Comparison of Extraction Methods of Chitin and Chitosan from Different Sources

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Abstract

Chitin and chitosan have become of great interest not only as underutilized resources, but also as new functional materials of high potential in various fields. The methods of isolating chitosan from different sources: shrimp (*Panaeus monodon*), crab (*Scylla olivicea* and *Scylla serrata*), locust (*Schistocerca gregaria*), honeybee *(Apis mellifera*), beelte (*Calosoma rugosa*) and fish (*Labeo rohita)* were compared. The same steps of demineralization and deproteinization were followed for the chemical extraction of chitin, but the concentration of reagents, the temperatures and reaction times were varied, which resulted in chitosans with different degrees of deacetylation.

Keywords: chitin, chitosan, extraction methods, different sources, deacetylation process

Introduction

Natural polymers are gaining more interest due to their biocompatibility and biodegradability in contrast to many synthetic polymers that have more limited properties. Chitin and chitosan are among the novel families of biological macromolecules that are studied as suitable functional materials, due to the excellent properties of these natural polymers, such as biocompatibility, biodegradability, non-toxicity and adsorption properties [Kumar, M.N.V.R., 2000].

Chitin and its deacetylated derivative chitosan are natural polymers composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Due to their natural origin, both chitin and chitosan

cannot be defined as a unique chemical structure but as a family of polymers which present a high variability in their chemical and physical properties. This variability is related not only to the origin of the samples but also to their method of preparation [Aranaz, I., *et al*., 2009].

Chitin is the second most abundant natural polymer in nature after cellulose and it is found in the structure of a wide number of invertebrates (crustaceans' exoskeleton, insects' cuticles) and the cell walls of fungi [Pillai, C.K.S., *et al*., 2009]. Chitin is a white, hard, inelastic, nitrogenous polysaccharide and is insoluble in acidic aqueous media because it has a low amount of 2-amino-2-deoxy-D glucose units, while chitosan is soluble in acidic conditions due to the high amount of 2-amino-2-deoxy-D glucose units [Brunner, E., *et al.*, 2009].

Chitosan is the *N*-deacetylated derivative of chitin, although this N-deacetylation is almost never complete. Chitosan is a non-toxic, biodegradable polymer, a fiber-like substance, very much similar to cellulose [Hossain, M.S., *et al*., 2014].

The only difference between chitosan and cellulose is the amine (-NH2) group in the C-2 position of chitosan instead of the hydroxyl (-OH) group found in cellulose. Unlike cellulose, chitosan has the ability to chemically bind with negatively charged fats, lipids, cholesterol, metal ions, proteins, and macromolecules due to its positive ionic charges [Rout, S.K., 2001]. The chemical structures of cellulose, chitin and chitosan are presented in Fig. 1.

