance of chitosan film was ameliorated by the incorporation of hydrophobic materials such as fatty acids to enhance the film's hydrophobicity [127]. Starch has been used to produce biodegradable films to partially or entirely replace plastic polymers because of its low cost and renewability. However,

a wide application of starch film is limited by its water solubility and brittleness [128, 129].

4.8. Gelling Properties. Hydrogels are three-dimensional networks that swell in water and aqueous solutions. These materials, based on both natural and synthetic polymers, are currently attracting a great deal of interest as bioactive molecules and in tissue engineering. Among natural biopolymers of interest, chitosan stands out due to its unique combination of favorable properties such as hydrogel forming. Chitosan hydrogels can be divided into two classes: physical and chemical. Chemical hydrogels are formed by irreversible covalent links, whereas physical hydrogels are formed by various reversible links. For various reasons, physically cross-linked hydrogels have attracted increasing attention as bioactive compounds.

The preparation and characterization of a few hydrogels of chitosan have been reported, such as thermoreversible chitosan-oxalate, chitosan-aldehyde gels [130–132], and chitosan-alginate [133]. So far, no simple ionic and nontoxic cross-linking agent has been found that gives reproducible chitosan gels at low concentrations, such as calcium ions for gelling of alginates. However, aqueous chitosan gels crosslinked with molybdate polyoxy-anions have been reported, resulting in transparent, thermoirreversible gels that are able to swell several times their original size in aqueous solutions, depending on the ionic strength [134]. Different chitosan gels made with covalent cross-linking have been reported, with cross-linking with glutaraldehyde being the most widely applied [135, 136]. In addition, an enzymatic gelling system with chitosan has been reported [137–139].

4.9. Ion Binding. Chitosan is proved to have the best chelating properties among other natural polymers [140]. Responsible for complex formation are amino groups of chitosan, in which nitrogen is a donor of electron pairs, although hydroxyl groups may also participate in sorption. The mechanism of combining these reactive groups with ions of metals is much differentiated and can depend on the ion type, pH, and also on the main components of the solution. The complexes formation could be also described based on Lewis acid-base theory: metal ion (acting as the acid) is the acceptor of a pair of electrons given by chitosan (acting as the base).

In relation to food applications of chitosan, its application as a cholesterol-lowering agent [141, 142] and more controversial use as a weight-reducing agent, knowledge on the selective binding of essential metal ions to chitosan is important. Most studies of ion binding to chitosan have been aimed at determining whether chitosan binds to a given ion, whereas only a few studies have involved determining the selectivity of binding of different ions to chitosan. Rhazi et al. [143] determined the selectivity of mixtures of the ions Cu²⁺ > Hg²⁺ > Zn²⁺ > Cd²⁺ > Ni²⁺ > Co²⁺ = Ca²⁺, using potentiometric and spectrometric methods. Vold et al. [144] reported the selectivity of different chitosans in binary mixtures of Cu²⁺, Zn²⁺, Cd²⁺, and Ni²⁺, showing that chitosan could bind Cu²⁺ in large excess of the other metal ions. Recent years, chitosan-metal complexes attracted

great interests for their potential use in agriculture, medical industry, and food industry [145–148]. It is well known that both chitosan and metals such as Ag^+ , Cu^{2+} , Ni^{2+} , and Zn^{2+} have the properties of disinfection and bactericide [147, 149]. After chitosan binds to metal ions through nitrogen and or oxygen, the bindings are likely to leave some potential donor atoms free and these free donor atoms enhance the antimicrobial activity [148]. So it stands a good chance that chitosan-metal complexes exhibit enhanced ability of the antimicrobial activity of chitosan molecule, which will be very favorable to their applications in agriculture, medical industry, and food industry [147, 148].

4.10. Emulsification. Even though chitosan alone does not produce emulsions, Cho et al. [150] reported that emulsifying capacity of egg yolk increased with the addition of chitosan. At a concentration of 0.5%, better emulsifying capacity was observed compared with at 0.1 or 0.3% chitosan. In general, chitosan emulsions tend to be very stable under temperature changes and aging. With viscosity, the DA is reported to be a determining factor in the emulsification properties of chitosan. The protein solution containing chitosan with intermediate DDA produces less effective emulsion compared with that containing chitosan with higher DDA. The optimum chitosan DDA for sunflower oil emulsification is 81 and 89 as reported by Del Blanco et al. [151] and Rout [152], respectively.

5. Modification of Chitosan Structure and Properties

Chemical modifications of chitosan are increasingly studied as it has the potential of providing new applications. With regard to its unique properties such as biocompatibility, biodegradability, and no toxicity to mammals, it is widely used in fields like biotechnology, pharmaceuticals, cosmetics and agriculture. In particular the antimicrobial activities of chitosan and its derivatives have aroused considerable recent interest. Unfortunately, in spite of the chitosan advantages, the poor solubility, low surface area, and porosity of chitosan are the major limiting factors in its utilization. Its solubility is limited at a pH higher than 6.5 where chitosan starts to lose its cationic nature. This problem is probably the major limiting factor for chitosan utilization, that is, its application in biology, since many enzyme assays are performed at neutral pH. If water-soluble chitosan would be easily accessible, it is expected that the biological and physiological potential would increase dramatically.

Chitosan can be modified by physical or chemical processes in order to improve the mechanical and chemical properties. Chitosan is a multinucleophilic polymer due to the presence of the amino group at C-2 and hydroxyl groups at C-3 and C-6 in the GlcN residue. Chitosan membrane is swollen in water; the amino groups may be protonated and leave the hydroxide ions free in water, which may contribute to the ionic conduction in the membrane. The initial sites where substitution occurs are the more nucleophilic amino groups. However, the experimental conditions and protection of the amino groups reduce the intermolecular

hydrogen bonding and creates space for water molecules to fill in and solvate the hydrophilic groups of the polymer backbone [75]. For introducing alkyl or substituted alkyl groups selectively at the amino groups, reductive alkylation is the most reliable procedure. Chitosan is treated with an aldehyde to give an imine (Schiff base), which is easily converted into an *N*-alkyl derivative by reduction with sodium borohydride or sodium cyanoborohydride [76, 78, 119, 120, 153, 154]. These reactions are facile; the DSs are generally high and the products are soluble in water or dilute acids. The chitosan derivatives mentioned in the literatures showed that one can differentiate specific reactions involving the $-\text{NH}_2$ group at the C-2 position or nonspecific reactions of $-\text{OH}$ groups at the C-3 and C-6 positions (especially esterification and etherification) [26, 155–158]. The positive charges on chitosan can also participate in ionic interactions, particularly with polyanions such as alginate and pectin. The complexes formed by electrostatic interaction between COO^- or SO_4^{--} and NH_4^+ [159] have been proposed for the recovery of suspended solids from aqueous food processing streams [160, 161] that can be used for animal feed.

Hydroxyalkyl chitosans are usually obtained in reactions of chitosan with epoxides. Depending on the reaction conditions (pH, solvent, and temperature); the reaction may take place predominantly at the amino or hydroxy groups giving *N*-hydroxyalkyl- or *O*-hydroxyalkyl chitosans or a mixture of both types. Under neutral and acidic conditions, *N*-hydroxyalkyl chitosan is preferred, leading to DS value < 2 . However, under alkaline conditions, the strongly nucleophilic oxygen ions will react much faster, resulting in *O*-hydroxyalkyl chitosan with DS values > 2 [26, 162, 163].

Acylation of chitosan was the usual method involving reacting chitosan under homogeneous reaction conditions with either an acid chloride or acid anhydride [164]. Acylation was shown to proceed smoothly at the free amino groups preferentially and then more slowly at the hydroxyl groups [164]. Complete *N*-acylation has been achieved by treating chitosan with cyclic acid anhydrides in aqueous homogeneous media at pH 4 to 8. Some of the resulting *N*-carboxyacyl chitosans were successfully converted into the corresponding imido forms by thermal dehydration [165]. *N*-acetylation of chitosan can be controlled when carried out in aqueous acetic acid solutions or in a highly swollen gel state in pyridine. With this gel, 50% *N*-acetylation was achieved, and the product was found to be soluble in neutral water [166, 167]. In case the swelling of chitosan is not sufficient, even the product with a similar DA does not give a homogeneous solution in water. Furthermore, no appreciable degradation is expected during the acetylation, and hence water-soluble chitosans with desired MWs can be prepared. Partial acetylation is also possible in homogeneous solutions in aqueous acetic acid/methanol [144] or in aqueous acetic acid [168] to give water-soluble products. The highest water solubility was again observed for a DA of 0.5. Under appropriate conditions similar to those for the benzoylation of chitin [169], chitosan was benzoylated (DS up to 2.5) with benzoyl chloride in methanesulfonic acid [170, 171].

