Chitosan: A review of sources and preparation methods

Mr Shijie Kou (Gabriel). School of Science, University of Waikato. ORCID: https://orcid.org/0000-0002-2934-0186 (First author)

Dr Linda Peters. School of Science, University of Waikato. (Co-author)

Associate Professor Michael Mucalo. School of Science, University of Waikato. (Corresponding author)
Email: michael.mucalo@waikato.ac.nz
Phone: +64 7 838 4404
Fax numbers:
Postal address: School of Science, University of Waikato, Gate 8 Hillcrest Road, Hamilton 3240, New Zealand.

Abstract

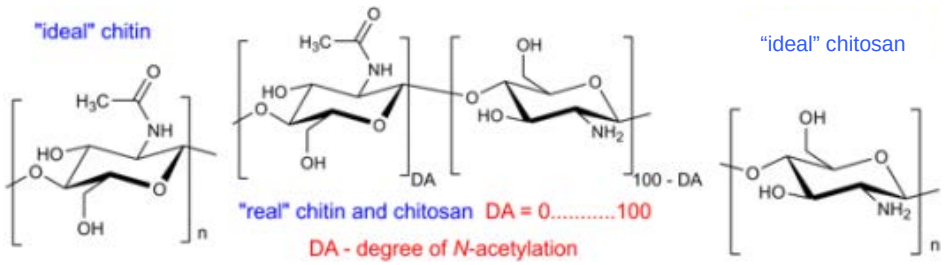
Chitosan, derived from chitin, has many desirable biomedical attributes.

This review aims to explore different sources of chitin and methods of chitosan production with industrial consideration. This article first discussed different sources of chitin for industrial scale production, with considerations given to both their environmental impacts and commercialization potential. Secondly, this article reviews the two categories of chitosan preparation – chemical methods and biological methods - based on existing publications which used lobster by-products as a feedstock source. The mechanisms of the chemical methods are firstly summarized, and then the different chemical agents and reaction parameters used are discussed. Next, both enzymatic and fermentation-based approaches are reviewed under biological methods and compared with chemical methodologies, with lactic fermentation methods as the major focus. This article concludes that lobster cephalothorax could be an ideal source for chitosan preparation on an industrial scale; and chemical methods involve simpler processing overall, while produce chitosan with stronger bioactivities because of lower molecular weight (MW) and higher degree of deacetylation (DD) achieved by the products. Moreover, due to biological methods inevitably necessitating further chemical processing, an approach involving some unconventional chemical methods has been regarded as a more suitable strategy for industrial scale chitosan production.

Keywords: chitosan, lobster, deacetylation.

1. Chitosan: structure and derivatives

As the precursor of chitosan, chitin is the most widely occurring biopolymer in nature after cellulose: it can be found in a range of eukaryotic species such as crustacea, insects and fungi [1, 2]. Chitin is a polymer of *N*-acetyl-D-glucosamine, and when it is subject to deacetylation and the repeating units in the polymer are predominantly without the acetyl functional group, i.e. as β-1,4-D-glucosamine, the polymer is known as chitosan [1, 3]. The mole fraction of the *N*-acetylated repeating units is defined as the degree of acetylation (DA), while the percentage of the repeating units of β-1,4-D-glucosamine in the polysaccharides is defined as the degree of deacetylation (DD) [3, 4]. Hence DA = 100% - DD as illustrated in Figure 1. Although current publications have no consensus regarding the cut-off of DD values between chitin and chitosan, it is usually between 40% and 75%, and most commercial chitosan have DD values between 70% - 90% [1, 4, 5].



100% - DD = DA

Figure 1. The relationship between DA and DD [6]. The repeating unit on the left is *N*-acetyl-D-glucosamine, while the one on the right is β-1,4-D-glucosamine. This figure is from an open access journal without copyright restriction for reuse, modification or republication.

DD is a critical parameter of chitosan, as prior research has reported that chitosan with a higher DD demonstrates stronger biological effects as well as increased water solubility [3]. This is because a higher DD indicates a higher concentration of amino groups in the molecule, and the protonation of the -NH_2 functional group is vital for manifesting chitosan's biological effects and water solubility [7].

Besides DD, molecular weight (MW) is another essential parameter which influences the bioactivity of chitosan. Like DA, lower MW chitosan usually shows more significant bioactivities than higher MW chitosan [3, 8, 9]. Previous studies have described different MW cut-off values to distinguish between high, medium, and low MW chitosan, and chitosan oligosaccharide [10, 11]. Nonetheless, irrespective of what the actual MW cut-off values are, the bioactivity of chitosan is usually found to be stronger when MW is lower (e.g. < 20 kDa) than higher (e.g. > 120 kDa) [12].

The correlation between chitosan MW and its solubility in water shows a comparable trend as per the relationship between MW and biological activities: the lower the MW, the higher the solubility the carbohydrate molecule will have [3, 13]. Chitosan with MW under 30 kDa is water-soluble in the free amine form without assistance needed from acidification [14, 15]. However, it should be noted that even within the under 30 kDa range, chitosan with MW greater than 22 kDa only shows limited solubility, while chitosan with MW under 9 kDa demonstrates significantly better solubility in water [16]. When the MW of chitosan is above 30 kDa, protonation of the amino group by acid is actually required to dissolve chitosan in water. Acetic acid is the most commonly used acid for this purpose, though many other acids that occur naturally in the human body e.g. HCl, lactic acid, citric acid, and pyruvic acid can also solubilize chitosan in water, with the exception of phosphoric acid [13].

