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Review

Modification of Chitosan for the Generation of Functional Derivatives

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Abstract: Today, chitosan (CS) is probably considered as a biofunctional polysaccharide with the most notable growth and potential for applications in various fields. The progress in chitin chemistry and the need to replace additives and non-natural polymers with functional natural-based polymers have opened many new opportunities for CS and its derivatives. Thanks to the specific reactive groups of CS and easy chemical modifications, a wide range of physico-chemical and biological properties can be obtained from this ubiquitous polysaccharide that is composed of β -(1,4)-2-acetamido-2-deoxy-D-glucose repeating units. This review is presented to share insights into multiple native/modified CSs and chitooligosaccharides (COS) associated with their functional properties. An overview will be given on bioadhesive applications, antimicrobial activities, adsorption, and chelation in the wine industry, as well as developments in medical fields or biodegradability.

Keywords: chitosan; polysaccharide; functional properties; bioactivity

1. Introduction

Chitosan (CS) is a copolymer of glucosamine and N-acetyl glucosamine branched by β -(1-4) linkages. It is derived from chitin, which is among the most abundant biopolymers on Earth. The word “chitin” is derived from the Greek language, meaning “envelope” or “tunic”. Chitin was the first polysaccharide identified by the French scientist Braconnot in 1811 and was fully described in 1884 as a natural poly- β -(1-4)-N-acetyl-D-glucosamine [1,2]. The unique chemical structures of chitin and CS led some authors to call them aminopolysaccharides [3]. Chitin is widely abundant as ordered crystalline microfibrils in several kinds of organisms, such as yeast and fungi (cell walls), crustacean shells, or insect cuticles, and is also produced by some green microalgae [4]. Two main polymeric forms of chitin have been described in the literature, namely α - and β -chitins, which are arranged as monoclinic and orthorhombic cells, respectively [5]. An allomorph γ -chitin is a combination of these two forms [5]. α -chitin (from yeast cell walls, exoskeleton of crustaceans, and arthropod cuticles) and β -chitin (from squid pens) correspond to anti-parallel and parallel arrangements of

polymer chains, respectively. The term “chitosan” (Kite-O-San) was firstly written by Hoppe-Seiler in 1894, to design deacetylated chitin [6]. Indeed, chitin is not soluble in water or other common organic solvents, but can be converted in CS after hot alkaline deacetylation in a solid state [2]. The degree of deacetylation (DD), which is the percentage of D-glucosamine units with respect to the total number of monomers (glucosamine and N-acetyl glucosamine), defines the frontier between chitin and CS. Conventionally, the DD value of CS is usually higher than 50%. The resulting CS, which is a polycationic polysaccharide, is soluble in dilute acidic media ($2 < \text{pH} < 6$), on the contrary to chitin [7]. In industrial processing, CS is mainly extracted from crab, shrimp shells, squid pens, and crustaceans by acidic treatment to eliminate calcium carbonates, followed by alkaline deproteinization [5]. The demineralized and deproteinized chitin is then submitted to a second alkaline treatment at high temperature before an optional decolorization step using hydrogen peroxide, sodium hypochlorite, or acetone [5]. All these acidic and alkali treatments are extremely hazardous for the environment and not sustainable. Enzymatic deacetylation is often considered as an ecofriendly alternative to alkaline deacetylation, but is not really industrially developed at the moment [6]. New commercial sources of CS from fungi (from Kitozyme company) and insects (from Ynsect company) have recently appeared on the market to valorize some by-products (mushroom wastes or cuticles of insects from new protein production chains). They are based on more green processes compared with those used by traditional CS production chains. The physico-chemical properties of CS depend on its molecular weight (from approximately 10 to 1000 kDa), DD (in the range of 50–95%), and sequence of the acetamido and amino groups. It has been used in a large range of applications due to its unique physicochemical properties, but also its low toxicity, biodegradability, biocompatibility, high adsorption capacity, and microbe resistance [4,8,9]. Indeed, the different functional groups of this polycationic polysaccharide can be modified with a wide diversity of ligands. Among them, the amino group ($-\text{NH}_2$) functionality is available for numerous chemical reactions, including reactions with aldehydes and ketones (Schiff's base), chelation of metals, alkylation, sulfonation, carboxymethylation, grafting acetylation, quaternization, etc. [10–12]. The numerous hydroxyl groups ($-\text{OH}$) are also, as for all polysaccharides, available for chemical modifications, such as sulfonation, carboxymethylation, phosphorylation, or hydroxyethylation [10–14]. All chemical groups along the backbone can be cross-linked using specific agents to give “chemical” hydrogels. They can also interact with each other due to ionic and hydrophobic interactions, molecular entanglements, or hydrogel bonds to generate physical hydrogels [9]. Moreover, macromolecules of CS can produce self-assembled structures based on hydrogen-bond networks formation in aqueous solutions, leading to fibers. Conformational variations of these CS assemblies have been reported to depend on local environmental changes around CS (e.g., pH, temperature, types of salt, and types of acids). All these reactions offer to CS a great potential as biosourced materials, biomaterials drug/enzyme delivery vehicles, tissue engineering scaffolds, adhesives, texturing agents, support for enzyme immobilization, bioactive agents etc. This review focuses on the fundamental uses of all forms of CSs, i.e., polymer, oligomer, native, and chemically modified, in a large variety of applications. Thus, bioadhesive applications, antimicrobial activities, adsorption, and chelation in the wine industry, as well as developments in medical fields or biodegradability, have been detailed for highlighting the potential of chitosan and derivatives.

2. Chitosan in Brief

2.1. Extraction and Structure of Chitosan from Natural Sources

Although chitin and CS have been known since the nineteenth century and the work of Henri Braconnot (1811) [15], research on these compounds really started around 1930 and was intensified after 1970. The major obstacle to their use lied in the difficulty of solubilizing them. However, research was encouraged by the fact that resources were abundant. Indeed, chitin is one of the most abundant polysaccharides on Earth, second only to cellulose [16–18]. It plays an essential structural role in the

cell wall of fungi and yeasts, and in cuticles of arthropods and insects. Chitin is a natural linear cationic polysaccharide consisting of β -(1,4) linked *N*-acetyl-D-glucosamine (GlcNac) (Figure 1). CS is obtained by the deacetylation of chitin with concentrated NaOH solution, and consists of a heteropolysaccharide of β -1,4 linked D-glucosamine and *N*-acetyl-D-glucosamine (Figure 1). Chitin and CS are characterized by the degree of acetamidation, denoted DA, and expressed as a percentage of acetamide groups present: it is greater than 50% in chitin and less than 50% in CS [18,19].

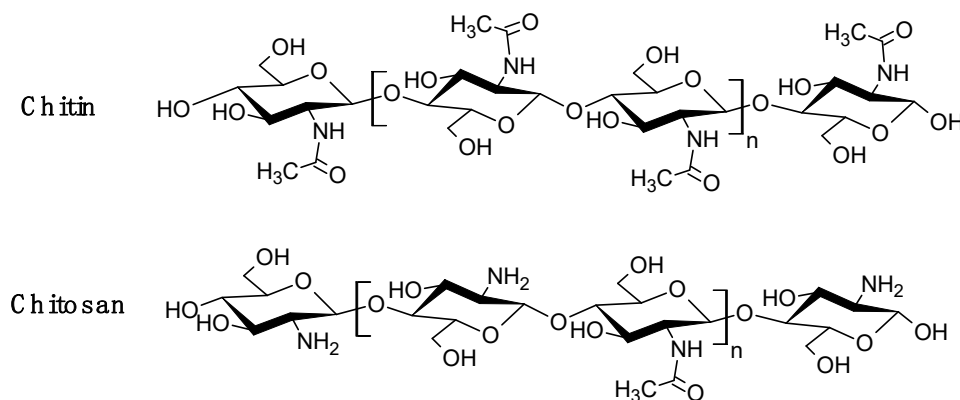


Figure 1. Chemical structure of chitin and chitosan.

In the case of CS, it is often preferred to mention the rate (%) of deacetylation, called DD, which corresponds to the relative amount of acetyl groups removed from chitin during the preparation of CS. Another definition considers that it is the solubility of the material in a solution of acetic acid, which defines the polymer as chitin or CS. In insects, fungi, diatoms, or marine animals, chitin is synthesized by chitin synthase (E.C. 2.4.1.16) [20]. In these organisms, chitin assembles in three distinct polymorphic forms named α , β , and γ (parallel, antiparallels, or mixture of both) [1,21]. The form of the chains is found to depend on the origin, and α -chitin is the most abundant form. Chitin deacetylase (E.C. 3.5.1.41) partially removes acetyl substituents and defines the acetylation degree of the final chitin [22]. CS is rarely found in nature, contrarily to chitin. Extraction of chitin (Figure 2) from fishery wastes (carapace of crustaceans and shellfish) requires strong chemical treatments, such as deproteinization with hot alkali (NaOH 1 N, at 60–100 °C for several hours) and demineralization with acid (HCl 0.3–2 N at about 100 °C for one or two days) to eliminate calcium carbonate, and discoloration [17].

Regarding fungal biomasses, chitin is covalently linked with glucan. The extraction process of the chitin-glucan is more recent (Figure 2) [23,24]. The extraction method includes hydrolysis steps, to separate the chitin-glucan from the rest of the mycelium, and lipid elimination by washing and drying. Then, CS is generally produced by partial deacetylation of chitin-glucan in a concentrated sodium hydroxide solution, for several hours at 110–115 °C, under inert atmosphere (N_2), in the presence of a reducing agent (NaBH_4). The deacetylation reaction is rarely complete, to avoid a sharp reduction in the molecular weight of the polymer. The use of high temperatures generally improves the reaction rates and yields [25]. Ultrasound and microwave technologies were also proposed to enhance the extraction and deacetylation steps [26–31]. Furthermore, biological treatments offer an alternative to such hard chemical reactions: lactic acid bacteria and bacterial protease can be used to remove proteins and deacetylation can also be performed with enzymes [32,33]. This produces higher quality products (better control of Mw and DA), but requires longer processes. The product is then dried and re-dissolved in an organic acid solution, in order to purify it. The CS obtained is in the form of an amorphous solid. It generally has a DD greater than 70% (between 70% and 80% in general), with a Mw which may reach 3×10^6 Da, but is generally comprised between 100 and 1000 kDa, with small amounts of smaller molecules (10–50 kDa). CS preparation mean Mw and polydispersity vary a lot from one preparation to another. Chitin, CS, and glucan-CS can be hydrolyzed by enzymes (chitinases,

chitosanases, glucanases) to prepare specific medium and low molecular weight (< 50 kDa) CS families without the glucan part [1,17].

