Chitin, alginate and others

Biopolymers
CHEM-E2155

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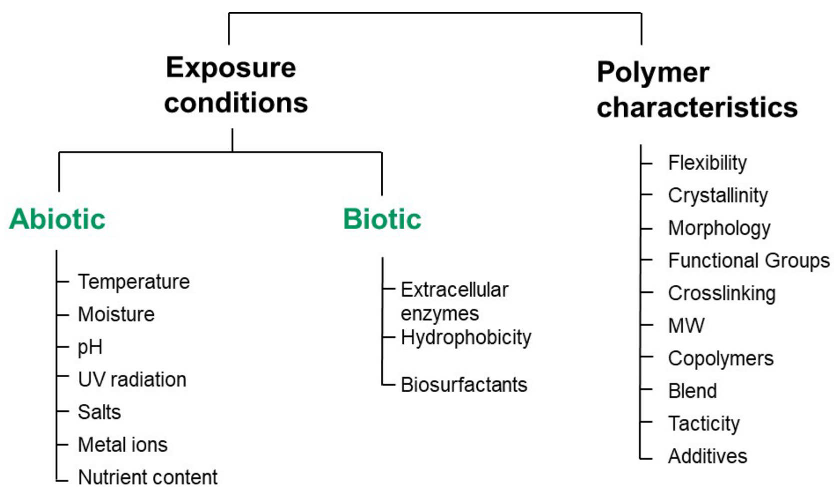
School of Chemical
Engineering

Previous lectures

1 week ago: Discussion day

3 weeks ago: Biodegradation Part 2

Factors affecting biodegradation



Biomaterials

Lumbar disc prosthesis PRODISC® L

Schedule

Day	Subject of lecture	Discussion part
08 January	Introduction to the course	
15 January	Biopolymers overview	Reading 1
22 January	Biopolymers for packaging	Reading 2
29 January	Discussion day	Reading 3 & Assignment 1
05 February	Biodegradation 1	Reading 4
12 February	Biodegradation 2	Reading 5
26 February	Discussion day	Reading 6 & Assignment 2
04 March	Chitin, alginates and others	Reading 7
11 March	Proteins	Reading 8
18 March	Discussion day	Reading 9 & Assignment 3
25 March	TBD	Reading 10

Learning Outcomes

After today's course you…

- know sources, harvesting methods and applications of chitin
- Understand the difference between chitin and chitosan
- know sources, harvesting methods and applications of alginate

Chitin & Chitosan

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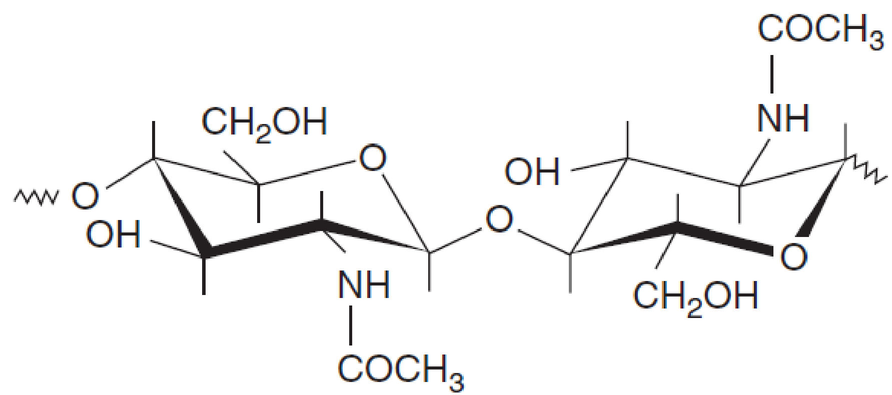
What is chitin?

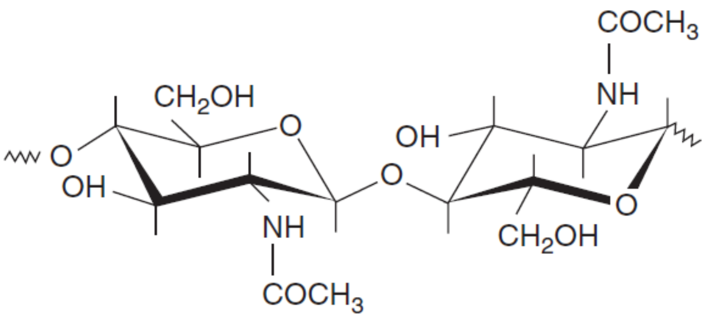
Second most abundant polysaccharide in nature after cellulose

Widely distributed in the animal and vegetal kingdom

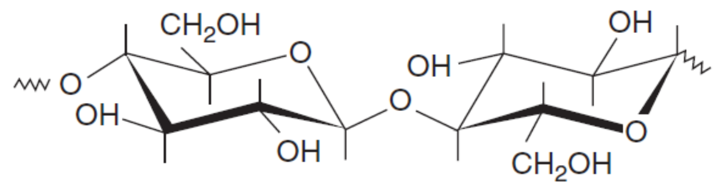
Linear polysaccharide composed of β(1 → 4) linked units of *N*-acetylglucosamine (GlcNAc)



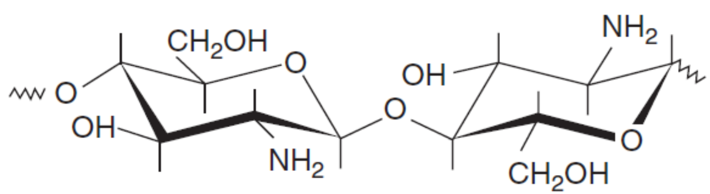
Similarities to cellulose



“Completely” acetylated chitin



Cellulose



Chitosan
(deacetylated chitin)

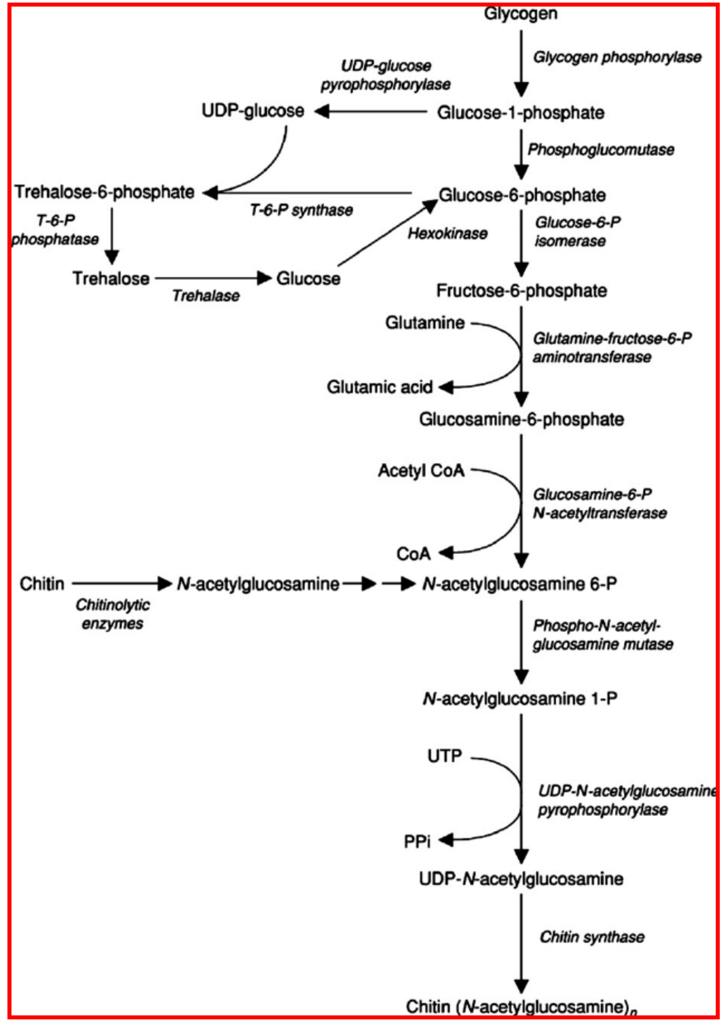
Biosynthesis

Similar to cellulose

Polymerized from activated monomers

Enzyme mediated

- Difficult to genetically engineer



Chitin biosynthesis is catalyzed by chitin synthase.
Exists in every chitin-synthesizing organism.
Chitin synthase remains bound to the growing polymer chain through many polymerization steps that sequentially adds single GlcNAc units to the non-reducing end of the extending chain.
The linear polymers of chitin spontaneously assemble into microfibrils of varying diameter and length.

Chitin - etymology

Greek “chitin” means cover or tunic. Present in the cell wall of fungi and in the exoskeletons of crustaceans, mushrooms and insects

Commercially it comes from wastes of the fishing industry. Crustacean shells (shrimp, crab, lobster, prawn and krill):

20-30% of chitin, 30-40% of protein, 30-50% of inorganic salts, 0-14% lipids.

