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review

Chitin Extraction from Crustacean Shells Using Biological Methods – A Review

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Summary

After cellulose, chitin is the most widespread biopolymer in nature. Chitin and its derivatives have great economic value because of their biological activities and their industrial and biomedical applications. It can be extracted from three sources, namely crustaceans, insects and microorganisms. However, the main commercial sources of chitin are shells of crustaceans such as shrimps, crabs, lobsters and krill that are supplied in large quantities by the shellfish processing industries. Extraction of chitin involves two steps, demineralisation and deproteinisation, which can be conducted by two methods, chemical or biological. The chemical method requires the use of acids and bases, while the biological method involves microorganisms. Although lactic acid bacteria are mainly applied, other microbial species including proteolytic bacteria have also been successfully implemented, as well as mixed cultures involving lactic acid-producing bacteria and proteolytic microorganisms. The produced lactic acid allows shell demineralisation, since lactic acid reacts with calcium carbonate, the main mineral component, to form calcium lactate.

Key words: chitin, crustacean shells, chitin extraction, biological methods

Introduction

Enormous amounts of chitin can be found in the biosphere; it is the major component of cuticles of insects, fungal cell walls, yeast or green algae (*1–3*). Fungi provide the largest amount of chitin in the soil (6–12 % of chitin biomass, which is in the range of 500–5000 kg/ha) (*4*). Chitin is also widely present in crab and shrimp shells (*5*).

A working estimate for the annual turnover is in the range of 10^{10}–10^{11} tonnes (*6,7*), making chitin one of the most abundant biopolymers. Chitin can be readily obtained by simple extraction (*8*). To date, the major source of industrial chitin comes from wastes of marine food production, mainly crustacean shells, *e.g.* shrimp, crab or krill shells (*9–11*).

In the processing of shrimps for human consumption, between 40 and 50 % of the total mass is waste. About 40 % of the waste is chitin, incrusted with calcium carbonate and astaxanthin, and containing meat and a small amount of lipid residues. A small part of the waste is dried and used as chicken feed (*11*), while the rest is dumped into the sea, which is one of the main pollutants in coastal areas (*12,13*). The utilization of shellfish waste has been proposed not only to solve environmental problems, but as a waste treatment alternative to

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the disposal of shellfish wastes (*14*). Crustacean shell waste consists mainly of 30–40 % protein, 30–50 % calcium carbonate, and 20–30 % chitin (Table 1) (*15–18*), with species and seasonal variations (*19*).

Seafood processing and consumption generate each year hundreds of tonnes of shellfish waste, like in Taiwan (*9*) or Indonesia (*20*), whereas in Germany only 22 616 tonnes of shrimp waste is discarded on the seashore (*11*). By-products from marine food production, mainly shrimp shells, comprise almost 40 % of total prawn mass and have become a major environmental concern due to their slow degradation (*8*). The major components (on dry mass basis) of shrimp waste are proteins, chitin, minerals and carotenoids (*20,21*).

To extract chitin from shrimp shells using traditional chemical treatment, 4 % NaOH is used for deproteinisation and 4 % HCl for demineralization. This process presents some drawbacks since it is expensive and environmentally unfriendly (*22*), and hence finding alternative processes would be really helpful. Biotechnological production of chitin has not been commercially available up to now, but it can offer new perspectives for the production of highly viscous chitosan, with a promising potential for applications in biomedicine and pharmacy (*23,24*). Fermentation of this biowaste using lactic acid bacteria for the production of chitin has been studied and reported (*25*). The use of organic acids such as lactic acid for the demineralisation process is a promising idea since organic acids can be produced by bacteria at low cost, are less harmful to the environment, can preserve the characteristics of the purified chitin and the resulting organic salts from the demineralisation process can be used as an environmentally friendly deicing/anti-icing agents and/or as preservatives (*22*).

Properties of Chitin

Chitin is one of the most abundant biopolymers in nature (*1*) and is a major component in the supporting tissues of organisms such as crustaceans, fungi and insects (*26*). It is a linear polysaccharide composed of α-(1–4)-linked 2-acetamido-2-deoxy-D-glucose units which may be de-*N*-acetylated to some extent (*27*). It is a structural polysaccharide similar to cellulose. Chitin molecules are known to be ordered into helicoidal, microfibrillar structures that are embedded into the protein material of the shells (*28*). Chitin is closely associated with proteins, minerals, lipids and pigments (*29*).

Several studies have clearly demonstrated that specific characteristics, namely degree of deacetylation and molecular mass of chitin and its deacetylated derivative, chitosan, vary with process conditions. The physicochemical characteristics of chitin and chitosan influence their functional properties such as solubility, chemical reactivity and biological activities (*30*), namely biodegradability (*2,31*), which differ depending on the crustacean species and preparation methods (*32*). Recent studies have revealed notable variability in the dye, water, and fat binding capacities of various chitins, chitosans and their derivatives produced from crustacean shell wastes at laboratory scale (*19*), as well as notable variability in the antibacterial activities (*32*), biodegradability and immunological activities (*33,34*).

The degree of deacetylation, defined as the molar fraction of deacetylated units in the polymer chain (*35*), is one of the most important factors influencing the properties of chitin and chitosan (*18*), such as solubility, flexibility, polymer conformation and viscosity (*16,24,36*). The degree of acetylation can be employed to differentiate between chitin and chitosan; in the case of chitin it is greater than a given value (*e.g.* >50 %) and insoluble, while in the case of chitosan it is smaller than that value and soluble (*37*). A number of methods have been reported to determine the degree of deacetylation of chitin (*2*).