N-saturated fatty acyl chitosan derivatives soluble in water, aqueous alkaline and acid solutions were prepared [117, 172, 173]. Acyl substitution was reported to take place on both *O*- and *N*-positions under a large excess of acid chloride. The successful preparation of *N,O*-acyl chitosans in MeSO₃H as solvent was performed by Sashiwa et al. [79, 174] and also in our laboratory [71]. In this method, chitosan was dissolved in MeSO₃H and the acid chlorides were added dropwise. The homogenous mixture was neutralized with NaHCO₃, then dialyzed and lyophilized to obtain the (*N,O*-acyl) chitosan derivatives with *O*-substitution as a major product. A noteworthy point is that both moderate substitution of *N,O*-acyl groups and moderate MW are important factors in obtaining highly biologically active compounds. Although the selective *O*-acylation of chitosan in MeSO₃H (owing to the salt formation of the primary amino group with MeSO₃H) was reported [175], the detailed chemical structure and the protecting effect of MeSO₃H on the amino group are not clear yet. The preparation of *O,O*-didecanoyl chitosan was also reported through a protected *N*-phthaloyl chitosan as intermediate [176]. However, this method needs several steps for the protection and deprotection of the *N*-phthaloyl groups [177]. Some *N*-carboxyacyl chitosans were also prepared by reaction of chitosan with intramolecular carboxylic anhydrides including maleic, glutaric, phthalic, and succinic [115, 165, 178]. As related compounds, some cyclic phthalimido derivatives of chitosan were reported [179]. In addition, *N*-carboxyacyl chitosans filaments were synthesized by suspended chitosan in methanol and carboxylic anhydrides were added [116, 180]. These compounds are usable as new functional materials in many fields because of their hydrophilic and acidic properties.

Grafting of chitosan allows the formation of functional derivatives by covalent binding of a molecule, the graft, onto the chitosan backbone [181]. The properties of the resulting graft copolymers are controlled by the characteristics of the side chains, including molecular structure, length, and number [182]. The cross-linking agents can be of varying length and contain other functional groups than those involved in cross-linking [183]. Partial cross-linking by di/polyfunctional reagents enables the use of chitosan for metal adsorption in acidic medium. Several bi- or polyfunctional cross-linking agents such as glutaraldehyde [184–187], ethylene glycol diglycidyl ether [188, 189], glyoxal [190], epichlorohydrin [191, 192], benzoquinone [193], and cyclodextrin [194–196] have been used. The fact that the cross-linking agents cited before are neither safe nor environment friendly has led to the use of water-soluble cross-linking agents such as sodium trimetaphosphate, sodium tripolyphosphate, or carboxylic acids [197].

One of the important strategies to increase both the solubility and positive charge density of chitosan is based on the introduction of quaternary ammonium groups into chitosan. This modification has got the commonly accepted term “quaternization of chitosan”. Thus, derivatives soluble in water and in both acidic and basic physiologic circumstances may be good candidates for the polycationic

biocides [198, 199]. Many efforts to synthesize quaternized chitosan derivatives have been reported. For example, Muzarelli and Tanfani [157] reported the formation of *N,N*-dimethyl chitosan and the preparation of *N,N,N*-trimethyl chitosan iodide with formaldehyde and sodium borohydride. Trimethyl chitosan ammonium iodide was also obtained by reaction of a low acetyl content chitosan with methyl iodide and sodium hydroxide under controlled conditions [200, 201]. Water-soluble quaternary ammonium salts of *N,N,N*-trimethyl, *N,N*-propyl-*N,N*-dimethyl, and *N*-furfuryl-*N,N*-dimethyl chitosans were also prepared by reacting of *N*-alkyl chitosan derivatives with methyl iodide [109, 202]. Stepnova et al. [203] discovered a new, smooth, and one-step method for preparation of quaternized chitosans by means of reaction with betaine in the presence of the coupling reagent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline in aqueous media at pH 5.5 ± 0.5. This reaction results in preparation of *N*-((trimethylammonio)acetyl) chitosan chloride and its amphiphilic derivatives. Other derivatives of quaternary chitosans as water-soluble and antimicrobial agents were recently prepared in our laboratory based on three-step process [70]. Schiff bases were firstly synthesized by the reaction of chitosan with aliphatic aldehydes followed by a reduction with sodium borohydride to form *N*-alkyl chitosans. *N,N,N*-dimethyl alkyl chitosans were then obtained by a reaction of chitosan containing *N*-butyl, (-pentyl), -hexyl, -heptyl, and -octyl substituents with methyl iodide.

The –OH and –NH₂ groups on the skeleton of chitosan are good ligands to coordinate with transition metal ions to get chitosan-metal complexes [146–148]. Moreover, the amine group of chitosan is modified using many chemical methods including chitosan 6-*O*-sulfate [204, 205], *N*-sulfated chitosan [206], and *N*-methylene phosphonic chitosans [207, 208]. So the functional groups of chitosan are easily modified by many organic reactions: tosylation [209], alkylation [210], carboxylation [211], sulfonation [205], Schiff base [212], and quaternary salt [157].

6. Application of Chitosan in Crop Protection

6.1. Chitosan and Its Derivatives as Antimicrobial Agents against Plant Pathogens (In Vitro Studies). The enormous increase in the number of relevant research papers and patents revealed a surprisingly high level of chitosan research activity from both academic and industrial scientists. Many literatures reported that chitosan and its derivatives have antimicrobial and plant-defense elicit function [6, 213–218]; therefore, these compounds are considered as useful pesticides in the control of plant diseases. The ideal antimicrobial polymer should possess the following characteristics: (1) easily and inexpensively synthesized, (2) stable in long-term usage and storage at the temperature of its intended application, (3) soluble in water or neutral media, (4) does not decomposed to and/or emit toxic products, (5) should not be toxic or irritating to those who are handling it, (6) can be regenerated upon loss of activity, and (7) biocidal to a broad spectrum of pathogenic microorganisms in brief times of contact [219].

Numerous studies on the antimicrobial activity of chitosan and its derivatives against most economic plant pathogens have been investigated [26, 70, 71, 76–78, 145, 220–227] and reviewed [6, 8, 10, 100, 218, 228–231]. Their antimicrobial activity has received considerable interest due to the problems associated with harmful synthetic antimicrobial agents [221, 232]. Chitosan's inhibition was observed on different development stages such as mycelial growth, sporulation, spore viability and germination, and the production of fungal virulence factors. It has been commonly recognized that the antimicrobial activity depends on the MW, DA, pH of chitosan solution and, of course, the target organism [202, 226, 232–244]. The natural antimicrobial characteristics of chitosan and its derivatives have resulted in their use in commercial disinfectants. Chitosan has several advantages over other types of disinfectants in that it possesses a high-antimicrobial activity, a broad spectrum of activity, and a low toxicity for mammalian cells [245]. Generally yeasts and moulds are the most sensitive group, followed by gram-positive bacteria and finally gram-negative bacteria [223]. The inhibitory activity was higher at pH 6.0 (pK_a value of chitosan = 6.2) than pH 7.5, when most amino groups are in the free base form.

Variation in sensitivity between closely related microorganisms was illustrated in an experiment in which plant pathogenic fungi were screened for sensitivity to chitosan in liquid media [246]. One *Cytosporina* sp. isolate was completely inhibited by 75 mg/L chitosan, while a second isolate of the same genus was unaffected by 1000 mg/L. Chien and Chou [247] noted that the antifungal activity of chitosan depends on the type, concentration and test organism. For example, at 0.1%, chitosan of 92.1 kDa showed a higher growth inhibition of 76.2% on *P. italicum* than did chitosan of 357.3 kDa (71.4%), while at 0.2%, the antifungal activity exerted by chitosan of 357.3 kDa was higher than chitosan of 92.1 kDa against *P. italicum*.

