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Comparative Physicochemical Characterization of Chitosan from Shells of Two Bivalved Mollusks from Two Different Continents

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Abstract The need to turn seafood wastes that hitherto constitute an environmental pollution into important pharmaceutical excipients is gaining ground worldwide. This study investigated the extraction and characterization of chitosan from two oyster shells: *Mytilus edulis* and *Laevicardium attenuatum* from different continents. Demineralization and deproteinization were carried out to obtain chitin from the shells, followed by deacetylation to obtain chitosan. Percent yield, degree of deacetylation and other physicochemical characteristics were determined for the extracted chitosan from the two oyster shells. The yield of chitosan from *Mytilus edulis* was 51.8% and 43.8% from *Laevicardium attenuatum*. The degree of deacetylation (DD) of chitosan from *Mytilus edulis* was 69.6% while that of *Laevicardium attenuatum* was 37.3%. The *Laevicardium attenuatum* chitosan had higher swelling ratio. Calcium was the predominant metal ion in the two chitosan. While the micrograph of *Mytilus edulis* chitosan showed a non-uniform particle distribution, *Laevicardium attenuatum* chitosan showed a brick-like structure. This work has shown that it is more economical to produce chitosan from *Mytilus edulis, and* the polymer has higher degree of deacetylation. The higher degree of deacetylation implies better solubility and the chitosan could be more suitable as a permeation enhancer. The *Laevicardium attenuatum* chitosan could be more suitable for sustained release formulation.

Keywords Chitosan, *Mytilus edulis, Laevicardium attenuatum*, Oyster shells

1. Introduction

Economic hardship globally calls for turning environmental waste products into useful pharmaceutical excipients such as chitin and chitosan. Chitosan is a polymer derived from deacetylation of chitin. Chitin is naturally obtained from several sources which include cell wall of fungi, exoskeleton of crustacean such as crabs and shrimp; insects, oyster shells and other marine animals [1]. Oyster shells are seen littering beaches, river banks and markets (after the animals have been removed and used as food). Attempts to burn them further constitute more environmental nuisance [2].

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. It is produced by treating the shells of the marine animals with the alkali sodium hydroxide. The amino group in chitosan protonates in acidic to neutral solutions making the polymer to be water-soluble at this pH range. It is a bioadhesive capable of binding to negatively charged surfaces such as mucosa membranes [3, 4]. Chitosan is biocompartible and biodegradable and it enhances the transport of polar drugs across epithelial surfaces. Therefore, chitosan has a number of commercial and biomedical uses. In medicine, it may be useful in bandages to reduce bleeding and as an antibacterial agent. It is also useful as a soluble dietary fiber.

In pharmaceutical industry, chitosan is useful in sustained delivery of drugs. It has also been employed in oral drug formulations in order to improve the dissolution of poorly soluble drugs [5]. Chitosan microspheres have also been produced for use in enhanced chromatographic separation [6], for the topical delivery of drugs [7], for drug targeting after injection [8] and for controlled release of drugs [9-11].

Mytilus edulis (Phylum mollusca, class bivalvia, Family Mytilidae) commonly called mussel, is an oyster animal which live on exposed shores in the intertidal zone (Figure 1). In most marine mussels, the shell is longer than it is wide, being wedge-shaped or asymmetrical. The external color of the shell is often dark blue, blackish, or brown, while the interior is silvery and somewhat nacreous. The word "mussel" is most frequently used to mean the edible bivalves of the marine family. Freshwater mussel species inhabit lakes, ponds, rivers, creeks, canals and they are classified in a different subclass of bivalves, despite some very superficial

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similarities in appearance. The shells of mussel are found scattered all over Long beach in the United States of America.