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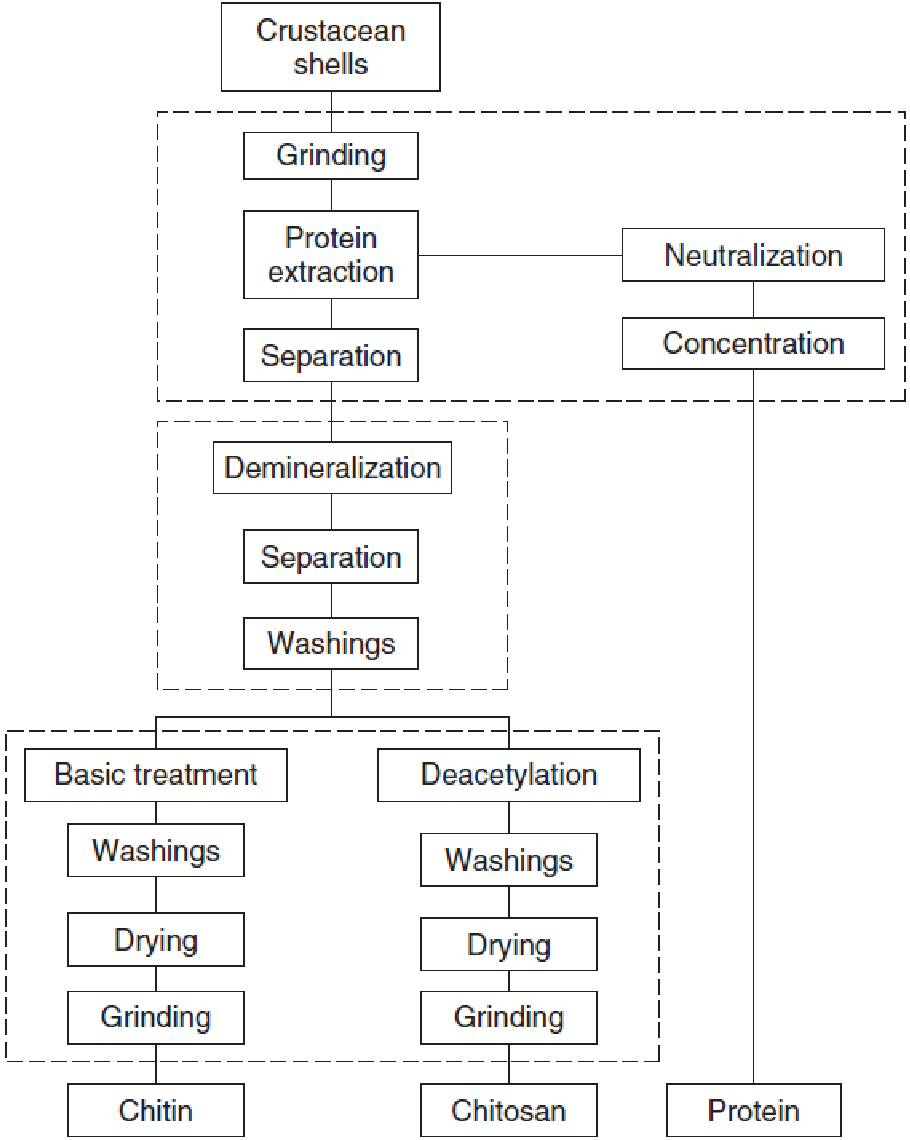
Isolation of chitin/chitosan

Mechanical processes
Steps of washing
Chemical treatments
Drying

Energy, chemicals, and water



Protein extraction

Sarcoplasmic calcium-binding protein (SCP), Ubiquitin

Diluted aqueous NaOH solutions (1–10 per cent) at temperatures ranging from 65 to 100°C.

The reaction time usually varies from 0.5 to 72 h.

Other reagents employed for the removal of the proteins include: Na_2CO_3 , $NaHCO_3$, KOH, K_2CO_3, $Ca(OH)_2$, Na_2SO_3, $NaHSO_3$, Na_3PO_4 and Na_2S*

The protein extracted can be recovered by lowering the pH of the solution to its isoelectric point for precipitation.

* Roberts G.A.F. , *Chitin Chemistry* , The Macmillan Press Ltd , London , 1992 . p. 350.

Demineralization

Calcium carbonate is the main mineral to be extracted

Usually extracted with **diluted** (1 to 10 wt.%) HCl solutions at **room temperature** (other acids: HNO_3, HCOOH, H_2SO_4, CH_3COOH)

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

Efficiency (or engineering) of the demineralization is dependent of the stoichiometric ratio of the reaction.

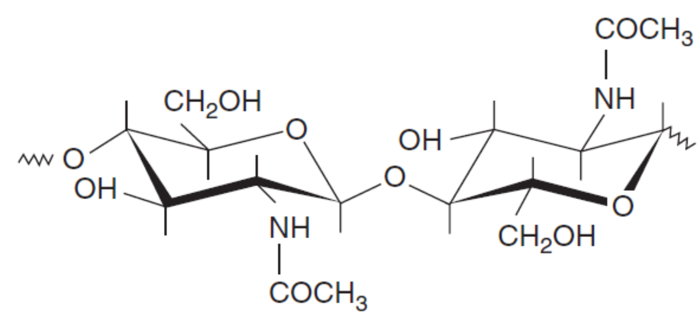
Processing route for chitin

Protein extraction

Demineralization

Bleaching (discoloration)

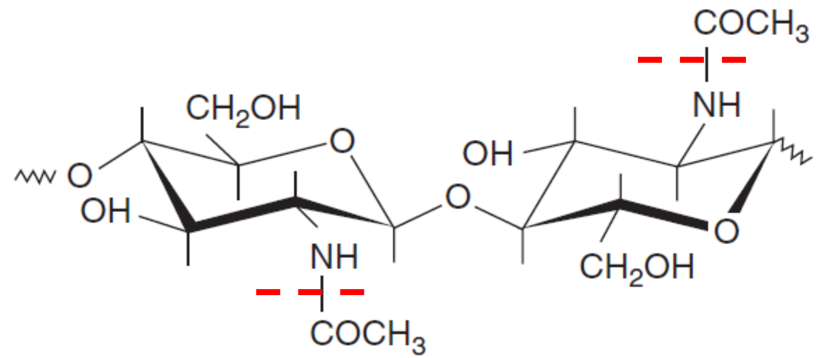
(reddish) Pigments are removed with organic solvents, but mostly common with NaClO (and sometimes mixtures of NaClO and acetone)



Processing route for chitosan

Protein extraction

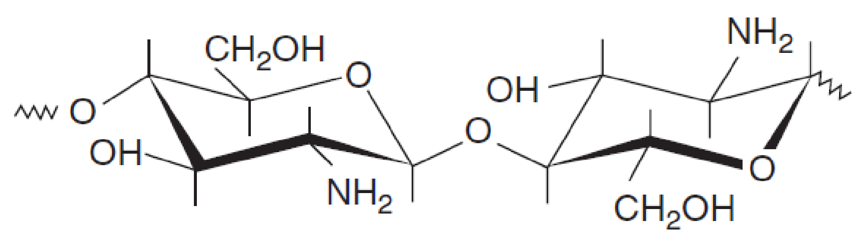
Demineralization



Deacetylation

Hydrolysis of the acetamide groups in strong alkaline medium

40-50 wt% NaOH or KOH at 100 °C



75-80% of deacetylation without high depolymerization

Mass production

Every year 6 - 8 million tonnes of waste crab, shrimp and lobster shells are produced globally

Example: 75% of the weight of a tuna fish can be extracted as fillets, but meat accounts for only around 40% of a crab's mass.

1.5 million tonnes

Nature 524, 155–157 (13 August 2015) Doi:10.1038/524155a

Mass production

Some challenges

Large volume of chemicals (NaOH and HCl)

