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*Review*

# Modification of chitosan: How generating new functional derivatives?

**Clément Brasselet[1], Guillaume Pierre[1,*], Pascal Dubessay[1], Marguerite Dols-Lafargue[2], Johana Coulon[3], Julie Maupeu[4], Amélie Vallet-Courbin[4], Hélène de Baynast[1], Thierry Doco[5], Philippe Michaud[1] and Cédric Delattre[1]**

[1] Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut Pascal, F-63000 CLERMONT-FERRAND, France ; guillaume.pierre@uca.fr
[2] ENSCBP Bordeaux INP-Unité de Recherche œnologie, 210 chemin de Leysotte, CS 50008, 33882 VILLENAVE D'ORMON CEDEX, France
[3] BIOLAFFORT, 11 rue Aristide Bergès, 33270 FLOIRAC, France
[4] ADERA-MICROFLORA, 210 chemin de Leysotte, CS 50008, 33882 VILLENAVE D'ORMON CEDEX, France
[5] UMR1083 Sciences pour l'œnologie, équipe BIO, INRA SupAgro, UM1, 2 place Viala, F-34060 MONTPELLIER CEDEX, France

* Correspondence: guillaume.pierre@uca.fr; +33473407422

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**Abstract:** Today, chitosan is probably considered as the biofunctional polysaccharide with the greatest 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 pointed the way towards chitosan and its derivatives. Thanks to specific reactive groups and easy chemical modifications, a wide range of physico-chemical and biological properties can be obtained from this ubiquitous polysaccharide composed of $\beta$-(1,4)-2-acetamido-2-deoxy-D-glucose repeating units. This review provides insights into multiple native/modified chitosans but also oligo-chitosans associated to their functional properties. Chemical and/or enzymatic strategies have been detailed to understand the methods of obtaining. Regarding the literature over the last 20 years, bioadhesive applications, antimicrobial activities, adsorption and chelation in wine industry but also developments in medical fields or biodegradability have been addressed.

**Keywords:** Chitosan; Polysaccharide; Functional properties; Bioactivity.

## 1. Introduction

Chitosan is a copolymer of glucosamine and N-acetyl glucosamine connecting by $\beta$-(1-4) linkages. It is derived from chitin which is among the most abundant biopolymers on earth. The word "chitin" is derived from 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-$\beta$-(1-4)-N-acetyl-D-glucosamine [1,2]. The unique chemical structures of chitin and chitosan 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), crustaceans shells or insects cuticules and also produced by some green microalgae [4]. Two main polymeric forms of chitin have been described in literature, namely $\alpha$- and $\beta$-chitins which are arranged as monoclinic and orthorhombic cells, respectively [5]. An allomorph $\gamma$-chitin is a combination of these two forms [5]. $\alpha$-chitin (from yeast cell walls, exoskeleton of crustaceans and arthropod cuticle) and $\beta$-chitin (from squid pen) correspond respectively to anti-parallel and parallel arrangements of polymer chains. 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 chitosan after hot alkaline deacetylation in 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 chitosan. Conventionally, the DD value of chitosan is usually higher than 50 %. The resulting chitosan, which is a polycationic polysaccharide, is soluble in dilute acidic media (2<pH<6) contrary to chitin [7]. In industrial processing, chitosan is mainly extracted from crab, shrimp shells, squid pens and crustaceans by acidic treatment to eliminate the 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 not really developed at the industrial scale at this time [6]. New commercial sources of chitosans from fungi and insects have appeared recently on the market to valorize some by-products (mushroom wastes or cuticules of insects from new protein production chains). They are based on more green processes compared with those used by traditional chitosan production chains. The physico-chemical properties of chitosan 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 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 (-$NH_2$) 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 (-OH) are also, as for all polysaccharides, available for chemical modifications such as sulfonation, carboxymethylation, phosphorylation or hydroxyethylation [10-14]. All these amine and hydroxyl groups along the chitosan chain can be cross-linked using cross-linking agents to give 'chemical' hydrogels. They can also interact each other due to ionic and hydrophobic interactions, molecular entanglements or hydrogel bonds to generate physical hydrogels [9]. Moreover, macromolecules of chitosan can produce self-assembled structures based on hydrogen-bond networks formation in aqueous solutions leading to fibers. Conformational variations of these chitosan assemblies have been reported to depend on local environment changes around chitosan (e.g., pH, temperature, types of salt, and types of acids). All these reactions offer to chitosan a great potential as biosourced materials, biomaterials drug/enzyme delivery vehicles, tissue engineering scaffold, adhesive, texturing agents, support for enzyme immobilization, bioactive agent and other. This review focuses on the fundamental uses of all forms of chitosans (polymer, oligomer, native and chemically modified) in a large variety of applications.

## 2. Chitosan in few words

### *2.1. Structure extraction and purification*

Although chitin and chitosan are 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 to solubilize them. But research was encouraged by the fact that resources were abundant. Indeed, chitin is the most abundant polysaccharide on earth after 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 $\beta$-(1,4) linked N-acetyl-D-glucosamine (GlcNac) (**Figure 1**). Chitosan is obtained by deacetylation of chitin with concentrated NaOH solution, and consists of a heteropolysaccharide of $\beta$-1,4 linked D-glucosamine and N-acetyl- D-glucosamine (**Figure 1**). Chitin and chitosan 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 chitosan [18,19].

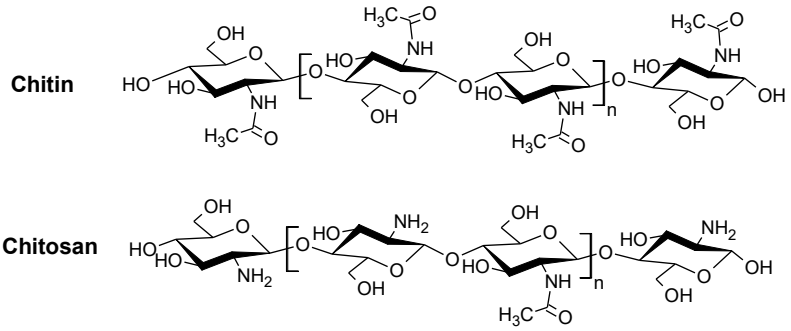



**Figure 1.** Chemical structure of chitin, and chitosan.

In the case of chitosan, 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 chitosan. Another definition considers that it is the solubility of the material in a solution of acetic acid, which defines the polymer as chitin or chitosan. In insects, fungi, diatoms or marine animals, chitin is synthesized by chitin synthase (EC. 2.4.1.16) [20]. In these organisms, chitin assemblies in three distinct polymorphic forms named $\alpha$, $\beta$ and $\gamma$ (parallel, antiparallels or mixture of both) [1,21]. The forms of the chains is found to depend on the orignin, and $\alpha$-chitin is the most abundant form. Chitin deacetylase (EC 3.5.1.41) partially removes acetyl substituents and defines de acetylation degree of the final chitin [22]. Chitosan 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 deproteinisation with hot alkali (NaOH 1N, at 60-100 °C for several hours) demineralization with acid (HCl 0.3-2 N at about 100 °C for 1 or two days) to eliminate calcium carbonate, and discoloration [17].

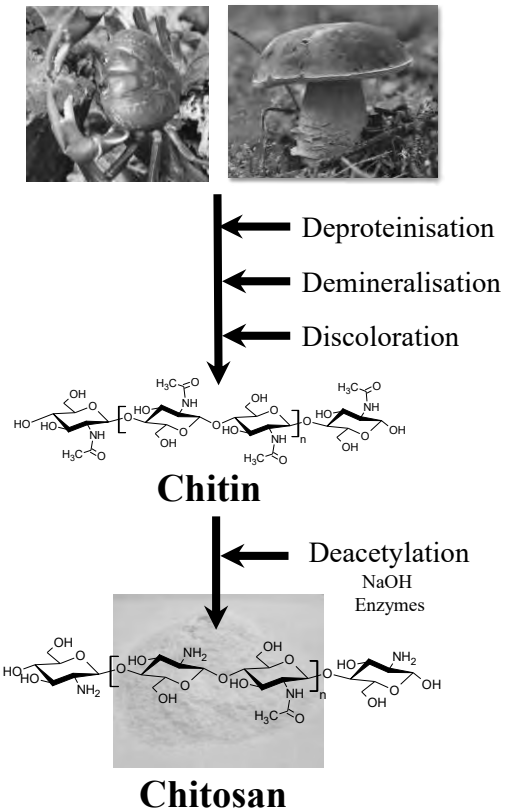



**Figure 2**. General steps for chitin and chitosan production.

The extraction process of the chitin-glucan from fungal biomass is more recent (**Figure 2**) [23,24]. The extraction method comprises hydrolysis steps, to separate the chitin from the rest of the mycelium and the lipid elimination by washing and drying. Then, chitosan is generally produced by partial