Figure 1. Photograph of *Mytilus edulis*

Laevicardium attenatum (Fig. 2) commonly called giant egg cockle, giant Pacific cockle and the yellow cardinal cockle is a species of saltwater clam, a cockle, a marine bivalve mollusk in the family Cardiidae. This species is found in the tropical Panamic Province, from Southern California south through the Pacific coast of Mexico and the Gulf of California, and as far south as Panama. It is also found on Lagos beaches in Nigeria. Giant egg cockles shells are ovate to trigonal, height greater than length; 45-48 low, smooth radial ribs; exterior color yellow to brown; interior white; anterior and ventral margins finely crenulate within; posterior margin smooth within; periostracum thick, dehiscent, tan to dark brown, silky; juveniles with commarginal ridges in periostracum [12].

Figure 2. Photograph of *Laevicardium attenuatum*

The amount of chitin or chitosan obtainable from a source is dependent on the source [13, 14]. In view of the need to turn the shell waste into a useful product, the enormous importance of chitosan and the dependence of quantity and quality of chitosan on the source, this work was carried out to extract chitosan from shells of *Mytilus edulis* and *Laevicardium attenatum* and to compare their physicochemical properties in the perspective of drug delivery.

2. Materials and Methods

2.1. Materials

Shells of *Mytilus edulis* were obtained from Long beach, California, USA where they were seen scattered all over the beach. *Laevicardium attenuatum* shells were picked on Lagos beach, Nigeria. Other materials used were sodium hydroxide pellets, potassium permanganate crystals and 36% $^{v}/_{v}$ hydrochloric acid (all from BDH Chemicals, England). Distilled water was prepared in the Process Laboratory of Department of Pharmaceutics and Pharmaceutical Technology, University of Uyo, Uyo, Nigeria. All other reagents used are of analytical grade.

2.2. Extraction of Chitosan

The two shells were washed separately and oven dried for four days. They were blended and then sieved to obtain 250 microns powder size. The 250 microns powder size was subjected to serial extraction. Extraction was carried out firstly by deproteination, followed by demineralization and then deacetylation processes using the method earlier reported by [2].

2.3. Determination of Yield of Chitosan

The percentage yield was calculated using equation 1.

$$\% \text{ Yield} = \frac{Mass\ of\ chitosan}{Mass\ of\ shell} X\,100 \qquad (1)$$

2.4. Determination of Degree of Deacetylaton (DD)

The degree of acetylation was determined using acid – base titration method with modification as described by [15].

2.5. Determination of Moisture Content

A 2 g sample of polymer was weighed and transferred into an electronic moisture analyzer (Type MB 35, OHAUS, Switzerland) and the percent moisture content was determined.

2.6. Determination of Viscosity

The viscosity of 2% $^{w}/_{v}$ dispersion of each polymer was determined using a DV1 prime viscometer (Brookfield Engineering, U.S.A.). About 80 ml of the polymer dispersion was placed inside the cup of the viscometer and the reading was taken at 60 rpm (spindle No.1).

2.7. Determination of Swelling Ratio

A 2 g sample of the polymer was placed inside a 10 ml measuring cylinder. The tapped volume was taken and the cylinder was filled with distilled water to the 10 ml mark. The mixture was mixed thoroughly and then allowed to stand for 6 h after which the swollen volume of the polymer was taken. The swelling ratio was calculated as the ratio of the volume of the swollen mass to the tapped volume.

2.8. Fourier Transform Infrared (FTIR) Spectroscopy

Infrared spectrophotometer (Shimadzu IR Prestige 21, China) was used in analyzing the sample of chitosan from the two different shells in the range of 400 to 4000cm^{-1}.

2.9. Scanning Electron Microscopy (SEM)

The structures of chitosan from the two shells were examined using electron microscope (Model SEM PROX, Phenomworld, Eindhoven, Netherlands).

2.10. X-ray Diffraction (XRD)

The degree of crystallinity was detected by XRD analysis at room temperature by the use of materials analyzer diffractometer (Model: EMMA, GBC Scientific Equipment Pty Ltd., Australia) equipped with Cu target X-ray tube with wavelength of 1.54059 Å and step size of 0.05. X-ray diffraction was taken in the 2θ range of 5° to 70°. The mineral search was carried out using the GBC X-ray analysis TRACES Software (Version 6) which operates on the full ICCD PDF-2 database file.