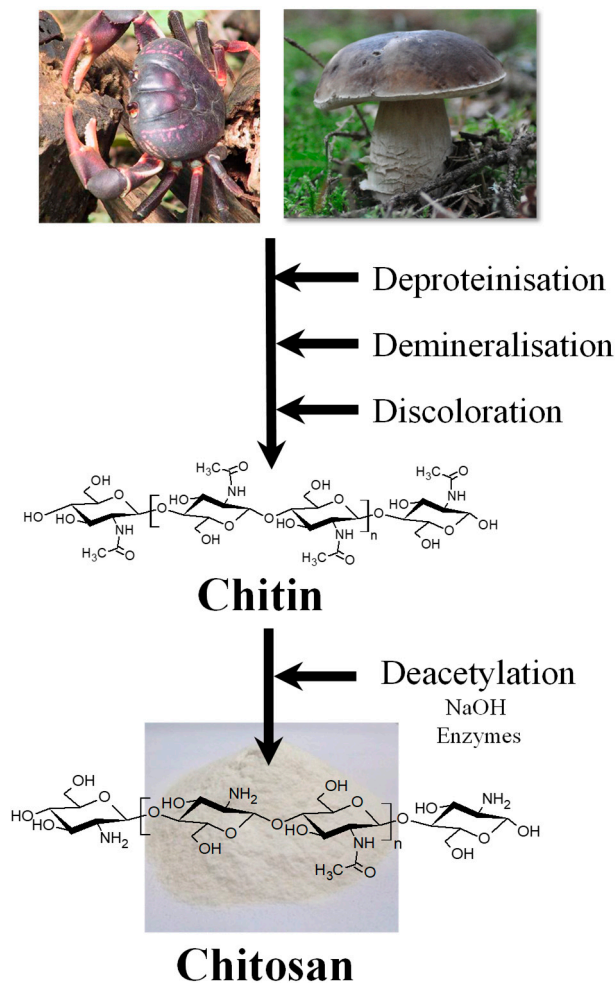


Figure 2. General steps for chitin and chitosan production.

CS is a weak base, with a pKa of 6.3–6.7. It is partially soluble in acidic aqueous solution when $\text{pH} < \text{pKa}$, and the solubility increases at $\text{pH} < 5.5$. The DD parameter affects (i) the solubility of acidic CS, due to the protonation of amine groups; (ii) the flexibility of the polysaccharide chains; (iii) the conformation of the polymer; and (iv) the viscosity of the solutions. The molecular chain length or mass is also an important property that can be expressed in weight (M_w) or number (M_n). M_n affects the solubility of the CS and the viscosity of solutions [1]. The CS characteristics (in terms of DD, M_n , polydispersity, and crystallinity) strongly depend on the extraction method and the source of isolation and they can vary widely from batch to batch [6,17,19].

2.2. Global Market

CS has several uses in the industry, such as cosmetics, water treatment, and agrochemicals [1,4]. CS application is mainly focused on waste water treatment, due to its biosorbent properties, in order to remove pollutants such as heavy minerals, oils, and phosphorous, which are responsible for deterioration of the water quality. Due to industrialization and the rising of the global population, the global CS market has increased lately, mainly in Asia and especially in Japan, representing 35% of the global market in 2013. Besides the main waste water treatment application, CS is expected to expand its use to the cosmetic industry because of its skin moisturizing properties. CS is also more and more thought of for hair care or dental care treatments, as well as in agriculture for stimulating plant

growth. The global CS market was valued at 1205 million US\$ in 2015 and will reach 2550 million US\$ by the end of 2022, with an increase of 10.7% between 2016 and 2022. Generally, ten to the power of ten tons of chitin are produced annually [1–4,34,35].

3. Chitosan Modification and Functionalization

Due to their exceptional properties and biological activities, CS and its derivatives are having growing success regarding the number of publications concerning their description and application in foods, environmental, material, cosmetic, pharmaceutical, and biomedical sectors. However, their applications are strongly limited by their solubility in many polar solvents and water. Overcoming this issue is possible by modifying CS through chemical/enzymatic methods to generate depolymerized and/or new derivatives.

3.1. Chitosan Chemistry

Chemical modifications of CS are well-documented in recent publications for the last few years [4]. Due to the presence of reactive amino ($-\text{NH}_2$) and hydroxyl ($-\text{OH}$) groups, CS is very easily modifiable. These modifications aim to enhance their biological and chemical properties and modify their solubility as a function of the final applications. The following section underlines the main modifications of CS described in the literature, i.e., (i) quaternization, (ii) *N*-alkyl modifications, (iii) *N*-acyl modifications, and (iv) C-6 oxidation.

3.1.1. Quaternized Chitosan Derivatives

Many publications [36–40] have shown the possibility to modify the positive (NH_3^+) charge of CS for making it soluble in a large range of pH values, but also in neutral or slightly alkaline medium. Quaternization is an example of a procedure to enhance the solubility of CS in water. CS is positively charged at pH under 6.5, whereas quaternized CS is still permanently positively charged at pH above 6.5. A quaternization reaction occurs between alkyl iodide and CS under basic conditions media. *N,N,N*-trimethylCS chloride (TMC) is the best known quaternized CS and has been described for numerous applications [4]. As shown in Figure 3, TMC is obtained after two consecutive reactions, firstly between methyl iodide CH_3I and CS in the presence of *N*-methyl-2-pyrrolidinone (NMP), which is used as a solvent in alkaline conditions (NaOH), and secondly by changing iodide ions with chloride ones thanks to an anionic exchange resin. Various types of quaternized CS can easily be obtained by modifying the carbon length of alkyl halides.

3.1.2. *N*-acyl Chitosan Derivatives

N-acylation gives hydrophobic properties to CS by grafting different fatty acids. The reaction is a specific amidation between $-\text{COOH}$ groups from fatty acids and $-\text{NH}_2$ groups from CS. Chemical reagents used for *N*-acylation are acyl halide or acid anhydride (Figure 3). This acylation is regularly performed in pyridine, chloroform/pyridine, or methanol/water/acetic acid. Nevertheless, this reaction can lead to *O*-alkyl CSs because of two reactive $-\text{OH}$ groups on the CS repeating unit. In order to avoid this *O*-acylation, many authors advise substituting primary hydroxyl groups of CS with trityl groups. This enhances the *N*-Acylation step owing to the formation of a CS chloroacyl [41]. Many types of acid anhydride have been tested to produce *N*-acyl CSs [42–45].

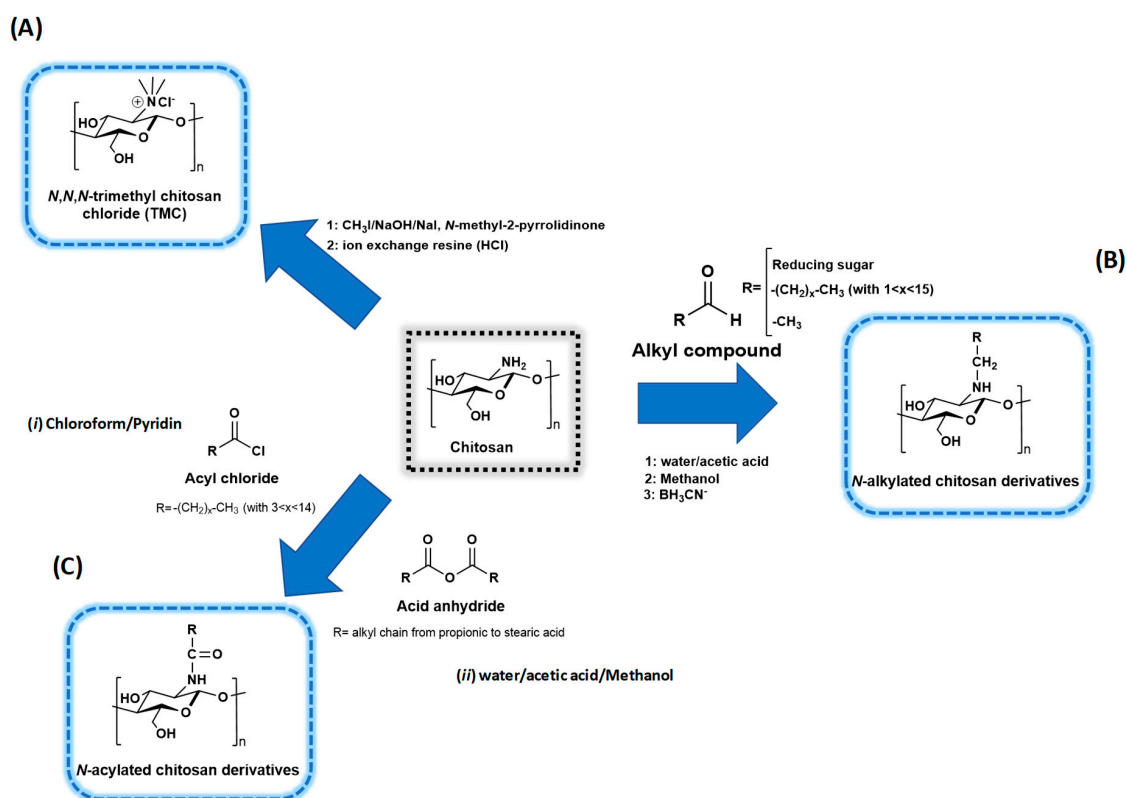


Figure 3. Production of chitosan derivatives by different methods: (A) Quaternization, (B) N-alkylation, and (C) N-acylation.

