

## *Intensifying chitin hydrolysis by adjunct treatments – an overview*

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# 1           **Intensifying chitin hydrolysis by adjunct treatments – an overview**

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## 12   **Abstract**

13   Chitin is, after cellulose, the most abundant organic natural polysaccharide on Earth,  
14   being synthesized as a dominant component in the exoskeletons of crustaceans,  
15   among other sources. In the processing of seafood for human consumption, between  
16   40 and 50% of the total raw material mass is wasted, causing a significant problem  
17   for the environment due to its slow degradation. Efforts to find uses for chitin  
18   derivatives, particularly their oligomers, have intensified since these chemicals are  
19   highly functional and offer a wide range of applications, especially as antimicrobial  
20   agent. As a consequence, some adjunct treatments, either chemical or physical in

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1 nature, have been employed to assist acid and enzymatic hydrolysis. This work  
2 provides a detailed review of the methods employed to intensify the formation of  
3 chitin oligomers, particularly focusing on the adjunct treatments used (microwave,  
4 ultrasonication, steam explosion and gamma irradiation), and evaluate the yield and  
5 characteristics of the oligomers formed. Adjunct treatments are more suitable for  
6 enzymatic hydrolysis since these treatments modify the chitin structure, and enhance  
7 the hydrolysis rate and yield of the oligomers, under milder reaction conditions. For  
8 future research, it would be worth trying pre-treatments like the application of high-  
9 pressure to chitin in order to lower its crystallinity.

10 **Keywords:** Chitin hydrolysis; Oligomers; acid hydrolysis; enzymatic hydrolysis;  
11 pretreatment

## 12 INTRODUCTION

13 Chitin - a  $\beta$ -(1 $\rightarrow$ 4)-linked polymer composed predominantly of *N*-acetyl-D-  
14 glucosamine (GlcNAc) units – is, after cellulose, the most abundant organic natural  
15 polysaccharide on Earth, offering a broad range of structural and protection  
16 functions, like cellulose in plants. The structure of chitin differs from that of cellulose  
17 in that the C-2 hydroxyl residues (-OH) are replaced by acetamide groups  
18 ( $\text{CH}_3\text{CONH-}$ ) (Figure 1). Chitin comprises three polymorphs:  $\alpha$ -chitin is the most  
19 abundant and  $\beta$ - and  $\gamma$ -chitin are very rare, which can be distinguished by their  
20 molecular chain arrangement and hydrogen bonding systems.  $\alpha$ - and  $\beta$ -Chitins have  
21 an antiparallel and a parallel chain arrangement, respectively, whereas  $\gamma$ -chitin  
22 consists of both parallel and antiparallel chains.<sup>1,2</sup> The antiparallel chain in  $\alpha$ -chitin  
23 are arranged in bonded piles or sheets linked together by the hydrogen and

1 acetamide groups running in opposite directions as compared to the  $\beta$ -chitin.<sup>2</sup> The  
2 intermolecular and hydrogen bonds present in  $\alpha$ -chitin make it difficult to melt and  
3 dissolve in common solvents at normal temperatures, which makes this material  
4 inconvenient for further processing.<sup>1</sup>

5 Chitin is synthesized as a dominant component in the exoskeletons of crustaceans  
6 and insects, as well as in the cell wall of fungi, yeast and algae. Nowadays, chitin  
7 extracted from crustacean shells, such as crab, shrimp, prawn, krill and lobster, are  
8 readily available in large quantities from shellfish processing industries in  
9 comparison with other sources. In the processing of seafood for human  
10 consumption, between 40 and 50 % of the total raw material mass is wasted.<sup>3</sup> This  
11 quantity of waste has been reported to be between  $10^{10}$  and  $10^{11}$  tons per year,  
12 which poses a significant problem for the environment due to its slow degradation.<sup>3-6</sup>