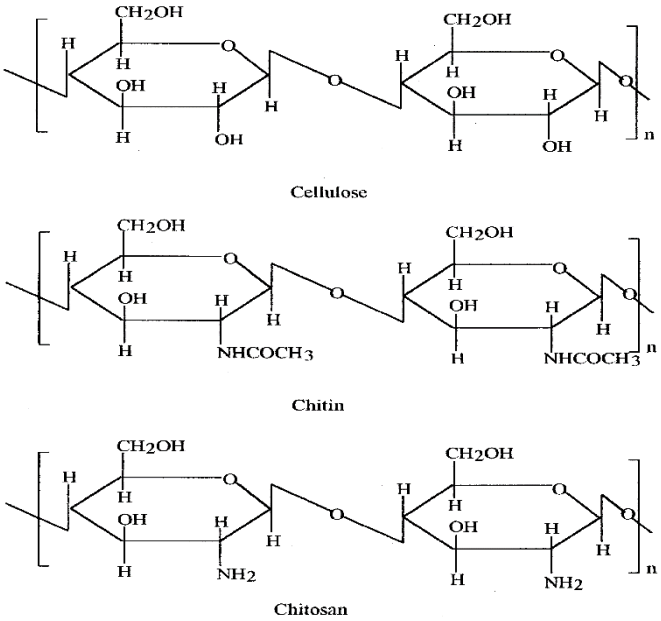

Fig.1. Chemical structures of cellulose, chitin and chitosan

Chitin can be extracted from various sources, however, commercial chitins are usually isolated from marine crustaceans, mainly because a large amount of waste is available as a by-product of food processing of marine products. Crustacean shells consist of 30-40% proteins, 30-50% calcium carbonate and calcium phosphate, 20-30% chitin and also contain pigments of a lipidic nature such as carotenoids (astaxanthin, astathin, canthaxanthin, lutein and β-carotene) and a high percentage of nitrogen (6.89%). These proportions may vary with species and with season and unfortunately crustacean shell wastes can be limited and subject to seasonal supply [Muxika, A., *et al*., 2017].

Fig. 2 briefly present the different sources used for chitin and chitosan extraction.

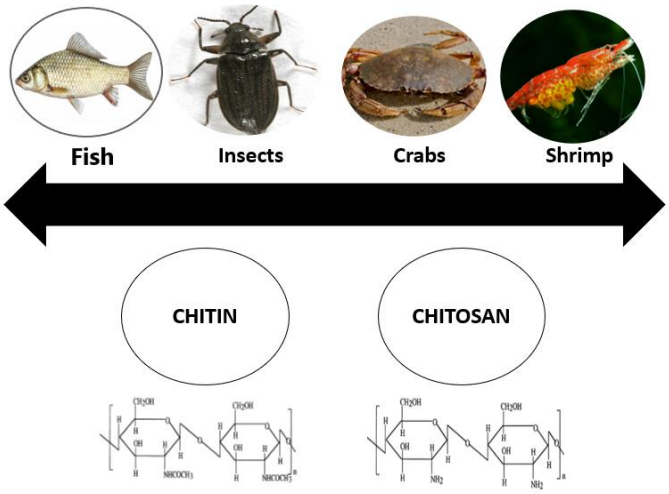

Fig.2. Different sources used for chitin and chitosan extraction

The chemical extraction of chitin involves a demineralization step in which the calcium carbonate is dissolved by acid treatment, followed by alkaline extraction to dissolve the proteins. A depigmentation step that removes the astaxantine is added in order to obtain a colourless product [Acosta, N., *et al*., 1993]. A brief scheme for obtaining chitin and chitosan is presented in Fig.3.

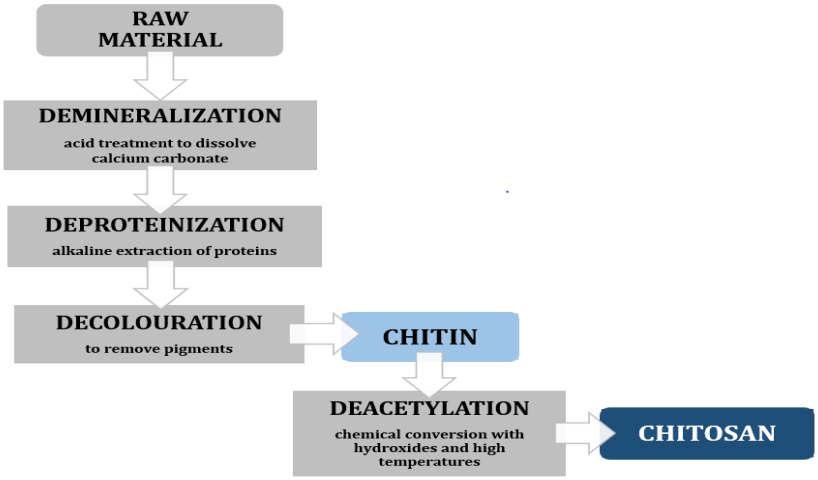

Fig.3. Scheme for obtaining chitin and chitosan

Chitin can be converted into chitosan by a deacetylation step. This step can involve enzymatic or chemical processes; however, the chemical conversion is preferred due to its lower cost and its suitability for mass production [Younes, I., *et al*., 2015]. Chemical deacetylation involves the treatment of chitin with hydroxides at high temperatures, usually above 80 ^{0}C. The treatments that use high NaOH concentrations (50–60%) and high temperatures (130–150 ^{0}C), have a very fast deacetylation step (2 h) [Lizardi-Mendoza, J., *et al*., 2016]. Fig.4 present the deacetylation process of chitin.

