United States Department of Agriculture
Agricultural Marketing Service | National Organic Program
Document Cover Sheet
https://www.ams.usda.gov/rules-regulations/organic/petitioned-substances

Document Type:

☒ **National List Petition or Petition Update**

A petition is a request to amend the USDA National Organic Program's National List of Allowed and Prohibited Substances (National List).

Any person may submit a petition to have a substance evaluated by the National Organic Standards Board (7 CFR 205.607(a)).

Guidelines for submitting a petition are available in the NOP Handbook as NOP 3011, National List Petition Guidelines.

Petitions are posted for the public on the NOP website for Petitioned Substances.

☐ **Technical Report**

A technical report is developed in response to a petition to amend the National List. Reports are also developed to assist in the review of substances that are already on the National List.

Technical reports are completed by third-party contractors and are available to the public on the NOP website for Petitioned Substances.

Contractor names and dates completed are available in the report.

CASCADE COLUMBIA DISTRIBUTION
Chitosan Petition Submission Package Cover Letter

RE: **Petition Requesting the National Organic Standards Board (NOSB) Reassess the Classification of Chitosan as a Nonsynthetic Material Under National Organic Program (NOP) Regulations**

TO: **US Department of Agriculture (USDA)**
Agricultural Marketing Service (AMS)
National Organic Program (NOP), Standards Division
Email: nosb@usda.gov

PETITIONER: **Joel Van Ornum**
Cascade Columbia Distribution (formerly DESI)

PREPARED BY: **Gordon Sargent**
Ag Consultant for Cascade Columbia Distribution

Date: **May 19,2025**

Cascade Columbia respectfully petitions the USDA to reassess its classification of chitosan as a **synthetic** material and instead recognize it as a **nonsynthetic** substance under the National Organic Program (NOP) Regulations. This petition does not seek to add or remove any substance from the National Organic Program's (NOP) National List of Allowed and Prohibited Substances (National List).

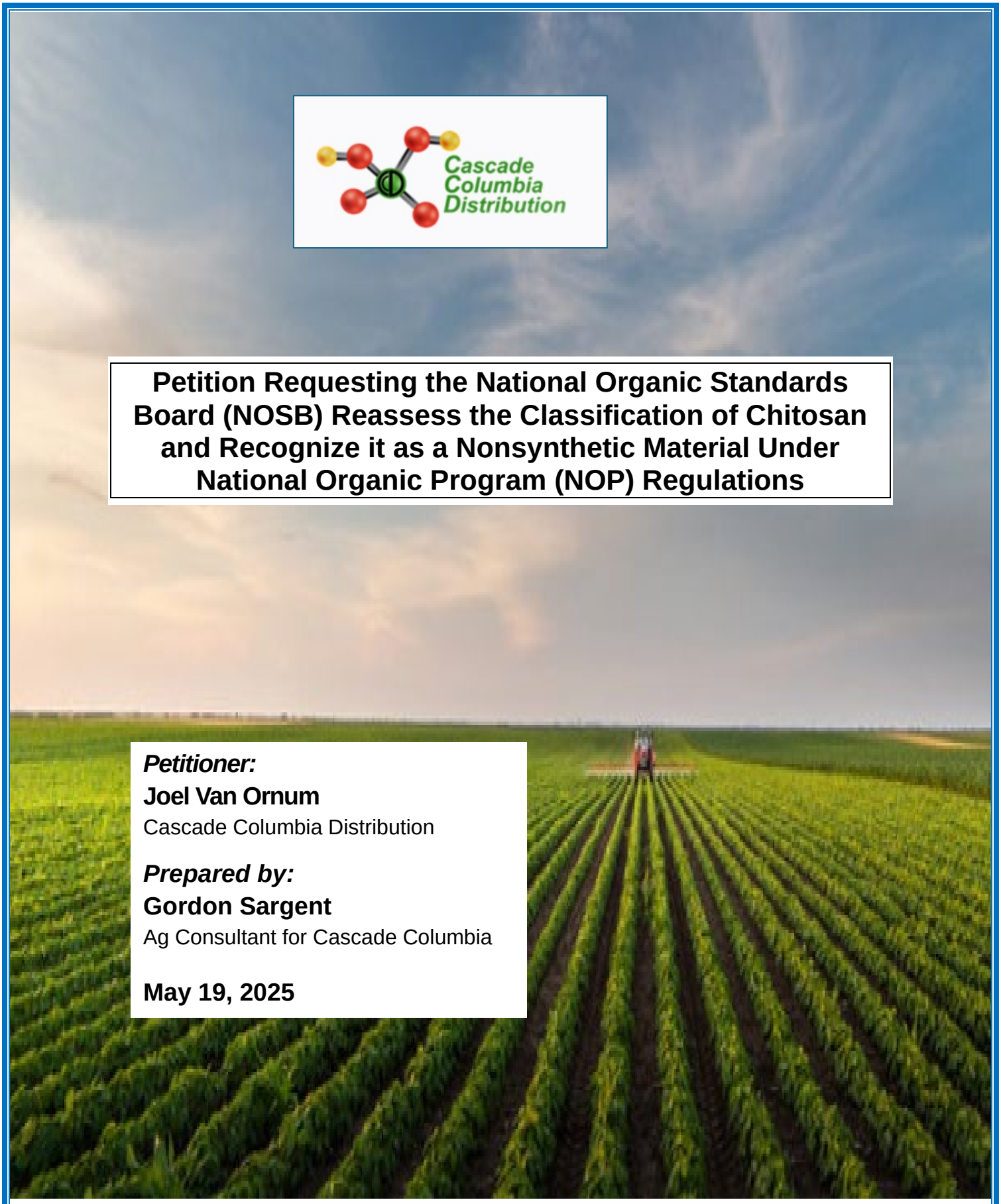
To support this request, Cascade Columbia is resubmitting its petition along with the following documents, which provide the additional information requested by USDA AMS:

1. **Chitosan Petition**:
2. **Chitosan Addendum 1:** Seven publications referenced in the Chitosan Petition.
3. **Chitosan Addendum 2**: Six hyperlinks to information referenced in the Chitosan Petition.
4. **Chitosan Addendum 3**: Six additional publications referenced in the Chitosan Petition.

The original petition was submitted in July 2024. Following an initial review by USDA AMS officials, certain deficiencies were identified, and additional information was requested. In response, a revised petition is provided including three addendums which provide further supporting information as well as addressing the USDA's specific requests.

If additional information is needed, feel free to contact me by phone at: 909-904-1582 or by email at: **sarge.gd@gmail.com**.

Respectfully,
Gordon Sargent
Ag Consultant for Cascade Columbia Distribution



Petition Requesting the National Organic Standards Board (NOSB) Reassess the Classification of Chitosan and Recognize it as a Nonsynthetic Material Under National Organic Program (NOP) Regulations

Petitioner:

Joel Van Ornum

Cascade Columbia Distribution

Prepared by:

Gordon Sargent

Ag Consultant for Cascade Columbia

May 19, 2025

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CHITOSAN-NONSYNTHETIC PETITION

Subject: **Petition Requesting the National Organic Standards Board (NOSB) Reassess the Classification of Chitosan and Recognize it as a Nonsynthetic Material Under National Organic Program (NOP) Regulations**

TO: **US Department of Agriculture (USDA)**
Agricultural Marketing Service (AMS)
National Organic Program (NOP), Standards Division
Email: nosb@usda.gov

From: **Gordon Sargent**
Ag Consultant for Cascade Columbia Distribution (formerly DESI)
email: sarge.gd@gmail.com

Date: **May 11, 2025**

This petition formally requests the NOSB reassess the classification of the natural biopolymer, **Chitosan** and recognize it as a **nonsynthetic material** under the NOP Regulations.

INTRODUCTION

The Organic Foods Production Act of 1990 (OFPA), as amended (7 U.S.C. § 6501-6523) provides the legal framework for establishing USDA regulations to evaluate and approve operations for the production of organic food. Accordingly, a substance that is “organic” and “nonsynthetic” can be used in the production of organic crops.

Efforts to gain approval for chitosan’s use in organic production date back to 1994. Proposals included its application both as an adjuvant and active ingredient in pesticide formulations. Despite these attempts, none have been successfully approved, as detailed in this petition.

In 2006, the National Organic Standards Board (NOSB) evaluated a petition to list chitosan as an adjuvant under the National Organic Program. During this process, the NOSB consulted with the Environmental Protection Agency (EPA) and determined that chitosan was already eligible for organic production due to its inclusion on EPA's Inerts List 4. Later that same year however, EPA reassessed all inert substances used in pesticide formulations. Consequently, EPA discontinued the use and maintenance of Inerts List 4 as well as all the other “Inerts Lists”. Furthermore, chitosan’s use as an inert in pesticide formulations was limited to **nonfood use only** thereby effectively preventing its use as an inert in the production of organic food.

More recently, Botry-zen’s 2019 submitted a petition to the NOSB to add chitosan to the NOP List as a pesticide active ingredient for disease control in the production of organic food. While the vote by the NOSB was a tie, the motion failed, lacking the required majority.

The petitioner assumed that chitosan is categorized as a synthetic substance which necessitates significant regulatory hurdles not the least of which it would have to go through the federal rulemaking process to add it to the list of synthetic substances approved for use in organic

production under the federal regulations (*7CFR § 205.601 Synthetic substances allowed for use in organic crop production).*

This petition however, submitted on behalf of Cascade Columbia Distribution to the NOSB, seeks to reclassify chitosan – a naturally occurring biopolymer **– from synthetic to nonsynthetic** within the framework of the National Organic Program (NOP) regulations. This proposal builds on scientific evidence supporting the reclassification, citing numerous developments over time that support this reclassification. Importantly, this proposal **does not** necessitate the need to amend the National Organic Program regulations under 7 CFR Part 205.

WHAT IS CHITOSAN?

Chitosan (poly-D-glucosamine; Figure 1) is a de-acetylated form of the polysaccharide, chitin (poly-N-acetyl-D-glucosamine; Figure 2). Both substances are found in nature and are comprised of chains of glucosamine molecules that are structurally related to cellulose (Figure 3). Chitin is a biopolymer second only to cellulose (Figure 3) in worldwide abundance.

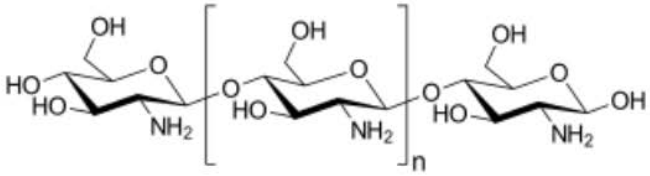


Figure 1. Structure of Chitosan

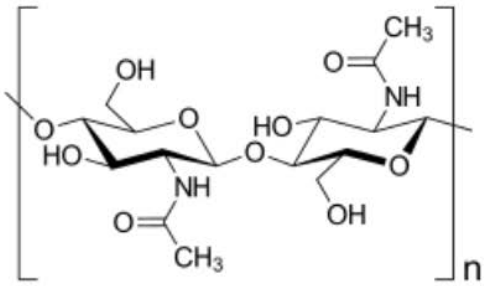


Figure 2. Structure of Chitin

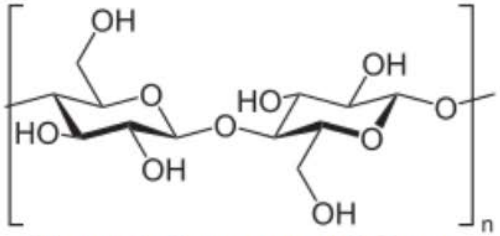


Figure 3. Structure of Cellulose

Commercially, chitosan is produced primarily from chitin that is extracted from the shells of marine crustaceans such as shrimp, crab, prawn, lobster, and krill. These crustacean shells serve as the essential raw materials in the production of chitosan. The chitosan material referenced in this petition is derived from shrimp shell in particular.

Marine crustaceans are obtained from the world oceans as a food source, with about 75% of the total weight ending up as seafood “waste” products which are disposed at sea, burned, landfilled, or left to spoil (Bellich et al., 2016; Hamed et. al., 2016; Younes and Rinaudo, 2015). Therefore, the commercial extraction of chitin and chitosan and their conversion into useful, natural commercial products provides a pathway for reducing this waste stream in the environment.

Chitosan is a heterogeneous substance and, depending on the degree and duration of deacetylation during the manufacturing process, can be defined as chitin that has been sufficiently deacetylated to form soluble amine salts (Jones, EPA, 2019-Aug).

In the Trends in Food Science & Technology journal paper titled *Industrial applications of crustacean by products (chitin, chitosan, and chitooligosaccharides): A review;* the following assertion is made on page 44:

> "The degree of deacetylation is generally defined as the glucosamine/N-acetyl glucosamine ratio, which goes up as chitin is converted to chitosan. Therefore, when the percentage of N-acetyl glucosamine is higher than glucosamine, the biopolymer is called chitin and when the percentage of glucosamine exceeds N-acetyl glucosamine the compound is called chitosan (Viarsagh, Janmaleki, Falahatpisheh, & Masoumi, 2010; Ramírez et al., 2010; Khor & Lim)."
>
> Hamed, et al.; 2016

Hamed et al. emphasized the intrinsic relationship between chitin and chitosan, explaining that their classification is solely based on their monomer ratios. Therefore, both polymers can be regarded as **natural and non-synthetic**.

COMMERCIAL USES OF CHITOSAN

Chitosan has a multitude of physiochemical properties that are currently utilized in a variety of commercial products.

Attribute	**Product examples**
Triggers plant defenses	Foliar, soil and seed treatment agricultural products
Sticker	For foliar applied pesticide products
Film forming	Hair and skin care products
Polycationic flocculant	Wine clarifier Water purification
Blood coagulation	Wound healing medical bandages
Binds fat and oils	Swimming pool and spa water flocculant/clarifier Fats and oil binder (dietary supplement)

An added benefit of chitosan is that it is biodegradable and sustainable – it is environmentally friendly and breaks down naturally without harmful residues.

CHITOSAN'S USE IN AGRICULTURE

This petition underscores the impressive agricultural benefits of chitosan. Its production not only transforms discarded shells into a valuable resource, contributing to waste reduction, but also offers an eco-friendly alternative to conventional chemicals. By enhancing plant defenses and improving soil health, chitosan provides a sustainable solution that aligns with modern environmental goals while boosting agricultural efficiency. It's a great example of how the synergy between science and nature can pave the way for a more sustainable future.

Systemic Resistance

Systemic Resistance in agriculture is an intriguing mechanism. Unlike traditional pesticides that directly target pathogens, materials like chitosan work by boosting the plant's innate immune system. Chitosan achieves this by activating plant defense signaling pathways, specifically stimulating **Induced Systemic Resistance (ISR)**. By stimulating these pathways, chitosan enhances the plant's defensive mechanisms against pathogens, contributing to improved resilience and overall health.

It's a remarkable example of harnessing natural compounds to strengthen agricultural practices.

Molecular Pathways

1. **Recognition and Activation:** Chitosan is recognized by plant receptors as a pathogen-associated molecular pattern (PAMP). This recognition triggers a cascade of immune responses.
2. **Signaling Molecules**: The ISR mechanism primarily involves jasmonic acid (JA) and ethylene (ET) signaling pathways. These pathways activate genes responsible for producing defensive compounds.
3. **Systemic Response**: Once activated, ISR signals travel throughout the plant, preparing distant tissues to respond more effectively to potential pathogen attacks. This systemic signaling enhances the plant's resilience without directly targeting pathogens.

Excerpt from the 2006-Jul Proposed Rule by the Agricultural Marketing Service (2006-Jul: Proposed Rule Page 37855):

> *…As a plant growth enhancer, the mode of action is believed to be that chitosan is taken up by plant cells where it enters the cell nucleus and stimulates messenger ribonucleic acid and enzyme production. This action stimulates the plant to produce more lignin in the stems, resulting in stronger stems.*

Benefits Across Crops

1. **Broad-Spectrum Protection**: ISR induced by chitosan provides defense against a wide range of pathogens, including fungi, bacteria, and viruses.
2. **Improved Crop Yield**: By reducing disease incidence, chitosan helps maintain healthier plants, leading to better yields.
3. **Eco-Friendly Alternative**: Chitosan reduces the need for synthetic pesticides, aligning with sustainable agricultural practices.
4. **Versatility**: It has shown effectiveness in crops like tomatoes, rice, wheat, and even ornamental plants, making it a valuable tool across diverse agricultural systems.

Regulatory Aspects

1. **EPA "Biochemical" Classification**: Chitosan is classified as a biochemical biopesticide by the Biopesticide and Pollution Prevention Division (BPPD) of the EPA's Office of Pesticide Programs (OPP). As such, the substance must:
    - be naturally occurring (or are synthetically derived equivalents to a naturally occurring chemical).
    - demonstrate a non-toxic mode of action against the target pest.
    - show minimal toxicity to humans and the environment

(Leahy et al., 2014).

2. **EPA-Registered Biopesticide Products:**
    - The first biopesticide product containing chitosan as an active ingredient was registered in 1986.
    - Currently, there are over 20 EPA-registered products containing chitosan as a biopesticide active ingredient.

3. **Addition of Chitosan to the "25 b List" in 2022**:
    - In 2022 a major milestone was reached with the inclusion of chitosan in the "25 b List" of Active Ingredients Eligible for Minimal Risk Pesticide Products under 40 CFR 152.25(f).
    - The updated list can be found at: 40 CFR 152.25(f) Table 1 Active Ingredients Permitted in Minimum Risk Pesticides Products Exempt from FIFRA
    - This marks the first addition of a new substance to the list in more than a decade.

4. **Chitin/Chitosan Products Listed by the Organic Materials Review Institute (OMRI) (products approved for use in organic production)**
    - **Crop Fertilizers and Soil Amendments:** Numerous OMRI Listed™ products containing **chitin** are "listed" by OMRI which permits their use as crop fertilizers and soil amendments used in organic production.
    - **Crop Pest, Weed, and Disease Control** Additionally, several pesticide products with **chitin** as an active ingredient are approved for applications for crop pest, weed, and disease control in organic production.
    - **Pesticide Adjuvant:** The OMRI Listed™ product, **Enhan-cer™ 1**, has **chitosan** as an active ingredient in a pesticide adjuvant formulation labeled for use on food crops. However, the EPA Office of Pesticide Programs (OPP) has not approved chitosan for use as an inert in adjuvant products applied to food crops. This was confirmed in a September 9, 2023 email from Dr. Evisabel Craig, Inert Ingredients Team Leader, OPP to Gordon Sargent (see attached copy).

      
      email 2023-09-27 EPA INERTS-Chitosar

      The **OMRI website** has access to their comprehensive databases of OMRI Listed™ products.

      Additional details on this issue are presented later in this petition.

5. **ChitoAg™ 80 Launch**
   - In 2023, **DESI** (recently acquired by **Cascade Columbia Distribution**), introduced **ChitoAg™ 80**, as a **"25 b" pesticide** with chitosan as its active ingredient.
   - ChitoAg™ 80 functions as a **Plant Growth Regulator**, aiding in the suppression of plant diseases and pathogens.
   - **Recognizing the safety of chitosan as a biopesticide**, EPA approved its inclusion on the list of minimum risk pesticides (40 CFR152.25(f), (also known as the **25(b) List**) **exempting it from FIFRA regulations**
   - **As a 25(b)-exempt pesticide product**, ChitoAg 80 is **exempt from FIFRA regulations**.
   - Customers of Cascade Columbia Distribution using ChitoAg 80 have expressed strong interest in its potential use on organic crops, a possibility that hinges on the NOSB classifying chitosan as a **nonsynthetic material** under NOP guidelines

CONTENTS AND FORMAT OF THIS PETITION

This petition is organized into two distinct sections:

- **Petition Part 1: Historical Review**
  Includes **Table 1: Chronological List of Regulatory Activities Related to Chitosan and Organic Use**
  Table 1 outlines the **timeline** of regulatory actions regarding chitosan and its role in organic production. A detailed discussion follows, expanding on each of the activities cited in **Table 1** which support the petitioner's argument that chitosan should be classified as "**nonsynthetic**" under the NOP guidelines.

- **Petition Part 2: Justification for Classification of Chitosan as "Nonsynthetic" under NOP Guidelines**
  This section provides additional evidence and analysis to support the claim that chitosan is indeed not "synthesized" as defined by NOP regulations – *meaning it is not artificially created*. Chitosan is *a* naturally occurring substance in nature.
  Moreover, it is an integral component of the **chitin biopolymer chain**, which is **already approved for use in organic production.**

*Note: Any highlighted or **bold** text has been added by the authors. Content enclosed within bordered sections has been directly excerpted from official documents.*

PETITION PART I: HISTORICAL REVIEW

TABLE 1: CHRONOLOGICAL LIST OF REGULATORY ACTIVITIES RELATED TO CHITOSAN AND ITS USE IN ORGANIC PRODUCTION REGARD TO THE NOP

Chronological List of Regulatory Activities Related to Chitosan and Organic Use rev 04

REF	YR	MO	ACTION	WHO-WHAT
A1	1994	Nov	Petition submitted to EPA to establish an exemption from the requirement of a tolerance for residues of Chitosan	Vanson L.P. submitted petition to EPA to establish an exemption from requirement of a tolerance
A2	1995	Apr	EPA establishes Tolerance Exemption for Chitosan used as a biological plant growth regulator	EPA established a tolerance exemption for chitosan as a pesticide active ingredient under 40 CFR 180.1072 used in the production of any raw agricultural commodity
B1	2004	Jan	Petition submitted petition to add chitosan to the NOP National List as an ADJUVANT	WSU submitted petition to add chitosan to NOP List as an adjuvant
B2	2004	Feb	Report issued: Technical Evaluation: Chitosan for Crops	ICF Consulting for USDA NOP re: WSU petition
na	2004	Aug	Australian govt issues report use of chitosan in organic systems with Aminogro from OPC	
B3	2005	Aug	NOSB VOTES to recommend adding Chitosan to the NOP List as an ADJUVANT	NOSB VOTES 13 YES 0 NO to add chitosan to the NOP List
B4	2006	Jul	PROPOSED RULE 71 FR 37854 - NOSB recommended adding chitosan to the National List for use of chitosan in organic production as an ADHESIVE ADJUVANT	Reccommendation not accepted. NOP consulted with EPA- chitosan is already approved for use in organic production under § 205.601(m) [EPA List 4 inerts are approved]
B5	2006	na	EPA completes reassessment of all inerts:	EPA: chitosan is not approved for food use as an inert
B6	2007	Dec	FINAL RULE Document ID: AMS-TM-07-0112-0001 issued by AMS clarifies use and prohibition of Chitosan in organic production	FINAL RULE: USDA/EPA conclude chitosan is already approved for use in organic production as an adjuvant under 205.601m as Chitosan is on EPA's List 4 *(however not approved for use on food)*
C1	2018	Oct	Petition submitted to EPA to add Chitosan to EPA's 25 b List of Minimal Risk Pesticides	Tidal Vision chitosan
C2	2019	Aug	Report issued: EPA Science Review	In Support of the addition of chitosan to the Minimum Risk Pestices
D1	2019	Oct	Petition submitted to NOSB to add Chitosan to NOP List for plant disease control	BotryZen/Biogro product: Armour-Zen 30% chitosan concentration)
E1	2020	Mar	Petition submitted_-to NOSB to add Chitosan to NOP List as a processing aid	Tidal Vision -Chitosan as a flocculent NOP *requested more information-never received - action dropped*
D2	2020	Jul	Report issued: Technical Evaluation Report-Nexight	Compiled by Nexight for the USDA NOP - review of BotryZen petition to add Synthetic Chitosan to the NOP List for plant disease control
D3	2021	Jun	USDA Crops Sub Committee Vote to List Chitosan for PLANT DISEASE CONTROL	4 to 4 = FAIL USDA Crops Sub Committee Vote to List Chitosan for plant disease control (re: Armour Zen 30%)
D4	2021	Oct	USDA/NOSB Vote to List Chitosan for PLANT DISEASE CONTROL	7 to 7 = FAIL USDA/NOSB Vote to List Chitosan for plant disease control (re: Armour Zen 30%)
C3	2023	Jan	EPA approves to add csn to 25(b) List of active ingredients permitted in exempted Minimum Risk Pesticide Products	EPA Final Rule Doc ID EPA-HQ-OPP-2019-0701

A1. 1994-Nov: Vanson LP submits petition to EPA to establish a tolerance exemption for Poly-D-glucosamine (chitosan)

A note of interest: the petition to establish a tolerance exemption for chitosan was submitted to the EPA in 1994 by the company, Vanson LP. Joel Van Ornum was head of the Vanson LP at the time the petition was submitted to EPA. He is now the chitosan product manager for Cascade Columbia Distribution.

A2. 1995-Apr: EPA establishes a tolerance exemption for Poly-D-glucosamine (chitosan)

> **40 CFR 180.1072 Poly-*D*-glucosamine (chitosan); exemption from the requirement of a tolerance.**
>
> *(a) An exemption from the requirement of a tolerance is established for residues of the biological plant growth regulator poly-D-glucosamine when used as a seed treatment in or on barley, beans, oats, peas, rice, and wheat.*
>
> *(b) An exemption from the requirement of a tolerance is established for residues of the biological plant growth regulator poly-D-glucosamine when used as a pesticide in the production any raw agricultural commodity.*

Consequently, chitosan, when used as an active ingredient in a biopesticide, is not required to establish a tolerance (Maximum Residue Limit) on the food crops it is used on.

REFERENCES B1, B2, B3, B4, B5

The activities listed in Section B references pertain to a 2004 petition submitted by Washington State University (WSU) to the USDA NOSB to add chitosan to the NOP List.

B1: 2004-Jan: Washington State University submits a petition to the NOSB to add chitosan to the NOP List as an adjuvant.

WSU proposed to add chitosan to the NOP List of materials approved for use by organic growers as an adjuvant. As an adhesive adjuvant, the substance would enhance the function of a pesticide (i.e. fungicide) acting as a sticker to plant surfaces. Specifically, the petition sought approval to test chitosan as a sticking agent for the fungicide copper sulfate pentahydrate in control of potato late blight. (Hadwiger, 2004).

B2: 2004-Feb: Poly-D-Glucosamine (Chitosan):Technical Evaluation Report Compiled by ICF Consulting for the USDA National Organic Program. (ICF Consulting, 2004)

The report provides a comprehensive analysis of chitosan and the WSU petition. Included are detailed descriptions of the chitosan's use, function, safety and regulatory status.

Below are selected comments from the report that are relevant to chitosan's classification as either synthetic or nonsynthetic under NOP guidelines.

Lines #14–19 [highlights and emphasis added]

Composition of the Substance ***Question #2: Is the petitioned substance formulated or manufactured by a process that chemically changes the substance extracted from naturally occurring plant, animal, or mineral sources? (From 7 U.S.C. § 6502 (21).) I***

*Chitosan (poly-D-glucosamine) is a polymer1 of glucosamine sugars, specifically glucosamine and N-acetylglucosamine (Hadwiger 2004). Its structure and composition are similar to both cellulose (i.e., the primary structural component of plant fiber) and chitin****. Like chitin, chitosan is found naturally in the shells of all crustaceans*** *and insects, as well as certain other organisms such as many fungi, algae, and yeast.* ***Chitosan is one of the most common polymers found in nature*** *(EPA 2003).*

Lines #98-114

Evaluation Question #2: Is the petitioned substance formulated or manufactured by a process that chemically changes the substance extracted from naturally occurring plant, animal, or mineral sources? (From 7 U.S.C. § 6502 (21).)

In the chitosan manufacturing process, the primary component of chitin (i.e., N-acetyl glucosamine) is chemically changed to the primary component of chitosan (glucosamine). ***Both the starting and ending chemicals in this process are natural components of both chitin and chitosan and are present in the natural animal byproduct source (e.g., crab shells)****.* ***The proportion of the two chemicals determines whether a mixture is chitin or chitosan.*** *According to the petitioner, chitosan is approximately 80 percent glucosamine and 20 percent N-acetyl glucosamine (Hadwiger 2004).* ***There are no precise definitions, however, of chitin and chitosan based on the percentage composition of glucosamine and N-acetyl glucosamine (Rabea et al. 2003).*** *In both chitin and chitosan, these two chemicals are linked together in chains, called polymers, of as many as 5,000 glucosamine and N-acetyl glucosamine molecules (i.e., monomers).*

B3: 2005-Aug: NOSB Review; Chitosan: Formal Recommendation by the NOSB to the NOP to List Chitosan as an Adjuvant.

The NOSB voted 13 yes, 0 no, and 1 absent to recommend: that chitosan be added to 205.601 for use in organic production with the following annotation, “as an adjuvant only.” (NOSB, 2005-Aug)

Excerpt from the minutes of the meeting:

FORMAL RECOMMENDATION BY THE
NATIONAL ORGANIC STANDARDS BOARD (NOSB)
TO THE NATIONAL ORGANIC PROGRAM (NOP)

Date: **August 17, 2005**

Subject: **Chitosan**

Chair: **Jim Riddle**
(sign)

Recommendation

The NOSB hereby recommends to the NOP the following:
Rulemaking Action: X
Guidance Statement:
Other:

Statement of the Recommendation (including Recount of Vote):

The NOSB recommends that chitosan be added to 205.601 for use in organic crop production with the following annotation, “as an adjuvant only.”

Board vote – August 16, 2005
13 yes, 0 no, 1 absent

The NOSB restricted the use of chitosan as an adjuvant only, due to the fact that chitosan could also be used as a plant defense booster.

B4: 2006-Jul: Proposed Rule to add chitosan to the NOP List: Federal Register Vol. 71, No. 127; Monday, July 3, 2006 (pp 37854-37857) (FR 2006 Jul 3)

FR-2006-07-03-11
Proposed Rule Csn-.

Proposed Rule online: Federal Register Vol. 71, No.127 July 3, 2006 Page 37854 Chitosan petition for use as an adjuvant

Excerpt from the Federal Register (highlight and emphasis added)

***SUMMARY**: This proposed rule would amend the U.S. Department of Agriculture's (USDA) National List of allowed and Prohibited Substances (National List) regulations to reflect recommendations submitted to the Secretary of Agriculture (Secretary) by the National Organic Standards Board (NOSB) on August 17, 2005. Consistent with the recommendations from the NOSB, this proposed rule would add two substances [chitosan and sucrose octanoate esters], along with any restrictive annotations, to the National List.*

Not Accepted as Chitosan is already permitted under §205.601(m) of the National List regulations

Recommendation Not Accepted

… Chitosan was petitioned for use in organic crop production as an adhesive adjuvant to be used with fungicides approved for use under the NOP regulations.

…In the petition for the use of chitosan, as an adjuvant, the proposed rate of application is 0.011 pounds of chitosan per 20 gallons of water; it is adequate to apply on 1 acre.

... In assessing risks to human health, the EPA has concluded that no risks to humans are expected when products containing chitosan are used according to label directions. In assessing risks to the environment, the EPA determined that no risks to the environment are expected because chitosan has not shown toxicity in mammals, it is abundant in nature, and it is used in tiny amounts.

At its August 17, 2005, meeting in Washington, DC, the NOSB recommended adding chitosan to the National List for use in organic crop production as an insecticide, with the restriction that it only be used as an adjuvant. In this open meeting, the NOSB evaluated chitosan against the evaluation criteria of 7 U.S.C. 6517 and 6518 of the OFPA, received public comment, and concluded that chitosan is consistent with the OFPA evaluation criteria.

… as an adjuvant, the application rate of chitosan would be approximately 0.011 pounds per 20 gallons of water. At such a rate, chitosan would be unlikely to act as a defense booster and plant growth enhancer. It is also unlikely that it would create unacceptable changes in oil temperature, water availability, pH levels, nutrient availability, or salt concentration. The NOP consulted with the EPA concerning the NOSB's recommendation to include chitosan on the National List. The EPA informed the NOP that for the petitioned use of chitosan, as an adjuvant, the substance would not be considered an active ingredient, but an inert ingredient. The EPA further stated that, in addition to chitosan being registered as an active ingredient, it is also approved as an EPA List 4B inert ingredient. ***The NOP regulations, at § 205.601(m), permits the use of EPA List 4 inert ingredients*** *with nonsynthetic substances or synthetic substances approved for use under the NOP regulations as an active pesticide ingredient. As a result,* ***the NOP will not propose to specifically add chitosan to the National List as an adjuvant; it is already permitted for use at § 205.601(m) of the National List regulations***]

As explained below, the conclusion that chitosan is "already permitted for use at § 205.601(m) of the National List regulations", is not completely valid.

B5: 2006: EPA Completes the Reassessment of All Inerts and Discontinues the use and Maintenance of List 4 referenced in the NOP under 205.601 (m)(1).

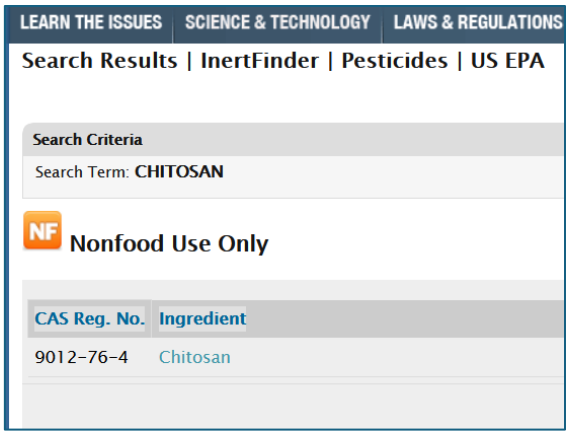
In 2006, the same year that the proposed rule referenced in B4 above was published in the Federal Register, the EPA completed the reassessment of all the listed inerts and discontinued the use/maintenance of all of the EPA Inerts Lists including List 4 which included chitosan.

Under EPA's new inerts classification, chitosan is approved for use as an adjuvant but restricted to **nonfood use** only. As a result, the conclusion reached by EPA and USDA in their Proposed Rule of July 3, 2006, **is not entirely valid. Chitosan is not approved** for use as an adjuvant in pesticide formulations **applied to food**. Consequently, even in its use as an adjuvant, chitosan is not approved for use on food crops in organic production.

This is where the USDA/EPA apparently failed to recognize the recent change of chitosan's inert status. In the same year as this Federal Register Notice, EPA classified the use of chitosan as an inert approved for **Nonfood Use Only** consequently, making such a product of no use in organic food production.

From EPA's Inerts Database:

EPA Inert Finder Database-searchable



B6: 2007-Dec: AMS Final Rule: Chitosan can be used as an inert. (FR 2007-Dec 10)

The Agricultural Marketing Service posted a final rule in response to NOSB's August 2005 recommendations. The final rule states that Chitosan was not added to the NOP List as it is already permitted under §205.601(m) of the NOP Regulations. This specific regulation includes all the inerts on EPA List 4. Again, as detailed previously, there was no recognition that EPA had reassessed the inerts ultimately approving Chitosan for nonfood use only.

2007-Dec 10 FR 72 No 236 AMS Docket No AMS-TM-07-0112

Excerpt from AMS Docket No. AMS-TM-07-0112 (highlight and emphasis added)

Summary

This final rule amends the U.S. Department of Agriculture's (USDA) National List of Allowed and Prohibited Substances (National List) regulations to reflect recommendations submitted to the Secretary of Agriculture (Secretary) by the National Organic Standards Board (NOSB) on August 17, 2005. Consistent with the recommendations from the NOSB, this final rule adds one substance, along with any restrictive annotations, to two sections of the National List. This final rule also clarifies the use and prohibition of chitosan.

***DATES**: This rule becomes effective December 11, 2007.*

Chitosan was petitioned for use in organic crop production as an adhesive "adjuvant" to be used with fungicides approved for use under the NOP regulations. The NOSB recommended adding chitosan to the National List for use in organic crop production as an "insecticide," with the restriction that it only be used as an "adjuvant." The EPA informed the NOP that data does not reveal chitosan having insecticidal properties. **[see NOTE below]** *Because the NOSB recommended the use of chitosan as an adjuvant, the recommendation restricts the use of the substance to the capacity of an inert ingredient. AMS, in consultation with EPA, has determined that chitosan, when used as an "adjuvant" (not demonstrating any pesticidal activity), is already allowed under the existing inert ingredient provisions of § 205.601(m) of the NOP regulations. However, chitosan, when used in combination with a fungicide, cannot be considered an inert or adjuvant, because chitosan has fungicidal properties and is considered an active ingredient in such cases. Accordingly, unless specifically added to § 205.601 of the National List as an active ingredient, chitosan cannot be used with a fungicide.*

Therefore, AMS has decided to refer the chitosan recommendation back to the NOSB so that it can reconsider the intended use of the substance and its inclusion on the National List (i.e., should it be considered a plant disease control; and should it be included on the National List as an approved active ingredient?). In the meantime, chitosan, under the inert ingredient provisions of § 205.601(m) of the NOP regulations, can be used as an "adjuvant" (not demonstrating any pesticidal activity) in combination with approved active ingredients on the National List, provided the approved active ingredient is not a registered fungicide. Chitosan, when used in combination with a fungicide, is an active ingredient and remains a prohibited substance that shall not be used in organic agriculture. Further, chitosan remains prohibited for use as a plant defense booster, a plant growth enhancer, and as an active ingredient in any other capacity.

NOTE: There has been ongoing confusion regarding references to chitosan's use as an insecticide. In WSU's 2004 petition to the NOSB, the use of chitosan was exclusively specified as an adhesive adjuvant not an insecticide. The misunderstanding likely occurred during the August 17, 2005 meeting of the NOSB when both chitosan and sucrose octanoate esters (SOE) were

evaluated for addition to the NOP List. SOE was petitioned for use in organic crop production as an insecticide/miticide which likely contributed to the misinterpretation.

§ 205.601 Synthetic substances allowed for use in organic crop production.

> *In accordance with restrictions specified in this section, the following synthetic substances may be used in organic crop production: Provided, That, use of such substances do not contribute to contamination of crops, soil, or water. Substances allowed by this section, except disinfectants and sanitizers in paragraph (a) and those substances in paragraphs (c), (j), (k), (l), and (o) of this section, may only be used when the provisions set forth in § 205.206(a) through (d) prove insufficient to prevent or control the target pest.*

205.601 (m)(1) allows synthetic inert ingredients for use in organic crop production if the substance is listed in EPA's List 4:

> *(m) As synthetic inert ingredients as classified by the Environmental Protection Agency (EPA), for use with nonsynthetic substances or synthetic substances listed in this section and used as an active pesticide ingredient in accordance with any limitations on the use of such substances.*
> *(1) EPA List 4—Inerts of Minimal Concern.*

Again, while the AMS stated chitosan can be used as an adjuvant (not demonstrating any pesticidal activity), there was no reference made to the fact that chitosan when used as an adjuvant, **is not approved for food use** by the EPA and therefore cannot be used on organic food crops.

C1: EPA 2018-Oct: Petition submitted to EPA by Tidal Vision proposal to add Chitosan to the list of minimum risk pesticides (MRPs)

Tidal Vision submitted a 48-page petition to the EPA to add chitosan to the list of active ingredients eligible for use in minimum risk pesticide products exempt from the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

Included were 27 letters of support from state and federal officials, university professors, seafood companies, trade associations as well as private companies.

C2: EPA 2019-Aug: Science Review in Support of the Addition of Chitosan to the list of minimum risk pesticides (MRPs) (Jones, EPA, 2019-Aug)

Excerpt from the Science Review document (highlight added):

> *Chitosan (Poly-D-Glucosamine; Figure 1) is a de-acetylated form of the naturally-occurring polysaccharide, Chitin (Poly-N-Acetyl-D-Glucosamine; Figure 2). Chitosan also occurs naturally and is formed by chitin deacetylases that have been identified in marine bacteria, fungi, and insects (review by Zhao et al., 2010). Chitosan is a heterogeneous substance and, depending on the degree and duration of deacetylation during the manufacturing process, can be defined as chitin that has been sufficiently deacetylated to form soluble amine salts (MRID 473622 and references therein).*

C3: EPA 2022-Nov: FINAL RULE: EPA adds chitosan to the List of Minimum Risk Pesticides.

The EPA added chitosan to the list of active ingredients eligible for use in minimum risk pesticide products exempt from registration and other requirements of the FIFRA.

Referenced in Document ID EPA-HQ-OPP-2019-0701-0026 posted in the Federal Register Vol. 87, No. 215/November 8,2022:

FR EPA-HQ-OPP-2019-0

D1: 2019-Aug: Botry-Zen submits petition to NOSB to add chitosan [synthetic] to the National List [for plant disease control]

In 2019, the NOSB received a second petition to add chitosan to the National List. The product was ARMOUR-Zen® 30, a liquid product containing 30% chitosan for plant disease control. The assumption in the petition was that chitosan is a synthetic material.

The petition did not clearly indicate a specific use for which chitosan is essential for organic production. Instead, it suggested that chitosan is an alternative to currently available materials and organic management practices, offering benefits related to toxicity and environmental safety.

This comment would be irrelevant if chitosan was classified as a nonsynthetic material. As such, chitosan would not have to be added to the NOP List as it would meet the requirements of being nonsynthetic and organic.

D2: 2020-Jul: TECHNICAL EVALUATION REPORT COMPILED BY NEXIGHT FOR THE USDA NATIONAL ORGANIC PROGRAM (Nexight, 2020-Jul)

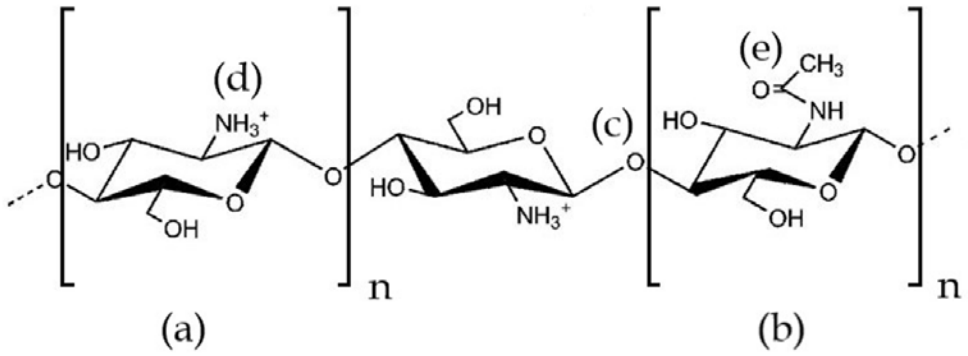
Characterization of Petitioned Substance

Composition of the Substance:

…There is no degree of deacetylation that officially defines when chitin becomes chitosan, but the lower limit described in literature is 40–60 percent (Hussain, Iman and Maji 2014). Typically chitin contains 85–95 percent N-acetylglucosamine and 5-15 percent glucosamine (Pillai, Paul and Sharma 2009)… Commercial chitosan usually contains at least 65 percent glucosamine and less than 35 percent N-acetylglucosamine (Pillai, Paul and Sharma 2009)…

The degree of deacetylation can vary, and so any given quantity of chitin or chitosan will typically contain both types of monomers. Below a pH of approximately 6, chitosan becomes positively charged by acquiring hydrogen ions…

Figure 1: Structure of chitosan under acidic conditions*



*Glucosamine monomers (a) are bonded to additional glucosamine monomers or N-acetylglucosamine monomers (b) via 1-4 glycosidic linkages (c). Glucosamine monomers possess an amine group (d), ionized due to low pH. N-acetylglucosamine monomers possess the acetamide (amino-acetyl) group (e). Drawing adapted from (Nilsen-Nygaard, et al. 2015).

In the above Figure 1 the structure of chitosan is illustrated.

(a) represents the **chitosan** monomer

(b) represents the **chitin** monomer.

The difference between “chitosan” and “chitin” is the number of acetamide (amino-acetyl) (e) groups.

D3: 2021-06-01 USDA Crops Subcommittee 4 to 4 vote to add chitosan to the NOP List (re: ARMOUR-Zen 30%)

https://www.ams.usda.gov/rules-regulations/organic/nosb/meetings

From the meeting minutes, the lead noted that there are over 200 compounds already listed in the OMRI. Other than that, there does not appear to be a major reason why chitosan should not be added to the NOP List.

NOSB voted unanimously to classify chitosan as a **synthetic for plant disease control** but the vote to add chitosan to the NOP List was a 4 to 4 tie = motion fails.

