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Comparison of chitins produced by chemical and bioprocessing methods

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Abstract: Lactic acid fermentation was used to extract chitin from prawn shell (*Nephrops norvegicus*) at two different scales of operation. The fermentation products were characterized and compared with chitin extracted from the same source by a chemical method. Chitosans produced from the obtained chitins were evaluated in terms of their intrinsic viscosity, molecular weight and degree of acetylation (DA). The fermentation removed 690 g kg^{-1} and 770 g kg^{-1} of inorganic matter, 490 and 440 g kg^{-1} of protein and 540 and 770 g kg^{-1} of lipids from the shells at laboratory and pilot plant scales, respectively. However, the functional properties such as the DA of the chitin, the molecular weight and the DA of the chitosans were similar to those obtained for the chemically-obtained chitin and its chitosan. Despite the incomplete extraction of chitin this biological process could be useful to produce chitin and chitosan in a more environment-friendly approach.
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Keywords: prawn shell; *Nephrops*; chitin; lactic acid fermentation; chitosan

INTRODUCTION

Chitin is a linear polysaccharide of *N*-acetyl-D-glucosamine (~50–100%) and D-glucosamine (~50–0%) linked by β, $1 \rightarrow 4$ glycosidic bonds (Fig 1). It is the second most abundant natural polymer after cellulose and also shares chemical and functional characteristics with it. Chitin is produced mainly by arthropods, the most abundant animal group, which includes insects and crustaceans. It is also produced by molluscs and fungi.[1] The main biological function of chitin is support, forming a structural part of the exoskeleton or cell walls of organisms. Nevertheless, chitin, chitosan (its deacetylated form) and their derivatives have been shown to have a range of biological activities and properties.[2–4] Because of this there has been a growing interest in chitin and its use in many applications.

Commercial chitin is extracted from crustacean waste provided by the seafood industry. The main sources are the shells of crab, shrimp, prawn, krill and lobster. The squid pen is the main source of β-chitin, an allomorph with different crystalline arrangement.[5] There are some reports of chitin extraction from insects and fungi, most of them at laboratory scale.[6–8] In general, crustacean shells are constituted mainly of a matrix made of chitin and protein, hardened by mineral salts. The amount of each component can vary widely among species and also in an intra-specific way as a function of season, age, gender and other environmental conditions. Depending on the species there are minor components such as lipids and pigments, among others. The chitinous waste could be considered hazardous as result of uncontrolled dumping. In the sea it leads to eutrophication and a high load of biological oxygen demand (BOD), on land it can result in pathogen-borne problems.[9] However, several schemes have been suggested for the utilization of this waste product, involving the extraction of chitin,[10–12] and the recovery of protein,[13,14] lipids (pigments)[15–17] and other compounds.[18,19] These substances could be valuable in a number of applications.[2–4,20]

Several techniques to extract the chitin from different sources have been reported. Most of them, including the main industrial method, rely on chemical processes for the hydrolysis of protein and removal of inorganic matter. Some include a decolourisation step to improve the colour of the extracted chitin, using solvent extraction or chemical oxidation of the remaining pigments. Generally these methods

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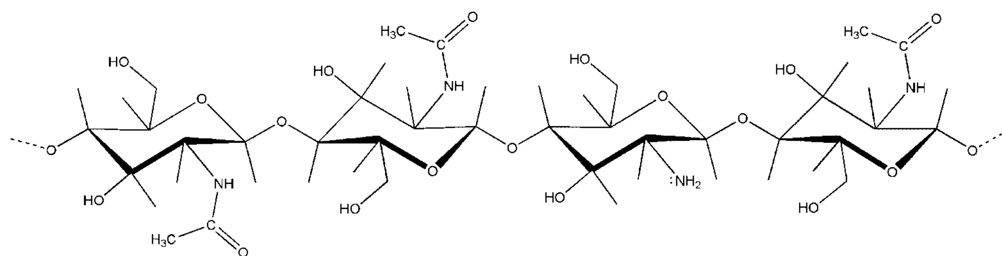

Figure 1. The chemical structure of chitin.

use large quantities of water and energy, and also often produce corrosive wastes. Moreover, these methods make the recovery of other products like protein and pigments relatively more complicated.[11] Enzymatic pathways are an alternative, which permit suitable extraction of chitin as well as a profitable recovery of other by-products. Processes using enzymatic extracts or isolated enzymes[14,21,22] and microbiological fermentations[10,23–26] have been used with various levels of success. This work compares the physicochemical properties and some quality aspects of chitin produced by a chemical technique against chitin extracted by lactic acid bacterial fermentation. This will help determine the suitability of biologically-produced chitin for various applications and reveal if any advantages are apparent in biologically-produced chitin.