Molecular mass determines the viscosity and the rate of degradation, which can be determined by viscometer,

Table 1. Contents of chitin in different organisms (*15–18*)

Organism	*w*(chitin)/%	Organism	*w*(chitin)/%
Crustaceans:		*Bombyx* (silk worm)	44.2[c]
Cancer (crab)	72.1[c]	*Galleria* (wax worm)	33.7[c]
Carcinus (crab)	64.2[b]	**Mollusks:**	
Paralithodes (king crab)	35.0[b]	clam	6.1
Callinectes (blue crab)	14.0[a]	shell oysters	3.6
Crangon and *Pandalus* (shrimp)	17–40	squid pen	41.0
Alaska shrimp	28.0[d]	krill, deproteinized shells	40.2
Nephro (lobster)	69.8[c]	**Fungi:**	
Homarus (lobster)	60–75[c]	*Aspergillus niger*	42.0[e]
Lepas (goose barnacle)	58.3[c]	*Penicillium notatum*	18.5[e]
Insects:		*Penicillium chrysogenum*	20.1[e]
Periplaneta (cockroach)	2.0[d]	*Saccharomyces cerevisiae*	2.9[e]
Blatella (cockroach)	18.4[c]	*Mucor rouxii*	44.5
Coleoptera (ladybird)	27–35[c]	*Lactarius vellereus*	19.0
Diptera	54.8[c]		
Pieris (butterfly)	64.0[c]		

[a]compared to the body fresh mass, [b]with respect to the body dry mass, [c]based on the mass of the organic cuticle, [d]compared to the total mass of the cuticle, [e]relative to the dry mass of the cell wall

light scattering and gel permeation chromatography (*38*). The chitins differ not only in molecular mass and degree of deacetylation, but also by their crystalline structure, which controls a number of properties (*16*). Chitin occurs in three polymorphic forms, α-, β-, and γ-chitins, which differ in the arrangement of molecular chains within the crystal cell (*35,39*). α-Chitin with its antiparallel chain arrangement is the most abundant chitin in nature (shrimps, crabs), β-chitin has parallel chains and occurs in squid pens (*11,40,41*), while γ-chitin presents the mixture of α- and β-chitins (*39*).

Similarly to cellulose, chitin is insoluble in water, aqueous solvents and common organic solvents, owing to its strong intra- and inter-molecular hydrogen bonds (*18,42*). Solubility, which is related to different parameters, is very difficult to control (*16,41*).

Chitosan, as a polyelectrolyte, is able to form electrostatic complexes under acidic conditions (*41*). It is a cationic polysaccharide and its cationic nature in acidic medium is unique among polysaccharides (*43*).

Owing to its chemical structure, chitosan is a substitute for biological media. Indeed, the glycosidic bond and the *N*-acetylglucosamine residues found in the chitosan macromolecules are also present in the structure of the extracellular matrix of most living tissues (*16*).

Methods of Chitin Extraction

Chemical methods

In the skeletal tissue, protein and chitin combine to form a protein-chitin matrix, which is then extensively calcified to yield hard shells. The waste may also contain lipids from the muscle residues and carotenoids, mainly astaxanthin and its esters (*8*).

A traditional method for the commercial preparation of chitin from crustacean shell (exoskeleton) consists of two basic steps (Fig. 1): (*i*) protein separation, *i.e.* deproteinisation by alkali treatment, and (*ii*) calcium carbonate (and calcium phosphate) separation, *i.e.* demineralisation by acidic treatment under high temperature, followed by a bleaching step with chemical reagents to obtain a colourless product (*44–46*).

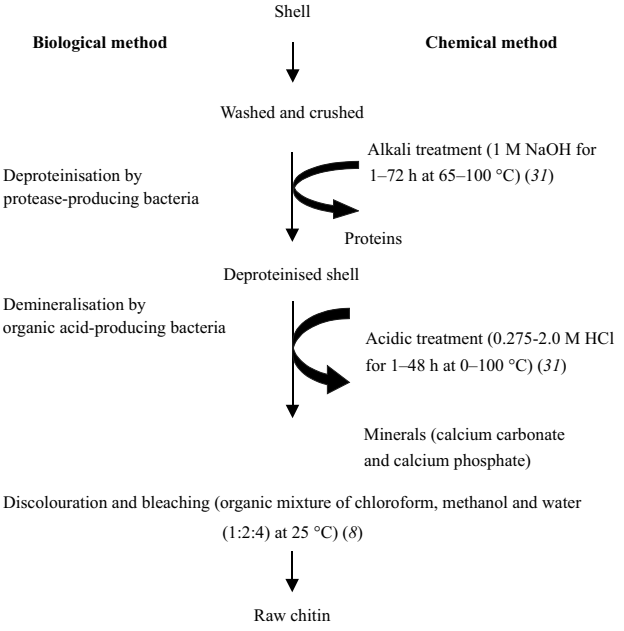

Fig. 1. Chitin recovery by chemical and biological methods (*8,31*)

Deproteinisation is usually performed by alkaline treatment (*29*). Demineralisation is generally performed by acid treatment including HCl, HNO_3, H_2SO_4, CH_3COOH, and HCOOH; however, HCl seems to be the preferred reagent (*29,44*). It was shown that the order of the two steps may be reversed for shrimp waste containing large protein concentrations, which stem primarily from the skeletal tissue and to a lesser extent from the remaining muscle tissue (*8*).