D4: 2021-10-20 Formal Recommendation from NOSB to NOP. 7 to 7 vote to add chitosan to the NOP List (re: ARMOUR-Zen 30%)

https://www.ams.usda.gov/rules-regulations/organic/nosb/meetings

NOSB voted unanimously to classify chitosan as a **synthetic for plant disease control** but the vote to add chitosan to the NOP List was a 7 to 7 tie (need 10 pass). The petition was submitted by Botry-Zen regarding their EPA BPPD registered product, Armour Zen (30% chitosan).

Excerpts from the meeting minutes:

- **Greenwood/chair of the Crop Subcommittee***: In going through the written comments, there's tremendous support for it being added to the National List, as people say another tool in the toolbox.*
- *I think where we had issues with it as a subcommittee was the fact that, do we really want to add another synthetic to the National List because there are alternatives for using chitosan.*
- **R. Epstein***: On chitosan, I do have written comments on it. We support adding it to the list.*
- **Greenwood***: I like it because it's a way to get something out of a commercial waste stream... there is a tremendous support for it being added to the National List, as people say another tool in the toolbox.*
- *…I think where we had issues with it as a subcommittee was the fact that, do we really want to add another synthetic to the National List because there are alternatives for using chitosan.*
- *I like the fact that it comes out of a waste stream, but back to Wood's comment, and mainly we don't need to look at this, but we are talking about global warming. It takes energy and some fairly toxic chemicals, sodium hydroxide and things, to produce it. So it may be benign in the in the field but I don't if the manufacturing itself if we create a market is going to have a bigger carbon footprint along the way.*
- ***Caldwell** [a farmer]: So, to me, I think that this is the kind of…if were going to be allowing synthetics for use in pest management, which I think we're are, this is really kind of the direction that we want to go in.*
- ***Bruce:** I do support tools in the tool boxes for farmers, however, I think there needs to be a lot more clarity with this tool.*
- ***Greenwood**:*
- *If you already have this crab shell powder, is there an advantage to adding another thing, if you already have something that's available?*
- *I have heard that, you know, the northern, like Northeastern area would produce it and actually produce good chitosan because they have thicker shells, so I think that actually might be a production area for that.*

Petition's author comments about the meeting minutes:

1. The comment regarding the use of "fairly toxic chemicals, sodium hydroxide…" seems peculiar. Sodium hydroxide is one of the most widely utilized chemicals in the world. It is produced from salt water. In the chitosan production process, the chemical is not incorporated in the chitosan itself as it is separated from the chitosan flakes after the deacetylation process. The spent sodium hydroxide rinse water is reused in the deproteinization of the crustacea shell. Any excess solution is neutralized with the acid rinse from the demineralization step resulting in the formation of sodium chloride salt.

2. There was a discussion about the efficacy of chitosan. However, it was not apparent that any scientific data/information was reviewed.
3. Throughout the meeting minutes, it seems it was more or less an informal discussion. There were a lot of "I don't knows", "I am not sure" and a number of unanswered questions.
4. It is important to note that there were three votes over the years regarding chitosan and organic status:
   4.1. 2005-Aug: NOSB votes 12 YES-0 NO recommending chitosan to be added to the NOP List (as an adjuvant).
   4.2. 2021-Jun: Crops Subcommittee votes 4 YES-4 NO to add chitosan to the NOP List (as a plant growth regulator/synthetic material).
   4.3. 2021-Oct: NOSB votes 7 YES-7 NO to add chitosan to the NOP List (as a plant growth regulator/synthetic material).

While the two latter tie votes failed to approve the motion, they, along with the NOSB unanimous vote in 2005, demonstrate there is significant interest in making chitosan available to organic growers.

If chitosan was classified as "nonsynthetic", there would be no need to go through the challenging petition formal review/approval process to amend the NOP List (=amending Federal Law).

E1: 2020-Mar Tidal Vision submits petition to add Chitosan as a flocculating agent to capture material that may be used in the production of fertilizer.

A 13-page petition with 151 pages of appendices. Tidal Vision makes a good case for Listing chitosan as a flocculating agent because there are currently no flocculating agents allowed on the USDA NOP's List.

> *Producers of organic processed foods and organic crop producers are missing the opportunity to retain a high-value fertilizer input in the organic value chain because there is currently no flocculating agent allowed on the USDA NOP's National List of Allowed and Prohibited Substances.*

(Tidal Vision Petition 2020-Mar 11)

However, their proposal was made with the assumption that their chitosan was classified as a "synthetic" material thereby significantly increased scrutiny by the NOSB as getting a synthetic substance added to the NOP List requires amending the NOP regulations.

The USDA Crops Subcommittee requested additional information about the manufacturing process of Tidal Vision's chitosan. Because there was no response from Tidal Vision, the action was abandoned.

PETITION PART 2: JUSTIFICATION FOR CLASSIFICATION OF CHITOSAN AS "NONSYNTHETIC" UNDER NOP GUIDELINES.

INTRODUCTION

Following are several sources of information referenced that support the argument that chitosan is "nonsynthetic" with regard to the NOP guidelines. Specifically, chitin and chitosan have the same polysaccharide backbone. The basic difference is the number of acetyl groups distributed along the chain,

1. Definitions:

National Organic Program (NOP)

> **7 CFR 205.2**
> ***Nonsynthetic (natural).***
> *A substance that is derived from mineral, plant, or animal matter and does not undergo a synthetic process as defined in section 6502(21) of the Act (7 U.S.C. 6502(21)). For the purposes of this part, nonsynthetic is used as a synonym for natural as the term is used in the Act.*
>
> ***7 USC 6502(21)***
> ***Synthetic****.*
> *A substance that is formulated or manufactured by a chemical process or by a process that chemically changes a substance extracted from naturally occurring plant, animal, or mineral sources, except that such term shall not apply to substances created by naturally occurring biological processes. (Pub. L. 101–624, title XXI, § 2103)*

The chitin polymer extracted from crustacea shells is not being chemically changed to make a new chemical substance. Chitosan occurs naturally in the crustacea shells. Additionally, the chitin polymer backbone structure is still in place after the deacetylation process – chitosan just has fewer acetyl groups than chitin.

The deacetylation process does occur naturally. However, the commercial production process speeds up the process and controls the outcome.

American Chemical Society (ACS)

> ***American Chemical Society (ACS),*** *In the context of chemistry,* ***synthesis*** *refers to the production of chemical compounds by reacting simpler materials****.*** *(Committee on Nomenclature, Terminology, and Symbols)*

In the world of organic chemistry, synthesis of new compounds is common practice. For example, a particular organic chemical is combined with another organic chemical to form a **new compound**. This is typically used in the process of developing new pharmaceuticals. New

pharmaceutical compounds are formed by combining different molecules to form bigger molecules that have particular properties.

2. NOP 5033-1: Decision Tree for Classification of Materials as Synthetic or Nonsynthetic (NOP 5033-1; 2016-Dec)

(see Appendix 1 for a copy of the NOP 5033-1 document)

Step 1. ls the substance manufactured, produced, or extracted from a natural source?

- **YES,** The chitin used to make chitosan under consideration in this petition is commercially produced from chitin that is **extracted** from crustacea shells – crab and shrimp shells which are an abundant byproduct of the food-processing industry.

Step 2b. At the end of the extraction process, does the substance meet all the criteria described at 4.6 of NOP 5033?

- **Not applicable.** 4.6 of NOP 5033 refers to the "Extraction of **Nonorganic** Materials".
- Chitin is organic and nonsynthetic and can therefore be used in organic production.
- Chitosan is a derivative of chitin. Both substances have the same polyglucosamine backbone. The difference is the number of acetyl groups along the chain.

Step 2. Has the substance undergone a chemical change so that it is chemically or structurally different than how it naturally occurs in the source material?

- **NO** The chitosan commercially produced using chitin, is the same the as the chitosan polymer naturally occurring in the shells of crustacea.
- The chitosan polymer commercially produced has the same polyglucosamine backbone as the chitin extracted from the crustacea shell remains. The difference is in the percentage of acetyl groups along the chain.
- The chitin polymer is not transformed into a different substance.

The definition of a chemical reaction/chemical change as found in the NOSB guidance for the review of synthetic and non-synthetic substances (August 17, 2005; Chair: Jim Riddle)
NOP Rec Guidance Review of Synthetic and Nonsynthetic substances.pdf (usda.gov)

4) Chemical reaction (chemical change) shall be understood to mean: A chemical reaction has occurred when one or more atoms are removed or added to a molecule. Examples of reactions include: 1) Addition or combination reaction: Two substances combine to form one: 2Na+Cl2----2NaCl; 2) Decomposition reactions: One compound breaks into two or more compounds or elements. CaCO3-----CaO + CO2; 3) Displacement reactions: Substances exchange parts. Two examples are hydrolysis (a hydroxyl ion from water is added to a substance and a hydrogen ion from water is added to a second molecule) and acid-base reactions (proton donation or acceptance) and 4) Protein configuration changes as a result of a physical association of an added substance.

(EPA Fact Sheet-Biopesticide Active Ingredient:Chitosan)

*The active ingredient is found in the shells of crustaceans..,Chitosan (poly-D-glucosamine) is one of the most common polymers found in nature. …**Like chitin, chitosan is found naturally in the shells of all crustaceans** …Commercially, chitosan is prepared from chitin, which is isolated from the shells of crustaceans after the edible parts have been removed.*

(ICF Consulting, 2004)

*Chitosan (poly-D-glucosamine) is a polymer1 of glucosamine sugars, specifically glucosamine and N-acetylglucosamine (Hadwiger 2004). Its structure and composition are similar to both cellulose (i.e., the primary structural component of plant fiber) and chitin**. Like chitin, chitosan is found naturally in the shells of all crustaceans** ….*

***Chitosan is one of the most common polymers found in nature**….). **Both the starting and ending chemicals in this process are natural components of both chitin and chitosan and are present in the natural animal byproduct source (e.g., crab shells)**. **The proportion of the two chemicals determines whether a mixture is chitin or chitosan.** According to the petitioner, chitosan is approximately 80 percent glucosamine and 20 percent N-acetyl glucosamine (Hadwiger 2004). **There are no precise definitions, however, of chitin and chitosan based on the percentage composition of glucosamine and N-acetyl glucosamine (Rabea et al. 2003).** In both chitin and chitosan, these two chemicals are linked together in chains, called polymers, of as many as 5,000 glucosamine and N-acetyl glucosamine molecules (i.e., monomers).*

The citations and comments provided above lead to a conclusion following the *NOP5033-2 Decision Tree for Classification of Materials as Synthetic or Nonsynthetic,* the chitosan substance referenced in this petition (commercially produced from crab and shrimp shells), is **nonsynthetic.**

3. Ash and charcoal: “nonsynthetic”?

Ash and charcoal are commonly permitted in organic farming due to their natural origin and beneficial properties, such as improving soil health and acting as soil amendments. Similarly, chitosan, derived from crustacean shells, has natural agricultural benefits, including its role as a plant growth regulator and disease suppressant.

The comparison highlights a key point: both ash and charcoal undergo processing but remain classified as nonsynthetic under organic regulations. Chitosan follows a similar path—it is extracted and refined, yet its fundamental composition remains natural. This raises an important discussion on **how processing affects classification** and whether chitosan should also be recognized as nonsynthetic under National Organic Program (NOP) guidelines.

From the website of the **Organic Materials Review Institute (OMRI):**

Home » Charcoal, Ash and Biochar as Soil Amendments
Charcoal, Ash and Biochar as Soil Amendments

Are charcoal, ash or biochar allowed as soil amendments in USDA organic farming?

By Brian Baker

The terms "charcoal," "ash" and "biochar" are often used interchangeably, especially within the context of material review. The answer to whether these materials are allowed in USDA organic production depends on how they're made. If these materials are produced by burning plants or animal materials (e.g., bones), they are permitted as a soil amendment on organic farms.

https://www.omri.org/omri-search?page=1&query=ash%20charcoal&exactMatch=false

Ash and charcoal, derived from the burning of plant or animal materials, are permitted as soil amendments in organic farming and, despite not being explicitly listed on the National List, are generally assumed to be "nonsynthetic" and naturally occurring under NOP standards.

However, unlike chitosan, ash and charcoal undergo a process that **fundamentally alters their molecular structure**. Initially composed of plant or animal matter with numerous hydrogen, oxygen, and carbon atoms, they are subjected to extreme temperatures that strip away easily removable molecules, **completely transforming** their original composition. For instance, wood undergoes combustion, producing ashes that bear **no chemical or physical resemblance** to the starting material. This transformation results from significant chemical reactions that entirely reshape the molecular structure.

In contrast, **the process of converting chitin to chitosan does not alter the polymer backbone** of the material—only the number of acetyl groups along the chain changes. The fundamental structure remains intact before and after processing. To produce chitosan, the **only modification** to the chitin molecule is the **removal of some (but not all) acetyl groups**, while the polymer itself remains chemically unchanged. Notably, chitosan is a **naturally occurring substance** in nature, further reinforcing its alignment with organic standards.

Because ash and charcoal (produced by burning plant or animal materials) are permitted for use as soil amendments used in organic production and they are not specifically listed on the National List, it is assumed that these materials are considered to be "nonsynthetic" and naturally occurring by NOP standards.

However, unlike chitosan, ash and charcoal go through a process that completely changes the starting molecules. They start out as plant or animal matter with many hydrogen, oxygen, and carbon atoms attached, However, the matter is subjected to extreme temperatures which strips off the easy to remove molecules thus completely changing the structure of the starting material. For example, the process takes wood through the burning process resulting in ashes. There is no resemblance chemically or physically to the starting material. Significant chemical reactions have occurred which completely changed the molecular structure of the starting matter.

The process of converting chitin to chitosan is fundamentally different from the burning of plant and animal matter. While combustion drastically alters the molecular structure of organic materials – eliminating hydrogen, oxygen, and carbon atoms and leaving behind ash with no resemblance to the original substance – the transformation of chitin into chitosan preserves the integrity of the polymer backbone.

In chitosan production, the **only modification** is the **removal of some (but not all) acetyl groups** along the polymer chain. This subtle adjustment does not chemically alter the polymer

itself, which remains intact and as can be found naturally occurring in nature. Unlike burned materials that undergo a complete molecular restructuring, chitosan maintains its original framework, reinforcing its classification as a **nonsynthetic** substance.

4. EPA Biopesticide Fact Sheet: Chitosan; Poly-D-glucosamine (128930)

Chitosan is used primarily as a plant growth enhancer, and as a substance that boosts the ability of plants to defend against fungal infections. It is approved for use outdoors and indoors on many plants grown commercially and by consumers. The active ingredient [chitosan] is found in the shells of crustaceans, such as lobsters, crabs, and shrimp, and in certain other organisms. Given its low potential for toxicity and its abundance in the natural environment, chitosan is not expected to harm people, pets, wildlife, or the environment when used according to label directions.

(EPA Biopesticide Active Ingredient Fact Sheet-Chitosan (128930)

5. Chitin and Chitosan Summary Document Registration Review: Initial Docket September 2007 (EPA 2007)

Because Chitosan is derived from Chitin, and because both compounds share basic physical characteristics, they have been combined into a single case - Registration Review Case 6063. As a matter of general similarity, Chitin and Chitosan are both naturally occurring polymers that are ubiquitous throughout nature. Structurally, they are related to cellulose and each consists of long chains of glucose molecules. Relationally, Chitosan is a deacetylated product of Chitin, whose chains of glucose are slightly modified.

6. Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials; (National Library of Medicine; National Center for Biotechnology Information; 2016-Mar)

Chitin is the most abundant aminopolysaccharide polymer occurring in nature, and is the building material that gives strength to the exoskeletons of crustaceans, insects, and the cell walls of fungi. Through enzymatic or chemical deacetylation, chitin can be converted to its most well-known derivative, chitosan. The main natural sources of chitin are shrimp and crab shells, which are an abundant byproduct of the food-processing industry, that provides large quantities of this biopolymer to be used in biomedical applications.

…Chitosan is the deacetylated form of chitin (that can have varying degrees of deacetylation)…In organisms like fungi and invertebrates, there have been reported varying degrees of deacetylation, giving a continuum of structure between chitin (fully acetylated) and chitosan (fully deacetylated)…The distinction between chitin and chitosan with different degrees of deactylation is not strict… In chitin, the acetylated units prevail and the degree acetylation is typically 0.90, while chitosan is a fully or partially N-deacetylated derivative with a typical degree of deacetylation of more than 0.65.

The publication can be found online:
Link: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5094803/

CONCLUSIONS

1.0 Misinterpretation of Chitosan Approval for Organic Farming

For years, the EPA and USDA National Organic Program (NOP) have maintained that chitosan is approved for use in organic farming as an adjuvant or inert. However, this claim is inaccurate.

1.1 **Incorrect Determination by USDA AMS and EPA**
The determination by the USDA Agricultural Marketing Service (AMS) and EPA, which claims that chitosan qualifies for use in organic production due to its inclusion on EPA's Inerts List 4, is factually inaccurate.

1.2 **Chitosan is not Approved by EPA for Food Use**
Chitosan, when used as an adjuvant or inert in a pesticide formulation, is not approved for "food use" by the EPA. Its approval is restricted to "nonfood use" applications. Consequently, it cannot be utilized as an adjuvant or inert used in pesticides/adjuvant products for use on food in neither organic nor nonorganic food crops.

1.3 **Impact on NOSB Petitions**
This misinterpretation has influenced the outcomes of chitosan-related petitions submitted to the National Organic Standards Board (NOSB).

1.4 **Implications of Recognizing Chitosan as "Nonsynthetic"**
If the NOSB were to recognize chitosan as a "nonsynthetic" material, as proposed in this petition, there would no need to seek its inclusion on the National Organic Program List. Once classified as organic and nonsynthetic, it would then meet the criteria required for approval for use in organic production.

2.0 Chitosan Should Be Recognized as "Nonsynthetic" Under NOP Guidelines and Regulations

2.1 **Chitosan is Not a Synthesized Substance**
The chitosan material discussed in this petition is not "synthesized" – i.e. two or more chemicals are not reacted to create a new substance.
Furthermore, the data, information and definitions provided by various government agencies including USDA NOSB, USDA AMS, EPA, and academic researchers – support the classification of chitosan as a nonsynthetic, organic substance. Consequently, chitosan should thereby not only be approved for use as adjuvant/inert, but also as a biopesticide active ingredient.

2.2 **Chitosan is Naturally Occurring**
Chitosan occurs naturally including the crustacea shells utilized in the commercial production of chitosan. The commercial process for mereaccelerates and controls this natural process.

2.3 **Comparison to Ash Approved for use in Organic production**
Ash, derived from burning plants or animal materials is allowed as a soil amendment in organic farming. This process alters both the physical and chemical properties of the resulting ash material, yet it remains approved for use in organic production.

2.4 **Chitin is approved for use in organic production**

Chitin is currently approved for use in organic farming as it is considered nonsynthetic. The only difference between the chitin and chitosan polymer backbone is the percentage of acetyl groups along the chain. The polymer chain itself remains unchanged.

3.0 The Benefits of Allowing Chitosan for use in Organic Production

3.1 The commercial production of chitosan not only repurpose shell that otherwise goes to waste but also provides a natural substance that can be used in place of; or to enhance the effect of conventional chemicals used in agriculture; offering benefits related to toxicity and environmental safety.

3.2 In the October 2021 meeting of the NOSB, there was significant interest in making chitosan available for use in organic production. Not only did it narrowly fail approval with a 7 to 7 vote, but also there were many comments stated in the meeting minutes supporting the approval of chitosan. For example the chair of the Crop Subcommittee, Rick Greenwood, stated, "*In going through the written comments, there's tremendous support for it being added to the National List, as people say another tool in the tool box.*"

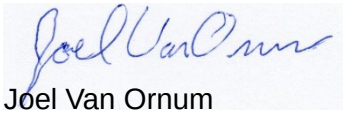
Of course, this review was conducted with the assumption that the chitosan material under consideration was "synthetic" and therefore required its approval/addition to the NOP List.

4.0 Final Comment

I appreciate the Board's commitment to maintaining the integrity of organic certification and respectfully urge consideration of Chitosan's reassessment to be classified as a **nonsynthetic material**. I would be grateful for the opportunity to provide further information and/or participate in discussions regarding this matter.

Thank you for your time and thoughtful consideration.

Sincerely,



Joel Van Ornum
Cascade Columbia Distribution

APPENDIX 1 Manufacturing Information

Item B.2 – Petitioner

Joel Van Ornum
Cascade Columbia Distribution*
6900 Fox Avenue South
Seattle, WA 98108
Website: https://cascadecolumbia.com/
Phone: 206-282-6334
* NOTE: *The original petitioner was a DESI (Dungeness Environmental Services, Inc.). In December 2024, DESI was wholly acquired by Cascade Columbia Distribution.*

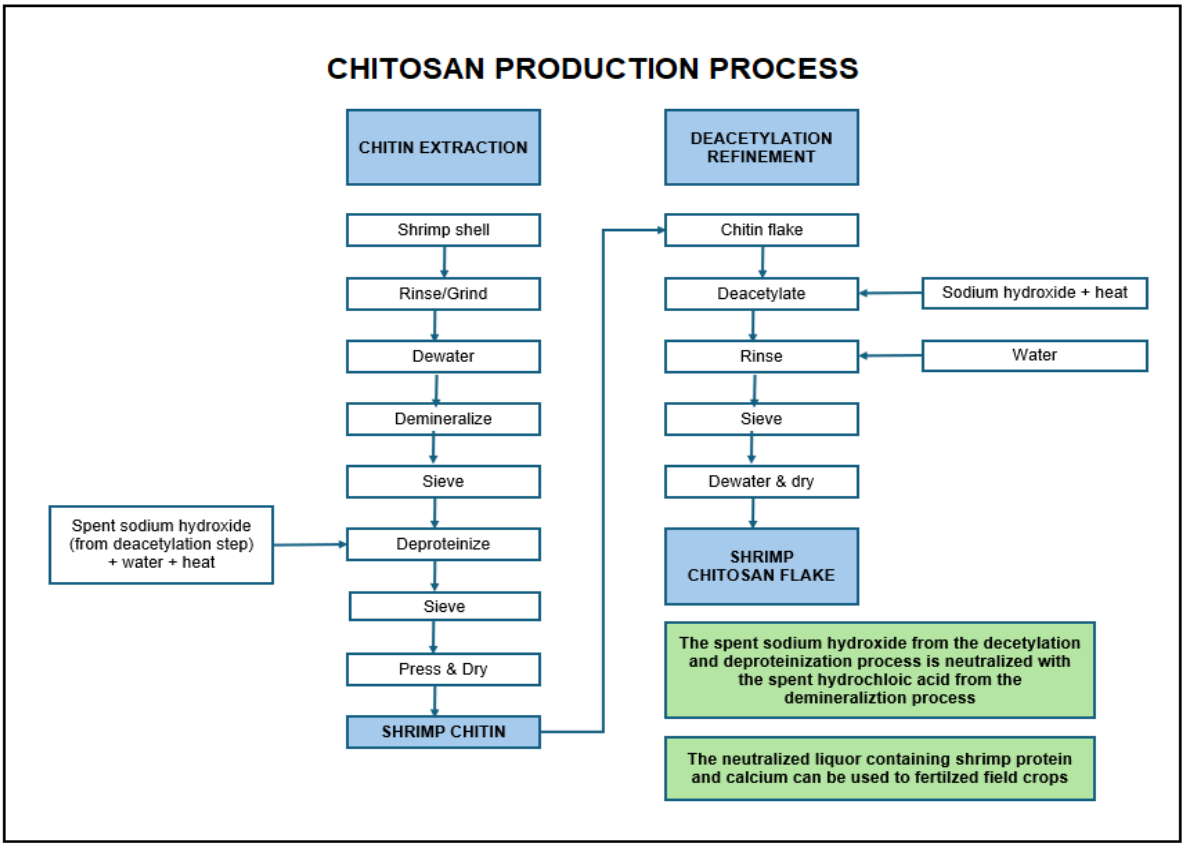
Item B.5 - Manufacturing Information and Process

The chitosan material referenced in the petition is produced by:

Panvo Organics Pvt. Ltd.
3rd Floor, Shakti Towers III
786 Anna Salai
Chennai – 600002 INDIA

Chitosan Production Process Flowchart

Panvo Organic's chitosan production process uses shrimp shell collected from area seafood processors. The process reflects the typical production process steps used by most commercial producers of chitosan that use crab, shrimp, and/or lobster shell as the raw material sources.



The species of shrimp processed by seafood processors that generate the shell used by Panvo Organic are:

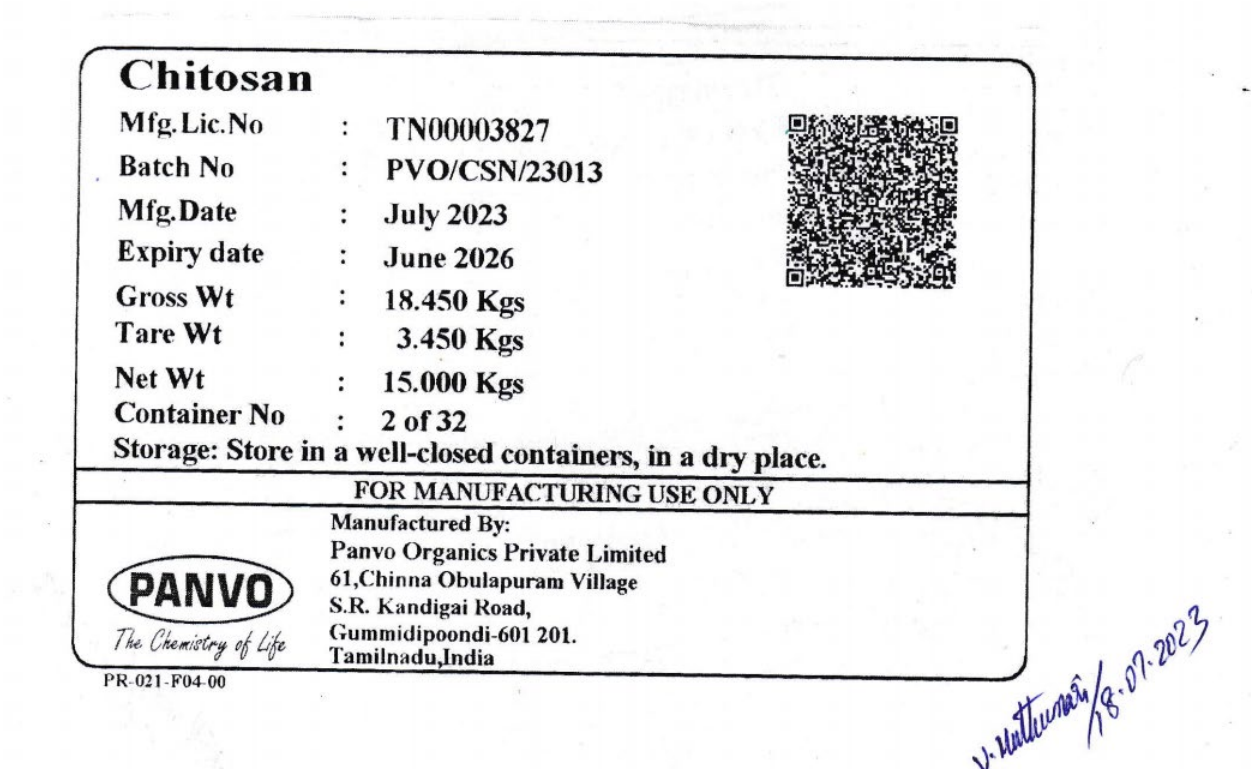
Tiger Prawn (*Penaeus monodon*): Known for its distinctive stripes and large size, it's one of the most popular species for export.

White Prawn (*Penaeus indicus*): Also known as Indian White shrimp, it's widely cultivated and processed.

Item B.9 - Chemical Abstracts Service (CAS) Registry Number and Product Labels

Chemical Abstracts Service (CAS) Registry Number: 9012-76-4

Product label:



Panvo Organics Safety Data Sheet for Chitosan:

SDS Panvo Chitosan.pdf

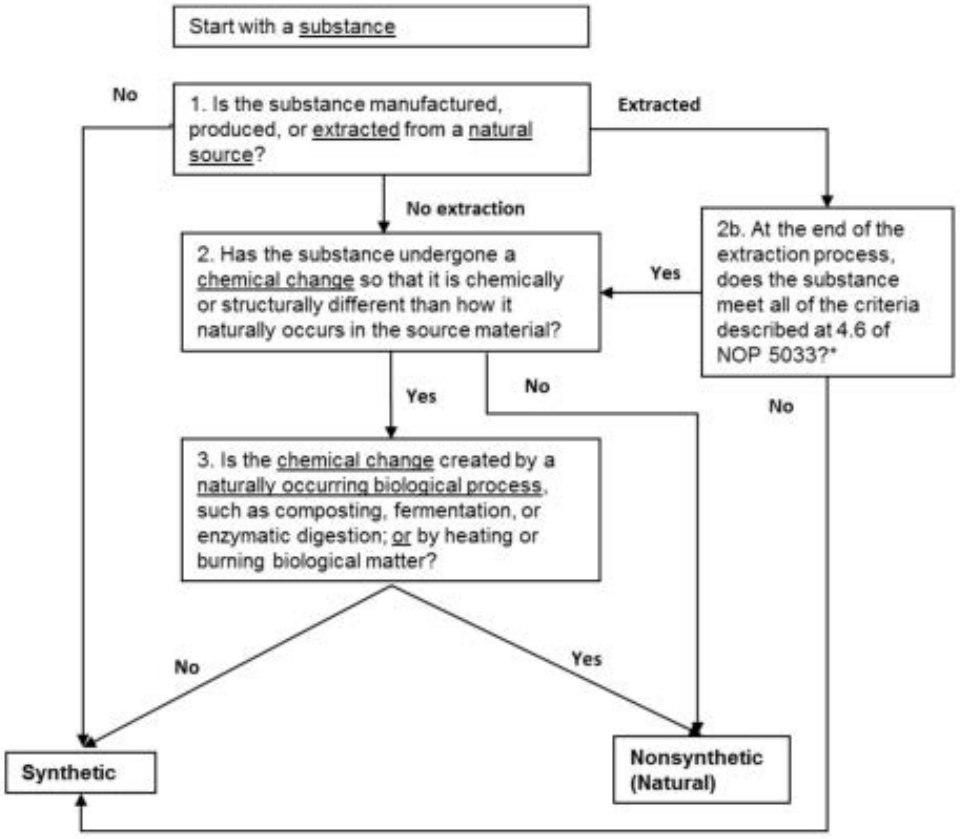
Panvo Organics Specification for Chitosan

SPEC SHEET PANVO CHITOSAN.pdf

APPENDIX 2: NOP 5033-1: Decision Tree for Classification of Materials as Synthetic or Nonsynthetic

Guidance
Decision Tree for Classification of Materials as Synthetic or Nonsynthetic

Underlined terms defined on page 2



* Excerpted from NOP 5033, 4.6 Extraction of Nonorganic Materials:

- At the end of the extraction process, the material has not been transformed into a different substance via chemical change;
- The material has not been altered into a form that does not occur in nature; and
- Any synthetic materials used to separate, isolate, or extract the substance have been removed from the final substance (e.g., via evaporation, distillation, precipitation, or other means) such that they have no technical or functional effect in the final product.

Definitions (bolded terms in 7 CFR 205.2)

Agricultural inputs. All substances or materials used in the production or handling of organic agricultural products.

Agricultural product. Any agricultural commodity or product, whether raw or processed, including any commodity or product derived from livestock, that is marketed in the United States for human or livestock consumption.

Allowed synthetic. A substance that is included on the National List of synthetic substances allowed for use in organic production or handling.

Chemical change. A process (i.e. chemical reaction) whereby a substance is transformed into one or more other distinct substances.

Enzyme. A protein that catalyzes a chemical reaction.

Extract. To separate, withdraw, or obtain one or more constituents of an organism, substance, or mixture by use of solvents (dissolution), acid-base extraction, or mechanical or physical methods.

Formulate. To combine different materials according to a recipe or formula.

Generic. The common and familiar non-proprietary name.

Manufacture. To make a substance from raw materials.

Natural source. Naturally occurring mineral or biological matter.

Naturally occurring biological process. A process that occurs due to the action of biological organisms or subcomponents of biological organisms, such as enzymes. Examples of naturally occurring biological processes include, but are not limited to, fermentation, composting, manure production, enzymatic processes, and anaerobic digestion.

Nonagricultural substance. A substance that is not a product of agriculture, such as a mineral or a bacterial culture, that is used as an ingredient in an agricultural product. For the purposes of this part, a nonagricultural ingredient also includes any substance, such as gums, citric acid, or pectin, that is extracted from, isolated from, or a fraction of an agricultural product so that the identity of the agricultural product is unrecognizable in the extract, isolate, or fraction.

Nonsynthetic (natural). A substance that is derived from mineral, plant, or animal matter and does not undergo a synthetic process as defined in section 6502(21) of the Act (7 U.S.C. 6502(21)). For the purposes of this part, nonsynthetic is used as a synonym for natural as the term is used in the Act.

Substance. A generic type of material, such as an element, molecular species, or chemical compound, that possesses a distinct identity (e.g. having a separate Chemical Abstracts Service (CAS) number, Codex International Numbering System (INS) number, or FDA or other agency standard of identity).

Synthetic. A substance that is formulated or manufactured by a chemical process or by a process that chemically changes a substance extracted from naturally occurring plant, animal, or mineral sources, except that such term shall not apply to substances created by naturally occurring biological processes.

Table 1. Classification examples of inputs:

Substance	Classification	Explanation
Ash (burned wood)	Nonsynthetic	Substance is created by burning biological matter.
Calcium carbonate (limestone)	Nonsynthetic	Substance is produced from a natural source (mined mineral) and does not undergo chemical change.
Calcium oxide (quicklime)	Synthetic	Substance is produced from a natural source (mined mineral), but undergoes chemical change caused by heating the mineral.
Citric acid	Nonsynthetic	Substance is created from a naturally occurring biological process (microbial fermentation of carbohydrate substances).
Gibberellic acid	Nonsynthetic	Substance is extracted from a natural source without further chemical change
Liquid fish products – pH adjusted with phosphoric acid	Synthetic	Substance is derived from a natural source, but is treated with synthetic acids for pH adjustment.
Molasses	Nonsynthetic	Substance is derived from a natural source and chemical change is due to heating or naturally occurring biological processes.
Newspaper	Synthetic	Substance is manufactured via a chemical process.
Raw manure	Nonsynthetic	Substance is from a natural source and used without further processing.
Rosemary oil	Nonsynthetic	Substance is extracted from a natural source.

APPENDIX 3: REFERENCES

*** Bellich, B., I. D'Agostino, S. Semeraro, A. Gamini, and A. Cesaro- 2016:** "The Good, the Bad and the Ugly" of Chitosans. Marine Drugs 14(5): 99; 31 p. DOI: 10.3390/md14050099.
**(A copy of this publication is provided in Addendum 1)*

*** EPA Fact Sheet: Biopesticide Active Ingredient Fact Sheet; Chitosan**
[NOTE: A date of June-2003 was previously mistakenly referenced)
The document is available online:
"Chitosan; Poly-D-glucosamine (128930) Fact Sheet" (PDF).
(A copy of this publication is provided in Addendum 1)

*** EPA 2007-Sep**: EPA Summary Document Registration Review CHITIN & CHITOSAN: Initial Docket
The document is available online:
EPA Summary Document Reg Review document No. EPA-HQ-OPP-2019-0701-0007
(A copy of the publication is provided in Addendum 1)

*** **FR 2006-Jul 3:** Federal Register Vol. 71, No. 127; July 3, 2006 (pp 37854) *Proposed Rule: Chitosan petitioned for use in organic crop production as an adhesive adjuvant.* [not accepted as Chitosan is already permitted under §205.601(m) of the National List Regulations].
The document is available online:
Federal Register Vol. 71, No.127 July 3, 2006 Page 37854 Chitosan petition for use as an adjuvant
(A copy of the publication is provided in Addendum 3)

***** FR 2007-Dec 10**: Federal Register Vol. 72, No. 236; (pp 69569–69572) Dept of Agriculture; Agricultural Marketing Service; 7 CFR Part 205; Docket No. AMS-TM-07-0112; Final Rule.
The document is available online:
2007-Dec 10 FR 72 No 236 Final Rule AMS Docket No AMS-TM-07-0112
(A copy of the publication is provided in Addendum 3)

***** Hadwiger-2004-Jan:** *Petition to add chitosan (poly-D-glucosamine) to the National List of materials approved for use by organic growers*. Submitted by Dr. Lee A. Hadwiger, Department of Plant Pathology, Washington State University, Pullman, WA.
The document is available online:
2004-Jan WSU Petition Add Chitosan to the National List as an adjuvant.pdf
(A copy of the petition is provided in Addendum 3)

* **Hamed, I., F. Ozogul, and J. M. Regenstein– 2016**: *Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review*. Trends in Food Science & Technology 48: 40-50.
(A copy of the publication is provided in Addendum1)

***** ICF Consulting; 2004-Feb:** Poly-D-Glucosamine (Chitosan)-Crops; Technical Evaluation Report Compiled by ICF Consulting for the USDA National Organic Program.

The document is available online:
2004-Feb ICF Chitosan Technical Report – Chitosan-Crops for AMS
(A copy of the publication is provided in Addendum 3)

*** Jones; EPA; 2019-Aug;** (EPA) Risk Assessment Branch, Biopesticides & Pollution Prevention Division (7511P); *Science Review in Support of the Addition of Chitosan (Poly-D-Glucosamine) to the list of minimum risk pesticides (MRPs) contained in 40 CFR 152.25(F)*
(A copy of the publication is provided in Addendum 1)

*** Leahy, J., M. Mendelsohn, J. Kough, R. Jones, and N. Berckes; 2014:** "Chapter 1: Biopesticide oversight and registration at the U.S. Environmental Protection Agency." In Biopesticides: State of the Art and Future Opportunities, Costs, et al.; ACS Symposium Series; American Chemical Society; Washington, DC: American Chemical Society.
(A copy of the publication is provided in Addendum 1)

***** Nexight 2020-Jul:** *Chitosan-Crops; Technical Evaluation Report Compiled by Nexight Group for the USDA National Organic Program*.
The document is available online:
2020-Jul Nexight Tech Evaluation Report.pdf
(A copy of the report is provided in Addendum 3)

***** NOSB 2005-Aug 17 NOSB Meeting Minutes & Transcripts**; *Formal Recommendation by the NOSB to the NOP;* "NOSB Meeting Minutes & Transcripts: 1992-2009." USDA Agricultural Marketing Service
The document is available online:
https://www.ams.usda.gov/rulesregulations/organic/nosb/meetings
(A copy of the report is provided in Addendum 3)

***** Tidal Vision Petition; 2020-Mar 11: National List Petition for Chitosan**
The document is available online:
2020-Mar Tidal Vision Petition (pdf)
(A copy of the publication is provided in Addendum 3)

*** Younes, I. and M. Rinaudo. 2015**: Chitin and Chitosan Preparation from Marine Sources. Structure, Properties and Applications. Marine Drugs 13(3): 1133-1174; doi:10.3390/ md13031133.
(A copy of the publication is provided in Addendum i)

Chitosan Petition

ADDENDUM 1

Publications (7 ea) Referenced in the Chitosan Petition (as requested by USDA AMS

TABLE OF CONTENTS

1. Bellich, B., I. D'Agostino, S. Semeraro, A. Gamini, and A. Cesaro- 2016: "The Good, the Bad and the Ugly" of Chitosans. Marine Drugs 14(5): 99; 31 p. DOI: 10.3390/ md14050099.

Review

"The Good, the Bad and the Ugly" of Chitosans

Barbara Bellich [1], Ilenia D'Agostino [2], Sabrina Semeraro [2], Amelia Gamini [1] and Attilio Cesàro [1,3,*]

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[3] Elettra-Sincrotrone Trieste, Strada Statale 14 km 163.5, Area Science Park, 34149 Trieste, Italy

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Academic Editors: Hitoshi Sashiwa and David Harding
Received: 14 March 2016; Accepted: 9 May 2016; Published: 17 May 2016

Abstract: The objective of this paper is to emphasize the fact that while consistent interest has been paid to the industrial use of chitosan, minor attention has been devoted to spread the knowledge of a good characterization of its physico-chemical properties. Therefore, the paper attempts to critically comment on the conflicting experimental results, highlighting the facts, the myths and the controversies. The goal is to indicate how to take advantage of chitosan versatility, to learn how to manage its variability and show how to properly tackle some unexpected undesirable features. In the sections of the paper various issues that relate chitosan properties to some basic features and to advanced solutions and applications are presented. The introduction outlines some historical pioneering works, where the chemistry of chitosan was originally explored. Thereafter, particular reference is made to analytical purity, characterization and chain modifications. The macromolecular characterization is mostly related to molecular weight and to degree of acetylation, but also refers to the conformational and rheological properties and solution stability. Then, the antimicrobial activity of chitosan in relation with its solubility is reviewed. A section is dedicated to the formulation of chitosan biomaterials, from gel to nanobeads, exploring their innovative application as active carrier nanoparticles. Finally, the toxicity issue of chitosan as a polymer and as a constructed nanomaterial is briefly commented in the conclusions.

Keywords: chitosan; physico-chemical properties; molecular weight; degree of acetylation; conformation; antimicrobial activity; from gel to nanobeads; drug delivery

1. Introduction

Chitosan is one of the most commonly cited polymers in the scientific research dealing with a wide range of biopharmaceutical and biomedical applications including food science and technology. It has been strongly indicated as a suitable functional material in view of its excellent biocompatibility, biodegradability, non-toxicity, and adsorption properties. Originally known as a marine polysaccharide from shrimps and crabs, chitosan was primarily thought to be an easily accessible substance from the food industry waste. The original samples of chitosan were often still raw materials, with few exceptions of purification applied to chitosan in medical and cosmetic uses. The main areas in its characterization were in terms of quality (either purity or harmfulness), intrinsic macromolecular properties and physical form. Independently of the final human use, the first and most important issue was, and is still, related to the presence of impurities, metals and other inorganics, proteins, pyrogenic, endotoxic and cytotoxic agents, bioburden, all of which have received over the years erratic attention. The macromolecular characterization is mostly related to molecular weight and to degree of

acetylation, but also refers to the rheological properties and solution stability (solubility, aggregation, and filterability). Last, but not less important in many applications, is the physical form of the powder or other morphology of the sample. Since nowadays there is real boom of interest in chitosan, it is worth mentioning that all these characteristics have been amply and often repeatedly reported in literature in original research and review articles. Indeed, more than a thousand papers (1150) were published in the decade 1981-1990, increasing to more than five thousand papers (5700) in the decade 1991-2000, to about 23,100 in the first decade of this century. A current search of "Chitin and Chitosan" in research article titles gives more than 1600 results, almost one-half of which were published in the last 15 years; so why write another review? The simple answer is that another view-point can still be worthwhile, provided that some properties are cross linked and related to some critical specificity of chitosan in a novel way, as reported schematically in Figure 1.

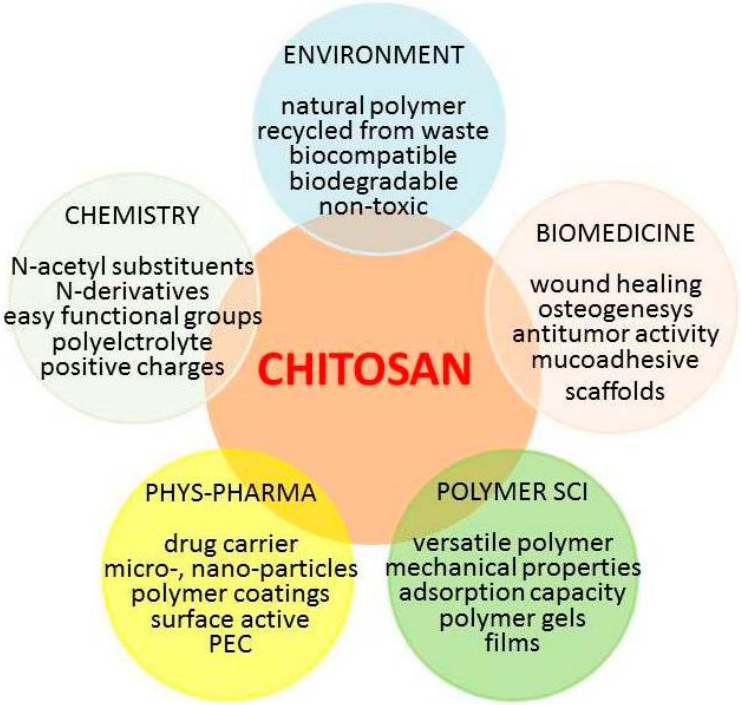

Figure 1. Schematic representation of relationship of chitosan properties with the pharmaceutical and biomedical applications.