2. The common sources for chitosan manufacturing

Shrimp and crabs are the most common sources cited in the literature as the raw material for chitosan preparation, while other species such as lobster, crayfish and

oyster have also been utilized [17-20]. Different organisms show different content (wt %) of chitin: crustacean shell waste on average consists of 30% - 50% by weight of calcium carbonate and 20% - 30% by weight of chitin, while in some genera of lobsters such as *Nephrops* sp. and *Homarus* sp., the shell consists of 60% - 75% by weight of chitin content, which is the highest among all chitin containing species [19, 21, 22].

Existing studies regarding the preparation of chitin or chitosan from crustacean by-products which contain 20% (wt %) or more of chitin have shown promising results as industrial feedstocks for chitosan production. For instance, *Procambarus clarkii* (crayfish) by-products (which included the complete animal body, thorax and claws) has been found to contain approximately 20% - 23% (by weight) of chitin, which already warrants its use as an economically viable source for chitin production on an industrial scale due to its ready availability and the low price of the source [23-25]. Existing literature has also recommended the economic and environmental merits of such crustacean sources for chitosan preparation, because 40% - 50% by weight of the total mass of the crustacean for human consumption ends up as waste, and most of such waste is dumped into the sea and becomes significant pollutants in coastal areas [21, 26]. Therefore, by-products of crustacea such as lobster cephalothorax can be identified as a suitable source for chitosan preparation on an industrial scale.

3. Commonly used methods to prepare chitosan

Existing literature has extensively covered the preparation of chitosan or chitin from marine sources. In general, the methodologies published can be categorized into

two types: chitosan extracted from the crustacea by-products via chemical methods, and via biological methods [3].

3.1 Chemical methods

Chemical methods of chitosan preparation mainly include three stages of reaction: demineralization ((the vast majority of the recent literature reports use of HCl in concentrations of up to 10% w/v to remove the $CaCO_3$ in the shell by reacting for 2 - 3 h with agitation), deproteinization [removing the protein and other organic components other than chitin in the shell by reacting with heated alkali solution, such as 1% - 10% (w/w) aqueous NaOH solution at temperatures of 65 - 100 ^oC for 0.5 - 12 h], and deacetylation [converting chitin to chitosan using 40% - 50% (w/w) heated alkali solution, for example, NaOH solution] [19, 25]. Most of the recently published literature adopted processes using the steps of demineralization → deproteinization → deacetylation, in that order for the reason that demineralization is a much easier reaction than deproteinization: if demineralization is used prior to deproteinization, it can create more surface area in the shell material by dissolving $CaCO_3$ and accelerate the deproteinization reaction which is later in the process [3]. Figure 2 demonstrates this process, and Figure 3 illustrates the chemical reactions of the demineralization and deproteinization stages. Despite this, some earlier studies have instead used the process where deproteinization is carried out first, i.e. via the steps of deproteinization → demineralization → deacetylation, as executing the process in this particular order has not been perceived to lead to any significant difference in the quality and yield of the chitin produced [3, 27, 28]. However, as chitin in lobster shells is tightly reticulated with minerals and other organic components such as proteins [29], demineralization should logically precede

deproteinization when lobster is used as the raw material to improve the efficiency of the process.