The ratio between the D-glucosamine and N-acetyl-D-glucosamine units of chitosan is considered as the degree of deacetylation [Bedian, L., *et al*., 2017], [Verlee, A., 2017]. In order for the polymer to become soluble in aqueous acidic media, the deacetylation degree of chitosan must reach 50%. When chitosan is dissolved in acidic environment, the amino groups in the chain protonate and the polymer becomes cationic, allowing it to interact with diverse types of molecules, thus turning chitosan into the only cationic marine polysaccharide. This positive charge may explain the antimicrobial activity of chitosan, because it interacts with the negatively charged cell membranes of microorganisms [Lizardi-Mendoza, J., *et al*., 2016].

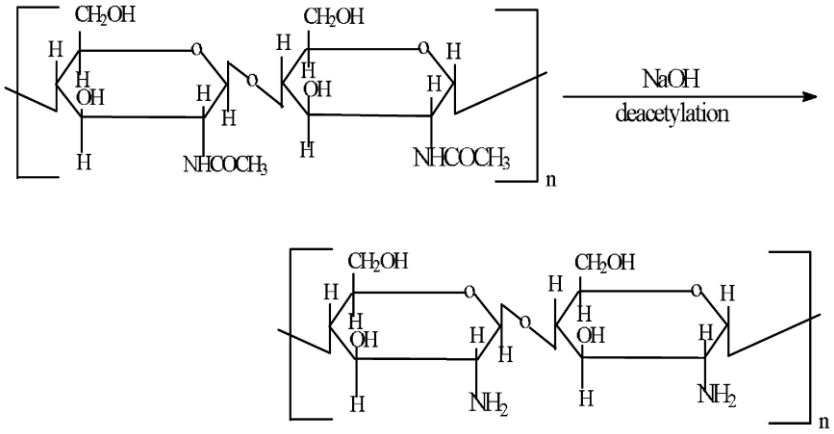

Fig.4. The deacetylation process of chitin

The characteristics of chitin and chitosan have a great effect on their properties and on their possible applications. Not every chitin or chitosan sample can be used for the same applications, and that is why a complete characterization of the samples is very important.

The main parameters affecting the properties of chitosan are the degree of deacetylation (DD) and the molecular weight (Mw). The degree of deacetylation is one of the most important chemical characteristics, which could influence the performance of chitosan in many of its applications [Baxter, A., *et al*., 1992], and the average Mw can have an influence on the viscosity development of aqueous solutions with a important role in the biochemical and biopharmacological applications [Tharanathan, R.N., *et al*., 2003].

Chitin and chitosan are currently receiving a great deal of interest as regards medical and pharmaceutical applications because of their interesting properties that make them suitable for use in the biomedical field, such as biocompatibility, biodegradability, non-toxicity and low immunogenicity [Harish, Prashanth K.V., *et al*., 2007], [Pillai, C.K.S., *et al*., 2009], [Nagahama, H., *et al*., 2008].

This paper present the chemical process for isolating chitin from different sources such as shrimp, locust, honey bee, beetle, crab and fish. The raw materials were subjected to demineralization and deproteinization to isolate the crude chitin. The obtained chitins were deacetylated to chitosan with different degrees of deacetylation. For the synthesis of chitosan from *P. monodon* shrimp two extraction methods were presented that differ by the order of the steps. Crab chitosan was obtained from two species of crab (*Scylla olivicea* and *Scylla serrata*) by two different methods that used different alkali solutions and conditions in the extraction steps, resulting in chitosans with different degrees of deacetylation.

Material and Method

The different sources used to extract chitin were shrimp (*Panaeus monodon*), crab (*Scylla olivicea* and *Scylla serrata*), locust (*Schistocerca gregaria*), honeybee *(Apis mellifera*), beelte (*Calosoma rugosa*) and fish (*Labeo rohita).*

The exoskeleton of the insects, of the shrimp and crab shells were scraped free of loose tissue, washed with tap and distilled water, oven dried until constant weight and grounded to pass through a 500 μm sieve.