13 Crustacean shells are composed of proteins, chitin, minerals, and carotenoids, which  
14 are the major components based on the dry mass.<sup>3</sup> In order to extract chitin from the  
15 crustacean shells, the following main steps must be employed: demineralization,  
16 deproteinization and decolouration.<sup>7</sup> The demineralization of shells can be achieved  
17 by extraction with dilute acid (hydrochloric acid, formic acid, acetic acid, sulfuric acid  
18 or EDTA – ethylenediamine-tetra-acetic acid) at room temperature. Deproteinization  
19 can be effected by treating the demineralized waste with aqueous alkali solution  
20 (sodium or potassium hydroxide) at the temperatures between 65 and 100°C. In this  
21 step, the most significant parameters to be considered for an efficient  
22 deproteinization are the concentration of alkali solution, processing time and  
23 temperature, and solid to solvent ratio. Benhabiles et al.<sup>8</sup> reported that the conditions  
24 for 96 % protein removal were: processing time of 120 mins at a temperature of 45

1 °C and the use of solid to solvent ratio of 1:2 (w/v) with the solvent (NaOH solution)  
2 concentration being 2 M. Finally, the decolouration was carried out by a bleaching  
3 with activated charcoal, or by using strong oxidizing agents such as sodium  
4 hypochlorite (NaClO) or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solutions.<sup>9</sup> Chitin separated from  
5 minerals, colourants and proteins is commercially available for industrial uses,  
6 although, it may require further purification in order to obtain regenerated chitin,  
7 which can be effectively used for producing chitosan and oligomers.<sup>10-12</sup>

8 Chitosan - a β-(1→4)-linked polymer composed predominantly of D-glucosamine  
9 (GlcN) units - is an *N*-deacetylated derivative of chitin formed by deacetylation under  
10 alkaline conditions at elevated temperature, which sodium hydroxide is a common  
11 alkaline media.<sup>4,13-15</sup> Chitosan is a semi-crystalline polymer which is insoluble in  
12 aqueous solutions above a pH value of 6.5, but fully soluble in diluted acids below  
13 pH of 5.<sup>16,17</sup> The difference between chitin and chitosan can be defined in terms of  
14 the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-  
15 glucopyranose units, which is commonly known as degree of *N*-acetylation (DA). The  
16 DA of chitosan is typically less than 0.35, whereas that of chitin is normally above  
17 0.90.<sup>15</sup> Higher and lower molecular weight chitosans may also possess excellent  
18 properties for specific applications. Numerous studies have reported that chitosan  
19 with higher molecular weight possess superior mechanical properties, such as higher  
20 tensile strength and better elongation of chitosan film,<sup>18,19</sup> higher antifungal  
21 activity,<sup>20,21</sup> and enhances nasal absorption of peptide drugs<sup>22</sup>. Lower molecular  
22 weight chitosans have a stronger superoxide scavenging activity,<sup>23</sup> greater  
23 antimicrobial activity,<sup>24</sup> and higher permeability of film.<sup>18</sup>

1 Chitin oligomers are derived from chitin by depolymerization in the presence of acid  
2 or enzymes.<sup>4,13,14</sup> Chitin oligomers are composed of GlcNAc units with approximately  
3 ten residues or less, and can be produced by depolymerization of chitin.<sup>4</sup> The  
4 depolymerization is commonly achieved by acid and enzymatic hydrolysis,  
5 employing hydrochloric acid (HCl) and chitinases, respectively. The chitin polymer  
6 chain is cleaved by this reaction to become oligomeric. Both these hydrolysis  
7 methods have been extensively reviewed by Jeon et al., Prashanth and Tharanathan  
8 and Ahmed et al.<sup>4,25,26</sup> Efforts to find uses for chitin oligomers have intensified since  
9 these chemicals are highly functional and offer a wide range of applications. Chitin  
10 oligomers have received increased research and commercial attention because  
11 these molecules are not only water soluble, nontoxic and biocompatible, but also  
12 exhibit numerous biological properties, such as antibacterial, antifungal, antitumor,  
13 and antioxidant activities.<sup>27,28-30</sup> According to the literature, antimicrobial action is one  
14 of the most important property of chitin oligomers due to its water solubility, and  
15 potent activity against bacteria and moulds. It has been reported that the oligomer  
16 possess low minimal inhibitory concentrations (MIC) values for gram-positive strain  
17 (*S.aureus*, *B. subtilis*, *L. monocytogenes* and *B. cereus*) and gram-negative strains  
18 (*E. coli*, *V. cholera*, *Shigella dysenteriae*, *B. fragilis*, *P. aeruginosa*, and *P.*  
19 *melaninogenica*) compared to chitin and chitosan.<sup>27,31</sup> In another research, the chitin  
20 oligomers were found to possess antifungal activity against *Aspergillus niger*.<sup>28</sup>  
21 Besides antimicrobial activity, some works also reported that the chitin oligomers,  
22 with molecular weight of 1-5 kDa, possess the ability to inhibit membrane protein  
23 oxidation and act as potent antioxidant in live cells.<sup>30,32</sup>