3.1.3. Oxy-Chitosan Derivatives

Many scientific publications have explored the production of water-soluble chitouronic acid sodium (carboxylated chitin or CS) with the use of TEMPO, which is an organic catalyst used for the oxidation of hydroxyl functions into aldehyde ones in NaOCl and NaBr conditions [46–49]. TEMPO is mainly known for its use for regioselectively oxidizing primary hydroxyl groups of various polysaccharides. Muzzareli et al. [50] have developed a method using TEMPO to produce oxy-CS derivatives, namely 6-oxyCS. Chitouronic sodium salts are mainly produced from pretreated (chemically or enzymatically) fungal or shrimp cell chitin. In their work, Muzarelli et al. [46] also used fungal biomass from *Trichoderma* and *Aspergillus* to produce a new range of carboxylated CS/chitin with a high degree of biocompatibility on human keratocytes, highlighting their potential use in drug delivery applications [51]. Pierre et al. [49] have synthesized a new bioactive C6 oxy-CS derivative. This new derivative showed good anti-parasitic properties against *Leishmania*. Very recently, an environmentally friendly process has been developed by Botelho da Silva et al. (2018) [52] for the C6 oxidation of CS through a TEMPO/laccase redox system in order to generate a water-soluble CS fraction (Figure 4).

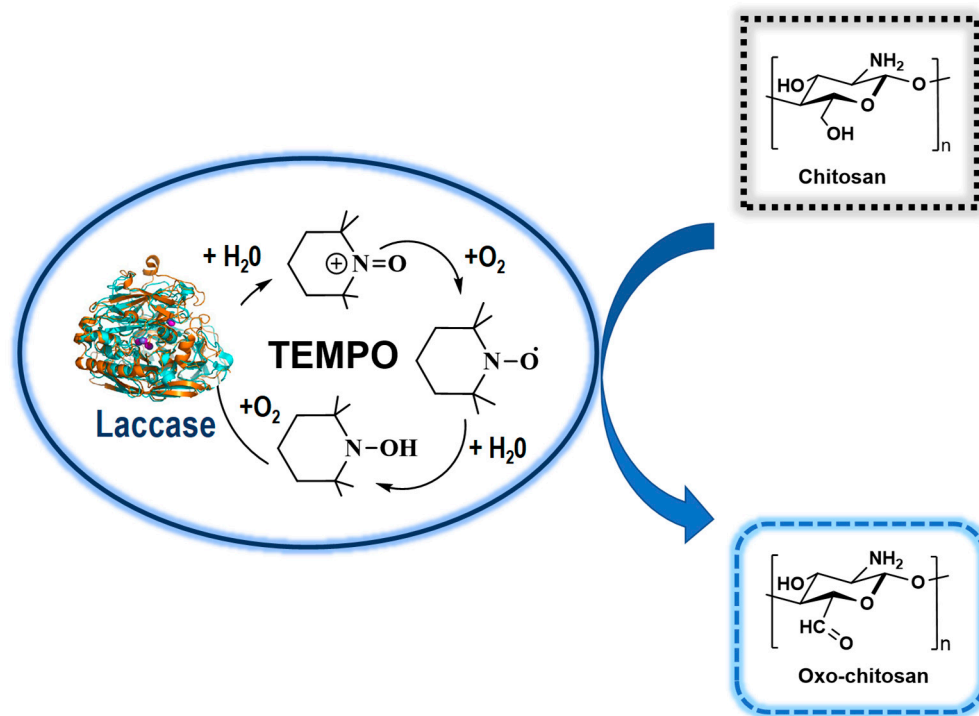


Figure 4. Environmentally friendly oxidation of CS *via* TEMPO/laccase system (adapted from [52]).

3.1.4. Cross-Linked Chitosan Derivatives

Making cross-linked CS requires the use of specific chemical agents for linking the chains together and thus creating a three-dimensional macromolecular network [1,2,9]. CS is most often crosslinked by covalent bonds in the presence of aldehyde derivatives, such as glyoxal, formalin, or glutaraldehyde, in acid or basic medium to generate CS-based hydrogel [9]. As a rule, the cross-linking reaction with CS consists of forming a Schiff base (imine) [2,4,9]. Glutaraldehyde (GTA) is the most studied crosslinking agent. It is synthetic, available, and inexpensive [1,9]. The reaction is a condensation reaction between the aldehyde function and a primary amine group from the CS chain in the presence of labile hydrogen [6,9,16]. However, GTA is toxic and natural alternatives to GTA are being studied to produce CS hydrogel, such as genipin [9], citric acid [53], and inorganic phosphate [54]. For example, Lusiana et al. [53] reported the use of citric acid as a cross-linking agent for the preparation of a CS/PVA membrane. This cross-link strategy was generally investigated to produce biomaterial as hemodialysis membranes. The cross-linking between citric acid and CS was expected to incorporate carboxylate groups (COO⁻) into biomaterial in order to increase the bioactive sites on the CS membrane for transporting biomolecules (urea, creatinine, etc.). Polyvinyl alcohol (PVA) was used to increase the mechanical efficiency and hydrophobicity of the cross-linked CS membrane [53]. In Figure 5, the main cross-linking CS strategies are presented.

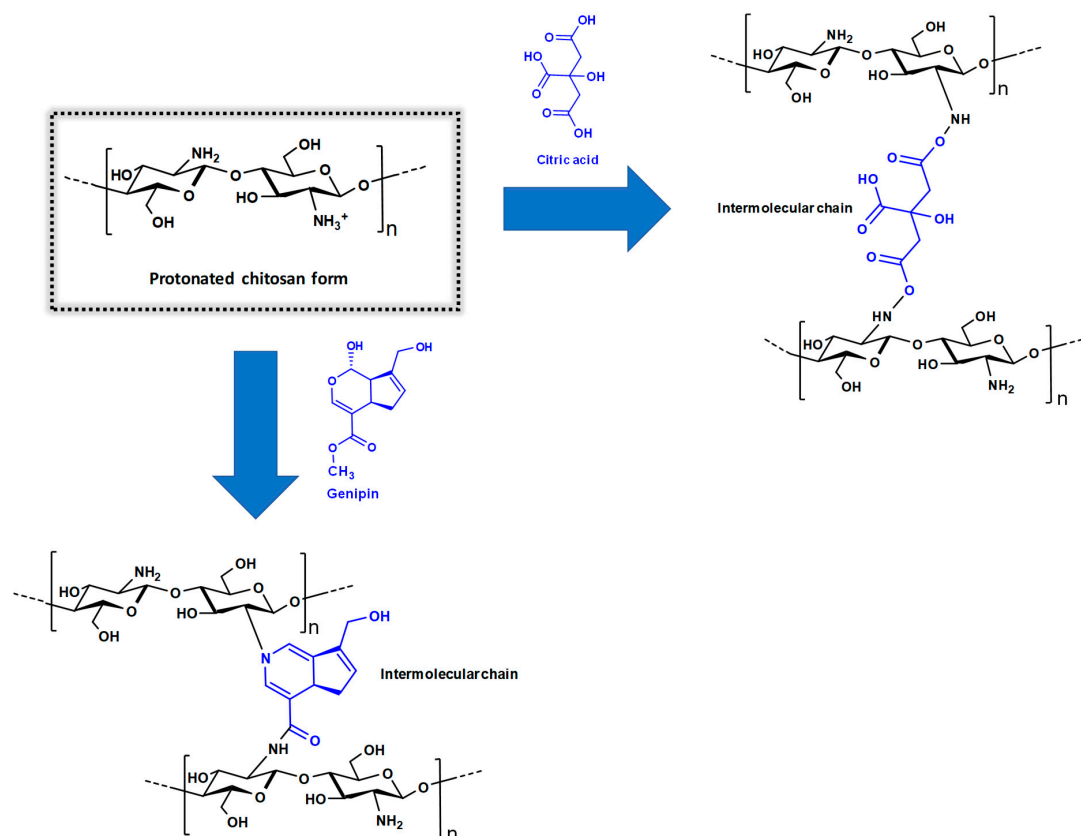


Figure 5. The mains cross-linking reactions using chitosan.

3.2. Chitooligosaccharide (COS) and Low Molecular Weight (LMW) Chitosan

High molecular weight CS is very difficult to use in commercial applications due to its high viscosity. Reducing the molecular weight of CS is a good way of decreasing viscosity issues and enhancing biological properties thanks to the production of chitooligosaccharides (COS) and low molecular weight CS (LMW). Generally, oligosaccharides are defined as oligomers with a degree of polymerization ranging from 2 and 10, but some higher DPs from 11 up to 30 are described as LMW in the literature [55]. The production of COS and LMW CSs is mainly achieved by physical, chemical, and enzymatic methods [55]. Table 1 gives an overview of these possible strategies, including the associated conditions for efficiently producing LMW CS or COS. The reduction of molecular weight by chemical, physical, or enzymatic processes has also been related to improving the solubilization of CS in water or acetic acid solutions [4,55]. Depolymerization of CS is principally performed by acid chitosan analysis, which is the most reported method for producing COS and LMW CSs [4]. Overall, chemical processes include chitosan analysis with HCl [56], HNO_2 [57], H_2O_2 [58], and potassium persulfate [59].