A report by Benhamou et al. [248] indicated that chitosan derived from crab-shell at concentrations of 500 and 1000 mg/L was effective in reducing disease incidence caused by *F. oxysporum* f. sp. *radicis-lycopersici*. At the same time El Ghaouth et al. [249] revealed that chitosan was effective in inhibiting mycelial growth of *P. aphanidermatum* completely at a concentration of 400 mg/L. In our laboratory, we found that chitosan concentration increased (750–6000 mg/L), the radial growth of *A. alternata*, *B. cinerea*, *Colletotrichum gloeosporioides*, and *Rhizopus stolonifer* was decreased [250]. The same effect was reported on *Sclerotinia sclerotiorum* when chitosan concentrations increased from 1% to 4% [251]. Other studies showed a linear decrease of growth of *R. solani* as the chitosan concentration gradually increased from 0.5 to 6.0 mg/mL [252]. However, a complete growth inhibition was recorded against *F. oxysporum*, *R. stolonifer*, *P. digitatum*, and *C. gloeosporioides* at concentrations of 3% [253, 254].

The fungicidal activity of three commercial chitosan samples (3.60×10^5 , 6.11×10^5 , and 9.53×10^5 Da) was tested in our laboratory against plant pathogenic bacteria of *Agrobacterium tumefaciens*, *Corynebacterium fascians*, *Erwinia amylovora*, *E. carotovora*, *Pseudomonas solanacearum*,

and *Sarcina lutea* [250]. The results indicated that chitosans of 6.11×10^5 and 9.53×10^5 Da were more potent in bactericidal activity than 3.60×10^5 Da chitosan and a chitosan of 9.53×10^5 Da exhibited a good antibacterial potency especially against *C. fascians* with MIC 500 mg/L. Moreover, three different MWs (0.5×10^4 , 3.7×10^4 , and 5.7×10^4 Da) chitosan were prepared in our laboratory from a commercial sample of chitosan (2.9×10^5 Da) and evaluated against bacteria of *A. tumefaciens* and *E. carotovora* and fungi of *A. alternata*, *B. fabae*, *F. oxysporum*, and *R. stolonifer* [255]. Chitosan of 0.5×10^4 Da exhibited a good antibacterial potency against *A. tumefaciens* with MIC 2600 mg/L, while chitosan of 3.7×10^4 Da was the most active against *E. carotovora* with MIC 950 mg/L. The antifungal activity was increased with decrease of the MW and chitosan of 0.5×10^4 Da exhibited a high antifungal potency against *B. fabae*, *F. oxysporum*, and *R. stolonifer* while the fungus of *A. alternata* was more sensitive to a chitosan of 3.7×10^4 Da. This fact is in agreement with studies of Kim and Rajapakse, [256] and Zhang et al. [257] who reported that oligochitosans, obtained by hydrolysis or degradation of chitosan, was not only water-soluble but also have shown to be more effective than chitosan. Interestingly, oligochitosan (hexamer unit) that elicited maximal pisatin formation also exhibited higher antifungal activity against *F. solani* than the lower with degree of polymerization [223]. Xu et al. [258] added that the oligochitosans prepared by enzymatic depolymerization were more effective than the original chitosan in inhibiting mycelial growth of nine phytopathogens *F. graminearum*, *Phytophthora capsici*, *Verticillium dahliae*, *A. solani*, *B. cinerea*, *C. orbiculare*, *Exserohilum turcicum*, *F. oxysporum*, and *Pyricularia oryzae* and their inhibition on different stages in life cycle was observed. Hirano and Nagao [259] testing high- and low-molecular-weight chitosan on different fungal species and they found that the best fungicidal activity on mycelia occurred in media supplemented with low-molecular-weight chitosan. However, Bautista-Baños et al. [260] indicated that no differences in the fungicidal pattern among the three different types of chitosan, whereas there was a higher fungicidal effect as chitosan concentration increased (0.5–2.0%). Meng et al. [261] reported that both of chitosan (350 kDa) and oligochitosan (6 kDa) strongly inhibited spore germination and mycelial growth of two phytopathogenic fungi *A. kikuchiana* Tanaka and *Physalospora piricola* Nose. El Ghaouth et al. [215] found that a chitosan at concentrations ranged from 750 to 6000 mg/L was very effective in inhibiting spore germination and germ tube elongation of *B. cinerea* and *R. stolonifer*. Furthermore, this biopolymer at a concentration greater than 1500 mg/L induced morphological changes in *R. stolonifer*. Hernández-Lauzardo et al. [262] confirmed that the spore morphology of *R. stolonifer* presented variations in area, form, and optical density in chitosan solutions.

Control of two sapstain fungi *Leptographium procerum* and *Sphaeropsis sapinea* by a combination of chitosan or chitosan oligomer and an albino strain of *Trichoderma harzianum* was tested by Chittenden and Singh [263]. There was no mycelial growth of the fungi regardless of chitosan concentrations used when either *L. procerum* or *S. sapinea* were simultaneously inoculated with *T. harzianum*.

However, the dose response of chitosan or chitosan oligomer was apparent when *T. harzianum* was not simultaneously inoculated with test fungi but introduced later. There was a greater growth reduction at higher concentrations (0.075–0.1%) of chitosan, and overall chitosan oligomer was more effective than chitosan aqueous solution. Chitosan alone was able to restrict or delay the germination of spores but the combination of chitosan and *T. harzianum* inhibited spore germination and hence colony formation of test fungi regardless of time delay.

In addition, chitosan shows an antiviral activity against plant viruses. It was shown that chitosan inhibited the productive infection caused by the bacteriophage, the efficiency of inhibition of bacteriophage depending directly on the final concentration in the medium [264]. Major factors of suppressing phage infections by chitosan are phage particle inactivation and inhibition of bacteriophage reproduction at the cellular level. Evidently, chitosan may be used for induction of phagoresistance in industrial micro-organism cultures to prevent undesirable phagolysis caused by inoculum contamination by virulent bacteriophages or by spontaneous prophage induction in lysogenic culture.

According to the data published on the antifungal and antibacterial activities of chitosan which indicated low activity observed against plant pathogens, several research groups have started to modify a chitosan molecule to produce high-antimicrobial active compounds. For examples, *N*-sulfonated and *N*-sulfobenzoyl chitosans [234], *N,N,N*-trimethyl chitosan [109], *N,O*-acyl chitosans [71, 79], *O*-acyl chitosans [77], hydroxyethyl acryl chitosan [265], dimethylpiperazine and trimethylpiperazine chitosans [266], carboxymethyl chitosans [267, 268], acyl thiourea chitosans [269], chitosan *N*-betainates [270], *N*-succinoyl chitosans [225], and *N*-heterocyclic chitosans [76]. We have prepared in our laboratory some of chitosan derivatives through the reductive amination reaction as described by Borch et al. [271] with various aldehydes. We noted that *N*-alkylation or -arylation of chitosan with aliphatic or aromatic aldehydes, respectively, effectively enhanced the antifungal activity of chitosan [77, 78, 119, 120]. For example, *N*-(*o,p*-diethoxybenzyl)chitosan was the most active one with EC₅₀ of 400 and 468 mg/L for *F. oxysporum* and *P. debaryanum*, respectively. With the same methods and techniques, but different kinds of aldehydes, we have synthesized a series of *N*-benzyl chitosan derivatives and the fungicidal assessment has been investigated against *B. cinerea* and *P. grisea* [119]. The data revealed that *N*-(*o,o*-dichlorobenzyl) chitosan was the most active compound against *B. cinerea* with an EC₅₀ of 520 mg/L. However, *N*-(benzo[d][1,3]dioxol-5-ylmethyl) chitosan, and *N*-(methyl-4H-chromen-4-one) chitosan as new *N*-(heterocyclic) chitosan derivatives were the most active against *P. debaryanum* and *F. oxysporum* [76]. Guo and coworkers [222] added that the Schiff bases of chitosan and the *N*-substituted chitosan derivatives had a slight activity against *B. cinerea* Pers., and the inhibitory indices were 26.8%, 33.5%, 39.3%, and 32.3% at 1000 ppm, respectively, compared with 45.4% at chitosan. Previously, we synthesized derivatives of *N,O*-acyl chitosans [71] and the data indicated that *N,O*-(*p*-chlorobutyl)

chitosan, *N,O*-decanoyl chitosan, *N,O*-cinnamoyl chitosan and *N,O*-(*p*-methoxybenzoyl) chitosan were the most active compounds against *B. cinerea* (EC₅₀= 430, 440, 450 and 500 mg/L, respect.) and were 12- to 13-fold more active than the native chitosan (EC₅₀ > 3000 mg/L).