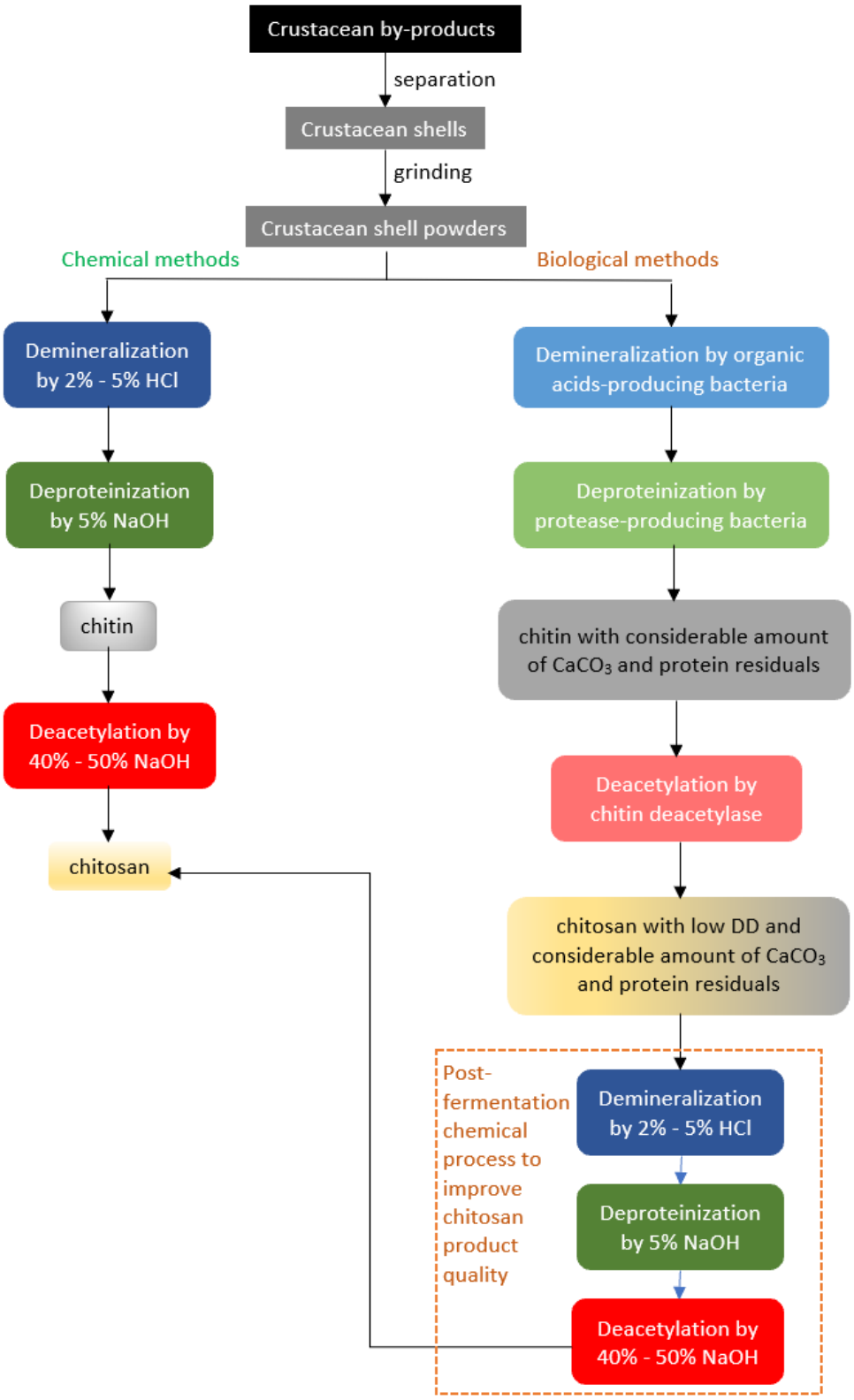


Figure 2. Chemical methods preparing chitosan from seafood byproducts as compared to biological methods of fermentation. Modified from [19].

$$CaCO_3 + 2H^+ \rightarrow Ca^{2+} + H_2O + CO_2 \uparrow$$

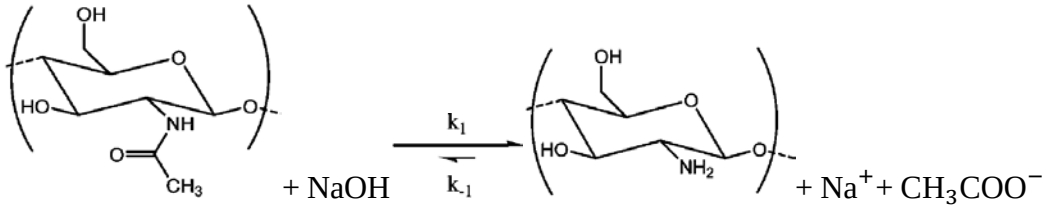
(A) demineralization

$$2(>CH\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}CH<) + 4OH^- \xrightarrow{\Delta} 3(>CH\text{-}CH_2\text{-}S^-) + >CH\text{-}CH_2\text{-}SO_2^- + 2H_2O$$

(B1) OH^- causing scission of the disulfide bond in protein

$$\text{-CO-NH-} + OH^- \rightarrow \text{-}COO^- + \text{-}NH_2$$

(B2) OH^- causing scission of the peptide bond in protein



(C) deacetylation

Figure 3. The reaction mechanisms of the chemical methods to prepare chitosan. (A) demineralization; (B1) OH^- causing scission of the disulfide bond in protein (modified from [30]); and (B2) OH^- causing scission of the peptide bond in protein (redrawn from [31]) as the two mechanisms of deproteinization; (C) deacetylation (modified from [32]).

3.1.1 Acids used for the demineralization stage

Younes and Rinaudo [3] published a comprehensive summary of chemical agents and reaction conditions used for chemical preparation of chitin and chitosan from a range of marine species. However, this literature review excluded studies which explored the use of chemical agents other than HCl and NaOH for chitin and chitosan preparation via chemical methods. For instance, some early research used ethylenediaminetetraacetic acid (EDTA) as the acid chosen for demineralization [33]. Demineralization of crustacean cuticles by HNO_3, H_2SO_4, and CH_3COOH has also been reported [2, 34]. Another study explored the demineralization of shrimp by a range of acids such as HCl, HCOOH, CH_3COOH, citric acid and combinations thereof [28]. This research found that under the same reaction conditions used (i.e. 0.25 M acid with a shell-to-acid ratio of 1:30 w/v at room temperature for 30 min

with agitation) HCl removed Ca most efficiently with a decalcification rate of 90.8%, although HCl is environmentally toxic at high concentration. In comparison, when 0.25 M acetic acid is employed under the same conditions, it showed the lowest decalcification rate of 78%. On the other hand, citric acid, when used, exhibited a decalcification rate of 86.1%, yet the by-product of this reaction, calcium citrate, is nearly insoluble in water at room temperature [35, 36]. Hence, using citric acid is undesirable as separating the insoluble calcium citrate by-product from the insoluble chitin product is difficult, especially on an industrial-scale production level. For the same reason H_3PO_4 should not be used for deproteinization, because tricalcium phosphate, $Ca_3(PO_4)_2$, is very insoluble in water and causes similar difficulties of separation [37]. In light of these issues, using acetic acid could be justified as the most desirable alternative to HCl rather than citric acid. This is due to several reasons. Firstly, calcium acetate has a high solubility in water [36]. Secondly, both Ca and acetate are essential for the normal human metabolism [38], and can be metabolized by microorganisms in the natural environment [39]. Thirdly, acetic acid is readily available given its industrial importance, and hence globally its price is significantly cheaper than the other possible alternative to HCl, which is lactic acid [40, 41]. Therefore, given all these reasons, acetic acid stands out as a very strong contender as an alternative to HCl, when comparing to other acids which potentially fulfil this role such as lactic acid.