Physical processes include depolymerization with sonication [26], electromagnetic irradiation, gamma irradiation [60,61], microwave irradiation, or a thermal procedure [62]. Finally, enzymatic processes use specific enzymes like chitinase [63] and chitosanase [64], but also non-specific enzymes, such as pepsin [65], cellulase [66], lipase, pronase, protease [67], lysozyme, papain, glucanase, hemicellulase, or pectinase. However, the main issues of enzymatic depolymerization are probably the cost of making it redhibitory for bulk use in commercial applications and the relative slowness of reactions. In contrast, the main drawbacks of chemical methods involve the use of non-green chemicals, the need of their removal, and the heterogeneity of final products [4]. New methods for reducing the molecular mass of CS have been described, e.g., high-pressure homogenization (HPH) [68], plasma [69], or using zeolithes adsorbents [70] to purify acid hydrolysis COS and LMW CS. Additionally, electrochemical processes have also been developed to efficiently depolymerize CS [71].

Table 1. Methods reported for producing LMW chitosan or COS.

Type of Method	Depolymerization Methods	Conditions	Mw ⁽¹⁾ DP ⁽²⁾	References
PHYSICAL	High pressure homogenization	1500 bars 1% CS in 1% acetic acid	30 kDa	[68]
	Sonication	Sonication at 35.2 W/cm ² , 30 min	140–143 kDa	[26]
	Gamma radiations	2% CS in 2% acetic acid, 200 KGy	3–5 kDa	[60]
		1% CS, 0.1% Tween 80 irradiation 50 kGy	75–77 kDa	[61]
	Autoclave	1% CS, 1% acetic acid, 121 °C, 60 min, 1 bar	313 kDa	[62]
CHEMICAL	Acid hydrolysis	0.5 M HCl, 1% CS, 30 h, 65 °C	-	[56]
		2% CS, 1.8 M HCl reflux 100 °C, 2 h	DP < 40	[70]
		0.976% CS, 50 mM HCl, 3.89 mM HNO ₃ , 35 °C, 30 min	< 16 kDa	[57]
	Free radical methods	1% CS in HCl 1.8 M, 100 °C, 2 h	DP > 6	[57]
		2% CS, 2% acetic acid, 1.5% H ₂ O ₂ (final) pH 3.0, 6 h	9.9 kDa	[58]
ENZYMATIC	Specific enzymes	Chitosanase from <i>Aspergillus</i> sp. 5U in 5.5% CS solution 45–50 °C, 68 h	DP < 10	[64]
		Chitinase from <i>Aeromonas hydrophila</i>	DP 1 to 5	[63]
	Non-specific enzymes	1% CS in 100 mM sodium acetate pH 4 with 1:100 Pepsin ratio, 2 h	9–13 kDa	[65]
		4% CS 1% acetic acid 50 °C E/S protease ratio 1:20	DP 1 to 8	[67]
		4.5% CS in 0.5 M acetic acid bicarbonate pH 5.6, cellulase, 50 °C, 14 h	DP 3 to 8	[66]

⁽¹⁾ Mw: Molecular Weight and ⁽²⁾ DP: Degree of Polymerization.

4. Functional Properties of Chitosan and Derivatives

4.1. Sedimentation and Flocculation in the Wine Industry

Chitin and CS have been allowed by the Codex Alimentarius since 2003 as coagulating/clarifying agents for fruit juices and nectars. Fungal CS extracted from *Aspergillus niger* is the only type of CS allowed in winemaking, since 2009, as specified by the Oenological Codex (OIV-OENO 368-2009). The process from which CS is obtained from chitin in fungi is protected by a patent [23] and its origin is guaranteed according to OIV-OENO 368-2009 by the three following properties: (i) residual glucans have to be lower than 2%; (ii) viscosity in 1% acetic acid has to be higher than 15 Cps; and (iii) the settled density has to be lower than 0.7 g/cm³. CS is a flexible polymer with several functional groups (amine, N-acetamide, and hydroxyl groups, as seen in the previous sections), which makes it a very reactive molecule in wine. Therefore, it has numerous potential applications in oenology,

and is allowed for fining must or wines (OIV-OENO 336A-2009 and 337A-2009) up to a maximal dose of 100 g/hL, but also for treating wines to remove the following contaminants (OIV-OENO 338A-2009): (i) ochratoxine A (up to a treatment limit of 500 g/hL) and (ii) iron, lead, cadmium, and copper (maximum dose: 100 g/hL), and finally to reduce the main wine spoilage yeast populations, *Brettanomyces* (maximum dose: 10 g/hL) [72]. Even though most CS is soluble in most organic acid solutions [73], it is not entirely soluble in wine. The sediment formed after CS treatment should be removed by racking. CS is described in the literature as being a promising agent to fine white wine in order to reduce the protein content and hence prevent the protein haze hazard, as an alternative to the commonly used bentonite [74]. In red wine, CS can be used to clarify wines, but reduces the total phenol content at high doses [75]. However, given the treatment doses required and the cost of the CS treatment for fining, this application is currently poorly used. Moreover, other fining agents exist on the market, even if alternatives to bentonite (which potentially can confer metals to the wine and whose organoleptic impact is not neutral) or other fining agents (such as the animal-derived gelatins) are needed. Likewise, CS is still poorly used for metal and ochratoxin A removal in wine. However, alternative treatments for the replacement of the traditional ferrocyanure potassium treatment used to remove copper and iron, as well as PVI/PVPP (for copper as well as other metals), would be useful. Practically, CS is rather widely used for its antimicrobial properties in wine and more precisely to control the spoilage yeast *Brettanomyces bruxellensis* [76,77]. In a context where sulphur addition is more and more limited and the emergence of sulphur resistant yeast populations has been demonstrated [78], the use of CS as a curative and preventive agent is increasing among winemakers. Moreover, the 10 g/hL maximal and efficient dose to reduce these spoilage yeast populations is compatible from both a practical and economical point of view. However, there is little knowledge about the biological reasons sustaining the anti-microbial activity of CS in wine and investigations still need to determine the impact of CS on other oenological microorganisms, whether wanted or not in wine. Moreover, the heterogeneity of CS batches (deacetylation degree and molecular weight for example) and large range of pH, turbidity, ethanol content, and other chemicals parameters encountered in wines will modulate the efficiency of CS treatments [79]. Strains of *B. bruxellensis* are more or less reactive to the same CS batch according to CS concentration, level of yeast population, and probably other oenological parameters [76,77,80]. The efficiency of CS is sometimes reinforced in oenological formulations by the addition other oenological products, such as enzymes or fining agents. With a very active and increasing market of these formulations, it is quite challenging to enumerate all the products available on the market.

4.2. Antimicrobial Functions

CS was shown to inhibit the growth of many microbial species bacteria, yeasts, or other fungi: pathogens, phytopathogens, and spoilage species, for food, medical, or agricultural applications. It displays a high antiseptic spectrum and a high activity compared to other molecules. As a result, it can be used to eliminate microbial contaminants in planktonic or biofilm form, or to simply prevent their multiplication or adhesion in bioactive and antiseptic materials (to wrap foods or seeds, for instance, to immobilize lytic enzymes, to encapsulate vaccines); in solutions to clean material or teeth; to treat plants and crops; or thanks to its high biocompatibility, directly in liquid foods such as fruit juices or wine (Table 2). Depending on the aim of CS employment, the mode and duration of CS treatment, and the total experiment, the medium of the test and the measured effects vary a lot. Minimal inhibitory or minimal lethal concentrations (MIC < MLC) are often determined in liquid or solid media, inhibition diameters are also frequently measured on agar plates, and biofilm prevention or elimination is tested via microplate assays or even directly on medical material, through microbial sedimentation (Table 2).

Table 2. Studies on antimicrobial activity of chitosan: diversity of target microbes, test media, and final aim of the treatment.

Effect	Medium/Method	Chitosan Form or Derivative	Microbial Species Targeted	References
Microbial growth inhibition	Liquid model medium (MIC)	Nanoparticles, many Mw/DA	Many species	[81–93]
	Beef slices			[93]
	Beer, wine			[94–96]
	Solid agar plates			[81,83,92,97,98]
	Medical catheter	Diverse viscosity	<i>K. pneumoniae</i> <i>E. coli</i>	[99]
	Liquid media	Distinct concentrations	Microbials cultures	[95,100]
Metabolism modification	Liquid medium	Distinct concentrations	<i>S. cerevisiae</i>	[101]
Biofilm inhibition	Liquid medium	Nanoparticles	<i>S. aureus</i>	[81,102]
Microbial elimination	Liquid medium, minimal lethal concentration	Many Mw/DA	Many species	[77,83–85,87–89,103]
Biofilm elimination	Elimination of biofilms, in flow cells/polystyrene wells	Nanoparticles	<i>S. mutans</i> <i>S. aureus</i>	[102,104]
Flocculation/sedimentation	Liquid medium	Many Mw/DA	Distinct species	[77,87,101,105–107]

The type of microorganism present (yeast, bacteria, genera, species, and even strain) and their concentration or way of life (biofilm or planktonic) will greatly change the efficient CS concentration needed [18,82,87,90,107]. Furthermore, the origin, Mw, and DA of the CS or CS derivatives and formulations (nanoparticles, gels or grafted CS) used vary a lot and the conclusions drawn are sometimes conflicting. As a result, the antimicrobial mode of action of CS in liquid media is still highly hypothetical. Microbial inhibition by CS may be the result of a sequence of molecular mechanisms which altogether lead to cell inhibition and death [79,86,90,108,109]. Besides, some report that CS activity is mostly growth inhibitory and resistant subpopulations exist [110]. Most studies agree that the cationic nature of solubilized CS interferes with the negatively charged residues of the bacterial surface (Figure 6).