1 As a consequence of growing interest for converting chitin into its oligomers, some  
2 adjunct treatments, either chemical or physical in nature, have been employed to  
3 assist acid and enzymatic hydrolysis, as well as enhance the yield of oligomers.  
4 Gamma irradiation, ultrasonication, microwave irradiation, steam explosion,  
5 supercritical water, grinding and depressurization are physical methods that have  
6 been employed previously to facilitate chitin hydrolysis.<sup>33-38</sup> Aqueous solutions such  
7 as phosphoric acid, hydrochloric acid, alkaline solution and methanol modify chitin  
8 structures prior to hydrolysis. These chemical treatments have been reported to  
9 decrystallise chitin, increase its solubility, and accelerate subsequent enzyme  
10 hydrolysis.<sup>11,12,39</sup>

11 This article aims to review the methods which have been employed to intensify the  
12 formation of chitin oligomers, particularly focusing on the adjunct treatments to  
13 improve the hydrolysis, and evaluate the characteristics of the chitin and oligomers  
14 formed.

## 15 **PREPARATION OF CHITIN OLIGOMERS**

16 This section reviews the different hydrolysis methods employed and adjunct  
17 treatments proposed to assist depolymerization of chitin.

### 18 **Methods based on acidic hydrolysis**

19 Chitin oligomers are generally manufactured by acid hydrolysis of chitin employing  
20 strong acids such as concentrated hydrochloric acid (HCl) to effect the cleavage of  
21 the chitin polymeric chain.<sup>27,30,34,40-42</sup> The concentration of HCl, incubation time and  
22 temperature are the key parameters in the process. HCl concentrations ranging from  
23 3 to 12 N have been used to hydrolyze chitin at temperatures ranging between 20

1 and 90 °C for time durations ranging between 5 min and 7 h.<sup>27,30,32,40-42,44,45</sup> The  
2 processing steps employed by earlier researchers for hydrolysing chitin are  
3 summarized in Figure 2. In general, a given amount of chitin powder is added to HCl  
4 solution and the mixture is constantly stirred under reflux in a water bath maintained  
5 at the desired temperature.<sup>32,40,42</sup> After incubating thus for the desired time period,  
6 the hydrolysis is stopped by cooling the reaction mixture in an ice bath or on dry  
7 ice,<sup>40,44</sup> following which, the chitin oligomers are isolated by: 1) freeze-drying the  
8 solution under vacuum, 2) redissolving the dried product in deionised water, and 3)  
9 neutralising the solution with NaOH followed by filtration to remove impurities from  
10 the oligomers. The freeze-drying and redissolving steps have been repeated twice  
11 by some researchers to remove any residual HCl left.<sup>42</sup> Some researchers have also  
12 proposed neutralisation with 25% sodium hydroxide (NaOH) to stop the  
13 hydrolysis.<sup>30,32</sup> The neutralized solution is centrifuged to separate the supernatant,  
14 and the unhydrolyzed chitin and the supernatant are desalted and purified prior to  
15 spray-drying in order to obtain the chitin oligomers as a light yellow powder.

16 Recently, Kazami et al.<sup>45</sup> developed an acetone precipitation method as a  
17 replacement to NaOH neutralisation to claim a simpler procedure for isolating the  
18 chitin oligomers. Acetone-insoluble material can be recovered by the following steps:  
19 1) adding acetone to stop the hydrolysis and stirring at a low temperature for a day;  
20 2) centrifuging the mixture of acetone and chitin; 3) repeatedly washing the acetone-  
21 precipitate with acetone to remove HCl (until the pH of supernatant reaches 4-5);  
22 and 4) suspending the acetone-precipitate in cold diethyl ether, centrifuging and  
23 drying to constant weight.