Many washing steps needed

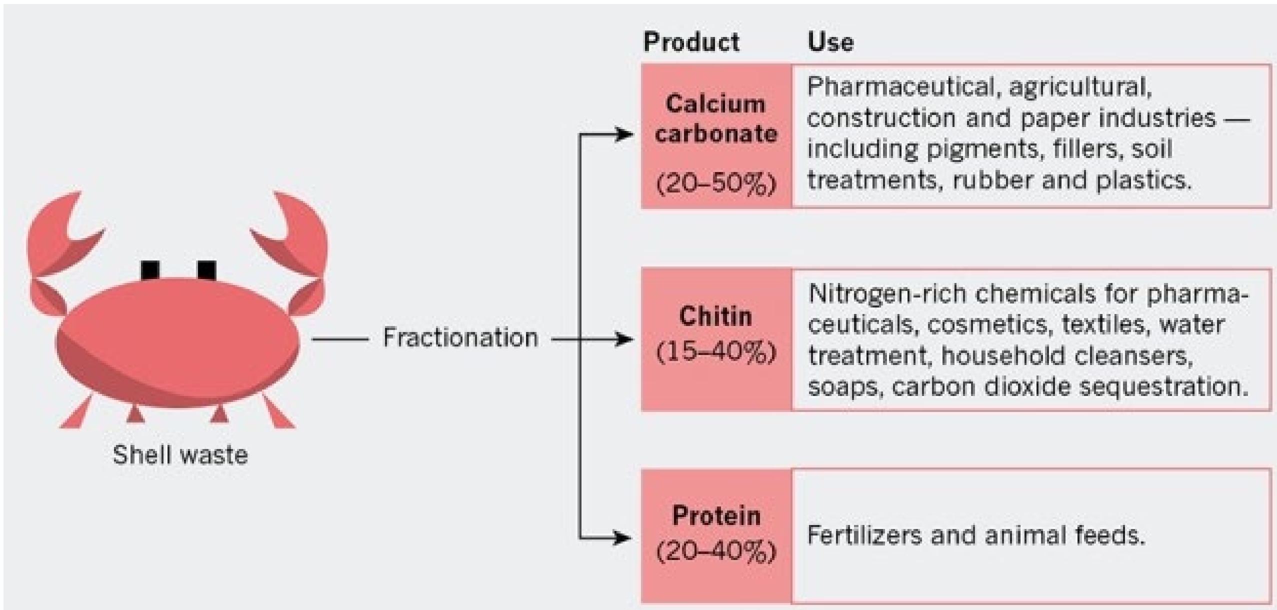
The production of 1 kg of chitosan from shrimp shells requires more than 1 ton of water

As a result, good quality chitin can cost up to **$200/kg**, although the starting material is cheap.

The global industrial use of refined chitin (in membranes, drug delivery, food and cosmetics) is low: around 10,000 tonnes/year

Nature 524, 155–157 (13 August 2015) Doi:10.1038/524155a

Biorefinery concepts



Properties

Degree of acetylation

Crystallinity

Solution properties

➡ **Applications**

Degree of (de)acetylation (DD)

Together with molecular mass, DD is the most important parameter to establish the chemical and physical identity of chitin and chitosan.

Degree of Deacetylation (DD): proportion of *N*-acetylated glycosidic units in chitin or chitosan.
Striking effect on the physicochemical properties of chitin/chitosan

IR, UV, ^{1}H NMR, ^{13}C (solid-state) NMR spectroscopy, elemental analysis, titration etc.

Degree of (de)acetylation (DD)

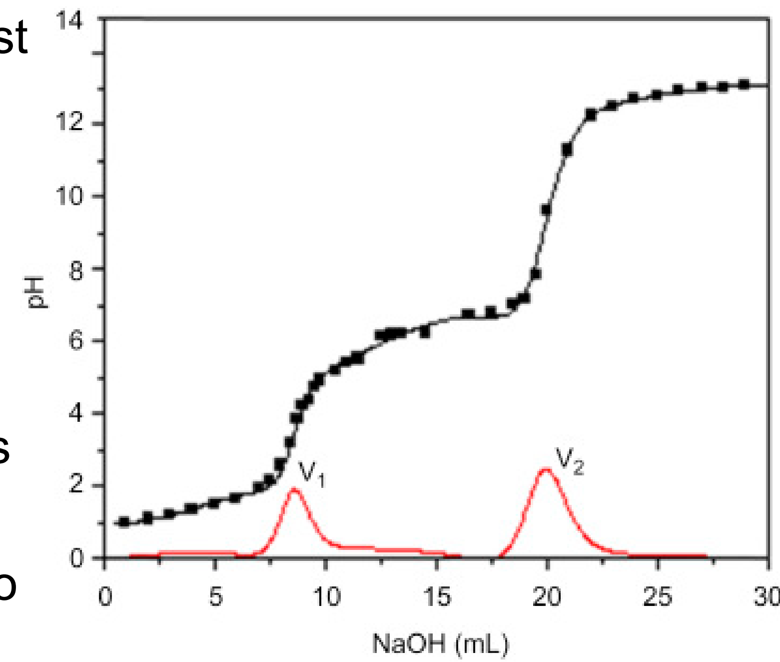
Potentiometric titration (most consistent and robust technique among the **non-NMR** methods)

Labor-intensive and time-consuming

A known mass of chitosan is dissolved in a known volume of HCl and titrated against NaOH

The titration curve of pH versus NaOH titration volume is generated and the curve's inflection points are found for each indicated transition

Volume of NaOH at each inflection point is applied to the equation to determine the amine content of sample.



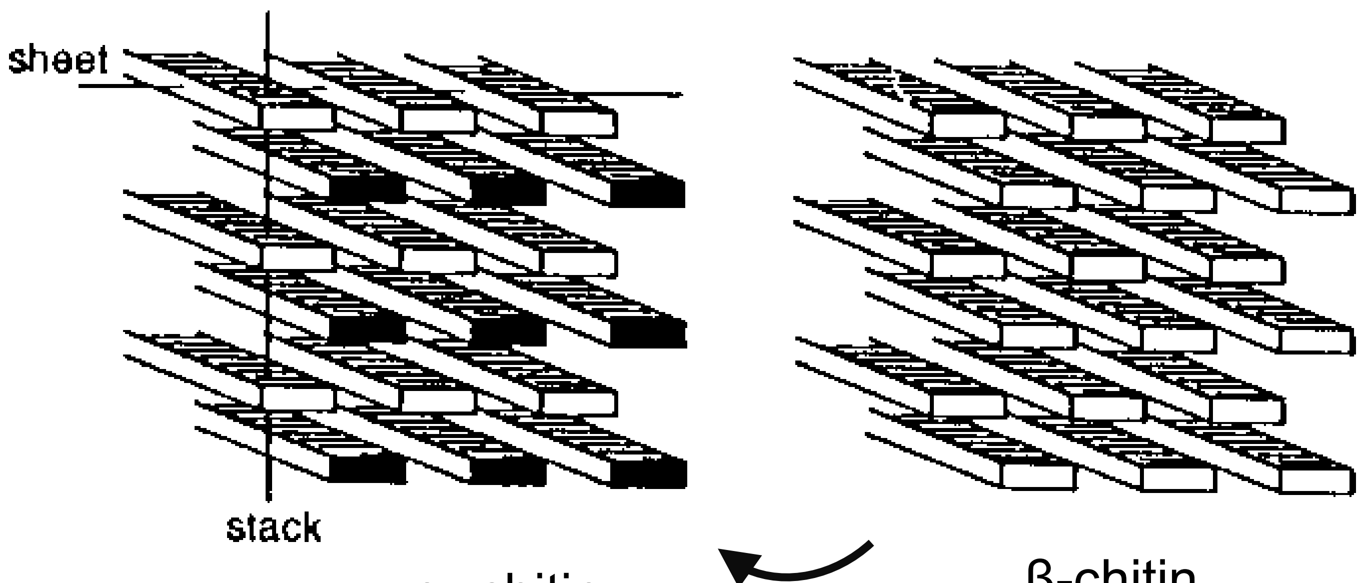
$$\mathbf{NH_2\% = 16.1 \cdot (Y\text{-}X)/M}$$

$$\mathbf{DDA\% = (NH_2\%/9.94\%) \cdot 100}$$

Crystal properties

Highly crystalline in nature

Found in two main polymorphs, α and β-chitin (γ-chitin also exists)