Thus, having in mind to classify the "properties" of chitosan in categories as "the good, the bad and the ugly" , a scrutiny of its characteristics is here overviewed in order to introduce the theme of this paper. Among all natural polysaccharides the presence of the monomer glucosamine, either fully acetylated or partially deacetylated, is unique in the chitin / chitosan polymers. Whether explicitly highlighted or only mentioned as a secondary aspect, this structural characteristic is probably the basis of its outstanding biophysical properties and the key for a rationale of its applications.

In summary, the most simple and salient characteristics that make chitosan a beneficial and valuable polysaccharide are:

1. Chitosan is linear natural polymer of glucosamine/acetylglucosamine that behaves as a polyelectrolyte with positive charge density at low pH.
2. Among the industrial polysaccharides, chitosan is an exception, being the only high molecular weight cationic polyelectrolyte, while polysaccharides are generally either neutral or anionic.
3. Chitosan is often claimed to be GRAS (Generally Recognized As Safe) and bioabsorbable.

The same versatile "good" characteristics are indeed also the reason for several undesired effects, starting from the most obvious one when dealing with copolymers, *i.e.*, the large variety of chemical composition in terms of comonomers. The large variety of chitosans differing in polymer size, degree of acetylation and possible chemical modifications increases exponentially. On the other hand, the simplicity of chemical modification is one of great strengths of chitosan. The "tunable" aspect of chitosan allows its optimization to give appropriate biomaterials for therapeutic applications, in principle enabling also the optimization of its biological profile.

The counterpart of the structural variability is in the difficult characterization of its (statistical) distribution of comonomers (glucosamine and acetyl-glucosamine) and in the unsolved question of how the compositional variability and the molecular weight distribution add complexity to the identification of beneficial properties of the final product (the "bad"). However, the studies of chitosan uptake, distribution and toxicity are indeed quite few, since most commonly in its drug delivery formulations, chitosan is the carrier or a functional excipient. Nonetheless, it is also evident that the inspection of chitosan interactions with some co-solutes or other biostructures may reveal some subtle effects both in classical solution thermodynamics as well as in biological applications, such as the "interactions with endotoxins". All these undesired and often hidden effects are most appropriately classified as "ugly", because they have frustrated scientists in their research efforts.

In the following sections the title issues are presented, relating and linking the chitosan properties, whenever possible, to some basic features and to advanced solutions for specific applications. Thus, after a first section in which tribute is paid to some pioneering work, the chemistry of chitosan is explored with reference, in particular, to purity, characterization issues and chain modifications. Then, the biocompatibility, antimicrobial activity and toxicity of chitosan in relation with its solution properties are reviewed. A section is dedicated to the formulation of chitosan biomaterials, from gel to nanobeads, exploring their innovative application as active carrier nanoparticles. Finally, the toxicity issue of chitosan as a polymer and as a constructed nanomaterial is briefly referred in the conclusions.

Particular attention is devoted to critical comments on the various conflicting experimental results, highlighting the facts, the myths and the controversies. The goal is to make the best use of the ***good versatility*** of chitosan, to learn as much as possible how to manage its ***bad variability*** and to tackle in the proper way its ***ugly undesirability***.

2. The Beginning

A short historical annotation may usefully illustrate the continuous efforts dedicated to increasing the knowledge of chitosans in terms of structure-properties relations and therefore to improving and developing their applications. Another pertinent preliminary comment is also necessary on the link, established from the very beginning, between academic research directly or indirectly related to commercial applications and the scientists involved in the R&D of companies present on the market in the fields of either generic chemicals or, more specifically, polymers. The scrutiny of papers dealing with chitosan properties gives rise to a permanent feeling that while its "potential" is continuously stressed, for many years this seemingly remained wishful thinking. Several factors concur with the idea that using chitosan as a market product could be beneficial. Some of these will be reported in the specific application discussed in this paper.

It is not strange that among the first studies on chitin and chitosan is the structural determination of the chain by diffractometry. The first X-ray diffactometric investigation of chitosan was carried out by Clark and Smith in 1936 [1] on fibers prepared from lobster tendon chitin by a solid state *N*-deacetylation. A subsequent X-ray pattern was obtained and fully analyzed [2] with a tendon chitosan prepared from a crab tendon chitin by similar deacetylation reported by Clark and Smith. Therefore, as mentioned by Ogawa in his review [3] "In spite of such early finding, the pattern was analyzed in 1997 [4], 60 years later."

The relevance of crystallographic studies lies in the possibility that the chain conformation of chitosan can be affected by the chemical environment. Indeed, it has been shown that in the two

polymorphs, hydrated (tendon) chitosan and anhydrous (annealed) chitosan, the crystalline structures consistently present antiparallel chains in extended two-fold helix, zigzag structure, with almost constant pitches of 1.034 and 1.043 nm, for the hydrate and anhydrous forms, respectively. This chain structure is similar to those found for chitin and cellulose. However, chitosan can easily change its solid state conformation from the extended two-fold to other structures by salt formation with acids. Although not comparable with other very flexible linear polysaccharides, such as amylose and pullulan, this moderate conformational flexibility is certainly due to the presence of free amino group on the monomer residue of chitosan; thus, the advantage of chitosan for its advanced tunable applications can be envisaged.

With regards to the more broad chemical and physico-chemical studies, very few papers were published on chitosan before 1970, some dealing with its characterization, a few on chemical reactions and derivative synthesis, and the largest number on its interaction with ionic species and the potential application in ion removal from waters. In this period, some research groups appear in the literature that dominated the later times. Between the 1970 and the 1980, some reactions (e.g., selective acylation) and properties of chitosan were assessed and many applicative properties of chitosan were explored. Among these works, it is particularly worth mentioning those on the hypocholesterolemic activity [5], the fungicidal effect [6], in addition to the reactions of chitosan to produce derivatives. A comprehensive review was offered by the First International Conference on Chitin/Chitosan held in 1977 in Boston [7], which signed the milestone for a continuous growing number of adepts in the chitin and chitosan forum. Thus, the great effort of the Muzzarelli group must be mentioned, starting with the characterization of metal chelating properties and the chromatographic uses of chitosan; as well as the contributions of the UK and Japan groups on the production and physicochemical characterization of chitosan gels.

In the following decade (1981–1990), an explosion of activities all around the world in the field of carbohydrate polymers gave rise to intensive studies in this area. In parallel, there was an increase in conferences dealing with industrial application of polysaccharides, with sessions in the very large meetings (e.g., International Carbohydrate Symposium, *etc.*) as well as some more specific thematic meetings (e.g., Grado 1981 [8] and Hoboken 1984 [9]). In the context of these meetings, several research groups emerged and the tie line for aggregation networks was marked.

In these and subsequent years, while continuous efforts were made on the preparation of well-defined samples (see, for example, [10]), many new ideas about the use of chitosan as biomaterial became explicit and other properties of chitosan and derivatives emerged in publications. The proposed uses of chitosan included the development of artificial skin, reconstruction of periodontal tissues, hemodialysis membranes, drug targeting and many other biomedical applications. The foreseen applications were always accompanied by sentences like "this novel biomolecule, biodegradable, and biocompatible, find applications in substituting or regenerating tissues" [11]. The similarity of chitosan structural characteristics with glycosamino-glycans, was interpreted as the reason for mimicking the functional behavior of the latter, while chitin was always structurally (or ancestrally?) associated with cellulose. In a slightly different direction, a derivative, *N*-carboxybutyl chitosan, was shown to display inhibitory, bactericidal, and candidacidal activities when tested against a few hundred cultures of various pathogens [12].

It is worth remembering that the first interest in the commercial applications of chitin grew in the 1930s and early 1940s, but took a backseat for many years because of competition from synthetic polymers. Large-scale production of chitin started in the mid-1970s when regulations were introduced to limit the dumping of untreated shellfish waste in coastal waters. Chitin was easily extracted from crab, lobster and prawn shells using solvents, and the production of chitin became an economical way to comply with the regulations and dispose of thousands of tons of shellfish waste. Although chitin has applications in creams and cosmetic powders and as a material for surgical stitches, the applications of chitosan expand to a multitude of areas: antibacterial lining for bandages and wound dressings, coating for seeds to boost disease resistance, agent to prevent spoilage in winemaking, in addition to its controversial use as dietary supplement (weight-loss by avoiding fat digestion). The specific

area of food applications has been shortly reviewed in a 2016 paper [13], in particular for antioxidant and antimicrobial effects, which are useful in the food industry to improve food safety, quality, and shelf-life. It has been estimated that about the order of 10^{10} tons are produced annually by living organisms, although chitin and chitosan are recovered exclusively from marine sources. Applications of these polymers continue to grow and an estimated global market of chitin and chitosan derivative business was forecasted to exceed € 70×10^9 by the year 2015 [14]. As reported in a news agency *"Not bad for bits of leftover lobster"* [15].

Before concluding this section, one point needs to be emphasized. The chain production of a "food-waste-recycling-biopolymer" is compatible with achievement of ecological benefits and deserves further attention and effort. For chitin and chitosan, the environmentally-friendly materials produced from these renewable sources are sustainable and contribute to the creation of a circular economy, which is the current goal of an ambitious package put forward by the European Commission in December 2015 [16]. For the more specific aspects here concerned, the waste produced each year by the shellfish processing industries represents a practical challenge, since about 75% of the total weight of crustaceans (shrimps, crabs, prawns, lobsters, and krill) ends up as by-products. The current lack of acceptable waste management options creates a potentially large environmental hazard concern. All around the world, seafood wastes are thrown away at sea, burned, landfilled, or simply left out to spoil. Therefore, the extraction of chitin from shells and its use as it is or after further processing not only is a way to minimize the waste, but also to produce compounds with valuable biological properties and specialty applications, provided that all the raw material is correctly remodeled in well characterized chemicals. As recently pointed out in literature ("don't waste seafood waste") [17], the three major components of crustacean shells are calcium carbonate (20%–50%), proteins (20%–40%) and chitin (15%–40%), which could be separated by using an integrated bio-refinery with solvent-free mechano-chemical processes. Although the main objective of this work is focused on the biopolymer chitosan, it is mandatory to mention that within this sustainability view-point, several useful chemicals and primers can be obtained from chitin [18–21], giving further value to an atom efficient economy. Once again, a parallel can also be envisaged between strategies used, or to be implemented, in cellulose and chitin exploitation.

3. A critical Examination of the Physico-Chemical Properties of Chitosan Polymers

In order to emphasize, once again, the relevance of a correct understanding of the relation structure-properties in chitosan, let's just mention in these first lines that chitosan composition has become a matter of great interest in patent claiming. Documentation for these claims lies on well-defined polymer chemical characterization and this concept will be further analyzed when discussing the difficulties encountered by regulatory agencies in approving chitosan uses.

The main outcome of a critical examination of the literature results is that often (although not always!) the correlation between properties of the nanoconstructs and the structure of the macromolecular components is missed. In particular, the impression is that the advantage offered by modulating the polymer structure is turned out in the disadvantage of having final products of remarkable variability. Therefore, aiming at exploitation of chitosan and chitosan nanoparticles in modern biomedical and pharmaceutical applications, some fundamentals of physicochemical properties of this biomacromolecule cannot be overlooked. The central "dogma" is that all useful applications can *in principle* be traced back to three molecular determinants: the degree of acetyl substitution, the molecular weight and its distribution, the nature and the fraction of substituents as pendant groups. In the more general applications, the additional step to be examined is the interplay of these molecular characteristics with the supramolecular interactions that provide size, shape and surface properties in the nanoconstructed patterns [22].

3.1. Chitosan as a Copolymer: Acetylation and Substitution

The first question concerns whether it is fully correct to speak about "chitosan" or better to use the term "chitosans", making explicit the central concept that the term chitosans implies a series

of copolymers that differ not only in the fraction of comonomers but also in the distributions and clength of the comonomer sequences. This aspect is well known in polymer sciences where the terms alternate-, block- and random copolymer have been introduced. It is also the case of alginate, a close polysaccharide, where the presence along the chain of the Guluronic (G) and Mannuronic (M) monomers with varying composition and sequence type, was amply recognized since long time [23], allowing to conclude, from ^{13}CNMR studies, that the relative occurrence of G-centered triads deviated significantly from those predicted by first-order Markovian statistics [24]. It is also known that in chitosan the two monomers, *N*-glucosamine and *N*-acetyl-glucosamine, display quite different solution properties, given the ionic character in acidic conditions of the first monomer and the slightly hydrophobic terminal in the other. This intrinsic difference, getting amplified for block-type sequences of acetyl substitution, may have dramatic consequences on chain conformation and aggregation, a fact that has encouraged the research on more hydrophobic substituents able to self-assembling and collapse in micelle-type lipospheres. Furthermore, derivatization of chitosan by grafting side chains, like is done in quaternization or glycosylation of amino groups, gives rise to a ter-polymer (namely, constituted by acetylated, de-acetylated and substituted monomers), worsening, therefore, the already complicated situation.

Thus, the essential issue of chitosan composition is the proper sample characterization and, first of all, the correct determination of the degree of acetyl substituents, DA, (some authors use the term DDA, degree of de-acetylation). Over the years, several papers have referred on the determination of the degree of acetylation by physical and chemical methods, although the preferred method is often simple and easy to use, not the more accurate. The long list of methods include among all, titration [25,26], IR spectroscopy [27–31], UV spectroscopy [32,33], circular dichroism (CD) [34], NMR spectroscopy [35–38] and *N*-acetyl group hydrolysis [39]. As a preliminary and important annotation, the comparison of data obtained with different techniques often show discrepancies in the DA values. Indeed, the difference in solubility of samples having DA from 1 (chitin) to 0 (fully deacetylated chitosan) affects sensibly the results and no single technique could be adopted to cover the full range, pointing out that solution methods can be used for soluble chitosans, whereas 13C CP/MAS NMR [36,37] and infrared spectroscopy (as recently summarized in a review [40]) are used for chitin and highly acetylated chitosans. Still, each method presents advantages and difficulties either in the sample preparation and/or in running the measurements. As a novel technique, the calorimetric (DSC) determination of the distinguishable decomposition of amino and acetyl groups was recently published, providing results that are independent of composition and molecular weight [41]. Thus, DSC emerged as an accurate technique to determine the degree of N-acetylation in chitin/chitosan samples, the main advantages lying on the possibility of determining the DA in the whole 0 to 1 range, without solubilization, at lower cost in relation to NMR, and more accurately than with IR. However, all techniques, except for NMR based measurements, require an accurate weighing of chitosan. Therefore, moisture needs to be eliminated carefully and the purity of the samples must be determined separately. The degree of acetylation of chitosan determined by NMR is based on the fact that the O-Ac group is easily recognizable in the spectrum of chitosan recorded at room temperature and can be integrated and normalized by the integral either of anomeric protons or of other ring protons. More important is the possibility of generalization of the NMR method to study chitosan derivatives. An interesting application is that of glycosylated samples prepared with cellobiose, maltose and lactose residues. The glycosylation introduced a pendant made by an open sugar ring C1-linked to the amino group and C4-linked to the terminal glycosyl residue. The NMR spectra show small differences for all signals of the pendant groups except for the carbon 4 and the carbon 5, which reflect the configurational change from glucose to galactose moieties. The NMR on these samples provided a DA = 0.13 and a glycosyl substitution ratio ranging from 0.43 to 0.50 for the three derivatives [42].

Not less important for chitosan varying in pendant group substitution is the knowledge of the substitution pattern of the groups (including the acetyl groups) along the chain, as it was referred as a relevant property in copolymers. This is illustrated in Figure 2, where it is schematically evidenced

why not only the fraction of comonomers (e.g., DA) but also their distributions and the length of the sequences of comonomers ultimately affect the polymer behavior, *i.e.*, each copolymer species is a "specific product". The relevance of these characteristics increases with the difference in the chemical properties of the two monomer species.

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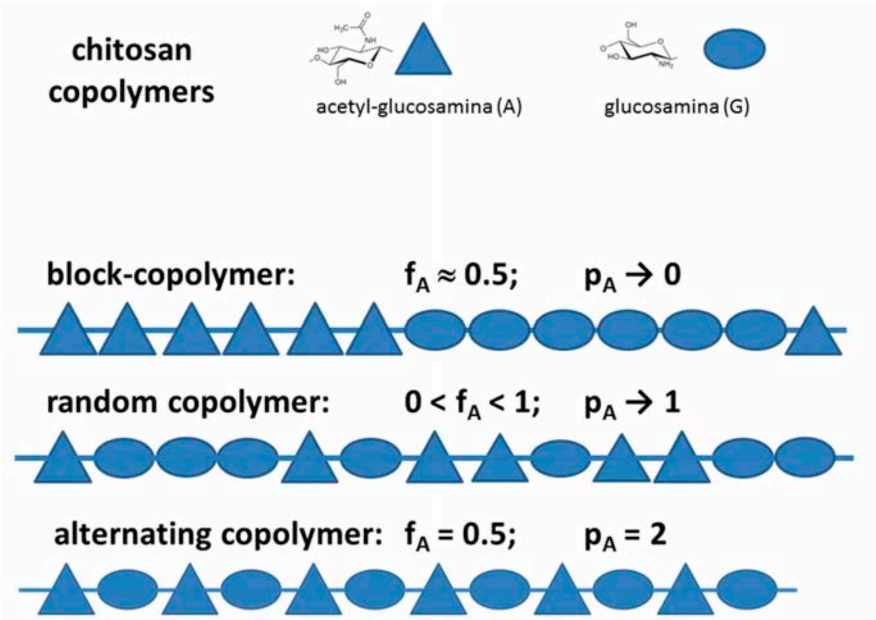

Figure 2. Schematic representation of three typical patterns of monomer distribution in a copolymer, here acetylglucosamine (A) and glucosamine (B). A perfect block-type copolymer with $f_A = 0.5$ should have a limiting p_A value = 0 for infinite length of blocks (top). A random distribution of the monomer (middle) is characterized a limiting p_A value = 0.5 for a value of f_A that can range around 0.5. The alternating copolymer, by definition, should have $f_A = 0.5$ and $p_A = 2$ (other values evidence structural irregularity). Thus, the scheme identifies limiting cases to interpret copolymer structure and underlines that nearest-neighbor effects give the three copolymer structures different physico-chemical properties.

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In general, a statistical copolymer consists of macromolecules in which the sequential distribution of the monomeric units obeys known statistical laws, such as the Markovian statistics (the so-called random copolymer obey a zero-order Markovian statistics, coinciding with a Bernoulli distribution of the monomers). The only very non-random distribution of monomers is the one where monomers A and B alternate, Poly(A-alt-B), which can be clearly defined as a "new" polymer, different from Poly(A) and Poly(B) and which has rigorously fraction values $f_A = f_B = 0.5$. Another example of a copolymer distribution is that of "block copolymer" (PolyA-block-polyB), which is characterized by sequences of one monomer (A) interrupted by sequences of the other monomer (B), the length of these sequences obeying to some *n*-order Markovian statistics (with $n > 1$). All these different copolymer sequences can be initially identified by the monomer fraction, $f_A = 1 - f_B$, and the probability of diads, f_{AA}, f_{BB}, f_{AB}. A compact mode of providing the sequence probability has been proposed for the general case of copolymers by Bovey & Mirau [43] and recently used for chitosan [44] with the definition of the parameter $p_A = f_{AB}/(2f_{AA} + f_{AB}) + f_{AB}/(2f_{BB} + f_{AB})$.

The relevance of this quantification can be traced back to the original findings of Aiba [45], who studied two samples of chitosan with similar DA but different solubility properties. This author set up the hypothesis that the samples of chitosan with DA > 0.5 prepared from highly deacetylated chitin had to be considered as random-type copolymers of *N*-acetyl-glucosamine and glucosamine units, whereas the samples with similar DA but prepared from partial deacetylation of chitin had to be considered as block-type copolymers.

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In conclusion, despite some different opinions in the literature and the generally assumed random statistics of residual acetyl substitution, the accurate determination of DA and of copolymer statistics is unavoidable. NMR determination of DA has been found to be precise and accurate also for the quantification of high DA, which is usually difficult to be measured with conventional techniques like IR or titration. Additionally DA can be calculated using different combinations of peaks in order to verify that the method is consistent. Some NMR techniques described in the

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3.2. Chitosan Copolymers: Molecular Weight Determination and Conformation

Before reporting on molecular weight (M) determination, another subtler question arises in these data for chitosans. Not only the problem of complete solubilization (1) or even the change in the macromolecular solvation (2) affect the M measurements, but also the correct interpretation of M can be vague if the degree of substitution is not known (3). Points (1) and (2) imply solution thermodynamic issues to be reviewed, since most of the experimental methods for M determinations deal with some solution properties measurements and a dependence of the solvent goodness through the Flory interaction parameter. Point (3) is of mere analytical origin and points out on the more classical definition of macromolecular chain length in terms of degree of polymerization (DP). As an example, a monodisperse chitin sample with DP = 1000, will display a molecular weight of 203.2×10^3, while the same sample fully deacetylated, *i.e.*, the corresponding chitosan without *any* degradation, has a molecular weight of 161.2×10^3, with a difference of about 20%. Therefore, even without degradation, a continuous change in the "measured" molecular weight is expected to occur upon deacetylation of chitin. This problem is much more impressive with large substituents as it occurs in the class of glycosylated chitosans [42,44]. The experimental determination of the M and M distribution of a series of samples of glycosylated chitosan with a constant DA = 0.13 and glycosyl substitution DS 0.42–0.50, is enlightening (Table 1).

Table 1. Physico-chemical properties of chitosan and chitosan derivatives (reported in [42]). Two commercial samples of Very-Low-MW and of Low-MW and three derivatives, a trimethyl-substituted (TM-chit), a galactosyl- (Gal-chit) and a glucosyl- (Glc-chit) substituted are reported. The molecular weight of the commercial LMW sample is given to be approximately 50,000–190,000 daltons based on viscosity. TM-chit contains also substitution on O6 and O3. The sugar substituent, gal or glc, is linked to ammine via an open sugar chain, since a disaccharide is the reactant. DS (non-acetyl substitution) determined by NMR. The column "Recovery" is a parameter relevant for the data to be representative of the sample investigated.

	DS	Mw	Mn	Mw/Mn	[η]	Rh	Rg	Recovery
		kg/mol	kg/mol		dL/g	nm	nm	%
VLMW-chit	-	30	18	1.7	0.7	6.4	-	94
LMW-chit	-	128	56	2.3	2.3	15.3	-	82
TM-chit	0.71	109	46	2.3	0.7	9.7	-	80
Gal-chit	0.50	473	102	5.1	1.9	21.3	50.8	99
Glc-chit	0.42	554	96	6.4	2.4	23.8	61.7	93

Molecular weight and conformation analysis of these derivatives were investigated at UFT—Centre for Environmental Research and Sustainable Technology, Bremen, with a "triple detectors" size exclusion chromatography Viscotek system. The array, with a differential refractometer, a right angle (90°) (RALS) and a low angle (7°) light scattering detector and a four capillary, differential Wheatstone bridge viscometer, provided as final data the Molecular weight (M) and Molecular Weight Distribution (MWD) of fractionated samples, including viscosity of each fraction related to a given dilute polymer concentration [42]. The analysis of data allow to extract the weight average M_w and the parameters a and k of the Mark–Houwink–Sakurada (MHS) equation, $[\eta] = kM^a$.

In particular, the most evident result is that, despite their overall similarity in the values and the trend of the viscosity, an increase (about doubling) of the molecular weight is observed in the glycosylated chitosans. This effect could be interpreted as a sign of chain dimerization, whereas simply arises from the derivatization reaction by which a pendant with M ≈ 320 is added on the chain about every two monomeric units, therefore doubling the original molecular weight in the absence of degradation. Therefore, these annotations claim for a re-examination of many published data of chitosan samples, in particular those with additional substitutions.

As far as the conformation of chitosan with low DA is concerned, it is worth reporting that SEC data with triple detectors provide the full plot of log [η] as a function of M with the interesting result that the exponent coefficient a increases from 0.5 to 1 with the molecular weight decreasing from 5×10^5 to 5×10^4, in line with the literature independent results (Figure 3) about the concurrent assignation of persistence length and mass per unit length of chitosan (see Table 3 of ref. [44]) and with the findings for the moderately stiff hyaluronan chain. These results are collected in a sort of master curve and contain the fundamental dependence of the exponent a as a function of the degree of polymerization (Figure 4).

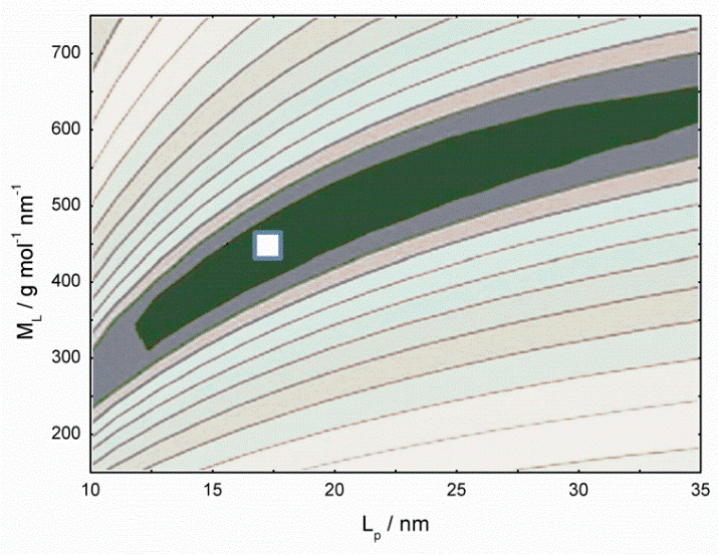
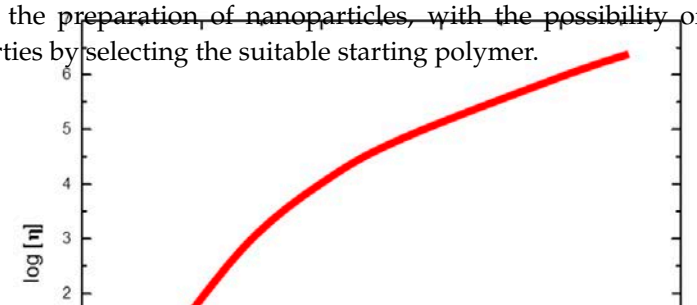


Figure 3. Descriptors of the chain conformation according to the Bushin–Bohdanecky equations. The solutions for chitosan are described in ref [48]. The diagram describes regions close to the target (dark color) and regions far from the target (pale color). The white square shows the coordinates of chitosans in the persistence length Lp (nm) vs. mass-per-unit length, M_L (g·mol^{-1}·nm^{-1}), plane (adapted and redrawn from ref [48]).

In conclusion, the correct polymer characterization and the robust interpretation of solution properties provide the basic knowledge for understanding the chain expansion behavior of the chitosans used for the preparation of nanoparticles, with the possibility of better tailoring the nanoparticle properties by selecting the suitable starting polymer.



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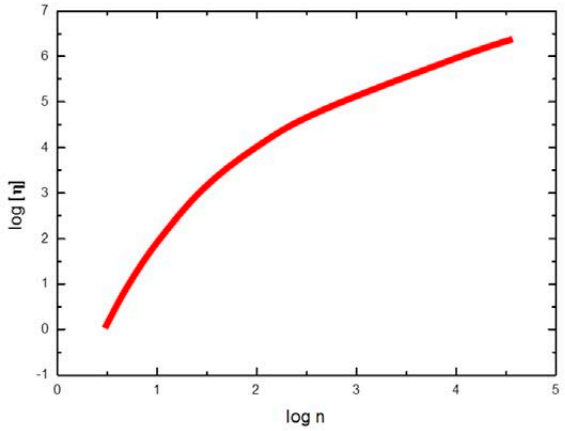

Figure 4. The dependence of intrinsic viscosity of chitosan and hyaluronan as a function of degree of polymerization (*n*) is reported as a master-plot (MHS log-log), normalized by the constant K (solvation constant). The emphasis is on the slope that increases with decreasing degree of polymerization n, *i.e.*, molecular weight M. The curve is drawn with the data of Figure 6b of ref. [47] for hyaluronan ($0.5 < n < 4$) and Figure 6.1 of ref [49] for chitosan ($2 < n < 4.5$).

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Returning to the solution properties, points (1) and (2), in addition to direct methods for molecular weight determinations (e.g., those based on light scattering measurements), other indirect methods are largely used because they are simple and ready to use. Among these methods, the most popular one is the determination of the intrinsic viscosity, [η] which is log-log proportional to the molecular weight by mean of the Mark–Houwink–Sakurada (MHS) equation, $[\eta] = k M^a$. The use of the equation implies, however, that a calibration is made on monodisperse fractions of the same polymer, under the same conditions of solvent and temperature. At this stage, it is clear that the calibration constants, k and a, depend on the very chemical structure of chitosan, starting from the value of DA and possibly on its distribution, since it has been recognized that the polymer solubility is affected. The solution to this problem was afforded in the past by changing the pH and the ionic composition. Empirical correlations have been reported to adapt the calibration constants of the MHS equation to a range of DA, pH and salt concentration. The use of the empirical relations, however, opens further questions about the compensation phenomena of chain expansion and solvation. Indeed, the analysis of the data of k produced by several authors, when plotted as a function of DA, shows the absence of a clear correlation that however could arise from some important difference in the acetylation patterns. The possibility that the samples may present a range of non-statistical sequence of acetylation patterns has been very recently presented [49–51].

3.3. Chitosan Solubility

On concluding this section on the intrinsic macromolecular properties of chitosan family, it is also necessary to add some words about the common practice of derivative preparation and the consequent changes in solubility that arise when substituents are inserted as pendant groups on the chain backbone. As a rule of thumb, polysaccharide solubility is affected by the chain linkage that governs the extension and the flexibility of the polymer. This is an important factor that makes the difference between polysaccharides otherwise undistinguishable on the basis of the rule "*similia similibus solvuntur*" (similar substances are miscible). This feature is clearly shown by comparing the configurational properties of several glucans, such as cellulose, amylose, pullulan, β-glucan [52,53], where flexibility and stability of the crystalline order play the major role. Therefore, for any given polysaccharide chain, solubility can increase (or decrease) depending on whether random (or regular) substitution is achieved and whether substituents can improve or not the interactions with the solvent. These concepts are schematically summarized in Table 2, where only a few examples are given to substantiate the above-mentioned rule of thumb. Chitosan is not an exception, as the changes

in the degree of acetyl substitution and the introduction of other substituents (ionic, hydrophilic, and non-polar) conform to the above scheme.

Table 2. Schematic role of structural and conformational features on solubility of polysaccharides.

Structural Features Change	Case-Polymers	Solution Behavior
chain linkage	cellulose	stiff and insoluble
	pullulan	flexible and soluble
side chains	curdlan	linear, insoluble
	scleroglucan	branched, soluble
non-sugar substituents	deacetylated gellan	gel with Ca, Mg
	native gellan (acetylated)	soluble with Ca, Mg
ionic groups (carboxyl)	Chitin	neutral, insoluble
pH > 6	hyaluronan	ionic, soluble, pH-depend.

Following these concepts, the issues of solubility of chitosan and chitosan derivatives can be focused in more detail and some issues of the past literature can be re-examined. Within the polysaccharide scientific community, the scarce water-solubility of chitosans, at neutral pH is a well-known and generally experienced fact. The solubility of chitosan(s) is an important concern representing a limit (if not an obstacle) not only to physical-chemical studies addressed to the structure-properties understandings, but also to its preparation and use as a polymer support or carrier material in biomedical applications, where neutral aqueous environment is often encountered.

It has already mentioned that the solubility of chitosan(s), *i.e.*, the entire family of partially deacetylated chitins with average degree of acetylation DA $\leqslant$ 50%, depends on molar mass, number and distribution of acetylation sites. These structural characteristics, in turn, are all related to the source of chitin and to the means of chitin extraction and deacetylation [54–56]. Besides the exploitation of other less common sources, the main industrial source of (α)-chitin is the shell (exoskeleton) of crabs or shrimps, while that of (β)-chitin is the squid pen. More recently, chitosan from chitin extracted from cultivated edible mushrooms, like *Agaricus bisporus*, has been made commercially available. This material, prepared by Kitozyme [57] and distributed by Sigma, is claimed to be a highly pure chitosan, ideal for wound healing and hemostasis, biosurgery and ophthalmology, scaffold and cell therapy, as well as drug delivery and vaccines. Since all commercial chitosans are far from an absolute purity, it is worth mentioning that a great difference in purity of chitosan may arise on whether the commercial product is used "as it is" directly in the human applications or it is subjected to physical and chemical modifications before end-uses. As a matter of fact, most procedures to modify chitosan by introducing pendant groups, or to process it by mixing with other polymers or chelating agents, imply "*de-facto*" manipulation that may introduce new contaminants or remove original contaminants. To the best of our knowledge, an analytical report on this particular aspect is lacking in the works published, while it will possibly be required in biomedical applications in addition to safety issues. Therefore, up-to-date information about accurate analytical reports is necessary and this specific point concerns both in-house purifications and commercial products. In particular, it would be desirable that data sheets of commercial products could be integrated with the macromolecular and characterization data discussed in this paper.

Indeed, keeping in mind that the amount of the residual *N*-acetyl glucosamine (GlcNAc) and the type of distribution, play a key role in determining the water solubility of chitosans [58,59], it is also necessary to analyze some solution molecular aspects. In particular, the role played by the glucosamine residues (GlcN) is generally referred to as improving solubility in acidic medium (*i.e.*, pH $\leqslant$ 6), due to protonation of amine group. On the other hand, the acetyl groups (and so the GlcNac residues) have been viewed as more hydrophobic entities that favor chain aggregation and negatively affect the water solubility [56,60–63]. However, while these concepts seem to be clear enough, the translation of the effects on a long chain is far to be naïve. Despite decades of the active research described, a clear relationship unambiguously linking the molar mass and the acetyl

content and distribution to water solubility has been still elusive. Although a poorer solubility is encountered in homogenously re-acetylated samples with high DA content with respect to commercial ones [64], the homogeneity of DA distribution is reported to be one of the factors that increases chitosan solubility in aqueous medium as long as the solution pH is kept below 6. This can be reworded by saying that, even when the acetyl groups are randomly distributed, a pH lower than 6 is a prerequisite for chitosan solubilization [61,65,66]. On the contrary, the dissolution of chitosan samples with high acetylation content remains troublesome at a molecular level especially at high molar masses [64,67,68]. These findings were originally found by Anthonsen *et al.* [69] on fractionated chitosan samples with f_A = 0.01 and 0.60; by using several characterization methods, a bimodal molecular weight distribution was observed in which about 5% of the sample had a very high molecular weight. The presence of supramolecular structures revealed by electron microscopy and the possibility of partially reducing this aggregated fraction by ultracentrifugation and filtration were consistent with the positive virial coefficients obtained earlier from osmotic pressure measurements. Worth mentioning is that the presence of concentration dependent aggregates are reported also in chitosan of relatively low molar mass at low DA (12%) [70,71].

At this point, it is necessary a *flashback* with some general comments, since it is not a novelty that for a given concentration and solvent the increase of a polymer molecular weight adversely affects its solubility. More specifically, polysaccharides are known, as well, as gelling or thickening agents irrespective of their having charged or neutral backbones (e.g., agarose, amylose, carrageenans, xanthan, alginate). Some of their final applicative properties linked to biological function are also often exalted by the physical form of storage samples after chemical manipulation and purification and by the procedure of solubilization. In particular, for many polysaccharides, including chitosan, these physical forms and solubilization procedures refer to solution, powder, freeze-dried, air dried and presence or addition of simple salts, heating procedure, respectively.

Solubilization processes imply that a solid form of the material has been obtained and the material "history" is involved, making evident that precipitation, gelation or freeze-drying are not equivalent. Therefore, it is clear that the question about whether the acetyl moieties favor aggregation because they impart hydrophobic characteristics to the chain, remains unclear when not controversial, until both the short range and the long range effects are properly studied. For example, although it is suggested that acetyl groups affect the chain conformation by a continuous enhancing of chain stiffness upon increasing acetyl content [72], the interpretation of the previous literature data remains somehow conflicting. It is reported for instance and in contrast with previous observations, that DA strongly influence the stiffness of the chain as evidenced by the increase in MHS exponent a and radius of gyration Rg with DA, above a certain molar mass.

Still, an apparently consistent and reliable picture of the dimensional properties of chitosan in solution can be achieved by re-analyzing some original data of viscosity and radius of gyration and properly plotting these values as function of degree of polymerization, n, (Figure 5). The controversial deductions on chitosan behavior, might likely result from disregarding that the overall chain length characteristics play the main role on viscosity and light scattering experiments, from which conformation or shape are deduced. The generic Debye relationship for an isolated chain is between the viscosity and the number of residues ($[\eta] \propto n^1$) and a direct correspondence between M and n exists only for chemically identical residues, as already stressed above. Indeed, taking into account the value n, neither the parameter a nor the exponent ν ($Rg \propto n^{\nu}$) seem to be much dependent on DA except for DA ~ 60%. Therefore, the fragmented picture that is given by the separated literature data [63,64,72,73] can be recomposed, as shown in the Figure 5.

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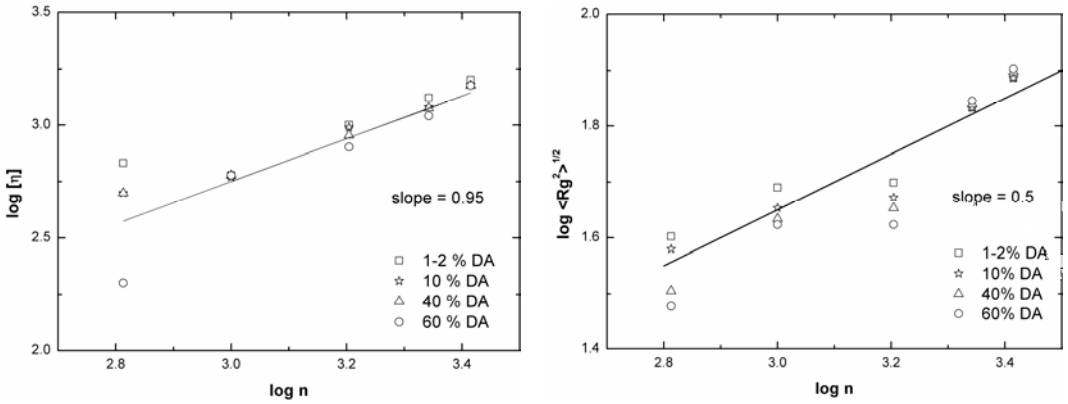

Figure 5. (**a**,**b**) Double logarithm plot of [η] and Rg values of chitosan samples with different DA values as a function of degree of polymerization, n. The data are recalculated from literature [63,64,72,73].

As before, here the values of $n = (M/Mru)$ are obtained from the experimental M and acetylation degree values [72,73] being Mru the average mass of repeating unit. The value of n can be identified as proportional to the chain contour length expressed in nm, being roughly 0.5 nm the average virtual bond length of the β(1,4)-D-glucose monomer (*i.e.*, the distance between two consecutive glycosidic oxygen atoms). On the other hand, the second virial coefficient is undeniably found to decrease with increasing DA and salt concentration, as expected for polyelectrolytes upon changing the effective charge density, being the effect the more evident the higher the M is [64,70].

Before concluding this section, a comment is still necessary of the mentioned changes of hydrophobicity as a function of DA. It should be clear that changes in local polarity by acetylation can scarcely affect chitosan properties as individual chains (very dilute solution). However, the influence of acetyl substitution can be more evident at high polymer concentration, where "hydrophobic" driven chitosan association can be observed. Under these circumstances, the presence of hydrophobic domains has been studied by mean of fluorescence using pyrene as hydrophobic probe, whose fluorescence spectrum is quite sensitive to the polarity of the microenvironment [71,74]. By ruling out the intrinsic acetyl contribution to the association tendency of chitosan chain, the authors assume that the formation of hydrophobic domains takes place at polymer concentrations close to and above the overlap concentration C*; for fully ionized samples with no added salt, this conclusion mainly indicates that the intermolecular character of the aggregation depends on the dimensional properties, *i.e.*, is independent from DA.

The scarce solubility of chitosan at neutral pH has been circumvented by modifying the polymer backbone with ionic or highly hydrophilic moieties. Chitosans bearing carboxylic, sulfate or *N*-alkyl groups have been synthesized for this purpose. Beside the enhanced solubility in aqueous medium, several additional features were disclosed by these modified chitosans. As a single mention, sulfate chitosan derivatives showed to be compatible with anionic polysaccharide like xanthan and the viscosity of the formed blends was found to be much greater than would have been expected from that of the individual components. Concerning the reductive alkylation route used to modify chitosan with the objective to reduce its intractability in neutral aqueous medium, the work of Yalpani and Hall [75–78] certainly deserves a specific mention; not only it showed the advantages of facility and versatility of the reaction procedure, but also it contained *in embryo* almost the entire "chitosan based biomaterials" research area whose development was still to come. It is not the scope of this review to discuss the synthetic approaches, since already reported in excellent comprehensive papers (for example, see ref [79] and references therein). However, the rationale of chitosan modification is that derivatization mainly aims at providing the following new performing properties. The first goal is to modulate physical-chemistry properties with the purpose to: (i) improve water solubility and blending ability with anionic biopolymers, enhancing and modulating rheological, hydration and compatibility

properties of the new materials; and (ii) increase hydrophobic or emulsifying properties. The second goal is on the high-rated field of biological applications, with particular attention to derivatives that can impart specific biological activities to chitosan-based constructs through conjugation, and to those that can enhance antimicrobial activity. The intertwined manner with which these purposes can be found in the more recent literature might explain the continuous growth of active research on this subject. Driven by the desire of tailoring specific and complex properties, these activities led to a boost of studies on chitosan derivatives for potential applications in a very large variety of fields.

4. Biocompatibility, Antimicrobial Activity and Toxicity (Chemistry *vs.* Material)

Resuming the introductory section, since the very beginning of chitosan investigation, the properties of chitosan were interpreted in terms of a very promising technological material for a wide spectrum of applications [12,80–83]. In particular, besides all its recognized and foreseen properties, chitosan was claimed to possess the appealing feature of antimicrobial and bacteriostatic activity. Solutions, films and composites made of chitosan have been reported since the 1980s to be antimicrobial against a wide range of micro-organisms like bacteria either Gram-positive or Gram-negative, human oral and gingival pathogens [84,85] yeasts [86,87], algae [88,89] and fungi [90]. However, its effectiveness is continuously debated about whether it acts as a *bactericidal* agent, *i.e.*, capable to kill live bacteria or as a *bacteriostatic* agent, *i.e.*, able to arrest the bacterial growth without killing the microorganisms [91–93].

In a recent review, the current research in the areas of food, medical and textile industries was analyzed in order to summarize the progress in the study of antimicrobial properties of chitosan [93]. The comparison of the antimicrobial activity in relation to the structural differences in chitosan or chitosan derivative, the polymer physical form and the microorganism tested, made evident the difficulties of rationalizing the results. The conclusion to be shared is the variety of results reported by researchers even under apparently identical conditions, often due to the lack of standardization of the assay conditions. A further more general comment deals with the ambiguities in some of the studies concerning the assessment of chitosan antimicrobial potential, mainly deriving from inadequate data on the polymeric characteristics that affects this ability. Pointing at this ambiguity, in another recent analysis it has been reported that "Given the large number of proclaimed medicinal benefits of chitosan, it comes as no surprise that the literature is filled with conflicting reports about these medical potentials." [94].