2.11. Energy Dispersive X-ray Fluorescence (XRF)

X-ray fluorescence (XRF) spectrometer (Model: EDX3600B, Skyray instrument Inc., USA) was used to determine the quantitative and qualitative elemental analysis of chitosan extracted from shells of *Mytilis edulis* and *Laevicardium attenuatum*. XRF detects elements between sodium (Na, Z = 11) and Uranium (U, Z = 92) with high resolution and fast analysis.

2.12. Statistical Analysis

Experimental data have been represented as the mean with standard deviation (SD) of different independent determinations. The significance of differences was evaluated by analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Some Physicochemical Properties

The results of the % yield, degree of deacetylation, moisture content, viscosity and swelling ratio of chitosan from *Mytilus edulis* and *Laevicardium attenuatum* are shown in Table 1. The yield varied from one animal to another within the same class. The higher yield from *Mytilus edulis* could be due to effective demineralization and deproteination steps. The lower yield from *Laevicardium attenuatum* may be as a result of the time length of the deacetylation process resulting in depolymerization of the chitosan polymer that could have led to loss of material from excessive removal of acetyl groups from the polymer during washing [16]. Divya *et al*., [17] extracted chitosan from crab shell and reported yield in the range 30-36.7%. They suggested that the difference in yield was due to the reaction time as well. Puvvada *et al*. [18] reported a yield of 34% which is lower than those of the two experimental shells.

Table 1. Some physicochemical properties of the polymers

Parameter	Chitosan from *Mytilis edulis*	Chitosan from *Laevicardium attenuatum*
% Yield	51. 80 ± 0.23	43.80 ± 0.43
Degree of deacetylation	69.60 ± 0.12	37.30 ± 0.31
Moisture content (%)	3.28 ± 0.12	3.84 ± 0.24
Viscosity of 2 % w/v dispersion (mPa.s)	3.24 ± 0.00	3.86 ± 0.00
Swelling ratio	1.19 ± 0.03	1.24 ± 0.04

The degree of deacetylation for *Mytilus edulis* was higher than for *Laevicardium attenuatum.* Al-Hassan [19] reported 54.65% which is intermediate of these two studied exoskeleton. The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin giving chitosan with a chemical reactive amino group –NH_2. This –NH_2 affects the chitosan's physicochemical properties and appropriate applications [20] as well as biodegradability and immunological activity [21]. The two main advantages of chitosan over chitin is that in order to dissolve chitin, highly toxic solvents such as lithium chloride and dimethylacetamide are used whereas chitosan is readily dissolved in dilute acetic acid. Also, chitosan possesses free amino groups as active sites in many chemical reactions [22].

Muñoz *et al.* [23] reported deacetylation degree of 73.6% and stated that the degree of deacetylation depends on the source of chitin and time, temperature and alkaline concentration used. The DD values are highly dependent on the process used [24, 25]. Hossain and Iqbal [16] concluded that the degree of deacetylation (DD) is influenced by NaOH concentration. The low DD in this work could be attributed to the conditions (alkali concentration, pressure and non pulverisation of chitin) used for the deacetylation. Extended heating time and high alkali concentration can be applied to drastically improve the degree of deacetylation.

Solubility is controlled by the degree of deacetylation and it is estimated that deacetylation must be at least 85% complete in order to achieve the desired solubility [26]. According to Bough *et al*. [27], a reaction time of 5 min with 45% NaOH may not be enough for chitin particles to be sufficiently swollen. A decrease in concentration of the NaOH concentration to 40% required increased time of > 30 min to obtain a soluble chitosan [28]. While chitin is insoluble in most organic solvents, chitosan is readily soluble in dilute acidic solutions below pH 6.0. Organic acids such as acetic, formic, and lactic acids are used for dissolving chitosan. The most commonly used is 1% acetic acid solution at about pH 4.0 as a reference. Chitosan is also soluble in 1% hydrochloric acid but insoluble in sulfuric and phosphoric acids. Solubility of chitosan in inorganic acids is quite limited. Concentrated acetic acid solutions at high

temperature can cause depolymerization of chitosan [24]. Above pH 7.0, chitosan solubility is poor. At higher pH, precipitation or gelation tends to occur and the chitosan solution forms poly-ion complex with anionic hydrocolloid resulting in the gel formation [29].