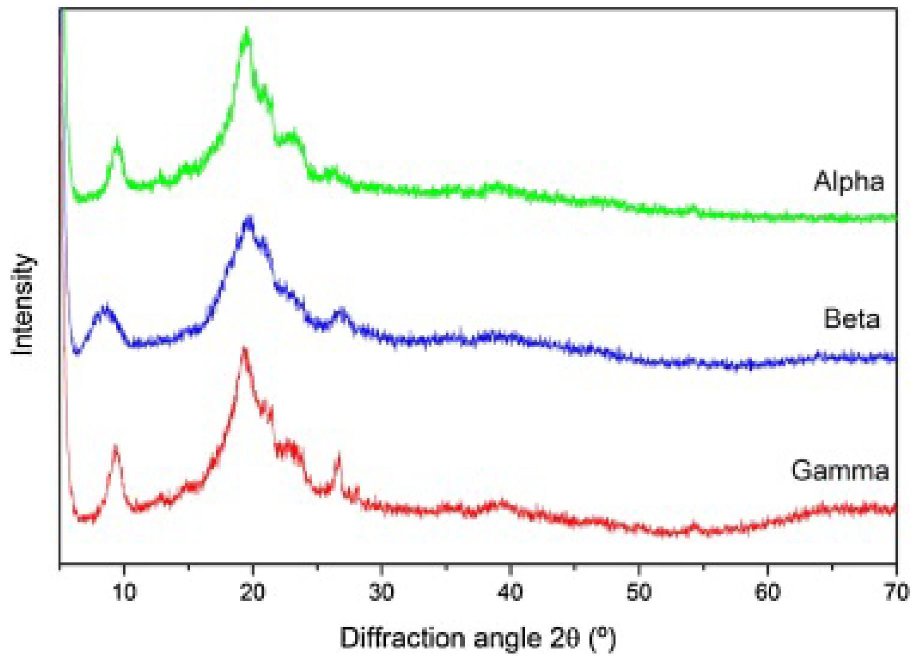


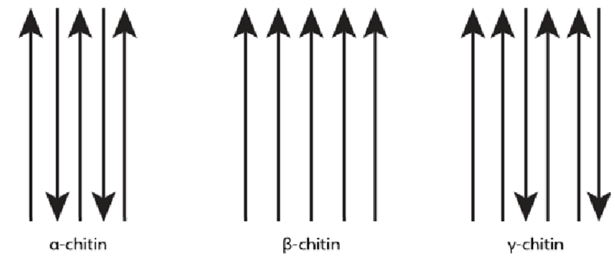
α -chitin

β-chitin

Precipitation from formic acid solution or treatment with cold 6M HCl

Crystal properties





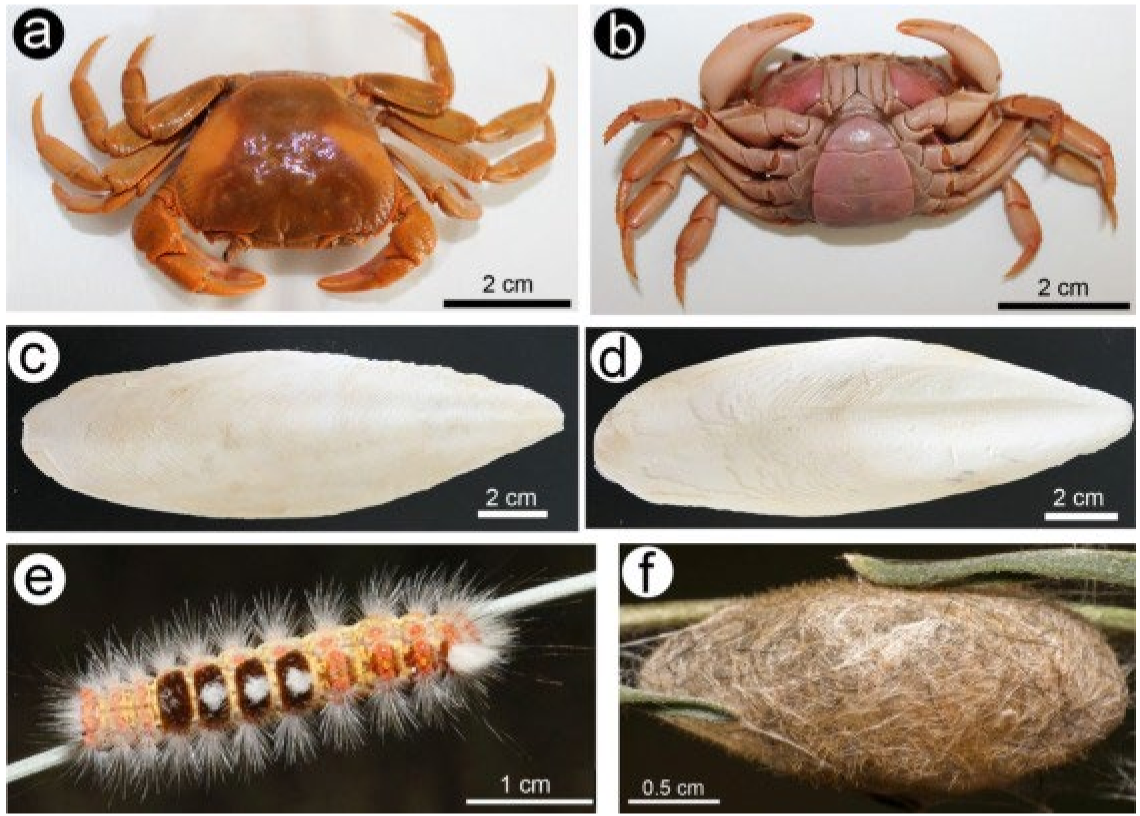
Fingerprint

Carbohydrate Polymers V.176 (15) p. 177-186, 2017

Crystal properties

α -chitin

β-chitin



γ-chitin

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Solution properties

Degree of Deacetylation (DD) has striking effect on the solubility and solution properties of chitin/chitosan

Chitin

semicrystalline polymer with extensive inter- and intra-molecular hydrogen bonds: difficult to dissolve in dilute acids or organic solvents under mild conditions

many solvents: toxic, corrosive, or mutagenic

Chitosan

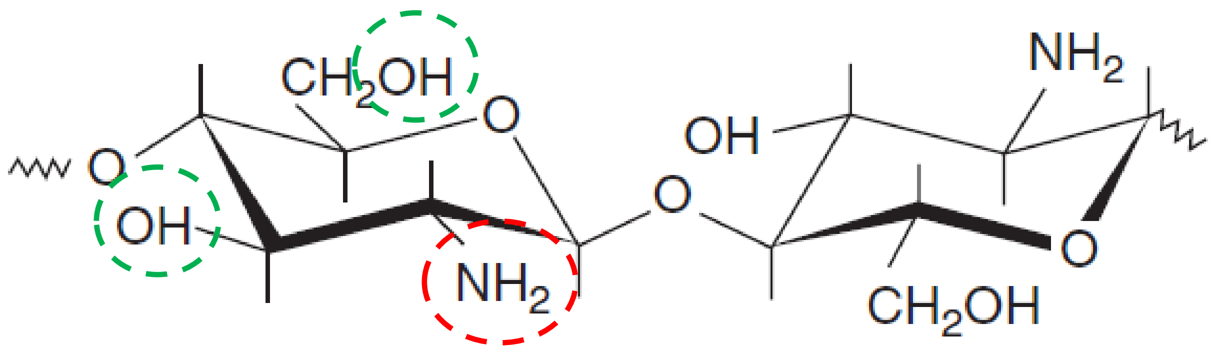
more tractable form than chitin

readily dissolves in dilute mineral or organic acids by protonation of free amine groups at pH below about 6.5

Engineering possibilities

Three reactive groups : primary (C-6) and secondary (C-3) hydroxyl groups, and amino (C-2) group

Etherification, esterification, *N*-alkylation, *N*-acylation, cross-linking, and graft copolymerization, etc.



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Due to its naturally “rare” positive charge chitosan is a good candidate for polyelectrolyte complexes

Degradation aspects (chitin/chitosan)

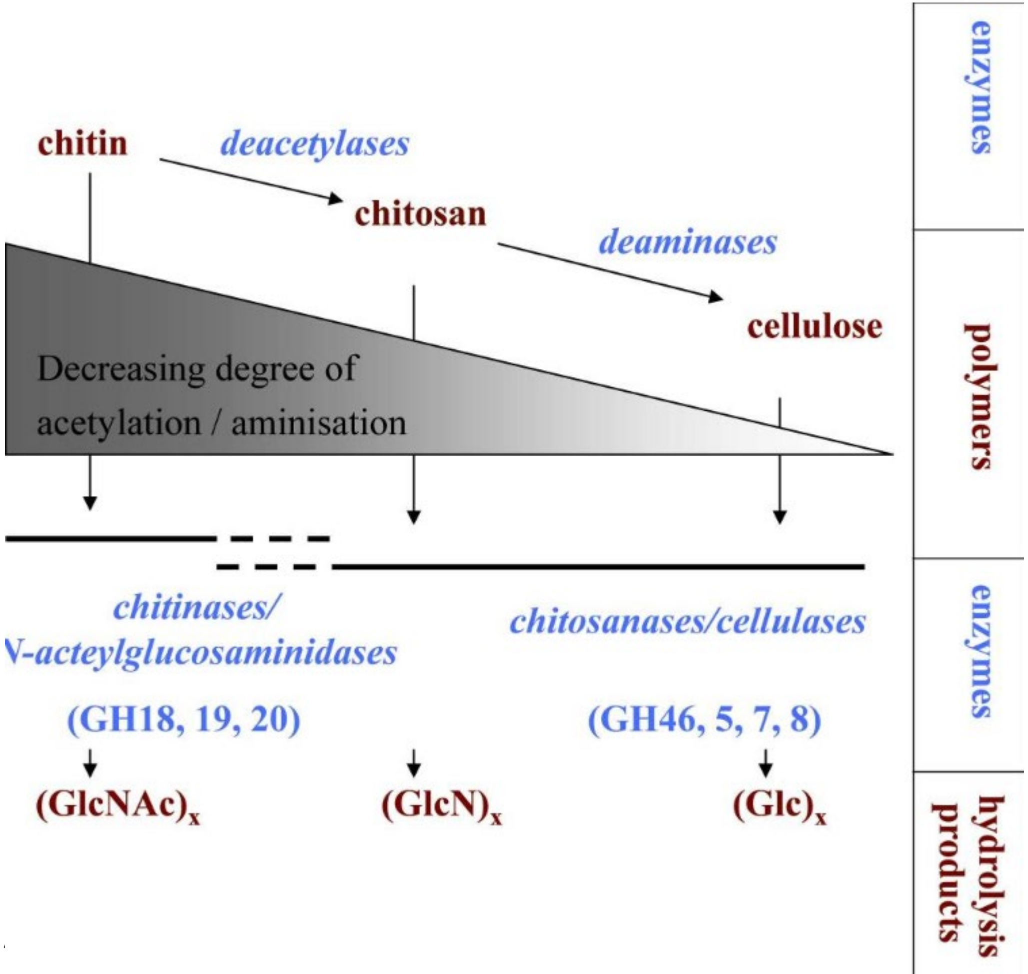
Biodegradable, and low toxicity.