deacetylation of chitin 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 ($NaBH_4$). 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 improve the extraction and deacetylation steps [26-31]. Furthermore, biological treatments offer 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 chitosan 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 $3x10^6$ Da, but generally comprised between 100 and 1000 kDa, with small amounts of smaller molecules (10-50 kDa). Chitosan preparation mean MW and polydispersity vary a lot from one preparation to the other. Chitin, chitosan and glucan-chitosan can be hydrolyzed by enzymes (chitinases, chitosanases, glucanases) to prepare specific medium and low molecular weight (<50 kDa) chitosan families [1,17]. Chitosan is a weak base, with a pKa of 6.3-6.7. It is partially soluble in acidic aqueous solution when pH<pKa, and the solubility increases at pH <5.5. The DD parameter affects (*i*) the solubility of acidic chitosan, 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 (MW) or number (Mn). Mn affects the solubility of the chitosan and the viscosity of solutions [1]. The chitosan characteristics (in terms of DD, Mn, polydispersity and crystallinity) strongly depend on the extraction method and the source of isolation and they can vary widely from batch to batch [17,19,34].

### *2.2. Global market*

Chitosan has several uses in the industry such as cosmetics, water treatment, and agrochemicals [1,4]. Chitosan application is mainly focused at waste water treatment, due to its bio sorbent properties, in order to remove pollutants such as heavy minerals, oils, and phosphorous which are responsible for the deterioration of the water quality. Due to industrialisation and rising of global population, global chitosan 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, chitosan is expected to expend its use to the cosmetic industry because of it's skin moisturizing properties. Chitosan is also more and more thought off for hair care treatments or dental care as well as in agriculture for stimulating plant growth. The global Chitosan market is valued at 1,205 million US$ in 2015 and would reach 2,550 million US$ by the end of 2022 with a increasing of 10.7 % between 2016 and 2022. Ten to the power of ten tons of chitin are produced anually [1-4,35,36].

## 3. Chitosan derivatives and functionalization

Due to their exceptional properties and biological activities chitosan and its derivatives has a growing success as judged by the number the publications mentioning them and their large application potential in foods, environmental, material, cosmetic, pharmaceutical and biomedical. However its applications are strongly limited by the poorly soluble behavior in many solvents and water of chitosan. To bypass this problem, chemical modifications and depolymerization of chitosan are proposed.

### *3.1. Chitosan chemistry*

Chemical modifications of chitosan are well documented in recent publications in last few years [4]. Due to the presence of reactive amino ($NH_2$) and hydroxyl (-OH) groups this polysaccharide is very easily modifiable. Those modifications aim to enhance biological and chemical properties of chitosan and modify its solubility in function of the desired applications. In this paragraph, we will

underline the principal modifications of chitosan described in literature that are: quaternization, N-alkyl modifications, N-acyl modifications and C-6 oxidation.

### 3.1.1. Quaternized chitosan derivatives

Many publications [37,38] have shown that it is possible to modify the positive ($NH_3^+$) charge of chitosan to make it soluble in a large range of pH but also in neutral or slightly alkaline medium. Quaternization is an example of enhanced solubility of chitosan in water. Actually, chitosan positive charge is present in only at pH under 6.5 but when chitosan is quaternized this one is permanently positively charged at pH above 6.5. Quaternization reaction occurred between alkyl iodide and chitosan under basic conditions media. N,N,N-trimethylchitosan chloride (TMC) is the best known quaternized chitosan and has it great spectrum of applications [4]. As shown in **Figure 3**, TMC is obtained after two consecutive reactions, on the one hand by the reaction between methyl iodide $CH_3I$ and chitosan with N-methyl-2-pyrrolidinone (NMP) as solvent in alkaline conditions (NaOH) and on the other hand by the replacement of iodide ion with chloride one with the intermediate of anionic exchange resin. Various types of quaternized chitosan can easily be obtained by changing the carbon length of alkyl halides.

### 3.1.2. N-alkyl chitosan derivatives

Production of N-alkylated chitosan is achieved by the reaction of $-NH_2$ groups with ketones or aldehydes in a binary solvent such as methanol/acetic acid to allow the solubilization of liposoluble alkyl molecules and water soluble chitosan [4]. This reaction between ketones or aldehydes and chitosan is a condensation with formation of Schiff-base intermediates (**Figure 3**).

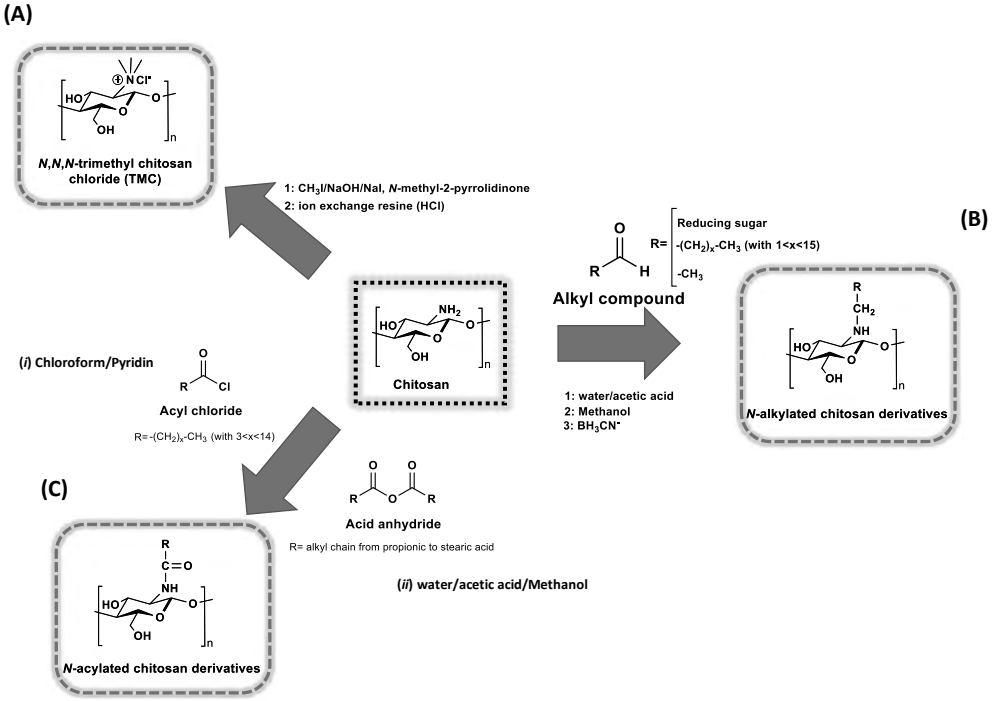



**Figure 3**. Production of chitosan derivatives by different ways: (A) Quaternization, (B) N-alkylation and (C) N-acylation.

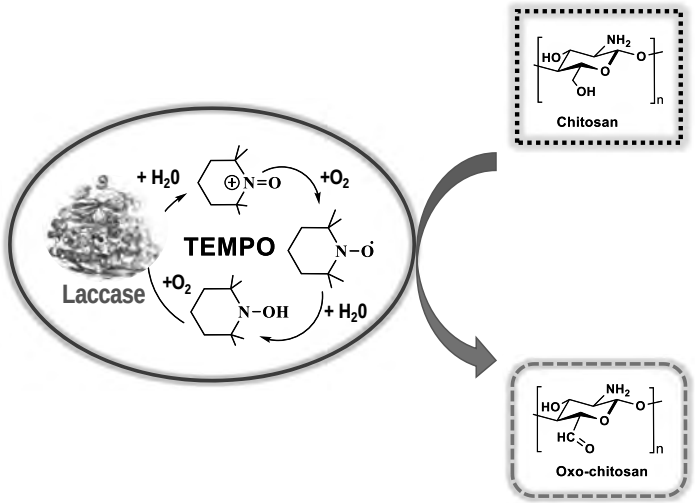
The transformation of those intermediates into N-alkylated chitosan derivatives is due the action of cyanoborohydride. Size of alkyl chain length can be modulated (generally between C3 and C12). In their publication Desbrieres et al. [39] showed that it is possible to synthesize N-alkyl chitosan with different chain length to be able to produce derivatives with a large rheological behavior. Some of others interesting publications clearly exhibit the importance of alkyl chain length and their substitution degree on chitosan on the interaction between transformed chitosan in water media [40,41].