MATERIALS AND METHODS

Nephrops norvegicus (Dublin Bay prawn) shell waste was obtained from a shellfish fishing company at Kilkeel harbour, Co Down, Northern Ireland. The shell waste consisted only of the shell covering the abdomen of the prawn and had very little meat attached to it. The prawn shell was kept frozen at −20 °C to prevent spoilage.

All reactants used were of analytical grade unless stated.

Chitin production

Biological method

At laboratory scale, 500 g of prawn shell was minced, using a food blender to 2–5 mm flakes. This was then added to a 5 dm^3 bioreactor (New Brunswick Scientific Co, Edison, USA) along with 5 dm^3 of a 150 g dm^{-3} glucose solution and 0.1 cm^3 dm^{-3} of inoculant (SIL-ALL 4 × 4® silage additive: *Lactobacillus salvarius, Enteroccus facium* and *Pediococcus acidilactici*). The reactor was run for at least 5 days at 30 °C and a stirrer velocity of 250 rpm. The solid fraction was separated from the liquid fraction and then was washed with water and dried to prevent spoilage. This chitin was given the abbreviation Cnbs.

This process was scaled up to the 500 dm^3 scale and the fermentation was carried out under the same conditions, except that the prawn shell was ground to a particle size of around 0.5 mm in an industrial mincer. The chitin obtained was given the abbreviation Cnbl.

Chemical method

In order to remove the inorganic matter the minced prawn shell was treated with 1 M hydrochloric acid in a 1:15 solid-to-liquid ratio. The reaction was carried out for 2 h at room temperature. After that the solids were washed until neutrality was reached.

Next the removal of proteins was carried out using 150 g dm^{-3} sodium hydroxide solution in a 1:10 solid-to-liquid ratio. The mixture was heated at 65 °C for 3 h in a reaction flask. The chitin was then washed until neutrality was reached and dried in an oven at 40 °C. The chitin obtained was given the abbreviation Cnc.

Chitosan production

A sample of each chitin was converted to chitosan using a thermochemical method. Samples of chitin (10 g) were added to reaction flasks and 100 cm^3 of 500 g kg^{-1} sodium hydroxide solution was added to each flask. The reaction was carried out at 100 °C for 2 h in a nitrogen atmosphere. The solid product was then removed, cooled and washed with water until the pH was neutral. The chitosans produced were given the abbreviations Csbs, Csbl and Csc respectively.

Physichemical analysis

The dried and ground prawn shell and the chitin samples (Cnbs, Cnbl, Cnc) were analysed to determine their contents of protein, chitin, inorganic matter, lipids and metals.

The protein and chitin contents were estimated from the nitrogen (N) data obtained using a Perkin-Elmer (Norwalk, USA) 2400 Series 2 CHNS Elemental Analyser. The stoichiometric relationships (1) and (2) were used to calculate the protein (P) and chitin (Q) content respectively (Lizardi J, Goycoolea FM and Argüelles-Monal W; unpublished):

$$P = (N \cdot Cq + K\text{-}100) \cdot Cp \cdot (Cq\text{-}Cp)^{-1} \quad (1)$$

$$Q = (N \cdot Cp + K\text{-}100) \cdot Cq \cdot (Cp\text{-}Cq)^{-1} \quad (2)$$

where: N is the total nitrogen content (%); K is the content of non-nitrogenous matter, equivalent to the total of the contents of inorganic matter (ash), lipids and water; Cp is nitrogen-protein conversion factor, a value of 6.25 was used and Cq is the nitrogen-chitin conversion factor that was calculated from the theoretical nitrogen content of chitin at the corresponding degree of acetylation (DA) of each sample.

The inorganic matter content and remaining moisture were determined gravimetrically.[27] The lipid content was determined using a Soxhlet extraction technique.