Their biodegradability is due to their susceptibility to enzymatic hydrolysis.

Chitin-degrading enzymes are detected in many types of organisms, such as fungi, bacteria, some algae, but also carnivorous plants or in digestional tracts of **higher animals**.

For example by lysozyme, a non-specific proteolytic enzyme present in all tissues of the human body. Lipase, an enzyme present in the saliva and in human gastric and pancreatic fluids, can also degrade chitosan.

Degradation aspects (chitin/chitosan)



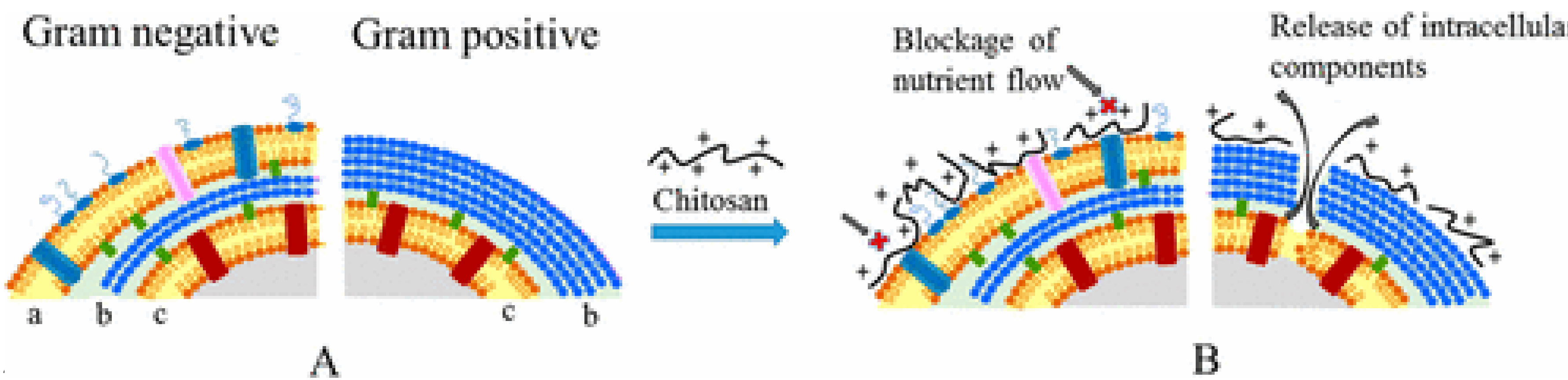
Processes involved in chitin degradation. If deacetylation and deamination processes are very active, chitosan or possibly even cellulose-like molecules might be produced. GH, glycoside hydrolase family; GlcNAc, N-acetylglucosamine; GlcN, glucosamine; Glc, glucose.

Front Microbiol. 2013; 4: 149.

Antimicrobial properties

Wide spectrum of **bacteria**, fungi and virus

Depends on: Molecular weight, degree of acetylation, distribution of the amine groups, hydrophobicity, cationic charge density, and other functional groups.



Biomacromolecules 2017, 18, 11, 3846-3868

Applications

Medicine

(Because bioactivity, antimicrobial, immunostimulation, chemotactic action, enzymatic biodegradability, mucoadhesion…)

Pharmacy

(formation of polyelectrolyte complexes, easy formation of capsules, low swelling in water, pH-sensitivity)

Agriculture

(fungicidal, antiviral, antibacterial, plant growth and regulation, plant protection…)

Food

(Chitosan is not FDA approved nor EU regulated, lots of attention as flocculant for the recovery of proteins, antimicrobial, emulsion stabilizer)

Cosmetics

(cationic character interacts with negatively charged biological surfaces – skin and hair - , metal ion complexing capacity, water retention)

Alginates

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Overview

"Alginate" is a collective term for a family of polysaccharides produced by brown algae and bacteria.

Structural component of marine brown algae… it comprises up to 40% of the dry mass

It is located mainly in the intercellular mucilage and cell walls as an **insoluble gel containing calcium, magnesium, potassium and/or sodium salts.**

Introduces mechanical integrity, flexibility and water retention.

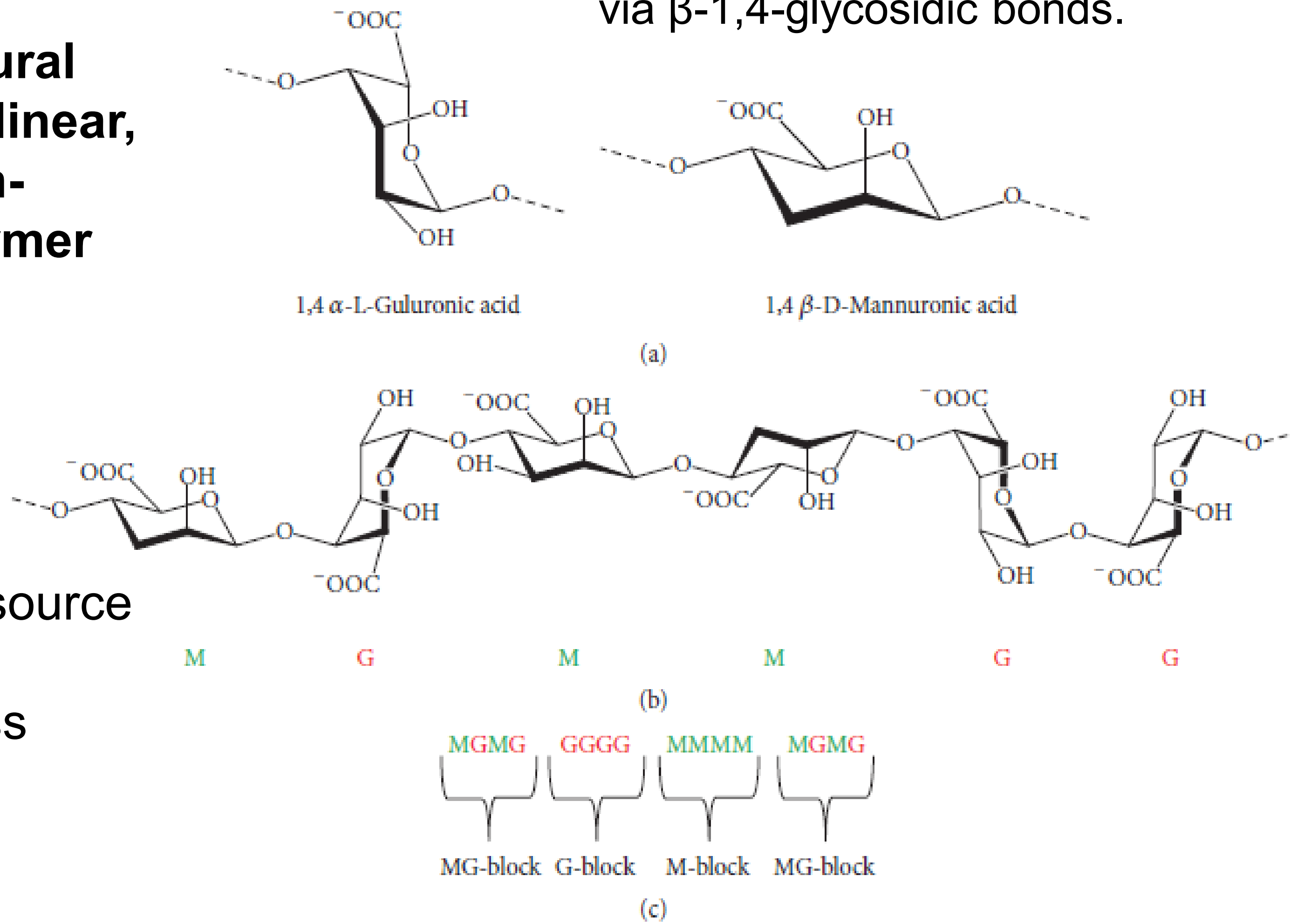
Laminaria digitata

Pseudomonas aeruginosa

Chemical structure

β-D-mannuronic acid (M) and its C5-epimer α-L-guluronic acid (G) linked via β-1,4-glycosidic bonds.