1 The dried acetone-precipitate (acetone-insoluble material) is then mixed with water  
2 in order to extract the water-soluble chitin oligomers as follows: 1) mixing the dried  
3 acetone-precipitate with water, stirring overnight, and centrifuging in order to  
4 separate the supernatant and water-precipitate; 2) repeating step (1) for the water-  
5 precipitate; 3) combining supernatant recovered from steps (1) and (2) as water-  
6 soluble chitin oligomers; and 4) suspending the water-precipitate in cold diethyl  
7 ether, centrifuging and drying to constant weight. Generally, the supernatant  
8 containing the chitin oligomers obtained appear brown or yellow in colour. Therefore,  
9 activated charcoal treatment is applied to yield a clear solution prior to the final  
10 drying process.<sup>34,42</sup>

11 Chitosan can also be potentially converted into chitin oligomers by employing a two-  
12 step process involving: 1) depolymerization of chitosan by hydrolysis in HCl to form  
13 chitosan oligomers; and 2) partial *N*-acetylation of chitosan oligomer in hydro-  
14 alcoholic solution of acetic anhydride in order to produce the oligomers.<sup>42</sup> This  
15 approach generally requires numerous steps and produces HCl and acetic anhydride  
16 residues, which are undesirable products and have a significant impact on the  
17 environment.

18 Figure 3 shows a possible mechanism of chitin hydrolysis by concentrated HCl that  
19 has been reported previously by Kazami et al.<sup>45</sup> The chitin before hydrolysis is  
20 assumed to consist of alternating crystalline and amorphous regions, and composed  
21 a number of polymeric chains. Initially, the amorphous regions are rapidly cleaved  
22 within 5 min of hydrolysis to produce regular-sized segments with a central  
23 crystalline region attached to amorphous tails at both ends. The amorphous tails are  
24 then gradually degraded, leading to the accumulation of chitin oligomers, as well as

1 a crystalline chitin core consisting of multiple chitin chains. Single chitin chains may  
2 then be slowly separated from the chitin core, and once separated, be rapidly  
3 hydrolyzed to yield chitin oligomers within 30 to 60 min.

4 Although the hydrolysis process described above is effective, some disadvantages  
5 have been reported such as the occurrence of deacetylation (that produces chitosan  
6 oligomers instead of chitin oligomers), production of acidic waste streams, high cost,  
7 lower yield of high degree of polymerization (DP) oligomers, and requiring skilled  
8 labor force for purification.<sup>4,11,25</sup> The lower yield and shorter chain length of the  
9 oligomers formed, particularly dimers, adversely influence bioactivity.<sup>46</sup> In addition,  
10 the process costs and the environmental impact of the process are also high mainly  
11 due to the use of strong acids during hydrolysis.<sup>6</sup>

## 12 **Methods based on enzymatic hydrolysis**

13 In contrast to acidic hydrolysis, higher DP chitin oligomers can be produced under  
14 milder reaction conditions by employing enzymatic hydrolysis. Enzymes, mainly  
15 chitinases, which have higher chitinolytic activity, are used for this purpose, and are  
16 commonly produced from microorganisms, plants, and insects. Some researchers  
17 have used bacterium *Serratia proteamaculans* 568,<sup>47</sup> *Serratia marcescens* 2170,<sup>48</sup>  
18 *Rhizobium* sp. GRH2,<sup>49</sup> *Bacillus cereus* TKU027,<sup>6</sup> to produce chitinases, while  
19 others have used hevamine,<sup>50</sup> a plant enzyme, having both chitinase and lysozyme  
20 activities. Chitinases can also be successfully extracted from fungi such as  
21 *Lecanicillium lecanii* and *Lecanicillium fungicola*, while *Trichoderma reesei* fungi is  
22 reported to have hydrolases (cellulases and  $\beta$ -glucanases).<sup>11,36,39</sup> The enzymes  
23 produced from various sources have to be purified before being used for hydrolysis.