A metal content profile was obtained using inductively coupled plasma optical emission spectroscopy analysis (ICP-OES, Thermo Elemental Iris Advantage, Massachusetts, USA). The samples were digested as described in method 200.3, US Environmental Protection Agency.[28]

The main functional properties were determined as follows. The DA of the chitin and chitosan samples were estimated from their Fourier transformed infrared spectra (FTIR) as described by Brugnerotto *et al*.[29] A Perkin-Elmer 'Spectrum RX FT-IR' Spectrometer was used to obtain transmission spectra from KBr-based pellets. The molecular weight (Mv, viscosity average) of the chitosan samples was calculated using the Mark–Houwink equation ($[\eta] = K Mv^{\alpha}$), where $[\eta]$ is the intrinsic viscosity and K and α are empirical constants.[30] Dilute solutions of the chitosan samples were prepared in 0.3 M acetic acid/0.2 M sodium acetate and filtered through 0.45 µm membranes. The intrinsic viscosities of the samples were determined using an Ubbelohde (BDH, Poole, England) viscometer. Since the three chitosans were prepared in the same way, their molecular weights suggested the molecular weights of their corresponding chitins.

RESULTS

The lactic fermentation bioprocess caused a drastic reduction of the pH as a result of the metabolic production of lactic acid. The pH evolution profile for the treatment (Fig 2) shows that acidic pH values were obtained within 24 h, and reached a pH value of less than 3.5 over 7 days. The rapid reduction of the pH could indicate adequate growth of the lactic acid bacteria. This promotes efficient inhibition of spoilage bacteria and enzymes.

The appearances of the obtained chitins from each method of production were compared visually.

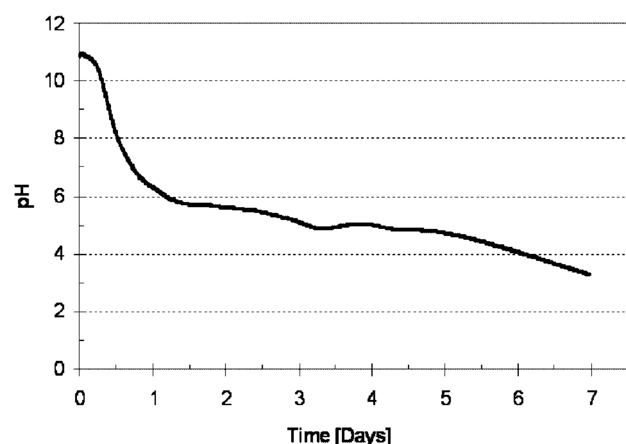

Figure 2. The change in pH during the laboratory-scale fermentation.

Table 1. Chemical composition of the raw material and products

Sample	Protein ($mg\,g^{-1}$)	Chitin ($mg\,g^{-1}$)	Inorganic ($mg\,g^{-1}$)	Lipids ($mg\,g^{-1}$)	DA (%)
PS	235	133	618	13	
Cnbs	119	679	196	6	67.0
Cnbl	132	723	142	3	70.5
Cnc	21	970	2	7	71.3

The Cnbs had a pale pink-orange colour; Cnbl was off-white with tan tones and Cnc had a light tan colour. Cnbl was the lightest and Cnbs the darkest; this is most probably due to the presence of impurities in this chitin such as pigments and proteins. The Cnbl sample looked whiter than the other samples because it had a smaller particle size. The Cnc method of production is a harsh chemical treatment so it is expected that most of the pigments in the sample were removed or degraded.

The chemical compositions of the source material, prawn shell (PS), and the obtained chitins are shown in Table 1. The quantity of inorganic matter was found to be above 600 $g\,kg^{-1}$ in the prawn shell. Prawns are in the group of crustaceans with the highest inorganic content in their shells.[31]

Cnc was found to have a low level of impurities and had 970 $g\,kg^{-1}$ chitin. Cnbs and Cnbl both had around 700 $g\,kg^{-1}$ chitin present. At the end of the fermentation both products retained around half of the original protein content of the shell. These results are comparable to those obtained by Cira *et al* in terms of final protein content.[24] Total deproteinization through a purely biotechnological process seems difficult to achieve.[10,14,21,22,26,32]

The acidic conditions in the biological process achieved the removal of more than 660 $g\,kg^{-1}$ of the inorganic content of the prawn shell in the 5 dm^3 reactor and at least 760 $g\,kg^{-1}$ in the 500 dm^3 bioreactor. These results are comparable with others reported for crustacean shell waste fermentations.[24,25,33] However, the process proposed by Bautista *et al* yielded a product with lower inorganic content after lactic acid fermentation.[23]