196 3.1.3. N-acyl chitosan derivatives

197 N-acyl chitosan derivatives bring hydrophobic properties to chitosan by grafting them with
198 different fatty acids. The reaction consists to a specific amidation between –COOH groups from fatty
199 acids and –$NH_2$ groups from chitosan. Chemical reagents used for N-acylation are acyl halide or acid
200 anhydride (**Figure 3**). This acylation is regularly performed in pyridine, chloroform/pyridine, or
201 methanol/water/acetic acid. Nevertheless, this reaction can lead to O-alkyl chitosans because of two
202 reactive –OH groups on the chitosan repeating unit. In order to avoid this O-acylation, many authors
203 advice to primary hydroxyl groups of chitosan by trityl groups and enhance the N-Acylation by the
204 creation of a chitosan chloroacyl [42]. Many types of acid anhydride have been tested to produce N-
205 acyl chitosans [43-46].

206 3.1.4. Oxy-chitosan derivatives

207 A large number of scientific publications have explored production of water soluble chitouronic
208 acid sodium (carboxylated chitin or chitosan) with the use of TEMPO an organic catalyst for oxidation
209 of hydroxyls functions into aldehyde in NaOCl and NaBr conditions [47-50]. TEMPO is mainly
210 known for his oxidation of primary hydroxyl group in a regio-selective manner of huge number of
211 polysaccharides. Muzzareli et al. (1999) [51] have developed a region-selective oxidation method
212 using TEMPO to produce oxy-chitosan derivatives namely 6-oxychitosan. Chitouronic sodium salts
213 are mainly produced from pretreated (chemically or enzymatically) fungal or shrimp cells chitin. In
214 their work, Muzarelli et al. [47] used fungal biomass from *Trichoderma* and *Aspergillus* to produce a
215 new range of carboxylated chitosan/chitin that shown biocompatibility to human keratocytes and
216 their potential use in drug delivery applications [52]. Pierre et al. [50] in their recent work have
217 synthesized a new bioactive C6 oxy-chitosan derivative. This new derivative showed good anti-
218 parasitic properties against Leishmania. Very recently, an environmentally friendly process has been
219 developed by Botelho da Silva et al. (2018) [53] for C6 oxidation of chitosan through a TEMPO/ laccase
220 Redox system in order to generate water soluble chitosan fraction (**Figure 4**).




221
222
223 **Figure 4**. Environmentally friendly oxidation of chitosan *via* TEMPO/laccase system (adapted from [53]).

224 3.1.5. Cross-linked chitosan derivatives

225 The crosslinking step of chitosan consists in creating a crosslinked structure through the use of
226 bridging that link the strings together and thus create a network macromolecular three-dimensional
227 more or less irreversibly crosslinked [1,2,9]. Chitosan is most often crosslinked by covalent bonds in
228 the presence of aldehyde derivatives such as for example: glyoxal, formalin or glutaraldehyde in an
229 acidic or basic medium to generate chitosan-based hydrogel [9]. As a rule, this cross-linking reaction
230 with chitosan consists in forming a Schiff base (imine) [2,4,9]. Glutaraldehyde (GTA) is the most
231 studied crosslinking agent. It is synthetic, available and inexpensive [1,9]. The reaction consists of a

condensation between the aldehyde and a primary amine group from chitosan chain in the presence of labile hydrogen [9,16,34]. However, the GTA is toxic and then, natural alternatives to the GTA are being studied such as the use of the genipin [9], and citric acid [54,55]. As for example, Lusiana et al. [54] study reported the use of citric acid as a cross-linking agent for preparation of chitosan/ PVA membrane. This cross-link strategy was generally investigated to produce biomaterial as hemodialysis membranes [55]. The cross-linking between citric acid and chitosan was expected to incorporate carboxylate group ($COO^-$) to biomaterial in order to increase bioactive sites on chitosan membrane for transporting biomolecules (urea, creatinine, etc.). Polyvinyl alcohol (PVA) was used to increase the mechanical efficient and increase hydrophobicity of cross-linked chitosan membrane [54]. In the **Figure 5** were presented the main cross-linking chitosan strategies.

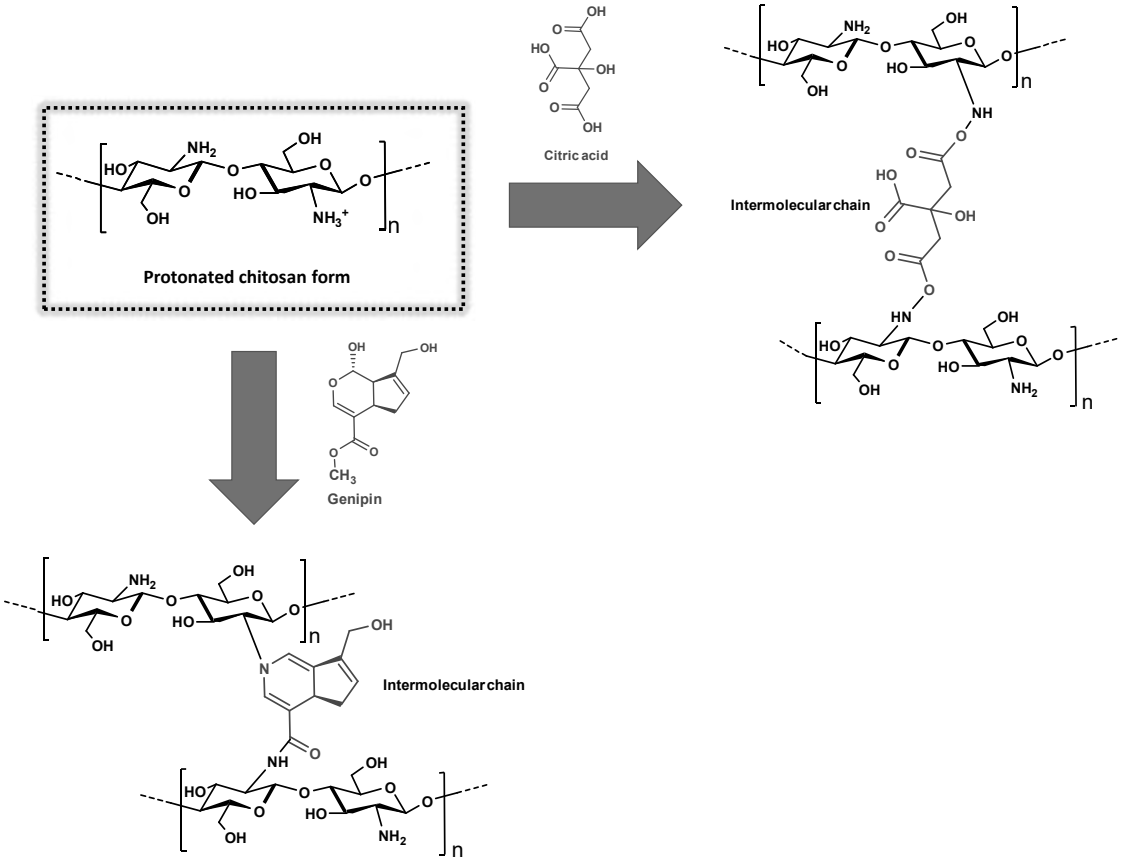



**Figure 5**. The mains cross-linking reactions using chitosan.

### *3.2. Oligochitosan and Low Molecular Weight (LMW) chitosan*

High molecular weight chitosan is very difficult to use in commercial applications due to high viscosity. Reducing molecular weight of chitosan is a good way to reduce viscosity and also to reinforce chitosan exceptional properties by the production of chitooligosaccharides (COS) and low molecular weight chitosan (LMW) described to have various biological properties. The production of COS and LMW chitosans is achieved principally by three ways: physical, chemical and enzymatic [56]. **Table 1** resumes the different possible ways including conditions to produce efficiently LMW chitosans or COS and DP or MW obtained after treatment when found in literature. The reduction of molecular weight by chemical, physical or enzymatic processes has been related to efficiently improve solubilization of chitosan in water or acetic acid solutions [4,56]. Depolymerization of chitosan is principally effected by chemical hydrolysis and precisely acid chitosanolysis is the most reported techniques to produce COS and LMW chitosans [4]. Then generally, chemical methods processes include chitosanolysis with HCl [57], $HNO_2$ [58], $H_2O_2$ [59] and potassium persulfate [60].