The subsequent (sometimes controversial) reported effects are:

- (i) The formation of a physico-chemical barrier (towards oxygen for example) by adhesion to the cell wall, especially on Gram positive bacteria [18,111]. As a result, the microbial envelope, which is known to be highly variable depending on the species and strain, particularly with bacteria, plays an important role in CS initial activity. All the elements, such as teichoic acids or external polysaccharides, that can be negatively charged will favor the interactions with CS. However, the exact nature of the surface components that interact with CS has not been accurately defined [83,103]. Species that contain chitin in their membrane would be less sensitive [82]. The membrane may not be the direct target as liposomes are poorly affected by CS [103,112]. Proteins or elements emerging from the membrane or the wall seem to be more likely to be recognized. However, the membrane composition and fluidity may influence the subsequent consequences of CS treatment [97,112];
- (ii) Some studies suggest a subsequent separation of the cell wall from the cell membrane, whilst others only mention a morphological change. Interaction with the membrane leads to altered cell permeability and may disrupt energy generation pathways [89,108,112–118];
- (iii) CS also causes agglutination and precipitation of the undesired microorganisms [77,106]. Indeed, *E. coli* was shown to protect itself by forming aggregates in the presence of chitoooligosaccharides

- (COS), which only displayed a bacteriostatic effect and the bacteria could rapidly grow after separation from the CS by membrane filtration [108,119]. In other studies, high Mw and low DD insoluble CS fractions were shown to act as fining agents, which eliminate such cell aggregates [105,106];
- (iv) The diffusion of low molecular weight CS into the cell and its interaction with DNA, RNA, and proteins is also suggested to contribute to the global mechanism [120–122];
 - (v) At sublethal doses, an induction of genes involved in stress regulation, arginine or glucose metabolism (energy), protein glycosylation, membrane synthesis, ion transport, wall construction, and autolysis is reported [84,85,110,122–124]. *S. cerevisiae* cells treated with sub-lethal doses of CS strengthen their wall and become resistant to beta-glucanase treatment [122,124];
 - (vi) Disruption of the membrane and release of cellular components are often reported, especially for Gram negative bacteria and for some yeasts [111], but depending on the dose used, this can or cannot be observed with some Gram positive bacteria, such as *S. aureus* [68,87,88,103,113,119,125–128];
 - (vii) The chelation and sequestration of metal ions and other nutrients in the broth have also been proposed [125].

In addition, several studies have focused on the parameters that modulate the antimicrobial activity of CS. Figure 7 gives an overview of the main parameters modulating the antimicrobial activity of CS.

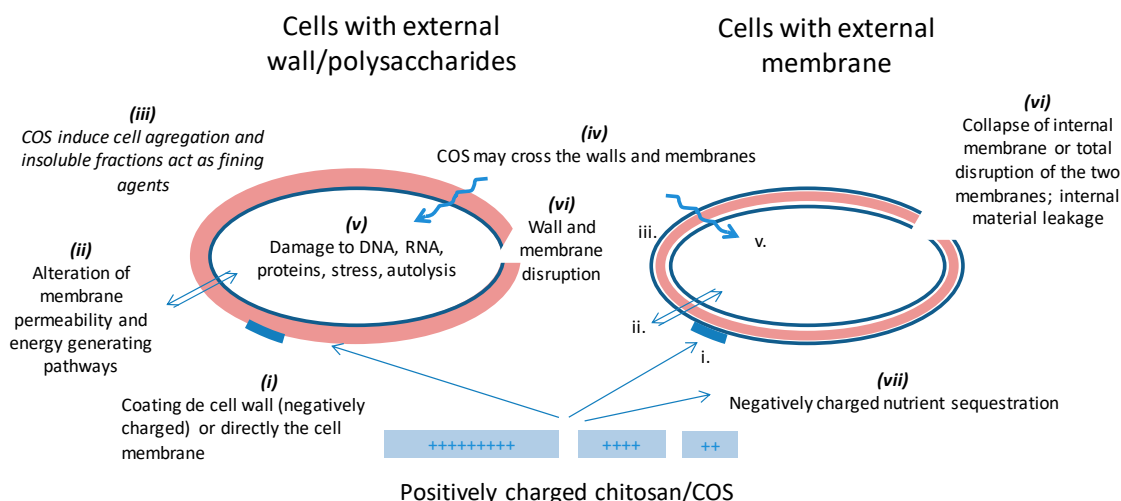


Figure 6. Inventory of the different molecular processes that may contribute to the chitosan antimicrobial activity (adapted from [79]). The numbers (i) to (vii) correspond to those used in the text (see above).

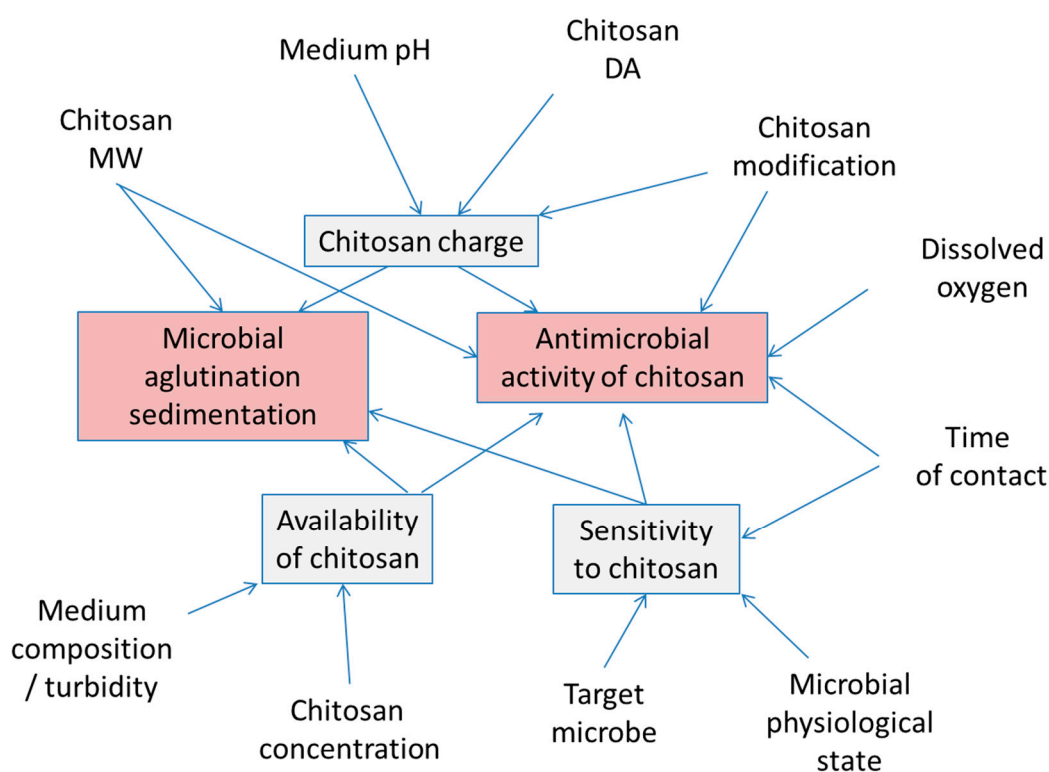


Figure 7. Parameters that modulate the antimicrobial activity of chitosan.

Regarding the intrinsic parameters, the CS Mw and DA are important parameters, more than the origin of the CS. Regarding the size of the active fractions, no consensus can be reached from the literature. The optimal active Mw may be species or even strain specific, and the opposite results are reported for various *E. coli* strains [83,108,120,129–132]. On the other hand, the antimicrobial activity is directly proportional to DD and inversely proportional to DA [83,84,129,133]. The activity is also modulated by the culture medium composition and it is different in laboratory media and in foods [95,98,134]: lipids, proteins, and divalent metal cations can bind to CS and prevent its interaction with target microbes [103]. Furthermore, Gyljenè et al. (2015) [135] suggest that dissolved oxygen can strongly increase the antiseptic activity of CS. The medium turbidity should also be considered, as CS binding to medium particles may render it inactive against microbes [93,95,98,136]. The medium pH is very important and CS loses its activity above pH 7, because of deprotonation and insolubility [83,120,127,130]. The use of CS derivatives such as carboxymethylCS, gallic acid grafted CS, or *N,N,N*-trimethyl CS enables higher antimicrobial activity at a higher pH [12,137–139]. The age of the microbial cultures, i.e., the physiological state of the microbes, and the nature of the species present, are also key elements modulating microbial sensitivity to CS [100,101,126,140]. Several studies mention the importance of CS concentration and time of contact regarding the aggregation and fining effects. Microbial flocculation seems to be more efficient with high Mw and low DD CSs, but this highly depends on the microbial species present [105,106]. Racking is essential to eliminate the still alive cell aggregates [108]. For example, in fruit juices and drinks such as beer or wine, CS is added directly in the beverage. If efficient racking is performed, CS treatment enables undesired microbes to be eliminated via two distinct activities: the killing one and the flocculating one [77,95,105,134]. However, racking is not always performed at the end of the test and the position (top, medium height, bottom, or whole homogenized medium) of medium sampling for microbial enumeration is not specified. This can greatly change the residual population measured and the risk of regrowth if live but flocculated individuals are maintained in the treated liquid [77,107,110].

4.3. Elicitation and Stimulation of Plants

As largely described in the literature, CS and derivatives also have applications as elicitors of plant growth defensive and stimulant responses [141–143]. In general, the idea that plant cells could release chemical substances during pathogen aggression was issued by the scientific community in the early 20th century. It was commonly referred to as phytoalexins to designate these plant antibiotics inducing a defense response against phytopathogens [141,142]. Later, these biomolecules resulting in the synthesis of phytoalexins were designated by the term “elicitors”. Thus, oligosaccharides derived from plants (endogens oligosaccharides: oligoxyloglucan and oligogalacturonate) or fungi (exogens oligosaccharides: oligo- β -glucan and oligochitin/COS) were widely described as active biological elicitors on biological mechanisms such as growth, cell development, symbiosis, and defense reactions [143,144]. During the aggression stage of a plant by a phytopathogen, different eliciting signals are emitted by both partners. First, in the early stages, oligogalacturonates, resulting from pectocellulosic wall degradation with fungal pectinase activities, set off acquired systemic resistance (ASR) in plants [145–147]. Several major components [147] can be distinguished to account for observed behaviors: (i) interaction with pecto-cellulosic walls of the host, (ii) induction of phytoalexins, (iii) specificity, (iv) hypersensitivity, (v) the action of toxins, (vi) the effect of ethylene, and (vii) the induction of pathogenesis-related proteins. Thus, ASR begins when all the different signals are perceived by a specific plant cell membrane receptor. Consequently, the plant then activates its natural defenses, such as the production of specific enzymes like chitinases, deacetylases, and β -(1,3)-glucanases, which will degrade the parietal constituents of the fungus to generate oligochitins, COS, and oligo- β -(1,3)-glucans [148]. Apart from all these oligo- β -(1,3)-glucan, oligochitins (β -(1,4)-*N*-acetyl-oligoglucosamines) and their deacetylated analogs (COS) are involved in the defense processes in many plant species, such as wheat (*Triticum*) and rice (*Oryza sativa*) [149,150]. The heptaoligochitin (DP 7) and octaoligochitin (DP 8) structures were found to be the most active elicitors [149,150]. Some examples of CS and oligochitin/COS elicitors derivatives are summarized in Table 3.