The major concern in chitin production is the quality of the final product, which is a function of the molecular mass (average and polydispersity) and the degree of acetylation. Harsh acid treatments may cause hydrolysis of the polymer, inconsistent physical properties in chitin and are source of pollution (*47*). High NaOH concentrations and high deproteinisation temperatures can cause undesirable deacetylation and depolymerisation of chitin (*8*). Percot *et al*. (*29*) reported that using inorganic acids such as HCl for the demineralisation of chitin results in detrimental effects on the molecular mass and the degree of acetylation that negatively affect the intrinsic properties of the purified chitin (Table 2). Similarly, according to Crini *et al.* (*16*) this method allows almost complete removal of organic salts, but at the same time reactions of deacetylation and depolymerisation may occur (Table 2). Quality improvement can be obtained by improving the contact of chemicals with the shrimp waste, for instance by using stirred bioreactors. This would allow reactions to proceed with the same efficiency at shorter exposure time and at lower temperature (*24*). Comparing different chitins (degree of acetylation, molecular mass and optical activity), variations of the characteristics of the obtained polymer were observed according to the acid used for the demineralisation (*16*).

In addition, chemical chitin purification is energy consuming and somewhat damaging to the environment owing to the high mineral acid and base amounts involved (*48*). These chemical treatments also create a disposal problem for the wastes, since neutralisation and detoxification of the discharged wastewater may be necessary (*49*). Another disadvantage of chemical chitin purification is that the valuable protein components can no longer be used as animal feed (*22,50*).

Biological methods

An alternative way to solve chemical extraction problems is to use biological methods. The use of proteases for deproteinisation of crustacean shells would avoid alkali treatment (Fig. 1). Besides the application of exoenzymes, proteolytic bacteria were used for deproteinisation of demineralised shells (*47*). This approach allows obtaining a liquid fraction rich in proteins, minerals and astaxanthin and a solid chitin fraction. The liquid fraction can be used either as a protein-mineral supplement for human consumption or as an animal feed (*25*).

Deproteinisation processes have been reported for chitin production mainly from shrimp waste using me-

Table 2. Comparison of chemical and biological methods for chitin recovery (*31,38*)

		Chemical method	Biological method
Chitin recovery			*in situ*
	Demineralisation	Mineral solubilisation by acidic treatment including HCl, HNO_3, H_2SO_4, CH_3COOH and HCOOH.	Carried out by lactic acid produced by bacteria through the conversion of an added carbon source.
	Deproteinisation	Protein solubilisation by alkaline treatment.	Carried out by proteases secreted into the fermentation medium. In addition, deproteinisation can be achieved by adding exo-proteases and/or proteolytic bacteria.
		Effluent treatment after acid and alkaline extraction of chitin may cause an increase in the cost of chitin.	Extraction cost of chitin by biological method can be optimised by reducing the cost of the carbon source.
			Solubilised proteins and minerals may be used as human and animal nutrients.
Chitin quality	The major concern in chitin production is the quality of the final product, which is a function of the molecular mass (average and polydispersity) and the degree of acetylation.	A wide range of quality properties of the final product.	Homogeneousness and high quality of the final product.
		Using inorganic acids such as HCl for chitin demineralisation results in detrimental effects on the molecular mass and the degree of acetylation that negatively affect the intrinsic properties of the purified chitin (*31*).	
		This method allows almost complete removal of organic salts, but at the same time the reactions of deacetylation and depolymerisation may occur (*38*).	
		The comparison of different chitins (degree of acetylation, molecular mass, optical activity) obtained with four different acids showed that the polymer characteristics varied according to the extraction method used (*38*).	

chanical (*47*), enzymatic (*51,52*) and microbial processes involving species like *Lactobacillus* (*25*), *Pseudomonas aeruginosa* K-187 (*53*) and *Bacillus subtilis* (*54*). Biological demineralisation has also been reported for chitin production from crustacean shells; enzymatically, using for instance alcalase (*52*), or by microbial process involving species like *L. pentosus* 4023 (*45*) or by a natural probiotic (milk curd) (*55*). In these biological processes, demineralisation and deproteinisation occur mainly simultaneously but incompletely (*47*). An overview of the various biological methods available in the literature is given in Table 3 (*11,25,45,46,48,49,53,56–70*).

Fermentation has been applied to fish for many years and represents a low-level (artisanal) and affordable (neither capital nor energy intensive) technology (*71*). It consists in the ensilation of crustacean shells and a low-cost *in situ* production of lactic acid from by-products such as whey, lignocellulose and starch. Lactic acid production by lactic acid bacteria induced a liquefaction of the semi-solid waste and led to a low pH and activation of proteases (*50*). The protein-rich liquid could be separated from the chitin, which remained in the sediment (*11*). This method might offer a commercial route for the recovery of chitin (*45*).

Lactic acid is formed from the breakdown of glucose, creating the low pH, which improves the ensilation that suppresses the growth of spoilage microorganisms. Lactic acid reacts with the calcium carbonate component in the chitin fraction, leading to the formation of calcium lactate, which precipitates and can be removed by washing. The resulting organic salts from the demineralisation process could be used as de- and anti-icing agents and/or preservatives (*56*). Deproteinisation of the biowaste and simultaneous liquefaction of the shrimp proteins occurs mainly by proteolytic enzymes produced by the added *Lactobacillus*, by gut bacteria present in the intestinal system of the shrimp, or by proteases present in the biowaste (*25*). It results in a fairly clean liquid fraction with a high content of soluble peptides and free amino acids (*71*).

Khanafari *et al*. (*22*) extracted chitin and chitosan from shrimp waste by chemical and microbial methods. Their results showed that the microbial method is more effective especially for the recovery of chitin, compared to chemical method.