3.1.2 The parameters impacting on the efficiency of demineralization reaction

To increase the efficiency of demineralization reaction, there are two measures which can be taken. The first one is to use both higher concentrations of acid and

lower shell-to-acid ratios to create an environment with excessive acid to encourage the reaction to progress towards the direction of demineralization [42]. The second measure is to increase the duration of the demineralization reaction, which allows more time for the interaction between acid and $CaCO_3$ [3]. However, the results of prior studies indicated that reaction duration beyond one hour only led to negligible further decreases in Ca content (e.g. less than 10% of further Ca removal in the second hour, and nearly all Ca has been removed in a 2 h duration demineralization reaction), while at the same time causing significant deleterious chitin degradation [25]. Therefore, the first measure is the more commonly used strategy for increasing the efficiency of demineralization.

3.1.3 Bases used for the deproteinization and the deacetylation stages

Even though NaOH, NaClO, KOH, $NaHCO_3$, Na_2CO_3, K_2CO_3, $Ca(OH)_2$, $NaHSO_3$, Na_2S, $CaHSO_3$, Na_3PO_4 and Na_2SO_3 have all been reported as being used for deproteinization of crustacea by-products [2, 28, 43], they are not ideal candidate bases to be used in chitosan production (especially for industrial scale production) for several reasons. Firstly, the adverse environmental effects of the by-products generated from using these chemicals are a concern. For instance, the high concentration of Na in the by-products generated by the reaction through using chemical agents containing the sodium cation can pollute soil and water systems [2]. In contrast, literature suggests that KOH could be deemed an environmentally and commercially viable alternative to NaOH and other bases for deproteinization [42]. This is firstly because KOH will not generate by-products with high concentrations of Na, but K instead. This is advantageous because K is an essential element for plant growth and a common component in fertilizer [44-47]. Secondly, KOH is an efficient chemical agent for deproteinization, since at concentrations as

low as 2% is reported to remove nearly all protein from crab shell in a two-hour deproteinization reaction at 90 ^{o}C, when the shell-to-base ratio is 1:20 (w/v) [25]. Another earlier study has also confirmed that using 10% or higher concentrations of KOH (such as 40%) can effectively remove all proteins in krill by-products down to a concentration level of 0.5% after only two hours of reaction at 90 ^{o}C [48]. Therefore, it can be concluded that KOH can be a credibly viable alternative chemical agent to NaOH for deproteinization, and this will be one of the focuses of the latter parts of this review.

Deacetylation is the last stage of preparing chitosan from marine by-products like crustacea, and it is achieved by using either heterogeneous or homogeneous reaction methodologies [3]. Heterogeneous methods use 40% - 50% (w/v) NaOH or KOH solution with the reaction being conducted at a temperature of approximately 100 ^{o}C for 1 - 12 hours, and produce water-insoluble chitosan possessing a DD of 85% - 99% [3, 25, 49]. In contrast, the homogeneous method while using 40% - 50% NaOH, prepares water-soluble chitosan in free amine form at ambient temperature [2]. However, such homogeneous methods involve dispersing chitin and NaOH in crushed ice, and the post reaction separation of chitosan from the reaction system is very complex because the chitosan produced is soluble in the residual NaOH solution, thus this method is difficult to scale up [50-52]. Therefore, this review has deliberately chosen to focus on the heterogeneous methodology, because of its being preferred by industry due to the relative ease of separating the insoluble chitosan product from the liquors of the residual NaOH and by-products [53, 54]. A summary of chitin prepared from lobster sources using heterogeneous methodologies for deacetylation as gleaned from the current literature is given in Table 1.

3.1.4 The parameters impacting on the efficiency of the deproteinization reaction

Prior publication found that the concentration of the alkali, the reaction temperature and the marine species used are the key variables to consider when attempting to increase the efficiency of the deproteinization reaction [25]. Earlier studies which have specifically used lobster by-products to prepare chitin have been summarized in Table 2. This table provides a contrast to the summaries from previously published reviews, which have mostly been dedicated to preparing chitosan from crab or shrimp species, but rarely focused on lobster and crayfish species.

3.1.5 The parameters impacting on the efficiency of the deacetylation reaction

Several parameters have been identified as important when attempting to increase the efficiency of the deacetylation reaction: DD rises mainly with increasing the reaction temperature, the concentration of the base used and the reaction duration [49, 55, 56]. However, although using higher reaction temperature can increase DD, it could also cause MW reduction of the chitosan products [25]. Previous research also reported that KOH aqueous solution is an efficient chemical agent for deacetylation at a concentration level of 40% - 60% (w/v) [27, 57]. Nonetheless, increasing KOH concentration to values higher than 60% has been reported to contribute little to the further increase of DD but rather to the reduction in the MW of the chitosan produced [57].