Extraction of chitin and chitosan from shrimp *Penaeus monodon* specimens

According to Puvvada Y.S., *et al*. (2012) the crushed shrimps shells were boiled in sodium hydroxide 2% (w/v) for one hour in order to dissolve the proteins and sugars [Lertsutthiwong, P., *et al*., 2002] and then cooled for 30 minutes at room temperature [Lamarque, G., *et al*., 2005].

The demineralization step was carried out using 1% HCl, 1:4 (w/v) for 24 h, to remove the calcium carbonate. Then, the shells were treated with 2% NaOH for 1 h and the obtained chitin was washed with deionized water [Trung, T.S., *et al*., 2006].

For the deacetylation process, the obtained chitin was boiled in 50% NaOH for 2 h and then cooled for 30 minutes at room temperature. The sample was washed with 50% NaOH, filtered and oven dried at 110 ^{0}C for 6 h [Huang, M. *et al*., 2004] to obtain chitosan.

The extraction method used by Marei N.H., *et al*. (2016) starts with the demineralization process which involves an acid treatment with 1M HCl solution, 1:15 (w/v), at 25 ^{0}C. The resulted sample was washed with distilled water until neutral pH.

The deproteinization step was perfomed with 1M NaOH at 100 ^{0}C for 8 h, several times. The resulted sample was washed with distilled water until neutral pH and then with hot ethanol and later boiled in acetone to remove all the impurities. The resulted chitin was dried in an oven at 50 ^{0}C to constant weight [Rødde, R.H., *et al*., 2008].

Deacetylation was perfomed with 50% NaOH, 1:15 (w/v), at 100 ^{0}C for 8 h and then filtered and washed with hot distilled water until neutral pH. The obtained chitosan sample was oven dried at 50^{0}C for 24 h [Abdou, E.S., *et al*., 2008].

Extraction of chitin and chitosan from fish *Labeo rohita*

The method proposed by Kumari S., et al., 2017 involves a deproteinization process with 3% NaOH, at 80 ^{0}C for 30 minutes, followed by washing the sample with distilled water until neutral pH.

The sample was treated with 3% HCl, at 25 ^{0}C for 30 minutes for demineralization, then washed with distilled water and dried at room temperature [Kumari, S., *et al*., 2015].

The obtained chitin sample was deacetylated with 40% KOH, at 90 ^{0}C, for 6 h [Kumari, S., *et al*., 2016].

Extraction of chitin and chitosan from insects: locust (*Schistocerca gregaria*), honeybee (*Apis mellifera*) and beetles (*Calosoma rugosa*)

The demineralization process uses 1M HCl solution, with a solution to solid ratio 15 mL/g, at room temperature. The samples were washed with distilled water until neutral pH [Marei N.H., *et al*. 2016].

For deproteinization, the samples were treated with 1M NaOH at 100 ^{0}C for 8 h, followed by washing with distilled water and hot ethanol.

Decolouration was achieved by boiling the samples in acetone. The obtained chitins were oven dried at 50 ^{0}C [Majtán, J., *et al*., 2007].

The chitin samples were treated with 50% NaOH, 1:15 (w/v), at 100 ^{0}C for 8 h (deacetylation). The rezulted chitosans were washed to neutrality with hot distilled water and oven dried at 50^{0}C for 24 h [Kaya, M., *et al*., 2015].

Extraction of chitin and chitosan from crabs *Scylla olivicea and Scylla serrata shells*

Chitin and chitosan were extracted from the mud crab (*Scylla olivicea)* by the method proposed by Shahidi and Synowiecki (1991).

Before the actual extraction process, a carotenoid extraction step was perfomed by mixing the gounded dried shells with cod liver oil and heating in a water bath at 60 ^{0}C for 30 minutes.

The dried shells, free of carotenoids, were treated with 2% KOH, 1:20 (w/v), for 2 h at 90 ^{0}C for deproteinization. The sample was washed with water until pH=7 and dried in the oven at 60 ^{0}C for 24 h.

The shells were demineralized with 2.5% HCl, 1:20 (w/v), at 20 ^{0}C for 6 h. The sample was washed with water until pH=7 and dried in the oven at 60 ^{0}C for 24 h.