With this statement out of the way, it is obvious to recognize that the mechanism of chitosan antimicrobial activity has not been completely understood so far, since it depends on many intrinsic properties of the polymer itself and extrinsic factors related to microorganisms and environmental conditions. Among a long list of factors, it is worth reporting the molecular weight values [95], degree of acetylation (DA) [96,97], pH, type of derivative, solvent composition (medium) and, in particular, presence of metal cations [98], in addition to microorganism type and its outer surface charge [99–101], growth conditions, *etc.*

In general, it can be stated that the absence of reliable experimental determination of molecular parameters makes presently impossible to pinpoint the dependence of antimicrobial activity of chitosan on M or DA. As discussed in a previous section, the reliability does not reside in an intrinsic accuracy of the determination of the molecular parameters, but rather in the poor selection of the appropriate methods or even in the absence of any determination. In order to shed light on this issue, some literature findings can be tentatively organized about the mode of action, the killing mechanism and the interaction with cell components.

Mode of action: There are many hypotheses about how chitosan could exhibit its bactericidal activity, but almost all studies underline the determinant contribution of the poly-cationic nature of chitosan. Therefore, the electrostatic interaction emerges as a fundamental feature of the killing potential, since the interaction with the negatively charged microbial surface would dramatically affect the bacterial vitality. However, in view of the variability of the microbial surfaces, different

organisms expose different more or less charged molecular patterns and respond to chitosan differently. This seems to be the case of gram-negative and gram-positive bacteria, although it is still uncertain which type is more sensitive. Some studies [12,102,103] indicate that the gram positive ones are more susceptible to chitosan than other micro-organisms, because of the presence of polyanionic teichoic acids on the outer surface [104,105]. Other studies reported that also gram negative are significantly affected by chitosan, implying a role of hydrophobic interactions by the exposed lipopolysaccharides [91].

Killing mechanism: Membrane structural perturbation by chitosan results from a series of studies by using a large number of different techniques, including the measurement of membrane polarity, evaluation of minimum inhibitory concentration, electron microscopy, transcriptome and proteome analysis. Based on the evidence of Raafat *et al.* it can be argued that the molecular mechanisms of action of chitosan in inhibiting or killing bacteria is definitely a complex process, which involves a series of interrelated events, that eventually lead to micro-organisms death [105]. First, the growth-inhibitory effect is shown in this study to be dose-dependent. A permeabilization of the cell membrane to small molecules and a significant membrane depolarization occur after treatments with the polymer, with consequent loss of cell integrity, stability and functionality. Moreover, a further implication on the cell components is disclosed by the changes observed in the expression profiles of the target organism (*Staphylococcus aureus* SG511 (*S. aureus*)), especially for those genes involved in regulation of stress, autolysis and energy metabolism [93,106].

Interactions with the cell components: Different opinions emerge in literature on the possibility that chitosan enters the cytoplasm. On one side chitosan is able to penetrate the cell and to interact with nucleic acid, thus interfering with the protein synthesis [107,108]. On the other side, the molecular mass of chitosan samples used in the experiments (not oligomers) has been questioned, making dubious its uptake. Alternatively, it has been suggested that high M chitosan deposition on cell surface could lead to a blockage of nutrients reducing microbial growth [98,109]. In a growth medium, chitosan might subtract micronutrients like essential metals (Ni, Zn, Co, Fe, Mg and Cu) to bacteria, inhibiting the production of toxin and important surviving molecules [93,110]. In conclusion, the proposed bacteriostatic activity of chitosan could arise from its well-known chelating ability.

5. Applications of Bacteriostatic Activity of Chitosan and Derivatives

Chitosan has been successfully used by researchers to exploit several applications in very diverse fields, in particular pharmaceutical (drug delivery, devices, and wound dressing), cosmetic, textile and food industries, as well as in agriculture and environment (waste-water purification). Some of the most interesting applications is based on the bactericidal potential of chitosan are summarized in the Table 3 of ref [93]. A summary of antimicrobial properties of chitosan and its derivatives is given, keeping in mind the general assertion that chitosan is considered as a GRAS (Generally Recognized As Safe) compound.

Multiple constructs/models of chitosan, and its derivatives, have been evaluated to express the bactericidal potential in medical field (e.g., post-surgery wound healing, patches and bandages as antimicrobial dressing after burning, antimicrobial coating of prosthesis) [111–116]. In the emerging field of nanobiopharmaceutics, antimicrobial chitosan nanoparticles have been studied for their enhancing effect of surface-to-size value. Thus, a vast number of works report the use of chitosan in form of nanoparticles, prepared in different conditions and blend, against various microorganisms [117–121].

In food industry, maintaining the quality and extending the shelf life of food products is mandatory. One of the most promising ways for effective preservation of food from alterations is using bioactive films as packaging. The use of active and/or edible bio-film based on chitosan alone, combined or enriched with different components such as plant extracts, other natural polysaccharides and antimicrobial peptides is emerging as an answer to such problems [122–127].

Finally, the use of antibacterial agents to prevent bacteria colonization is becoming a popular procedure in textile production, especially for goods employed in medical or hygienic services or

sport-wears (odor-control textile) [128]. This antimicrobial supplementation is especially needed for natural fibers, which are more vulnerable to microbial attacks. Two examples, by Gupta and Haile [129] and Ye *et al.* [130], reported that a sensitive reduction of *S. aureus* (99%) was observed in modified chitosan embedded cotton.

6. Application of Chitosan as Delivery Systems

6.1. Chitosan Microparticles

The use of chitosan in pharmaceutical technology was originally conceived as an excipient for solid dosage forms, being used as coating, film-forming, mucoadhesive, disintegrant, tablet binder and viscosity-increasing agent [131]. The first investigations on the suitability of chitosan as drug carrier date back over twenty years [132]. Since the very beginning the use of chitosan was claimed to offer numerous advantages, such as high availability in nature, relatively low toxicity, and, above all, the presence of positively charged amino groups that enable both physical and chemical cross-linking [133]. In addition, after the seminal works of Yalpani and Hall [75–78], both the amino and hydroxyl groups have been exploited for a huge number of chemical modification [134].

Although among the several formulations the initial attention was focused on chitosan microspheres [135]. The spreading of nanotechnology shifted very fast the attention towards the nanoparticles, due to the numerous advantages offered by their size (in the nanometer range) [136–138]. Nowadays the choice of formulating micro- or nanoparticles is motivated by the type of performance required. For example, for intravenous delivery, only nanoparticles can be injected since microparticles would cause obstruction of blood vessels, while in case of pulmonary delivery, would microparticles have better efficacy, since nanoparticles would be exhaled [139].

Micro- and nanoparticle production is the preliminary step for the physical generation of spherical domains, independently of the final stage of preparation. Thereafter, an appropriate gelation process is applied to stabilize the micro- or nano-domains. The most common procedures for micro-particle production are extrusion, emulsion and spray-technologies (Figure 6). The "extrusion" technique is widely used and, in the simplest case, can be performed by using a syringe with a needle. Several parameters, such as the diameter of the orifice, the flow rate, the viscosity of the solution, the distance between the hardening solution and the orifice, the polymer concentration and the temperature [140], control the size of the droplets and thus the final dimensions of the particles. The emulsion technique consists of dispersing a liquid in another one that is not miscible. Most importantly, it is possible to tune the size of the droplets by selecting the appropriate composition of the two phases, *i.e.*, the type and concentration of polymer and surfactant [141,142]. The spray-technologies are based on the atomization in heated gas (air or nitrogen) of a fluid material (solution, emulsion or suspension), which is followed by a fast removal of the solvent (usually water). The concentration of the polymer and the temperature of the system influence the final dimension.

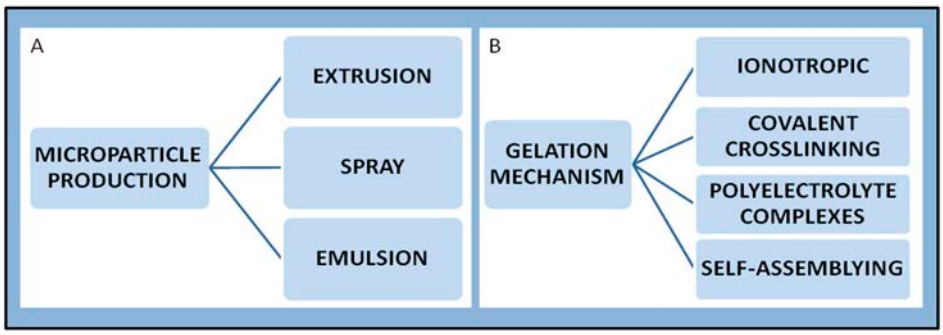

Figure 6. Graphical classification of the particles production (**A**) and mechanisms of gelation (**B**).

6.2. Chitosan Nanoparticles

Over the years the interest in the formulation of chitosan nanoparticles dramatically increased, exploring new and innovative preparation methods [143–145]. Basically, the main mechanisms for chitosan nanoparticles preparation rely on a crosslinking process (either chemical or physical [146]), formation of polyelectrolyte complexes [147] and self-assembly of hydrophobically modified chitosan [145] (Figure 6). Other possible processes include reverse micelle, desolvation,

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Chitosan crosslinking, such as a dropwise addition of a cross-linker to a chitosan solution, is considered an easy production technology to immobilize protein [149] or encapsulate different molecules [150,151]. Figure 7 summarizes the main characteristics of the gelation mechanisms. A classical covalent cross-linker is the glutaraldehyde; it acts as a bridge between two glucosamine units belonging to the same polymeric chain or to different chains. The amine group of the chitosan reacts with the aldehyde group of the linker leading to an imine bond via a Schiff reaction [147]. The concentration of glutaraldehyde strongly affects the textural properties of the final product [152]. The final structure results in an irreversible network with high mechanical properties, such as rigid network structures and high resistance to dissolution also in extreme pH conditions. One of the main drawback encountered is the high toxicity of the aldehydes [153]; the use of such systems is actually limited due to insufficient biocompatibility of the cross-linkers. Similar considerations can be done for epichlorohydrin [154]. A new crosslinking agent, genipin, which is found in gardenia fruit extract, is becoming an interesting alternative to glutaraldehyde [154–162].

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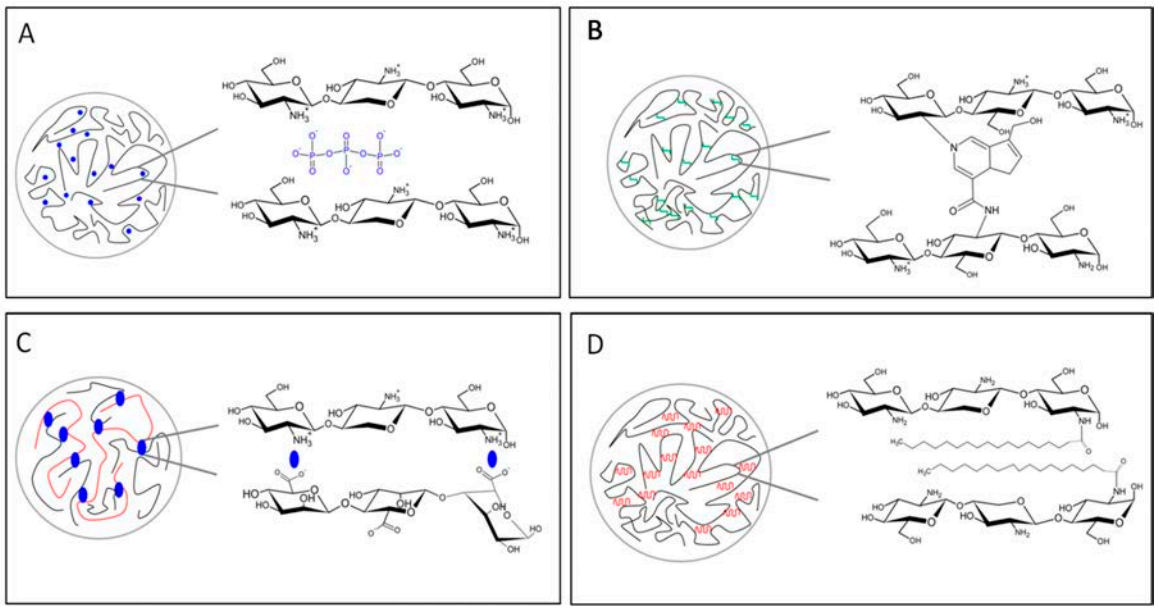

Figure 7. Gelation mechanisms for chitosan nanoparticles preparation: (**A**) ionic crosslinking; (**B**) covalent crosslinking; (**C**) polyelectrolyte complexation (PEC); and (**D**) self-assembly.

Another possibility is the use of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), similar compounds, which enable the formation of an amide bond via an intermediate derivative, between the amino group of chitosan and the carboxyl group of different molecules. EDC is also known as zero-length cross-linker [163]. A valuable alternative is represented by carboxylic acids (especially from natural origin) [133,164]. All the linkers mentioned above can link chitosan with different polymers, such as gelatin [165], or other compounds, such as hydroxyapatite [166]. Attempts have been addressed to perform covalent modification using dextran sulfate [167]. Alginate too is potentially able to interact covalently with chitosan [168], producing resistant hydrogel. When choosing a covalent crosslinking method, it is important to check that the cross-linker does not bind also to the drug, to avoid formation of drug–polymer conjugates.

However, such covalent derivatives of chitosan are not considered as the best choice for drug delivery due to their lack of swelling [147] and absence of pH-dependence drug release. Chitosan is indeed appealing since it is a stimulus-responsive polymer [169].

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The ionic crosslinking is also very appealing due to the presence of charged amino groups on chitosan in acidic conditions that allow to crosslink the polymer by using negatively charged ions, such as tripolyphosphate (TPP), which is the most widely used (note that this name entered incorrectly in the common language and is difficult to remove; chemically speaking it is a trimer and not a poly-phosphate, and even less a "tri-poly-something"). The interaction, commonly referred as ionotropic gelation, was firstly reported by Bodmeier [170] for the preparation of beads by dropping chitosan in a TPP solution and further extensively studied and described by Calvo *et al.* [171]. The ionic gelation takes place spontaneously via electrostatic interactions between chitosan chains and TPP [172]. This methodology of nano-particles preparation allows to encapsulate several types of biomolecules, from protein [173–175], to small drugs [176,177] and DNA fragments [178], and, because of its versatility, it was extensively studied and optimized [179,180].

Since the beginning, such mild conditions of gelation, appeared immediately very attractive for the encapsulation of labile drugs, such as proteins or peptides, characterized by poor bioavailability upon oral administration [181]. Within this frame, numerous studies have been conducted for the oral delivery of insulin [182], for the pulmonary delivery [183] and also for ocular delivery [184]. In addition to drug delivery, chitosan-TPP nanoparticles have been explored for many other uses. For example Du *et al.* explored the loading of various metal ions to obtain chitosan nanoparticles characterized by antibacterial activity [117]. This study evidenced that the antibacterial activity was significantly enhanced by the metal ions load.

Another very popular mechanism for the easy formation of nanoparticles is through polyelectrolyte complexations (PEC). Chitosan, the only natural polymer with a positive charge, is an attractive polymer for its ability to complex with a wide range of negatively charged polysaccharides by electrostatic interaction, forming PEC. More specifically, the interaction involves a phase separation where the solvent is excluded from the hydrophilic colloids. This can be achieved by adding a competing hydrophilic compound, such as a salt or an alcohol [185], and it is referred as simple coacervation, while complex coacervation occurs in a solution of two oppositely charged polyelectrolytes, giving rise to two immiscible liquid phases [186]. The phase rich in colloids is the coacervate, while the other phase is the equilibrium solution [187]. The interaction is likely to occur first via the long-range Coulomb forces, which provide for the primary strong binding energy, followed by the more directional short-range hydrogen bonds. The optimum condition for complex coacervation requires a constant control of pH. The mechanical properties and permeability of the products are strongly influenced by the properties of the starting material and conditions of reaction.

Chitosan is able to form PEC with both natural and synthetic polymers and since the early work published on PECs by Fuoss and Sadek (1949) [188], different studies and reviews have been published on this topic. PEC complexes obtained using natural biopolymers with opposite charge have been formulated for peptide [189,190] and protein [191] carriers but represent also an ideal colloidal carrier for DNA delivery [192]. Another emerging field of application is the delivery of antibiotics. Tobramycin loaded nanoparticles functionalized with dornase alfa demonstrated DNA degradation and improved nanoparticles penetration, thus increasing the efficacy of tobramycin [193].

In particular, among natural polymers, chitosan can interact ionically with several polyanion [194], such as alginate where the coacervation is due to interactions between the carboxyl functions of guluronic/mannuronic units of the alginate and the positively charged amine groups of the chitosan. A slight variation of this method is known as ionotropic pre-gelation reaction, as reported by Sarmento in a study for oral delivery of insulin [195,196]. This method, firstly reported by Rajaonarivony [197], involves an interaction among alginate, calcium and poly-lysine. The reaction consists in a first step

characterized by an interaction between alginate and calcium ions, due to the gelling properties of alginate for the presence of guluronic residues [196], that are able to exchange sodium ions with divalent cations such as Ca^{2+}, Sr^{2+} and Ba^{2+}, forming the characteristic egg box structures. This step in the original study was followed by the crosslinking of pre-gelled alginate with poly-lysine, but in several subsequent works chitosan substituted poly-lysine [191,195,196,198–200]. Hyaluronan, another biocompatible polymer negatively charged that can be used to form complexes with chitosan [201,202] has the ability to bind several receptors as CD44 [203]. Hyaluronan can also be used as coating for chitosan-TPP complexation [180]. Further examples of complexation are represented by the reaction of chitosan with carrageenan [204], dextran sulfate [195,205], and xanthan [206]. Studies on similar interactions using negatively charged macromolecules, such as arabic gum [207], gelatin [208] and pectin [209] have been published.

A recent review [210] reports on the advances made in this field, focusing the attention on chitosan PEC's with natural polysaccharides, such as alginate, hyaluronic acid, pectin, carrageenan, xanthan gum, gellan gum, arabic gum, and carboxymethyl cellulose, *etc.*, discussing also *in vitro* and *in vivo* data. On the other hand, synthetic polymers, such for example polyacrilic acid, have also been considered for polyeletrolytes complexation [211,212].

A sub-category of complex coacervation is represented by polyelectrolyte-colloids coacervate that has some advantage with respect to polyelectrolyte-polyelectrolyte coacervation in retaining the structure of colloids and in reducing the heterogeneity and configurational properties of the final system. In particular polymers-protein coacervates represent an interesting system for preservation of bio-functionality and are particularly important in enzyme immobilization, protein purification, antigen delivery and food stabilization [186]. Polyelectrolyte-protein coacervation [213] can occur between the amino groups of the chitosan and the reactive groups of heparin [214], BSA [186] and casein [215]. A change in the proportion between polyelectrolyte and protein can increment or suppress the coacervate.

Another appealing macromolecule is DNA, whose complexes with chitosan can be formed by simple mixing [216,217] and represent a promising non-viral vehicles for gene delivery [192,218]. A very recent review [219] reports on the promising use of chitosan as a non-viral nucleotide delivery system in spite of viral vectors that, although characterized by a high transfection efficiency, pose some safety concerns regarding immunogenicity and insertional mutagenesis. Thus, achievements towards this direction would be of great benefit for gene therapy.

Chitosan can be also modified with hydrophobic groups, resulting in grafted polymers that show a tendency to form inter- and intramolecular interactions in polar solvents by forming polymeric micelles that can be used to encapsulate hydrophobic drugs. The long polymeric chains interconnected with the hydrophobic molecules help stabilize the micelles protecting the internal drug. An abundant literature is available on this topic, investigating chitosan amphipatic behavior after the crosslinking with cholesterol [220,221], deoxycholic acid [222], stearic acid, and linoleic acid [223,224].

Finally, it is worth to stress that a full knowledge of the physicochemical properties of the chitosan is required to obtain nanoparticles with tailored characteristics. The final structure of the network is the result of the contribution of different parameters, such as molecular weight and degree of acetylation, which affect the hydrophobic interactions and the network of hydrogen bonds. Xu and Du reported a direct correlation between chitosan MW and both encapsulation efficiency and release of BSA [173]. Similarly, formulation parameters, such as the ratio between chitosan and TPP [225] and operative parameters, such as stirring time and speed, affect the properties of the nanoparticles and their yield [226]. The release profile is a consequence of the behavior of the nanoparticles in the aqueous environment; more specifically the drug release occurs basically by three main mechanisms, diffusion, swelling and erosion (Figure 8), and it is strictly dependent on the type and degree of the crosslink. In case of a covalent cross-link, the permanent network obtained prompt for a drug release, which is mainly by diffusion, and the overall release profile will depend on the cross-linking degree. Similarly, the cross-linking density and the pH of the environment, as clearly reported by

Berger *et al*. [146], influence also the swelling capacity. The mechanism of erosion is instead possible in presence of an ionic cross-linker. The kinetic of swelling and erosion will affect the initial part of the release curve determining a characteristic lag-phase. A huge amount of studies have been done in the field of model equations to describe the different several release profiles [227,228].

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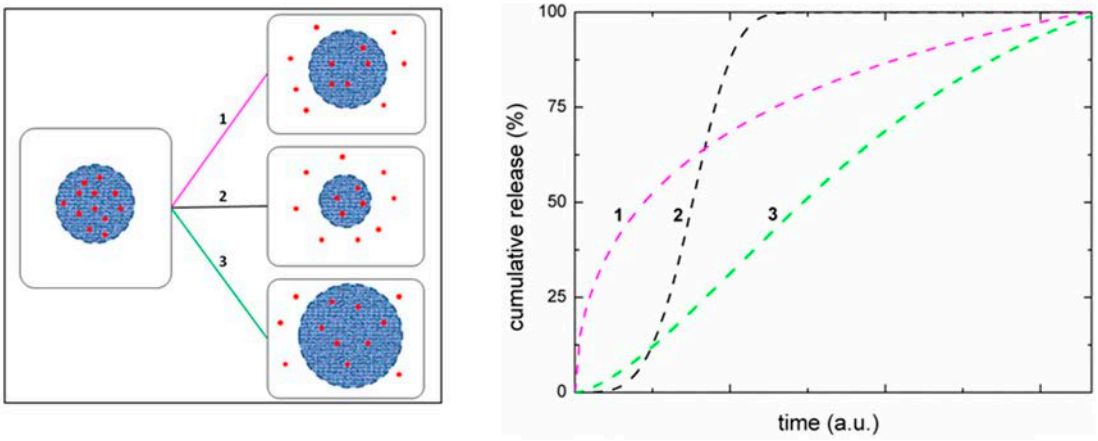

Figure 8. Example of release mechanisms (1: diffusion, 2: erosion and 3: swelling) and some related graphical trends according to mathematical models. Each mechanism is driven by the chemical structure of the network: elastic swelling and/or hydrolysis of cross-link, but also chitosan depolymerization by lysozyme action, in addition to the basic diffusion from the porous nanoconstruct.

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The improvements and modifications described so far will lead further progresses in pharmaceutical applications [229]. Many papers claim the potentiality of chitosan as carrier for drug delivery. Has this promise been fulfilled? Chitosan has been recognized as safe (GRAS) and approved for dietary use in Italy, Japan and Finland and only in 2008 a specific monograph was introduced in the European Pharmacopeia and in 2011 in the US National formulary. This of course has limited its use for the development of new drug formulations. Currently, chitosan is approved for wound dressing applications and cartilage repairing formulation. A recent review [230] on the use of chitosan as an absorption enhancer in nasal drug delivery formulations describes the ChiSys™ technology for the delivery of peptides, proteins and small hydrophylic drugs. A chitosan-based formulation for nasal administration of morphine (Rylomine™), at the moment in phase 2 (UK and EU) and phase 3 (USA) clinical trials, is expected to be released on the market in the near future.

7. Conclusive Remarks

The challenging point after reading so many articles and blending literature results with our own experience is recurrent in many lines of this article. It can be synthetized in two simple questions: How are ugly and bad tunable to good? How much we are willing to bet on a chitosan future? This final section attempts to resume the main issues that, to the best of our knowledge, should be addressed in the near future.

We would emphasize that the difficulties encountered in chitosan development as a marketed product in drug delivery and bioactive material could actually reside in the poor correlation between accurate chemical structure determination and its effective biological responses. An explicit reference can be made to references [42–44], where this issue has been recently reformulated. However, the main characteristics of chitosan to be considered, for example in the development of drug delivery systems, are not only the acetylation degree (DA), molecular weight (M), and purity, but also the relationships between internal and external parameters. Indeed, by stating that bioactivity and biocompatibility are both substrate- and host-dependent, it is clear that responses depend on investigated materials and on the experimental read-out used. In the case of chitosan, the understanding of material biocompatibility is hindered not only by the limited knowledge on the biological processes involved in material-cells interactions, but also by the poor assessment of the polymer characteristics. A recent review [222] addresses the general problem of the safety issue on chitosan uses, suggesting a more careful assessment of its safety in non-oral formulations.

A naive answer is given by the fact that chitosan approval by the American Food and Drug Administration (FDA) is not a general definition as GRAS, but FDA and other regulatory agencies evaluate and approve materials with respect to specific applications. Not to forget that other relevant properties beyond the polymer itself are size, morphology, crystallinity, surface characteristics, degradation profile and additional products.

On the polymer side, the reported chitosan versatility and the variety of formulations add confusion to inexperienced researchers and regulatory scientists. For this reason, a thorough and systematic description of the chitosan used in a study should be either provided by the producer or be complemented by the research laboratories. Needless to say, the existence of expertise spread around the word and of large infrastructures should also be usefully utilized.

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2. EPA Fact Sheet: Biopesticide Active Ingredient Fact Sheet "Chitosan; Poly-D-glucosamine (128930) Fact Sheet" (PDF).

Chitosan; Poly-D-glucosamine (128930) Fact Sheet

Summary

Chitosan is used primarily as a plant growth enhancer, and as a substance that boosts the ability of plants to defend against fungal infections. It is approved for use outdoors and indoors on many plants grown commercially and by consumers. The active ingredient is found in the shells of crustaceans, such as lobsters, crabs, and shrimp, and in certain other organisms. Given its low potential for toxicity and its abundance in the natural environment, chitosan is not expected to harm people, pets, wildlife, or the environment when used according to label directions.

I. Description of the Active Ingredient

Chitosan (poly-D-glucosamine) is one of the most common polymers found in nature. Structurally, it is related to cellulose, which consists of long chains of glucose molecules linked to each other. In chitosan, the building block of the chains is a slightly modified form of glucose. [For another pesticide active ingredient structurally related to chitosan and cellulose, see chitin, also called poly-N-acetyl-D-glucosamine.] Like chitin, chitosan is present in the shells of all crustaceans and insects, and in certain other organisms including many fungi, algae, and yeast. Commercially, chitosan is prepared from chitin, which is isolated from the shells of crustaceans after the edible parts have been removed.

OPP Chemical Code: 128930 ; (CAS# 9012-76-4)

II. Use Sites, Target Pests, And Application Methods

- **Use Sites:** Many field crops, ornamentals, and turf grown in fields, home gardens, nurseries, and other sites.
- **Uses:** Plant defense booster; plant growth regulator (enhancer).
- **Target pests:** Helps plant defend against certain fungal diseases, including early and late blight, downy and powdery mildew, and gray mold.
- **Application Methods:** Spray on leaves throughout growing season, with applications every one to two weeks as needed.

III. Assessing Risks to Human Health

No risks to humans are expected when products containing chitosan are used according to label directions. In toxicity tests, the only effect seen was slight skin irritation after chitosan was applied to skin.

IV. Assessing Risks to the Environment

Risks to the environment are not expected because chitosan has not shown toxicity in mammals, it is abundant in nature, and it is used in tiny amounts.

V. Regulatory Information

Year registered (licensed for sale) as active ingredient: 1986

Number of end products, February 2001: 4

VI. Manufacturers

August Bjornson, DCV, Inc., 3521 Silverside Rd., Wilmington, DE 19810
SafeScience Products, Inc, 31 St James Avenue, 8th floor, Boston, MA 02116-4101

Agent: Bruce Jaeger, ph 301-261-8491

VII. Additional Contact Information

Ombudsman, Biopesticides and Pollution Prevention Division (7511P)
Office of Pesticide Programs
Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, D.C. 20460

3. EPA 2007-Sep: Chitin and Chitosan Summary Document Registration Review: Initial Docket

Docket Number: EPA-HQ-EPA-2006-0566
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Chitin and Chitosan Summary Document
Registration Review: Initial Docket
September 2007

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I. PRELIMINARY WORK PLAN

Introduction:

The Food Quality Protection Act of 1996 mandated the continuous review of existing pesticides. All pesticides distributed or sold in the United States must generally be registered by EPA, based on scientific data showing that they will not cause unreasonable risks to human health, workers, or the environment when used as directed on product labeling. The new registration review program is intended to make sure that, as the ability to assess risk evolves and as policies and practices change, all registered pesticides continue to meet the statutory standard of no unreasonable adverse effects. Changes in science, public policy, and pesticide use practices will occur over time. Through the new registration review program, the Agency periodically reevaluates pesticides to make sure that as change occurs, products in the marketplace can be used safely. Information on this program is provided at: http://www.epa.gov/oppsrrd1/registration_review/.

The Agency has begun to implement the new Registration Review program pursuant to FIFRA Section 3(g) and intends to review each registered pesticide approximately every 15 years to determine whether it continues to meet the FIFRA standard for registration. The public phase of registration review begins when the initial docket is opened for each case. The docket is the Agency's opportunity to state clearly what it knows about the pesticide and what additional risk analyses and data or information it believes are needed to make a registration review decision. Chitin and Chitosan are among the first biochemical pesticides to undergo the registration review process and to be reassessed using the most current standards of risk assessment.

Because Chitosan is derived from Chitin, and because both compounds share basic physical characteristics, they have been combined into a single case - Registration Review Case 6063. As a matter of general similarity, Chitin and Chitosan are both naturally occurring polymers that are ubiquitous throughout nature. Structurally, they are related to cellulose and each consists of long chains of glucose molecules. Relationally, Chitosan is a deacetylated product of Chitin, whose chains of glucose are slightly modified. As biochemical active ingredients, they are applied in different use patterns; however, they both demonstrate a low toxicity profile, and are both known for their role in bolstering plant resistance. Based on these factors and the data submitted, no adverse human health or ecological risks are expected as a result of exposure to these compounds, if they are used as labeled. However, if additional information is submitted that warrants further risk assessments, the Agency will then conduct any necessary risk assessment(s). Further information regarding use sites, summaries of health, ecological effects data reviews and endangered species assessments, as well as a list of registered pesticide products containing these active ingredients, is found in Parts II and III in this document.

Anticipated Risk Assessment and Data Needs:

Ecological Risk Assessment: Ecological effects for both Chitin and Chitosan were duly considered in the course of reviewing the applications for the first products containing these active ingredients. In each case, non-target data and/or various non-target waiver requests were sufficient to determine that the proposed uses of the pesticides containing these active ingredients posed negligible to non-existent ecological risk. These determinations were captured in both summary science memos and regulatory decision documents. EPA's ecological risk assessments are undergirded by the understanding that both Chitin and Chitosan are naturally occurring, ubiquitous, and regarded as essentially non-toxic to animals. Additionally noteworthy, applications of pesticides containing Chitin or Chitosan are not expected to result in concentrations of the compounds measurably greater than common background concentrations. Accordingly, the Agency anticipates that no additional ecological effects or environmental data are required for either Chitin or Chitosan.

Endangered Species Act: Endangered species assessments for Chitin and Chitosan are expected to be completed in October of 2007. The assessment documents will be added to the docket at that time. Potential commenters will also have full opportunity to make comments during the 60 day comment period when the assessment documents are posted in the docket for the *Proposed Registration Review Decision for Chitin* .

Human Health: Based on reviews of human health exposure and summary risk assessments that were conducted when the first pesticides containing these active ingredients were originally registered, the Agency anticipates that no additional human health effects data will be required for either Chitin or Chitosan. The reviews indicate that data were sufficient to fulfill all current biochemical pesticide toxicology data requirements for both Chitin and Chitosan. Moreover, all the data reviewed by the Agency indicate that both Chitin and Chitosan are so low in toxicity as to be considered virtually non-toxic to humans or animals. In further support of Chitin's and Chitosan's lack of toxicity, it is also noted that both ingredients are regularly present in many foods; and both have an exemption from the requirement of a tolerance – 40 CFR 180.1089 and 40 CFR 180.1072, respectively. In sum, the Agency anticipates that no new data or human health risk assessments will be necessary for either Chitin or Chitosan.

Incidents:

The OPP Incident Data System (IDS) indicates that there have been no reports of human and domestic animal incidents for products containing either Chitin or Chitosan. The Agency will consider any additional incidents data or comments submitted in response to this docket.

Efficacy and Label Claims

The Agency requires and reviews efficacy data for products making labeling claims to control public health pests. A review of the pesticide product labels containing Chitin and Chitosan

reveals no claims against public health pests. Accordingly, no efficacy data will likely be required. Notwithstanding, a full review of label claims remains incomplete pending the final registration review decision. As appropriate, registrants will be required to resolve any outstanding efficacy/labeling concerns in response to that final decision.

Timeline:

EPA has created the following estimated timeline for the completion of the Chitin and Chitosan Registration Review case. This schedule is subject to revision should there be a need for a Data Call-in during the registration review process or should other issues arise.

Activities	**Estimated Month/Year**
Open Public Comment Period for Chitin and Chitosan Docket	September 2007
Close Public Comment Period	November 2007
Develop Final Work Plan (FWP)	January 2008
Open Public Comment Period for Proposed Reg. Review Decision	FY 2008 3rd Quarter
Close Public Comment Period	FY 2008 3rd Quarter
Final Decision	FY 2008 4th Quarter
Total (years)	1

Guidance for Commenters:

The public is invited to comment on EPA's preliminary registration review work plan. Stakeholders are specifically asked to provide any additional information regarding use information and any incident data not already reported to the agency. In addition, the Agency welcomes any comments regarding EPA's risk assessments and its anticipated decision not to conduct further risk assessments. The Agency will carefully consider all comments as well as any additional information or data provided prior to issuing a final work plan for the Chitin and Chitosan case.

Water quality concerns for Chitin and Chitosan are expected to be non-existent since these pesticides are ubiquitous in nature and have a very low toxicity profile. Public comment is invited on any water quality concerns. Similarly, trade irritants aren't expected for this pesticide since it is exempted from the requirements for a tolerance in the U.S. Growers and other stakeholders are asked to comment on any trade irritant issues for this pesticide.

Next Steps:

After the comment period closes, the Agency will prepare a Final Work Plan for this pesticide.

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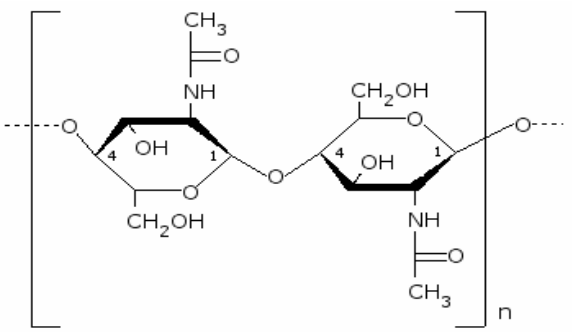
II. CHITIN FACT SHEET

Chitin Background Information:

- Registration review case number: 6063
- Chemical Name: Poly-N-Acetyl-D-Glucosamine
- PC Code: 128991
- CAS#: 1398-61-4
- Source material: Shells of crustaceans
- Sole (and original) registrant: Igene Biotechnology
- First approved for use in a registered product in 1988
- Not subject to reregistration (no Reregistration Eligibility Decision [RED])
- Exemption from The Requirement of a Tolerance: 40 CFR 180.1089; was reassessed September 12, 2003.
- Registration Review Lead: Chris Pfeifer; pfeifer.chris@epa.gov

Description of the Active Ingredient:

Chitin is a naturally occurring chain of glucose molecules that is structurally related to cellulose. It is ubiquitous in nature. Chitin is most commonly derived from crustacean shells, particularly from crabs and shrimp. Historically, it has been used as a food additive and a fertilizer. As a pesticide active ingredient, it acts by stimulating the growth of certain microorganisms in soil, which release substances that kill pathenogenic nematodes and their eggs. The compound is also reputed to play a role as a plant growth regulator by bolstering plant defenses against disease.



Chitin

Chitin Use Information:

- Chitin is used for controlling soil nematodes on crops (food and non-food), ornamentals and turf.
- Use sites are residential and commercial. They include: agricultural fields, nurseries, greenhouses, sod farms, commercial turf grasses, golf courses, home lawns and gardens.

- Application methods include: pre-plant soil incorporation and post-plant surface application followed by saturation.
- Mode of action: Chitin acts in soil to stimulate the growth of naturally occurring microorganisms in the soil, which then release substances that kill pathogenic nematodes and their eggs.
- Chitin is the active ingredient in CLANDOSAN 618, which is the sole registered pesticide product containing this active ingredient.

Recent Actions:

There have been no recent significant regulatory activities regarding the sole registered Chitin product (i.e. tolerance related actions, changes of use patterns, submission of toxicology studies or incident reports).

Ecological Risk Assessment Status:

Ecological effects for Chitin were fully considered in the course of reviewing the application for the first product containing this active ingredient (CLANDOSAN 618). A registration decision document, issued in March of 1988, concurred that Chitin posed negligible to non-existent ecological risk. In that decision document, the Agency granted data waivers for all nontarget data requirements relating to the application of the Chitin-based pesticide, CLANDOSAN 618. It was determined that under normal conditions, the proposed end uses would pose minimal hazards to nontarget organisms. EPA noted the following as grounds for a rationale: 1) historical data on Chitin demonstrating negligible toxicity on humans and animals; 2) a ubiquity of Chitin in nature such that applications of Chitin would likely fall within the existing range of background concentrations; and 3) the ability of Chitin to degrade. As a result of these considerations, the Agency does not anticipate the need for new data or the need to conduct a new ecological risk assessment for Chitin.

Endangered Species Act: An endangered species assessment for Chitin is expected to be completed in October of 2007. The assessment document will be added to the docket at that time. Potential commenters will also have full opportunity to make comments during the 60 day comment period when the assessment document is posted in the docket for the *Proposed Registration Review Decision for Chitin* (FY 2008, 3rd Quarter).

Human Health Risk Assessment Status:

All biochemical pesticide toxicology data requirements applicable to a human health effects determination for Chitin were considered and fulfilled for the Chitin-based pesticide CLANDOSAN 618 in 1988. Acute Oral Toxicity and Acute Eye Irritation studies specific to the pesticide were both accepted as Toxicity Category IV. The balance of the Tier I biochemical toxicology data requirements were satisfied through a public literature submission, which further supported the case for Chitin's low toxicity profile. Additional information used

in making a human health effects determination for Chitin included: 1) a bridging of data used to establish an exemption from the requirement of a tolerance for Chitosan; 2) an approval by the FDA for the use of Chitin as a food additive; 3) a history of unrestricted use of Chitin as a soil amendment, without a record of incident; and 4) a recognition that any exposure gains relative to pesticidal applications would be negligible, given Chitin's ubiquity in nature. Altogether, the aforementioned information provided sufficient grounds for section 3 registration and an exemption from the requirement of a tolerance for Chitin – 40 CFR 180.1089. As a result of these considerations, the Agency does not anticipate the need for new data or the need to conduct a human health risk assessment for Chitin.

Incidents:

The OPP Incident Data System (IDS) indicates that there have been no reports of human and domestic animal incidents for products containing Chitin. The Agency will consider any additional incidents data or comments submitted in response to this docket.

Labels and Products:

The sole registered product with Chitin as an active ingredient follows:
CLANDOSAN 618 (25% a.i.) EPA Reg. No. 54137-1 (formerly EPA Reg. No. 58200-9)

Labels for the above products can be obtained from the Pesticide Product Label System (PPLS) website: http://oaspub.epa.gov/pestlabl/ppls.home.

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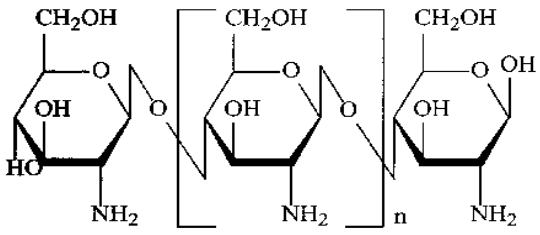
III. CHITOSAN FACT SHEET

Chitosan Background Information:

- Registration review case number: 6063
- Chemical Name: Poly-D-Glucosomine
- PC Code: 128930
- CAS#: 9012-76-4
- Source material: Chitin derived from the shells of crustaceans
- End-use product registrants: Plant Defense Boosters, Inc; Chem-tex, Laboratories, Inc.
- Chitosan was first approved for use as a Plant Growth Regulator (PGR) in a registered product in 1986.
- Chitosan was registered for use as an antimicrobial agent in a registered product in 2003.
- Not subject to reregistration (no Reregistration Eligibility Decision [RED])
- Exemption from The Requirement of a Tolerance: 40 CFR 180.1072; was reassessed July 3, 2002.
- Registration Review Lead: Chris Pfeifer; pfeifer.chris@epa.gov

Description of the Active Ingredients:

Chitosan is a naturally occurring chain of glucose molecules that is structurally related to cellulose. It is one of the most common compounds in nature. Commercially, Chitosan is prepared through the deacetylation of Chitin. Chitosan has several biomedical applications. It is considered to be a hemostatic agent that is hypoallergenic and is known to possess anti-bacterial properties. These properties also allow for its use as an active ingredient in anti-microbial pesticides. However, as a pesticidal active ingredient, Chitosan is best known as a plant growth regulator that boosts the ability of plants to defend against fungal infections.



Chitosan

* This diagram of the chemical structure of Chitosan is presented for illustrative purposes, and is intended primarily as a generalized point of comparison with Chitin. The actual chemical structure of Chitosan is subject to substantial variations based on the degree of deacetylation of Chitin.

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Chitosan Use Information:

- As a pesticide, Chitosan is used as a Plant Growth Regulator (PGR), fungicide and an antimicrobial agent.
- As a PGR, Chitosan's mode of action is characterized by its ability to bolster plant resistance against fungal diseases. Specifically, it purports protection against downy mildew, powdery mildew, early blight, late blight, gray mold, leaf spot, anthracnose and blast.
- PGR use sites include: agricultural fields, nurseries, greenhouses, commercial turf grasses, and home gardens (food and ornamental).
- As a PGR, it is produced in aqueous form and is applied by spray to plants throughout the growing season, with applications every one to two weeks as needed.
- Chitosan is the active ingredient in ELEXA-4, which is the sole registered PGR product containing this active ingredient.
- As an antimicrobial pesticide, Chitosan employs anti-bacterial properties to protect fabrics from bacterial and fungal growth.
- The antimicrobial pesticide is produced as an aqueous solution and is either applied by spray or added to laundry's wash cycle.
- Chitosan is the active ingredient in ChitoSante, which is the sole antimicrobial pesticide containing this active ingredient.

Recent Actions:

There have been no recent significant regulatory activities regarding either Chitosan product, (i.e. tolerance related actions, changes of use patterns, submission of toxicology studies or incident reports).

Ecological Risk Assessment Status:

As an active ingredient, Chitosan is employed in three distinct use patterns – as a PGR, as a fungicide on plants, and as an antimicrobial agent. Ecological effects were considered with regard to each use pattern.

With regard to Chitosan's initial use as a PGR, ecological effects were first considered in 1986 when the applicant Natural AG submitted a mix of general animal toxicity data and waiver requests to fulfill their non-target data requirements in support of EPA Reg. No. 56437-1. Subsequent registrations for Hyga, ELEXA, and ELEXA-4 were all granted waivers for their non-target requirements based on like rationales. The most recent review of non-target waiver requests occurred for ELEXA-4 in March of 2000. The review took place in the Biopesticides and Pollution Prevention Division (BPPD) and reflects the most current thinking on ecological effects relative to Chitosan. In that review, it was determined that under normal conditions, the proposed end uses would pose minimal hazards to non-target organisms. BPPD noted the following as grounds for its waivers: 1) copious amounts of historical data on Chitosan

demonstrating negligible toxicity on humans and animals; 2) a ubiquity of Chitosan in nature such that proposed application rates would likely fall within the existing range of background concentrations; and 3) the ability of Chitosan to decompose. As a result of these considerations, the Agency does not anticipate the need to conduct an ecological risk assessment for the use of Chitosan as a PGR, nor as a fungicide used on plants.