Table 3. Oligochitins/COS as biostimulators and elicitors of plants defenses.

Plants	Effects	References
Rice	Induction of phytoalexin	[149]
Wheat	Increase phenolic compounds	[150,155]
Pea	phytoalexin production	[151]
Tomato	Proteinase inhibitor synthesis	[152]
Soybean	Synthesis of callose	[153]
Parsley	Synthesis of callose	[154]
Potato	Enhance tuber size	[156]
Strawberry	Increase fruits yields	[156]
Barley	Increase phenolic compounds	[156]
Maize	Increase seed weight	[156]
Rape	Increase chlorophyll	[156]
Basil	Increase phenolic compounds	[156]

COS also exhibit activity on pea (*Pisum sativum*) and tomato (*Solanum lycopersicum*) leaves defenses, but at concentrations higher than those described for *N*-acetylated forms (oligochitins) [151,152]. Some other COS fractions were described to induce: (i) the synthesis of callose, which is a β -(1,3)-glucans during the defense responses of plants such as parsley (*Petroselinum crispum*) and soybean (*Glycine max*) [153,154] and (ii) lignin deposition and phenolic acid increasing in leaves of wheat [155]. More, CS and COS were also shown to highly stimulate positive plant effects on Potato (*Solanum tuberosum* L.), strawberry (*Fragaria ananassa* Duch.), basil (*Ocimum ciliatum*), rape (*Brassica rapa* L.), maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) [156]. Generally speaking, these bioelicitor activities from oligochitins/COS seem to be essentially modulated by ionic interactions between

these polycationic derivatives and the negatively charged compounds of the plant membrane, such as phospholipids [141,142].

Moreover, oligochitins/COS and their derivatives have also been extensively described as molecular messengers strongly involved in establishing the symbiosis between Rhizobia and legumes. Indeed, the Nodulation Factors (Nod Factors) are bacterial glycolipids involved in the formation of atmospheric nitrogen (N_2) fixing nodules on the roots of legumes. Some Nod factors have already been purified from culture supernatants of mutant *S. meliloti* strains [157].

All Nod factors produced by rhizobia have a main chain consisting of several β -(1,4)-linked *N*-acetyl-D-glucosamine residues (most commonly four to five residues). In *S. meliloti*, the Nod factor is a β -(1,4)-linked D-GlcNAc tetrasaccharide. Three of the four amine functions are substituted with acetates and one is substituted with a bi-unsaturated C-16 fatty acid (Figure 8). Therefore, we can usually talk about Lipo-ChitoOligosaccharide (LCO).

Many other Nod factors were subsequently isolated; they differ in the number of glucosamine residues, degree of acetylation or the presence of a more unsaturated and/or longer chain of fatty acids, or by different carbohydrate substitutions [158–160]. This work makes it possible to highlight the high level of specificity and recognition of oligosaccharides by the plant cell. Nod factors play a critical role in the ability of rhizobia to induce root nodules and many other infection-related responses in the host plant, at concentrations in the order of 10^{-7} – 10^{-11} M [161–163]. In fact, at low concentrations, the LCOs induce deformation of the plant's absorbent hairs, whereas at high concentrations, they induce the division of the cells of the plant's internal cortex, thus allowing the formation of the nodule [162,163].

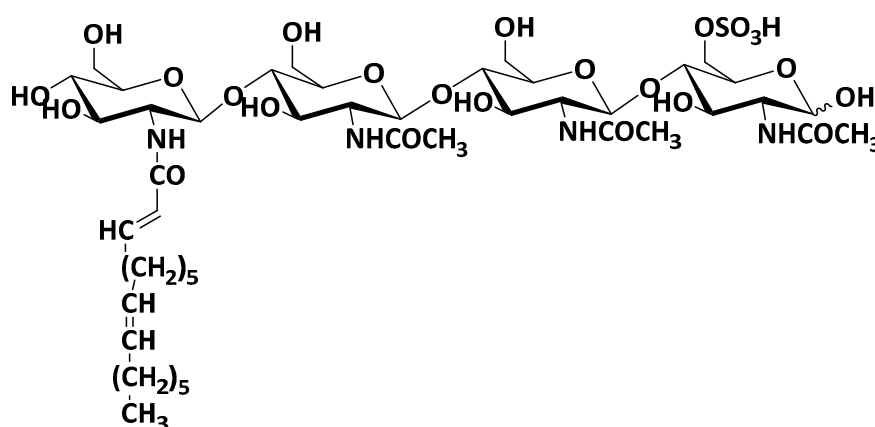


Figure 8. Structure of lipo-chitoooligosaccharides produced by EJ355 strain from *S. meliloti* [159].

4.4. Biomedical and Pharmaceutical Functions

Regarding the previous section, CS is a unique cationic biocompatible and biodegradable (see Section 5) polysaccharide that can be modified, as required, according to the needed end-use application. This is particularly true for biomedical and pharmaceutical applications ranging from drug delivery systems [164] to functional biomaterials [165], also considering tissue engineering [166], cell culturing [167], regenerative scaffolds [168], wound healing [169], smart hydrogels [170], active nanoparticles [171], anticoagulants [172], gene therapy [173], etc. (Figure 9). This list is obviously non-exhaustive regarding a short search on Scopus with more than 120 recent documents (2018–2019) using “biomedical” AND “pharmaceutical” AND “chitosan” AND “derivative” keywords.

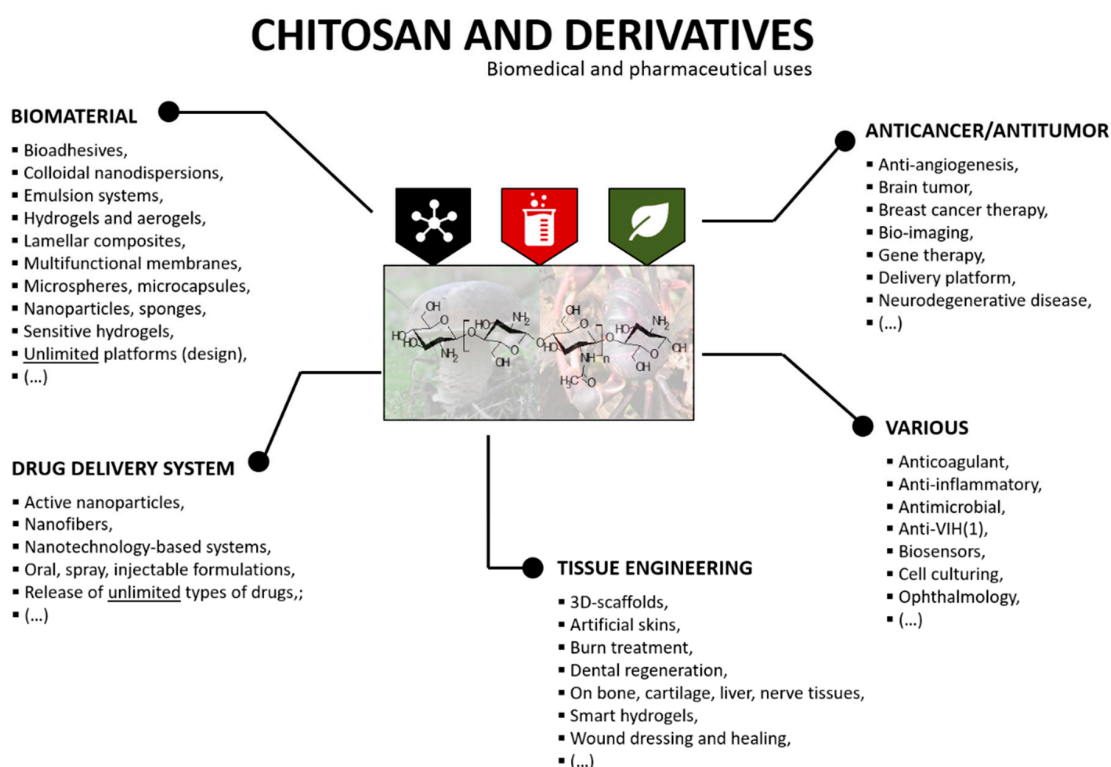


Figure 9. Various applications of chitosan and derivatives in biomedical and pharmaceutical fields.