The moisture contents of the two forms of chitosan are not significantly different. The moisture content of chitosan value varies depending on its season, relative humidity and intensity of sunlight. The report generated by the Korean Food and Drug Adminisration [30] indicated that the moisture content of chitosan powder should be < 10%. The moisture contents obtained for chitosan from the two sources are within the range.

Even though the viscosity of dispersion containing chitosan from *Laevicardium attenuatum was* higher, the value was not significantly different from that of *Mytilus edulis*. The values are significantly lower than those of plant gums [31]. Viscosity enhancement is crucial in the indication of a polymer as a suspending or emulsifying agent. Therefore, chitosan is less suitable than plant gums for use as suspending and emulsifying agents.

The polymers from the two sources have low swelling ratios showing they are not strong hydrogels. Chitosan affects drug delivery in different ways. It is a bioadhesive agent, a matrix former for sustained delivery, a disintegrant and a permeation enhancer [32]. Since the swelling ratios are low, chitosan from these sources could be more effective as a permeation enhancer since the polymer must be in solution to be effective as such.

3.2. FT-IR Spectra of Chitosan from the Two Sources

The FTIR spectra of chitosan from *Mytilus edulis* and *Laevicardium attenuatum* are shown in Fig. 3 and Fig. 4 respectively.

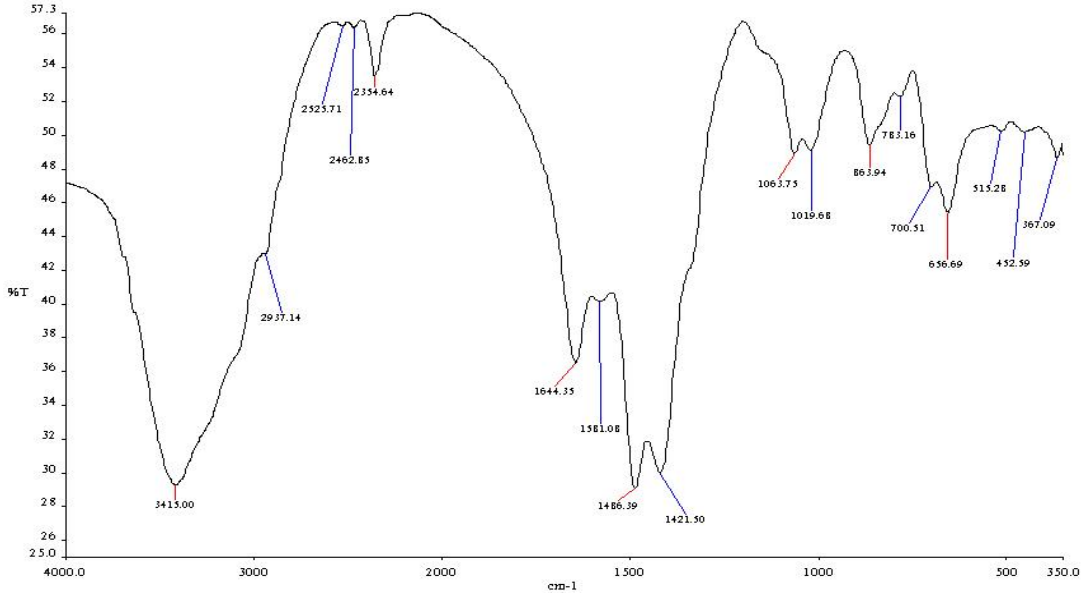

Figure 3. FTIR Spectrum of *Mytilis edulis*

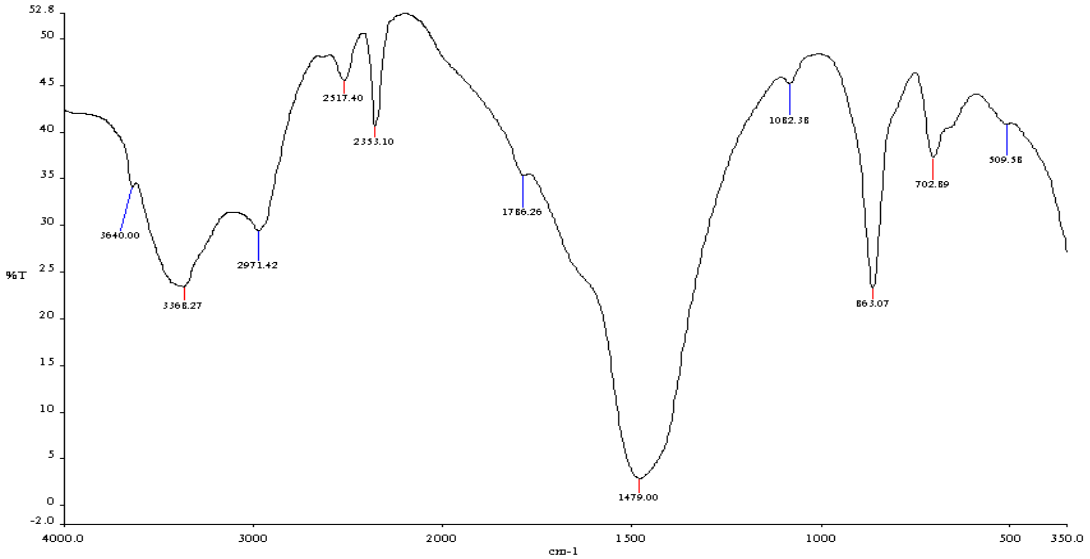

Figure 4. FT-IR Spectrum of *Laevicardium attenuatum*