261 **Table 1.** Methods reported for producing LMWC or COS.

| Type of method | Depolymerization methods | Conditions | MW* DP** | References |
|---|---|---|---|---|
| **PHYSICAL** | High Pressure Homogenization | 1500 bars 1% chitosan in 1% acetic acid | 30 kDa | [70] |
| | Sonication | Sonication at 35.2 W/cm², 30 min | 140-143 kDa | [61] |
| | Gamma radiations | 2% chitosan in 2% acetic acid, 200 KGy | 3-5 kDa | [62] |
| | | 1% Chitosan, 0.1% Tween 80 irradiation 50 kGy | 75-77 kDa | [63] |
| | Autoclave | 1% Chitosan, 1% acetic acid, 121°C, 60 min, 1 bar | 313 kDa | [64] |
| **CHEMICAL** | Acid hydrolysis | 0.5 M HCl, 1% chitosan, 30 h, 65°C | - | [57] |
| | | 2% chitosan, 1.8 M HCl reflux 100°C, 2h | DP<40 | [72] |
| | | 0.976 % chitosan, 50 mM HCl, 3.89 mM $HNO_3$, 35°C, 30 min | < 16 kDa | [58] |
| | | 1% Chitosan in HCl 1.8 M, 100°C, 2h | DP > 6 | [58] |
| | Free radical methods | 2% chitosan, 2% acetic acid, 1.5% $H_2O_2$ (final) pH 3.0, 6h | 9.9 kDa | [59] |
| | | 1.5% chitosan in 2% acetic acid solution, 1.08 g KPS, 70°C | 17.4 kDa | [60] |
| **ENZYMATIC** | Specific enzymes | Chitosanase from *Aspergillus* sp. 5U in 5.5 % chitosan solution 45-50°C, 68h | DP<10 | [66] |
| | | Chitinase from *Aeromonas hydrophila* | DP 1 to 5 | [65] |
| | Nonspecific enzymes | 1% Chitosan in 100 mM sodium acetate pH 4 with 1:100 Pepsin ratio, 2h | 9-13 kDa | [67] |
| | | 4 % chitosan 1% acetic acid 50°C E/S protease ratio 1:20 | DP 1 to 8 | [69] |
| | | 4.5% chitosan in 0.5M acetic acid bicarbonate pH 5.6, cellulase, 50°C, 14h | DP 3 to 8 | [68] |

262 *Molecular Weight (MW) and *Degree of polymerization (DP).

263 Physical processes include depolymerisation with sonication [61], electromagnetic irradiation,
264 gamma irradiation [62,63] and microwave irradiation or thermal procedure [64]. Finally, enzymatic
265 processes use specific enzymes like chitinase [65] and chitosanase [66] but also non specific enzymes
266 like pepsin [67], cellulase [68], lipase, pronase, protease [69], lysozyme, papaïn, glucanase,

hemicellulase and pectinase. Many studies have related that the use of non specific methods like physical and enzymatic degradation of chitosan produce high COS and LMW chitosans yields. However, the main problem of enzymatic depolymerisation is the enzyme cost, making it redhibitory for bulk use in commercial applications and also the relative slowness of reactions whereas, chemical methods have the drawbacks of using non green chemicals, their removal and the non-uniformity of final products [4]. New methods for reducing molecular mass of chitosan have been found like High pressure homogenization (HPH) [70], plasma [71] or using zeolithes adsorbents [72] to purify acid hydrolysis COS and LMWC. Note to mention that electrochemical processes have also been developed to efficiently depolymerize chitosan [73].

## 4. Functional properties of chitosan

### *4.1. Sedimentation and flocculation in wine industry*

Chitin and chitosan are allowed by the Codex Alimentarius since 2003 as coaguling/clarifying agents for fruit juices and nectars. Fungal chitosan extracted from *Aspergillus niger* is the only type of chitosan allowed in winemaking, since 2009, as specified by the Oenological Codex (OIV-OENO 368-2009). The process from which chitosan is obtained from chitin in fungi is protected by a patent [74] and it's origin is guaranteed according to OIV-OENO 368-2009 by the three following properties: residual glucans have to be lower than 2 %; viscosity in 1 % acetic acid higher that 15 Cps and the settled density lower than 0.7 g/cm$^3$. Chitosan is a flexible polymer with several functional groups (amine, N-acetamide and hydroxyl), which makes it a very reactive molecule in wine. It hence 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 treat wines to remove the following contaminants (OIV-OENO 338A-2009): (*i*) ochratoxine A (up to a treatment limit of 500 g/hL) but also (*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) [75]. Even though most chitosan is soluble in most organic acid solutions [76], it is not entirely soluble in wine. The sediment formed after chitosan treatment should be removed by racking. Chitosan 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 [77]. In red wine, chitosan can be used to clarify wines but reduces the total phenol content at high doses [78]. However, given the treatment doses required and the cost of the chitosan treatment for fining, this application is today poorly used. Moreover, other fining agents exist on the market even if alternatives to replace 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, chitosan 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 cooper and iron as well as PVI/PVPP (for cooper as well as other metals) would be useful. Practically, chitosan is rather widely used for its antimicrobial properties in wine and more precisely to control the spoilage yeast *Brettanomyces bruxellensis* [79-80]. In a context where sulphur addition is more and more limited and the emergence of sulphur resistant yeast populations has been showed [81], the use of chitosane 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 both from a practical and economical point of view. However little is known about the biological reasons sustaining the anti-microbial activity of chitosan in wine and investigation still need to precise the impact of chitosan on other oenological microorganisms, whether wanted or not in wine. Moreover, heterogeneity of chitosane batches (deacetylation degree and molecular weight for example) and large range of pH, turbidity, ethanol content and others chemicals parameters encountered in wines will modulate the efficiency of chitosan treatments [82]. Strains of *B. bruxellensis* are more or less reactive to a same chitosane batch according to chitosan concentration, level of yeast population and probably others oenological parameters [79,80,83]. The efficiency of chitosan 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*

Chitosan 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 in 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 chitosan employment, the mode and duration of chitosan treatment and of 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, or biofilm prevention or elimination are tested via microplate assays or even directly on medical material, 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 | [84-96] |
| | Beef slices | | | [96] |
| | Beer, wine | | | [97-99] |
| | Solid agar plates | | | [84,86,95,100,101] |
| | medical catheter | Diverse viscosity | *K. pneumoniae* *E. coli* | [102] |
| | Liquid media | Distinct concentrations | microbials cultures | [98,103] |
| metabolism modification | Liquid medium | Distinct concentrations | *S cerevisiae* | [104] |
| Biofilm inhibition | Liquid medium | Nanoparticles | *S. aureus* | [84,105] |
| Microbial elimination | Liquid medium, minimal lethal concentration | many Mw/DA | many species | [80,86-88,90-92,106] |
| Biofilm elimination | Elimination of biofilms, in flow cells/ polystyrene wells | Nanoparticles | *S. mutans* *S. aureus* | [105,107] |
| Floculation/sedimentation | Liquid medium | Many Mw/DA | Distinct species | [80,90,104,108-110] |

The type of microorganism present (yeast, bacteria, genera, species and even strain), their concentration or way of life (biofilm or planktonic) will change a lot the efficient chitosan concentration needed [85,90,93,110,111]. Furthermore, the origin, MW and DA of the chitosan or chitosan derivatives (nanoparticles, gels or grafted chitosans) used vary a lot and the conclusions drawn are sometimes conflicting. As a result, the antimicrobial mode of action of chitosan in liquid media is still highly hypothetical. Microbial inhibition by chitosan may be the result of a sequence of molecular mechanisms which altogether lead to cell inhibition and killing [82,89,93,112,113]. Besides, some report that chitosan activity is mostly growth inhibitory and resistant subpopulations exist [114]. Most studies agree to say that the cationic nature of solubilized chitosan interferes with the negatively charged residues of the bacterial surface (**Figure 6**).