1 Additionally, non-chitinase commercially available enzymes, like cellulase,  
2 hemicellulase, pepsin, papain, lysozyme, and pectinase have also been reported to  
3 hydrolyse chitin.<sup>12,31,51</sup>

4 A flow diagram for the enzymatic hydrolysis of chitin is showed in Figure 4. Prior to  
5 hydrolysis, the substrate or chitin suspension is prepared by adding chitin powder to  
6 a phosphate or acetate buffer solution, so that its concentration is between 0.5 and  
7 2.0 % w/v.<sup>6,12,33,48,51</sup> Buffers strength in the range of 0.01 to 0.05 M and pH 5.0 to 5.5,  
8 are reported to provide optimum condition for the substrate preparation.<sup>6,12,31,36,51</sup>  
9 The enzyme is subsequently mixed with the substrate at an appropriate amount, so  
10 that its chitinolytic activity in the system is under the standard assay condition. In the  
11 hydrolysis, one unit of enzyme activity (1 U) is defined as the amount of enzyme  
12 releasing 1  $\mu\text{mol}$  GlcNAc per minute.<sup>52</sup> After mixing, the mixture is incubated at  
13 various temperature-time combinations, depending on the enzyme action. Hydrolysis  
14 by chitinases, lysozyme, pectinase, and pepsin have been reported to require  
15 incubation at temperatures between 37 and 44 °C.<sup>6,12,36,47,48,50,51</sup> To stop the reaction,  
16 the hydrolysis mixture is heated to 90 °C or boiled for 10 min, and subsequently  
17 centrifuged and filtered to separate the supernatant which contains the oligomers,  
18 and unhydrolysed chitin.<sup>11,31,36,51</sup>

19 Earlier studies have reported that the yield of high DP chitin oligomer resulting from  
20 enzymatic hydrolysis is greater compared to acidic hydrolysis.<sup>4</sup> This may be due to  
21 the enzyme acting selectively on the crystalline and otherwise inaccessible parts of  
22 chitin. During chitin hydrolysis, the enzymes which degrade the polysaccharide chain  
23 can be either endo-acting or exo-acting. Figure 5 shows the mechanism of endo-  
24 acting enzymes which randomly cleave glycosidic linkages of chitin, generate free

1 ends and chitin oligomers, while exo-acting enzymes release dimers (two units of  
2 GlcNAc) from the reducing (C1) or non-reducing (C4) ends.<sup>47</sup>

3 Of course, the use of enzymes also has its fair share of disadvantages: specific  
4 enzymes such as chitinase and chitosanases are not readily available commercially  
5 and, even if available, tend to be very expensive.<sup>37</sup> Further, the presence of protein  
6 residues after hydrolysis potentially limit biomedical application due to possible  
7 allergen and pyrogenicity<sup>25</sup> effects, which will warrant significant further purification  
8 that will make the whole process economically unviable. However, this method has a  
9 key advantage because minimum chemical wastes are produced during hydrolysis.<sup>6</sup>

#### 10 **Use of chemical and physical adjunct treatments to intensify chitin hydrolysis**

11 Irradiation of chitin has recently been used to assist acid or enzymatic hydrolysis.  
12 Ultrasonic irradiation or ultrasonication has been reported to be advantageous for  
13 depolymerization, because it preserves the chemical nature of the polysaccharide by  
14 simply splitting the most susceptible chemical bonds and lowering its molecular  
15 weight.<sup>53</sup> Takahashi et al.<sup>54</sup> and Ajavakom et al.<sup>34</sup> determined the effectiveness of  
16 ultrasonication during acid hydrolysis. In these investigations, the mixture of chitin  
17 and HCl was sonicated at various wavelengths for different durations. It has been  
18 found that the chitin powder completely dissolved within 30 min in the HCl solution  
19 during sonication at 50 or 60 Hz (275 W).<sup>34</sup> Takahashi et al.<sup>54</sup> noted that the amount  
20 of oligomers up to DP 7 (seven units of GlcNAc in each chain) increased after 120  
21 min of ultrasonication, which was 2 to 4 times greater than oligomers produced  
22 without ultrasonication. On the other hand, the chitin could be degraded during  
23 demineralization in an ultrasound-assisted extraction process. The depolymerization