In the spectrum of *Mytilus edulis* chitosan, the peak at 863.94 cm^{-1} can be assigned to C-N stretching. The absorption peak at 1019.68 and 1063 cm^{-1} can be assigned to C-O bending of glucose molecule while those at 1421.50 and 1486.39 cm^{-1} can be assigned to C-H bending of the side chain $–CH_2OH$. The peaks at 1581.08 and 1644.35 cm^{-1} are characteristic of bending vibration of N-H while 3415 cm^{-1} is characteristic of N-H stretching vibration of amides [33]. The high intensity of peaks that are associated with N-H vibration suggests deacetylation of the chitin. When deacetylation occurs, there is an increase in intensity of peak indicating the prevalence of NH_2 groups [34]. All these absorption peaks are typical of chitosan molecule.

In the spectrum of *Laevicardium attenuatum* chitosan, the peak at 863.07 cm^{-1} can be assigned to C-N stretching. The absorption peak at 1062.38 cm^{-1} can be assigned to C-O bending of glucose molecule while 1479.00 cm^{-1} can be assigned to C-H bending of side chain $–CH_2OH$. The absorption at 1786.20 cm^{-1} is typical of an amide while 3640 cm^{-1} is characteristic of free O-H groups [33]. All these absorption peaks are typical of chitosan molecule. The lower intensity of vibration due to N-H in the spectrum of this chitosan signifies lower level of deacetylation [34].

3.3. Scanning Electron Microscope Analysis

The surface morphology of the two shell samples is shown in Fig 5. The particle surface of *Mytilus edulis* chitosan became rougher as the magnification was increased. The micrograph shows a non-homogeneous particle distribution while the particle size and shape are uneven at low magnification. The SEM image of *Laevicardium attenuatum* shows an agglomeration of uneven particles. At high magnification, the particles were observed to have a brick-like structure.