Alginate is a natural polysaccharide, linear, unbranched, non-repeating copolymer



Depends on the source
Part of the algae
Extraction process

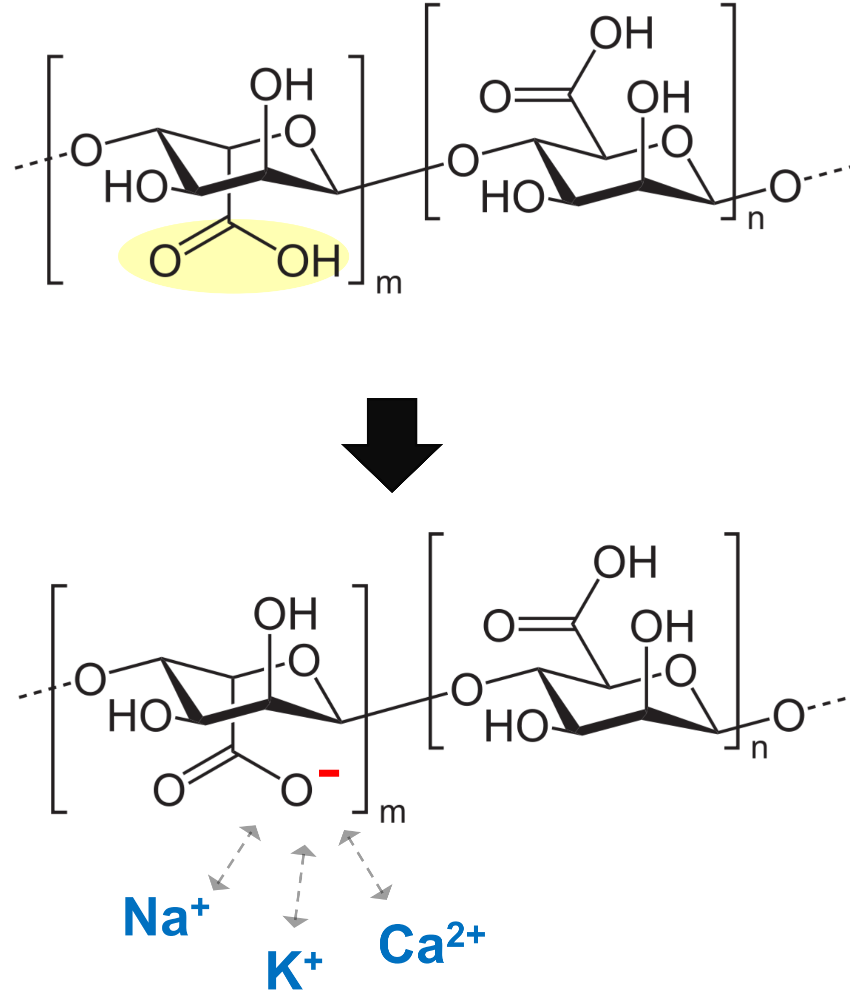
Alginate derivatives

Alginic acid

Sodium alginate

Calcium alginate

Potassium alginate



Sources

Brown Seaweed

Class *Phaeophyceae*

Broad size depending on the specie (0.6 to 20 m long. Usually thick

Temperate coastline

Currently mostly wild (They cannot be grown by vegetative means, but must go through a reproductive cycle involving an alternation of generations)

Farming is starting slowly

Polymer is found as homopolymeric blocks of MM-units, GG-Units, or MG-units alternated.

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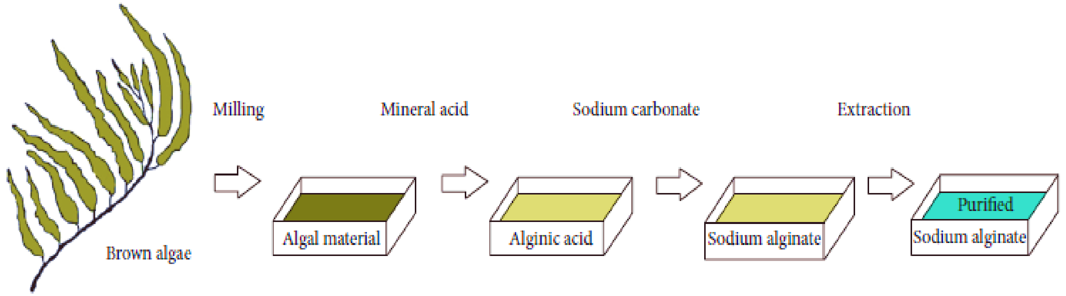
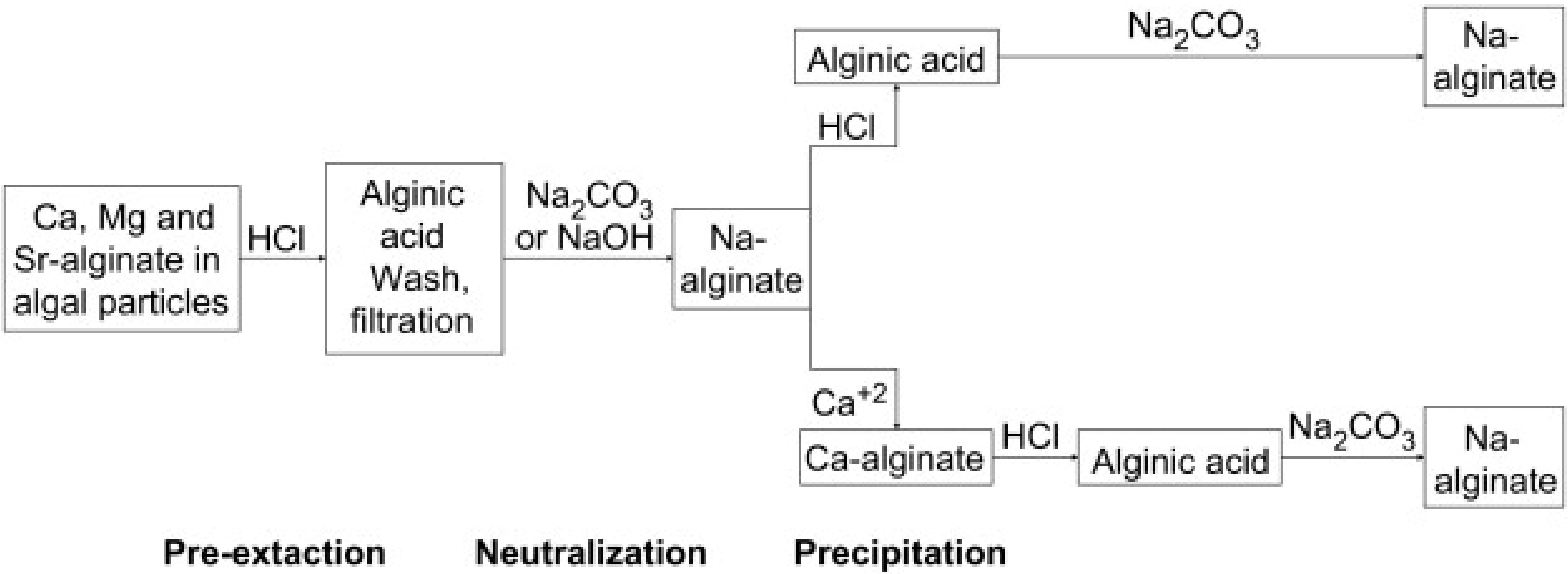
Isolation from seaweed



Isolation from seaweed



Sources

Bacteria

Azotobacter vinelandii produces alginate as part of the encystment process, as a mechanism to promote desiccation-resistance under adverse environmental conditions to maintain hydration of the cell, being a structural element in the cell.

Pseudomonas aeruginosa produces alginates in order to survive in water-limited conditions. Alginates are part of the thick biofilms that protect the bacteria from harmful organims

Azotobacteri vinelandii

Pseudomonas aeruginosa

Bacterial biosynthesis pathway

The production mechanism of alginate in *P. aeruginosa* and *A. vinelandii* is very similar