1 occurring may be due to the application of high intensity irradiation, which results in  
2 breaking covalent bonds in the polymeric chain.<sup>55</sup> Ultrasonication has also been  
3 applied to the chitin suspension prior to enzymatic hydrolysis. In this process, the  
4 treated chitin depolymerized to a lesser extent, which was detected by the lower  
5 amount of reducing sugars measured.<sup>36</sup> All these studies agreed that ultrasonication  
6 facilitated acidic and enzymatic hydrolysis without drastically changing the degree of  
7 acetylation (DA) of the chitin. However, if high intensity ultrasonication was applied,  
8 the covalent bond in the polymeric chains of chitin could break due to cavitation by  
9 temporarily dispersing aggregates.<sup>55</sup>

10 Some researchers have measured ultrasonic intensity directly, while others have not.  
11 When the intensity is not measured, it is calculated by measuring the transient rise in  
12 temperatures during ultrasonication, and after it is switched off, as follows:

$$13 \quad I = \frac{mc_p}{\pi r^2} \left[ \left( \frac{dT}{dt} \right)_a - \left( \frac{dT}{dt} \right)_b \right] \quad (1)$$

14 where:  $I$  is the ultrasonic intensity,  $(dT/dt)_a$  is the slope of the initial rise in  
15 temperature,  $(dT/dt)_b$  is the slope of heat loss after the ultrasonic processor was  
16 turned off,  $m$  is the sample mass,  $c_p$  is the heat capacity of the solvent, and  $r$  is the  
17 radius of ultrasonic probe.<sup>55</sup>

18 Microwave irradiation has been established as a patented technique for producing  
19 chitin oligomers. Chitin is added to HCl and subjected to a conventional microwave  
20 device at 700 to 2100 W for up to 24 h.<sup>56</sup> This technique has been repeated by other  
21 researchers with a slight modification, where 38% HCl was initially pre-warmed at  
22 850 W by conventional microwave oven for a shorter time and the pre-warmed HCl

1 was quickly added to chitin powder for further irradiation at various reaction times.<sup>34</sup>  
2 Microwave irradiation has also been applied prior to the enzymatic hydrolysis of  
3 chitin. Roy et al.<sup>33</sup> used a microwave with built-in magnetic stirrer and non-contact  
4 infrared continuous feedback temperature system for chitin pre-treatment. In this  
5 study, the chitin suspended in acetate or phosphate buffer was irradiated with the  
6 microwave at optimum temperature and time reported to be 57.5 °C and 38 min,  
7 respectively; this was followed by hydrolysis with chitinase. This study found that the  
8 polar molecules in the chitin suspension align with the magnetic field generated by  
9 microwave, and have a tendency to accelerate the hydrolytic reaction rate. The  
10 microwave pre-treatment is comparable to ultrasonication, when the treated chitin is  
11 insignificantly deacetylated after irradiation.<sup>33</sup>

12 Improvement on the hydrolysis rate and chitin properties could be accomplished by  
13 gamma irradiation, which is one of the physical methods requiring no chemical  
14 additive and no temperature control during reaction.<sup>37,38</sup> Gamma irradiation, applied  
15 at different doses ranging from 15 to 210 kGy, to the solid form of chitin, prior to  
16 hydrolysis with chitinases, has been investigated.<sup>37</sup> Previously, this method was  
17 effectively applied as the adjunct treatment in the production of chitosan oligomer,  
18 using irradiation doses ranging between 2 and 500 kGy.<sup>57-59</sup> These authors  
19 suggested that the application of gamma irradiation at various doses may reduce the  
20 molecular weight of oligomers due to the breaking of glycoside bond.

21 The adjunct treatments discussed above are physical methods which influence the  
22 reaction through non-thermal effects. However, thermal treatments, such as steam  
23 explosion (SE), has also been investigated to influence the enzymatic hydrolysis of  
24 chitin. Steam explosion treatment consists in heating of chitin with saturated steam,

1 followed by a sudden decompression of the pressurized system to produce insoluble  
2 solid fraction and a liquid fraction of soluble sugars.<sup>60,61</sup> Villa-Lerma et al.<sup>36</sup> applied  
3 steam explosion to the mixture of chitin powder and deionized water at 180 °C and 1  
4 MPa for various reaction times. The treated chitin mixture was then added to  
5 phosphate buffer and this combination acted as substrate for hydrolysis with  
6 chitinase. It has been reported that steam explosion can significantly reduce chitin  
7 crystallinity without significant depolymerization occurring during treatment.