Lactic acid fermentation of shrimp waste

Recently, biological processes for chitin production have been reported using bacteria that produce organic acids and enzymes for the demineralisation and deproteinisation of crustacean shells. Fermentation of shrimp (*Penaeus monodon*) waste with lactic acid bacteria for chitin recovery was studied with added carbohydrates such as lactose or cassava extract as a natural energy source (*56*) and date juice for extraction of chitin from head and shell of shrimp *Parapenaeus longirostris* using *Lactobacillus helveticus* (*57*). Raw heads of African river prawn (*Macrobrachium vollenhovenii*) were fermented with *Lactobacillus*

Table 3. Overview of the biological methods for chitin recovery

Waste source	Strains and/or proteolytic enzymes	Carbon source	Duration / day	Efficiency/%		Refs.
				deprotein-isation	demineral-isation	
Lactic acid fermentation						
Penaeus sp.	*Lactobacillus* spp. B2	sucrose whey	6	85	87.6	(*59*)
demineralised *Nephrops norvegicus*	Stabisil: *Streptococcus faecium* M74, *L. plantarum, Pediococcus acidilactici*	lactose	7	40	n.d.	(*60*)
Nephrops norvegicus	Sil-All$^{4\times4}$: *L. plantarum, L. salivarius, S. faecium, P. acidilactici*	glucose	7	n.d.	90.99	(*48*)
Nephrops norvegicus	*L. paracasei* A3	glucose	5	77.5	61	(*58*)
one-step shrimp fermentation	*L. plantarum* 541	glucose	–	75	86	(*25*)
pretreated *Procambarus clarkii* (crayfish)	*L. paracasei* A3	dextrose	3	94	97.2	(*61*)
Procambarus clarkii	immobilized *Lactobacillus pentosus* 4023	whey	2.1	81.5	90.1	(*45*)
Chionoecetes japonicus	*L. paracasei* ssp. *tolerans* KCTC-3074	glucose	1	54.7	55.2	(*56*)
Parapenaeus longirostris	*L. helveticus*	date juice	14	91	44	(*57*)
Non-lactic acid fermentation						
Metapenaeus dobson	*Bacillus subtilis*	jaggery	–	84	72	(*62*)
shrimp and crab shell	*Pseudomonas aeruginosa* K-187	–	5	82	–	(*49*)
shrimp and crab shell powder	proteases of *P. aeruginosa* K-187	–	7	72	–	(*53*)
natural shrimp shells	immobilized proteases of *P. aeruginosa*			78		
acid treated natural shrimp shell				45		
shrimp and shell crab powder				67		(*53*)
crab shell powder	*P. aeruginosa* F722	–	7	63	92	(*63*)
Chionoecetes opilio (natural crab shell waste)	*Serratia marcescens* FS-3		7	47	84	(*64*)
	Delvolase®			90		
	Combination of Delvolase® and *Serratia marcescens* FS-3			85		
	S. marcescens FS-3 supernatant culture			81		
shrimp shell waste	*Bacillus cereus*		–	97.1	95	(*65*)
	Exiguobacterium acetylicum			92.8	92	
squid pen	*Bacillus* sp. TKU 004			73	n.d.	(*66*)
Penaeus monodon	*Pediococcus acidilactici* CFR2182			97.9±0.3	72.5±1.5	(*70*)
shrimp shells	*Pediococcus* sp. L1/2	sucrose	1.5	n.d.	83	(*46*)
Cofermentation						
two-step fermentation of *Penaeus monodon* and *Crangon crangon*	first step: anaerobic deproteinisation by autochthonous flora of Indonesian shrimp shells and/or proteolytic bacteria			97.4	99.6	(*11*)
	second step: *L. casei* MRS1			90.8	99.7	
prawn waste	*Lactobacillus lactis*			66.5	78.8	(*67*)
	Teredinibacter turnerae			77.8	23.3	
	co-fermentation of both species			95	95	
red crab shell waste	one-step fermentation: *L. paracasei* ssp. *tolerans* KCTC-3074 and *S. marcescens* FS-3		7	52.6	97.2	(*68*)
				94.3	68.9	
	successive two-step fermentation					
	two *Bacillus licheniformis* strains with treatment of the final fermentation product with 0.9 % lactic acid		2	99	98.8	(*69*)

n.d.=not determined

plantarum using cane molasses (*71*). Lactic acid bacteria fermentation for demineralisation has also been reported for shrimp waste (*50*), crayfish exoskeleton (*45*), scampi waste (*58*) and prawn waste.

Lactic acid fermentation combined with chemical treatments has been studied as an alternative to chemical extraction of chitin, reducing the amount of alkali and acid required (*59*). It was considered as a pretreatment of shrimp waste followed by demineralisation and deproteinisation using low concentrations of HCl (0.5 M) and NaOH (0.4 M).

The production of chitin from prawn (*Nephrops norvegicus*) shell waste by fermentation was investigated by Healy *et al*. (*60*). In that study, previously demineralised shells were incubated for deproteinisation with commercial bacterial inocula (Stabisil and Nutrimink, both from Nutrimix Ltd, Lankashire, UK; and Sil-All$^{4\times4}$, Sil-All, Alltech, Stamford, Lincolnshire, UK) together with lactose (Nutrimink) under both aerobic and anaerobic conditions for 7 days (Table 3). Stabisil is used mainly for the ensiling of fish offal and consists of active bacterial cultures (*Streptococcus faecium* M74, *Lactobacillus plantarum*, and *Pediococcus acidilactici*). In general, protein depletion of approx. 40 % was achieved irrespective of the considered conditions (aerobic or anaerobic).

The anaerobic fermentation of prawn (*Nephrops norvegicus*) shell waste using lactic acid bacteria, in combination with the inoculant Sil-All$^{4\times4}$ (Table 3), a powdered grass silage inoculant containing a mixture of selected proteolytic enzyme-producing bacteria (*L. plantarum, L. salivarius, S. faecium* and *P. acidilactici*), has been established as an effective method to break down shell waste and isolate the valuable components contained within the shell structure. The fermentation process employed in this study for the bioprocessing of prawn shell waste has achieved several desirable effects. The prawn shells were demineralised to a considerable degree. On average, the shells had 90.99 % of their original calcium removed. Elemental analysis showed that N-values were within a close range of the theoretically ideal chitin nitrogen content proposed in the literature (*48*).