With regard to Chitosan's use as an antimicrobial agent, the Agency has also found that ecological risk is unlikely. An August 2007 memo from the Risk Assessment and Science Support Branch of the Antimicrobials Division notes the following in support of this position: 1) Most uses are indoors and allow for minimal environmental exposure. 2) Available information and the prevalent use of Chitosan in food and drugs support the case for Chitosan's relative nontoxicity. 3) Chitosan is a naturally occurring compound that is common in nature. In sum, the Agency anticipates that no ecological risk assessment will be necessary for Chitosan when it is used as an antimicrobial active ingredient on fabrics.

Endangered Species Act: An endangered species assessment for Chitosan is expected to be completed in October of 2007. The assessment document will be added to the docket at that time. Potential commenters will also have full opportunity to make comments during the 60 day comment period when the assessment document is posted in the docket for the *Proposed Registration Review Decision for Chitin* (FY 2008, 3rd Quarter).

Human Health Risk Assessment Status:

Human health effects were considered with regard to each use pattern of Chitosan. As a PGR and fungicide applied to plants, the Agency anticipates that no additional human health effects data will be required for Chitosan. Summary reviews indicate that data were sufficient to fulfill all current biochemical pesticide toxicology data requirements for Chitosan. Health effects data for Chitosan were first found sufficient for the registration of EPA Reg. No. 56437-1 and the issuance of an associated exemption from the requirement of tolerance in 1986. The exemption, found under 40 CFR 180.1072, was reaffirmed once more in a 1995 review. The review based its approval on the following information: 1) the original literature and data submitted in 1986 characterizing Chitosan's lack of toxicity; 2) approval by the FDA for use of Chitosan as a food additive; 3) an extensive history of human use and exposure, without record of incident; and 4) the relatively low application rates. With regard to the recent standards of the Food Quality Protection Act, a tolerance reassessment involved product specific toxicological data and all applicable biochemical pesticide toxicology data requirements. Studies were submitted and approved for all Tier I biochemical toxicology data requirements. Waiver requests were made and accepted for all other toxicological data requirements. The Agency completed tolerance reassessment for Chitosan on July 3, 2002. Accordingly, the Agency does not anticipate that a human health risk assessment will be needed for Chitosan when it is used as a PGR or as a fungicide used on plants.

With regard to Chitosan's use as an antimicrobial agent, the Agency has considered all

applicable toxicology data requirements. A July 2003 summary memo notes the following toxicological profile for the sole end-use product, Chitosante: Acute Dermal Toxicity, Acute Oral Toxicity, Acute Inhalation Toxicity and Skin Irritiation are accepted as Toxicity Category IV; Acute Eye is accepted as Toxicity Category III; and Chitosante is deemed a Non-sensitizer. Based on the toxicity profile of the end-use product and summary reviews characterizing the health effects of Chitosan, the Agency anticipates that no human health risk assessment will be necessary when Chitosan is used on fabrics as an antimicrobial active ingredient.

Incidents:

The National Pesticides Information Center (NPIC) database indicates that there have been no reports of human and domestic animal incidents for products containing Chitosan. The Agency will consider any additional incidents data or comments submitted in response to this docket.

Labels and Products:

The following products have Chitosan as an active ingredient:

ELEXA-4 (4% a.i.)	EPA Reg. No. 81045-2
ChitoSante (6% a.i.)	EPA Reg. No. 81446-1

Labels for the above products can be obtained from the Pesticide Product Label System (PPLS) website: http://oaspub.epa.gov/pestlabl/ppls.home.

4. Hamed, I., F. Ozogul, and J. M. Regenstein– 2016: Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. Trends in Food Science & Technology 48: 40-50.

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Review

Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review

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ABSTRACT

Background: Food processing produces large quantities of by-products. Disposal of waste can lead to environmental and human health problems, yet often they can be turned into high value, useful products. For example, crustacean shell wastes from shrimp, crab, lobster, and krill contain large amounts of chitin, a polysaccharide that may be extracted after deproteinisation and demineralization of the exoskeletons.

Scope and approach: This review summarizes the current state of knowledge of these crustacean shellfish wastes and the various ways to use chitin. This biopolymer and its derivatives, such as chitosan, have many biological activities (e.g., anti-cancer, antioxidant, and immune-enhancing) and can be used in various applications (e.g., medical, cosmetic, food, and textile).

Key findings and conclusions: Due to the huge waste produced each year by the shellfish processing industry and the absence of waste management which represent an environmental hazard, the extraction of chitin from crustaceans' shells may be a solution to minimize the waste and to produce valuable compound which possess biological properties with application in many fields. As a food waste, it is important to also be aware of the non-food uses of these wastes.

1. Introduction

Modern food production generates a large quantity of by-products most of which are still underutilized. Yet, these food wastes may often contain several usable substances of high value including some that may have important health benefits. Generation of waste during the processing of food is unavoidable and disposal can be one of the major problems for those industries and for society. Especially if not done properly it can have negative impacts on the environment (i.e., pollution), create risks to human health, and a loss of income to the waste generator. For example, the seafood processing industry produces a large quantity of by-products and discards (heads, tails, skins, scales, viscera, backbones, and shells). These residual materials may be an excellent source of proteins, lipids, pigments, and small molecules. In addition the shell materials may be a source of chitinous materials.

There has been an interest with respect to better use of chitin, which is the second most available polysaccharide after cellulose. It is a fairly ubiquitous compound produced by many organisms: fungi and algae cell walls, insects' exoskeletons, mollusks (endoskeleton of cephalopods) and crustaceans' shells. Annually, it has been estimated that on the order of 10^{10}–10^{11} tons are produced by living organisms (Revathi, Saravanan, & Shanmugam, 2012). However, commercially chitin is mainly recovered from marine sources, i.e., the crustaceans processing industries. In fact more than 10,000 tons could be available every year from shellfish waste (Merzendorfer, 2011), which would provide sufficient raw material if the appropriate commercial procedures for value-added processes were developed.

Chitin is obtained from crustaceans' exoskeletons after demineralization and deproteinisation treatments. However, one of the limitations in the use of this biopolymer on a large-scale is its water insolubility. Therefore, water-soluble derivatives have been produced. Chitosan is the most important of these. It is obtained after

Abbreviations: BHA, Butylated hydroxyanisole; BHT, Butylated hydroxytoluene; CN, Chitin nanofibrils; COS, Chitooligosaccharide; COX-2, Cytooxygenase-2; IL-6, Interleukine 6; LPS, Lipopolysaccharide; PGE2, Prostaglandin E2; ROS, Reactive oxygen species; TBARS, Thiobarbituric acid reactive substances; TBHQ, Tertiary butylhydroquinone; TNF-α, Tumor necrosis factor-α.

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0924-2244/

deacetylation of chitin and it is the only natural cationic polysaccharide known (Du et al., 2014). Chitin and its derivatives are renewable, biocompatible, biodegradable, and non-toxic compounds that have many biological properties such as: anti-cancer (Salah et al., 2013), antioxidant (Yen, Yang, & Mau, 2008), antimicrobial (Goy, de Britto, & Assis, 2009) and anti-coagulant (Vongchan, Sajomsang, Kasinrerk, Subyen, & Kongtawelert, 2003) properties. In addition, they are used as biomaterials in a wide range of applications: for biomedical purposes such as for artificial skin, bones, and cartilage regeneration (Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Parvez et al., 2012), for food preservation such as for edible films (Muzzarelli & Muzzarelli, 2005), and for pharmaceutical purposes such as for drug delivery (Riva et al., 2011).

The waste produced each year by the shellfish processing industries represent a practical challenge. With approximately 75% of the total weight of crustaceans (shrimp, crabs, prawns, lobster, and krill) ending up as by-products (Kuddus & Ahmad, 2013) and the current lack of acceptable waste management options there is a potentially large environmental hazard concern. Usually, seafood wastes are thrown away at sea, burned, landfilled, or simply left out to spoil (Xu et al., 2013). Therefore, the extraction of chitin from crustaceans' shells and its use as is or after further processing may be a way to minimize the waste and to produce valuable compounds with remarkable biological properties and crucial application in various fields.

This paper reviews the efforts to add value to crustacean shell wastes by producing high value substances, mainly chitin and its derivatives, which are starting to become used commercially.

2. Crustaceans processing shell wastes as a source of valuable products

The amounts of chitin from shell wastes vary with species (Table 1) and seasons, but, in general, the exoskeletons contain about 30–40% proteins, 30–50% minerals (mainly calcium carbonate), and 20–30% chitin along with others compounds such as pigments (e.g., astaxanthin) and lipids (Vani & Stanley, 2013; Hayes, 2011).

3. Chitin

After cellulose, chitin $(C_8H_{13}O_5N)_n$ is the second most abundant biopolymer on earth (Synowiecki & Al-Khateeb, 2003; Xu et al., 2013). Cellulose and chitin are polysaccharides with structural similarity; chitin has an acetamide group ($NH-CO-CH_3$) at C-2 in place of the hydroxyl group in cellulose (Thirunavukkarasu, Dhinamala, & Moses Inbaraj, 2011). Chitin can be obtained from various sources (Table 2), it is mainly found in the exoskeletons of arthropods (insects, crustaceans, and arachnids) and mollusks (beaks and endoskeleton of cephalopods). However, various microorganisms also produce chitin including the cell walls of fungi and yeasts, and the spines of diatoms (Sharp, 2013; Merzendorfer & Zimoch, 2003; Jothi & Nachiyar, 2012). The crustacean shells such as those from crabs and shrimps are the most important chitin source for commercial use due to the availability of wastes from the seafood processing industry (Kaur & Dhillon, 2013). Chitin gives living organisms a strong structure.

The first discovery of chitin was made in 1811 by Professor Henri Braconnot. The polysaccharide was isolated from mushrooms and was named fungine. Later, in 1823, Odier found the same substance in the exoskeleton of insects and named it chitin. This term comes from the Greek words "chiton" which means tunic or envelope (Souza, Almeida, Colwell, & Rivera, 2011; Ferraro et al., 2010). In 1859, Roughet discovered that chitin could be transformed into a water soluble form after chemical manipulation. After that in 1870, this modified chitin was named chitosan. Despite, the early discovery of chitin, the amount and quality of recent research has expanded the information available (Cheba, 2011).

Table 1
Examples of chitin content from different sources.

Organisms	Chitin content (%)	References
Crustaceans		
Nephro (lobster)	69.8[a]	Synowiecki & Al-Khateeb, 2003
Euphausia superb (krill)	24[a]	Arbia et al., 2013
Homarus (lobster)	60–75[a]	
Crangon crangon (Shrimp)	17.8[a]	
Lepas (goose barnacle)	58.3[a]	
Chionoecetes opilio (Crab)	26.6[a]	
Insects		
Blatella (cockroach)	18.4[a]	Kaur & Dhillon, 2013
Coleoptera (ladybird)	27–35[a]	
Diptera	54.8[a]	
Pieris (butterfly)	64.0[a]	
Bombyx (silk worm)	44.2[a]	
Galleria (wax worm)	33.7[a]	
Fungi		
Aspergillus niger	42.0[b]	Synowiecki & Al-Khateeb, 2003
Penicillium notatum	18.5[b]	
Penicillium chrysogenum	19.5–42[b]	
Saccharomyces gutulata	2.3[b]	
Mucor rouxii	9.4[b]	
Siboglinidae	33[c]	Crini, Guibal, Morcellet, Torri, & Badot, 2009
Cnidaria	3–30[d]	Crini et al., 2009
Brachiopod	4–29[a]	Crini et al., 2009
Mollusks	6–40[e]	Crini et al., 2009
Squid, cuttlefish, octopus		

[a] chitin % compared to the dry mass of exoskeletons.
[b] chitn % relative to the dry mass of mycelium.
[c] chitin % is given compared to the dry mass of tube worm.
[d] chitin % is given compared to the dry mass of membranes and egg capsules.
[e] chitin % is based on the dry mass of cuttlebone, pen and beaks.

Table 2
Chitin sources.

Kingdom/Phylum	Subphylum/Class	Groups/Species	References
Arthropods	Insects	Beetles, silkworm (*Bombyx mori*), *Aedes aegypti*,	Merzendorfer & Zimoch, 2003
	Crustaceans	Crabs, shrimps, lobsters, prawns, krill	Rinaudo, 2006
	Arachnids	Scorpions, spiders	Grupa & Cody, 2010; Rinaudo, 2006.
Mollusks	Gastropods	Opisthobranchia	Martin et al., 2007
	Bivalves		Weiss & Schönitzer, 2006
	Cephalopods	Squid pen beaks, cuttlefish bones	Jothi & Nachiyar, 2012; Sharp, 2013
Fungi	Eurotiomycetes	*Aspergillus niger*	Rinaudo, 2006
	Mucormycotina	*Mucor rouxii*	
	Saccharomycetes	*Candida albicans*	Walker et al., 2008
Algae	Bacillariophyceae	Diatoms	Rinaudo, 2006; Sharp, 2013.
	Phaeophyceae	brown algae	
	Chlorophyceae	green algae	

3.1. Chitin structure and physicochemical properties

Chitin, a cellulose-like polysaccharide (Fig. 1) is a linear, poly-β-(1,4)-N-acetyl-D-glucosamine (Vani & Stanley, 2013; Giyose, Mazomba, & Mabinya, 2010; Thirunavukkarasu et al., 2011; Dutta, Dutta, & Tripathi, 2004). Chitin occurs in nature as ordered crystalline microfibrils (Merzendorfer, 2006). It is found in three polymorphic forms: α chitin; β-chitin; and γ-chitin. α-Chitin is the most stable form of the three crystalline variations and is arranged in anti-parallel strands. It serves as the resistance structure in insect cuticles, shells of crabs, lobsters and shrimp, and in fungal and yeast cell walls. It is also found in marine sponges. The α configuration is the most abundant form found in nature. β-Chitin is less stable than the α form. The β form is arranged in parallel chains. It has been found in the extracellular fibers of diatoms, the pens of squid (Lavall, Assis, & Campana-Filho, 2007), and the spines and chaetae of certain annelids. The least common form is γ-chitin which is a mixed form, i.e., a combination of α and β structures. It has been found in the stomachs of squid and in the cocoons of two genera of beetles (Rinaudo, 2006; Souza et al., 2011; Cheba, 2011).

Chitin is a nitrogenous polysaccharide which in its pure state is white or yellowish. It is also odorless and tasteless. It has excellent biocompatibility and biodegradability (Ramírez et al., 2010; Cheba, 2011). Chitin is highly hydrophobic, and therefore it is insoluble in water and even most organic solvents. It can be solublized in a few solvents such as hexafluoroisopropanol, hexafluoroacetone, and chloroalcohols in conjunction with aqueous solutions of mineral acids, and dimethylacetamide containing 5% lithium chloride (Dutta et al., 2004). This limited solubility is believed to be due to the strong intermolecular hydrogen bonding that makes chitin a rigid material (Wilson & Omokanwaye, 2013).

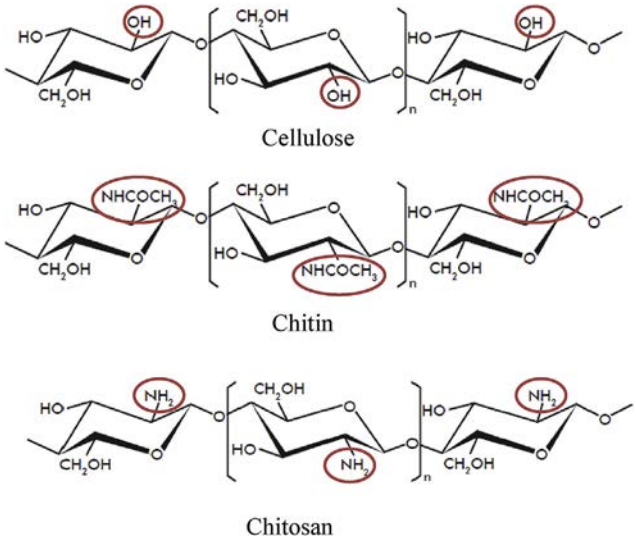

Fig. 1. Comparison between the chemical structures of cellulose, fully acetylated chitin and fully deacetylated chitosan (Ramírez et al., 2010).

3.2. Extraction of chitin

Chitin from crustacean shell wastes represents approximately half of the total weight of shellfish. During processing the heads, shells, and tails are considered inedible (Islam, Khan, & Tanaka, 2004). The uncontrolled dumping of this waste biomass has a negative impact on the environment. Due to their abundance, crustacean shells are considered the source of choice for chitin isolation (Ramírez et al., 2010). Two types of methods are used to obtain chitin: chemical and biological (microbial) methods (Fig. 2).

Despite the fact that chemical methods have many disadvantages (uneconomical, eco-unfriendly, and negatively affect the physico-chemical properties of chitin), the short processing time still makes it the most commonly used treatment commercially. Another drawback of chemical chitin purification is that the removed proteins and minerals, although potentially valuable supplements for human foods and animal feeds, are sufficiently damaged that they are no longer appropriate for these applications (Arbia, Arbia, Adour, & Amrane, 2013). Therefore, the interest in biological extraction is increasing since it is a safer and cheaper treatment for chitin recovery. But to date, it has been limited to laboratory scale studies (Kaur & Dhillon, 2013).

A comparative study (Table 3) done by Khanafari, Marandi, and Sanatei (2008) who used shrimp wastes to extract chitin and chitosan by chemical and microbial methods. They showed that the microbial method yields more chitin than the chemical method. Beaney, Lizardi-Mendoza, and Healy (2005) using prawn shells got similar results. They highlighted also the more environment friendly effects of the microbial methods.

3.2.1. Chemical methods

After obtaining the shells from different sources, they are washed, dried, and size reduced (crushed or grounded into a powder) (Abdou, Nagy, & Elsabee, 2008). The traditional chemical methods involve three steps: demineralisation, deproteinisation, and decoloration. The first step consists of processing the powdered raw material with an acidic treatment with HCl, the preferred reagent. The purpose is to remove mineral constituents (calcium carbonate and calcium phosphate). An alkali treatment is used for deproteinisation of the demineralized shells. Proteins are eliminated with NaOH. A decoloration step is added if a colorless product is wanted. Acetone or an organic solvent mixture are used to remove the pigments such as carotenoids (Kaur & Dhillon, 2013;

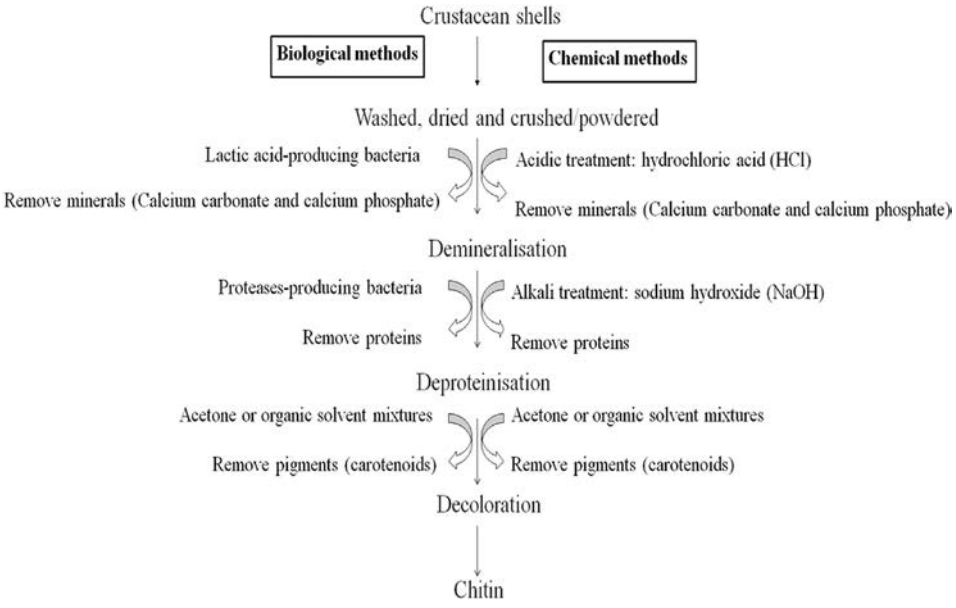

Fig. 2. Chitin extraction by chemical and biological methods.

Table 3
Comparison between chemical and biological methods for chitin extraction (Arbia et al., 2013; Kaur & Dhillon, 2013).

Extraction methods	Treatment	Advantages	Disadvantages	Quality of chitin
Traditional chemical	**extraction**	**Acidic treatment:** Demineralisation with decalcifying agents (HCl, HNO_3, H_2SO_4, CH_3COOH, and HCOOH) **Alkali treatment:** Deproteinisation with NaOH	- Used at the industrial scale for chitin preparation - Short processing time	Environmentally unfriendly, high cost because of the effluent treatment generated after acid and alkaline reagents. Removed proteins and minerals cannot be used as human and animal food supplements after being damaged by the acidic and alkali treatment
Despite the complete removal of organic salts, there can be	deacetylation and depolymerisation reactions			
Biological	**extraction**	**Lactic acid treatment**: Demineralisation with lactic acid-producing bacteria **Proteases treatments:** Deproteinsation with proteases-producing bacteria	- Environmentally safe, low cost because of the absence of effluent - Solubilized proteins and minerals may be used as human and animal nutrients	Limited to laboratory scale studies only
	Homogeneous and high quality final products			

Abdulkarim, Isa, Abdulsalam, Muhammad, & Ameh, 2013; Benhabiles et al., 2012; Mohammed, Williams, & Tverezovskaya, 2013).

3.2.2. Biological treatments

To avoid acidic and alkali treatments that could be a source of environmental problems, biological treatments offer an alternative way to extract chitin from crustacean shells. Lactic acid-producing bacteria have been used for demineralisation of crustacean shells. In fact, the lactic acid produced by bacteria reacts with the calcium carbonate component in the biomass waste resulting in the formation of calcium lactate, which can be precipitated and removed by washing (Arbia et al., 2013).

For the deproteinisation, proteases from bacteria will eliminate proteins. The biological treatment consists in a fermentation of the crustacean biowaste by different species of lactic or non-lactic bacteria such as *Pseudomonas aeruginosa* K-187, *Serratia marcescens* FS-3, *Bacillus subtilis* (Jo et al., 2008). Two other bacteria *Bacillus cereus* and *Exiguobacterium acetylicum* have shown high activity with both the deproteinisation and demineralization steps. The fermentation of shrimp shell wastes resulted in 97.1 and 92.8% deproteinisation, and 95 and 92% demineralisation, respectively (Sorokulova, Krumnow, Globa, & Vodyanoy, 2009).

4. Chitosan

Chitosan is the *N*-deacetylated derivative of chitin. Again, marine crustacean shells are widely used as the primary source for the production of chitosan (Venkatesan & Kim, 2010) from chitin (Varum & Smidsrod, 2012). Because chitosan is soluble in water, it is a more convenient polymer to work with (Ramírez et al., 2010). Chitosan has been widely used in different fields ranging from pharmaceuticals (Varshosaz, Jaffari, & Karimzadeh, 2006) through food industry and agriculture (Boonlertnirun, Boonraung, & Suvanasara, 2008) to medicine (e.g., artificial skin and orthopedic tissue-engineering) (Vani & Stanley, 2013).

4.1. Chitosan structure and physicochemical properties

Chitosan is a linear polysaccharide that contains copolymers of D-glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units) linked by β (1,4) glycosidic bonds. This biopolymer is obtained by the partial deacetylation of chitin (Fig. 1). Chitosan is considered to be polycationic, nontoxic, biocompatible, and biodegradable (Yuan, Chesnutt, Haggard, & Bumgardner, 2011; Zhang, Andresen, et al., 2010; Zhang, Xia, et al., 2010; Teng, 2011; Anaya, Cardenas, Lavayen, Garcia, & O'Dwyer, 2013).

The degree of deacetylation is generally defined as the glucosamine/N-acetyl glucosamine ratio, which goes up as chitin is converted to chitosan. Therefore, when the percentage of N-acetyl glucosamine is higher than glucosamine, the biopolymer is called chitin and when the percentage of glucosamine exceeds N-acetyl glucosamine the compound is called chitosan (Viarsagh, Janmaleki, Falahatpisheh, & Masoumi, 2010; Ramírez et al., 2010; Khor & Lim, 2003).

Chitosan is the only commercially available water soluble cationic polymer due to the positive charges on its amino groups. This unique property makes it appropriate for a wide range of applications in foods, cosmetics, and pharmaceuticals. The cationic biopolymer can interact with anionic molecules such as glycosaminoglycans (GAG) and proteoglycans. Many cytokines/growth factors are linked to GAG. A complex of chitosan-GAG may retain and concentrate those substances (Ferraro et al., 2010). Chitosan, due to the D-glucosamine, is insoluble at neutral and alkaline pH in aqueous solution but it is soluble in dilute acids (pH < 6–6.5) such as acetic acid, formic acid, succinic acid, lactic acid, and malic acid along with dilute HCl (No, Meyers, Prinyawiwatkul, & Xu, 2007; Sogias, Khutoryanskiy, & Williams, 2010; Tungtong, Okonogi, Chowwanapoonpohn, Phutdhawong, & Yotsawimonwat, 2012).

4.2. Deacetylation of chitin

Chitosan can be obtained by deacetylation of chitin through alkaline or enzymatic methods (Fig. 3). The purpose is to remove acetyl groups ($COCH_3$) however the N-deacetylation is almost never complete (Abdulkarim et al., 2013).

4.2.1. Alkaline methods

Raw materials obtained after chitin demineralization, deproteinisation, and discoloration are mixed with 40–50% NaOH. Chitosans with different degree of deacetylation are generated depending on the reaction temperature, time, and the concentration of the alkali solution (Teng, 2011; Yuan et al., 2011). The alkaline treatments hydrolyze the acetyl groups and transform the N-acetyl-D-glucosamine units into D-glucosamine units with free $-NH_2$ groups (Jung & Zhao, 2011). The alkali treatment can cause environmental pollution, requires significant energy, and produces poor quality chitosan. Therefore enzymatic methods could replace it to overcome those limitations (Teng, 2011; Raval, Raval, & Moerschbacher, 2013).

4.2.2. Enzymatic methods

Chitin acetyl groups are removed by chitin deacetylase. This enzyme was first found in *Mucor rouxii* in 1794 (Teng, 2011). Chitosan occurs naturally in the cell walls of some fungi. The presence of this enzyme activity had also been reported in some insects, presumably to allow them to hydrolyze some of the materials in their food supply. However, there are also some problems with the use of enzyme-producing fungi such as a low yield of deacetylase-producing strains, low enzyme activity, and fermentation requirements that are complicated. Therefore chitin deacetylase-producing bacteria (*Serritia* sp. and *Bacillus* sp.) may replace the current fungal strains (Zhou, Zhang, He, & He, 2010). Bacteria are easier and faster than fungi to grow in large-scale fermentation systems (Kaur, Dattajirao, Shrivastava, & Bhardwaj, 2012).

5. Chitooligosaccharides

Chitooligosaccharides (COS) are the depolymerized products of chitosan (Xu et al., 2008). Chitosans with degrees of polymerization less than 20 and an average molecular weight less than 3900 Da are called COS (Lodhi et al., 2014). During the preparation of chitosans, viscosity is generally used to estimate the molecular weight (Xia, Liu, Zhang, & Chen, 2011). There is an increasing interest in COS because they are more suitable for some industrial applications due to their low molecular weights, low viscosity, and short chain lengths (Harish Prashanth & Tharanathan, 2007; Zhang, Andresen, et al., 2010; Zhang, Xia, et al., 2010; Mourya, Inamdar, & Choudhari, 2011). Besides their water solubility properties, COS are also reported to have excellent biological properties: cholesterol lowering (Choi et al., 2012), antibacterial (Wang et al., 2007), antitumor, and immuo-enhancing effects (Xu, Jiang, et al., 2012; Xu, Lei, et al., 2012).

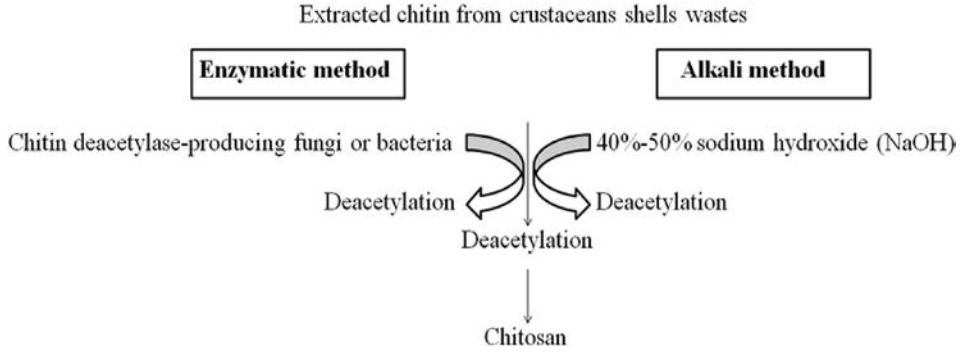

Fig. 3. Deacetylation of chitin to produce chitosan.

5.1. Depolymerisation of chitosan

The very high molecular weight and high viscosity of chitosan make it difficult to use in many applications. Hydrolyzed products of chitosan such as COS were found to be much easier to manipulate in large-scale commercial applications. To obtain those degradation products various methods have been developed: chemical and enzymatic (Fig. 4). Usually enzymes are preferred to chemical reagents, which are associated with higher environmental risks. Acceptable methods should be able to reduce the molecular weight of chitosan without altering its chemical structure.

5.1.1. Enzymatic methods

COS can be produced by specific enzymes such as chitosanases and non-specific enzymes such as cellulases, lipases, papain, lysozyme, hemicellulases, pectinases, pepsin, and pronase (Abdel-Azi et al., 2014). Chitosanases are widely present in nature. They have been found in bacteria, viruses, fungi, and plants (Zhang, Zhou, Wang, Wang, & Tang, 2013; Zhang & Zhang, 2013; Sinha, Tripathi, & Chand, 2012). Theses specific enzymes catalyze the cleavage of β-1,4-glycosidic linkages in chitosan in a random way to form oligomers (Zhang, Andresen, et al., 2010; Zhang, Xia, et al., 2010). However, there is a limitation to their use because of their high cost and lack of availability in large quantities (Rodriguez-Herrera et al., 2008). For that reason, researchers have looked for non-specific commercial enzymes that have been shown to degrade chitosan with essentially the same efficacy as chitosanases at a relatively lower cost (Struszczyk et al., 2009).

5.1.1.1. Non-specific enzyme for COS preparation: cellulase-treated chitosan. For the preparation of COS, cellulase has been shown to be able to hydrolyze chitosan (Wu & Tsai, 2004). The products were lower molecular weight polymers with increased water solubility and no modification of their chemical structures (Qin et al., 2004). Industrially these non-specific enzymes are cheaper, commercially available, and efficient (Feng, Gong, Du, & Huang, 2009). Cellulases are very common in nature. They are found in bacteria, fungi, and plants. The chitosanolytic activity is due to the similarity between chitin, chitosan, and cellulose which are all polymers of D-glucose linked by β-1,4-glycosidic linkages. In chitosan, the C-2 hydroxyl groups of cellulose are replaced by amino groups while in chitin acetamide groups are found at the same position. These latter groups do not seem to interfere with the enzyme–substrate complex (Xia, Liu, & Liu, 2008).

5.1.1.2. Specific enzymes for COS preparation: Chitosanase-treated chitosan. Chitosanases are hydrolases that catalyze the cleavage of the β-(1,4)-glycosidic bonds of chitosan. These enzymes have been found in microorganisms including viruses, bacteria, and fungi. Chitosanase activity has also been detected in some plants and animals (Ghinet et al., 2010; Kim & Ji, 2001; Cheng, Wang, & Hong, 2012). Despite the excellent results when used for COS production, the use of chitosanases is limited due to the high cost of their extraction, purification, and concentration. Therefore, the quantities produced are not sufficient to deal with the huge amount of marine shell wastes (Pagnoncelli et al., 2010).

5.1.2. Chemical methods

Chitosan can be chemically hydrolyzed by acidic or oxidative-reductive depolymerization. Acidic depolymerization is done using different reagents: HCl, hydrofluoric acid, nitrous acids, acetic acid, or sulfuric acid. However, the use of chemical solutions has been shown to have several disadvantages such as low yields of low molecular weight chitosan (COS) and the need to remove these strong acids which leads to environmental pollution (Zamani & Taherzadeh, 2010). Free radicals can also be used to reduce the molecular weight of chitosan. H_2O_2 or potassium persulfate degradation have been proved to decrease the viscosity of chitosan (Mourya et al., 2011).

6. Food applications

Chitin and its derivatives are known to have wide range of biological activities including antioxidant effects, antimicrobial effects, and many other properties that could be used in the food industry to improve food safety, quality, and shelf-life. Annually, over 80, 000 tons of chitin are produced from industrial wastes (Kim, Mendis, & Shahidi, 2007), especially from the seafood processing industry.

6.1. Antioxidant activity

Reactive oxygen species (ROS) such as H_2O_2, hydroxyl radicals, and superoxides lead to oxidative stress which is correlated with various pathologies: cancer (Manda, Nechifor, & Neagu, 2009), cardiovascular disease (Zhang, Andresen, et al., 2010; Zhang, Xia, et al., 2010), premature aging (Cui, Kong, & Zhang, 2012), rheumatoid arthritis, and inflammation (Filippin, Vercelino, Marroni, & Xavier, 2008). Chitin is among various compounds (vitamin C and

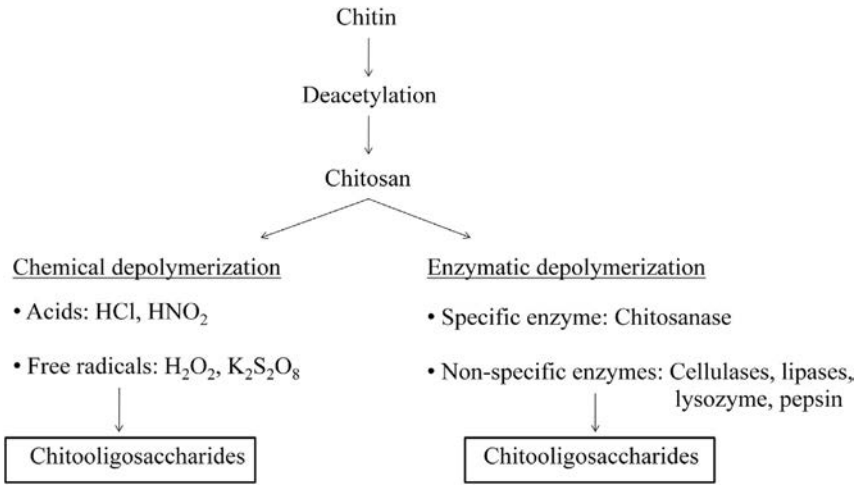

Fig. 4. Depolymerization of chitin and production of COS.

E) that have shown antioxidant effects (Park & Kim, 2010). Thus it could be added as an ingredient for the production of functional food which could prevent age-related and diet-related diseases (Kerch, 2015).

The oxidation of highly unsaturated food lipids causes off-favors and rancidity. Usually, synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used. However, because of potential health hazards associated with such compounds (Younes & Rinaudo, 2015), more safe and natural antioxidants have become preferred; especially if a lack of toxicity can be demonstrated (Harish Prashanth and Tharanathan, 2007). The addition of 1% chitosan resulted in a decrease of 70% in 2-thiobarbituric acid reactive substances (TBARS) values of meat after 3 days of storage at 4 °C. Chitosan's inhibition seems to be related to its chelation of the free iron that is released from the heme proteins of meat during heat processing (Tharanathan & Kittur, 2003).

Like meats, lipid oxidation of unsaturated fatty acids occurs in seafood. This oxidation is catalyzed by the high concentrations of pro-oxidants such as hemoglobin and metal ions in the fish muscle (No et al., 2007). The antioxidant effect of chitosan varies with its molecular weight, concentration, and viscosity as shown by Kamil, Jeon, and Shahidi (2002). Chitosans obtained from crab shell wastes were tested on herring flesh (*Clupea harengus*). Chitosan with different viscosity were used (360, 57, and 14 cP) to treat the fish samples. The corresponding viscosity average molecular weights were 1.8×10^6, 9.6×10^5, and 6.6×10^5 Da. All three biopolymers showed antioxidant effects, i.e., they lowered peroxide values, TBARS, and total volatile aldehydes. However, the highest activity was observed with the low viscosity chitosan (14 cP). These results were compared with those obtained with commercial antioxidants (BHA, BHT, and tertiary butylhydroquinone (TBHQ)) and it seemed that the 14 cP sample was more effective than the higher viscosity chitosan because of the similarity of its action with the conventional antioxidants one. Chitosans may retard lipid oxidation by chelating ferrous ions present in the fish model system, thus eliminating prooxidant activity of ferrous ions or preventing their conversion to ferric ion (No et al., 2007).

Kim and Thomas (2007) also observed similar results with chitosan with different molecular weights (30, 90, and 120 kDa) in Atlantic salmon (*Salmo salar*). The antioxidant activity was measured as decreased TBARS and increased 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity. The scavenging effect of chitosan was compared to that of BHT and an equivalent efficiency of 85% was obtained. Moreover, the lowest molecular weight chitosan (30 kDa) showed the highest activity.

6.2. Food preservatives

Chitin and its derivatives can be used in the food industry as food preservatives (Sethulekshmi, 2014; Barikani, Oliaei, Seddiqi, & Honarkar, 2014). They have an antimicrobial activity which allows them to protect foodstuff from microbial deterioration. The antibacterial activity of chitinous materials has been linked to their positive charges which interact with the negative charges of bacterial cell walls leading to leakage of the intracellular molecules of the microorganisms (Khoushab & Yamabhai, 2010). The addition of chitosan to food inhibits microorganisms' growth and avoids poor appearance, off flavors, and economic losses. El-Diasty, Nesreen, and Hoda (2012) noticed that by adding chitosan to cheese it improved its mycological quality. Mould and yeast growth were inhibited and shelf-life was extended.

6.3. Antimicrobial packaging

The antibacterial properties of chitosan have also been used as active edible packaging (Muzzarelli & Muzzarelli, 2005). Biofilms have been formed from chitosan that allow long term storage of food products. Antimicrobial coating of vegetables, fruits, grains, and fish retard microbial invasion as chitosan acts as a protective barrier to enhance the sensory and nutritional quality of the food (Sinha, Chand, & Tripathi, 2014; Aranaz et al., 2009). Besides being a protective barrier, edible biopolymer films can be used as carriers of bioactive compounds to enhancing food quality. Polymeric bioactive films could be combined with different antimicrobial agents such as organic acids, bacteriocins (nisin and lacticin), plant extracts (thymol, p-cymene, and cinnamaldehyde), proteins (e.g., conalbumin), antibiotics, fungicides, and chelating agents (EDTA) to reduce food spoilage by pathogenic microorganisms and enhance shelf-life (Dutta, Tripathi, & Dutta, 2012). Chitosan-based edible films are biodegradable and can be consumed along with the product in the package. Moreover, they form transparent films with good mechanical properties that constitute a shell protecting the appearance and the quality of the food inside (Bourbon et al., 2011; Park, Byun, Kim, Whiteside, & Bae, 2013).

6.4. Dietary fiber

Chitosan and COS have been used as a source of dietary fiber (Xia, Liu, Zhang, & Chen, 2010). They are considered to be functional foods because of their non-digestibility by intestinal enzymes, which allows their as prebiotics. They stimulate beneficial bacteria in the gastrointestinal tract (*Bifidobacterium* and *Lactobacillus* sp.) (Morganti, Morganti, & Morganti, 2011; Harish Prashanth & Tharanathan, 2007). They can also act as thickener and stabilizing agents (Rinaudo, 2006; Arvanitoyannis, 2008).

7. Other applications

Table 4 summarizes the potential uses of chitin and its derivatives. Due to their properties (biocompatibility, biodegradability, nontoxicity, and gel-forming) and biological activities (antimicrobial, immunological, and antioxidant) these biopolymers can be considered as a novel source of functional materials.

7.1. Pharmaceuticals

For the delivery of pharmaceutical ingredients chitin, chitosan, and their derivatives have been used. Due to their biocompatibility, biodegradability, and low toxicity they are highly valuable polymer which can encapsulate pharmaceutical actives, protect them against degradation, and permit their release over a prolonged period (Dash et al., 2011). Different polymeric carrier systems such as nanoparticles, matrices, microsphere, hydrogels, capsules, or micelles have been investigated (Bansal, Kumar Sharma, Sharma, Prakash Pal, & Malviya, 2011). The release of the active agents usually occurs by diffusion through the polymer, by degradation of the polymer, or by disorganization of the supramolecular structure of the delivery system (Coelho et al., 2010). The Delivery of gene and drugs has been realized over the past years. Chitosan is one of the most commonly studied polymers among non-viral vectors for gene delivery (Lee et al., 2001). It is considered as a good candidate for gene transfection due to electrostatic interaction between cationic chitosan and negatively charged DNA (Sinha et al., 2004). Many nucleic acid delivery vehicles for gene therapy have been studied. For instance, viral vectors have been widely used for encapsulation of genes. However, these carriers have some side effects (oncogenic, immunogenic, and cytotoxic) (Mazumder, Zahir,

Table 4
Application fields and potential uses of chitin and its derivatives.

Fields	Examples of application	References
Pharmaceuticals	- **Pharmaceutical ingredients' carriers**	Dash et al., 2011
	- **Non-viral vectors for gene delivery**	Riva et al., 2011
	- **Gene and active constituents' encapsulation and protection**	Sinha et al., 2004
	- Gradual release of drugs	Lee et al., 2001
	- **Improve the arrival of nucleic acids to the therapeutic targets**	
Biomedical	- **Scaffolds fabrication for tissue regeneration**	Yang, 2011
	- Barrier of chitin-based dressing to microbial infections	Jayakumar et al., 2010 Pillai et al., 2009
	- Surgical stitches development	Xin-Yuan & Tian-Wu & Tsai, 2004
Cosmetic and dermatological	**Active cosmetic product transporters**	Morganti et al., 2008 Gautier et al., 2008
	Skin, hair, and oral care products	
	Moisturizer and anti-aging agents	Dutta et al., 2004 Khoushab & Yamabhai, 2010 Aranaz et al., 2009
Foods	Protective barrier against food spoilage	
	Food preservative additives and biofilm formation (edible packaging)	Harish Prashanth & Tharanathan, 2007
	Stabilizers and thickeners compounds	
	Prebiotics (dietary fibers) ingredients	Arvanitoyannis, 2008
Textiles	- Antimicrobial and non-allergenic fibers	Pillai et al., 2009
	- **Coagulant and flocculant agents for dyes and chemicals removal from textile wastewaters**	Abu Hassan et al., 2009 Dutta et al., 2004
	- Chelating agent that scavenges heavy metals from polluted water	
Agriculture	- Seeds-coating agents	Abu Hassan et al., 2009 Dutta et al., 2004
	- Enhancing of plant defenses and protection against pests and microorganisms	
	- Chemical pesticides replacement	Zeng et al., 2012
	- **Seeds germination acceleration**	
Paper	- **Confer strength to paper against moisture**	Dutta et al., 2004
	- Biodegradable packaging for food wrapping	Cheba, 2011
Enzymes-**immobilization**	- Enzymes immobilization support on chitin-and chitosan-based materials (wine, sugar, and wastewaters treattnent for pollutant removal)	Krajewska, 2004.
	- **Biosensors manufacturing for in situ measurements of environmental contaminants and metabolite control in artificial organs**	

& Zaman, 2014). In addition Coating of phannaceuticals with chi-tosan and its derivatives are used to provide controlled release of many drugs (anti-cancer, antibiotic, anti-inflammatory, anti-dia-betic, and gastro-intestinal agents) (Sinha et al., 2004). The active ingredients are more efficient with a decrease of side effects and gradual release of active constituents (Park & Kim, 2010).