8 Another physical treatment that has been proposed to enhance and increase the  
9 oligomers yield is a combination of the use of supercritical water and mechano-  
10 chemical grinding with a ball mill.<sup>35</sup> Supercritical water is water at temperatures near  
11 or above 374 °C, meanwhile, the mechano-chemical grinding is a term used for the  
12 chemical reaction that occurs during mechanical treatment of the sample, typically  
13 grinding by ball mill.<sup>62-64</sup> After being treated with supercritical water, the chitin  
14 undergoes mechano-chemical grinding. This pre-treatment results in chitin flakes  
15 which are fragile and easy to grind, and form an effective substrate for hydrolysis  
16 with reduced particle size and molecular weight.<sup>35</sup>

17 In addition, chitin structures can also be pre-treated chemically by using aqueous  
18 solution, such as phosphoric acid, HCl, alkaline solution (mixture of sodium  
19 hydroxide and sodium dodecylsulfate), and methanol. These pre-treatments have  
20 reportedly decrystallized chitin, increased its solubility, and accelerated subsequent  
21 enzyme hydrolysis.<sup>11,12,39</sup> Ramírez-Coutiño et al.<sup>39</sup> deacetylated  $\alpha$  and  $\beta$ -chitin by  
22 deacetylation with alkali solution, thereby partially transforming chitin into chitosan  
23 (degree of deacetylation 55 and 50%, respectively) prior to hydrolysis. Reduction in

1 hydrogen bonds caused by the elimination of acetyl group increases the solubility of  
2 partially deacetylated chitin in aqueous media.<sup>65,66</sup>

### 3 **CHARACTERISTICS AND CHARACTERIZATION OF CHITIN AND ITS** 4 **OLIGOMERS**

5 This section summarizes the various methods used in literature to characterize chitin  
6 oligomers produced by chitin hydrolysis with and without the use of adjunct  
7 processes. Chemical structure and composition, degree of N-acetylation (DA),  
8 degree of polymerization (DP), molecular weight, and crystallinity are important  
9 properties characterizing chitin oligomers. The methods employed to determine  
10 these characteristics are discussed below.

#### 11 **Chemical structure and composition**

12 Fourier transform infrared (FT-IR) and proton nuclear magnetic resonance H NMR  
13 spectroscopies can be reliably used to record the composition and chemical  
14 structure of the oligomers.<sup>30,32,34,36,37,39,41-43,55</sup> These methods require simpler  
15 procedures to prepare samples, and provide information on the chemical structure  
16 faster than conventional methods.<sup>67</sup>

#### 17 **Fourier transform infrared spectroscopy (FT-IR)**

18 FT-IR spectroscopy is a very attractive technique suitable for both soluble and  
19 insoluble samples.<sup>68</sup> The technique is based on the vibrations of the atoms of a  
20 sample molecule. Infrared spectrum is obtained by passing infrared radiation through  
21 the sample and determining the fraction of the incident radiation absorbed at a

1 particular energy. The energy at any peak in an absorption spectrum corresponds to  
2 the frequency of a vibration of a part of the sample.<sup>69</sup>

3 The infrared spectrum can be divided into three main frequencies which is far-  
4 infrared ( $< 400 \text{ cm}^{-1}$ ), mid-infrared ( $4000 - 400 \text{ cm}^{-1}$ ) and near-infrared ( $13000 - 4000$   
5  $\text{cm}^{-1}$ ). To obtain the FT-IR spectrum of the chitin and its oligomers, mid-infrared  
6 frequency is used as explained by Lambert-Beer Law.<sup>27,30,36,55,70</sup> The law states that  
7 the absorbance values at a given frequency of  $4000$  to  $400 \text{ cm}^{-1}$  are proportional to  
8 the concentrations of the sample. Measurements are initiated by preparing KBr  
9 (potassium bromide) pellet, which is a small amount of chitin sample well mixed with  
10 the KBr (approximately  $0.1$  to  $1.0 \%$  w/w) and pressed into a pellet-forming die. The  
11 measurement is taken by scattering the infrared light onto the KBr pellet using FT-IR  
12 system.<sup>30,70,71</sup> This is the most common method used, which utilizes the property of  
13 KBr as alkali halide that becomes plastic when subjected to pressure and forms a  
14 transparent sheet in the infrared region.<sup>72</sup>