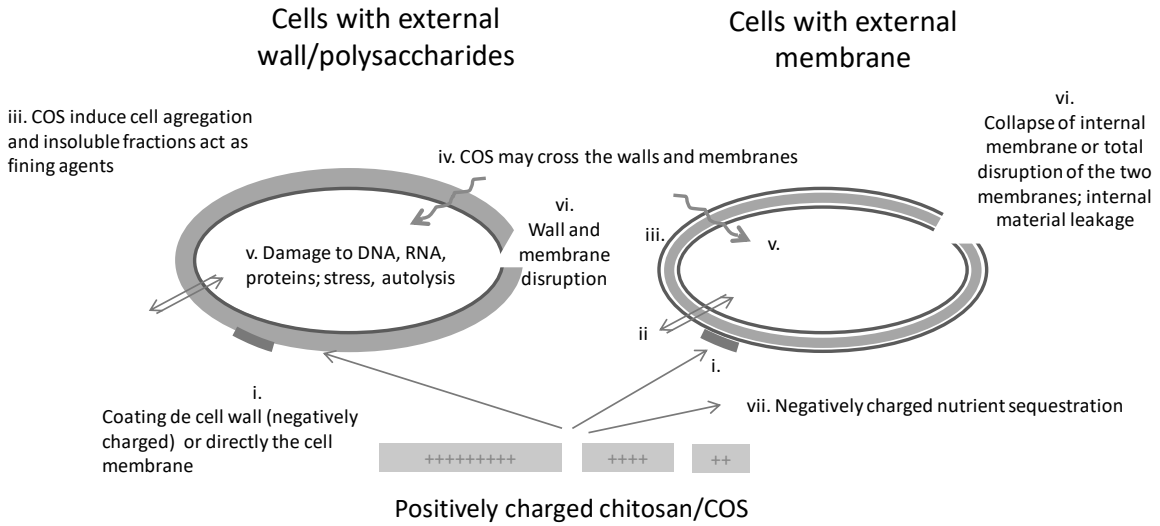



**Figure 6**. Inventory of the different molecular processes that may contribute to the chitosan antimicrobial activity (adapted from [82]). The numbers *i* to *vii* correspond to those used in the text (see above).

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 [111,115]. 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 chitosan initial activity. All the elements such as teichoic acids or external polysaccharides that can be negatively charged will favor the interactions with chitosan. However, the exact nature of the surface components that interact with chitosan has not been accurately defined [88,106]. Species that contain chitin in their membrane would be less sensitive [85]. The membrane may not be the direct target as liposomes are poorly affected by chitosan [106,116]. Proteins or elements emerging from the membrane or the wall seem to be more likely recognized. However the membrane composition and fluidity may influence the subsequent consequences of chitosan treatment [100,116].

(*ii*) Some studies suggest a subsequent separation of the cell wall from the cell membrane, others only mention a morphological change. Interaction with membrane leads to altered cell permeability and may disrupt energy generation pathways [92,112,116-123,92].

(*iii*) Chitosan also causes agglutination and precipitation of the undesired microorganisms [109,120]. Indeed, *E. coli* was shown to protect itself by forming aggregates in the presence of chitooligosaccharides (COS), which displayed only a bacteriostatic effect and the bacteria could rapidly grow after separation from the chitosan by membrane filtration [112,124]. In others studies high MW and low DD insoluble chitosan fractions were shown to act as fining agents which eliminate such cells aggregates [108,109].

(*iv*) The diffusion of low molecular weight chitosan into the cell and its interaction with DNA, RNA and proteins is also suggested to contribute to the global mechanism [125-127].

(*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 [87,88,114,127-129]. *S. cerevisiae* cells treated with sub-lethal doses of chitosan strengthen their wall and become resistant to beta-glucanase treatment [127,129].

(*vi*) Disruption of the membrane and release of cellular components are often reported especially for Gram negative bacteria and for some yeasts [115], but depending on the dose used this can be observed or not with some Gram positive bacteria such as *S. aureus* [69,90,91,106,117,124,130-133].

(*vii*) The chelation and sequestration of metal ions and other nutrients in the broth has also been proposed [130].

In addition, several studies have focused on the parameters that modulate the antimicrobial activity of chitosan. **Figure 7** summarizes the main parameters modulating the antimicrobial activity of chitosan.

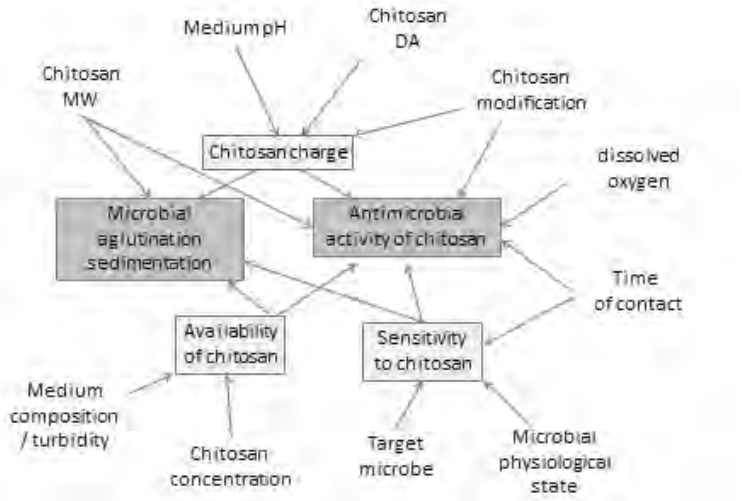



**Figure 7**. Parameters that modulate the antimicrobial activity of chitosan.

Regarding the intrinsic parameters, the chitosan MW and DA are important parameters, more than the origin of the chitosan. 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 opposite results are reported for various *E. coli* strains [86,112,124,134-137]. On the other hand, the antimicrobial activity is directly proportional to DD and inversely to DA [86,87,134,138]. The activity is also modulated by the culture medium composition and it is different in laboratory media and in foods [98,101,139]: lipids, proteins and divalent metal cations can bind to chitosan and prevent its interaction with target microbes [106]. Furthermore, Gyliene et al (2015) [140] suggest that dissolved oxygen can strongly increase the antiseptic activity of chitosan. The medium turbidity should be considered also, as chitosan binding to medium particles may render it inactive against microbes [96,98,101,141]. The medium pH is very important and chitosan loses its activity above pH 7, because of deprotonation and insolubility [86,125,132,135]. The use of chitosan derivatives such as carboxymethylchitosan, gallic acid grafted chitosan or N,N,N-trimethyl chitosan enables higher antimicrobial activity at higher pH [12,142-144]. 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 chitosan [103, 104,121,145]. Several studies mention the importance of chitosan concentration and time of contact regarding the aggregation and finning effects. Microbial flocculation seems more efficient with high MW and low DD chitosans, but this highly depends on the microbial species present [108,109]. Racking is essential to eliminate the still alive cell aggregates [112]. For example in fruit juices and drinks such as beer or wine, chitosan is added directly in the beverage. If efficient racking is performed, chitosan treatment enables to eliminate undesired microbes via two distinct activities: the killing one and the flocculating one [80,98,108,139]. But 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 highly change the residual

population measured and the risk of regrowth if live but flocculated individuals are maintained in the treated liquid [80,110,114].

### *4.3. Elicitation*

As largely described in the literature, chitosan and derivatives also has applications as elicitors of plant growth defensive and stimulant responses [146,147]. In general rule, the idea that plant cells could release chemicals substances during pathogen aggression was issued by the scientific community in the early 20$^{th}$ century. It was commonly referred to as phytoalexins (alkaloid, flavonoids...) to designate these plant antibiotics inducing a defense response against phytopathogens [146,147]. Later, these biomolecules resulting in the synthesis of phytoalexins have been designated by the term "elicitors". The concept of oligosaccharins was proposed by Albersheim in the 1990s to characterize oligosaccharides having an active role, called "hormone-like", in the regulation of biological processes. Thus, oligosaccharides derived from plants (endogens oligosaccharides: oligoxyloglucan and oligogalacturonate) or fungi (exogens oligosaccharides: oligo-$\beta$-glucan and oligochitin) were widely described as active biological regulators at nanomolar concentrations, on mechanisms such as growth, cell development, symbiosis and defense reactions [148,149]. 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 [150,151]. Several major components [152] can be distinguished to account for observed behaviors: (1) interaction with pecto-cellulosic walls of the host, (2) induction of phytoalexins, (3) specificity, (4) hypersensitivity, (5) the action of toxins, (6) the effect of ethylene and (7) 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 chitinases and $\beta$-(1,3)-glucanases, which will degrade the parietal constituents of the fungus to generate oligochitin and oligo-$\beta$-(1,3)-glucan [153]. Apart from all these oligo-$\beta$-(1,3)-glucan, oligochitins ($\beta$-(1,4)-N-acetyl-oligoglucosamines) and their deacetylated analogs (oligochitosans) are involved in the defense processes in many plant species such as wheat (*Tricicum*) and rice (*Oryza sativa*) [154,155]. The heptaoligochitin (DP 7) and octaoligochitin (DP 8) structures were found to be the most active elicitors [154,155]. In the **Table 3** some examples of chitosan and oligochitin/oligochitosans elicitors derivatives are summarized.