In the prawn shell the most abundant metallic elements were Ca, Mg, Na, Sr, K and Fe, respectively (Table 2). Calcium was by far the most abundant and there was around 17 times more calcium present than magnesium. When the prawn shell was converted to chitin in the large bioreactor the calcium content in the chitin was reduced from 7.777 to 1.741 $mg\,g^{-1}$. This corresponds to a reduction in calcium of 880 $g\,kg^{-1}$. In the small bioreactor the calcium content was reduced to 3.087 $mg\,g^{-1}$, corresponding to a reduction of 600 $g\,kg^{-1}$. After chemical processing the calcium content was reduced to 20 $\mu g\,g^{-1}$; this corresponds to a reduction of 99.75%. A similar pattern prevailed for the other most abundant metallic elements. The data obtained from this analysis confirm the data obtained from the gravimetric analysis. The contents of the regulated heavy metals (lead, cadmium and

mercury) were all below the permitted levels stated for crustacean foodstuff.[34]

The lipid content of the shell was reduced to a great extent by fermentation. The Cnbs and Cnbl had 53.8% and 76.9% lower lipids contents than the original prawn shell, whereas the chemical process only removed 46.1% of the lipids in Cnc. There are reports indicating that the acid fermentation could facilitate availability or extraction of carotenoproteins, carotenoid pigments and valuable fatty acids.[15,35]

The FTIR spectra of the processed samples (Fig 3) were used to estimate their DA.[29] There were only minor differences between the three chitin spectra, despite the differences in chemical composition, mainly in protein content. That differences were reflected in the weak absorption band centred at 1630 cm^{-1}. In the prawn shell spectrum (Fig 3) a strong wide absorption band centred at 1410 cm^{-1} masked the bands used to estimate the DA. On the basis of obtaining the purest possible chitin the chemical process may be the most effective. However, biotechnological processes have the potential to be a viable alternative. This concurs with a trend to overcome environmental and safety disadvantages associated with the chemical process, such as the use of highly corrosive reagents and the management of their contaminant residuals to avoid environmental issues.

Table 2. Metal content profile of prawn shell and chitin samples[a] ($\mu g\, g^{-1}$)

	PS	Cnbl	Cnbs	Cnc
Ca	7777	1741	3087	19.69
Mg	468.6	8.92	14.3	2.849
Na	218.2	27.15	28.62	14.79
Sr	121.9	21.25	38.16	0.199
K	90.72	3.93	4.46	0.904
Fe	30.75	13.93	10.3	1.787
Mn	23.49	2.52	26.23	0.082
Sn	3.59	4.41	4.03	3.946
Si	2.42	3.6	2.49	1.355
Al	1.96	10.04	9.25	1.594
B	1.95	2.24	4.41	0.054
Zn	1.73	1.64	3.93	0.173
Se	1.12	0.63	0.3	0.037
Cu	0.76	0.74	1.98	1.361
Ba	0.59	0.74	0.38	0.032
Pb	0.45	0.51	0.55	0.039
Cr	0.39	0.73	0.23	0.028
Ti	0.16	0.1	0.05	0.026
Ni	0.08	0.36	0.08	0.035
Co	0.08	0.02	0.81	0.002
Cd	0.05	0.01	[b]	0.007

[a] The contents of V, As, Sb and Hg were below the limit of detection.
[b] Below the limit of detection.

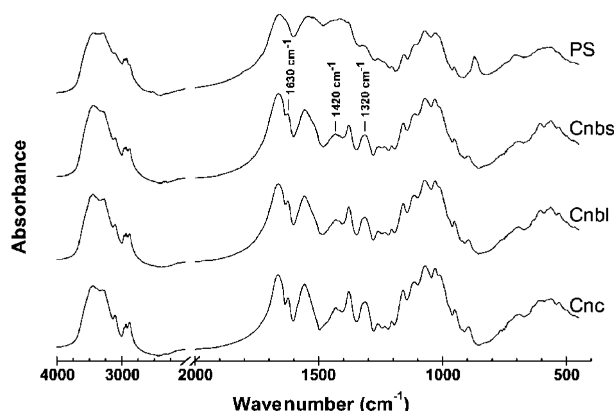

Figure 3. FTIR spectra of the prawn shell (PS) and obtained chitins (Cnbs: chitin obtained by laboratory-scale fermentation. Cnbl: chitin obtained by pilot plant-scale fermentation. Cnc: chitin obtained by chemical extraction).