3.4. XRD Diffraction Patterns of *Mytilus edulis* and *Laevicardium attenuatum*

The X-ay diffraction patterns of chitosan from *Mytilus edulis* and *Laevicardium attenuatum* are shown in Fig. 6 and 7 respectively. Different mineral phases of $CaCO_3$ were identified in the samples. For *Mytilus edulis,* it was calcite and for *Laevicardium attenuatum*, it was aragonite. Aragonite is one of the two common, naturally occurring crystal forms of calcium carbonate, the other form being the mineral calcite. Aragonite forms naturally in almost all mollusks. Traces of Coesite (SiO_2) were also found in both samples.

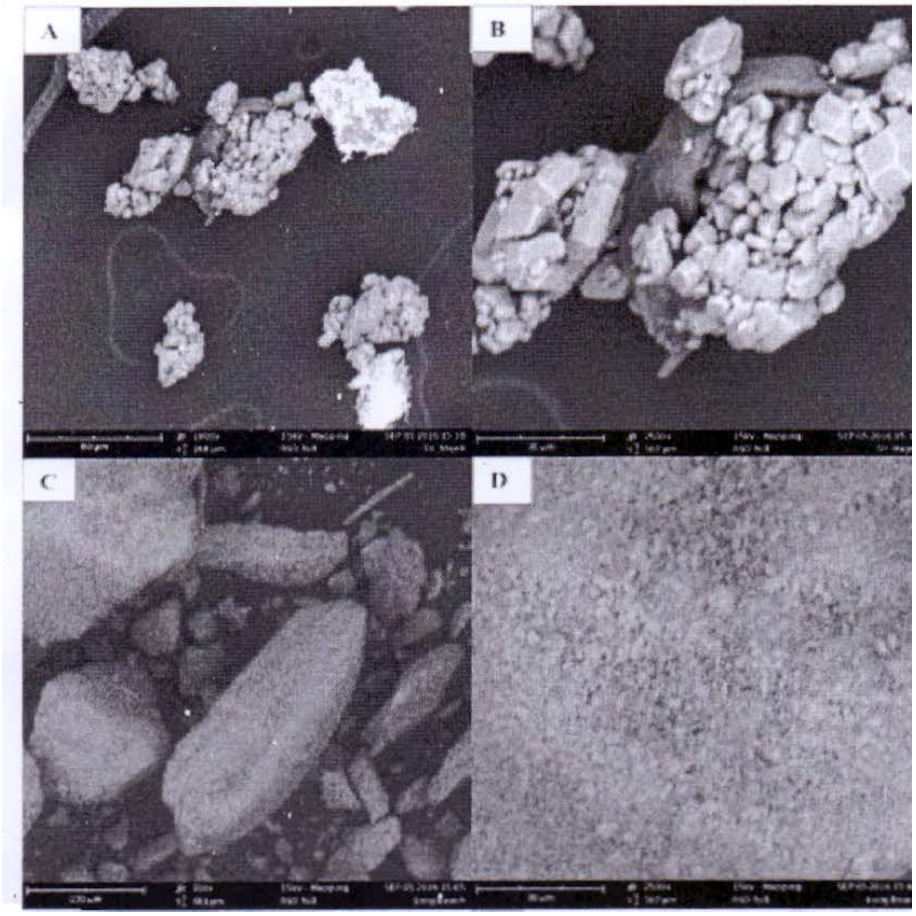

Figure 5. SEM Micrograph of (A) *Laevicardium attenuatum* x 1000; (B) *Laevicardium attenuatum* x 2500, (C) *Mytilus edulis* x 700, (D) *Mytilus edulis* 2500