In terms of the reaction duration of deacetylation, acetyl groups in chitin are mostly removed at the early stages of the reaction (< 1 h); but after 1 h, deacetylation only

further progresses very slowly by a constant rate of 1.2 - 1.8% per hour [58]. This trend is consistent with the findings of No and Meyers [25], who noted that DD proceeded rapidly to about 68% in the first hour of alkali treatment in a 50% NaOH solution conducted at 100°C, but then progressed only gradually to reach a total DD of 78% after five hours of reaction. Moreover, the deacetylation reaction is unable to achieve significant removal of acetyl group beyond two hours of reaction. Instead, the increase of reaction time over two hours contributes more to the degradation of the chitin molecular chain than to further deacetylation [25, 59]. This trend is observed when using KOH for deacetylation as well [57]. Juang, Tseng, Wu and Lin [58] explained such a drop of the reaction efficiency of deacetylation over time. They stated that this decrease is probably due to the increase in the viscosity of the alkaline solution comprising the reaction system: the culprit being K_2CO_3 buildup as a result of the fast deacetylation process occurring in the initial stages of reaction, which increases the viscosity of the alkaline solution in the reaction system and hinders the diffusion of OH^- into the pores of the chitin particles where the deacetylation reaction taking place. At the same time, the chitosan MW decreases because of the hydrolysis reaction of the polysaccharide chain due to the attack of OH^-occurring principally outside the pores of the chitin particles. This viscosity-based reason could also explain the observed decrease in the observed chitosan hydrolysis reaction rate by alkali over time [60]. In fact, further support for viscosity being the cause can be found from the observation that with intermittent washing, high MW chitosan can be produced with nearly 100% DD [25]. However, if an intermittent washing approach were used to remove K_2CO_3 generated to reduce the viscosity of the reaction solution, the yield would reduce, as unintended product removal usually occurs during a washing process [42].

In regard to the effects of other parameters on the deacetylation reaction, existing literature has not yet demonstrated a clear consensus regarding the significance of most such parameters [53]. For example, Chang, Tsai, Lee and Fu [49] reported that the chitin-to-base ratio was insignificant for DD, while Moorjani, Khasim, Rajalakshmi, Puttarajappa and Amla [61] claimed the opposite. Similar disagreement regarding the effects of the reaction atmosphere is also reported: while earlier literature suggested that exposing chitin to O_2 during the deacetylation reaction promotes the degradation of chitin, most existing research still reported the execution of the reaction in an air environment instead of under a N_2 atmosphere when KOH was employed for deacetylation [25].

On the other hand, research has suggested that microwave processing can considerably increase the reaction efficiency of deacetylation, particularly in terms of reducing the reaction time needed [53], by providing three-dimensional heating to the reaction mass [62]. Similarly, use of ultrasound can also promote the efficiency of the deacetylation reaction, although this is at the cost of significantly reducing product MW in most cases [62].

Table 1. Parameters of heterogeneous chitin deacetylation for lobster sources or using KOH as an alternative to NaOH.

Raw material	Base	Temperature (ºC)	Time (h)	Chitin to base ratio (w/v)	Reaction atmosphere	Specifications of the chitosan product	Reference
Lobster	50% NaOH	130	5	1:10	N_2	DD 92.4%, MW 220 kDa.	Juang, Tseng, Wu and Lin [58].
Ground lobster carapaces	55% KOH	100 - 140	0.5 - 15	1:100	N_2 or Air	DD 65.0% - 82.1%	Lusena and Rose [57].
Crab and krill	39% KOH dissolved in 95% ethanol and ethylene glycol	reflux	20	1:17	Air	Viscosity 67 cps for chitosan from crab, 60 cps for chitosan from krill (1% product in 5% acetic acid)	Anderson, De Pablo and Romo [63] as cited in No and Meyers [25].
Prawn	60% KOH	100	1	1:65	Air	Viscosity 309 cps (1% product in 2% acetic acid)	Moorjani, Achyuta and Khasim [64] as cited in No and Meyers [25].

Table 2. A summary of the parameters of demineralization and deproteinization reactions by using lobster as the source of chitosan.

Demineralization				Deproteinization			Reference
Acid (concentration w/w)		Temperature (°C)	Duration (h)	NaOH (w/v) %	Temperature (°C)	No. of repeats × duration (h) per repeat	
6.17% HCl	First repeat: Shell to acid ratio (w/v) 1:9	Ambient	5	4%. Shell to base ratio (w/v) 1:5.5	100	5 × 12	Hackman [65] as cited in No and Meyers [25].
	Second repeat: Shell to acid ratio (w/v) 1:5.5	0	48				
37% HCl		-20	4	10%	Room	3 × 24	BeMiller and Whistler [66] as cited in No and Meyers [25].
6.17% HCl		Ambient	5	4%	100	5 × 12	Hackman and Goldberg [67] as cited in Younes and Rinaudo [3].
5% HCl		70	2 × 2 repeats	5%	80-85	2 × 0.5	Blumberg, Southall, Van Rensburg and Volckman [68] as cited in and No and Meyers [25].
90% formic acid. Shell to acid ratio (w/v) 1:10		Ambient	18	10%. Shell to base ratio (w/v) 1:50	100	1 × 2.5	Horowitz, Roseman and Blumenthal [69] as cited in No and Meyers [25].
1.7% HCl		25	2 × 3 repeats	1.2%	80	3 × 3	Tolaimate [70] as cited in Younes and Rinaudo [3].
3% HCl. Shell to acid ratio (w/v) 1:10		Ambient	15	8%. Shell to base ratio (w/v) 1:10	90	1× 4	Zhang and Wang [71]. [1]