Biological treatment of minced scampi (*Nephrops norvegicus*) waste supplemented with glucose in a bioreactor using the lactic acid bacterium *Lactobacillus paracasei* strain A3 was performed by Zakaria *et al*. (*58*). After 5 days of batch culture at 30 °C, high-protein liquid was produced as a result of proteolysis, with solubilisation of 77.5 and 61.0 % of protein and calcium respectively, initially present in the waste material, while the solid fraction contained 17.5 % chitin (on dry mass basis) (*58*) (Table 3).

The fermentation of shrimp waste by *Lactobcillus plantarum* 541 with and without pH control was examined by Rao *et al*. (*25*). Among four acids tested (glacial acetic, citric, hydrochloric and lactic acids) to control pH at the start and during fermentation, acetic and citric acids proved to be the most effective. In the presence of an additional carbon source, glucose, 75 % deproteinisation and 86 % demineralisation of biowaste were achieved at pH controlled at 6.0 with acetic acid, while without pH control, 68.1 and 64.1 % deproteinisation and demineralisation respectively, were achieved.

Crayfish (*Procambarus clarkii*) by-products were also used to recover chitin (*61*). The material was fractionated by sedimentation and flotation into two fractions: a proteinaceous and a chitinous fraction. Deproteinisation and demineralisation yields of the latter fraction by semi-solid state fermentation with *L. paracasei* and supplemented with dextrose were 94.0 and 97.2 %, respectively.

Demineralisation of crayfish (*Procambarus clarkii*) chitinous fraction (exoskeleton) has also been investigated by fed-batch fermentation using immobilized cells of *Lactobacillus pentosus* 4023 (*49*). Jung *et al*. (*56*) investigated the demineralisation of red crab (*Chionoecetes japonicus*) shell wastes by a lactic acid bacterium (*Lactobacillucs paracasei* ssp. *tolerans* KCTC-3074) fermentation and compared these results with the chemical treatment efficiency using lactic acid. Relative residual ash content rapidly decreased to 49.1 and 16.4 % after 12 h in 5 and 10 % lactic acid, respectively. With 2.5, 5 and 10 % inocula of lactic acid bacteria, the residual ash content rapidly decreased on the first day to 55.2, 40.9 and 44.7 %, respectively. The protein content after demineralisation ranged from 51.3 to 54.7 % after chemical treatment and decreased to 32.3 % after lactic acid fermentation. These results suggested that lactic acid fermentation can be an alternative for crab shell demineralisation, even though demineralisation rate and efficiency were lower than those recorded during chemical treatment (*56*).

These authors also showed that microbial growth was mainly affected by the concentration of an additional carbon source like glucose instead of increasing the inoculum level. About 80 % of the relative residual ash content was removed over 5 days under the optimal conditions (10 % starter inoculum, 10 % glucose supply, along with 10 % red crab shells at 30 °C). These results confirmed that lactic acid fermentation could provide an alternative to chemical treatment, although a second run or milder acid treatment (such as 0.5 M HCl) may be needed (*72*).

Use of non-lactic acid bacteria for chitin recovery

Non-lactic acid bacteria have also been tested for chitin recovery. Fermentation of shrimp (*Metapenaeopsis dobsoni*) shell in jaggery broth using *Bacillus subtilis* for the production of chitin and chitosan showed that the level of acid produced as well as the proteolytic activity of *B. subtilis* allowed shell demineralisation and deproteinisation (*62*). About 84 % of the protein and 72 % of minerals were removed from the shrimp shell after fermentation (Table 3).

Pseudomonas aeruginosa K-187 strain isolated from the soil of northern Taiwan is a producer of protease and chitinase/lysozymes when cultured in a medium containing shrimp and crab shell wastes as the sole carbon sources (*49*). It was shown that *P. aeruginosa* K-187 is capable of shell waste deproteinisation in either solid-state, liquid-solid or liquid fermentation. Higher deproteinisation yield was recorded in solid-state fermentation, 82 % after 5 days, showing that *P. aeruginosa* K-187 is more efficient than the proteolytic bacterium *P. maltophilia*, known to be highly efficient in the deproteinisation of prawn shell waste. The use of protease produced by *P. aeruginosa* K-187 was therefore promising in deproteinisation of crustacean wastes (*49*).

Proteolytic enzymes for deproteinisation of crustacean wastes have also been investigated. Oh *et al*. (*53*) demonstrated that the proteases of *P. aeruginosa* K-187 led under optimal culture conditions to 72 % protein removal for shrimp and crab shell powder after a 7-day incubation, while that of natural shrimp shell and acid-treated shrimp and crab shell powder was 78 and 45 %, respectively. In the case of enzyme immobilization, 67 % deproteinisation of shrimp and crab shell powder was achieved (Table 3). After 7 days of incubation at the optimal temperature of 30 °C, 92 % demineralisation and 63 % deproteinisation of crab shell waste samples inoculated with the newly isolated *P. aeruginosa* F722 were achieved (*63*).

Deproteinisation and demineralisation of crab (*Chionoecetes opilio*) shell wastes was carried out by Jo *et al*. (*64*) using *Serratia marcescens* FS-3 isolated from environmental samples (seaside soil in the southwestern area of Korea) which exhibited strong protease activity. The demineralisation and deproteinisation of natural crab shell wastes with 10 % *Serratia marcescens* FS-3 as inoculum was 84 and 47 % after 7 days of fermentation. When the shell waste was treated with 1% Delvolase® (Gist-Brocades, DSM, Heerlen, The Netherlands) as a reference, deproteinisation rate was 90 %. With a combination of 10 % *Serratia marcescens* FS-3 culture supernatant and 1 % Delvolase®, deproteinisation rate of the shell waste was 85 %, while the rate was 81 % in 10 % *Serratia marcescens* FS-3 culture supernatant only (Table 3).