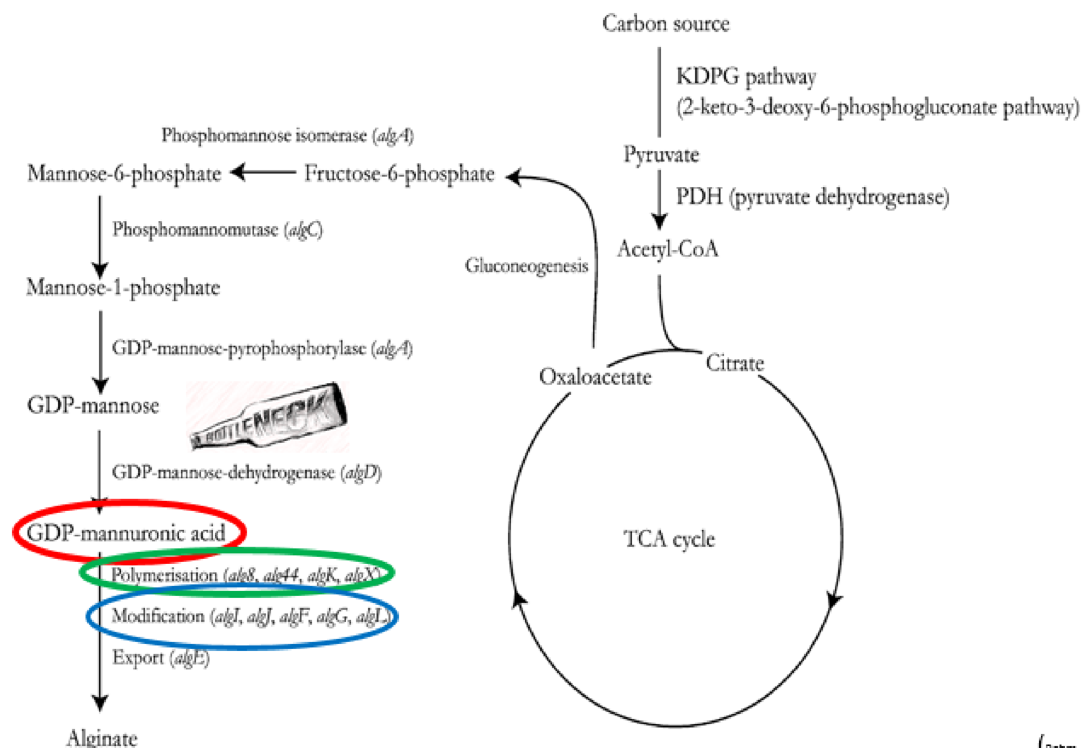
Precursor synthesis

Polymerization

Modification

- Modification at polymer level
    - Transacetylases (AlgI, AlgJ, AlgF)
    - C5-epimerases (AlgG)
    - Lyases (AlgL)

Secretion (AlgE)



(Rehm

World J Microbiol Biotechnol. 7, 33(11), p.198, 2017

Bacterial production

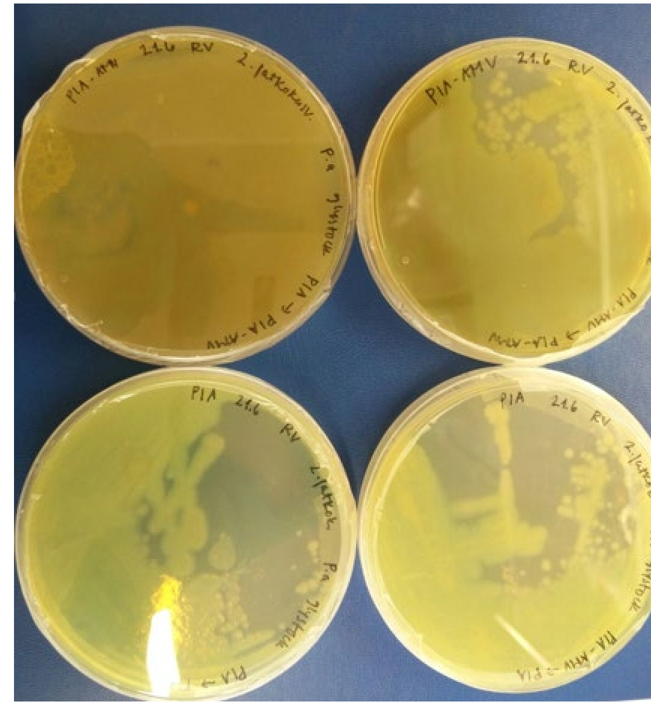
Incubation of the bacteria strain

Organism can be genetically engineered (yields, substrate, functionalities, G to M ratios)

Produces a jelly, boggy mucous layer rich in alginate

Collection

Isolation and Purification



Material from M.Sc. Riikka Virtanen

Bacterial production (detailed)

Day 1
1. Plating
2. Incubation 72h, 37 ◦C

Day 3
3. Mucoid layer harvested to 10 ml of phosphate buffered saline (PBS x 1, pH 7,2)
4. Stirred with magnetic stirrer, 1h
5. Centrifuged 4h, 17700 x g, 4 ◦C
6. Enzyme digestion with DNase I (10 µg/ml) for 4h, 37 ◦C
7. Enzymes inactivated and denatured by heating, 30 min, 80 ◦C
8. Centrifuged 30 min, 17700 x g, pellet discarded

Day 4
9. Precipitated with cold absolute ethanol to a final concentration of 80% (vol/vol), 1-2 h, 4 ◦C
10. Precipitated alginate collected with a bent glass-rod
11. Squeezed dry, and washed 2x2,5ml 80 % (vol/vol) ethanol and once in 2,5 ml 96 % (vol/vol) ethanol
12. Precipitate dissolved in 10 ml sterile 0,9 % saline
13. Precipitated with cold absolute ethanol 80% (vol/vol), 1-2 h, 4 ◦C
14. Precipitated alginate collected with a bent glass-rod
15. Squeezed dry, and washed twice in 80 % (vol/vol) ethanol and once in 96 % (vol/vol) ethanol
16. Precipitate dissolved in 10 ml PBS supplemented with 10 mM $MgCl_2$ and 1 mM $CaCl_2$
17. Centrifugation

Day 5
18. Precipitated with cold absolute ethanol 80% (vol/vol), 1-2 h, 4 ◦C
19. Precipitated alginate collected with a bent glass-rod
20. Squeezed dry, and washed twice in 80 % (vol/vol) ethanol and once in 96 % (vol/vol) ethanol
21. Ion-exchange chromatography - DEAE-Sephacel column with an ammonium carbonate gradient
22. Precipitate dissolved in XX ml 0,025 M ammonium carbonate before loading onto the anion-exchange column
23. Loading 1 ml of alginate solution per ml of anion exchanger
24. Gradient of 0,025 to 1,0 M ammonium carbonate was run with a peristaltic pump to a volume 10 times the bed volume
25. Eluate continuously monitored for A(280)
26. Fractions (5 ml) collected and assayed for uronic acid content with the carbazole-borate method (Internal standards D-mannurolactone and seaweed alginate, A530 measured)
27. Positive fractions pooled and dialyzed 3 x 24 h against demineralized sterile water

Day 8
28. Lyophelized

Day 9-10
29. Drying, 120 ◦C, 4 h
30. P_2O_5 exsiccation in vacuum for 2 days

Roughly 30 steps! Doable, but it needs good planning, setup, biotech expertise and management.

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Pedersen, 1989

Issues in the production

Algal alginates are not ideal sources for commercial demands.

Marine pollution and climate conditions (e.g. El Niño) affect the seaweed alginate supply

Production has to be located on shore areas.

Some environmental concerns are associated with the seaweed harvesting and processing.

Competition with the food sector (algae as a food product in Asia limits its refinery to alginate)

Issues in the production

The low price of the traditional algal alginates acts as a deterrent for the establishment of commercially feasible bacterial production processes.

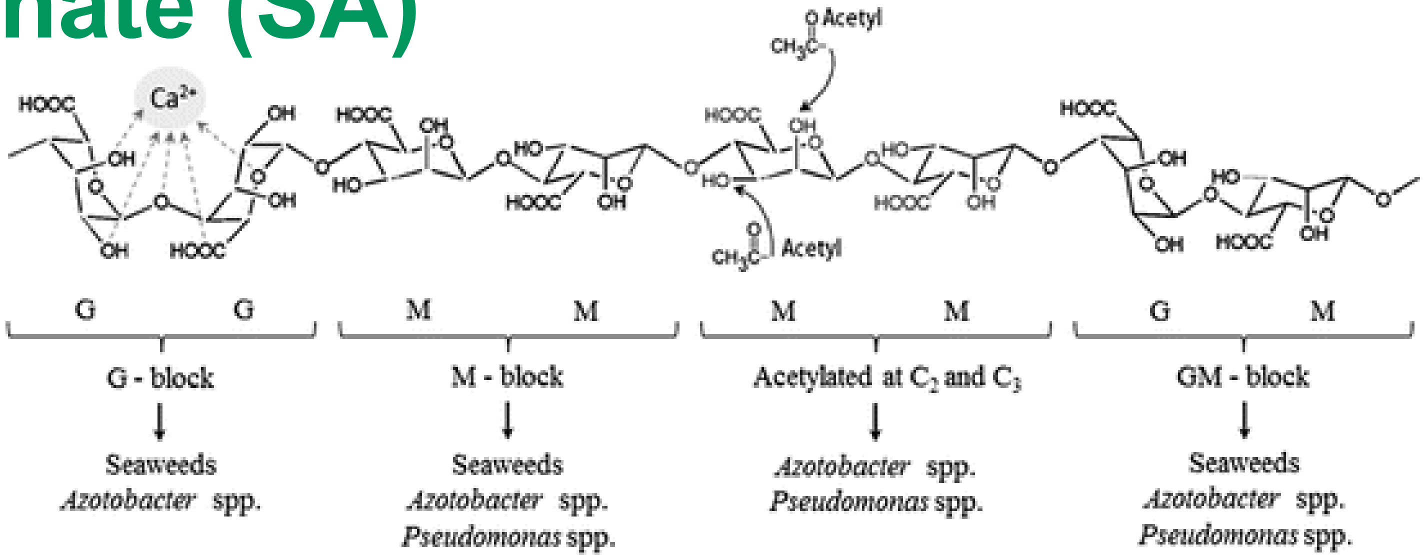
A. vinelandii is the most used host for bacterial alginate production, but the yields are rather low (4 g/L).