Previously Muzzarelli et al. [272] prepared five chemically modified chitosans and tested their antifungal activities against *Saprolegnia parasitica*. Results indicated that, as for the chitosan-bearing broth assay, *S. parasitica* did not grow normally; on the first day for methylpyrrolidinone chitosan and *N*-phosphonomethyl chitosan and on the second day for *N*-carboxymethyl chitosan, a tightly packed precipitate was present at the bottom of the test tubes instead of the fluffy fungal material as in the control. In contrast, *N*-dicarboxymethyl chitosan seemed to favor fungal growth, while dimethylaminopropyl chitosan did not significantly differ from the control.

N,N,N-dimethylalkyl chitosans as quaternary and water-soluble chitosan compounds were recently prepared in our laboratory to test their antimicrobial activities against the most economic plant pathogenic bacteria *A. tumefaciens* and *E. carotovora* and fungi *B. cinerea*, *F. oxysporum*, and *P. debaryanum* [70]. Quaternary chitosans enhanced the antibacterial activity and *N,N,N*-dimethylpentyl chitosan was the most active with MIC 750 and 1225 mg/L against *A. tumefaciens* and *E. carotovora*, respectively. However, both of *N,N,N*-dimethylpentyl chitosan and *N,N,N*-dimethyloctyl chitosan were significantly the highest in fungal mycelial growth inhibition of *B. cinerea*, *F. oxysporum* and *P. debaryanum*. In addition, spore germination of *B. cinerea* and *F. oxysporum* was significantly affected with the compounds at the tested concentrations and the inhibition activity was increased with an increase in the chain length of the alkyl substituent. Previously Hernández-Lauzardo et al. [236] reported that spore germination of *R. stolonifer* was affected by different MWs chitosan (1.74×10^4 , 2.38×10^4 and 3.07×10^4 Da). They found that chitosan of 1.74×10^4 and 2.38×10^4 Da markedly reduced spore germination, but no significant effects were found among the tested concentrations (1.0, 1.5, and 2.0 mg/mL). However, they observed a complete inhibition of spore germination with a chitosan of 3.07×10^4 Da. Recently, the effect of *N*-(benzyl) chitosan derivatives on spore germination of *F. oxysporum* was evaluated in our laboratory at 250, 500, and 1000 mg/L [78]. All the derivatives had better inhibition of spore germination, about threefold compared with chitosan. *N*-(*p*-dimethylaminobenzyl) chitosan, *N*-(*p*-ethylbenzyl) chitosan, *N*-(*o*-methoxybenzyl) chitosan, and *N*-(*o,p*-diethoxybenzyl) chitosan significantly exhibited high inhibition percentage (>90%) of spore germination at 1000 mg/L.

6.2. Chitosan and Its Derivatives in Plant Disease Control (In Vivo Studies). The plant protection activity of chitosan compounds have been well documented in many different plant systems [10]. The control diseases of chitosan include bacteria, fungi, and viral diseases. In this section, the effects of these compounds on several plants will be discussed.

In 1980, Professor Hadwiger at Washington State University reported that oligochitosan can induce soybean against *F. solani* [273]. Oligochitosan from the *F. solani* f. sp. *phaseoli* cell walls could elicit defense reaction in pea pod tissue. Concentrations of oligochitosan as low as 0.9 and 3 mg/mL elicited phytoalexin induction and inhibited the germination of *F. solani*, respectively. This was the first publication of chitosan-induced plant resistance. From then, a series of excellent work was conducted in his laboratory. These creative researches led to an increased tide on the study of chitosan-induced plant defense.

The soilborne phytopathogenic fungi *F. solani* and *C. lindemuthianum* were inhibited by chitosan and *N*-(carboxymethyl) chitosan [274–276]. Benhamou et al. [248] added that chitosan concentrations of 0.5 and 1 mg/mL showed high plant protection from *F. solani*, when seed coating and soil amendment were performed. Although chitosan at 0.1 mg/mL induced a delay in disease development (root lesions visible by 4 days after inoculation), emergence of wilting symptoms occurred between 7 and 10 days after-inoculation, while death of about 80% of the plants was recorded 1 week later. *F. acuminatum*, *Cylindrocladium floridanum*, and other plant pathogens of interest in forest nurseries were inhibited by chitosan [277]. Similarly, *Aspergillus flavus* was completely inhibited in field-growing corn and peanut [278]. Generally, chitosan has high-antifungal activity, but it is less effective against fungi with a chitin or chitosan component in their cell walls [246]. Part of the effect observed by chitosan on the reduction of soilborne pathogens comes from the fact that it enhances plant defense responses. The other part is linked to the fact that it is composed of polysaccharides that stimulate the activity of beneficial micro-organisms in the soil such as *Bacillus*, fluorescent, *Pseudomonas*, actinomycetes, mycorrhiza, and rhizobacteria. This alters the microbial equilibrium in the rhizosphere disadvantaging plant pathogens. Beneficial organisms, on the other hand, are able to compete them through mechanisms such as parasitism, antibiosis, and induced resistance [279–284].

Great advancement of chitosan on rice disease control has been achieved in recent years. In 2002, Agrawal and coworkers reported the effect of chitosan in initiating defense response in the leaves of rice for the first time [285]. After treatment with 0.1% chitosan, necrotic streaking was clearly observed on the upper side of rice leaves. Enhanced defense against rice blast pathogen, *Magnaporthe grisea* 97-23-2D1, was observed in *H7S* rice seedlings treated with oligochitosan. In this experiment, 5 mg/L oligochitosan solution showed the best effect and the disease control was more than 50% [286]. Chitosan was tested in rice production by Boonlertnirun et al. [287] and its application by seed soaking and soil application four times throughout cropping season significantly increased rice yield over the other treatments. However, application by seed soaking and spraying the foliar four times tended to show ability on disease control. Zeng and Shi [288] developed a new type of organic rice seed coating agent using liquid-based polymeric adhesives. By using chitosan as the main raw material, modified with sodium hydroxide and polymerised with plant

growth regulators and other additives, the novel seed coating agent is a safer, cheaper, and more environmentally friendly alternative. Results of antifungal tests showed that the antifungal efficiency of the seed-coating agent was increased with increasing amount of dosage; a 1 : 20 concentration was the best for inhibiting growth of the two phytopathogens *R. solani* and *F. moniliforme*.

Wheat is another important source of staple food, especially in cold countries; therefore, Russian scientists conduct more research in this area. Studies conducted on wheat infection with *Bipolaris sorokiniana* indicated that oligochitin with a MW of 5–10 kDa and the DA of 65% has good effect on controlling wheat disease [289, 290]. The ability of oligochitosan to promote wheat resistance to pathogenic toxin was also validated [291]. Chitosan treatment (2–8 mg/mL) of wheat seeds (two cultivars of spring wheat (Norseman and Max)) significantly improved seed germination to recommended seed certification standards (>85%) and vigour at concentrations >4 mg/mL by controlling seed-borne *F. graminearum* infection. The germination was <80% in the control and >85% in chitosan-treated seeds. The reduction of seed-borne *F. graminearum* was >50% at higher chitosan treatments compared with control [292].

Tobacco is an important economic crop and a model plant for research. Many reports reveal that chitosan can induce tobacco's resistance to tobacco mosaic virus, tobacco necrosis virus, and *Phytophthora parasitica*. For example, Falcon studied the effect of different sizes and DA of chitosan derivatives on tobacco protection against *P. parasitica* [293]. The results of their experiment showed that different chitosans have distinct effects on this disease control, though less acetylated chitosan were better for inhibition of *P. parasitica* growth, partially acetylated chitosan were more effective in protecting tobacco against this pathogen by systemic induction of plant immunity.