Two bacterial cultures were isolated and tested for the degradation of shrimp shell waste, *Bacillus cereus* and *Exiguobacterium acetylicum* (*65*). Fermentation of 3 % shell waste at 37 °C with *B. cereus* and *E. acetylicum* allowed to obtain 97.1 and 92.8 % deproteinisation and 95 and 92 % demineralisation, respectively. The protein content was reduced from 18.7 to 5.3 % with *B. cereus* and to 7.3 % with *E. acetylicum*. The high activity of the isolated strains in the decomposition of shrimp shell waste suggests broad potential for environmentally friendly application of these bacteria for chitin extraction from chitin-rich wastes (*65*).

In the preparation of β-chitin, deproteinisation of squid pen was carried out by means of a protease-producing bacterium isolated and identified as *Bacillus* sp. TKU004 (*68*). Under optimal conditions for protease production by *Bacillus* sp. TKU004, a deproteinisation yield of 73 % was achieved (Table 3).

Mahmoud *et al*. (*73*) proposed the use of organic acids (lactic and acetic) produced by cheese whey fermentation to demineralise northern pink shrimp (*Pandalus borealis*) shell wastes biologically deproteinised by fungus *Aspergillus niger* ATCC 16513. This study demonstrated that the effectiveness of lactic and/or acetic acids for shrimp shell demineralisation is similar to that of hydrochloric acid.

Cofermentation of various bacteria for chitin recovery

Chitin purification of *Penaeus monodon* and *Crangon crangon* shells was also tested in a two-step fermentation process involving anaerobic deproteinisation by means of the autochthonous flora of Indonesian shrimp shells and/or proteolytic bacteria from various sources, followed by homofermentative lactic acid fermentation using *L. casei* MRS1 in the second step (*11*). After deproteinisation and decalcification of *Penaeus monodon* and *Crangon crangon* shells, the protein content was 5.8 and 6.7 % and the calcium content was 0.3 and 0.4 %, respectively.

Different strategies were applied to extract chitin from prawn waste using a lactic acid-producing bacterium, *L. lactis*, and a protease-producing marine bacterium, *Teredinibacter turnerae* (*67*). Both bacteria were cultivated individually and cofermented. *L. lactis* removed the inorganic materials efficiently (66.5 % of deproteinisation, 78.8 % of demineralisation, and 52.2 % process yield), while *T. turnerae* was more efficient in deproteinisation (77.8 % of deproteinisation, 23.3 % of demineralisation and 49.2 % process yield) (Table 3). However, the highest process yield (95.5 %) was obtained during cofermentation of both bacteria. Although biological chitin extraction was incomplete compared to the chemical method, the biological treatment employed could still be considered as an alternative, more environmentally benign method (*67*).

A cofermentation of *L. paracasei* ssp. *tolerans* KCTC-3074 with *Serratia marcescens* FS-3, a protease-producing bacterium, was also tested in order to extract chitin from red crab shell wastes in one step (*68*). The cofermentation resulted in the highest level of demineralisation (97.2 %), but the level of deproteinisation was only 52.6 % after 7 days (Table 3), showing that cofermentation is applicable in one-step extraction of crude chitin from red crab shell waste, but the improvement of deproteinisation is needed (*68*). These authors also tested the biological extraction of chitin from red crab shell wastes in successive two-step fermentation involving *L. paracasei* ssp. *tolerans* KCTC-3074 and *Serratia marcescens* FS-3. The successive fermentation in the combination of KCTC-3074 and FS-3 strains gave the best result in the coremoval of $CaCO_3$ and proteins from crab shells. In this combination, final demineralisation and deproteinisation yields were 94.3 and 68.9 %, respectively (*47*).

Proteolytic but chitinase-deficient microbial cultures were isolated from shrimp shell waste and characterized. The most efficient isolate was found to be a mixed culture consisting of two *Bacillus licheniformis* strains. Fermentations were carried out at 42, 50 or 55 °C, 500 rpm and 2 volumes of air per volume of liquid per minute. After 48 h, the fermented shrimp shells were harvested and washed. The final fermentation product was demineralised with 0.9 % lactic acid for 3 h and then oven dried. The results showed more than 99 % deproteinisation and 98.8 % demineralisation (*69*) (Table 3).

Key factors for shrimp waste demineralisation

Many factors, such as inoculum size, initial pH value, carbon concentration and carbon to nitrogen ratio have been reported to influence the fermentation process and consequently the demineralisation efficiency (*46,50*).

Fermentation of shrimp (*Penaeus monodon*) biowaste was conducted using different lactic acid bacteria to select the most efficient starter culture based on pH reduction and acid production. *Pediococcus acidilactici* CFR2182 was found to be the most efficient among the five starter cultures tested. Fermentation conditions, inoculum, sugar level and incubation time were optimized using response surface methodology and led to an optimal pH of 4.3±0.1. The optimized conditions for fermentation of shrimp biowaste with *P. acidilactici* were found to be 5 % (by vol-

ume per mass) inoculum, 15 % (by mass) glucose and 72 h incubation time at (37±1) °C (final pH=4.3), leading to (97.9±0.3) % deproteinisation and (72.5±1.5) % demineralisation (*70*).