Table 2 continued

Demineralization			Deproteinization			Reference
Acid (concentration w/w)	Temperature (°C)	Duration (h)	NaOH (w/v) %	Temperature (°C)	No. of repeats × duration (h) per repeat	
3.08% HCl	Not reported	Not reported	4%	105-110	Not reported	Abdou, Nagy and Elsabee [24].
0.5% HCl	Ambient	16	3%	80	1 × 1.5	Juang, Tseng, Wu and Lin [58].
3.08% HCl. Shell to acid ratio (w/v) 1:10	Ambient	2	15%. Shell to base ratio (w/v) 1:10	65	1 × 3	Acosta, Jiménez, Borau and Heras [72].
7.5% Lactic acid. Acid to shell ratio is 18 mL/g	Microwave 1200 W to reach 50, 75 or 100°C	0.4	2% 25 mL	100	1× 0.5	Nguyen, Barber, Smith, Luo and Zhang [29]. [1]

[1] When the diameter of shell pieces is no more than 5 mm, the chitosan produced can achieve the specifications of food grade products (i.e. the products exhibit residual ash ≤ 1.5%)

Note: Some of the literature cited above used M instead of (w/w) % as the unit of concentration. Hence the units from M to % has been converted based on 12 M HCl = 37% HCl, and 40 g NaOH per 1 L of solution equals 4 grams per 100 (mass/volume) or 4% (m/v).

3.2 Biological methods

Besides chemical methods, biological methods (i.e. enzymatic methods and fermentation methods) are also available to prepare chitosan from crustacean by-products.

3.2.1 Enzymatic methods

Enzymatic methods share the same demineralization mechanism as per the chemical methods, i.e. using acid to remove the $CaCO_3$ in shell as per discussed earlier [73, 74]. Nevertheless, this method replaces alkaline and high reaction temperature with enzymes for deproteinization and deacetylation reaction at a mild temperature, usually around 25 – 59 ^{o}C [74, 75]. Various proteinases have been developed for enzymatic deproteinization [2, 19], and these enzymes are usually extracts from microbes or fish entrails, such as intestines of sardinella (*Sardinella aurita*) and grey triggerfish (*Balistes capriscus*) [74]. Likewise, deacetylases can also be extracted from fish intestines or microbes [2, 20, 76, 77], for instance, Alcalase® obtained from *Bacillus licheniformis* [3]. Genetically-modified microorganisms have also been reported as another source of enzymes for deproteinization and deacetylation reactions [78].

Enzymatic methods use much milder reaction conditions by enabling the reactions via sophisticated reaction mechanisms. For example, CE4 in the carbohydrate esterase enzyme family can remove *N*-acetyl functional groups by an acid/base reaction mechanism facilitated by metal ions (of usually zinc or cobalt) bound to the reaction site of the enzyme. which has been discussed in more detail by van den Broek, Boeriu and Stevens [54]. Despite the milder reaction parameters, enzymatic methods have significant limits compared to chemical methods. The biggest one is

the cost of the operation, particularly for industrial-scale production. This is because enzymes used for deproteinization and deacetylation are considerably more expensive than the generic bases used in chemical methods; additionally, the deproteinization reaction usually requires different enzyme(s) from those used for deacetylation reactions even in one production process [2, 53]. Moreover, enzymatic methods are less efficient than chemical methods, because of their inability to eliminate the final residual 10% of the proteins in shells during deproteinization [3], and the DD value by enzymatic deacetylation is even lower [53, 79].

To resolve the issue of the expensive cost of the enzymes, fermentation methods have been developed as the alternative, because microbes can rapidly multiply themselves while continuing to secrete enzymes into the reactors under the optimized reaction conditions so reducing the high enzyme cost [3, 19]. Nevertheless, the problem of reaction inefficiency (e.g. the issue of the unreacted residual proteins and the low DD value) can only be addressed by adding a cycle of chemical reactions to refine the product following the completion of all enzymatic reactions [3, 80]. Furthermore, some prior research of enzymatic chitosan production has used chemical deacetylation rather than enzymatic deacetylation to resolve the reaction inefficiency issue [62]. This means “enzymatic methods” *per se* should be regarded as a *de facto* combination of techniques involving chemical methods and biological methods, and hence involves the inevitable use of generic chemicals for demineralization before the enzymatic reactions as well as after due to product refinement [62].

3.2.2 Fermentation methods

Due to the limitation of enzymatic methods discussed above, most publications using biological methods for chitin/chitosan preparation have hence used fermentation methods instead [81, 82]. Fermentation methods can be subdivided into two sub-categories, namely lactic acid fermentation methods and non-lactic acid fermentation methods, depending on whether the microbial strains used in the studies secrete lactic acid or other organic acids as the acid(s) for the demineralization reaction [21]. Existing research has predominantly used lactic-acid fermentation methods when lobster by-products are used as the sources of chitosan, and the fermentation processes have usually been reported to take approximately seven days or more to complete [21], with the separation of calcium lactate by-products (generated by the lactic acid fermentation) from the chitosan product being identified as a challenging issue [19]. Table 3 summarizes only the features of lactic acid fermentation methods which require fewer than seven days of fermentation [21], while those requiring longer periods of time have not been included.