Sclerotinia rot is the most harmful disease on oilseed rape production. The inducing resistance of oligochitosan to *Sclerotinia sclerotiorum* on *Brassica napus* was studied [294]. However, oligochitosan did not affect the radial growth of *S. sclerotiorum* colonies; it reduced the frequency and size of rot compared with controls when applied to oilseed rape before inoculation. The best pretreated time was 3 days before inoculation, and the best inducing resistance concentration of oligochitosan was 50 μ g/mL. Oligochitosan can be modulated into steady colloid solution, so it can be used as a seed-coating agent. It does not influence the seed sprout and emerge, but can obviously suppress the emergence of *S. sclerotiorum*; the control rate of three species of rape was 34.19–44.10% [295].

Chitosan was shown to inhibit the systemic propagation of viruses and viroids throughout the plant and to enhance the host's hypersensitive response to infection. Potato is a tuberous crop from the perennial *Solanum tuberosum* of the *Solanaceae* family. It is an essential crop in the world. The effect of the chitosan-induced resistance to viral infection was investigated in potato plants. The plants were sprayed with different molecular weights of chitosan solution (1 mg/mL) and the greatest antiviral activity was shown by chitosan of 120 kDa. In another experiment, potatoes were

infected with potato virus X after chitosan pretreatment. It was found that chitosan treatment significantly decreased the number of systemically infected plants compared to control, and the treated leaves also accumulated less amount of virus than the control leaves [296]. The antiviral activity of chitosan depends on the average degree of polymerization, the degree of *N*-deacetylation, the positive charge value, and the character of the chemical modifications of the molecule. Possible mechanisms of suppressing viral infections by chitosan are also discussed [264, 296–301]. Chitosan applied by spraying or inoculating leaves protected various plant species against local and systemic infection caused by alfalfa mosaic virus, tobacco necrosis virus, tobacco mosaic virus, peanut stunt virus, cucumber mosaic virus, and potato virus X [300, 301]. The ability of chitosan to suppress viral plant infections does not depend on the virus type because chitosan affects the plant itself by inducing resistance to the viral infection. Imitating the contact of the plant with a phytopathogen, chitosan induces a wide spectrum of protective reactions in the plant, which limit a systemic spread of the viruses and viroids over the plant and lead to the development of systemic acquired resistance [296–301].

The effect of oligochitosan and oligochitin on gray mould caused by *B. cinerea* in cucumber plants were evaluated by Ben-Shalom et al. [302]. It was shown that oligochitosan and oligochitin had different effects on this cucumber-pathogen interaction. Although complete inhibition of *Botrytis* conidia germination was found at 50 ppm chitosan solution *in vitro*, chitosan also controlled the gray mould in treated plants compared with control plants. But there was no effect of oligochitin on both pathogen growth on PDA and leaves. Besides this fungicidal effect, spraying chitosan 1, 4, and 24 h before inoculation with *B. conidia* decreased gray mould by 65, 82, and 87%, respectively. However, spraying chitosan on the leaves decreased gray mould incidence only by 52% 1 h after inoculation. These results suggest that the antifungal and elicitor activity of chitosan are both necessarily for the control of gray mould in cucumber [302]. *P. aphanidermatum* (Edson) Fitzp is an aggressive and economically important pathogen in greenhouse-grown cucumbers. Especially in substrates like rockwool which exhibit high water retention capacity, it can flourish and spread rapidly by zoospores. The pathogen causes severe root and crown rot, which result in wilting and death of plants. A recent paper showed that chitosan cannot induce defense to this pathogen when used alone. But the application of chitosan in combination with *Lysobacter enzymogenes* 3.1T8 (a biocontrol bacterium) reduced the number of diseased plants by 50%–100% in four independent experiments relative to the *Pythium* control. [303, 304].

Vasyukova et al. [305] reported that low-molecular-weight water-soluble chitosan (5 kDa), obtained after enzymatic hydrolysis of native crab chitosan, was shown to display an elicitor activity by inducing the local and systemic resistance of *S. tuberosum* potato and *Lycopersicon esculentum* tomato to *P. infestans* and nematodes, respectively. Chitosan induced the accumulation of phytoalexins in tissues of host plants; decreased the total content; changed the composition of free sterols producing adverse effects on infesters; activated

chitinases, β -glucanases, and lipoxygenases; stimulated the generation of reactive oxygen species. The activation of protective mechanisms in plant tissues inhibited the growth of taxonomically different pathogens (parasitic fungus *P. infestans* and root knot nematode *Meloidogyne incognita*). In addition, the potential of *B. pumilus* strain SE 34 in combination with chitosan, for inducing defense reactions in tomato plants inoculated with *F. oxysporum*, was studied [306]. A substantial increase in the extent and magnitude of the cellular changes induced by *B. pumilus* was observed when chitosan was supplied to bacterized tomato plants. These changes were characterized by a considerable enlargement of the callose-enriched wall appositions deposited onto the inner cell wall surface in the epidermis and the outer cortex.

Chitosan of 350 kDa was more effective at 25°C than oligochitosan (6 kDa) in controlling of the disease in pear fruit caused by two phytopathogenic fungi of *A. kikuchiana* Tanaka and *Physalospora piricola* Nose [261]. When treated with oligochitosan, pear fruit increased the activities of chitinase and b-1,3-glucanase. Differently, chitosan treatment significantly increased peroxidase activity of pear fruit. The results suggested that chitosan and oligochitosan triggered different mechanism for pathogenicity inhibition and disease control.

Chitosan compounds are used as biopesticides in many grape-producing countries. It was reported that oligochitosan (1500 Da and a DA of 20%) at 200 $\mu\text{g}/\text{mL}$ dramatically reduced the infection of grapevine leaves by *Plasmopara viticola* and *B. cinerea*. Dose-response experiments showed that maximum defense reactions and control effect of *B. cinerea* were achieved with 75–150 $\mu\text{g}/\text{mL}$ [307–309]. Similarly, on apple and watermelon, chitosans can induce plant defense against canker and anthracnose.

Guan et al. [310] examined the use of chitosan to prime maize seeds. Although chitosan had no significant effect on germination under low temperatures, it enhanced germination index, reduced the mean germination time, and increased shoot height, root length, and shoot and root dry weights in two tested maize lines. In both tested lines, chitosan induced a decline in malonyldialdehyde content, altered the relative permeability of the plasma membrane and increased the concentrations of soluble sugars, proline, peroxidase, and catalase activities. In other studies, seed priming with chitosan improved the vigor of maize and wheat seedlings. It was also reported that such treatment led to an increase of seed resistance to certain diseases and improve their quality and/or their ability to germinate [292, 311]. Similarly, peanut seeds soaked in chitosan were reported to exhibit an increased rate of germination and energy, lipase activity, and gibberellic acid and indole acetic acid levels [312]. Ruan and Xue [313] showed that rice seed coating with chitosan may accelerate their germination and improve their tolerance to stress conditions. In carrot, seed coating helps restrain further development of *Sclerotinia* rot [251]. It has also been reported that chitosans can activate plant defense to disease on several other plants such as barley [314], pearl millet [315], carrot [316], sunflower [317], and coconut [318].

6.3. Chitosan and Its Derivatives in Postharvest Application.

Fruits and vegetables deteriorate rapidly after harvest and in some cases do not reach consumers at optimum quality after transport and marketing. The main causes of their deterioration are dehydration, with the subsequent weight loss, color changes, softening, surface pitting, browning, loss of acidity, and microbial spoilage, among others. One of the potential approaches to extend the storability of these perishable commodities is to apply edible coatings or films on the surface, followed by a cold storage [319]. Therefore, the use of bioactive substances such as chitosan to control postharvest microbial diseases has attracted much attention due to imminent problems associated with chemical agents [215, 216, 320]. It has become a promising alternative treatment for fruits and vegetables due to its natural character, antimicrobial activity, and elicitation of defense responses [321, 322]. Indeed, chitosan is an ideal preservative coating for fresh fruits and vegetables because of its film-forming and biochemical properties [323] and has led to prolonged storage life and controlled decay of several fruit crops [324]. Chitosan coating is likely to modify the internal atmosphere without causing anaerobic respiration, since chitosan films are more selectively permeable to O₂ than to CO₂ [325].