**Table 3.** Oligochitin/oligochitosan as biostimulator and elicitor of plants defenses.

| Plants | Effects | References |
|---|---|---|
| Rice | Induction of phytoalexin | [154] |
| Wheat | Increase phenolic compounds | [155,160] |
| Pea | phytoalexin production | [156] |
| Tomato | Proteinase inhibitor synthesis | [157] |
| Soybean | Synthesis of callose | [158] |
| Parsley | Synthesis of callose | [159] |
| Potato | Enhance tuber size | [161] |
| Strawberry | Increase fruits yields | [161] |
| Barley | Increase phenolic compounds | [161] |
| Maize | Increase seed weight | [161] |
| Rape | Increase chlorophyll | [161] |
| Basil | Increase phenolic compounds | [161] |

Oligochitosan 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) [156,157]. Some other oligochitosans 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*) [158,159] and (*ii*) lignin deposition and phenolic acid increasing in leave of wheat [160]. More, chitosan and oligochitosans were also shown to 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.*) [161]. As generally speaking, these elicitor activities from oligochitins/oligochitosans seem to be essentially modulated by ionic interactions between these polycationic derivatives and the negatively charged compounds of the plant membrane such as phospholipids [146,147].

More, oligochitins/oligochitosans 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 [162]. All Nod factors produced by rhizobia have a main chain consisting of several β-(1,4)-linked N-acetyl-D-glucosamine residues (most commonly 4 to 5 residues). In *S. meliloti*, the Nod factor is a β-(1,4)-linked D-GlcNAc tetrasaccharide. C-6 of the reducing end is sulfated. The absence of this sulfate group causes the loss of activity of this Nod factor vis-à-vis alfalfa. Three of the four amine functions are substituted with acetates and one is substituted with a bi-unsaturated C-16 fatty acid (**Figure 8**). So we can usually talk about Lipo-ChitoOligosaccharide (LCO).

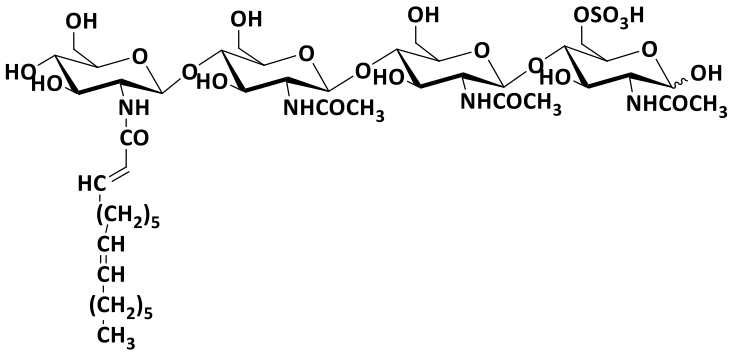



**Figure 8**. Structure of lipo-chitooligosaccharides produced by EJ355 strain from *S. meliloti* [162].

Many other Nod factors were subsequently isolated; they differ in the number of glucosamine residues or the presence of a more unsaturated and / or longer chain of fatty acids, or by different carbohydrate substitutions [163-165]. This work makes it possible to highlight the high level of specificity and recognition of oligosaccharides by the plant cell. All of these Nod factors are produced in response to secreted biological inducers by the roots of some plants. 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 [166,167]. 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 [167, 168].

### *4.3. Biomedical and pharmaceutical*

Regarding the previous section, chitosan is a unique cationic biocompatible and biodegradable polysaccharide (**see section 5**) that can be modified, as wish, according to the needed end-use application. This is particularly true for biomedical and pharmaceutical applications ranging from drug delivery system [169] to functional biomaterials [170], considering also tissue engineering [171], cell culturing [172], regenerative scaffolds [173], wound healing [174], smart hydrogels [175], active nanoparticles [176], anticoagulant [177], gene therapy [178], etc. (**Figure 9**). This list is obviously non exhaustive regarding a short search on Scopus with more than 120 recent document results with "biomedical" AND "pharmaceutical" AND "chitosan" AND "derivative" keywords.

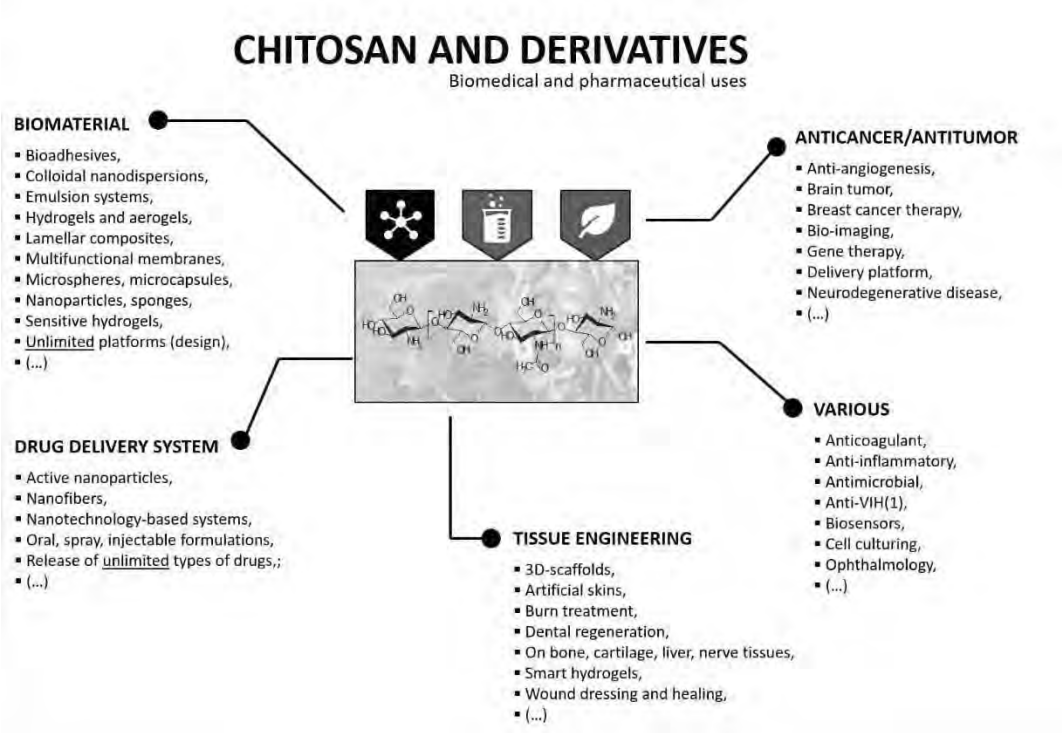



**Figure 9.** Various applications of chitosan and derivatives in biomedical and pharmaceutical fields.

Very recently, Mittal et al. [179] published a deep and comprehensive review that scientist readers should adress to fully understand the recent progress of chitosan chemistry for a use in biomedical fields, as well as the paper of Laroche et al. [4] which highlighted the need of integral approach to comprehend all the potential of chitosan and its derivatives. Additionnaly, Khan et al. [180] detailed in their review the implications of molecular diversity of chitin, chitosan and some derivatives. The authors suggested the strong potential of chitosan-based nanomaterials to enhance nanobiotechnology in the future. Phil et al. [181] gave an emphasis on various biological activities of chitooligosaccharides (COS). COS with low DP (< 20) seemed to be the most prefered bases for prospecting biomedical properties due to their excellent solubility, absorbability and capacity to cross physiological barriers [182]. Additional lipophilic groups were described to greatly increase biocompatibilty [183]. COS and associated derivatives were reported for their uses in DNA/drug delivery system [184], tissue regeneration [182], anticancer/antitumor [185], anti-HIV(1) [186], anti-hypertensive [185] or Alzheimer's disease [187]. N,N,N-trimethyl chitosan (TMC) was reported as a quaternized hydriophilic derivative for assembling new pharmacaeutical nano-structures [187] but also for applications in tissue engineering [188]. 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 platform for proliferation of soft tissue, extracellular matrix and specific cells involved in adipogenesis. Beside, some authors developed with TMC derivative new nanoparticulate formulations, such as Sheng et al. [189] who loaded LMW protamine on TMC-coated nanoparticles for oral administration. This formulation clearly allowed an increase of intestinal permeability and efficient effects on intestinal mucus layer. As another example, TMC micelles can be prepared to overcome subasorption and solulibitly problems of specific active molecules such as insoluble alkaloid, osthole, etc. [190,191]. The use of nanoparticles is not new and papers deal today with specific derivatives such as carboxymethyl chitosan (CMCS), which are soluble in both acidic and alkaline solutions, for designing nanotechnology-bases systems based on stimulus-based, diffusion, swelling or erosion-controlled release [192]. Beside, Hakimi et al. [193] recently showed the potential of thiolated methylated dimethylaminobenzyl chitosan as delivery vehicle. This statement was validated on Human Embryonic Kidney cells (Hek293) and the results

revealed here again an improvement of solubility, disponibility and no significant cytotoxicity. Cross-linking reactions between chitosan, COS or chitosan 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 [194], poly-γ-glutamic acid (γ-PGA) [195], Poly(ethylene glycol) (PEG) and cyclodextrin [196,197], C-phycocyanin [198] or Poly(acrylamide-co-acrylic acid) [199]. Finally, chitosan 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*).