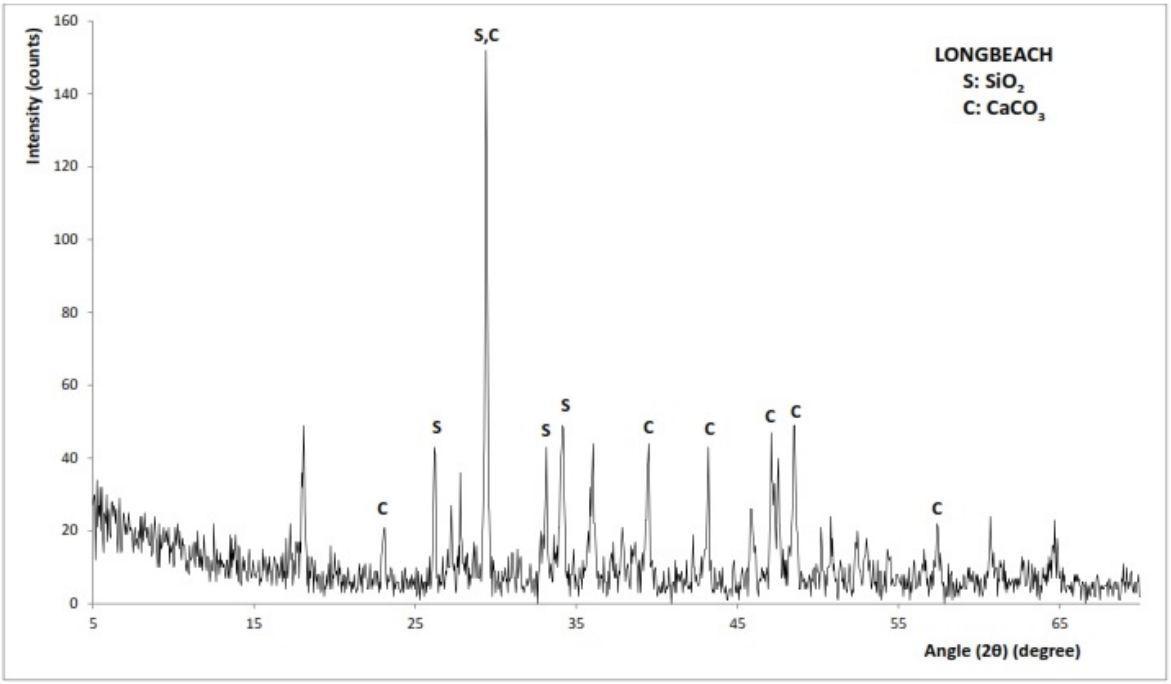

Figure 6. XRD pattern of *Mytilis edulis*

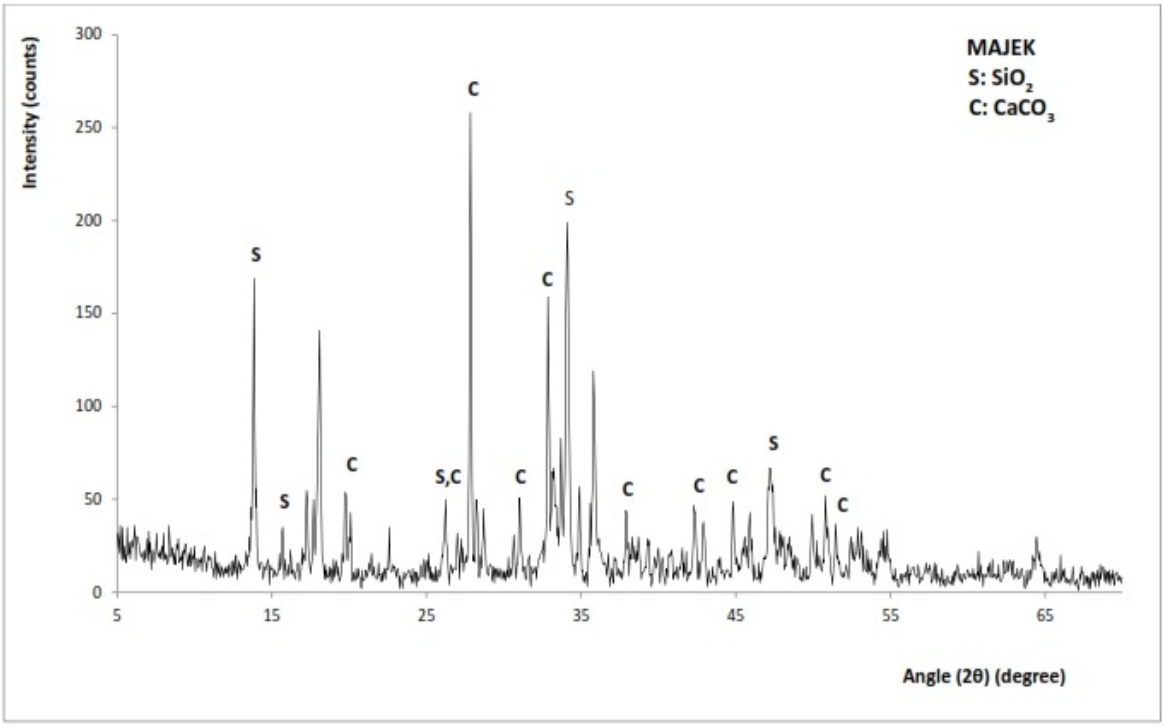

Figure 7. XRD pattern of *Laevicardium attenuatum*

3.5. X-Ray Flourescence (XRF) Patterns of *Mytilus edulis* and *Laevicardium attenuatum*

The intensity of peaks and elemental composition of the two chitosan as revealed by the XRF are shown in Table 2. Calcium is the major element in the two chitosan. The *Mytilus edulis* chitosan contained 69% calcium while *Laevicardium attenuatum* chitosan contained 54.3%. Lead is absent in the two chitosan indicating the non-toxicity of the polymer.

With an XRF spectrometer both very small concentrations of very few ppm and very high concentrations of up to 100% can be analyzed directly without any dilution process. With an XRF spectrometer, both very small concentrations of very few ppm and very high concentrations of up to 100% can be analyzed directly without any dilution process.

Table 2. Elemental Composition of Chitosan from Shells of *Mytilus edulis* and *Laevicardium attenuatum*

Element	Intensity		Content	
	Mytilis edulis	*Laevicardium attenuatum*	*Mytilis edulis*	*Laevicardium attenuatum*
Mg	0.0001	0.0001	0.1506	0.0785
Al	0.0007	0.0006	0.2137	0.1683
Si	0.0013	0.0012	0.0331	0.0165