The type of the carbohydrate was also examined (*59*). Shrimp (*Penaeus* spp.) waste was fermented with *Lactobacillus* B2 at different amounts (0, 5 and 10 % by volume per mass), as well as different sources (lactose, sucrose and milk whey powder) and levels (10 or 20 % by mass) of carbohydrates. Sucrose was selected as the carbohydrate source in further experimental work due to its better acid production potential as compared to lactose and milk whey powder; it resulted in 56.8 and 63.5 % demineralisation and deproteinisation, respectively.

Various sucrose supplementations were also tested for shrimp shell demineralisation (*46*), showing that the addition of 5 % of pretreated shrimp shells with a chlorine solution into the culture broth of *Pediococcus* sp. L1/2 containing 50 g/L of sucrose can achieve 83 % demineralisation within 36 h. Further studies indicated that different surface areas of shrimp shell did not affect ash reduction (*46*).

The effect of the initial glucose concentration and the inoculation level of *L. casei* strain A3 and *Lactobacillus* sp. strain B2 in shrimp (*Penaeus* spp.) fermentation were examined by Shirai *et al*. (*50*). They showed that high initial glucose (10 %) and starter (5 %) mass fractions reduced the time and increased the amount of lactic acid produced (*50*).

However, on a commercial scale, glucose addition is an expensive alternative. The use of cheaper sources of carbon may be useful. With this aim, cassava flour in combination with amylolytic lactic acid bacteria was tested (*74*). Fermentation of shrimp biowaste was conducted with two *L. plantarum* strains under various salt concentrations. The non-amylolytic strain *L. plantarum* 541 and the amylolytic strain *L. plantarum* A6 showed reasonable growth in biowaste in the presence of 6 % salt (*74*). Higher deproteinisation and demineralisation efficiencies were recorded with strain 541, and were at the utmost 81.4 and 59.8 % under 2 % salt conditions, whereas strain A6 led to 65.5 and 52.2 % demineralisation and deproteinisation, respectively (*74*).

As another cheaper alternative, date juice was also tested as fermentation medium (*57*) and compared to glucose. The use of date juice with 208 g/L of total sugar led at best to 44 % demineralisation at 35 °C, but with 80 g/L of total sugar deproteinisation was improved at 30 °C until an almost total removal (91 %) (*57*).

The effect of temperature on chitin recovery from a mixture of cephalothoraxes of shrimp species *Litopenaeus vannamei, Litopenaeus stylirostris* and *Litopenaeus setiferus* using *L. plantarum* was examined (*75*). The fermentation was conducted in bed-column reactors at temperatures ranging from 15 to 45 °C. Response surface methodology showed the highest demineralisation and deproteinisation yields in the range of 27–36 and 30–40 °C. These results corroborate those of Adour *et al*. (*57*), who found an optimal demineralisation at 35 °C.

The effect of crab shell size on biodemineralisation by means of *L. paracasei* ssp. *tolerans* KCTC-3074 was also investigated (*76*). Demineralisation was performed using samples with four different particle sizes (0.84–3.35, 3.35–10, 10–20 and 20–35 mm) with 10 % inoculum, 5 % shell and 10 % glucose at 30 °C and 180 rpm for 7 days. Shell size had a minor effect on demineralisation efficiency (*76*).

Applications of Chitin and Chitosan

Natural and non-toxic biopolymers chitin and chitosan are now widely produced commercially from crab and shrimp shell waste. During the past few decades, chitin and chitosan have attracted significant interest in view of a wide range of proposed novel applications (*19*). Their unique properties, biodegradability, biocompatibility and non-toxicity make them useful for a wide range of applications (Table 4; *1,11,18,29,40,41,44,69,73,75,77–131*).

Chitin is mainly used as the raw material to produce chitin-derived products, such as chitosans, oligosaccharides, and glucosamine (*1*). There are now over 2000 concrete applications, and the field of nutrition is the largest user of chitosan with 1000 tonnes consumed in 2000. The worldwide industrial production of these derivatives in year 2000 is estimated to be above 10 000 tonnes (*18*).

Wastewater treatment with chitin and chitosan

Chitin and chitosan can be used for the adsorption or fixation of heavy metals (*77*) and dyes (Table 4). Chitosan is a polycation polymer effective in coagulation, flocculation and dehydration of activated sludge, and hence used in wastewater treatment (*16,18*). Another recent application is immobilization of microorganisms or sludge in chitosan matrices for wastewater treatment in extreme environmental conditions (extreme pH, presence of organic solvents), allowing the reuse of cells and hence their implementation in continuous process.

Applications of chitin and chitosan in food

Only limited attention has been paid to food application of these versatile biopolymers (*96*). They offer a wide range of unique applications, which are non-exhaustively listed in Table 4. The use of chitosan in the food industry is related to its functional properties, and nutritional and physiological activities. Chitosan exhibits water-, fat- and dye-binding capacity, as well as emulsifying properties (*132*); it was shown to be useful in the preparation of stable emulsions without any other surfactant (*133*). It has been used as a dietary supplement due to some interesting properties (Table 4).

Biomedical application of chitin and chitosan

Chitin and chitosan show excellent biological properties such as non-toxicity (*134,135*), which is illustrated by a dose limit per day of 17 g/kg (*16*), biodegradation in the human body (*136–138*), biocompatibility (*139,140*), and immunological, antibacterial, wound-healing (*102, 141,142*) and haemostatic activity (*143,144*), in cell culture, tissue engineering (*103,145–147*) and drug delivery (*148,149*), since it is highly biocompatible and biodegradable in physiological environment (*150*). Chitin is also used as an excipient and drug carrier in film, gel or powder form for applications involving mucoadhesivity.