Table 3. A summary of previously reported chitin preparation processes by lactic fermentation.[1]

Bacterial strain and raw materials	Pre-fermentation and fermentation processes	Post-fermentation process	Final product	Reference
Lactobacillus spp. strain B2. Health risk: Group 1. [2] Starting raw materials: Shrimp waste. Carbohydrate sources: Sucrose, lactose and spray dried cheese whey.	Pre-fermentation process: None. Special reactor or fermentation facilities required: No. Fermentation parameters: 30 ºC, for 4, 6 and 90 days.	Separation required: One process. Purification required: Demineralization by HCl from 1.0 M to 0.2 M for 2 h at 25 ºC. Then deproteinization by NaOH from 1.0M to 0.2 M for 2 h at 25 ºC.	Chitin yield: 2 kg scale = 29.6%. 30 kg scale = 25.7%. Efficiency: Demineralization = 87.6%. Deproteinization = 85%.	[83]
Streptococcus faecium M74, *Lactobacillus plantarum*, and *Pediococcus acidilactici*. Health risk: *S. faecium* = Group 2. [3] *L. plantarum* and *P. acidilactici* = Group 1. Raw materials: Demineralized prawn (*Nephrops norvegicus*) shell. Carbon source: Lactose ('Nutrimink').	Pre-fermentation process: Finely ground shell to 2 mm particle size. Special reactor or fermentation facilities required: No. Fermentation parameters: 25 ºC for 7 days.	Not recorded.	Chitin yield: No data. Efficiency: Demineralization = No data. Deproteinization = 40%.	[84]
L. plantarum, L. salivarius, S. faecium and *P. acidilactici*. Health risk: As per above. Starting raw materials: Fresh shell (*N. norvegicus*). Carbon source: Glucose.	Pre-fermentation: Grounding by domestic food blender, then dissolve into glucose solution. Special reactor or fermentation facilities required: Yes. Fermentation parameters: 30 ºC, 7 days.	Separation required: Three processes. Purification required: No.	Chitin yield: 20.43%. Efficiency: Demineralization = 90.99%. Deproteinization = No data.	[85]
Lactobacillus parucasei strain A3. Health risk: Group 1. Starting raw materials: *N. norvegicus.* Carbon source: Glucose.	Pre-fermentation: Mincing. Special reactor or fermentation facilities required: Yes. Fermentation: Inoculum for 3 days, then fermentation for 5 days at 30 ºC.	Separation required: Centrifuge. Purification required: No.	Chitin yield: The solid fraction comprised 17.5% chitin (dry weight basis). Efficiency: Demineralization = 61%. Deproteinization = 77.5%.	[86]
Lactobacillus parucasei strain A3. Health risk: Group 1.	Pre-fermentation: Mincing, then or air-drying at 50 °C, then separation.	Separation required: No.	Chitin Yield: 25.75% Efficiency:	[87]

Table 3 continued

Bacterial strain and raw materials	Pre-fermentation and fermentation processes	Post-fermentation process	Final product	Reference
Raw materials: *P. clarkii*, whole body included. Carbon source: Dextrose.	Special reactor or fermentation facilities required: Yes. Fermentation: 30 °C for 3 days.	Purification required: 0.5 M HCl for 6 h, 0.3 M NaOH for 6 h, and a 1:5 dilution of ClO^- at room temperature for 6 h.	Demineralization = 97.2%. Deproteinization = 94%.	
Lactobacillus paracasei subsp. tolerans KCTC-3074. Health risk: Group 1. Raw materials: Red crab (*Chionoecetes japonicus*) shell waste. Carbon source: Glucose.	Pre-fermentation: Drying then mincing. Special reactor or fermentation facilities required: Yes. Fermentation: 30 °C for 5 days.	Separation: No data.	Chitin Yield: No data. Efficiency: Demineralization = 81%. Deproteinization = 30% - 40%.	[88]
Lactobacillus plantarum 541. Health risk: Group 1. Raw materials: frozen shrimp waste. Carbon source: Glucose	Pre-fermentation: Mincing. Special reactor or fermentation facilities required: No. Fermentation: 30 °C for 26 h.	Separation required: Three processes.	Chitin Yield: No data. Best efficiency achieved: Demineralization = 87.97%. Deproteinization = 90.76%.	[89]
Immobilized *Lactobacillus pentosus*-4023. Health risk: Group 1. Raw materials: Crayfish (*P. clarkii*). Carbon source: Concentrated acid whey	Pre-fermentation: Separation. Special reactor or fermentation facilities required: Yes. Fermentation: Inoculation for 2 days, then fermentation at 35 °C for 2.1 days.	Separation: No data. Purification required: Demineralization by boiling with 0.5 M HCl, then deproteinization by boiling with 0.25 M NaOH.	Chitin Yield: 20.6% Efficiency: Demineralization = 90.1%. Deproteinization = 81.5%. Degree of deacetylation = 26%.	[23]
L. acidophilus SW01. Health risk: Group 1. Raw materials: Shrimp waste. Carbon source: Glucose.	Pre-fermentation: No. Special reactor or fermentation facilities required: No. Fermentation: Anaerobic fermentation at 37 °C for 7 days.	Separation: Not recorded. Purification required: No.	Chitin Yield: No data. Efficiency: Demineralization = 99.3%. Deproteinization = 96.5%.	[90]

[1] Most prior studies cited here which used biological methods and prepared from fishery by-products either stopped the preparation process when chitin was produced, or used chemical methods to convert chitin to chitosan.