Although the product obtained from this process was not as rich in chitin as that produced from the chemical process it was still a useful product. Ryachi and Bencheikh have suggested that chitin can be used to remove metal ions from water.[36] For this application the chitin produced in this bioprocessing would be ideally suited as one of the prime considerations in this application is low cost. Also the impurities in this chitin would not adversely affect the removal of the ions to a large degree.

If purer chitin were required, for instance to make chitosan for medical applications, the fermented waste could be further treated with mild chemical treatment to remove the residual protein and minerals.

The functional properties of the chitosan obtained from the biological process (Table 3) were reasonably similar to those obtained through the chemical method. Thus it could be used in an application where the impurities (manly inorganic) would not pose any significant problems. Water treatment applications such as the one described by Chung *et al* are examples of such applications.[37]

Solutions prepared with Csbl and Csbs were clear and had a low amount of insoluble material. Therefore a lack of solubility should not be an issue for any application. The chitosan obtained from chitin in the large bioreactor had similar functional properties to the chitin used by Wang *et al* to make a cross-linked collagen chitosan matrix for artificial livers.[38] The chitosan used in their experiment was 80% deacetylated and had a molecular weight of 5.3×10^4. The chitosan produced from the chitin in the large bioreactor was 81.92% deacetylated and had a molecular weight of 4.84×10^4. It is likely that a further step, to reduce protein in particular might be required to meet the standards of this application.

The molecular weight of chitosan from the chemical process was the highest; this could be due to the action of putrefying enzymes, which could act on the chitin before the pH dropped low enough to prevent them being active.[33] Another factor that could interfere with the molecular weight determination is the residual

Table 3. Physicochemical characteristics of the chitosans obtained

	$[\eta]$ ($cm^3\, g^{-1}$)	*Mv*	DA (%)
Csbs	395.625	70.3×10^3	17.78
Csbl	269.016	48.4×10^3	19.08
Csc	464.137	99.2×10^3	17.81

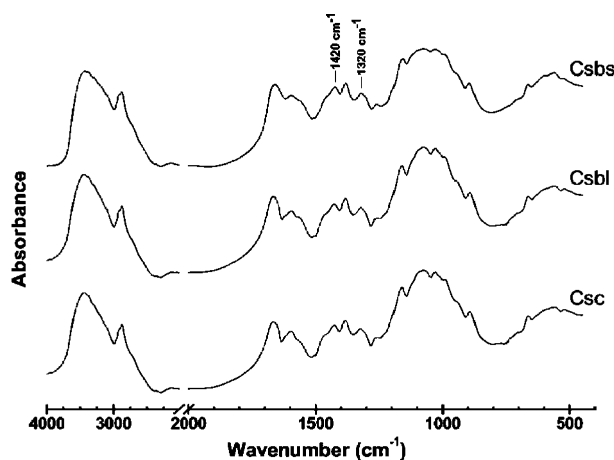

Figure 4. FTIR spectra of the obtained chitosans.

inorganic content. For instance, the Csbl and Csbs samples contained 372.1 and 658.4 $\mu g\,g^{-1}$ of calcium, respectively. Such a quantity of additional inorganic salts could cause underestimation of the intrinsic viscosity. The FTIR spectra of the obtained chitosans are shown in Fig 4; there are only minor differences. This would seem to indicate that the content of inorganic impurities present in chitosans from the biological process does not affect their spectra.

CONCLUSIONS

The lactic acid fermentation of prawn shell reduces its content of inorganic material and, to a lesser extent, the quantity of protein and lipids. This biological process produces a low quality chitin, compared with the usual chemical extraction method. Thus the applications where this biological chitin would be suitable are limited to those where its impurities do not interfere with its function. However, this environment-friendly method could be an effective pre-treatment, in order to reduce the use of corrosive reactants and the cost of waste management, to produce a high quality chitin. On the other hand it also could be seen as an effective preservation method of prawn shell. This is important as the chitin produced depends to a large extent on the quality of the raw material. Due to large seasonal fluctuations in the raw material supply, the effective preservation of the raw material quality is important as without it the shells will spoil quickly and become worthless. If the large-scale production of chitin is to be carried out viably then these seasonal fluctuations must be evened out in order to maximize the efficiency.

It was possible to obtain chitosan from chitin extracted by the proposed biological method using a common type of thermochemical method. This chitosan had similar functional physicochemical properties to those of chitosan from the chemically extracted chitin. The main difference was the residual inorganic matter content, mainly calcium. In order to obtain a higher quality chitosan a further purification step (dialysis or sequential filtration–precipitation[39]) should be included to remove the inorganic mineral content.

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