### *4.4. Adhesive*

Chitosan is an interested candidate for adhesive applications, especially in wood field. Chitosan has various deacetylation degrees (DD) and a large spectrum of molecular weights (Mw). It has been reported that its adhesive properties increase when DD and Mw increase [200,201]. The mechanisms of adhesion are multiple [1,202]. 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, viscosity of chitosan solution increases with concentration. For example, viscosity is of 90.2 Pa.s for chitosan solution of 4% (w/v) and increases to 7132 Pa.s for a solution of 9 % (w/v) [203]. Surface tension needs to be low to easily spread out upon all type of adherent materials. Surface tension is around of 38 $mN.m^{-1}$ for 2 % (w/v) chitosan concentration in 1 at 2 % (v/v) acetic acid [204]. Kutnar et al. [205] estimated that surface tension of viscoelastic thermal compressed wood is ranged between 28.6 and 35.5 $mN.m^{-1}$. Chain link analogy for an adhesive bond in wood was proposed by Marra [206]. 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 chitosan solutions into wood or porous biosourced materials is discussed by Patel et al. [207] and Mati-Baouche et al. [208]. No penetration is observed respectively into wood [207] and into sunflower [208].

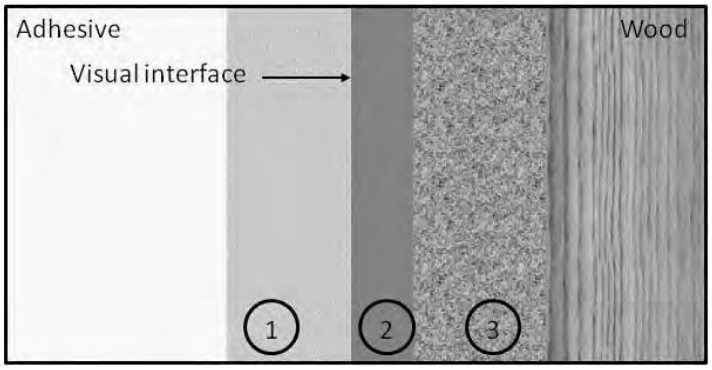



**Figure 10.** Schematic representation of the interfacial zone between adhesive and wood. 1: adhesive boundary layer, 2: interface between boundary layer and wood substrate which constitutes the adhesion mechanism (mechanical interlocking, covalent bonding ou secondary chemical bonds, 3: adhesive penetration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

But for water based adhesive, water is adsorbed by the wood cell wall and the high molecular weight polymer molecules are trapped bit the pit membrane [209]. For Pizzi et al., secondary forces appear to be the dominated mechanism for bonding wood [210]. Chitosan carries polar and H-bonding functional groups. At acidic pH, positively charged chitosan in wet condition interacts more strongly with negative charged surface via electrostatic forces, H-bonds and van der Waal's forces

568 between glucosamine and hydrated surface of adherend [7]. The bonding strength of chitosan was
569 evaluated on three plywood veneer sheets with various amounts of chitosan before and after water
570 immersion treatment [211]. Water treatment consisted on immersion during 3 h at 30 °C. Specimens
571 were cooling in water and tested in the wet condition. The dry bond strength increased with
572 increasing chitosan to 16 $g.m^{-2}$ and decreases slightly. Before water immersion, the optimum bond
573 strength was 2.13 MPa for 16 $g.m^{-2}$ chitosan and after immersion, the maximum value of the bond
574 strength was 1.7 MPa in the condition of 32 $g.m^{-2}$. Umemura et al. [212] shown that the dry bond
575 strength of chitosan is in the range 1.1 MPa – 1.6 MPa for Mw varying between 35 000 and 350 000
576 Daltons. With glucose addition (70 wt%), the bond strength increased to 1.75 MPa for low molecular
577 weights chitosans. In contrast, the bond strength tended to decrease at greater amounts of added
578 glucose for high molecular weight chitosan. Maillard reaction in above formulation formed brownish
579 melanoidins which occurred between COOH of glucose and $NH_2$ of chitosan that was improved
580 adhesive properties of glucose cross-linked low molecular weight chitosan. Patel et al. [207] evaluated
581 the potential of chitosan as wood adhesive using a double lap shear test. Three formulations were
582 tested: chitosan 4 % (w/v), chitosan 6 % (w/v) and a formulation chitosan 6 % (w/v), glycerol 1 % (v/v)
583 and trisodium citrate dihydrate 5 $mmol.L^{-1}$. Dry bond strength were respectively 4.2, 6.1 and 6.0 MPa.
584 Paiva et al. [213] obtained the same results concerned the influence of the concentration of chitosan
585 on cork adhesive performances. They mixed chitosan with oxidized xanthan gum to increase the
586 adhesive power. Combination of oxidized xanthan gum with chitosan had the potential to improve
587 the adhesion properties due to crosslinking of the aldehydes with the amino groups to form an imine
588 linkage. To reduce water affinity and to improve mechanical properties of chitosan, hydrophilic
589 material such as stark can be incorporated. It forms intermolecular hydrogen bonds between the
590 amino and hydroxyl groups of chitosan and the hydroxyl groups of starch [214]. Chitosan is a basic
591 linear polysaccharide. Its performances can be improved with the chemical cross-linking technique.
592 For example, glutaralhedyde converts chitosan into a network structure for medium-density
593 fiberboard applications [215]. Others authors proposed to formulate chitosan with konjar
594 glucomannan [211] or lignin [216]. Chitosan can be used as adhesive with others materials, for metal
595 for example. Patel et al. [217] tested chitosan adhesive with aluminum adherents using double-lap
596 shear configuration. They studied different surface treatments and they shown that aluminum
597 adherents chemically treated by NaOH presented the best bonding strength. Formulated with
598 glycerol (1 % v/v) as plasticizer, chitosan (7 % w/v) in 2 % (v/v) acetic acid obtained a maximum shear
599 strength of 40.8 MPa.
600
601 *4.5. Others*

602 Chitosan is a versatile polysaccharide with many different other applications, some of the most
603 important ones are detailed below. Owing to his chemical properties earlier described, chitosan is
604 also a promising adsorbent easily modifiable (by grafting, cross-linking, functionalization or coating).
605 Due to its unique polycationic behavior, chitosan can strongly interact with negatively charged
606 molecules or ions. These adsorption and chelation properties are pH dependant and also depend on
607 chitosan molecular weight and acetylation degree. These characteristics make chitosan a polymer of
608 choice of fighting water pollution and control the quality of water effluents and notably attract metal
609 ions such as copper, zinc, lead or cadmium [218]. Coagulation and flocculation properties of chitosan
610 are also crucial in wastewater treatment plants [219] to reduce chemical oxygen demand (COD),
611 chlorides, turbidity and proteins [220]. In order to enhance absorptive properties of chitosan for
612 metals and organic textiles dyes many types of derivatives emerged, non exhaustically: zeolites,
613 EDTA or montmorillonite. Chitosan is also more and more used in the creation of innovative
614 packaging and material science owing to its remarkable barrier properties especially against water
615 vapor and low permeability to oxygen [221]. These properties help to maintain product quality by
616 keeping it away from oxidation or moisture. The same study showed an important resistance to UV
617 light of chitosan when modified with adequate amount of glycerol. Paper industry is using chitosan
618 film as a paper finisher to improve paper strength to moisture. Due to its non toxicity and
619 biocompatibility, this polysaccharide has also numerous food applications by providing texturing,

gelling and foaming agents and helping the stabilization of emulsions. Chitosan is also a super efficient lipid binder and can be used in supplemented food for obesity or dietary destination [218]. In agriculture, it is used for seed coating and can act as a frost protective [220]. Finally promising solid state batteries including modified chitosan has been reported by some authors [219,221].