Very recently, Mittal et al. [174] published a deep and comprehensive review that scientist readers should address to fully understand the progress of CS chemistry for use in biomedical fields, as well as the paper of Laroche et al. [4], which highlighted the need for an integral approach to comprehend all the potential of CS and its derivatives. Additionally, Khan et al. [175] detailed in their review the implications of the molecular diversity of chitin, CS, and some derivatives. The authors suggested the strong potential of CS-based nanomaterials to enhance nanobiotechnology in the future. Phil et al. [176] placed an emphasis on various biological activities of chitooligosaccharides (COS). COS with low DP (< 20) seemed to be the most preferred bases for prospecting biomedical properties due to their excellent solubility, absorbability, and capacity to cross physiological barriers [177]. Additional lipophilic groups were described to greatly increase biocompatibility [178,179]. COS and associated derivatives were reported for their uses in DNA/drug delivery system, [180], tissue regeneration [177], anticancer/antitumor [180], anti-HIV(1) [181], anti-hypertensive [180], or Alzheimer's disease [182]. *N,N,N*-trimethyl CS (TMC) was reported as a quaternized hydrophilic derivative for assembling new pharmaceutical nano-structures [182], but also for applications in tissue engineering [183]. These authors prepared a multifunctional nanohybrid scaffold able, on one hand, to *in vitro* load/release bioactive molecules (e.g., LMW heparin), and on the other hand, to play the role of a platform for the proliferation of soft tissue, extracellular matrix, and specific cells involved in adipogenesis. Furthermore, some authors have developed new nanoparticulate formulations with a TMC derivative, such as Sheng et al. [184], who loaded LMW protamine on TMC-coated nanoparticles for oral administration. This formulation clearly allowed an increase of intestinal permeability and efficient effects on the intestinal mucus layer. As another example, TMC micelles can be prepared to overcome subabsorption and solubility problems of specific active molecules, such as insoluble alkaloid, osthole, etc. [185,186]. The use of nanoparticles is not new and current papers deal with specific derivatives such as carboxymethyl CS (CMCS), which are soluble in both acidic and alkaline solutions, for designing nanotechnology-based systems based on stimulus-based, diffusion, swelling, or erosion-controlled release [187]. Beside, Hakimi et al. [188] recently showed the potential of thiolated methylated dimethylaminobenzyl CS as a delivery vehicle. This statement was validated on Human Embryonic

Kidney cells (Hek293) and the results revealed an improvement of solubility and disponibility, and no significant cytotoxicity. Cross-linking reactions between CS, COS, or CS derivatives with other polymers, synthetic and/or natural oligo- or polymers, open the way to unlimited applications, as reported by many authors, with recent examples for pectin [189], poly- γ -glutamic acid (γ -PGA) [190], Poly(ethylene glycol) (PEG) and cyclodextrin [191,192], C-phycoerythrin [193], or Poly(acrylamide-co-acrylic acid) [194]. Finally, CS users interested in biomedical and pharmaceutical applications should keep in mind that the possibilities of design are unlimited, obviously maintaining the essential physicochemical, biocompatibility, biodegradability, and biosolubility properties (in particular *in vivo*).

4.5. Adhesive Properties

CS is an interesting candidate for adhesive applications, especially in the wood field. CS has various DD and a large spectrum of Mw. It has been reported that its adhesive properties increase when DD and Mw increase [195,196]. The mechanisms of adhesion are multiple [1,197]. However, the surface tension and the viscosity of the liquid adhesive are important because they influence the interlocking mechanisms and modify the interactions with the adherent. First, the viscosity of the CS solution increases with concentration. For example, the viscosity is 90.2 Pa.s for a CS solution of 4% (w/v) and increases to 7132 Pa.s for a solution of 9% (w/v) [198]. Surface tension needs to be low to easily spread out upon all types of adherent materials. Surface tension is around of 38 mN.m⁻¹ for a 2% (w/v) CS concentration in 1 at 2% (v/v) acetic acid [199]. Kutnar et al. [200–202] estimated that the surface tension of viscoelastic thermal compressed wood ranged between 28.6 and 35.5 mN.m⁻¹. Chain link analogy for an adhesive bond in wood was proposed by Marra [203]. He considered a succession of links between adhesive and wood, especially in the interface between the boundary layer and the wood structure. This interface constitutes the adhesion mechanisms: mechanical interlocking, covalent bounding, and secondary chemical bounds due to the electrostatic forces through the adhesive penetration in wood cells (Figure 10). The penetration of CS solutions into wood or porous biosourced materials is discussed by Patel et al. [202] and Mati-Baouche et al. [203]. No penetration is observed respectively into wood [202] and sunflower [203].

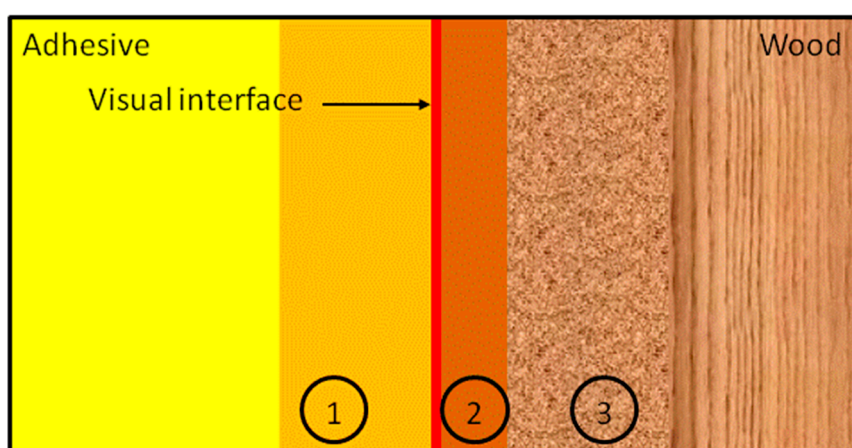


Figure 10. Schematic representation of the interfacial zone between adhesive and wood. 1: adhesive boundary layer, 2: interface between boundary layer and wood substrate which constitutes the adhesion mechanism (mechanical interlocking, covalent bonding, or secondary chemical bonds), and 3: adhesive penetration zone.

However, for water-based adhesive, water is adsorbed by the wood cell wall and the high molecular weight polymer molecules are trapped by the pit membrane [204]. For Pizzi et al., secondary forces appear to be the dominated mechanism for bonding wood [205]. CS carries polar and H-bonding functional groups. At acidic pH, positively charged CS in wet condition interacts more strongly with

the negatively charged surface *via* electrostatic forces, H-bonds, and van der Waals forces between glucosamine and the hydrated surface of adherend [7]. The bonding strength of CS was evaluated on three plywood veneer sheets with various amounts of CS before and after water immersion treatment [206]. Water treatment consisted of immersion for 3 h at 30 °C. Specimens were cooled in water and tested in the wet condition. The dry bond strength increased with increasing CS to 16 g.m⁻² and then decreased slightly. Before water immersion, the optimum bond strength was 2.13 MPa for 16 g.m⁻² CS and after immersion, the maximum value of the bond strength was 1.7 MPa in the condition of 32 g.m⁻².

Umemura et al. [207] showed that the dry bond strength of CS is in the range 1.1 MPa–1.6 MPa for Mw varying between 35 and 350 kDa. With glucose addition (70 wt%), the bond strength increased to 1.75 MPa for low molecular weights CS. In contrast, the bond strength tended to decrease at greater amounts of added glucose for high molecular weight CS. The Maillard reaction in the above formulation formed brownish melanoidins, which occurred between the COOH of glucose and NH₂ of CS, demonstrating improved adhesive properties of glucose cross-linked low molecular weight CS. Patel et al. [202] evaluated the potential of CS as wood adhesive using a double lap shear test. Three formulations were tested: CS 4% (w/v); CS 6% (w/v); and a formulation of CS 6% (w/v), glycerol 1% (v/v), and trisodium citrate dihydrate 5 mmol.L⁻¹. Dry bond strengths were respectively 4.2, 6.1, and 6.0 MPa. Paiva et al. [208] obtained the same results concerning the influence of the concentration of CS on cork adhesive performances. They mixed CS with oxidized xanthan gum to increase the adhesive power. The combination of oxidized xanthan gum with CS had the potential to improve the adhesion properties due to crosslinking of the aldehydes with the amino groups to form an imine linkage. To reduce water affinity and to improve the mechanical properties of CS, hydrophilic material such as starch can be incorporated. It forms intermolecular hydrogen bonds between the amino and hydroxyl groups of CS and the hydroxyl groups of starch [209]. CS is a basic linear polysaccharide. Its performance can be improved with the chemical cross-linking technique. For example, glutaraldehyde converts CS into a network structure for medium-density fiberboard applications [210]. Others authors have proposed formulating CS with konjar glucomannan [208] or lignin [211]. CS can be used as an adhesive with other materials, for metal, for example. Patel et al. [212] tested CS adhesive with aluminum adherents using a double-lap shear configuration. They studied different surface treatments and showed that aluminum adherents chemically treated with NaOH presented the best bonding strength. Formulated with glycerol (1% v/v) as plasticizer, CS (7% w/v) in 2% (v/v) acetic acid obtained a maximum shear strength of 40.8 MPa.

4.6. Other Potential Applications

Owing to the chemical properties earlier described, CS is also a promising adsorbent that is easily modifiable. Due to its unique polycationic behavior, CS can strongly interact with negatively charged molecules or ions. These adsorption and chelation properties are pH-dependent and also depend on CS molecular weight and acetylation degree. These characteristics make CS a polymer of choice for water pollution issues and controlling the quality of water effluents through the chelation of metal ions such as copper, zinc, lead, or cadmium [213]. Coagulation and flocculation properties of CS are also crucial in wastewater treatment plants [214] to reduce chemical oxygen demand (COD), chlorides, turbidity, and proteins [215]. In order to enhance absorptive properties of CS for metals and organic textile dyes, many types of derivatives have emerged, non exhaustively: zeolites, EDTA, and montmorillonite. CS is also being more and more used for creating innovative packaging owing to its remarkable barrier properties, especially against water vapor, and its low permeability to oxygen [216]. These properties help to maintain product quality thanks to the control of oxidation or moisture. The same study showed an important resistance to the UV light of CS after modification with an adequate amount of glycerol. The paper industry is using CS film as a paper finisher to improve paper strength to moisture. Due to its non toxicity and biocompatibility, this polysaccharide also has numerous food applications by providing texturing, gelling, and foaming properties and helping the stabilization

of emulsions. CS is also a super efficient lipid binder and can be used in supplemented food for obesity or dietary destination [213]. In agriculture, it is used for seed coating and can act as a frost protective [215]. Finally, promising solid state batteries including modified CS have been reported by some authors [214,216].