Strawberry is among the most perishable fruits and is vulnerable to physical injuries and microbial infection. El Ghaouth and coworkers [215, 326] investigated the effect of chitosan coating on decay and quality of strawberries. Fruits were inoculated with spore suspension of *B. cinerea* or *R. stolonifer* and subsequently dipped in chitosan solutions (1.0 and 1.5% in 0.25 N HCl). In both studies, chitosan coating significantly reduced the decay of strawberries compared with the control. Chitosan coating decreased the respiration rate of strawberries with a greater effect at higher concentration. The improved storability of fresh strawberries by chitosan-based coating also has been documented [319, 327–330].

Li and Yu [331] reported that chitosan significantly delayed the postharvest development of brown rot disease caused by *Monilinia fructicola* on peach fruit. Effects of chitosan coating on browning of litchi (*Litchi chinensis*) fruit were also investigated by several workers [332–334]. Chitosan coating, irrespective of concentration 1 and 2% dissolved in 2% glutamic acid delayed changes in contents of anthocyanins, flavonoids, and total phenolics. It also delayed the increase in polyphenol oxidase (PPO) activity, and partially inhibited the increase in peroxidase activity [332, 335]. Jiang et al. [333] also similarly observed that chitosan of 2% in 5% acetic acid coating delayed the decrease in anthocyanin content and the increase in PPO activity. Such effects of chitosan coating were also observed with peeled litchi fruit [336], longan fruit [337], and fresh-cut Chinese water chestnut vegetable [338]. Dependence of browning rate of chitosan-coated litchi fruit on the initial pericarp water content [332], pericarp pH, and dehydration rate during storage [334] has been reported.

Penicillium is the most harmful citrus fruit postharvest pathogen and infects the fruit through microinjuries generated in the flavedo, during harvesting and processing. However, nowadays, consumers around the world demand

high-quality food, without chemical preservatives, leading to increased effort in discovering new natural antimicrobials. Accordingly, the fungistatic effects of chitosan have been investigated. For example, coating of citrus fruit with chitosan was effective in controlling fruit decay caused by *P. digitatum* and *P. expansum* and chitosan of 15 kDa at 0.2% was more effective in controlling the growth of fungi than chitosan of 357 kDa [339].

Moreover, edible coatings can be used as a vehicle for incorporating functional ingredients such as antioxidants, flavors, colors, antimicrobial agents, and nutraceuticals [327, 340–342]. Several workers have endeavored to incorporate calcium [327, 328], vitamin E [327, 343], potassium [319], or oleic acid [330] into chitosan film formulation to prolong the shelf life and to enhance the nutritional value of fruits.

Gray mold and blue mold rots caused by *B. cinerea* and *P. expansum*, respectively, in sweet cherry fruit were reduced by preharvest spraying or postharvest dipping of chitosan [344]. Liu et al. [345] added that the control effects of chitosan on both fungi significantly decreased in tomato fruit at 5000 and 10000 mg/L and the gray mold was better controlled than blue mold. Previous investigations on chitosan coating of tomatoes have shown that it delayed ripening by modifying the internal atmosphere that reduced the decay [215, 216, 320]. Recently we investigated the effectiveness of different molecular weights chitosan on the gray mold caused by *B. cinerea* as *in vivo* in tomato fruit (*Solanum lycopersicum* L. var. *lycopersicum*) stored at different temperatures [220]. The treatments significantly reduced fungal decay and all compounds at concentrations of 2000 and 4000 mg/L exhibited complete fungal control in wound-inoculated fruit. In addition, chitosan had potential for the elicitation of defense markers, including total soluble phenolics, PPO activity and total protein content. This finding suggests that the effects of chitosan may be associated with direct fungitoxic properties against the pathogen, and the elicitation of biochemical defense responses in fruits [220]. After treatment with chitosan, various defense responses have been induced, including the elicitation of phenylalanine ammonia lyase (PAL) activity in grape berries [324, 346], chitinase, and β -1,3-glucanase in oranges, strawberries, and raspberries [345, 347, 348].

7. Factors Affecting Antimicrobial Activity of Chitosan

The extent of the antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as MW, DDA, pH, temperature, solubility, derivatization, type of organism and so on. It is necessary to understand these factors for the effective application of chitosan and its derivatives as antimicrobial agents against plant pathogens.

7.1. Molecular Weight. Effect of chitosan MW on the antimicrobial activities has been explored and most investigators used the uncertain term of low MW (LMW) chitosan for a partially depolymerized chitosan not indicating exactly its MW [349]. Only a few data on the bactericidal activity of LMW chitosan could be compared depending on bacteria

tested, conditions of biological test and chitosan MW, but even in this case the results did not correspond to each other. Thus, chitosan of 9300 Da restricted growth of *E. coli*, while chitosan of 2200 Da promoted growth of this bacterium [350]. Increase in the MW led to a decrease in chitosan activity against *E. coli* in some studies [351, 352], while in the others an increased activity for a high MW (HMW) in comparison with LMW chitosan have been found [353]. In contrast to the above mentioned results, no differences in HMW and LMW chitosan activities were found against *E. coli* [354, 355] and *Bacillus subtilis* [351, 354].

It has been demonstrated that LMW chitosans (of less than 10 kDa) have greater antimicrobial activity than native chitosans. However, a DP of at least seven is required; lower MW fractions have little or no activity [227]. Chitosan with a MW ranging from 10,000 to 100,000 Da would be helpful in restraining the growth of bacteria. In addition, chitosan with an average MW of 9300 Da was effective against *E. coli*, while that with a MW of 2200 Da accelerated growth of the same bacteria [356]. Tanigawa et al. [357] reported that D-glucosamine hydrochloride (chitosan monomer) did not show any growth inhibition against several bacteria, whereas chitosan was effective. This suggests that the antimicrobial activity of chitosan is related to not only its cationic nature but also to its chain length. Shimojoh et al. [358] also found that chitosan of 220,000 Da was most effective, whereas chitosan of 10,000 Da was the least effective in their bactericidal activities. However, the antimicrobial activity of chitosan of 70,000 Da was better than of 426,000 Da for some bacteria, but for the others, the effectiveness was reversed. Yalpani et al. [359] reported that medium MW chitosans showed higher antimicrobial activities against *B. circulans* than chitoooligosaccharides (DP 2–30), whereas they were less effective against *E. coli* than chitoooligosaccharides. From the results of Shimojoh et al. [358] and Yalpani et al. [359] one can notice that the relationship between MW of chitosan and the antimicrobial activity can be affected by the test organisms. Numerous researchers have reported that the antibacterial activity of chitosan is a MW dependant [291, 354, 357, 360, 361]. Hwang et al. [362] concluded that chitosan with MW about 30,000 Da exhibited the highest bactericidal effect on *E. coli* from their investigation of MW range of 10,000–170,000 Da. Jeon et al. [354] suggested that the MW of chitosan is critical for the inhibition of microorganisms and suggested the required MW be higher than 10,000 Da for better antimicrobial activity.