7.2. Biomedicals

Chitin and chitosan exist in different applied forms (hydrogels, membranes, nanofibers, beads, micro/nanoparticles, scaffolds, and sponges) and are used in numerous types of biomedical applica-tions such as wound healing, tissue engineering, and in lenses fabrication for ophthalmology (Xu, Jiang, et al., 2012; Xu, Lei, et al., 2012; Kavitha, Keerthi, & Tamizh Mani, 2011; Yang, 2011; Wan & Tai, 2013; Kumar et al., 2011).

Biopolymers have been used to develop biological substitutes for damaged tissues, and to restore, maintain and improve bio-functions (Dash et al., 2011). Chitin and its derivatives have been used as membranes and scaffolds (three dimensional porous structures) for tissue regeneration (bones, cartilage, livers, kidneys, and nerves) (Dhandayuthapani, Yoshida, Maekawa, & Kumar, 2011; Patel, Bonde, & Srinivasan, 2011; Diban & Stamatialis, 2014; Sudha, Aisverya, Rose, Jayachandran, & Kim, 2013).

Wound healing can be delayed because of the antibacterial resistance of microorganisms (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010). As a consequence of the infection, wounds are un-able to heal properly and leave disfiguring scares. A chitin-based dressing has been shown to protect burns and wounds from bac-terial infection. Chitin and its derivatives act as artificial skin ma-trixes that are able to promote a rapid dermal regeneration (Khor & Lim, 2003). After being applied for burn treatments, chitosan is degraded by endogenous enzymes. This is a major advantage as removing the wound dressing can cause damage and trauma to the injuries (Dutta et al., 2004). Chitin and its derivatives are an ideal dressing as they moisten the wound interface, are permeable to oxygen and permit gaseous exchange, act as a barrier to microor-ganisms, and are capable of removing excess secretions (Azuma et al., 2015).

Chitin and its derivatives possess unique properties such as immunological compatibility, optical clarity, transparency, gas permeability, wettability, mechanical stability, and flexibility that can be exploited for contact lenses fabrication (Thomas & Thomas, 2013). Chitosan has replaced synthetic polymers in ophthalmo-logical applications as it ensures security and comfort during use (Dutta et al., 2004). Besides, due to their antimicrobial and wound healing characteristics, chitosan-based lenses are useful for injured eyes (Wysokowski et al., 2015).

7.3. Cosmetic and dermatological applications

Chitin has been used as an active carrier for cosmetic products. In a study conducted by Morganti et al. (2008), chitin-nanofibrils (CN) were embedded with antioxidant ingredients (melatonin, lutein, and ectoin). CN were able to improve the penetration of the active ingredients through the skin layer. Due to their radical scavenging activity, the combination of chitin and antioxidant components could be used to prevent the hannful effects of solar radiation responsible for photo-aging and wrinkles. Tests with volunteers showed a tolerance for the CN, the absence of erythema, and improved skin barrier functions. These biopolymers are able to retain water and maintain a permeability barrier at the skin's sur-face. Chitin polymers are used in skin care (creams, lotions, nail lacquers, and makeup), hair care (shampoos, hair colorant, and hair spray) and oral care (toothpaste, mouthwashes, and chewing gum) products (Gautier, Xhauflaire-Uhoda, Gonry, & Pierard, 2008; Dutta et al., 2004). CN are readily used in cosmetic dermatology as they have a backbone similar to hyaluronic acids, which means that

endogenous enzymes can metabolize the CN (Morganti, 2009).

7.4. Textile applications

Chitinous compounds have been interacted with natural fibers, such as cotton and wool, and synthetic fibers such as polyesters (Gao & Cranston, 2008). Due to their water-solubility and bio-adhesiveness along with their pos1t1ve charges, and their biocom-patibility, biodergradability, non-toxicity, and antimicrobial activ-ity, it has been suggested that they could be used as an antimicrobial in textiles (Mourya, Inamdar, & Tiwari, 2010; Grupta, 2007). Growth of microorganisms such as bacteria and fungi on textiles can decrease the fabric mechanical strength and cause unpleasant odors not only on the textile itself but on the wearer (Liu, Ren, & Jie Liang, 2015). Chitin fibers, which are known for their anti-microbial and non-allergenic properties, have also been used in clothes fabricated for babies and the elderly, both of whom have sensitive skin (Pillai, Paul, & Sharma, 2009; Chen, Wang, Hua, & Du, 2007).

Wastewater treatment is another emerging application of chi-tosan and its derivatives in the textile industry. These biopolymers are considered as eco-friendly coagulants compared to synthetic coagulants and flocculants. The textile industry is among the most polluting because the industry generates colored wastewaters that contain dyes, textile auxiliaries, and other chemicals. Besides, due to its polycationic nature, chitosan can be used as flocculating and chelating agents to scavenge heavy metals (Abu Hassan, Pei Li, & Noor, 2009; Dutta et al., 2004).

7.5. Agriculture

Chitosan has been used as a seed-coating agent to control pests and improve plant defenses against microorganisms (Zeng, Mei, & Wu, 2010). Positive results have been obtained in a study conducted by Mahdavi and Rahimi (2013). They tested the effect of chitosan on germination and growth of ajowan (Trachyspermum ammi). Stim-ulation of germination and growth was observed with a decrease in the harmful impact of abiotic stress such as high salinity. Sigriifi-cance benefits were observed for soybean yield, seed germination, and plant growth in another study of Zeng, Luo, and Tu (2012) in which soybeans were coated with chitosan. Usually chemical pes-ticides are used against crop pests but because of their high toxicity and the possibility of the development of resistance against them, chitin and its derivatives might be used instead (Badawy & Rabea, 2011). The plant protective properties of chitin, chitosan, and their derivatives are highly desirable as they offer protection against fungi, viruses, bacterial diseases, and nematodes (Ramirez et al., 2010).

8. Conclusions

Chitin is readily available since it is the second most abundant polysaccharide after cellulose on Earth. Chitin, chitosan, and their derivatives are valuable compounds that meet the needs of con-sumers for natural products that impact positively on health. There is a great deal of interest in these polysaccharides due to their appealing properties of biodegradability, biocompatibility, and non-toxicity. Therefore, they can be used in a wide range of in-dustrial applications in biomedicines, pharmaceuticals, agriculture, foods, cosmetics, textiles, and enzynies-immobilization. This versatility made them valuable and provides a potentially lucrative outlet for seafood processing wastes. They also provide highly valuable components with health benefits such as anti-microbial, antioxidant, and anti-inflammatory effects. Besides chitin and its derivatives are eco-friendly. The shellfish processing industries are a large source of by-products, predominantly chitin. Despite their potential value, crustacean shell wastes are still underutilized and their use in all of the above applications needs to be further researched. At the same time the various industries need to be encouraged to incorporate many of these applications commercially.

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5. Jones; EPA; 2019-Aug; (EPA) Risk Assessment Branch, Biopesticides & Pollution Prevention Division (7511P); Science Review in Support of the Addition of Chitosan (Poly-D-Glucosamine) to the list of minimum risk pesticides (MRPs) contained in 40 CFR 152.25(F)

Active Ingredient(s): Chitosan
PC Code(s): 128930
Type of Review: Product Chemistry, Human Health, Non-target Organisms and Environmental Fate

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MEMORANDUM

DATE: 08/23/2019

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

SUBJECT: Science review in support of the addition of Chitosan (Poly-D-Glucosamine) to the list of minimum risk pesticides (MRPs) contained in 40 CFR 152.25(f)

Chemical Class:	Biochemical
PC Code:	128930
CAS Number:	9012-76-4
Active Ingredient Tolerance/Exemption:	40 CFR 180.1072

FROM: Russell S. Jones, Ph.D., Senior Scientist
Risk Assessment Branch
Biopesticides & Pollution Prevention Division (7511P)

THROUGH : Shannon L. Borges, Chief
Risk Assessment Branch
Biopesticides & Pollution Prevention Division (7511P)

TO: Linda Hollis, Chief
Biochemical Pesticides Branch
Biopesticides & Pollution Prevention Division (7511P)

ACTION REQUESTED

Tidal Vision Products, LLC submitted a petition requesting the addition of Chitosan (Poly-D-Glucosamine) to the list of minimum risk pesticides (MRPs) contained in 40 CFR 152.25(f). In support of the petition, the applicant submitted a letter outlining the seven criteria that were considered in the creation of the MRP list and how Chitosan fulfills each of the seven criteria.

Active Ingredient(s): Chitosan
PC Code(s): 128930
Type of Review: Product Chemistry, Human Health, Non-target Organisms and Environmental Fate

BACKGROUND

Chitosan (Poly-D-Glucosamine; Figure 1) is a de-acetylated form of the naturally-occurring polysaccharide, Chitin (Poly-N-Acetyl-D-Glucosamine; Figure 2). Both substances are comprised of chains of glucosamine molecules that are structurally-related to cellulose (Figure 3). Chitin is a biopolymer second only to cellulose (Figure 3) in worldwide abundance. Chitin is found in numerous organisms, the most plentiful of which are the shells of marine crustaceans; it is also found in insect exoskeletons and fungal cell walls (FDA GRN No. 443, 2012; review by Kurita, 2006). Chitosan also occurs naturally and is formed by chitin deacetylases that have been identified in marine bacteria, fungi, and insects (review by Zhao et al., 2010). Chitosan is a heterogeneous substance and, depending on the degree and duration of deacetylation during the manufacturing process, can be defined as chitin that has been sufficiently deacetylated to form soluble amine salts (MRID 47362201 and references therin).

Chitin is the sole raw material used for the commercial manufacture of Chitosan. The largest commercial sources of Chitin are marine crustaceans (e. g., shrimp, crab, prawn, lobster, and krill) obtained from the world oceans as a food source, with about 75% of the total weight ending up as seafood "waste" products which are disposed at sea, burned, landfilled, or left to spoil (Bellich et al., 2016; Hamad et. al., 2015; Younes and Rinaudo, 2015). Therefore, the commercial extraction of chitin and chitosan and their conversion into commercial products provides a pathway for reducing this waste stream in the environment.

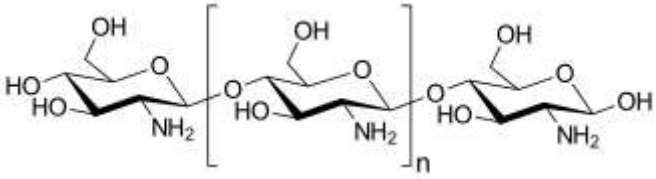

Figure 1. Structure of Chitosan

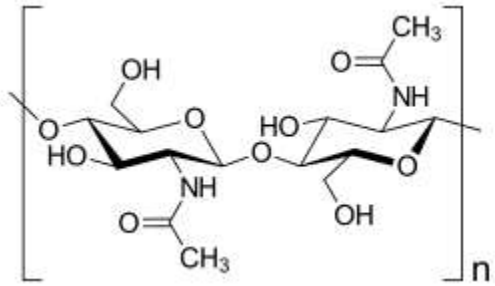

Figure 2. Structure of Chitin

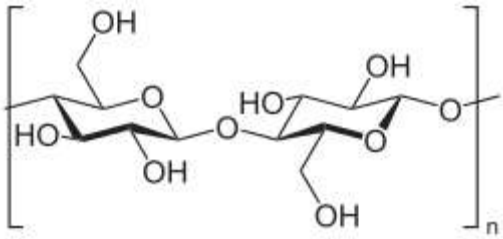

Figure 3. Structure of Cellulose

Extraction of Chitin and its conversion to Chitosan: The percentages of the three major components of crustacean shells are variable, depending upon species and seasons, but they are comprised of calcium

carbonate (20%–50%), proteins (20%–40%) and chitin (15%–40) (review by Bellich et al., 2016; Bolat et. al. 2010; Hamad et. al., 2015; Hong et. al., 1989). Chitin is first extracted from crushed/powdered crustacean shells by (1) demineralizing the shells enzymatically (via lactic acid) or with hydrochloric acid to remove calcium carbonate and calcium phosphate; (2) treatment with proteases or with sodium hydroxide to remove proteins; and (3) a decolorization extraction with organic solvents to remove pigments (e. g., carotenoids). Chitosan is prepared from chitin by de-acetylation, either enzymatically (via naturally-occurring deacetylases) or by treatment with 40-50% sodium hydroxide (review by Hamed et. al., 2016).

IA. FACTORS CONSIDERED FOR AMENDING 40 CFR 152.25 TO EXEMPT SUBSTANCES FROM REGULATION UNDER FIFRA (61 Federal Register 8876, March 6, 1996)

The following seven factors described at 61 FR 8876 (March 6, 1996) are considered when substances are added to the list of substances exempted from FIFRA requirements as pesticides:

1. Whether the pesticidal substance is widely available to the general public
2. If it is a common food or a constituent of a common food
3. If it has a nontoxic mode of action
4. If it is recognized by the Food and Drug Administration as safe
5. If there is no information showing significant adverse effects
6. If its use pattern will result in significant exposure
7. If it is likely to be persistent in the environment.

Additionally, this FR notice clarified that the factors are not meant to be absolute criteria and certain ones may be unsupported for some substances. However, taken as a whole, the EPA believes that these factors indicate that substances proposed for this list will not pose a risk that warrants regulation under FIFRA.

IB. THE FOLLOWING SPECIFIC CRITERIA WERE USED TO QUALIFY SUBSTANCES TO BE EXEMPT FROM THE REQUIREMENTS OF FIFRA, AND THEIR APPLICATION TO CHITOSAN

1. <u>Widely available to the general public</u>.

   Chitosan has a wide range of agricultural. biopharmaceutical, biomedical, cosmetic, textile, and food additive applications (review by Bellich et. al., 2016; review by Hamad et. al., 2016; US

EPA, 2007). Chitosan is used as a food preservative to inhibit microorganism growth and extend shelf life (El-Diasty et. al. 2012); as a component of edible, biodegradable, antimicrobial films applied to foods and food packaging (Sinha et. al., 2012). As an antimicrobial pesticide, chitosan employs anti-bacterial properties to protect fabrics from bacterial and fungal growth (US EPA, 2007). It also is used in flocculating agents for water and waste treatment, chelating agents for removal of traces of heavy metals from aqueous solutions, coatings to improve dyeing characteristics of glass fibers, wet strength additives for paper, adhesives, photographic and printing applications, thickeners, and fibers and films (review by NTP, 1999). Chitosan has several biomedical applications and is a hemostatic agent that is hypoallergenic and is known to possess anti-bacterial properties (US EPA, 2007), and it also is useful in stimulating plants to resist pathogens. The diverse routes of exposure to the general public via agricultural. biopharmaceutical, biomedical, cosmetic, textile, and food additive applications demonstrates that chitosan is a common substance in the environment. The lack of reports of adverse human health effects, despite the widespread exposure to chitosan, supports the conclusion that there are minimal risks to humans and the environment if chitosan is to be used as a minimum risk pesticide.

2. Common foods or constituents of common foods.

Although chitosan is not a food, it has numerous food related uses. It may be present in foods as a food preservative to inhibit microorganism growth and extend shelf life (El-Diasty et. al. 2012); as a component of edible, biodegradable, antimicrobial films applied to foods and food packaging (Sinha et. al., 2012); as a dietary supplement and excipient in medicines (NTP, 1999); and numerous uses in dairy production (Evdokimov et. al., 2015). Chitosan has been widely used as an over-the-counter dietary supplement as a putative “fat blocker” and as a diluent and vehicle for orally administered medicines (NTP, 1999) with no reported adverse health effects. Chitosan was granted an exemption from the requirements of tolerances on all food commodities in 1995, and this decision was re-assessed in 2002; no changes were made to the tolerance exemption at that time.

3. Nontoxic mode of action.

In agricultural practice, Chitosan acts as an induced resistance promoter characterized by its ability to stimulate internal mechanisms of treated plants to resist plant pathogens (US EPA, 2007). As an antimicrobial pesticide, Chitosan employs antibacterial properties to protect fabrics from bacterial and fungal growth (US EPA, 2007). Antibacterial modes of action may include, but are not limited to, disruption of bacterial cell membranes or cell wall integrity, inhibition of respiration, interactions with bacterial DNA/RNA, and/or physical deposition (smothering) (review by Malerba and Cerana, 2016). No known adverse effects have been reported for humans and other non-target organisms following agricultural, biopharmaceutical, biomedical, cosmetic, textile, and food additive applications of products containing chitosan.

4. Recognized by the Food and Drug Administration as safe.

A search of the FDA inventory of Generally Recognized As Safe (GRAS) Notices indicates that Chitosan does not have FDA GRAS status under 21 CFR 170.36. Three notices were made to FDA's CFSAN/Office of Food Additive Safety for shrimp-derived Chitosan (GRN Nos. 73, 170, and 443) but FDA ceased to evaluate the notices at the request of the notifier in 2002, 2005, and 2013, respectively. One notice was made to FDA's CFSAN/Office of Food Additive Safety for Chitosan from *Aspergillus niger* (GRN No. 397) in 2011, for which FDA had no questions. As of the 06/14/2019 update of the FDA GRAS Notices web page, FDA has not made a final determination on the GRAS status of the GRN Nos. 73, 170, 397, and 443. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=Chitosan) (Accessed 08/15/2019).

5. No significant adverse effects to humans or the environment.

Chitosan has minimal acute toxicity and is classified in Toxicity Category IV for acute oral toxicity, acute dermal toxicity, acute inhalation toxicity, acute eye irritation, and acute dermal irritation; it is not a skin sensitizer (EPA, 2016). No toxic effects were observed in a six-month study wherein male and female rats were dosed with up to 5200 mg Chitosan/kg-bw in the diet, although decreased fat digestion and depletion of some fat-soluble vitamins was reported (NTP, 2017); this observation is consistent with the dietary supplement use of Chitosan as a "fat blocker." In addition, chitosan has been shown to have anti-genotoxic and anti-carcinogenic properties (NTP, 1999). Intentional oral ingestion of chitosan containing foods, dietary supplements, and medicines has been estimated to be up to 20 g per person per day (NTP, 1999).

Although the source material for chitosan (chitin) is obtained from shellfish, industrially-manufactured chitosan is not likely to have allergenicity concerns, provided that all animal proteins are removed during the extraction and purification process from chitin. The harsh manufacturing process that involves demineralization with hydrochloric acid, protein removal with sodium hydroxide and a final extraction with organic solvents (review by Hamed et. al., 2016) is likely sufficient to remove and/or denature any proteins, fats and other contaminants of allergenic or other toxic concern (review by Muzzarelli, 2010). There are few credible reports of allergic responses to Chitosan exposure. One study in Japan noted that a "47-year-old female developed systemic urticaria and difficulty breathing" following oral ingestion of a chitosan-containing dietary supplement, but the purity of the Chitosan used in the study was unclear, and it appeared to be derived from a non-shellfish source (Kato et. al., 2005). Conversely, it has more recently been reported that oral administration of chitosan has been demonstrated to protect mice against peanut-induced allergic response (Bae et. al., 2013). Widespread use of chitosan in agricultural, pharmaceutical, biomedical, cosmetic, textile, and food additive applications (review by Bellich et. al., 2016; review by Hamad et. al., 2016; NTP, 1999; Sinha et. al., 2012; US EPA, 2007) without reports of adverse effects, indicates that there is little concern for allergenic response following exposure to highly purified chitosan.

No adverse effects are expected when non-target organisms are exposed to chitosan. Chitosan is produced naturally in several species of marine bacteria, fungi, and some insects and may serve as a protective mechanism against pathogen infection (Zhao, et. a., 2010). Positive effects on plant growth and the suppression of plant pathogens in the soil rhizosphere and plant foliage have been reported by numerous authors (reviews by Hassan and Chang, 2017; and Malerba and Cerana, 2018). A search of the EPA's Incident Data System database did not reveal any human health or ecological incidents pertaining to the use of chitosan as a pesticide.

6. Negligible human or environmental exposure.

Non-pesticidal exposure of humans and the environment to Chitosan is likely to far exceed exposure from pesticidal sources. Non-pesticidal exposure to Chitosan is relatively widespread and continuous based on a diverse range of uses in agriculture, pharmaceuticals, medicine, cosmetics, textiles, as a food additive (review by Bellich et. al., 2016; El-Diasty et. al. 2012) and as an anti-microbial agent (US EPA, 2007).

In the Chitin and Chitosan Summary Document for Registration Review (US EPA 2007), it was determined that based on reviews of human health and non-target organism exposure, and the summary risk assessments that were conducted when the first pesticides containing these active ingredients were originally registered, the Agency anticipates that no additional human health effects or non-target organism data will be required for either Chitin or Chitosan. The reviews indicate that data were sufficient to fulfill all current biochemical pesticide toxicology data requirements for both Chitin and Chitosan and that that no new data or human health risk and environmental risk assessments will be necessary for either Chitin or Chitosan.

7. Non-persistence.

The environmental fate of chitosan (poly-D-glucosamine) has not been thoroughly investigated, although many soil types have been reported to contain chitosan-degrading bacteria. The apparent ubiquitous presence of Chitosan-degrading bacteria in diverse soil types indicates that Chitosan may be readily degraded in soils and may be important it the biocycling of chitin and chitin derivatives in the environment (Davis and Eveleigh, 1984; Sawaguchi et. al., 2015; review by Somashekar and Joseph, 1996). Few studies were identified that assessed the rates of Chitosan biodegradation in soil. In one study using sandy and silty soils (Sawaguchi et. al., 2015), it was observed that Chitosan added to silty soil dissipated by 50% after 10 days incubation at 25°C, and was non-detectable after 30 days, and the dissipation was associated with the presence of actinobacteria belonging to genera *Streptomyces* and *Kitasatospora,* populations of which increased proportionally to the decrease in chitosan content. The chitosan content of the sandy soil as well as that of the soil microorganisms was relatively unchanged under the same experimental conditions, suggesting that the sandy soil contained fewer chitosan-degrading microorganisms. Another study, using sheep-grazed

agricultural soils in the UK (Roberts, Bol, and Jones, 2007) and radio-labeled glucosamine it was reported that glucosamine removal from soil solution was primarily by microbial processes and that the half-life in soil solution ranged from 1-3 hours. The results of the two studies suggest that the low "free" concentrations of glucosamines (such as Chitosan) in soils is due to rapid degradation by soil microbes.

II. RISK ASSESSMENT

Chitosan is widely used in agricultural, biopharmaceutical, biomedical, cosmetic, textile, and food additive applications with no known reports of adverse effects to humans and the environment. Based on information obtained from studies submitted to the Agency in support of product registrations, and from the open technical literature, chitosan has been shown to have minimal acute and subchronic toxicity, is not a sensitizer or an allergen, and is not genotoxic, mutagenic, or carcinogenic. In addition to exposure to humans and the environment via intentional uses of chitosan-containing products, naturally-occurring chitosan, as well as its sole natural source, chitin, are widespread in the environment in the form of shells of aquatic organisms (e. g. marine and freshwater crustaceans and molluscs), soil microorganisms, and insect exoskeletons. The apparent ubiquitous presence of chitosan-degrading bacteria in soils indicates that chitosan is readily biodegraded in soils and may be important in the biocycling of chitin and chitin derivatives (likely derived from soil fungi) in the environment. Chitosan applied as a minimum risk pesticide likely would not persist in the environment due to ubiquitous presence of chitosan-degrading microorganisms.

This assessment agrees with the Chitin and Chitosan Summary Document for Registration Review (US EPA, 2007), where it was determined that the Agency anticipates that no additional human health effects or non-target organism data will be required for either Chitin or Chitosan. Based on all of the information available to the Agency, there are no risk concerns for human health or the environment if chitosan is intended for use as a minimum risk pesticide.

III. CONCLUSIONS

There is sufficient scientific evidence to support a conclusion of minimum risk for chitosan. This conclusion is supported by information contained in Agency reviews for the registration of EPA-registered products containing Chitosan as an active ingredient, and human health and environmental information contained in the open technical literature.

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Active Ingredient(s): Chitosan
PC Code(s): 128930
Type of Review: Product Chemistry, Human Health, Non-target Organisms and Environmental Fate

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Chapter 1

Biopesticide Oversight and Registration at the U.S. Environmental Protection Agency

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The Environmental Protection Agency (EPA) is committed to encouraging the development and use of biopesticides and considers them inherently reduced-risk pesticides. Biopesticides (microbial pesticides, biochemical pesticides, and plant-incorporated protectants) are required to be evaluated by EPA. The Agency must make findings of "no unreasonable adverse effects" to man and the environment to support its registration decision to permit sale and distribution under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as well as a "reasonable certainty of no harm" under the Federal Food, Drug, and Cosmetic Act (FFDCA) to permit residues in food and/or feed. This chapter will review areas including how EPA views the benefits of biopesticides, related laws and legal requirements, biopesticide registration, and biopesticide data requirements. EPA's commitment to low risk biological pesticides as alternatives to conventional chemical pesticides will also be emphasized.

What are Biopesticides?

Biopesticides, also known as biological pesticides, are pesticides derived from natural materials such as animals, plants, bacteria, and certain minerals. Typically, biopesticides have unique modes of action and are considered reduced risk pesticides. Biopesticides fall into three major classes:

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ACS Symposium Series; American Chemical Society: Washington, DC, 2014.

- Biochemical pesticides;
- Microbial pesticides; and
- Plant-incorporated protectants.

Biochemical Pesticides

Biochemical pesticides are naturally occurring substances or are synthetically derived equivalents that have a non-toxic mode of action to the target pest(s), and have a history of exposure to humans and the environment demonstrating minimal toxicity. Synthetically derived biochemical pesticides are equivalent to a naturally occurring chemical with such a history. Biochemical pesticides include, but are not limited to: semiochemicals (insect pheromones and kairomones), natural plant and insect regulators, naturally occurring repellents and attractants, induced resistance promoters, and enzymes. Biochemical pesticides typically degrade rapidly and are not persistent in the environment.

Biochemical pesticides, with the exception of pheromones, tend to have much less species-specificity and are broader spectrum pesticides than the microbials. They also may have lethal effects upon the target pest. Lethal but non-toxic biochemical pesticides include suffocating agents (e.g., soybean oil), dessicants (e.g., acetic acid), and abrasives (e.g., diatomaceous earth).

Microbial Pesticides

Microbial pesticides are microorganisms that produce a pesticidal effect. They have pesticidal modes of action that often include competition or inhibition, toxicity and even use of the target pest as a growth substrate. They may be:

- Eukaryotic microorganisms including, but not limited to, protozoa, algae, and fungi;
- Prokaryotic microorganisms, including, but not limited to, bacteria;
- Autonomous replicating microscopic elements, including, but not limited to, viruses.

Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest(s). For example, there are fungi that control certain weeds and other fungi that kill specific insects.

The most widely used microbial pesticides are subspecies and strains of *Bacillus thuringiensis*, or *Bt*. Each strain of this bacterium produces a different mix of proteins, and specifically kills one or a few related species of insect larvae. While some *Bt* strains control moth larvae feeding on plants, others are specific for larvae of flies and mosquitoes. The target insect species are determined by whether the particular *Bt* produces a protein that can bind to a larval gut receptor, thereby causing the insect larvae to starve.

Plant-Incorporated-Protectants (PIPs)

Consistent with the Coordinated Framework for Regulation of Biotechnology issued by the U.S. Office of Science and Technology Policy in 1986 (51 FR 23302) genetically modified (GM) crops with pesticidal traits fall under the oversight of EPA, the U.S. Department of Agriculture, and the U.S. Food and Drug Administration. EPA's oversight focuses on the pesticidal substance produced (e.g., *Bt Cry* proteins) and the genetic material necessary for its production in the plant (e.g., *Cry* genes). EPA calls this unique class of biotechnology-based pesticides plant-incorporated protectants (PIPs).

PIPs are pesticidal substances that plants produce and the genetic material that has been added to the plant. For example, scientists can take the gene for the *Bt* pesticidal protein and introduce the gene into the plant's own genetic material. Then the plant, instead of the *Bt* bacterium, manufactures the substance that destroys the pest. EPA regulates the protein and its genetic material, but not the plant itself.

How EPA Views Benefits of Biopesticides

In 1994, the Biopesticides and Pollution Prevention Division (BPPD) was established in EPA's Office of Pesticide Programs (OPP) to facilitate the registration of biopesticides. BPPD promotes the use of safer pesticides, including biopesticides, as components of integrated pest mangement (IPM) programs.

EPA is committed to encouraging the development and use of low risk biological pesticides as alternatives to conventional chemical pesticides (*1*). The Agency recognizes that these pesticides are often different in their mode of action and has employed numerous measures to facilitate the application process. These include distinct data requirements for microbial and biochemical biopesticides, consolidation of biological pesticide application processing to a single group within OPP, and regulatory relief activities (*2*) . EPA is committed to the efficient, effective approval of safer pesticides as well as a transparent, predictable process in decision making.

Since biopesticides tend to pose fewer risks than conventional pesticides, EPA generally requires much less data to register a biopesticide than to register a conventional pesticide, and EPA's review times are shorter for biopesticides.

While biopesticides require less data and are registered in less time than conventional pesticides, EPA always conducts rigorous reviews to ensure that pesticides will not cause unreasonable adverse effects on human health or the environment. For EPA to be sure that a pesticide is safe, the Agency requires that registrants submit a variety of data about the composition, toxicity, degradation, and other characteristics of the pesticide. These data requirements are described in more detail later in this paper.

There are several benefits to using biopesticides, including:

- Decreased risk without affecting yield. Biopesticides—when used as a component of an IPM program—can greatly decrease the use of conventional pesticides, without affecting crop yield.

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- Often less toxic. Generally, biopesticides are inherently less toxic than conventional pesticides and are safer to those using them.
- Often effective in very small quantities and decompose quickly. This can result in lower exposures and avoid pesticide pollution problems.
- Targeting of specific pests. Biopesticides generally affect only the target pest and closely related organisms, in contrast to broad spectrum, conventional pesticides that may affect non-target organisms such birds, insects, and mammals.
- When used in rotation with conventional products, biopesticides can help prevent development of pest resistance problems.
- Improved residue management. Buyers and consumers are becoming increasingly selective in their purchasing habits. Illegal pesticide residues left on produce can result in loss of markets, fines, and other consumer avoidance. Biopesticides often contain natural products that are normally consumed and do not have residue concerns.

Many microbial and biochemical biopesticides are not intended to function as "stand-alone" pest control products to completely replace conventional pesticides. Instead, these biopesticides are most effective when used as a component of an IPM program because they generally affect only the target pest and closely related organisms.

Additionally, for agricultural use products, biopesticides typically qualify for a reduced restricted entry interval and have no pre-harvest interval. Restricted entry intervals are requirements that limit the time that workers can return to a field once it has been treated with a pesticide. Restricted entry intervals can delay or obstruct time-sensitive cultural practices. Many biopesticides also do not have harvest restrictions. A harvest restriction is a waiting period between when a pesticide is applied and when the treated crop can be harvested and marketed. The waiting period after treatment can often be several days. Biopesticides without harvest restrictions give a grower much greater flexibility during harvest.

Microbial and biochemical biopesticides are generally labeled for use on a wide range of crops. As a result, for some minor crops or obscure pest problems, a biopesticide may be available when no conventional product is registered for the use. In addition, for larger crops such as corn, soybean, and cotton, PIP biopesticides have reduced the use of more toxic conventional insecticides.

Overview of OPP and BPPD's Role

EPA's Office of Pesticide Programs (OPP), along with the Office of Chemical Safety and Pollution Prevention (OCSPP), works with 10 Regional Offices and other EPA program offices on a wide range of pesticide issues and topics, such as:

- Evaluating potential new pesticides and uses;
- Providing for Special Local Needs and emergency situations;
- Reviewing safety of older pesticides;
- Registering pesticide producing establishments;

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- Enforcing pesticide requirements; and
- Pesticide field programs, such as the frontline implementation activities carried out by states, tribes, and EPA Regional pesticide experts.

OPP is comprised of nine divisions, three of which are divisions responsible for the registration of pesticides. The Biopesticides and Pollution Prevention Division (BPPD) is responsible for all regulatory activities associated with biologically-based pesticides. Within BPPD, the Biochemical Pesticides Branch and Microbial Pesticides branch are responsible for registering biochemical and microbial pesticides, respectively. Additionally, the Microbial Pesticides Branch registers PIPs and other biotechnology-related products.

BPPD also is working to reduce pesticide risk by promoting Integrated Pest Management (IPM) initiatives and coordinating the Pesticide Environmental Stewardship Program (PESP). BPPD's vision is to be a world leader in biopesticide regulation and pollution prevention. The mission of BPPD is to protect human health and the environment by reducing the risks of pesticides through registering biopesticides and through encouraging pollution prevention practices.

Main Statutes and Legal Requirements

EPA regulates the use of pesticides under the authority of two federal statutes: the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA) (*3*)(*4*). Additionally, the Food Quality Protection Act of 1996 (FQPA) amended FIFRA and FFDCA setting tougher safety standards for new and old pesticides and to make uniform requirements regarding processed and unprocessed foods (*5*). Finally, the Pesticide Registration Improvement Act (PRIA) establishes pesticide registration service fees for registration actions in the three registering divisions of EPA's Office of Pesticide Programs (*6*).

Other statutes that play roles in the regulation of biopesticides include:

- Endangered Species Act;
- Migratory Bird Treaty Act; and
- Clean Water Act

The following descriptions give brief overviews of the main statutes, though such descriptions are not intended to be comprehensive.

FIFRA

FIFRA provides the basis for regulation, sale, distribution and use of pesticides in the U.S. FIFRA authorizes EPA to review and register pesticides for specified uses. EPA also has the authority to suspend or cancel the registration of a pesticide if subsequent information shows that continued use would pose unreasonable risks. Some key elements of FIFRA include:

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- Is a product licensing statute; pesticide products must obtain an EPA registration before manufacture, transport, and sale
- Registration based on a risk/benefit standard
- Strong authority to require data--authority to issue Data Call-ins
- Ability to regulate pesticide use through labeling, packaging, composition, and disposal
- Emergency exemption authority--permits approval of unregistered uses of registered products on a time limited basis
- Ability to suspend or cancel a product's registration: appeals process, adjudicatory functions, etc.

Microbial, biochemical, and plant-incorporated protectant biopesticides are considered pesticides under FIFRA, and generally are required to be evaluated and registered by EPA under Section 3 of FIFRA. EPA must make a finding of no unreasonable adverse effects to man and the environment from use of the pesticide in order to support its registration decision.

FFDCA and FQPA

The Federal Food, Drug, and Cosmetic Act (FFDCA) authorizes EPA to set maximum residue levels, or tolerances, for pesticides used in or on foods or animal feed. Under FFDCA and amendments to both FFDCA and FIFRA under the FQPA, EPA must make a similar finding of a reasonable certainty of no harm if the use of such agents results in residues in food or feed. If the submitted information supports this safety finding, EPA may establish a numerical tolerance or an exemption from the requirement of a tolerance regarding those residues. As of this writing, no microbial pesticides or plant-incorporated protectants registered for food use have been required to obtain a numerical tolerance. Rather, exemptions from the requirement of a tolerance have been granted based on the finding of no significant adverse effects in the supporting data.

PRIA

In 2004, Congress passed the Pesticide Registration Improvement Act (PRIA) and established a registration fee-for-service system with specific fees and decision times by type of action. PRIA 3 is the second five-year extension of the original Act and was the result of support and collaboration from a coalition of industry, grower, environmental groups, and farm worker advocates. As biopesticides are usually inherently less toxic than conventional pesticides, biopesticide registrations require a significantly reduced data set compared to conventional registrations. Additionally, biopesticides can follow truncated decision review timelines as well as reduced registration fees.

Experimental Use Permits, Emergency Exemptions, and State and Local Need Registrations

In the process of pesticide development, field testing is often necessary to evaluate the efficacy of a pesticide. Title 40 CFR Part 172 describes when it is necessary to obtain an Experimental Use Permit (EUP) under Section 5 of FIFRA for testing unregistered pesticides. Briefly, the size of the outdoor test acreage is greater than a cumulative 10 acres of land or 1 surface acre of water, an EUP is required. Any food or feed crops involved in or affected by the tests must be destroyed or consumed only by experimental animals unless a tolerance or exemption from a tolerance has been established. These acreage limitations are applicable only for outdoor terrestrial and aquatic uses. For those pesticides being tested on sites for which acreage is not relevant (e.g., tree stumps, rodent control, structural treatments or bird repellents), the determination of the need for an EUP is made on a case-by-case basis.

Other criteria to determine when an EUP must be obtained are set forth in 40 CFR Part 172.3. An EUP is of limited duration and requires that the test be carried out under controlled conditions. For small-scale field tests of genetically modified microbial pesticides or non-indigenous microbial pesticides that USDA has not previously acted upon, applicants must submit a notification to EPA for determination of whether an experimental use permit is necessary, even if the testing is on less than 10 acres

In addition to registration under Section 3 of FIFRA, there are two additional means under FIFRA whereby a pesticide product may be distributed in the absence of a Section 3 registration or an experimental use permit. One is pursuant to an emergency exemption under Section 18 of FIFRA. Under this section, Federal or State agencies may request limited approval for an unregistered use of a currently registered pesticide product or the use of an unregistered pesticide product. Such a request can only be granted when there is a potentially severe economic or human health impact and no other alternatives are available for pest control. A Section 18 exemption usually allows use of the particular pesticide product for a year; however, the duration of the exemption may be limited or expanded depending on the situation (*7*).

Cases also exist where a particular pesticide product may be registered for one or more uses, but not for a particular use which is determined by the State as being a special local need. In these cases, the State may register that use or formulation needed for the special local need under Section 24(c) of FIFRA provided that appropriate tolerances or exemptions from tolerance exist if food or feed uses are involved. The EPA has 90 days to disapprove of such State registrations.

Biopesticide Registration

Before a pesticide can be marketed and used in the United States, FIFRA requires that EPA evaluate the proposed pesticide to assure that its use will not pose unreasonable risks of harm to human health and the environment, including non-target species. This involves an extensive review of health and safety information.

Pesticide registration is also the process through which EPA examines the ingredients of a pesticide; the site or crop on which it is to be used; the amount, frequency, and timing of its use; and storage and disposal instructions. A pesticide cannot legally be used, sold, or distributed if it has not been registered with EPA's Office of Pesticide Programs. FIFRA Section 2 (u), defines the term "pesticide" as:

(1) any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest;
(2) any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant; and
(3) any nitrogen stabilizer.

EPA makes online resources, such as the Pesticide Registration Manual (also known as the Blue Book), available to assist applicants through the registration process (*8*).

As biopesticides are usually inherently less toxic than conventional pesticides, biopesticide registrations may require a significantly reduced data set compared to conventional registrations. Additionally, there are reduced associated timelines and fees to help expedite registration processes. Timeframes to register pesticide products vary dependent on the PRIA code assigned to the submission. Based on PRIA 3 decision review timelines and fees for FY 14/15, biopesticide submissions can range from 7 months and $6,079 USD for a new non-food use (PRIA 3 code: B650) to 19 months and $48,621 USD for a new food use active ingredient with a petition to establish a tolerance (PRIA 3 code: B580). This is compared to 12 months and $12,156 USD for a conventional new non-food indoor use (PRIA 3 code: R260) and 24 months and over $590,000 USD for a new food use active ingredient (PRIA 3 code: R010).

Additionally, the Agency recommends that registrants request a pre-submission meeting with the appropriate registering branch. The pre-submission meeting is an excellent opportunity to discuss products in development and steps to take to ensure a timely registration decision. All information exchanged at these meetings is held confidential until a pesticide registration submission is made.

Pheromone Regulatory Relief

The Agency acknowledges that use of certain types of pheromone products presents lower risk than conventional pesticides, and also acknowledges the unique properties of these niche-type products regarding their inherently narrow host range (*9*). To promote the use of pheromone products, the Agency initiated a regulatory relief program that allows flexible confidential statements of formula for pheromone experimental use permits (EUPs) to allow for active ingredient adjustments during the course of experimentation. The Agency has also published generic tolerances and relaxed the acreage cut-off when an EUP is required for pheromones.

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EPA established the following exemptions from the requirement of a tolerance as a result of the pheromone regulatory relief program: 1) for inert materials in polymeric matrix dispensers (40 CFR 180.1122); 2) for pheromones in retrievably-sized polymeric matrix dispensers (40 CFR 180.1124); 3) for straight-chained lepidopteran pheromones (sprayables) (40 CFR 180.1153); and 4) for inert polymers in sprayable formulations, (40 CFR 180.1162). EPA further set forth certain policies raising the acreage limit to 250 acres for experimental use permit requirements for the testing of pheromones in polymeric matrix dispensers (59 FR 3681), for testing of non-food use broadcast pheromones (59 FR 34182), and for straight-chained pheromones (sprayables) (60 FR 168).

Products Exempt from Registration

EPA has determined that pest control organisms such as insect predators, nematodes, and macroscopic parasites are exempt from the requirements of FIFRA (40 CFR 152.20(a)). In addition, pheromones (and identical or substantially similar compounds) labeled for use only in pheromone traps for monitoring and pheromone traps in which those chemicals are the sole active ingredients are not subject to regulation under FIFRA (40 CFR 152.25(b)). However, the use of pheromones in traps in conjunction with conventional pesticides, in other application methods (other than traps), or for purposes other than monitoring, is subject to regulation under FIFRA.

Minimum risk pesticides that meet certain criteria are a special class of pesticides that are not subject to federal registration requirements because their ingredients, both active and inert, are demonstrably safe for the intended use. They are exempt from federal registration under section 25(b) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). EPA does not review or register pesticides that satisfy the 25(b) criteria (40 CFR 152.25(f)), though registration of these products is required by most states.

International Partnerships, Involvement, and Outreach

To streamline agency resources and promote international biopesticide registration, EPA and Health Canada's Pest Management Regulatory Agency (PMRA) have established a process for the joint review of biopesticide products. The procedure entails a joint pre-submission consultation to establish specific data requirements.

Joint reviews increase the efficiency of the registration process, facilitate simultaneous registration in Canada and the U.S., and increase access to new pest management tools in both countries. Efficient work-sharing requires a mutual understanding of the responsibilities of each agency, as well as common procedures and time frames (*10*).

EPA has been an active member of the Organisation for Economic Co-operation and Development's (OECD) Biopesticide Steering Group (BPSG) which meets annually to discuss harmonization of guidelines and principles of

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risk assessment. Comparisons and modifications of guidelines for toxicity and pathogenicity studies are vetted within the BPSG to reach consensus on risk assessment procedures for a variety of microorganisms used in pest management. In addition, specific organisms are reviewed to ensure that the latest scientific information on their biology is considered when evaluating their safe use in pest management. Production of toxins or secondary metabolites by some microbial pest control agents (MPCA) are of concern and it is critical that risk managers understand the prevalence of these compounds in products intended for environmental release.

While the BPSG brings together a broad range of scientists from many countries, not all aspects of dossier formating, concerns over aspects of study guidelines and which studies are critical for risk assessment will be agreed upon by all members. Despite this, the BPSG provides an important forum for discussion on a wide range of topics and is the only such venue to reach such a broad range of MPCA developers and regulators. The greater the degree of harmonization of data requirements among member countries resulting from these interactions, the more likely reduced-risk biopesticides will find widespread use in agriculture.

Regarding international outreach, EPA's Office of Pesticide Programs meets periodically with representatives from several countries to discuss products of biotechnology and their impact on trade of agricultural commodities. Updates on regulatory approvals and assessment of novel traits are presented to U.S. and foreign governmental representatives for consideration and discussion. Asynchronous approval of biotechnology products by trading partners has led to occasional rejections of shipments of commodities at great expense and disruption of trade. These meetings provide a forum for direct interaction between regulators and a greater understanding of the risk assessment process as the U.S. is often seen as the lead country in the development and regulation of genetically engineered crops. The ultimate goal of these exchanges is the acceptance of risk management decisions (i.e., approvals) from one country by an importing country without the need for a separate additional review process.