5. Biodegradability of Chitosan Derivatives and Life Cycle Assessment (LCA)

Since the last decade, the biodegradability of CS has been extensively studied, notably to produce COS, which present varying bioactivities and numerous potential applications in food, agriculture, biomedicine, pharmaceuticals, and cosmetics [217,218]. The combination of chemical (e.g., acidic depolymerization) and physical processes constitute the well-known way of producing COS [18,219–221], but these treatments nevertheless yield poorly defined oligosaccharide combinations varying in their DP, pattern of acetylation (PA), and fraction of acetylation (FA). Alternatively, CS depolymerization using enzymatic hydrolysis seems to be more relevant for COS production since it involves a more gentle and controlled procedure (pH, temperature), leading to a better control of Mw distribution of COS [221] and generation of more defined products [222,223]. However, as the efficiency of enzymatic hydrolysis of CS remains dependent on PA and FA, the chemical states of CS used as a substrate may influence the composition of enzymatic products [224,225].

CS has been reported to be susceptible to numerous enzymes, including specific (chitosanases, E.C. 3.2.1.132; chitinases, E.C. 3.2.1.14) and non-specific (glycosidase, lipase, proteases, etc.) CS hydrolyzing enzymes [226]. Non-specific chitosanolytic enzymes belong to heterogeneous enzyme families such as cellulase [227], amylase [228], pectinase [229], papain [230], lysozyme [231,232], or lipases [233] (Table 4). Although chitinases and chitosanases are very effective, the utilization of non-specific enzymes is more suitable for the low-cost production of COS [234]. Among non-specific enzymes, cellulases showing bifunctional activities (cellulase-chitosanases) have been well-documented and were isolated from various organisms, such as *Bacillus* sp., *Trichoderma* sp., and *Lysobacter* sp. [235–239]. With activities and reaction conditions varying according to the sources, some cellulase lead, by an endo-type cleavage, to final hydrolysis products distributed from dimers to tetramers [227]. Chitosanolytic activity associated with bifunctional cellulase may represent 15–40% of cellulase activity [236] and be enhanced with increasing deacetylation degree [240,241]. Furthermore, chitosanases are generally recognized as enzymes degrading specifically CS, but not chitin, and have been classified into three subclasses according to the nature of the cleavage positions: GlcN-GlcN and GlcNAc for subclass I, GlcN-GlcN for subclass II, and GlcN-GlcNAc for subclass III [222]. These enzymes, belonging to five Glycoside hydrolase families (GH-5, -8, -46, -75, and -80), degrade CS *via* an endo-type mechanism. However, new enzymes with exochitosanase activity have been reported, notably exo- β -D-glucosaminidase able to cleave CS from non-reducing termini, releasing GlcN residues [242,243]. Recently, the identification of a carbohydrate binding domain (CBM) for some chitosanases may suggest additional interaction with the CS polymer, involving a different mode of CS hydrolysis [244,245]. The chitosanases described are issued from many organisms, including bacteria, cyanobacteria, fungi, and plants [222]. Although the performance of chitosanases on CS depolymerization is largely dependent on enzyme sources and reaction conditions, it has the advantage of being able to design a selected enzyme mixture to generate the controlled production of COS with selected DP or perform the complete CS hydrolysis to GlcN free [222,239].

Table 4. Non-exhaustive list of enzymes biodegrading chitosan.

Enzyme/Microorganism	Mode of Action on Chitosan	Distribution of Reaction Products	Substrate Specificity	References
Cellulase				
<i>Bacillus cereus</i> D-11	GlcN-GlcNAc, GlcNAc-GlcN, GlcN-GlcN	Chitobiose, chitotriose and chitobiose	CMC, CS	[235]
<i>Bacillus sp.</i> 65	GlcN-GlcN	ND	CMC, CS	[238]
<i>Bacillus cereus</i> S1	GlcN-GlcN	Dimer, trimer and tetramer	CMC, Colloidal and soluble CS	[239]
<i>Lysobacter sp.</i> IB-9374	Endo-type cleavage	Chitobiose, chitotriose, chitotetraose	CMC, Colloidal CS, CS, Glycol CS	[236]
<i>Trichoderma reesei</i>	GlcN-GlcN GlcN-GlcNAc,	Oligomers	CMC, Avicel, CS	[241]
<i>Trichoderma viride</i>	GlcNAc-GlcN, GlcN-GlcN cleavage from the non-reducing end	Oligomers	CMC, CS	[227]
Chitosanase				
<i>Bacillus circulans</i> WL-12	GlcN-GlcN, GlcN-GlcNAc	(GlcN) ₂ , (GlcN) ₃ , (GlcN) ₄ , oligomers	Lichenan, colloidal CS	[246]
<i>Bacillus subtilis</i> str168	NA	(GlcN) ₂ to (GlcN) ₆	Low weight CS	[247]
<i>Amycolatopsis orientalis</i>	Exo-type chitosanase (Exo-β-D-glucosaminidase)	NA	CS	[248]
Chitinase				
	Random hydrolysis GlcNAc	Oligomers	CS	[249]
Lipase				
	NA	Mainly (GlcN) ₂ to (GlcN) ₆ , complete hydrolysis (GlcM) when increasing reaction time	CS	[237]
Papain				
	GlcN-GlcN, GlcN-GlcNAc	GlcN, (GlcN) ₃ , (GlcN) ₄ in soluble fraction, and oligomers in insoluble fraction	CS	[230]
Pectinase				
<i>Aspergillus niger</i>	NA	Dimer to hexamer with predominance of dimer, oligomers	CS	[229,250]
Lysozyme				
	GlcNAc-GlcNAc	NA	CS film	[231,232]

NA: Data not available, CMC: Carboxymethylcellulose, CS: Chitosan.

On the other hand, the biodegradation of CS derivatives relative to chemically modified or grafted-CS copolymers was also investigated using enzymatic hydrolysis, e.g. C6-oxidized CS [133], CS phenolic [251], CS/hyaluronan [231], or CS/alginate [252]. As an example, a commercialized enzymes mixture (Glucanex[®], Macerozyme R-10) and crude extract from *T. reesei* IHEM 4122 have shown the best performance for C6-oxidized CS degradation, with final hydrolysis yields ranging from 12.9 to 36.4% (*w/w*) [245]. In summary, the biodegradation of CS and derivatives has been proved efficient, mainly thanks to the availability of a large panel of enzymes.

Today, many studies focus on the improvement of these enzymes by genetic engineering, or the use of microorganisms producing chitosanolytic enzymes for degrading in situ CS bio-based products, notably in environmental and medical (CS-based systems used for drugs release) applications.

The benefits of CS, including its large availability, low-cost, biocompatibility, and biodegradability, make it attractive for industrial processing in a context of multiple applications (bio-based material and adhesives, tissue engineering, . . .) [253]. In the actual initiative of the establishment of the ecological impact in industrial processes development, studies of life cycle assessment (LCA) for CS utilization (from the extraction to the manufacturing product) have emerged for the last year. However, these

studies remain restricted to a few applications. As an example, Leceta et al. [219,249] has launched an LCA study to estimate the impact of manufacturing CS from crustacean waste to a bio-based film. A comparative analysis with propylene-based films (PBF) allowed it to be demonstrated that PBF had significant disadvantages associated with the polluting nature, the consumption of higher energy, and the release of carcinogen products. In support of these data, a schematic diagram of the life cycle for the CS-based adhesive was proposed by Mati-Baouche et al. [1], including the presentation of the main steps leading to the production of CS-based adhesive from crustacean waste. In a different context, after demonstrating the potential of grafting phenol and catechin on a CS polymer to generate a functionalized biopolymer, the relative impact of CS derivatives was compared with other water-soluble polymers using the framework of LCA. In conclusion, LCA constitutes an indispensable approach to generating important data on CS manufacturing environmental impacts and may contribute to strengthening the stimulation/interest of the industrial sector in CS processing development.

6. Conclusions

CS and their derivatives are bio-based, biodegradable, and biocompatible polysaccharides, having specific physico-chemical properties that can be exploited in numerous application fields. Indeed, they can be considered as a backbone rich in -OH and -NH₂ groups available for chemical reticulation and modifications with the objective of giving them specific functional properties. Chemical modification of CS is the main way to increase its solubility in aqueous solutions or organic solvents, leading to the formation of CS-based materials. In this context, recent research has focused on the use of this non-toxic linear polysaccharide on native or modified forms for several applications in the food area (dietary ingredients, food preservative, and/or techno-functional agent), biomedical applications (wound healing, gene delivery, tissue engineering, scaffold and hydrogels, pharmaceutical excipient), waste treatment (adsorption of heavy metal, coagulation of pollutants and bactericide agents), agriculture (elicitor of plant defense reactions), adhesives (wound bonding), and biotechnology (cells and enzymes immobilization). The major part of these applications is real, and products are currently on the market. However, in the future, their development on a large scale should consider the availability of commercial CS sources, which is constrained and limited by the volumes of raw materials for its production at an industrial scale. In this context, the development of new CS-producing chains exploring new and easily accessible sources of chitin has appeared as fundamental to increasing the volumes of production and proposing to the market low-cost CS. These new sources of CS, as the traditional ones, should be treated by innovative and ecological processes to avoid the use of strong acids and bases which are very hazardous for the environment, but also to limit the water consumption. For that, biological treatments of chitin and CS with enzymes (proteases or chitin deacetylase) or microorganisms producing them offer an alternative to traditional treatments combined or not combined with new technology (microwave for example), replacing the conventional deacetylation at high temperature. The actual research on new sources of proteins, notably exploring the large-scale production of insects and microalgae, could generate new chitin-rich by-products available for the industrial community to produce more sustainable and low-cost CS.

Information: All the chemicals cited in the paper could be easily purchased from suppliers such as Sigma-Aldrich, etc.

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