The antimicrobial activity of different MWs chitosan and chitosan oligomers (DP 2–8) against several plant pathogens were examined by Hirano and Nagao [259]. It was observed that the increases in MW increased the number of inhibited fungi. The strongest growth inhibition was observed with LMW and the weakest was observed with HMW chitosan. Kendra and Hadwiger [223] examined the antifungal effect of chitosan oligomers on *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli*. The antifungal activity was found to increase as the polymer size increased. Monomer and dimer units did not show any antifungal activity at 1000 µg/mL. However, heptamer (DP = 7) showed maximal antifungal activity and the minimum concentrations were

identical to those observed for both native chitosan and the acid-cleaved chitosan. We examined the antimicrobial activity of 3.60×10^5 , 6.11×10^5 and 9.53×10^5 Da chitosans against plant pathogenic bacteria *A. tumefaciens*, *C. fascians*, *E. amylovora*, *E. carotovora*, *P. solanacearum* and *S. lutea* and fungi *A. alternata*, *B. fabae*, *F. oxysporum*, *P. digitatum*, *P. debaryanum* and *R. solani*. The results indicated that chitosans of 6.11×10^5 and 9.53×10^5 Da were more potent in bactericidal activity than chitosan of 3.60×10^5 Da and chitosan of 9.53×10^5 Da exhibited a good antibacterial potency especially against *C. fascians* with MIC 500 mg/L. The data demonstrated that the fungicidal activity was increased as MW increase and chitosan of 9.53×10^5 Da was the most potent one against all the tested fungi [250]. Recently we also investigated the antifungal activity of depolymerized chitosans (0.5×10^4 , 3.7×10^4 , 5.7×10^4 , and 2.9×10^5 Da) on the gray mold caused by *B. cinerea* as *in vitro* and *in vivo* on tomato fruit [220]. In an *in vitro* experiment, the result demonstrated that the antifungal activity increased as the chitosan MW decreased. In an *in vivo* study, chitosan with MW of 5.7×10^4 Da was the most effective among those tested.

It is difficult to find a clear correlation between MW and antimicrobial activity, generally the antimicrobial activity increases as the MW of chitosan increases. However, the activity decreases over a certain high MW. The discrepancies between data may result from the different DDA and MW distributions of chitosan. The evaluation of only the MW dependence of the antimicrobial activity requires a wide MW range of chitosan samples with the same DDA. It is almost impossible to obtain this because chitosan is a natural polymer. From the existing data, it is difficult to determine what the most optimal MW for the maximal antimicrobial activity is. The selection of MW of chitosan could be thought to be more dependent on its application.

7.2. Degree of Deacetylation. The antimicrobial activity of chitosan is directly proportional to the DDA of chitosan [291, 357, 358, 363]. The increase in DDA means the increased number of amino groups on chitosan. As a result, chitosan has an increased number of protonated amino groups in an acidic condition and dissolves in water completely, which leads to an increased chance of interaction between chitosan and negatively charged cell walls of micro-organisms [224]. Variation of deacetylation process yielded chitosan with significant differences in DDA% as well as variation of the MW. Simpson et al. [364] reported that chitosan with a DDA of 92.5% was more effective than chitosan with a DDA of 85%. On the contrary, Ikinici et al. [365] reported that change in DDA (73, 84 and 95%) did not have any effect on the antimicrobial activity of chitosan against *Porphyromonas gingivalis*.

Hongpattarakere and Riyaphan [366] prepared chitosan from black tiger shrimp carapace by deacetylation process performed in 50% NaOH at 100°C under vacuum, nitrogen, and regular atmospheres. Each condition was maintained for 0.5, 1, and 2 h. MIC values of chitosans varied depending on conditions of deacetylation processes, reaction times, and test micro-organisms. In general, chitosan prepared

under atmosphere showed the lowest MIC value or highest inhibitory effect on test micro-organisms, whereas that deacetylated under nitrogen showed the least inhibitory effect. Chitosan obtained from 1 h of deacetylation under regular atmosphere showed the lowest MIC value (625 ppm) against *E. coli* and *S. aureus*, while *Candida albicans* was inhibited at MIC value of 312.5 ppm due to its higher DDA and lower MW compared to chitosan deacetylated under vacuum and nitrogen atmospheres.

7.3. The pH. The antimicrobial activity of chitosan is strongly affected by the pH [226, 243, 244, 291]. Lower pH increases the antimicrobial activity for much the same reasons, in addition to the “hurdle effect” of inflicting acid stress on the target organisms. Tsai and Su [226] examined the antimicrobial activity of chitosan (DDA 0.98) against *E. coli* at different pH values of 5.0, 6.0, 7.0, 8.0, and 9.0. The greatest activity was observed at pH 5.0. The activity decreased as the pH increased and chitosan had little antibacterial activity at pH 9.0. Other researchers [243, 291] reported that chitosan had no antimicrobial activity at pH 7.0 due to the deprotonation of amino groups and poor solubility in water. This suggests that the antimicrobial activity comes from the cationic nature of chitosan.

7.4. Temperature. Temperature also has an effect on the antimicrobial activity of chitosan. Higher temperature (37°C) has been shown to enhance its antimicrobial activity compared to refrigeration temperatures. However, the greatest single influence on antimicrobial activity is the surrounding matrix. Tsai and Su [226] examined the effect of temperature on the antibacterial activity of chitosan against *E. coli*. The cell suspensions in phosphate buffer (pH 6.0) containing 150 ppm chitosan were incubated at 4, 15, 25, and 37°C for various time intervals and the surviving cells were counted. The antibacterial activity was found to be directly proportional to the temperature. At the temperatures of 25 and 37°C, the *E. coli* cells were completely killed within 0.5 and 1 hr, respectively. However, at lower temperatures (4 and 15°C) the number of *E. coli* declined within the first 5 hrs and then stabilized. The authors concluded that the reduced antimicrobial activity resulted from the decreased rate of interaction between chitosan and cells at a lower temperature.

7.5. Cations and Polyanions. Young et al. [367] observed that chitosan-induced leakage of UV-absorbing material from *Glycine max* was strongly inhibited by divalent cations in the order of $Ba^{+2} > Ca^{+2} > Sr^{+2} > Mg^{+2} > Na^{+} > K^{+}$. It was assumed that the cations displaced Ca^{+2} released from the cell surface, formed complexes stabilizing the cell membrane, and consequently reduced the chitosan-induced leakage. Young and Kauss [368] reported that chitosan caused the release of Ca^{+2} present on *Glycine max* cell and/or plasma membrane, which destabilized the cell membrane and further induces leakage of intracellular electrolytes. They suggested that the cross-linking of chitosan (polycation) with phospholipids or protein components in the cell membrane affects the membrane permeability, which

further causes leakage of intracellular substances and finally causes the death of cell. Tsai and Su [226] also reported that reducing bactericidal effect of chitosan against *E. coli* by the addition of salts containing alkaline earth metals such as $MgCl_2$, $BaCl_2$, and $CaCl_2$ was observed. The order of effectiveness was $Ba^{+2} > Ca^{+2} > Mg^{+2}$. The authors proposed that the cations form complexes with chitosan and consequently the reduced available amino groups of chitosan led to the reduced bactericidal effect unlike Young's assumption [367]. In addition to the reduced chitosan-induced leakage by cations, the leakage was also reduced by the addition of polyanions such as sodium polygalacturonate and sodium poly-L-aspartate. The complete prevention of electrolyte leakage was observed when the number of carboxyl groups in the polyanions was equal to that of the amino groups of chitosan. It was attributed to the formation of polycation (chitosan)-polyanion complexes, which were observed by formation of precipitate. However, monomeric galacturonate and aspartate did not show any effect on the leakage and no precipitation of chitosan was observed. The explanation of this provided by the authors was that individual ionic bonds between anionic monomers and polycations could dissociate, but the multiple bonds between polyanion and polycation would not dissociate at the same time.

8. Mode of Antimicrobial Action of Chitosan

Chitosan is a natural polymer and has no antigenic properties, and thus is perfectly compatible with living tissue. Its antithrombogenic and hemostatic properties make it very suitable for use in all fields of biology. The exact mechanisms of the antimicrobial activities of chitosan and its derivatives are still unknown. It is known that chitosan antimicrobial activity is influenced by a number of factors that act in an orderly and independent fashion. Because of the positive charge on the C-2 of the glucosamine monomer below pH 6.0, chitosan is more soluble and has a better antimicrobial activity [6, 234]. The polycationic structure forms unnecessarily in acidic conditions because the grafted groups of specific derivatives may change the pK_a of chitosan and cause protonation at higher pH value. When the positive charge density of chitosan strengthens, the antibacterial property will increase consequently, as is the case with quaternized chitosans [70, 109, 369, 370] and chitosan metal complexes [145, 148, 371]. On the contrary, if the polycationic property of chitosan is deprived or reversed, the corresponding antimicrobial capacity will be weakened or lost. Therefore, large amounts of amino groups are able to enhance the antimicrobial activity. Accordingly, native chitosan with higher DDA shows a stronger inhibitory effect than that a molecule with a lower DDA. Moreover, it has been reported that asparagine *N*-conjugated chitosan oligosaccharide that possesses two positively charged sites provides strong interaction with carboxyl-negative charges on the bacteria cell wall [354, 372].

Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular

constituents [6, 234, 241, 373–376]. Papineau et al. [239] and Sudarshan et al. [243] reported that chitosan acts mainly on the outer surface of the bacteria. At a lower concentration (<200 mg/L), the polycationic chitosan does probably bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension. Chitosan interacts with the membrane of the cell to alter cell permeability. For example, fermentation with baker's yeast is inhibited by certain cations, which act at the yeast cell surface to prevent the entry of glucose. UV-absorption studies indicated that chitosan caused considerable leakage of proteinaceous material from *P. oaroecandrum* at pH 5.8 [377]. Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth [274, 275]. It also activates several defense processes in the host tissue [215], acts as a water-binding agent, and inhibits various enzymes.

Another mechanism is that the positively charged chitosan interacts with cellular DNA of some fungi and bacteria, which consequently inhibits the RNA and protein synthesis [243, 378]. In this mechanism, chitosan must be hydrolyzed to low MW to penetrate into the cell of micro-organisms however, this mechanism is still controversial. Tokura et al. [350] examined the antimicrobial action of chitosan with average MW of 2200 and 9300 Da having DDA of 0.54 and 0.51, respectively. It was observed that chitosan of 9300 Da was stacked on the cell wall and inhibited the growth of *E. coli*. However, the chitosan of 2200 Da, which permeated into the cell wall, accelerated the growth of *E. coli*. They suggested that the antimicrobial action is related to the suppression of the metabolic activity of the bacteria by blocking nutrient permeation through the cell wall rather than the inhibition of the transcription from DNA. Chitosan also inhibits toxin production by *A. alternata* and macerating enzyme production by *Erwinia* in addition to eliciting phytoalexin production [379, 380]. The effects of chitosan on growth inhibition of plant pathogenic fungi in fruits and vegetables were correlated with the reduction of aflatoxin, elicitation of phytoalexin and phenolic precursors, enhanced production of chitinases, and other factors relevant to the plant defenses [220, 381, 382]. El Ghaouth et al. [215, 216, 320] added that the fungistatic properties of chitosan against *R. stolonifer* were related to its ability to induce morphological changes in the cell wall. In addition to the direct effect of chitosan on different plant pathogens, it also activates several defense processes in treated plants or their products at postharvest stage. These defense mechanisms include accumulation of chitinases, synthesis of proteinase inhibitors, and lignification and induction of callous synthesis [320, 325]. For example, when applied on wounded wheat leaves, chitosan-induced lignifications, and consequently restricted the growth of nonpathogenic fungi in wheat. Chitosan inhibited the growth of *A. flavus* and aflatoxin production in liquid culture, preharvest maize, and groundnut, and it also enhanced phytoalexin production in germinating peanut [274, 275].

Unfortunately, in spite of the chitosan advantages, it is only soluble in acidic aqueous solutions with pH values lower than 6.5. At higher pH values, amino groups of chitosan macromolecules become unprotonated and chitosan forms insoluble. This problem is probably the major limiting factor for its utilization, that is, its application in biology, since many enzyme assays are performed at neutral pH. If water-soluble chitosan would be easily accessible, it is expected that the biological and physiological potential of chitosan would increase dramatically. One of the strategies to increase both the solubility and positive charge density of chitosan macromolecule is based on the introduction of quaternary ammonium groups in chitosan. This modification has got the commonly accepted term "quaternization of chitosan". Thus, water-soluble chitosan derivatives soluble to both acidic and basic physiologic circumstances may be good candidates for the polycationic biocides [70, 383, 384]. The antimicrobial action of such compounds is believed to occur when the compounds are absorbed onto the bacterial cell surface, increasing the permeability of the lipid cell membrane and causing death through the loss of essential cell materials. In addition, these derivatives of chitosan are generally more active against gram-positive bacteria than their corresponding monomers. Antimicrobial activity generally increases as the content of the quaternary ammonium moiety increases. The antimicrobial activities of quaternary chitosan derivatives were evaluated against some of gram-positive, gram-negative bacteria, and fungi. It was found that the activity increased with increasing chain length of the alkyl substituent, and this was attributed to the contribution of the increased hydrophobic properties of the derivatives. These results clearly demonstrated that hydrophobicity and cationic charge of the introduced substituent strongly affect the antibacterial activity of quaternary chitosan derivatives [70, 110].

It can be concluded that the exact mechanism of the antimicrobial action of chitosan is still ambiguous, although six main mechanisms, none of which are mutually exclusive, have been proposed, as follows: (1) interactions between the positively charged moieties on the chitosan molecules and those negatively charged ones on the microbial cell outer membranes leads to changes in the cell membrane structure and permeability inducing the leakage of proteinaceous and other intracellular constituents and so challenging the biochemical and physiological competency of the bacteria leading to loss of replicative ability and eventual death; (2) chitosan acts as a chelating agent that selectively binds trace metals and subsequently inhibits the production of toxins and microbial growth; (3) chitosan activates several defense processes in the host tissue, acts as a water binding agent, and inhibits various enzymes; (4) LMW chitosan penetrates the cytosol of the micro-organisms and, through the binding of chitosan with DNA, results in the interference with the synthesis of mRNA and proteins; (5) Chitosan on the surface of the cell can form an impermeable polymeric layer which alters the cell permeability and prevents nutrients from entering the cell; (6) finally, since chitosan can adsorb the electronegative substances in the cell and flocculate them, it

disturbs the physiological activities of the micro-organism leading to their death.

9. Concluding Remarks

The recourse to naturally occurring products with interesting antimicrobial and eliciting properties such as chitosan has been getting more attention in recent years. This product can be used in a number of ways to reduce plant disease levels and prevent the development and spread of pathogens, thus preserving crop yield and quality. The potent effect of chitosan on plant diseases control is from its antimicrobial properties and plant innate immunity elicited activity. The antimicrobial activity depend on several factors such as MW, DDA, solubility, positive charge density, chemical modification, pH, concentration, hydrophilic/hydrophobic characteristic, chelating capacity, and type of micro-organism. Chitosan has also become a postharvest promising treatment for fruits and vegetables due to its natural character, antimicrobial activity, and elicitation of defense responses. It possesses film-forming and barrier properties, thus making it a potential raw material for edible films or coatings and can be used to improve the storability of perishable foods. In spite of the chitosan advantages, the poor solubility, low-surface area, and porosity of chitosan are the major limiting factors in its utilization. Therefore, several research groups have started to modify a chitosan molecule to produce high-antimicrobial active derivatives.

Though there are many papers focused on chitosan and its derivatives in plant protection, there are still many problems that need to be studied. Examination of better ways to incorporate these products into Integrated Pest Management strategies remains to be pursued in many major crops especially against plant pathogenic bacteria and fungi. Interesting theoretical and applied findings were gathered in recent years, whereas more are needed to examine the mechanisms governing the mode of action of these compounds when applied at large scales. In the case of antimicrobial mode of action, future work should aim at clarifying the molecular details of the underlying mechanisms and their relevance to the antimicrobial activity of chitosan. Moreover, further investigations in this area, in particular with regard to microorganism resistance mechanisms against this compound, are warranted. In addition, participation and collaboration of research institutes, industry, and government regulatory agencies will be the key for the success of the antimicrobial mechanism.

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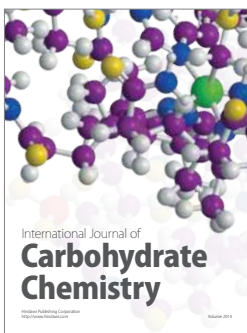
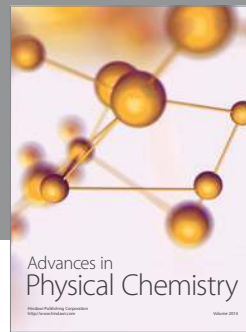
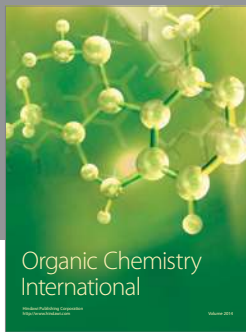
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