Table 4. Applications of chitin, chitosan and their derivatives

Application area	Specific use	Refs.
Wastewater treatment	removal/recovery of metal ions from wastewaters, copper, chromium, cadmium, lead, nickel, mercury, iron, silver, zinc, cobalt and arsenic	(*78*,*80*–*89*)
	removal and binding of dyes	(*90*–*93*)
	removal and binding of heavy metals	(*1*,*11*,*44*, *77*,*78*)
	sludge treatment and dehydration agent	(*18*)
	biological denitrification	(*94*,*95*)
Food	food and nutrition	(*73*)
	bioconversion for the production of value-added food products	(*96*)
	preservation of food	(*94*)
	filmogenic properties – food wrapping	(*96*,*97*)
	filtration and clarification of fruit juices	(*97*)
	hypolipidemic and hypocholesterolimic agent (slimming agent)	(*98*,*99*)
	antioxidant	(*100*)
	phenolic compound adsorption	(*101*)
	chitosan hydrogels for cell immobilization (lactic acid production) and for pigment encapsulation (astaxanthin) used in aquaculture to give typical salmon colour	(*18*)
	iron extract (to help in preventing bad odours in cooked meat)	(*18*)
Biomedicine	burn and wound dressings for humans and animals	(*29*,*69*)
	antitumour activity	(*102*)
	drug delivery, gene delivery	(*103*–*105*)
	artificial skin, pharmacy	(*106*)
	immunostimulating properties in mammals and plants	(*107*–*109*)
	antiviral and anti-*Candida albicans* activities	
	enhancing specific immunity (adjuvant properties) and stimulation of cytokine production	
	ocular drug delivery vehicles in ophthalmology	(*110*)
	as nerve conduit for nerve regeneration due to its ability to facilitate nerve cell attachment	(*111*)
	therapeutic agents in the treatment of tumours (chitin and chitosan conjugates of 5-fluorouracil)	(*112*,*113*)
	encapsulation applications due to chitosan ability to form gels in the presence of certain divalent cations such as calcium, barium and strontium	(*114*)
	nutraceutical value as a potent antioxidant and matrix metalloproteinase inhibitor *via* alleviations of radical-induced oxidative damage (water-soluble carboxymethyl derivatives of chitin and chitosan)	(*115*)
	self-hardening paste for guided tissue regeneration in the treatment of periodontal bone defects (hydroxyapatite-chitin-chitosan composite bone-filling material)	(*40*)
	spermicide	(*112*)
Agriculture	plant elicitor	(*116*)
	stimulation of chitinase and glucanase production (increased response to pathogen attack)	(*116*)
	stimulation of chitinase activity in compost (change of bacterial and fungal genetic diversity)	(*117*)
	antimicrobial (antifungal) agent and biopesticide	(*118*,*119*)
	enhancing plant vitality and ability to degrade walls of fungi upon entry	(*120*,*121*)
	fertilizer and biocontrol agent	(*118*,*122*)
	enhancing biocontrol efficiency by addition to plant growth-promoting rhizobacteria	(*123*–*125*)
Textile and paper	textile fibres	(*75*)
	paper manufacture (additive)	(*112*)
Biotechnology	chitin affinity chromatography to selectively adsorb chitinase from a fermentation broth	(*112*,*126*)
	affinity matrix (chitosan) for the separation of wheat germ agglutinin	(*112*)
	enzyme and whole cell immobilizer	(*41*,*90*,*127*)
	N-acetyl chitobiose production from chitin using commercial hydrolytic enzymes	(*125*)
	chitinase and chitosanase production from *L. paracasei*, *Pseudomonas* and *Streptomyces* species	(*128*–*131*)
	microorganism immobilization for bioremediation of seawater polluted with crude oil	(*79*)
	support for biosensors	(*41*)
	bioseparation	(*90*,*91*)
Cosmetics	ingredients for hair and skin care (moisturizer)	(*112*)

Other applications of chitin and chitosan derivatives

Chitin and chitosan derivatives may effectively reduce soil-borne diseases. In addition, chitin exhibits several functions, including retention of nutrients in the soil, and contributes to the nitrogen cycle (*4*). Chitin and chitosan have a versatile application potential in agriculture (Table 4). In addition, they have found various other applications (*151*). Chitin can also be transformed into saccharides under certain conditions (*152*). It can also be used as a slowly degrading substrate in microbial fuel cells (*153*).

Conclusions

The importance of biopolymers chitin and chitosan resides in their biological (biodegradability, biocompatibility and non-toxicity) and physicochemical properties (degree of acetylation and molecular mass). These unique properties offer many potential applications in different fields. Recently, they have been widely applied in agriculture, medicine, pharmaceutics, food processing, environmental protection and biotechnology.

The recovery of chitin by chemical method using concentrated acids and bases in order to deproteinise and to demineralise shellfish waste (the most industrially exploited) at high temperature can deteriorate the physicochemical properties of this biopolymer and consequently its biological properties, which results in products of varying quality that are neither homogeneous nor reproducible. Biotechnology offers the opportunity to preserve the exceptional qualities of chitin and its derivatives. Nowadays, a new method based on the use of lactic acid bacteria and/or proteolytic bacteria has been used for chitin extraction. This method allows to produce a good quality chitin; it also leads to a liquid fraction rich in proteins which can be used for human and animal feed, and also produces pigments, mainly asthaxanthin.

Although the biological method seems to be a promising approach for demineralisation and deproteinisation, the use of this method is still limited to laboratory scale because demineralisation and deproteinisation have not yet reached the desired yields if compared to the chemical method. The physicochemical conditions that influence the fermentation are the keyfactors of this bioprocess. Determination of the optimal conditions for biodeproteinisation and biodemineralisation of shells, the use of an effective bacterium and an inexpensive carbon source are the main factors which have to be considered to optimize chitin recovery from shellfish waste by fermentation.

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