## 5. Biodegradability of chitosan derivatives and Life Cycle Assessment (LCA)

Since last decade, the biodegradability of chitosan has been extensively studied, notably for the production of COS which present varying bioactivities and numerous potential applications in food, agriculture, biomedicine, pharmaceutics and cosmetics [222,223]. The combination of chemical (e.g. acidic depolymerization) and physical processes constitute the well-known way of producing COS [224-226], but these treatments nevertheless yield poorly defined oligosaccharide combinations varying in their DP, pattern of acetylation (PA) and fraction of acetylation (FA). Alternatively, the chitosan 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 molecular weight distribution of COS [227] and the generation of more defined products [228,229]. However, as the efficiency of enzymatic hydrolysis of chitosan remains dependent on PA and FA, the chemical states of chitosan used as substrate may influence the composition of enzymatic products [230,231].

Chitosan 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.) chitosan hydrolyzing enzymes [232]. Non-specific chitosanolytic enzymes belong to heterogeneous enzyme families such as cellulase [233], amylase [234], pectinase [235], papain [236], lysozyme [237,238] or lipases [239] (**Table 4**). Although chitinases and chitosanases are very effective, the utilization of non-specific enzyme is more suitable for low-cost production of COS [241]. Among non-specific enzymes, cellulases showing bifunctional activities (cellulase-chitosanase) have been well documented and were isolated from various organisms such as *Bacillus* sp., *Trichoderma* sp. and *Lysobacter* sp. [240, 242-245]. 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 [233]. Chitosanolytic activity associated to bifunctional cellulase may represent 15-40% of cellulase activity [242] and be enhanced with increasing deacetylation degree [246-247]. Furthermore, chitosanases are generally recognized as enzymes degrading specifically chitosan but not chitin and have been classified in 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 [228]. These enzymes, belonging to five Glycoside hydrolase families (GH-5, -8, -46, -75 and -80) degrade chitosan via endo-type mechanism. However, new enzymes with exochitosanase activity have been reported, notably exo-β-D-glucosaminidase able to cleave chitosan from non-reducing termini, releasing GlcN residues [257, 258]. Recently, the identification of carbohydrate binding domain (CBM) for some chitosanases may suggest additional interaction with chitosan polymer, involving to a different mode of chitosan hydrolysis [259,260]. The chitosanases actually described are issued from a large number of organisms including, bacteria, cyanobacteria, fungi and plants [228]. Although the performance of chitosanases on chitosan depolymerization is largely dependent on enzyme sources and reaction conditions, it has the advantage to design selected enzyme mixture to generate the controlled production of COS with selected DP or perform the complete chitosan hydrolysis to GlcN free [228, 254]. On the other hand, the biodegradation of chitosan derivatives relative to chemically modified or grafted-chitosan copolymers was also investigated using enzymatic hydrolysis, as for example for C6-oxidized chitosan [138], chitosan phenolic [261], chitosan hyaluronan [237] or chitosan alginate [262]. As example, commercialized enzymes mixture (Glucanex®, Macerozyme R-10) and crude extract from *T. reesei* IHEM 4122 have shown the best performance for C6-oxidized chitosan degradation with final hydrolysis yields ranging from 12.9 to 36,4 % (w/w) [260]. In summary, the biodegradation of chitosan and derivatives has been proved efficient thanks mainly to the availability of large panel of enzymes.

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

| Enzyme /microorganism | Mode of action on chitosan | Distribution of reaction products | Substrate specificity | References |
|---|---|---|---|---|
| **Cellulase** | | | | |
| *Bacillus cereus* D-11 | GlcN-GlcNAc, GlcNAc-GlcN, GlcN-GlcN | Chitobiose, chitotriose and chitobiose | CMC, chitosan | [240] |
| *Bacillus sp*. 65 | GlcN-GlcN | ND | CMC, chitosan | [244] |
| *Bacillus cereus* S1 | GlcN-GlcN | Dimer, trimer and tetramer | CMC, Colloidal and soluble chitosan | [245] |
| *Lysobacter sp. IB-9374* | Endo-type cleavage | Chitobiose, chitotriose, chitotetraose | CMC, Colloidal chitosan, chitosan, glycol chitosan | [242] |
| *Trichoderma reesei* | GlcN-GlcN | Oligomers | CMC, avcel, chitosan | [247] |
| *Trichoderma viride* | GlcN-GlcNAc, GlcNAc-GlcN, GlcN-GlcN cleavage from the non-reducing end | Oligomers | CMC, chitosan | [233] |
| **Chitosanase** | | | | |
| *Bacillus circulans* WL-12 | GlcN-GlcN, GlcN-GlcNAc | $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$, oligomers | Lichenan, colloidal chitosan | [267] |
| *Bacillus subitilis* str168 | NA | $(GlcN)_2$ to $(GlcN)_6$ | Low weight chitosan | [269] |
| *Amycolatopsis orientalis* | Exo-type chitosanase (Exo-β-D-glucosaminidase) | NA | Chitosan | [258] |
| **Chitinase** | Ramdom hydrolysis GlcNAc | Oligomers | Chitosan | [270] |
| **Lipase** | NA | Mainly $(GlcN)_2$ to $(GlcN)_6$, complete hydrolysis (GlcM) when increasing reaction time | Chitosan | [239] |
| **Papain** | GlcN-GlcN, GlcN-GlcNAc | GlcN, $(GlcN)_3$, $(GlcN)_4$ in soluble fraction, and oligomers in insoluble fraction | Chitosan | [236] |
| **Pectinase** | | | | |
| *Aspergillus niger* | NA | Dimer to hexamer with predominance of dimer, oligomers | Chitosan | [235,268] |
| **Lysozyme** | GlcNAc-GlcNAc | NA | Chitosan film | [237,238] |

NA: Data not available, CMC: Carboxymethylcellulose.

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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 chitosan bio-based products, notably in an environmental and medical (chitosan-based systems used for drugs release) applications.

The benefits of chitosan by 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, …) [263]. In the actual initiative of the establishment of ecological impact in industrial processes development, studies of life cycle assessment (LCA) for chitosan utilization (from the extraction to the manufacturing product) have emerged for last year. However, these studies remain restricted to few applications. As example, Leceta et al. [264,265] has launched LCA study to estimate the impact of manufacturing chitosan from waste crustacean to bio-based film. A comparative analysis with propylene-based films (PBF) allowed demonstrating that PBF had significant disadvantages associated to the polluting nature, the consumption of higher energy and the release of carcinogen products. In support of these data, a schematic diagram of life cycle for the chitosan-based adhesive was proposed by Mati-Baouche et al. [1], including the presentation of the main steps leading to the production of chitosan-based adhesive from crustacean waste. In a different context, after demonstrating the potential of grafting phenol and catechin on chitosan polymer to generate functionalized biopolymer, the relative impact of the chitosan derivatives was compared with other water-soluble polymers using the framework of LCA [266]. In conclusion, the life cycle assessment constitutes an indispensable approach to generate important data on chitosan manufacturing environmental impacts and may contribute to strengthen the stimulation/interest of industrial sector for the chitosan processing development.

## 6. Conclusion

Chitosan and their derivatives are bio-based, biodegradable and biocompatible polysaccharide having specific physico-chemical properties that can be exploited in numerous applications fields. Indeed, they can be considered as a backbone rich in –OH and $–NH_2$ groups available for chemical reticulation and/modifications with the objective to give to them specific functional properties. The chemical modifications of chitosan are the main way to increase its solubility in aqueous solutions or organic solvents, leading afterwards to the formation of chitosan-based materials. In this context recent research has focused on the use of this non-toxic linear polysaccharide on this native or modified forms for several applications in 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 agent), agriculture (elicitor of plant defense reactions), adhesive (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 a next future, their development at large scale should consider the availability of commercial chitosan sources which is constrained and limited by the volumes of raw materials for its production at industrial scale. In this context, the development of new chitosan producing chains exploring new and easily accessible sources of chitin appeared as fundamental to increase the volumes of production and propose to the market low-cost chitosan. These new sources of chitosan, 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 environment but also to limit the water consumption. For that biological treatments of chitin and chitosan with enzymes (proteases or chitin deacetylase) or microorganism producing them offer an alternative to traditional treatments combined or not with new technology (microwave for example) replacing the conventional deacetylation at high temperature. The actual research of new sources of proteins, exploring notably 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 chitosan.

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