P	0.0058	0.0045	0.2726	0.2115
S	0.0051	0.0047	0.3515	0.3162
K	0.0000	0.0000	0.0000	0.0000
Ca	0.7116	0.5538	69.8695	54.2746
Ti	0.0000	0.0000	0.0000	0.0000
V	0.0000	0.0001	0.0000	0.0023
Cr	0.0000	0.0001	0.0000	0.0033
Mn	0.0009	0.0001	0.0598	0.0012
Co	0.0000	0.0002	0.0000	0.0022
Fe	0.0024	0.0024	0.3079	0.3081
Ni	0.0003	0.0008	0.0131	0.0477
Cu	0.0008	0.0014	0.0171	0.0303
Zn	0.0013	0.0023	0.0413	0.0782
As	0.0000	0.0000	0.0000	0.0000
Pb	0.0000	0.0000	0.0000	0.0000
W	0.0001	0.0003	0.0000	0.0838
Au	0.0000	0.0000	0.0000	0.0000
Ag	0.0000	0.0000	0.0000	0.0000

4. Conclusions

A higher yield of chitosan with higher degree of deacetylation can be obtained from shells of *Mytilus edulis*. The higher degree of deacetylation could enhance the solubility of the chitosan and increase the suitability of the polymer as a permeation enhancer. The *Laevicardium attenuatum* chitosan with higher swelling ratio could be more suitable for sustained release formulation. Calcium is the predominant metal ion in the two chitosan. While the *Mytilus edulis* chitosan has a rough surface and non-homogeneous particle distribution, *Laevicardium attenuatum* chitosan is an agglomeration of particles which exists as a brick-like structure.

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