The decolouration step used acetone to treat the samples, for 10 minutes, followed by drying at room temperature for 2 h. The decolourized shells were washed with tap water, and dried at 60 ^{0}C for 24 h in the oven [Sarbon, N.M., *et al*., 2015].

The resulted chitin was deacetylated with 40% NaOH, 1:15 (w/v) at 105 ^{0}C for 2 h, then washed with deionized water until pH=7. The obtained chitosan was dried at 60 ^{0}C for 24 h [Yen, M.T., *et al*., 2009].

Extraction of chitin from the black crab *Scylla Serrata* shells is performed by the method of Kumari S., et al., (2017).

The grounded shells were subjected to a alkaline treatment with 3% NaOH, at 80 ^{0}C, for 30 minutes. The protein free sample were then washed with distilled water until neutrality and oven dried.

Demineralization was achieved with 3% HCl, at 25 ^{0}C, for 30 minutes. The sample was washed with water to remove the excess HCl and dried at 25 ^{0}C.

Chitosan was obtained by deacetylation of the chitin sample with 40% KOH, at 90^{0}C, for 6 h [Hajji, S., 2015].

Determination of degree of deacetylation (DD)

The direct titration method was used to determine the degree of deacetylation of chitosans extracted from different sources [Kucukgulmez, A., 2011].

Dried chitosan samples (0.2 g) were dissolved in 20 cm^3 0.1 M HCl and 25 cm^3 deionized water. After 30 minutes of continuous stirring, the second portion of deionized water (25 cm^3) was added and stirring continued for 30 minutes. When the chitosan samples were completely dissolved, the obtained solutions were titrated with a 0.1 mol·dm^{-3} NaOH solution using an automatic burette (0.01cm^3 accuracy). The degree of deacetylation (DD) of chitosans was calculated using the formula [Tolimate, A., *et al*., 2000]:

$$\boldsymbol{DD}[\%] = \mathbf{2.03}\frac{\boldsymbol{V_2 - V_1}}{\boldsymbol{m + 0.0042(V_2 - V_1)}}$$

where: *m* – weight of sample, *V1*, *V2* – volumes of 0.1 mol·dm^{-3} NaOH solution corresponding to the deflection points, 2.03 – coefficient resulting from the molecular weight of chitin monomer unit, 0.0042 – coefficient resulting from the difference between molecular weights of chitin and chitosan monomer units.

Results and discussion

The degree of deacetylation (DD) may range from 30 to 95% depending on the source and preparation procedure [Martino, A.D., *et al*., 2005]. The degree of deacetylation values are highly dependent on the source and method of purification [No, H.K., & Meyers, S.P., 1995], as well as the type of analytical methods employed, sample preparation and type of instrument used, and various other conditions that may influence the degree of deacetylation analysis.

The degree of deacetylation (DD) is an important parameter that affects the properties, such as solubility, chemical reactivity and biodegradability of the obtained chitosan [Lamarque, G., et al., 2005].

Chitosan extracted from shrimp *Penaeus monodon* by two methods with similar reaction conditions showed two different degrees of deacetylation. The extraction conditions for each method are presented in Table 1.

The method used by Puvvada Y.S., *et al*., 2012 led to a chitosan with a DD of 89%, while Marei N.H., *et al*., 2016 obtained a chitosan with a DD of 74%. This is probably because Marei N.H., *et al*., 2016 used a decolouration step, involving boiling the chitin sample in acetone, that may have affected the yield of chitin.

The deacetylation step was carried out in the same conditions of temperature and NaOH concentration, but a longer reaction time resulted in a lower DD value, due to the excessice removal of the acetyl groups from the polymer during deacetylation.

Table 1. Extraction conditions of chitosan from shrimp *Penaeus monodon*

[Puvvada Y.S., *et al*., 2012], [Marei N.H., *et al*., 2016]

	Extraction step	Reagent	Temperature	Time	DD
Shrimp *Penaeus monodon* (M1)	Deproteinization	NaOH 2%	100 ^{0}C	1h	89%
	Demineralization	HCl 1%	25 ^{0}C	24h	
	Decolouration	-	-	-	
	Deacetylation	NaOH 50%	100 ^{0}C	2h	
Shrimp *Penaeus monodon* (M2)	Demineralization	HCl 1M	25 ^{0}C		74%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	

Table 2 shows the extraction conditions of the method used by Kumari S., *et al*., 2017. It can be observed that no decolouration step was used, and the temperatures and reaction times are lower than in the case of shrimp extraction. Also, the alkali solution used for deacetylation is KOH in a concentration of 40%. In this conditions, the resulted DD value was 75%, higher than the value obtained from shrimp by the method of Marei N.H., *et al*., 2016.