[2] Risk group 1 microbes are unlikely to cause human, plant or animal diseases.

[3] Risk group 2 microbes can cause disease but are unlikely to be serious.

When one compares the fermentation methods as summarized in Table 3 with the chemical methods discussed earlier, the pros and cons of chemical methods versus biological methods can be identified and contrasted to support the conclusion that chemical methods have several advantages over biological methods. Chemical methods have the advantages of employing shorter process durations, simpler production processes (particularly in terms of pre-reaction processes and post-reaction processes, such as product purification), and the chitosan produced having medium to lower MW and higher DD (which shows stronger biological properties). On the other hand, the comparison also shows that the disadvantages surrounding chemical methods are that traditionally the reaction process uses some toxic or corrosive chemicals such as HCl and NaOH, and consequently, the by-products of the chemical process (such as the reaction liquors, which contain high concentrations of Na^+ from the use of NaOH) may be significant pollutants if not disposed of prudently or if they cannot be readily reused or recycled [20, 21]. Currently, only one prior publication has entertained some discussion regarding the issues of by-products from the perspective of the total processing system if the traditional chemical methods were scaled up for industrial-level production, and provided one possible option of optimizing the design of the chemical processing system to better utilize the by-products generated from each stage of the reaction [25].

In contrast, biological methods have the merit of producing high MW chitosan product (which exhibits better mechanical properties). However, biological methods have some drawbacks. For instance, although fermentation methods have the advantages of lowering the cost of operation and not using generic acids for demineralization reaction when comparing with enzymatic methods, fermentation

usually requires some specific microbial strains, and such strains are usually not available in countries with enhanced biosecurity placed at their borders to prevent the introduction of foreign biological agents or species into their domestic environment e.g. New Zealand [91]. In such cases, the logistics of importing such strains are time consuming, costly, and furthermore, involve necessary access to accredited contained facilities (which are mandated under enhanced biosecurity regulations). Other issues include the risks of contamination during fermentation, the necessary inclusion of higher risk strains such as *Streptococcus* for fermentation, the fermentation media, and the specialized equipment required, which can all be expensive. Moreover, biological methods not only usually take a much longer time to complete the entire process comparing to chemical methods, but also frequently involve more complicated procedures than chemical methods, particularly at the pre-fermentation and post-fermentation stages e.g. the separation process and the purification process [62]. Earlier studies which comprehensively summarized the chitin sources and strains/enzymes used for chitin/chitosan preparation [2, 19, 21] found that the chitin/chitosan prepared via biological methods usually only reach 70% - 90% $CaCO_3$ removal and 40% - 94% protein removal, with the whole process taking about 3-7 days to complete. As biological methods tend not to remove $CaCO_3$, protein impurities and acetyl functional groups in shells as thoroughly as chemical methods do, an additional stage of product purification by a cycle of chemical methods must be added after the completion of the biological reaction process in order to further remove the residual $CaCO_3$ and proteins from the chitin/chitosan products [19] and hence reach the desirable quality, as shown in Figure 2. This means that biological methods are ironically not completely free from using hazardous chemicals, such as HCl and NaOH, which are routinely used in chemical methods, in contrast to what existing publications have claimed [19].

Considering all these factors, the unconventional chemical methods using KOH as the alternative of NaOH enhanced by microwave or ultrasound can actually have more advantages than biological methods from the perspective of:

1. Manufacturing chitosan with stronger bioactivities (i.e. lower MW and higher DD value) on an industrial scale; and

2. Minimizing serious impacts on the environment due to the use of inherently simpler production processes without involving chemical agents containing high concentrations of Na.

3. Such methods can also make use of microwave or ultrasound technology to significantly reduce the reaction time required for the whole process, as such technology has been available on industrial scale productions [92-95]

Hence, due to these advantages, such unconventional chemical methods warrant further research and development for industrial scale chitosan production.

4. Conclusion

Chitosan exists in a wide range of species, and crustacea by-products containing at least 20% of chitin, such as lobster cephalothorax, are promising sources for chitosan production on an industrial scale. It is clear that when selecting an appropriate chitosan preparation method, the one leading to chitosan with stronger bioactivities (i.e. the products with lower DA value and lower MW) should be the preferred method.

Chemical and biological methods constitute the two major categories of chitosan preparation. This review argues that chemical methods have more advantages for chitosan preparation over biological methods, because the processes involved are

inherently simpler and quicker, and the products are shown to have stronger bioactivities due to lower MW and higher DD. In contrast, despite the reaction parameters being comparatively milder, it is clear that biological methods require the usage of not only hazardous microbes, specialized equipment, and complex post-fermentation purification process, but also a cycle of chemical processing as the final stage to refine the chitosan products to achieve the optimal quality. Therefore, this review has developed the opinion that unconventional chemical methods, such as using KOH as an alternative to NaOH and enhancing the reactions by microwave or ultrasound, could be the better strategies to adopt rather than biological methods for industrial scale chitosan production. However, further development of these unconventional chemical routes is recommended before they are considered and taken on by industry.

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