Biopesticide Data Requirements

Looking at the data that is required for biopesticide registration, biochemical and microbial pesticides are subject to a different set of data requirements for registration than conventional chemicals. These Data Requirements for Registration, which are tiered, are listed in 40 CFR Part 158: Subpart U Biochemical Pesticides 158.2000 and Subpart V: Microbial Pesticides 158.2100. EPA has published guidance for developing these data in the Biochemical Pesticides Test Guidelines, OSCPP Series 880 and the Microbial Pesticides Test Guidelines, OSCPP Series 885.

The current regulations allow for flexibility in fulfilling the required data. This can be accomplished through providing a rationale as to why a specific test is not practical to perform, or by providing scientific rationale to address the particular

endpoint. In addition, the Agency has the authority to invoke additional testing requirements if a potential risk has been identified and needs to be investigated. This flexible approach ensures that potential risks presented by biopesticides will be properly assessed.

Biochemical Data Requirements

Product Analysis and Mammalian Toxicology

In general, the product characterization information required for biochemical pesticides is the same as required for conventional chemical pesticides. These include:

- Data/information on product identity and composition;
- Information on manufacturing process; and
- Discussion of the formation of impurities, enforcement analytical methods, analysis for certification of limits, and physical/chemical properties.

The Agency has adopted a tiered testing scheme to assure the safety of biochemical pest control agents toward mammalian species, similar to that used for microbial pesticides, and is comprised of three tiers. Adverse effects in a lower tier will trigger additional testing in the next higher tier (*11*) (*12*).

The mammalian toxicology studies generally required for registration in or on a terrestrial food crop include, in Tier I, acute toxicity tests (oral, dermal, and inhalation exposures, & primary dermal and primary eye irritation studies).

In addition, a battery of genotoxicity studies, 90- day oral, dermal, and inhalation studies (depending upon likely routes of repeated exposure), an immunotoxicity study, and a developmental toxicity study may be required. Hypersensitivity incidents are to be reported, if they occur. The Agency has, on a case by case basis, considered scientifically valid information or peer reviewed literature in lieu of guideline studies. In many cases, lack of significant exposure serves as a basis for not requiring active ingredient or product specific data.

Non-Target Organism Testing

The unique nature of biochemical pesticides has led to a reduction in the data requirements for these products, as compared to synthetic chemical pesticides. Maximum hazard or limit dose testing of the technical grade of the active ingredient (TGAI) is used in assessing hazard to non-target wildlife. The TGAI is the purest and highest concentration form of the biochemical pesticide active ingredient.

There are three tiers of biochemical pesticide data requirements with regards to non-target organism testing. If adverse effects are not observed in Tier I testing (short term studies on non-target birds, aquatic organisms, plants, and insects), no further testing will be required. Should adverse effects be observed in Tier

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I studies, Tier II environmental fate studies will be triggered. If Tier II studies indicate that the biochemical active ingredient will persist in the environment, potentially resulting in longer exposure periods, longer term Tier III non-target wildlife studies will be required. Rarely are biochemical pesticides subjected to testing above Tier I.

Once the potential hazards to non-target wildlife have been determined via the tiered testing scheme, risk to non-target wildlife can be assessed based on expected exposure to a biochemical active ingredient via its application in an end-use product (EP) according to its proposed product label use directions.

Product Performance

Product performance data must be developed for all biochemical pesticides. However, such data is typically not required to be submitted unless it relates to a public health pest or is requested by the agency.

Microbial Data Requirements

Product Analysis and Mammalian Toxicology

Crucial to any evaluation of the hazards presented by a microbial pest control agent is correct identification. This identification allows the Agency to ascertain possible hazards associated with the proposed microbial agent and any closely related organisms, and to utilize published literature to facilitate the review. The Agency expects a registrant to provide the most accurate, current taxonomic information to verify the identity of their active microbial agent. For bacteria this information can include genetic DNA homology, morphology, biochemical tests and antibiotic sensitivity. Information for other types of microbes such as fungi, viruses and protozoa is usually less extensive, and may therefore involve other identification methodologies such as serotyping, DNA homology, restriction mapping or isozyme analysis when available. Any adverse effects known to be associated with the microbe, or closely related species, (such as toxin production and pathogenicity in species other than the target pest) should also be reported.

Additionally, the method used to manufacture microbial products is examined to determine whether adequate quality controls are in place to insure a pure product. This quality control review includes an examination for methods to verify purity and stability of the seed or stock cultures and to ensure that the final product is not contaminated with mammalian pathogens. Consideration is also given to final quality control measures for the microbial product that determine potency to insure that these tests relate to bioactivity and label claims (*13*).

The purpose of reviewing mammalian toxicology data for microbial pesticides is to ensure that the use of these products causes no unreasonable adverse effects to human health or non-target mammals. In order to do this the Agency must verify that the microbial product is correctly identified, presents little possibility of pathogenicity or toxicity to humans or other mammals, and is manufactured in a manner to prevent contamination with human pathogens.

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To assure the safety of microbial pest control agents toward mammalian species, the Agency has adopted a tiered testing scheme similar to the tiered scheme used for biochemical pesticides. Tier I is designed to expose the test animal, mice or rats, to a single acute, maximum hazard or limit dose of the live microbial pesticide. Tests involving mammalian tissue cultures are required for viral pest control agents to insure there is no possibility of mammalian infection given optimal conditions for expression of viral pathogenesis.

The major endpoints for the toxicity/pathogenicity tests are to observe any adverse effects on the test animals and to establish that the microbial test substance is being cleared from the exposed animals. The animals are observed for any unusual clinical signs during the test, and for gross abnormalities at necropsy. Specific organs are isolated from sacrificed animals during the course of the test to determine the level of microbial test substance present. This is done to assure that a high dose was administered and track the normal mammalian response which recognizes the test substance as foreign and clears it from the system. Unusual persistence of the test microbe in an organ is also considered an adverse effect. Replication of the test microbe in organs is also an adverse reaction, indicating potential for infectivity.

If any adverse effects are noted in the Tier I of the toxicity/pathogenicity tests, further testing is indicated using a tier progression to verify the observed effects and clarify the source of the effects. These Tier II tests could involve a subchronic toxicity/pathogenicity test or, if the adverse effect was believed to be due to a toxic reaction rather than pathogenicity, an acute toxicity test to establish an LD50 value for the toxin. Residue data are required if significant human health concerns arise from the toxicology testing. The majority of biopesticide products screened to date have not indicated any adverse effects to warrant testing further than Tier I.

In addition to testing the safety of the purified microbial agent, the safety of the marketed pesticide product, including inert ingredients, is ascertained. Acute oral, dermal, and inhalation toxicity as well as eye irritation, and dermal irritation testing may be required. However, rationales for no further testing may be appropriate depending on the nature of the inert ingredients and results of the initial toxicity/pathogenicity tests with the microbial agent. Any incidents of hypersensitivity in production workers, applicators or the general public must be reported to the Agency.

Genetically Modified Microbial Pesticides

Genetically modified microbial pesticides may be subject to different data or information requirements on a case-by-case basis, depending on the particular microorganism, the parent microorganism, the proposed use pattern, and the manner and extent to which the organism has been genetically modified. Additional data requirements may include:

- Information on the genetic engineering techniques used;
- The identity of the inserted or deleted gene segment (base sequence data or enzyme restriction map of the gene);

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- Information on the region controlling expression of the gene in question;
- A description of the new traits or characteristics that are intended to be expressed;
- Tests to evaluate genetic stability and exchange of the new traits; and/or
- Selected Tier II environmental expression and toxicology tests.

It is important for applicants to work closely with the Agency regarding data requirements to ensure that the proper tests are done and any unique characteristics of the microbial pesticide are taken into account in specific testing procedures.

Non-Target Organism Testing

The unique nature of microbial pesticides has led to changes in the data requirements for these products as compared to synthetic chemical pesticides. This is particularly evident in assessing risk to non-target wildlife (*14*). The testing requirements have been set up to test not only toxicity but also pathogenicity. This was accomplished by increasing the length of the tests (up to 30 days), and looking for signs of infection during and after the testing period. Beneficial insect testing was added in order to ensure that potential risks from insect pathogens used as pesticides had been adequately assessed.

For microbial pesticides used to control post-harvest diseases, the non-target organism data requirements to assess potential risks would also follow this case by case procedure. For example, in many instances the use of these products would be in enclosed areas (i.e., packing houses, storage buildings, etc.) and would be considered an indoor use. If this were the case, then testing of non-target organisms would probably not be required because of a lack of exposure. However, if the proposed use was determined to be outdoor and to have potential exposure to non-target organisms, then the ecological testing requirements would need to be addressed.

Tier I short term testing utilizes maximum hazard or limit dosing of non-target organisms. If no adverse results are observed in Tier I, then further testing is not warranted and environmental fate data are not required. In the first tier of non-target organism testing, avian oral, freshwater fish, freshwater aquatic invertebrate, and honeybee testing are required. In addition, tests to evaluate microbial pesticide effects on wild mammals, plants, and beneficial insects are required depending on the proposed use site, target organism, and degree of anticipated exposure. If adverse effects are observed in the first tier, then potential exposure to non-target organisms is evaluated in Tier II studies.

Product Performance

Product performance data must be developed for all microbial pesticides. However, such data is typically not required to be submitted unless it relates to a public health pest or is requested by the agency (*15*).

Plant-Incorporated Protectant Data Requirements

In general, the data requirements for PIPs are based on those for microbial pesticides (*16*). The reason for this situation is that PIP traits registered to date have been developed from genes found in microorganisms. The exact data requirements for each product have been developed on a case by case basis. The majority of products EPA has seen have been proteins, either related to plant viruses or based on proteins from the common soil bacteria *Bacillus thuringiensis* (*Bt*). The general data requirements include product characterization, mammalian toxicity, allergenicity potential, effects on non-target organisms, and environmental fate. For the *Bt* products, insect resistance management is included to prevent the loss of benefits of both the microbial sprays and the *Bt* PIPs from overuse and selection for resistant pest populations.

Conclusion

EPA is committed to encouraging the development and use of low risk biological pesticides as alternatives to conventional chemical pesticides. This commitment is shown by having a division dedicated to the registration of biopesticides, as well as distinct review timelines, fees, and required data. The efficient, effective approval of safer pesticides as well as a transparent, predictable process in decision making are top priorities for EPA, OPP, and BPPD.

Every day, the management and staff of BPPD focus on protecting human health and the environment by reducing the risks of pesticides through regulating biopesticides and encouraging pollution prevention practices. These safer options maximize the benefits of pesticides while helping to protect the air we breathe and the water we drink for generations to come. EPA looks forward to a continued role in helping to bring a broad array of safer pesticide options to market.

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Review

Chitin and Chitosan Preparation from Marine Sources. Structure, Properties and Applications

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Abstract: This review describes the most common methods for recovery of chitin from marine organisms. In depth, both enzymatic and chemical treatments for the step of deproteinization are compared, as well as different conditions for demineralization. The conditions of chitosan preparation are also discussed, since they significantly impact the synthesis of chitosan with varying degree of acetylation (DA) and molecular weight (MW). In addition, the main characterization techniques applied for chitin and chitosan are recalled, pointing out the role of their solubility in relation with the chemical structure (mainly the acetyl group distribution along the backbone). Biological activities are also presented, such as: antibacterial, antifungal, antitumor and antioxidant. Interestingly, the relationship between chemical structure and biological activity is demonstrated for chitosan molecules with different DA and MW and homogeneous distribution of acetyl groups for the first time. In the end, several selected pharmaceutical and biomedical applications are presented, in which chitin and chitosan are recognized as new biomaterials taking advantage of their biocompatibility and biodegradability.

Keywords: chitin; chitosan; chemical and enzymatic deproteinization; demineralization; characterization; deacetylation; biological activities; biomedical applications

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1. Introduction

Chitin or poly (β-(1→4)-*N*-acetyl-D-glucosamine) is a natural polysaccharide of major importance, first identified in 1884 (Figure 1). This biopolymer is synthesized by enormous number of living organisms [1] and it belongs to the most abundant natural polymers, after cellulose. In the native state, chitin occurs as ordered crystalline microfibrils which form structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast. So far, the main commercial sources of chitin are crab and shrimp shells. In industrial processing, chitin is extracted by acid treatment to dissolve the calcium carbonate followed by alkaline solution to dissolve proteins. In addition, a decolorization step is often added in order to remove pigments and obtain a colorless pure chitin. All those treatments must be adapted to chitin source, owing to differences in the ultrastructure of the initial material (the extraction and pre-treatments of chitin will be described later), to produce first a high quality chitin, and then chitosan (after partial deacetylation). Chitin is infusible and sparingly soluble during transformation into different conformations. The question of its solubility is a major problem in the development of both processing and use of chitin as well as its characterization.

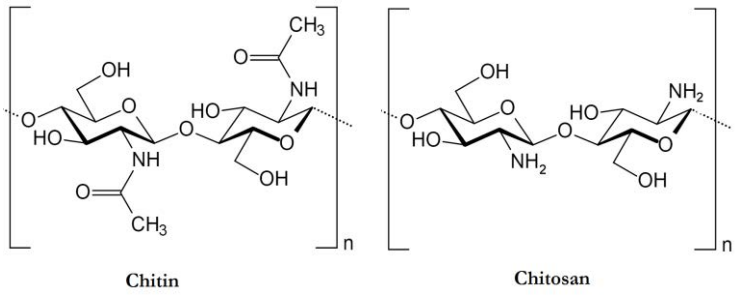

Figure 1. Chemical structure of chitin and chitosan

Chitin has more applications while transforming to chitosan (by partial deacetylation under alkaline conditions) [2–4]. Chitosan is a random copolymer with a molar fraction DA (degree of acetylation) of β-(1→4)-*N*-acetyl-D-glucosamine (Figure 1) and a fraction (1-DA) of β-(1→4)-D-glucosamine (Figure 1). The degree of acetylation of chitosan is characterized by the molar fraction of *N*-acetylated units (DA) or as a percentage of acetylation (DA%).

This review aims to present the state-of-the-art knowledge on the morphology of chitin and chitosan, the main techniques applied to chitin isolation and chitosan production. Then, the best methods for characterization in solution or solid state are also indicated. It is pointed out that for biomedical products, chitin and chitosan need to be highly purified, since residual proteins and pigments can cause side effects. Finally, the main biological properties will be analyzed in relation with the chemical structure (degree of acetylation and molecular weight of chitosan).

Concerning applications of chitin and chitosan, several examples used for drug release, wound dressing or biofilms are described. It is important to recall that chitin is a natural polymer as well as biocompatible and biodegradable in the body, thus widely used for biomedical and pharmaceutical applications. Additionally, good film forming properties are valuable for wound dressing, artificial skin or packaging.

2. Chitin Preparation and Characterization

2.1. Morphology of Chitin

Depending on its source, chitin occurs as two allomorphs, namely the α and β forms [5,6], which can be differentiated by infrared and solid-state NMR spectroscopies together with X-ray diffraction. In the solid state, chitin chains are assembled by the H-bonds network which controls the solubility, swelling and reactivity.

α-Chitin isomorph is by far the most abundant; it occurs in fungal and yeast cell walls, in krill, lobster and crab tendons and in shrimp shells, as well as in insect cuticle. In addition to the native chitin, α-chitin is systematically formed by: recrystallization from chitin solution [7,8], by *in vitro* biosynthesis [9,10] or enzymatic polymerization [11] due to high thermodynamical stability of this isomorph.

The rarer β-chitin is found in association with proteins in squid pens [5,12] and in the tubes synthesized by pogonophoran and vestimetiferan worms [13,14]. The crystallographic parameters of the two isomorphs allow us to conclude that there are two antiparallel molecules per unit cell in α-chitin but only one in β-chitin in a parallel arrangement. In these two structures, the chains are organized in sheets and held by intra-sheet hydrogen bonds. In addition, in α-chitin, inter-sheet hydrogen bonds prevent diffusion of small molecules into the crystalline phase. No inter-sheet hydrogen bonds are found in the crystal structure of β-chitin. This may explain its swelling in the presence of polar guest molecules (ranging from water to alcohol and amines) which penetrate the crystal lattice without disturbing the sheet organization and the crystallinity of the sample. The removal of the guest molecule allows us to revert to the original state of anhydrous β-chitin. The reactivity of β-chitin isomorph is larger than the α-isomorph, which is important for enzymatic and chemical transformations of chitin [15]. To conclude, both α and β forms are insoluble in all the common solvents. This insolubility is a major problem in the view of the development of processing and applications of chitin.

2.2. Chitin Extraction

The main sources of raw material for the production of chitin are cuticles of various crustaceans, principally crabs and shrimps. In crustaceans or more specifically shellfish, chitin is found as a constituent of a complex network with proteins onto which calcium carbonate deposits to form the rigid shell. The interaction between chitin and protein is very intimate and there is also a small fraction of protein involved in a polysaccharide-protein complex [16]. Thus, chitin isolation from shellfish requires the removal of the two major constituents of the shell, proteins by deproteinization and inorganic calcium carbonate by demineralization, together with small amounts of pigments and lipids that are generally removed during the two previous steps. In some cases, an additional step of decolorization is applied to remove residual pigments. Many methods have been proposed and used over the years to prepare pure chitin; however, no standard method has been adopted. Both deproteinization and demineralization could be carried out using chemical or enzymatic treatments. The order of two steps mentioned before may be reversed with some benefit, especially when

enzymatic treatment is considered. Microbial fermentation is also employed; in that case deproteinization and demineralization steps are processed simultaneously.

Regardless to the selected treatment, the isolation of chitin begins with the selection of shells. For example, for lobsters and crabs, the selection has important bearing on the subsequent quality of the final isolated material. Ideally, shells of the same size and species are chosen. In the case of shrimps, the wall of shell is thinner, thus the chitin isolation is easier than from other types of shells. The selected shells are then cleaned, dried and ground into small shell pieces.

2.2.1. Chemical Extraction

2.2.1.1. Chemical Deproteinization

The deproteinization step is difficulty due to disruption of chemical bonds between chitin and proteins. This is performed heterogeneously using chemicals which also depolymerize the biopolymer. The complete removal of protein is especially important for biomedical applications, as a percentage of the human population is allergic to shellfish, the primary culprit being the protein component.

Chemical methods were the first approach used in deproteinization. A wide range of chemicals have been tested as deproteinization reagents including NaOH, Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, $Ca(OH)_2$, Na_2SO_3, $NaHSO_3$, $CaHSO_3$, Na_3PO_4 and Na_2S. Reactions conditions vary considerably in each study. NaOH is the preferential reagent and it is applied at concentration ranging from 0.125 to 5.0 M, at varying temperature (up to 160 °C) and treatment duration (from few minutes up to few days). In addition of deproteinization, the use of NaOH invariably results in partial deacetylation of chitin and hydrolysis of the biopolymer lowering its molecular weight.

2.2.1.2. Chemical Demineralization

Demineralization consists in the removal of minerals, primarily calcium carbonate. Demineralization is generally performed by acid treatment using HCl, HNO_3, H_2SO_4, CH_3COOH and HCOOH [17,18]. Among these acids, the preferential reagent is dilute hydrochloric acid. Demineralization is easily achieved because it involves the decomposition of calcium carbonate into the water-soluble calcium salts with the release of carbon dioxide as shown in the following equation:

$$2\ HCl + CaCO_3 \longrightarrow CaCl_2 + H_2O + CO_2 \uparrow$$

Most of the other minerals present in the shellfish cuticle react similarly and give soluble salts in presence of acid. Then, salts can be easily separated by filtration of the chitin solid phase followed by washing using deionized water.

Demineralization treatments are often empirical and vary with the mineralization degree of each shell, extraction time, temperature, particle size, acid concentration and solute/solvent ratio. The latter depends on the acid concentration, since it needs two molecules of HCl to convert one molecule of calcium carbonate into calcium chloride. In order to have a complete reaction, acid intake should be equal to the stoichiometric amount of minerals, or even greater. [19,20]. Since, it is difficult to remove all minerals (due to the heterogeneity of the solid), larger volume or more concentrated acid solution is used. Demineralization can be followed by acidimetric titration: the evolution of pH towards neutrality

corresponds to acid consumption but the persistence of acidity in the medium indicates the end of the reaction [21].

Several demineralization treatments were previously used, involving various reaction conditions. Conventionally, demineralization is accomplished using dilute hydrochloric acid at different concentrations (up to 10% w/v) at room temperature, during different incubations time (Table 1). Among such methods are those of Muzzarelli *et al.* [22], Hackman [23,24], Anderson *et al.* [25] (Table 1).

Exceptions to the above are seen in the methods of Horowitz *et al.* [26] and Synowiecki *et al.* [27] where demineralization was accomplished with 90% formic acid and 22% HCl, respectively, at room temperature. Most of the aforementioned methods include drastic treatments that may cause modifications, such as depolymerization and deacetylation of native chitin [28]. In order to overcome this problem, other methods have been developed using mild acids (to minimize degradation). For instance, Austin *et al.* [29] used ethylenediaminetetracetic acid (EDTA), Brine and Austin [30] applied acetic acid. Peniston and Johnson [31] studied a sulfurous acid process, *etc.* However, these treatments resulted in chitins with high residual ash content.

Demineralization using HCl usually can be achieved in 2 to 3 h under stirring [19]. However, reaction time varies with preparation methods from 15 min [18] to 48 h as seen in Table 1. Longer demineralization time, even to several days, results in a slight drop in the ash content but also causes polymer degradation [32,33].

Moreover, it was reported that the use of high temperature accelerates the demineralization reaction by promoting the penetration of the solvent into the chitin matrix. Thus, some demineralization reactions were carried out at higher temperature [34]. Furthermore, it was reported that the penetration of solvent into the chitin matrix strongly depends on the particles size. According to Marquis-Duval [35], the decisive factor in the demineralization is related to the contact area between the chitin matrix and the solvent. However, it was reported that high temperatures, longer incubations, high acid concentrations and granulometry affect the final physico-chemical properties of the resulting chitin.

In conclusion, although many experimental conditions can be found in the literature for the removal of minerals, effects on the molecular weight and acetylation degree cannot be avoided. Only Percot *et al.* [18] studied the extraction of chitin from shrimp shells using mild conditions allowing them to obtain chitin with a high DA. The demineralization was performed under the following conditions: at room temperature, in the presence of stoichiometric amount of 0.25 M HCl with regards to the calcium carbonate content, for 15 min incubation time. Deproteinization was later classically processed at 70 °C for 24 h using 1 M NaOH. These conditions well preserve the chitin structure, with a high DA remaining above 95%. Unfortunately, the amount of residual proteins and minerals were not determined and the influence on MW was not studied.

Table 1. Comparison of conditions for chitin production according to literature.

Source	Deproteinization				Demineralization			References
	NaOH Concentration *	Temperature (°C)	Number of Baths	Duration (h)	HCl Concentration*	Temperature (°C)	Duration (h)	
12 species of crustaceous and cephalopods	0.3 M	80–85	From 2 to 7 according to the source	1 h for each bath	0.55 M	Room	15 mn to 1 h by bath repeated 2–5 times according to the source	[21]
Shrimp	0.125 M 0.75 M	100 100	1 1	0.5 -	1.25 M	Room	1	[36]
Shrimp	1.25 M	100	1	0.5	1.57 M	20–22	1–3	[37]
Crab	0.5 M	65	1	2	1.57 M	Room	5	[22]
Crab	1 M	80	1	3	1 M	Room	12	[38]
Crab	1 M	100	1	36	2 M	Room	48	[32]
Crab	1 M	100	3	72	1 M	Room	-	[23]
Crab	1.25 M	85–90	3	24	1.37 M	Room	24	[39]
Crab/Lobster	2.5 M	Room	3	72	11 M	−20	4	[40]
Krill	0.875 M	90–95	1	2	0.6 M	Room	2	[25]
Lobster	1 M	100	5	12	2 M	Room	5	[24]
Squid	2 M 2 M	Room 100	2	One night 4	1 M	Room	One night	[41]
Lobster	10%	100	1	2.5	10% HCl 90% formic	Room	18	[26]
Krill	3.5%	25	1	2	3.5%	20	1.5	[42]
Lobster	5%	80–85	2	0.5	5%	70	4	[43]
Crawfish	3.5%	65	1	2	1 M	Room	0.5	[44]
Crab	1 M	50	1	6	1 M	20	3	[30]
Shrimp	1%	65	1	1	0.5 M	Room	-	[45]
Shrimp	3%	100	1	1	1 M	Room	0.5	[46]
Shrimp	4%	100	1	1	5%	Room	-	[47]

* Reactant concentrations are expressed in molarity or w/v %.

2.2.1.3. Processes Preserving Chitin Structure

To the best of our knowledge, there have been no studies which conduct a production of chitin with the highest DA and MW, free of minerals and proteins.

However, only the partial deacetylation may be controlled (using solid state ^{13}C-NMR). Furthermore, the chain degradation can be also evaluated by viscometry but after additional treatment, *i.e.*, solubilization of residual chitin in specific solvent, or after conversion to a soluble product (chitosan). Nevertheless, in the last case, the deacetylation process is usually accompanied by polymer degradation. Thus, to estimate the influence of chitin extraction process, only DA is determined as indication of the degree of the chitin degradation. It may be assumed that the higher the DA is obtained for an extracted chitin, the less the polymer is degraded.

Optimized extraction method of pure chitin production with maximum preservation of its structure (MW, DA) allows us to get chitin corresponding to the native chitin in the cuticle structure. This approach was proposed by Tolaimate *et al*. [21] who used chemical treatments for both demineralization and deproteinization.

In the study of Tolaimate *et al*. [21], a new approach was proposed using successive baths of lower HCl (0.55 M) and NaOH (0.3 M) concentrations. The number of baths for each step was dependent on the tested animal species. This method has proved a good efficacy on the reduction of proteins and minerals as well as preservation of the native chitin form for 12 different species of crustaceous and cephalopods (Table 2). The DA of the prepared chitins, determined with ^{13}C-NMR, was varying between 96% and 100% for all the species. For example, for shrimp shells, extracted chitin was 100% acetylated. So far, such high degree of deacetylation has never been mentioned in the literature.

Table 2. Comparison of chitin production from different sources according to Tolaimate *et al.* [48].

Source		**Number of Deproteinization Baths**	**Number of Demineralization Baths**	**DA**
		0.3 M; NaOH 80 °C; 1 h	**0.55 M HCl; 25 °C; 2 h**	
Cirripedia	Anatife	4	2	100
Reptantia Brachyura	Red crab	3	5	97
	Marbled crab	3	3	99
	Spider crab	3	3	96
Reptantia Macrura	Lobster	3	3	-
	Crayfish	7	3	100
	slipper lobster	3	2	-
	Freshwater crayfish	3	2	-
Natantia	Pink shrimp	3	3	100
	Grey Shrimp	2	2	100
Stomatopoda	Squilla	3	3	100
Cephalopoda	Squid	2	2	100

2.2.2. Biological Extraction of Chitin

The extraction by chemical treatments has many drawbacks: (i) it harms the physico-chemical properties of chitin and leads to MW and DA decrease that negatively affects the intrinsic properties of the purified chitin; (ii) it affects wastewater effluent that contains some chemicals (iii) it increases the cost of chitin purification processes. Furthermore, the development of the green extraction techniques based on the concept of 'Green chemistry' is gaining greater attention, favoring the application of enzymes and microorganisms for chitin extraction. A comparative study was carried out by Khanafari *et al.* [49] for extraction of chitin from shrimp shells by chemical and biological methods. The results indicated that the biological method (using microorganisms) was better than the chemical one because it preserves the structure of chitin. Bustos and Healy [50] also demonstrated that chitin obtained by the deproteinization of shrimp shells with various proteolytic microorganisms has higher molecular weights in comparison with chemically prepared shellfish chitin. The biological extraction of chitin offers high reproducibility in shorter time, simpler manipulation, smaller solvent consumption and lower energy input. However, the biological method is still limited to laboratory scale studies.

Recently, two reviews have reported the most common biological methods used for chitin extraction [51,52], *i.e*., the use of proteolytic enzymes in order to digest the proteins or a fermentation process using microorganism which allows a digestion of both proteins and minerals.

The use of enzymes in the deproteinization step was first mentioned in the original Rigbv patent from 1934 but there has been a renewed interest in this approach since 1977. This work has led to the lactic acid bacterial fermentation process, studied more extensively later by Guerrero Legarreta *et al*. [53] and Cira *et al*. [54].

2.2.2.1. Enzymatic Deproteinization

Chitin extraction requires the use of proteases. Proteolytic enzymes are mainly derived from plant, microbial and animal sources. Many proteases such as alcalase, pepsin, papain, pancreatine, devolvase and trypsin remove proteins from crustacean shells and minimize the deacetylation and depolymerization during chitin isolation. This treatment may be performed either after, or before demineralization step of the solid material, which modifies the accessibility for the reactants.

Both purified and crude extracted proteases are used in the deproteinization step. However, commercially purified enzymes are expensive in contrast to crude proteases, which are not only cheaper but also more efficient due to the presence of coexisting proteases. Crude proteases are mainly derived from bacteria and fish viscera, bacterial proteases being the most common. Marine animals possess the same functional classes of enzymes, which are present in animal tissues and may be recovered in both active and stable forms for commercial use. In several of the major fish producing countries, the by-products represent about 50% of the seafood harvest [55]. These materials are largely underutilized and discarded as waste. Thus, application of these crude enzymes in the chitin extraction process could be interesting in decreasing the costs of this process as well as in preserving the environment.

It must be noted that the efficiency of enzymatic methods is inferior to chemical methods with approximately 5%–10% residual protein typically still associated with the isolated chitin. The final

isolated chitin could be then treated with an additional NaOH treatment (under milder conditions and for a shorter time) to increase its purity and preserve the structure of chitin.

Many reports have demonstrated the application of bacterial proteases in deproteinization step. For example, Synowiecki and Al-Khateeb [56] applied enzymatic deproteinization on previously demineralized shrimp waste in order to produce chitin and a nutritionally valuable protein hydrolyzate. Alcalase 2.4 L (Novo Nordisk A/S), a serine endopeptidase obtained from *Bacillus licheniformis,* was used. This enzyme was selected due to its specificity for terminal hydrophobic amino acids, which generally leads to the production of non-bitter hydrolyzate and allows an easy control of the hydrolysis degree. The obtained hydrolyzate is a good source of essential amino acids in food applications. However, the effectiveness of deproteinization was limited by the presence of residual small peptides and amino acids attached to chitin molecules which persist after enzymatic hydrolysis. This method allows the isolation of chitin containing about 4% of protein impurities. Such purity is sufficient for many non-medical applications of chitin. Gilberg and Stenberg [57] also used alcalase 2.4 L for chitin, protein hydrolyzate and asthaxanthin recovery.

Manni *et al.* [58] compared the isolation of chitin from shrimp waste using *Bacillus cereus* SV1 crude alkaline proteases to the use of 1.25 M NaOH. Shrimp shells were demineralized after deproteinization using dilute HCl treatment. The residual protein content was significantly higher in the chitin isolated with the enzymatic deproteinization than that obtained with alkali treatment (10% compared to 6%).

In another study, enzymatic deproteinization was optimized by Younes *et al.* [59] before demineralization. In this study many microbial proteases were compared on the basis of their efficiency in shrimp shells deproteinization. Six alkaline crude microbial proteases from *Bacillus mojavensis* A21, *Bacillus subtilis* A26, *B. licheniformis* NH1, *B. licheniformis* MP1, *Vibrio metschnikovii* J1 and *Aspergillus clavatus* ES1, were used. The highest deproteinization degree was obtained with *B. mojavensis* A21 proteases, being at about 76%. Then, the effect of reaction conditions, *i.e*., mainly enzyme/substrate ratio, temperature and incubation time, on the deproteinization degree were optimized using response surface methodology to reach 88% deproteinization under the optimized conditions.

Recently many fish and marine invertebrate alkaline crude proteases have been applied for shrimp shell deproteinization. Mukhin and Novikov [60] studied the possibility of using crustacean waste both as a substrate and as a source of proteases. The shell proteins were degraded with crude proteases isolated from the hepatopancreas of crab. The objective was to optimize the protein hydrolyzate yield. However, even under the best conditions, *i.e*., temperature = 50 °C, time = 12 h, pH = 8.4, Enzyme/Substrate ratio of 6 g/kg, the degree of hydrolysis was never higher than 80%.

Younes *et al*. [61] used alkaline proteases from the red scorpionfish *Scorpaena scrofa* for shrimp waste deproteinized up to 85%. Activities of these crude alkaline proteases are probably related to the fish feeding mainly on crustaceans and mollusks inducing the nature and the specificity of its enzymes.

By contrast, when extraction is carried out by chemical process, the order of two steps (deproteinization and demineralization) does not have significant effect on the quality and the yield of the final chitin [62]. However, if enzymatic deproteinization is applied, the minerals presented in the cuticles may decrease the accessibility of the proteases and affect shrimp shells deproteinization efficiency. Thus, demineralization should be performed firstly.

2.2.2.2. Fermentation

The cost of using enzymes can be decreased by performing deproteinization by fermentation process, which can be achieved by endogeneous microorganisms (called auto-fermentation) or by adding selected strains of microorganisms. This latter can be achieved by single-stage fermentation, two-stage fermentation, co-fermentation or successive fermentation.

Many microorganism species were proposed for crustacean shells fermentation as summarized by Arbia *et al.* [51]. Fermentation methods could be separated into two major categories: lactic acid fermentation and non-lactic acid fermentation.

(a) Lactic Acid Fermentation

Fermentation of crustacean shells can be performed by selected *Lactobacillus* sp. strain as inoculum which produces lactic acid and proteases. Lactic acid is obtained by conversion of glucose resulting in lower pH condition of silage suppressing the growth of spoilage microorganisms. Lactic acid reacts with the calcium carbonate, leading to the formation of a precipitate of calcium lactate separated from lighter shells which are recovered and rinsed with water. This process may be realized either on purified crustaceous shells, or on complete shrimp waste (including heads and viscera). Thus, deproteinization and simultaneous liquefaction of the proteins could occurred by action of proteases produced by added strains, or by gut bacteria present in the intestinal system of the treated shrimps, or by proteases present in the biowaste itself. The efficiency of lactic acid fermentation depends on many factors, mainly the species and quantity of inoculums, carbon source and its concentration, initial pH and pH evolution during fermentation, temperature and the duration of fermentation [63–65].

For example, Choorit *et al.* [66] used response surface methodology to optimize demineralization efficiency in fermented shrimp shells. Following variables were tested: sucrose concentration, initial pH value and soaking time, using *Pediococcus* sp. L1/2. Results showed an increase in demineralization degree (caused by higher sucrose concentration and soaking time) as well as an important effect of the initial pH. Demineralization degree reached at about 83% at pH 7, compared to 68% at pH 6 (sucrose concentration 50 g/L and soaking time 72h).

Otherwise, Rao *et al*. [64] studied the effect of different fermentation parameters (initial pH, initial glucose concentration and inoculation with different quantities of *Lactobacillus*) on deproteinization and demineralization degrees. Combined treatment with *Lactobacillus* and reduction of initial waste pH by addition of acetic acid produced lower deproteinization and higher demineralization degrees than treatment with *Lactobacillus* or acid individually. In addition, inoculation with *Lactobacillus* resulted in a high-quality protein liquor output, whereas autofermented waste (due to the presence of shrimp microflora) gave a strong stinky protein fraction. In the fermentation with lactic acid bacteria, the demineralization efficiency and the quality of the derived product are high, and the addition of commercial proteases may even increase deproteinization.

(b) Non Lactic-Acid Fermentation

In non-lactic acid fermentation, both bacteria and fungi were used for crustacean shells fermentation, for example: *Bacillus* sp. [67–69], *Pseudomonas* sp. [65,70,71] and *Aspergillus* sp.[72].

Ghorbel-Bellaaj *et al*. [69] evaluated six proteolytic *Bacillus* strains on the fermentation of shrimp waste: *Bacillus pumilus* A1, *B. mojavensis* A21, *B. licheniformis* RP1, *B. cereus* SV1, *B. amyloliquefaciens* An6 and *B. subtilis* A26. Results showed that all the *Bacillus* strains were able to deproteinize shrimp waste. The highest deproteinization degree was obtained using *B. cereus* SV1. These authors had also tested the role of additional amount of glucose on fermentation and they concluded that glucose had no significant effect on deproteinization degree and improved demineralization.

Sini *et al*. [68] had studied fermentation of shrimp shells in jaggery broth using *B. subtilis.* About 84% of the proteins and 72% minerals were removed; after this step the residue was treated with 0.8 N HCl and 0.6 N NaOH to reduce residual proteins and minerals to satisfactory level at about 0.8% proteins and 0.8% minerals.

Many factors have been reported to influence the fermentation process and consequently deproteinization and demineralization efficiencies [65,66,73]. Ghorbel-Bellaaj *et al*. [70] used Plackett-Burman factorial design to screen the main factors influencing fermentation efficiency using *P. aeruginosa* A2. This method is thus quite useful in preliminary studies, in which the main objective is to select variables that can be fixed or eliminated in further optimization process. Only four variables were reported to be effective on deproteinization and demineralization degrees: shrimp shell concentration, glucose concentration, inocculum size and incubation time. Under these conditions: initial medium pH, temperature, speed of agitation and volume of culture no effect on fermentation efficiency was observed. Then, from response surface methodology, under optimal conditions for fermented shrimp shells, maximum demineralization was 96%, and deproteinization was 89% [70].

Proteolytic enzymes released from fungus *A. niger* were also tested for their deproteinization and demineralization efficiency of crustacean shells. Teng *et al*. [74] evaluated concurrent production of chitin from shrimp shells and fungi in a one-pot fermentation process where proteases from the fungi hydrolyze proteins into amino acids that in turn act as a nitrogen source for fungal growth. Results showed that residual proteins in the isolated shrimp chitin were below 5%. The protein content in the fungal chitin was higher (10%–15%). They concluded that fungi and shrimp shells supplementation with glucose in a single reactor led to release of protease by the fungi and enhance the deproteinization of shrimp shells. The hydrolyzed proteins in turn were utilized for fungal growth, leading to lower pH of the medium and further demineralization of the shrimp shells.

The various biological methods of chitin extraction by microorganisms are simple, more productive and environmentally friendly when compared to chemical processes. However, microbial fermentation has its drawbacks such as: longer processing time compared to chemical methods, poorer accessibility of proteases (caused by the presence of minerals which lead to high residual proteins). Nevertheless, deproteinization rate could be ameliorated depending on the end use requirements in particular for biomedical applications. This could be achieved by using simultaneous or successive processes such as two-step fermentations or co-fermentation of microorganisms. In order to obtain highly purified chitin, biotechnological process must be completed by further mild chemical treatment to remove the residual protein and minerals.

Recently, Gortani and Hours [52] concluded that a cost-effective, fast, and easily controllable industrial process for producing chitin of high MW and DA still requires further development and optimization of the extraction process, such as: minimization of chitin degradation and decrease the impurity levels to a satisfactory level highly desirable for specific applications.

2.3. Chitin Characterization and Solubility

In the solid state, the chains are parallel in β-chitin and antiparallel in α-chitin [75]. Their crystalline structures were reviewed in different papers using X-ray diffraction method [76–79], IR spectroscopy [80–86], and NMR [87–90]. Solid state ^{13}C-NMR is a commonly used technique for differentiation of the two isomorphs [88], for determination of the deacetylation degree of chitin (DD) and for control of purification conditions. Figure 2 shows typical spectra for α-chitin and chitosans with different acetylation degrees [90].

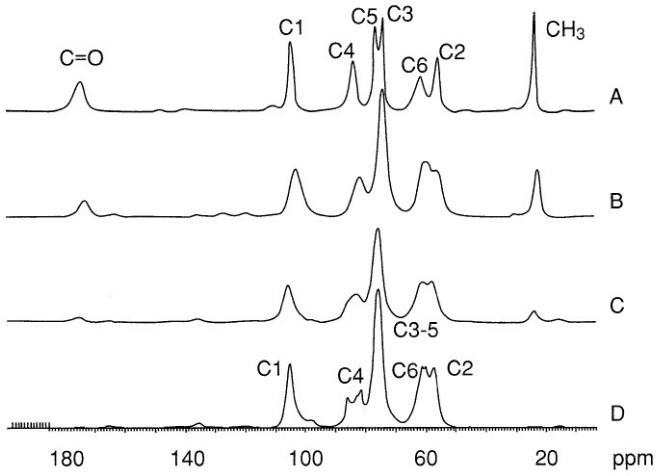

Figure 2. ^{13}C NMR spectra for (**A**) chitin and chitosans (**B**) obtained by homogeneous reacetylation DA = 0.60; (**C**) commercial chitosan from Pronova DA = 0.2; (**D**) fully deacetylated chitin. Reproduced with permission from [90]. Copyright 2000 American Chemical Society.

Unfortunately, physical properties of chitin in solution cannot be analyzed correctly due to poor data caused mainly by difficulties in dissolution of this polymer. Dissolution is desired to estimate the molecular weight but also to process chitin (chitin cannot be processed in molten state). Presence of aggregates in solution precludes light scattering measurements and overestimates the molecular weights. Therefore, the most applicable technique here is viscometry where the Mark-Houwink parameters are known under defined thermodynamic conditions used (solvent, temperature). One of the mostly known system is based on complex formation between chitin and LiCl (at 5 wt% in the solvent DMAC) in dimethylacetamide solvent. Experimental values of parameters *K* and *a* relating intrinsic viscosity [η] and molecular weight M for chitin in this solvent are estimated from well-known Mark-Houwink equation according to:

$$[\eta]\ (\mathrm{mL/g}) = KM^{a} \quad (1)$$

with $K = 7.6 \times 10^{-3}$, a = 0.95 at 30 °C [91] and $K = 2.4 \times 10^{-1}$, a = 0.69 at 25 °C [92].

A review on chitin and chitosan including the question of their solubility was recently published in relation to fiber processing [93]. It has been demonstrated that chitin is able to be processed from solutions. Additionally, chitin, like other polysaccharides derived from cellulose, has good film-forming properties and good stability promoted by the establishment of a hydrogen bond network

between extended chains. Chitin gives original properties to the new materials due to its biocompatibility, biodegradability and non-toxicity, with antimicrobial activity and low immunogenicity.

3. Chitosan Preparation and Characterization

3.1. Chitosan Preparation

The term chitosan usually refers to a family of polymers obtained after chitin deacetylation to varying degrees. In fact, the acetylation degree, which reflects the balance between the two types of residues (Figure 1), differentiates chitin from chitosan. When the DA (expressed as molar percentage) is lower than 50 mol%, the product is named chitosan and becomes soluble in acidic aqueous solutions [94]. During deacetylation, acetyl groups are removed but also depolymerization reaction occurs, indicated by changes in MW of chitosan.

Chitin can be converted to chitosan by enzymatic preparations [95–98] or chemical process [99,100]. Chemical methods are used extensively for commercial purpose of chitosan preparation because of their low cost and suitability to mass production [100].

3.1.1. Chemical Deacetylation

From a chemical point of view, either acids or alkalis can be used to deacetylate chitin. However, glycosidic bonds are very susceptible to acid; therefore, alkali deacetylation is used more frequently [100,101].

The *N*-deacetylation of chitin is either performed heterogeneously [102], or homogeneously [103]. Commonly, in the heterogeneous method, chitin is treated with a hot concentrated solution of NaOH during few hours, and chitosan is produced as an insoluble residue deacetylated up to ~85%–99%. According to the homogeneous method, alkali chitin is prepared after dispersion of chitin in concentrated NaOH (30 g NaOH/45 g H_2O/ 3 g Chitin) at 25 °C for 3 h or more, followed by dissolution in crushed ice around 0 °C. This method results in a soluble chitosan with an average degree of acetylation of 48%–55% [99]. This process produces deacetylation with acetyl groups uniformly distributed along the chains, for example chitosan with DA = 10% after 580 h at 25 °C [103].

Rinaudo and Domard [104] reported that the solubility of chitosan can be characterized not only by the fraction of 2-acetamido-2-deoxy-D-glucose units in the molecule but also by the *N*-acetyl group distribution. Aiba [105] showed that deacetylation reaction performed under heterogeneous conditions gives an irregular distribution of *N*-acetyl-D-glucosamine and D-glucosamine residues with some blockwise acetyl group distribution along polymeric chains. Thus, solubility and degree of aggregation of chitosan can vary in aqueous solutions leading to changes in their average characteristics. For instance, physico-chemical properties of such chitosans may differ from those of randomly acetylated chitosans obtained under homogeneous conditions.