Table 2. Extraction conditions of chitosan from fish *Labeo rohita* [Kumari S., *et al*., 2017]

	Extraction step	Reagent	Temperature	Time	DD
Fish *Labeo rohita*	Deproteinization	NaOH 3%,	80 ^{0}C	30 min.	75%
	Demineralization	HCl 3%	25 ^{0}C	30min.	
	Decolouration	-	-	-	
	Deacetylation	KOH 40%,	90 ^{0}C	6h	

The degree of deacetylation of the chitosans derived from insect chitins, under similar conditions are showed in Table 3. All three samples presented high values of DD, over 90%.

The DD of the chitosan extracted from locust *Schistocerca gregaria* is the highest, with a value of 98%, followed by the chitosan isolated from honey bee *Apis mellifera*, 96% and from beetles *Calosoma rugosa*, 95%.

The DD values of chitosan obtained from these insects are the highest for all the samples compared, meaning that insects are a valuable source for chitosan extraction.

Table 4 presents two methos for isolating chitosan from the shells of two crabs, namely *Scylla olivicea* and *Scylla serrata*.

It can be observed that the method proposed by Sarbon N.M., *et al*., 2015 resulted in a chitosan with a lower DD value (53%), than that of the chitosan obtained by the method of Kumari S., *et al*., 2017. This low DD may be influenced by the different alkali solution used in the deproteinization step and also by the reaction time, which is longer in this case. The acetone used in the decolouration step of the chitin extracted from *Scylla olivicea* crab may have influenced the low value of DD.

Table 3. Extraction conditions of chitosan from locust *Schistocerca gregaria,* honeybee *Apis mellifera* and beetle *Calosoma rugosa* [Marei N.H., *et al*., 2016]

	Extraction step	Reagent	Temperature	Time	DD
Locust *Schistocerca gregaria*	Demineralization	HCl 1M	25 ^{0}C		98%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	
Honey bee *Apis mellifera*	Demineralization	HCl 1M	25 ^{0}C		96%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	
Beetle *Calosoma rugosa*	Demineralization	HCl 1M	25 ^{0}C		95%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	

Table 4. Extraction conditions of chitosan from crab *Scylla olivicea* [Sarbon N.M., *et al*., 2015] **and *Scylla serrata*** [Kumari S., *et al*., 2017]

	Extraction step	Reagent	Temperature	Time	DD
Crab *Scylla olivicea*	Deproteinization	KOH 2%	90 ^{0}C	2h	53%
	Demineralization	HCl 2,5%,	20 ^{0}C	6h	
	Decolouration	Acetone		10 min.	
	Deacetylation	NaOH 40%	105 ^{0}C	2h	

Crab *Scylla serrata*	Deproteinization	NaOH 3%	80 ^{0}C	30 min.	70%
	Demineralization	HCl 3%	25 ^{0}C	30min.	
	Decolouration	-	-	-	
	Deacetylation	KOH 40%,	90 ^{0}C	6h	

Following the comparison made between the extraction methods for obtainig chitosan from different sources, it can be observed that a valuable source of chitosan are insects, with DD values of over 95%. In the case of shrimp and crab chitosan, the differences in DD was due to the reaction conditions and the decolouration step used to remove the pigments. This step is necessary to obtain a colourless product, but influences the characteristics of the polymer.

Conclusion

Taking into account all the samples compared in this review it can be observed that good quality chitosan can be extracted for a variety of natural sources, such as marine crusteceans (shrimp, crabs), fish scales and insects (locusts, bees and beetles). The degree of deacetylation is very much dependent on the source and preparation procedures and can be easily influenced by the reaction conditions. The highest DD values were observed for the samples prepared from insects (DD = 95%-98%).

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