One additional trouble with the production of bacterial alginate is that the production organisms are pathogenic

Biocompatibility and purity have to been taken into consideration, especially when the desired applications related to medical or pharmaceutical fields (purification steps $$)

https://newrepublic.com/article/150775/humans-created-new-natural-disaster

Bacterial alginate (BA) vs. Seaweed alginate (SA)



- SA high content of G blocks, alginate by *P. aeruginosa* does not possess G blocks.
- Bacterial M-residues are O-acetylated to various degrees at the O2 and/or O3 position
- BA more flexible gels whereas SA produces more rigid ones.

M/G ratio determination

Very important structural feature
Physicochemical properties
Material's properties

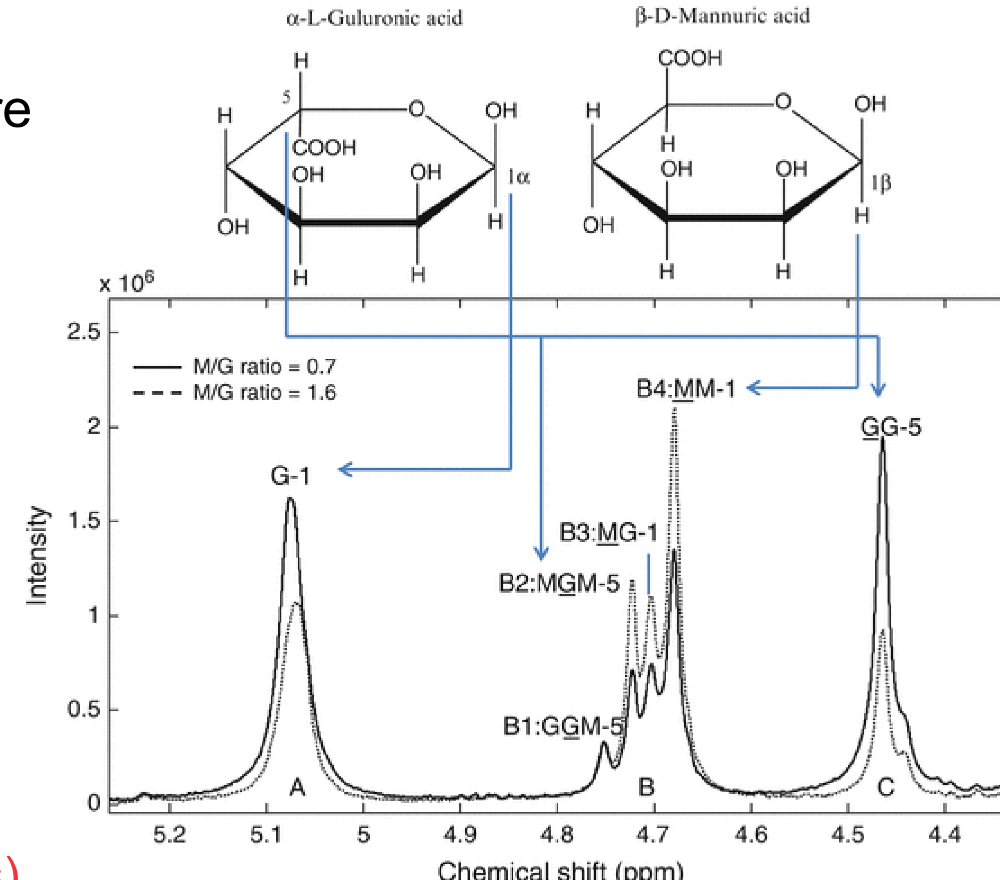
NMR methods (very precise)

^{1}H NMR

^{13}C NMR

Vibrational spectroscopy:
Raman, IR, NIR

(often indirect or calibrated methods)



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Natural Products From Marine Algae pp 347-363

Gelling properties

Aligned **G-blocks** form diamond shaped holes in which the bivalent counter ions bind forming a structure called EGG-BOX, leading to viscous solutions

Regarding the amount of calcium ions present, these interactions can be either permanent or temporary

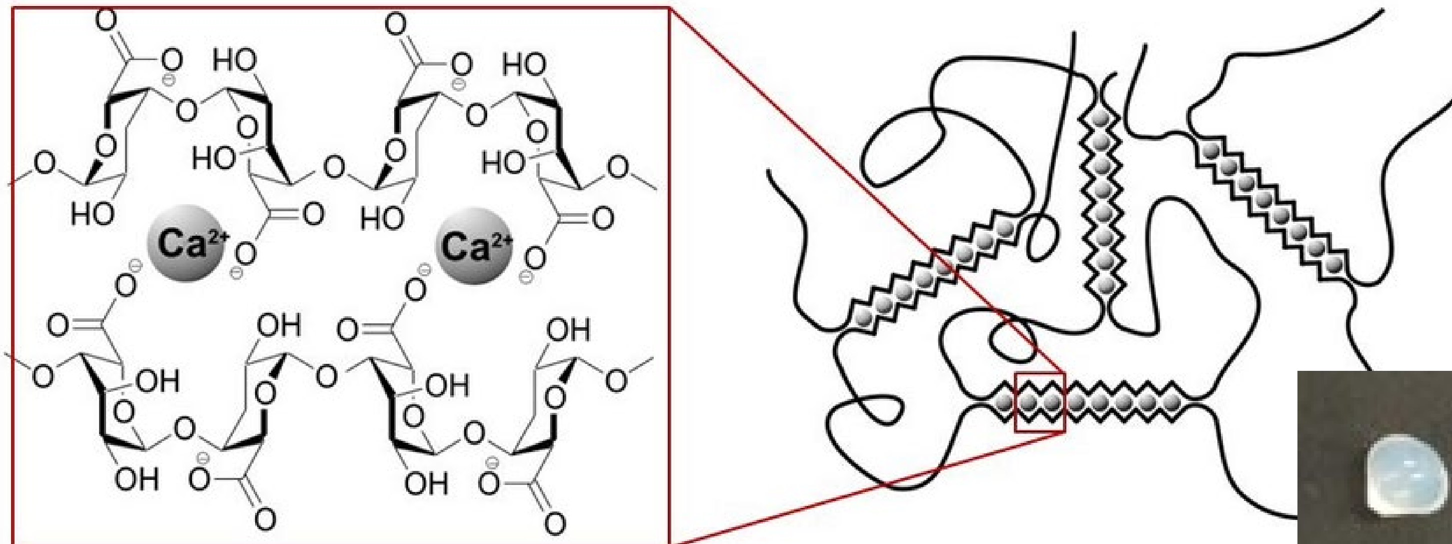
Achieved by **ionic**, covalent cross-linking, or with thermal gelation.

Cationic metal ions

Graft copolymerization of NIPAAm

Example: Poly(ethylene glycol)-diamines

Gelling properties



Engineering possibilities

-COOH mediated modifications
Ammonium alginate
Propylene glycol alginate

Mw of alginates produced by *A. vinelandii* varies with respect to the dissolved oxygen levels. *A vinelandii* is capable of modifying the ratio of M- and G-blocks, and thus the material properties of the given alginate

Genetically engineered bacteria to manipulate M/G ratio

- G-blocks are responsible of gelling.
- M-residues trigger immunogenic response.
- MG-blocks form flexible chains and are more soluble in lower pH
- High amount of G-blocks provide heat-stable and strong (rigid) gels
- High amount of M-blocks grant weaker elastic gels (softer) that are less heat-stable but are more durable to freeze-thaw processing.

Applications

Food industry (thickening agent, gelling agent, emulsifier, stabilizer, texture-improver)… It is FDA approved

Textile printing (substrate of color paste)

Animal food (binder and thickening agent)

Pharmaceutical (wound dressing, dental impression material, tablet binder, controllable drug release, immobilization)

Cosmetics (thickener, moisture retainer)

Sodium alginate, water, calcium lactate

Sodium alginate, fruit juice and calcium solution expunged through a syringe

Recap questions

- What are the major steps of chitin isolation?
- How do chitin and chitosan differ and how does this affect their properties?
- What are alginates?
- Where do we find aliginates and how do they differ depending on their source

Reading 7 discussion

Title: Chapter 3 - Bioplastics, Biocomposites, and Biocoatings from Natural Oils
From: R.L. Quirino, R.C. Larock. ACS Symposium Series 2011, Vol. 1063, Renewable and Sustainable Polymers

Discuss and summarize:

- **how triglycerides and fatty acids can be turned into bioplastics**
- **the properties of these bioplastics**

Instructions:
Write your names and summary of your discussion in e.g., PowerPoint. Save the text as image file (.jpg) and upload it to the Padlet page:
https://padlet.com/michaelhummel/CHEME2155_2024