Furthermore, variations in chitosan preparation may also result in changes of: DA, distribution of acetyl groups along the chains, MW and viscosity in solution [106,107].

In fact, many parameters in the deacetylation reaction can impact the characteristics of the final chitosan [108]. For instance, Rege and Block [109] had investigated the effect of temperature, processing time and mechanical shear on chitosan characteristics, and found that temperature and

processing time have a significant effect on DA and MW. Tolaimate *et al.* [110] reported that chitosan DA is greatly affected by temperature and repetition of alkaline steps. Wu and Bough [45] studied the effects of time and NaOH concentration. Tsaih and Chen [111] also examined the effect of time reaction and temperature. All these studies were conducted using a classical one-variable-at-a-time experimentation. These reports indicate that MW and DA of chitosan are mainly affected by NaOH concentration, reaction time, temperature and repetition of alkaline steps. Additional factors such as reaction reagent, atmosphere, particle size, chitin and solvent ratio, and source of raw material were also tested in others studies [100,102,110,112].

Weska *et al.* [113] attempted to optimize chitin deacetylation by response surface methodology (controlling MW and/or DA) using temperature and reaction time variables. Hwang *et al.* [114] studied the effect of temperature, time and NaOH concentration on the deacetylation. Chang *et al.* [102] reported the influence of NaOH concentration, temperature and solution/chitin ratio and found that chitosan DA was decreasing with increase of temperature and NaOH concentration. Other parameters, such as: the use of alkali successive baths, atmospheric conditions and presence of different additives could influence deacetylation but were not considered previously in optimization studies.

Deacetylation was investigated using seven factors: the alkali reagent, its concentration, temperature, reaction time, the use of successive baths, atmospheric conditions and the use of sodium borohydride, a reducing agent [115]. For that purpose, a fractional factorial design was applied and a mathematical model was established to allow optimizing experimental conditions for chitosan of desired DA. Results clearly revealed a significant effect of temperature and the alkali reagent nature (NaOH treatment is much more efficient than KOH). It has been found that DA is significantly affected by the use of successive baths, reaction time and alkali concentration. By contrast, the atmospheric conditions (nitrogen or air) and the use of a reducing agent ($NaBH_4$) do not have significant effect on the DA of chitosan but MW of chitosan was higher under atmospheric nitrogen and addition of sodium borohydride which prevents polymer degradation. These results are in agreement with previous ones obtained with thiophenol and $NaBH_4$ used as oxygen scavenger and reducing agent, respectively [116].

3.1.2. Enzymatic Deacetylation

Chemical deacetylation has also disadvantages: energy consumption; waste of concentrated alkaline solution, thus an increase of environmental pollution, broad and heterogeneous range of soluble and insoluble products.

In order to overcome these drawbacks in the chitosan preparation, an alternative enzymatic method exploiting chitin deacetylases has been explored. The use of chitin deacetylases for the conversion of chitin to chitosan, in contrast to the currently used chemical procedure, offers the possibility of a controlled, non-degradable process, resulting in the production of novel, well-defined chitosan [117]. This method is specially used to prepare chitosan oligomers.

Chitin deacetylase (EC 3.5.1.41) catalyzes the hydrolysis of *N*-acetamido bonds in chitin to produce chitosan. The presence of this enzyme activity has been reported in several fungi [118–123] and insect species [124]. The mostly well-studied enzymes are those extracted from the fungi *Mucor rouxii* [95,118,119], *Absidia coerulea* [120], *Aspergillus nidulans* [121] and two strains of *Colletotrichum*

lindemuthianum [122,123]. All the enzymes are glycoproteins and are secreted either into the periplasmic region or into the culture medium. Furthermore, all enzymes exhibit a remarkable thermal stability at their optimal temperature (50 °C), and exhibit a very strong specificity for β-(1,4)-linked *N*-acetyl-D-glucosamine polymers. However, the enzymes vary significantly in their MW and carbohydrate content and display a wide range of pH optima. It is interesting to notice that chitin deacetylases, produced by *C. lindemuthianum* and *A. nidulans,* are not inhibited by acetate (a product of the deacetylation) which make them suitable for potential biotechnological applications [121–123].

The efficiency of chitin deacetylase, isolated from the fungus *M. rouxii*, on the chitosan preparation was tested using chitin as a substrate (both in its crystalline and amorphous morphology) [125]. Deacetylation degrees remain very low (<10%) indicating that the enzyme is not really effective on insoluble chitins. Similar results were also obtained using chitin deacetylases isolated from other sources [120,122,123]. Thus, pretreatment of chitin substrates before enzyme addition seems to be necessary in order to improve the accessibility of the acetyl groups to the enzyme and therefore to enhance the deacetylation yield. For that purpose, experiments have been performed in homogeneous conditions using chitin deacetylase from *M. rouxii* with partially deacetylated water-soluble chitosans [126]. In selected conditions, the enzyme is able to deacetylate chitosan up to 97% (deacetylation from an initial chitosan with DA = 0.32 and a number-average degree of polymerization of 30) [126].

These findings suggest that the development of a controllable process using the enzymatic deacetylation on chitinous substrates is an attractive alternative process that can result in the preparation of novel chitosan polymers and more interestingly oligomers.

3.2. Chitosan Characterization and Solubility

Chitosan obtained from partial deacetylation of chitin becomes soluble in aqueous acidic medium when the average degree of acetylation DA is lower than 0.5. In fact, this limit depends on the distribution of acetyl groups along the chains. At this stage, it is possible to obtain a complete characterization of the polymer but it may differ from the starting material, specially its molecular weight is reduced during deacetylation in strong alkaline medium, as mentioned previously.

The physical properties of chitosan in solution depend strongly on DA and on the acetyl group distribution along the chains. Block-wise distribution of acetyl groups, caused by heterogeneous deacetylation performed on solid state chitin, causes chain association even in dilute solutions and formation of aggregates as well as difficulties in molecular weight determination [127,128]. In addition, fully deacetylated chitosan may be reacetylated in homogeneous phase [129] in order to get samples soluble in acidic conditions up to DA∼0.6 in relation with a random distribution of the acetyl groups. Under these conditions, the existence of free -NH_2 groups, available on C-2 position of D-glucosamine units along the chains, allows to perform specific reactions in homogeneous conditions [130–136].

The first step in chitosan characterization is the determination of their molecular weights (after dissolution), then DA and eventually the distribution of acetyl group along the chain (by NMR). Additionally, different solvents based on acetic acid have been proposed, for instance: 0.3 M acetic acid added with sodium acetate (up to 0.1 or 0.2 M) in aqueous solution. The presence of an external

salt is needed to screen the long-range electrostatic repulsions between the charged chains. Chitosan is also soluble in acetic acid or hydrochloric acid at pH lower than 6 (its intrinsic pK being around 6.5) [1].

The determination of average DA for chitosan may be performed by different techniques: infrared spectroscopy [86], elementary analysis, and potentiometric titration, but ^{1}H liquid state [89] and solid state ^{13}C-NMR [90,137,138] are preferred. Infrared spectroscopy has to be used carefully because the result interpretation is related to the difficulty in adopting a convenient base line. This problem was also discussed previously for samples with different DAs [86]. At present, ^{1}H NMR seems to be the most convenient technique to get the correct DA for soluble samples. An example is given in Figure 3. Additionally, ^{13}C NMR is also convenient for DA determination in the case of pure chitin up to fully deacetylated chitosan with a good agreement between measurements in solid state and liquid phase (Figure 2) [90].

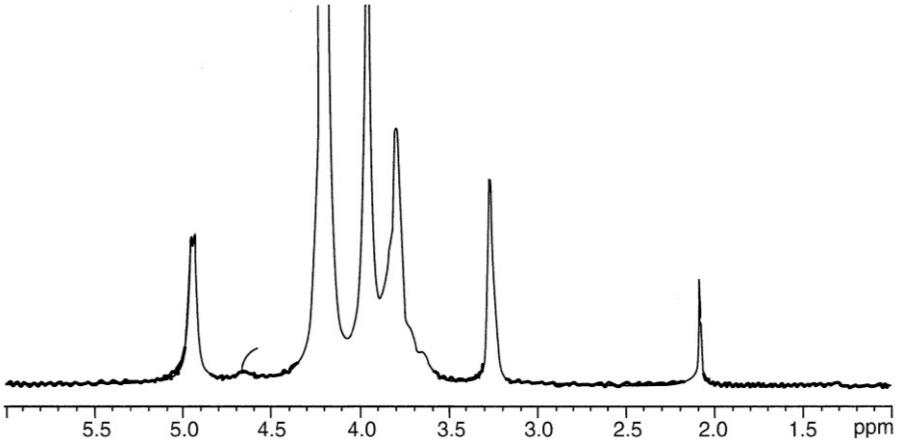

Figure 3. ^{1}H-NMR spectrum of chitosan with DA~0.06 in D_2O at pH~4, T = 85 °C and polymer concentration 5 g/L. Signals at 4.9 ppm is for H-1 of D-glucosamine unit, at 4.7 ppm is for H-1 of *N*-acetyl-D-glucosamine, at 3.2 ppm is for H-2 and at 2.1 ppm is for -CH_3 of the acetyl group allowing to get DA.

It is important to recall that high field ^{13}C-NMR spectroscopy is important to establish the distribution of acetyl groups along the chitosan chains [139,140].

Furthermore, the molecular weight distribution and the average molecular weight as well as the intrinsic viscosity also play a significant role. However, chitosan solution must be free of aggregates, thus the solvent for chitosan must be chosen carefully. The viscometric-average molecular weight has been usually calculated from intrinsic viscosity (lower impact of small fractions of aggregates) using the Mark-Houwink relationship [1]. In order to determine *K* and *a* parameters, an absolute MW has to be calculated using light scattering technique; nevertheless, the obtained value is usually overestimated due to high sensitivity to aggregate formation [127,128]. These artifacts may be omitted for instance by the use of 0.3M acetic acid/0.2M sodium acetate (pH = 4.5) solvent which does not form aggregates in this mixture [141]. Under these conditions, the absolute M values were obtained from steric exclusion chromatography (SEC) equipped with viscometer and light scattering detector on line allowing to determine the Mark-Houwink parameters without fractionation and also to obtain the relation between the radius of gyration and the molecular weight [142]. The *K* (mL/g) and *a* parameters at 25 °C are 7.9×10^{-2} and 0.796 respectively.

The relatively high values obtained for the parameter a are in agreement with the semi-rigid character of this polysaccharide which controls their dimensions, hydrodynamic volume and viscometric contribution. The stiffness is related to the persistence length (L_t) of the chain: chitosan in acid medium behaves like a polyelectrolyte, so the actual total persistence length L_t at a given ionic concentration is equal to the intrinsic contribution L_p and the electrostatic contribution L_e, calculated following the Odijk treatment [143]. The conformational analysis of chitin with different degrees of deacetylation confirms that chitin and chitosan are semi-rigid polymers characterized by a persistence length which depends moderately on the degree of acetylation of the molecule. From this analysis, chitosan, free of acetyl groups, has an intrinsic persistence length L_p of 9 nm in salt excess [144]. L_p increases when DA increases up to 12.5 nm for DA = 0.6, remaining constant up to pure chitin at 25 °C. These predictions are in agreement with the experimental values obtained by SEC [142].

3.3. Processing and Main Properties of Chitosan-Based Materials

Solutions of chitosan prepared in acidic medium are processed to the needed conformation (casted for a film, spun for fibers, freeze dried for sponges, *etc.*), immersed in an alkaline solution (in which they precipitate), washed and dried. The processing of chitosan is easier than that of chitin but the stability of the materials is lower due to the larger hydrophilic character and especially the pH sensitivity. For better stability, chitosan may be crosslinked using reagents such as epichlorohydrin, diisocyanate, 1,4-butanediol diglycidyl ether, or glutaraldehyde [145–147]. Many chitosan hydrogels were obtained by treatment with multivalent anions as oxalic acid [148,149] or citric acid [150–152] or tripolyphosphate [153]. Blends and composites are sometimes produced taking advantage of the polycationic properties of chitosan in acidic conditions.

In fact, chitosan being a polyelectrolyte is able to form interesting electrostatic complexes (hydrogels) with oppositely charged macromolecules. The properties of these complex materials depend on the polymer concentration, temperature, pH and ionic concentration. Electrostatic polyelectrolyte complexes (PEC) are mentioned in the literature involving chitosan complexed with synthetic or natural polymers [4]. Electrostatic interactions between chitosan and lipidic vesicles are also important in the biological and pharmaceutical fields due to bioadhesive and permeabilizer roles of chitosan [154–156]. Coating of liposomes with chitosan also increases their biocompatibility, and stabilizes the composite membrane against pH as well as ionic concentration [155].

Nowadays, these electrostatic interactions are applied for preparation of layer-by-layer polyelectrolyte capsules or films based on charged biocompatible polysaccharides or chitosan/synthetic polyelectrolytes [157–159]. Core-shell phospholipid nanoparticles were stabilized via layer-by-layer self-assembly of anionic alginate and cationic chitosan and were proposed for protein release [157].

Chitosan and alginate electrostatic complexes have been mostly used so far for biological applications [160–162]. Complexes formed between DNA or RNA and chitosan (oligomers or polymers) are actually under further investigation in many laboratories; the charge density and DA of chitosan are essential for the complex stability [163–167].

4. Relation between Chemical Structure and Biological Activities

Because chitosan and derivatives possess many beneficially properties such as biocompatibility, biodegradability, safety and also interesting biological activities, much attention has been paid to their applications especially in biomedical, food, biotechnology and pharmaceutical fields [168,169]. Among their attractive biological activities, antimicrobial, antioxidant and antitumor activities will be discussed in detail below. These properties are specially recognized in the field of food preservation and packaging to avoid the use of chemical preservatives and to produce edible antimicrobial films due to the good film forming properties of chitosan. Chitosan, as a polymeric ingredient with a good antimicrobial and antioxidant properties, does not migrate easily out of the protecting film and has better barrier properties. This was previously demonstrated in many studies, such as those of Friedman *et al.* [170], Kardas *et al.* [171] and Alishahi and Aider [172].

Friedman *et al.* [170] studied the antimicrobial activities of chitosan in solution, powders and edible films and coating against foodborne pathogens, spoilage bacteria, and pathogenic viruses and fungi in several food categories. These include fruit juices, eggs and dairy, cereal, meat and seafood products. They suggest that low-molecular-weight chitosans at a pH below 6.0 presents optimal conditions for achieving desirable antimicrobial and antioxidative-preservative effects in liquid and solid foods.

The use of chitosan and derivatives in the food industry was also described in the review of Kardas *et al.* [171]. They demonstrated that these biopolymers offer a wide range of unique applications including preservation of foods from microbial deterioration and formation of biodegradable films.

Alishahi and Aider [172] reported that chitosan films in packaging application tend to exhibit resistance to fat diffusion and selective gas permeability. But, inconvenience comes from their low resistance to water and water vapor transmission. This behavior is due to the strongly hydrophilic character mainly of chitosan, a property that leads to high interaction with water molecules [173]. For this reason, polymer blending or the use of biocomposites and multilayer systems are potential approaches to prepare chitosan-based bioactive coatings.

4.1. Antimicrobial Activity

Chitosan was shown to have several advantages over other disinfectants, as it possesses a higher antimicrobial activity, a broader spectrum of activity, a higher kill rate, and lower toxicity towards mammalian cells [174,175]. Many studies have demonstrated that chitosan have an important antimicrobial activity. However, the actual mechanism of inhibition is not yet fully understood. The most feasible hypothesis is a change in cell permeability due to interactions between the positively charged polysaccharide (chitosan at pH lower than 6.5) and the negatively charged membrane. The mechanism underlying the inhibition of bacterial growth should be that the positively charged polymer combines with anionic components such as *N*-acetylmuramic acid, sialic acid and neuraminic acid, on the cell surface.

Firstly, concerning the antibacterial activity of chitosan, possible actions of chitosan and its derivatives have been proposed. Chitosan (especially low-MW particles) could penetrate cell wall of bacteria, combine with DNA and inhibit synthesis of mRNA and DNA transcription [176]. High MW chitosan could interact with cell surface and consequently alter cell permeability [177], or form an

impermeable layer around the cell, thus blocking the transport of essential solutes into the cell [178,179]. Chung *et al.* [180] confirmed that antibacterial mechanism includes hydrophilicity and the negative charge of cell surface and the adsorption of chitosan onto the bacterial cell. They show that cell wall hydrophilicity and negative charge of the cell surface were higher in gram-negative bacteria compared to gram-positive and that, in addition, the distribution of negative charges on their cell surfaces was quite different from that of gram-positive. Then, the more negatively charged cell surfaces interact more with positively charged chitosan, in acidic conditions. Their results showed a high value of correlation coefficient between adsorbed chitosan and inhibition efficiency. Moreover, many other studies showed that chitosans are more effective for gram-negative bacteria than gram-positive bacteria [181–183].

It was also indicated that adsorbed amounts of chitosan were related to environmental pH values (pH < 6.5) and degree of acetylation of chitosan [183–185]. Chitosan is more absorbed by bacterial cells at lower pH in relation with the increase of the chitosan positive ionic charge in relation with the fraction of deacetylated groups (1 − DA). From literature, it is clearly shown that there is a direct relationship between the antibacterial activity of chitosan and its characteristics especially DA. Effect of DA on chitosan antimicrobial activity has been clearly demonstrated in our previous study [186]. In these data, it is clearly shown that the lower DA, the lower MW and the lower pH give the larger efficiency.

In addition, influence of the MW was introduced by Zheng *et al.* [187]. They differentiated the effect of chitosan on *Staphylococcus aureus* (gram-positive) and on *Escherichia coli* (gram-negative) and demonstrated that, for gram-positive *S. aureus*, the antimicrobial activity increases with increase of the molecular weight of chitosan. On the opposite, for gram-negative *E. coli*, they indicate that the antibacterial activity increased with decrease in molecular weight. These authors suggested the two following mechanisms for the antimicrobial activity: in the case of *S. aureus*, the chitosan on the surface of the cell forms a polymeric membrane, which inhibits nutrients from entering into the cell and, for *E. coli*, chitosan with a lower molecular weight entered the cell through pervasion.

Effect of MW was also discussed by Benhabiles *et al.* [188] preparing oligomers of chitin and chitosan. Their antimicrobial activities against four gram-positive and seven gram-negative bacteria were compared to initial chitosan and chitin. They conclude that chito-oligomers would have advantages as new antimicrobial agents due to their higher activity and larger water solubility than the native polysaccharides.

Concerning the antifungal activity, it has been reported that chitosan can reduce the infection of *Fusarium oxysporum* f. sp. apii in celery and inhibits the spread of *Sphaerotheca pannosa* var. rosae, *Peronospora sparsa* and *Botrytis cinerea* on roses [189–191]. Treating tomato plants with chitosan solution reduced mycelial growth, sporangial production, release of zoospores and germination of cysts of *Phytophthora infestans* which resulted in significant disease protection [192]. In addition, chitosan seed treatment could reduce *Colletotrichum* sp. infection and improve performance of chilli seedling [193]. Concerning the mechanism, it is suggested that chitosan forms a permeable film at interface [194] and has two functions: direct interference of fungal growth and activation of several defense processes. These defense mechanisms include accumulation of chitinases, synthesis of proteinase inhibitors, lignification and induction of callous synthesis [195].

In fact, dependence of activities on chitosan characteristics was reported to depend on the particular fungal species. For example, it was shown that fungal growth decreased with increasing MW for *F. oxysporum* and with decreasing DA for *Alternaria solani*, but no MW or DA dependences were observed with *A. niger* [186].

4.2. Antioxidant Activity

Oxidative stress belongs to the main causes of many diseases, mainly cancer and cardiovascular problems, which significantly increase the worldwide mortality [196–202]. Dietary antioxidants, which inactivate reactive oxygen species and provide protection from oxidative damage [196–202], are considered as important preventive strategic molecules.

Once the lipid oxidation occurs in food products, off-flavors and undesirable chemical compounds are formed and this may be dangerous for health. Therefore, in order to minimize this risk, some antioxidants (like synthetic antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ) and propyl gallate) are added to delay the deterioration, caused by lipid oxidation. However, these antioxidants create potential health hazards, and their use has been restricted in some countries. Therefore, there has been a growing interest in natural antioxidants rather than in synthetic ones.

In recent years, much more attention has been paid to study the antioxidant activity of chitosan and its derivatives [203]. It was reported that chitosan and its derivatives act as antioxidants by scavenging oxygen radicals such as hydroxyl, superoxide, alkyl as well as highly stable DPPH radicals tested *in vitro* [204]. Sun and collaborators [205] reported that chitosan and their derivatives act as hydrogen donors to prevent the oxidative sequence.

Furthermore, it was observed that the radical scavenging properties of chitosans depend on their DA and MW. Park *et al.* [204] demonstrated that low-MW chitosans are more active than those with higher MW. Chitosan samples with low MW (1∼3 kDa) revealed higher potential to scavenge different radicals. Other examination showed that low-MW chitosans can exhibit more than 80% of superoxide radical scavenging activity at 0.5 mg/mL concentration [206]. The influence of chitosan MW (30, 90, and 120 kDa) on the antioxidant activity in Salmon skin was also studied [207]. The results of this study showed that all chitosans present antioxidant activities, which reduce Salmon lipid oxidation, the 30-kDa chitosan sample having the higher antioxidant activity. In addition, highly deacetylated (90%) chitin are more preferable for scavenging DPPH, hydroxyl, superoxide and carbon-centered radicals [208]. Even though the precise mechanism of radical scavenging activity is not clear, it is attributed to amino and hydroxyl groups (attached to C-2, C-3 and C-6 positions of the pyranose ring) reacting with unstable free radicals, which facilitate formation of stable macromolecule radicals.

4.3. Antitumor Activity

Chitosan and its derivatives possess also antitumor activities investigated by both *in vitro* and *in vivo* method [209]. Some *in vivo* studies reported that chitosan inhibits the growth of tumor cells by exerting immunoenhancing effects. They concluded that the observed antitumor activity was not due to a direct killing of tumor cells, but by an increase of lymphokines production resulting in proliferation

of cytolytic T-lymphocytes [210]. Chen *et al.* [211] demonstrated that intratumoral administration of a chitosan gel in animals reduces metastatic breast cancer progression. Chitosan also stimulates macrophages maturing into cytotoxic macrophages and suppresses tumor growth in mice [212]. It has been suggested that elevated secretion of IL-1 and IL-2 causes the anti-tumor effect through maturation and infiltration of cytolytic T-lymphocytes [213].

Other studies demonstrated that chitosan also exhibits a direct effect on tumor cells; it inhibits tumor cell proliferation by inducing apoptosis [214]. For instance, Hasegawa *et al.* [215] showed that chitosan may cause apoptotic death of bladder tumor cells via caspase-3 activation. Further studies revealed that chitosan nanoparticles could also induce necrotic death, which had been tested on liver cancer cells via neutralization of cell surface charge, observed as a decrease in mitochondrial membrane potential and induction of lipid peroxidation [216]. Moreover, chitosan may inhibit Ehrlich ascites tumor growth by reduction of glycolysis causing a decrease in glucose uptake and ATP level in the tumor cells [217]. This study also found that chitosan administered orally at a dose of 1 mg kg^{-1} in mice reduces tumor growth by ~62%, without any toxicity to the liver. Thus, chitosan *per se* possesses potential activity against cancer, even when it is administered orally. A similar study, using a chemically-induced tumor model, showed that addition of chitosan to the diet enables to suppress aberrant crypt tumor lesions in the colon of mice [218]. Interestingly, such protection with chitosan additive in feed lasts only up to 6 weeks. This investigation highlighted that chitosan is responsible for an increase in expression of p21/Cip and p27/Kip and consequently a decrease of expression of proliferating cell nuclear antigen in a human gastric cancer cell line. Nevertheless, further studies are required in order to better understand all mechanisms involved in chitosan-based tumor stasis.

Chitosan activity depends not only on the chitosan structural characteristics, such as DA and MW, but also on tumor species. Jeon and Kim [219] studied the antitumor activity of chitosan oligosaccharides with different molecular weights. They found that chitosan oligosaccharides with MW ranging from 1.5 to 5.5 kDa may effectively inhibit the growth of Sarcoma 180 solid (S180) or Uterine cervix carcinoma No. 14 (U14) tumor in BALB/c mice. Studies on mice examining chitosan samples with different MW revealed significant antimetastatic effects of chitosan against Lewis lung carcinoma. It was shown that the activity increases with decreasing the molecular sizes suggesting an immunostimulating effect which activates peritoneal macrophages and stimulates non-specific host resistance. It was also shown that chitosan samples with higher MW exhibits lower antitumor activity [220]. However, other authors found that decrease of MW of chitosan from 213 to 10 kDa does not affect its *in vitro* cytotoxicity on human lung carcinoma cell line A549 [221]. Additionally, chitosan samples with different MW ranging from 42 to 135 kDa were also evaluated in terms of their cytotoxicity on human bladder cancer RT112 and RT112cp cells and no effect of MW was observed [222]. The same study attempted to examine also the effect of chitosan DA (homogeneous chitosans with DA ranging from 2% to 61%) on cytotoxicity. The results from this experiment indicated that all chitosan samples were active on bladder carcinoma cells, with better activity for samples with higher DA.

It has been shown that chitosan reveals anticancer activity, thus it may be used for encapsulation of anticancer agents. However, before its incorporation into new drugs, pre-tests are required.

5. Pharmaceutical and Biomedical Applications of Chitin and Chitosan

The main properties of chitin and chitosan, applied for specific applications, have been already described such as: biocompatibility, renewable origin, non-toxicity [223], non-allergenicity and biodegradability in the body [224]. In addition, due to their attractive biological activities (antifungal, antibacterial, antitumor, immunoadjuvant, antithrombogenic, anticholesteremic agent) and bioadhesivity (especially of chitosan and its derivatives [225]), they are widely used as absorption promoters and hydrating agents, as well as for film production and wound healing [1–4,226]. Chitin and more easily chitosan may be processed, depending on the intended application, into different conformations such as fibers, powders, films, sponges, beads, solutions, gels and capsules [171]. Consequently, chitosan may be used in oral, nasal as well as ocular routes, for drug delivery in both implantable and injectable forms. Chitin and chitosan in fiber or film state, are mainly applied for tissue engineering and wound care dressing [227–229]. Additionally, transmucosal absorption promoter effect of chitosan is especially important for nasal and oral delivery of polar drugs to administrate peptides and proteins and for vaccine delivery [230,231]. Cationic chitosan may affect transport of ions through an interaction with the cell surface (inducing perturbation of membrane phospholipids bilayers). Chitin is also used as excipient and drug carrier in film, gel or powder form for applications involving mucoadhesivity [52]. Actually, the main promising developments are aimed to pharmaceutical and biomedical domains [232–245]. Several selected applications for chitin and chitosan will be described below and summarized in Table 3.

Applications of chitin are less developed compared to those of chitosan due to its large insolubility and difficulties in processing. Therefore, chitin is very often combined with chitosan which gives in fact similar applications.

Chitin accelerates wound-healing in spray, gel and gauze [246–249]. It is used as support of medicaments or to control drug release [238] taking into account the biodegradability, low toxicity, physiological inertness, antibacterial properties, hydrophilic character, gel forming properties, affinity for proteins and mucoadhesivity [250].

Great attention had been paid to a composite material made of hydroxyapatite-chitin-chitosan which may be used as bone filling material for guided tissue regeneration (treatment of periodontal bony defects). This composite forms a self-hardening paste [251–261]. Chitin was also used for enzymes and whole cells immobilization [262] as well as for tissue engineering [263,264].

Chitosan (the only pseudo-natural polycationic substance) and its electrostatic complexes formed with synthetic or natural polymers (as alginate) are used as antithrombogenic materials for: controlled release, drugs encapsulation, enzymes and cells immobilization and also as gene carriers. Advantage of chitosan-based materials is related to their biodegradability, antibacterial activity, hydrophilic property, as well as presence of polar groups which are able to form secondary interaction with other polymers (-OH and -NH_2 groups involved in hydrogen bonds and the *N*-acetyl groups in hydrophobic interactions).

Table 3. Main applications of chitin and chitosan in pharmaceutical and biomedical domains.

Forms	Applications
Beads Microspheres [265] Nanoparticles	Drug delivery [266] Enzyme immobilization Gene delivery vehicle [267] Encapsulation of sensitive drugs [172]
Coatings	Surface modification Textile finishes
Fibers Nanofibers [268] Nonwonen bioactive fibers [269]	Medical textiles Suture Guided bone regeneration Scaffold for nerve tissue regeneration Wound healing
Films	Wound care Dialysis membrane Antitumoral [270] Semi-permeable film for wound dressing [271]
Powder	Adsorbent for pharmaceutical and medical devices Surgical glove powder Enzyme immobilization
Sponge [272]	Mucosomal hemostatic dressing Wound dressing Drug delivery [272] Enzyme entrapment Artificial skin [271]
Shaped objects	Orthopedics Contact lenses
Solutions	Cosmetics Bacteriostatic agent Hemostatic agent Anticoagulants Antitumor agent Gene delivery [267] Spermicide [245]
Gels	Delivery vehicle Implants, coating Tissue engineering Wound dressing for wet treatment [271]
Tablets	Compressed diluent Disintegrating agent Excipient [273]
Capsules	Delivery vehicle

Materials for wound dressing and tissue engineering are important but still under development [274–282]. New adhesives were also proposed [283,284]. The Az-chitosan derivative is non-toxic, cytocompatible and mechanically suitable for peripheral surgeries [285]. Chitosan films, like

many other polysaccharide-based films, exhibit resistance to fat diffusion and selective gas permeability but they are relatively poor in terms of resistance to the transmission of water and water vapor. This behavior is observed due to their hydrophilic character leading to high interaction with water molecules [173]. In order to overcome this problem, polymer blending or biocomposites and multilayer systems are used for preparation of chitosan-based bioactive and stable coatings.

Mucoadhesivity of chitosan and its cationic derivatives is recognized and proved to enhance the adsorption of drugs especially at neutral pH. *N*-trimethyl chitosan chloride interacts with the negatively charged cell membranes [286]. *N*-lauryl-carboxymethylchitosan being an amphiphilic polymer forms micelles solubilizing taxol which becomes more efficient. This type of chitosan derivative is safe in terms of membrane toxicity and it could be useful as carrier for hydrophobic cancer drugs [287,288]. Chitosan or its derivatives were used for gene transfection. It was shown for *N*-alkylated chitosan that transfection efficiency increases upon elongation of the alkyl side chains up to eight carbons in the side chain [289]. Quaternized chitosan was also used for the same purpose [290]. Porous chitosan (and derivatives) microspheres were prepared in order to deliver antigens in a controlled way [291]. This type of particles was loaded with Newcastle disease virus vaccine and tested *in vitro* and *in vivo* [291,292].

An interesting application of the chitosan- calcium phosphate cement was found. Chitosan or chitosan glycerophosphate was mixed with calcium phosphate and citric acid and an attractive injectable self-hardening system for bone repair or filling indications was formed [253–255].

At the end, several examples of applications for drug delivery are mentioned [265,293–296]. Chitosan may be processed more easily than chitin to different forms: in sponge, capsule or nanoparticle depending on the tested system and the goal of its administration.

6. Conclusions

In this review, the characteristics of chitin and chitosan are described. This was followed by a discussion on the solubilization required to process the polysaccharides to obtain new materials. Their fiber and film-forming abilities are recognized on the basis of the H-bonds network formation in the solid state which may be useful for new potential applications.

However, the most important applications come from their hydrophilic character and antimicrobial properties, especially desired for production of new biomaterials.

Chitosan in comparison with chitin is soluble in acidic media, which is applied for improvement of processing methods. In fact, chitosan may be easily processed as fiber, film, sponge, bead, gel or solution. Additionally, its cationic charge provides the possibility to form electrostatic complexes and/or multilayer structures. The presence of free -NH_2 groups along chitin and chitosan chains allows to perform specific modifications (performed on the C-2 position of the D-glucosamine unit) under pretty mild conditions (even in aqueous conditions with chitosan). Furthermore, chitin and chitosan can be blended with synthetic or natural polymers (proteins, DNA, alginate, hyaluronan, *etc.*).

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Author Contributions

This work was performed in strong cooperation between the two co-authors.

Conflicts of Interest

The authors declare no conflict of interest.

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Chitosan Petition
ADDENDUM 2

Hyperlinks as Requested by USDA AMS

Information requested by USDA AMS in their December 5, 2024 letter to G. Sargent:

"The following items referenced in your petition can be excluded from the above request to submit PDFs. However, please add hyperlinks to these references to support the Board's review of your petition."

The information/links provided in this Addendum sorted:

First: **CHRONOLOGICAL ORDER**
Second: **BY ISSUE**

The text in blue text boxes signify the documents/links that USDA AMS requested in their December 5, 2024 email to G. Sargent.

The capital letters i.e. A, designate the 5 individual issues with regard to organic use approval for chitosan:
A 2004-Jan Petition WSU-Chitosan as adjuvant/sticker.
B 2019-Oct Petition Botryzen-Chitosan for plant disease control.
C 2020-Mar Petition Tidal Vision-Chitosan as adjuvant/flocculant
D Additional records*** (section added by current petitioner)
E Previous petitions (section added by current petitioner)
F Other pertinent information (section added by current petitioner)

***** Addendum II : 7 PDFs of requested documents**
***** Addendum III: Manufacturing Information.**

Table of Contents

A 2004-Jan WSU Petition Chitosan adjuvant/sticker

2. 2004-Jan: WSU Hadwiger petition to NOSB.

2004-Jan WSU Petition Add Chitosan to the National List as an adjuvant.pdf

The use of chitosan specified in this petition was as an adjuvant/sticker.

3. 2004-Feb: ICF Technical Evaluation Report: Chitosan-Crops

2004-Feb ICF Chitosan Technical Report - Agricultural Marketing Service

2005-Aug Crops Subcom Votes 3-0 add chitosan to the National List

2005-Aug NOSB Votes 3-0 to Recommend Chitosan (pdf)

WSU/Hadwiger's petition was the first effort to get chitosan approved for use in organic farming. There are however, three significant errors realized at the outcome of the Crops Subcommittee meeting in February, 2005.
As stated on the last page of the Committee's August, 2005 Evaluation Criteria form:

> *"Poly-D-Glucosamine (Chitosan) [CAS NO. 9012-76-4] is synthetic and allowed as an insecticidal soap [205.505.(e)6]"*

1. First of all, on the last page of the Evaluation Form, the reference to *"insecticidal soap"* is an obvious error. It conflicts with the purpose referenced on the same page, that is *as "an adjuvant only".* Furthermore, if the material is used as an insecticide, it cannot, obviously be classified/regulated as an adjuvant.
2. The reference to *insecticidal soap* was most likely mistaken with the petition submitted for the substance, sucrose octanoate ester in review with the Committee during the same time frame. The applicant claimed sucrose octanoate acts as an insecticidal soap.
3. At the time this Addendum was drafted, there are no applicable regulations under "*205.505.(e)6"* of Title 7 as referenced in the same form. (perhaps it should be *205.601 Synthetics allowed for use in organic crop production (m)(1): EPA List 4.*

Additional comments about the mistaken classification of the use of chitosan as insecticidal soap and adjuvant only, was carried over into subsequent reviews by NOP representatives in their evaluation of whether or not the chitosan can be added to the NOP List. One can question whether these inaccuracies influenced the outcome of their reviews.

1. 2006-Jul 3: FR Proposed Rule Chitosan-adjuvant

FR Vol. 71, No.127, July 3, 2006 - Document in Context
(click on **Agricultural Marketing Service** and PDF. (See page 37855)

Page FR page 37855

Chitosan (Poly-D Glucosamine) (CAS #9012-76-04). Chitosan was petitioned for use in organic crop production as an adhesive adjuvant to be used with fungicides approved for use under the NOP regulations…

…At its August 17, 2005, meeting in Washington, DC, the NOSB recommended adding chitosan to the National List for use in organic crop production as an insecticide, with the restriction that it only be used as an adjuvant.

Page FR 37856

…The NOP regulations, at § 205.601(m), permits the use of EPA List 4 inert ingredients with on synthetic substances or synthetic substances approved for use under the NOP regulations as an active pesticide ingredient. As a result, the NOP will not propose to specifically add chitosan o the National List as an adjuvant; it is already permitted for use at 205.601(m) of the National List regulations…

Page FR37857.

General Notice of Public Rulemaking

This proposed rule reflects recommendations submitted to the Secretary by the NOSB. The 2 substances proposed to be added to the National List were based on petitions from the industry. The NOSB evaluated each petition using criteria in the OFPA. Because these substances are critical to organic production and handling operations, producers and handlers should be able to use them in their operations as soon as possible. A 30-day period for interested persons to comment on this rule is provided.

So the mistaken claim that chitosan is an insecticide as an adjuvant only, was carried over from the Crops Subcommittee in their August, 2005 meeting to the Proposed Rule posted in the July 3, 2006 Federal Register.

However, a more significant issue involves changes the EPA made to the status of inerts used in pesticide (and adjuvant) formulations. During the same time period, 2006, EPA completed the reassessment and classification of all the inerts used in pesticides (and adjuvants) formulations. EPA discontinued the use and maintenance of all of its Inerts Lists.

Concurrently the EPA established classifications for use of inerts as either **Food Use or Nonfood Use.** Consequently, Chitosan was (and still is) EPA-approved for **Nonfood Use only**. This means Chitosan cannot be used in pesticide and adjuvant formulations for products applied to food crops.

Therefore, while NOSB contends that because chitosan is, as an inert, on EPA's Inerts List 4 and thereby is approved for use in organic production under 205.601(m) of the National List, it is not EPA-approved for use on food crops.

In other words, chitosan is not EPA-approved for use on food crops (i.e. berries, corn, soybean, citrus fruit, etc. It is only approved for use on non-food crops (i.e. turf, Christmas trees, ornamentals, flowers, etc.),

Therefore the use of chitosan as an inert is severely limited to nonfood use which seems rather useless in organic production.

It is interesting to note that OMRI errantly does List adjuvant products formulated with chitin, for use on food crops. The people I communicated with at OMRI do not fully understand the regulations.

2007-Dec Final Rule (USDA NOP): Chitosan is not an insecticide

https://www.regulations.gov/document/AMS-TM-07-0112-0001

As a result, for the purpose of the NOP regulations, chitosan would be better characterized as a plant disease control.

…At its August 17, 2005, meeting in Washington, DC, the NOSB recommended adding chitosan to the National List for use in organic crop production as an insecticide, with the restriction that it only be used as an adjuvant.

Chitosan (Poly-D Glucosamine) (CAS #-9012-76-04) was petitioned for use in organic crop production as an adhesive adjuvant to be used with fungicides approved for use under the NOP regulations. At its August 17, 2005, meeting in Washington, DC, the NOSB recommended adding chitosan to the National List for use in organic crop production as an insecticide, with the restriction that it only be used as an adjuvant.I

In addition to the concerns raised about chitosan's use as an adjuvant in combination with another fungicide, the issue of whether chitosan should be considered an insecticide (as recommended by the NOSB) or a plant disease control was mentioned. The EPA informed the NOP that data does not reveal chitosan having insecticidal properties. Instead, chitosan is considered more of a systemic acquired response inducer and demonstrates fungicidal activity. As a result, for the purpose of the NOP regulations, chitosan would be better characterized as a plant disease control.

…"The NOSB recommended adding chitosan to the National List for use in organic crop production as an "insecticide," with the restriction that it only be used as an "adjuvant.",,, AMS, in consultation with EPA,… has determined that chitosan, when used as an "adjuvant" (not demonstrating any pesticidal activity), is already allowed under the existing inert ingredient provisions of § 205.601(m) of the NOP regulations."

B. 2019-Oct BioGro/Botryzen Petition Chitosan for plant disease control

2019-Oct BioGro-Botryzen Petition: petition requesting chitosan be added to the NOP List for use in organic crop production for plant disease control. The product referenced is Armour-Zen®.

2019-Oct BioGro-Botryzen Armour Zen petition (pdf) chitosan for plant disease control

4. 2020-Jul Nexight Tech Evaluation Chitosan-Crops;
2020-Jul Nexight Tech Evaluation Report.pdf

2021-Jun NOSB Crops Subcommittee Notes page Vote 4-4 to add Chitosan plant disease control.pdf
The motion did not receive the required two-thirds majority required Motion

2021-Oct NOSB Formal Recommendation Vote 7-7 add Chitosan for plant disease control.pdf
The motion did not receive the required two-thirds majority required Motion fails

C. 2020-Mar: Petition by Tidal Vision as an adjuvant/flocculant

5. 2020-Mar Tidal Vision Petition Chitosan as a Flocculant
Tidal Vision Petition; Add Chitosan to National List Under §205.601 for Chitosan for use in Organic Crop Production as a Production Aid (flocculant)
2020-Mar Tidal Vision Petition (pdf)

2021-Jun Crops Subcommittee pg 58 Petition insufficient.pdf
Chitosan - coagulant for fertilizer (RG). The lead summarized the substance, which is being petitioned as a value-added product. The CS sent follow-up questions to the petitioner in September 2020 and got no response. Without responses to its questions, the CS deemed the petition insufficient at this time and will send the it back without review. The NOP will transmit the information to the petitioner.

E. Additional records.

6. Any reference to NOSB recommendations or meeting minutes.
7. Formal recommendations, subcommittee proposals, subcommittee notes written by the NOSB.
8. Any previous petition for chitosan reviewed by the NOSB.

2007-Sep Chitin and Chitosan Summary Document; Registration Review (EPA)
https://ww 2007-Sep Chitin and Chitosan Summary Document; Registration Review (EPA)

2007-Sep EPA Registration review Chitin & Chitosan

E. Previous petitions

8. Any previous petition for chitosan reviewed by the NOSB

2019-Oct BioGro-Botryzen Petition: petition requesting chitosan be added to the NOP List for use in organic crop production for plant disease control. The product referenced is Armour-Zen®.

F. Other pertinent information

1995-Apr: EPA Final Rule: TOLERANCE EXEMPTION

Establishes an exemption from the requirement of a tolerance of the biochemical growth regulator chitosan when used as a seed treatment in or on rice.

https:1995-Apr Final Rule Tolerance exemption for chitosan

2023-Jan: EPA Final Rule: Chitosan added to Minimum Risk List Pesticides (exempt from FIFRA)

Establishes HEPA Adds Chitosan to the List of Active Ingredients Eligible for Minimum Risk Pesticide Exemption
Released on November 8, 2022

e final rule is available in docket **EPA-HQ-OPP-2019-0701**

Chitosan Petition

ADDENDUM 3

Additional Publications (6 ea) Referenced in the Chitosan Petition

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*** **FR 2006-Jul 3: Federal Register Vol. 71, No. 127; July 3, 2006 (**pp 37854) Proposed Rule: Chitosan petitioned for use in organic crop production as an adhesive adjuvant. [not accepted as Chitosan is already permitted under §205.601(m) of the National List Regulations].

*** **FR 2007-Dec 10: Federal Register Vol. 72, No. 236;** (pp 69569–69572) Dept of Agriculture; Agricultural Marketing Service; 7 CFR Part 205; Docket No. AMS-TM-07-0112; Final Rule.

*** **Hadwiger-2004-Jan: Petition to add chitosan (poly-D-glucosamine) to the National List of materials** approved for use by organic growers. Submitted by Dr. Lee A. Hadwiger, Department of Plant Pathology, Washington State University, Pullman, WA.

*** **ICF Consulting; 2004-Feb: Poly-D-Glucosamine (Chitosan)-Crops; Technical Evaluation Repor**t Compiled by ICF Consulting for the USDA National Organic Program.

*** **Nexight 2020-Jul: Chitosan-Crops; Technical Evaluation Report** Compiled by Nexight Group for the USDA National Organic Program.

*** **NOSB 2005-Aug 17 NOSB Meeting Minutes & Transcripts; Formal Recommendation by the NOSB to the NOP;** "NOSB Meeting Minutes & Transcripts: 1992-2009." USDA Agricultural Marketing Service