15 In a chitin molecule, the acetamido group at the position C2 (Figure 5),  
16 intramolecular hydrogen bonds ( $\text{C6-OH}\cdots\text{O}=\text{C}$ ) and intermolecular hydrogen bonds  
17 ( $\text{NH}\cdots\text{C}=\text{O}$ ) provide polymer stability.<sup>73</sup> In  $\alpha$  form, the absorption bands of carbonyl  
18 group (amide I) stretching are split at  $1660 \text{ cm}^{-1}$  and  $1620 \text{ cm}^{-1}$ , which are attributed  
19 to intermolecular and intramolecular hydrogen bonds respectively.<sup>73,74</sup> Unlike  $\alpha$   
20 crystalline form, a single absorption band of amide I  $\beta$ -chitin is observed at  $1560 \text{ cm}^{-1}$   
21 which is attributed to the stretching of carbonyl group hydrogen bonded to amide  
22 group of the intra-sheet chain.<sup>74</sup> In previous research,  $\alpha$ -chitin extracted from  
23 *Daphnia magna* resting egg (zooplankton genus in aquatic ecosystems) exhibited  
24 absorption bands at  $1652 \text{ cm}^{-1}$  and  $1622 \text{ cm}^{-1}$  for amide I.<sup>73</sup> Recently, the chitin

1 extracted from six different aquatic invertebrates presented the spectrum of amide I  
2 at 1652-1656  $\text{cm}^{-1}$  and 1619-1620  $\text{cm}^{-1}$ , thus allowing them to be characterized as  $\alpha$   
3 crystalline form.<sup>75</sup> These two strong absorption bands were also observed in  
4 ultrasonication and steam explosion treated chitin. However, an additional shoulder  
5 was present at 1633  $\text{cm}^{-1}$  in ultrasonication treated chitin which might be due to a  
6 reduction in amino-based hydrogen bonding.<sup>36</sup> In chitin oligomers structure, the  
7 spectrum generated by FT-IR exhibits intense absorption bands at 3358  $\text{cm}^{-1}$  (OH  
8 stretch), 2917  $\text{cm}^{-1}$  (C-H stretch), 1651  $\text{cm}^{-1}$  (C=O stretch, amide I), 1548  $\text{cm}^{-1}$  (N-H  
9 bend, amide II), 1313  $\text{cm}^{-1}$  (C-N stretch, amide III), and 1150-1000  $\text{cm}^{-1}$  (pyranose),  
10 as shown in Figure 6.<sup>30</sup> All the reported FT-IR spectra indicate that the crystalline  
11 structure formation of intact chitin, treated chitin and its oligomers are not affected by  
12 the types of processes.

### 13 **Proton nuclear magnetic resonance ( $^1\text{H}$ NMR) spectroscopy**

14  $^1\text{H}$  NMR spectroscopy is a powerful and reliable technique for polymer structural  
15 analysis by exploiting the magnetic properties of certain atomic nuclei. The spectra  
16 of chitin and its oligomers can be obtained by cross-polarization or magic-angle-  
17 spinning method using deuterated oxide ( $\text{D}_2\text{O}$ ) as solvent.<sup>31</sup>

18 In chitin, the chemical composition obtained by  $^1\text{H}$  NMR resonated between 3.6 and  
19 4.4 ppm, which are assigned to H-2 to H-6 protons, while acetyl protons are found at  
20 2.6 ppm.<sup>74,43</sup> The anomeric region of  $\alpha$ - and  $\beta$ -anomer (H-1) generated peaks at 5.43  
21 and 5.05 ppm, respectively.<sup>43</sup> As observed in chitin oligomers, the spectra present  
22 two singlets at 2.06/2.08 ppm assigned to the N-acetyl protons, and a broad signal at  
23 5.20 ppm assigned to H-1 protons of the reducing end  $\alpha$  anomer residues.<sup>42</sup> The

1 chitin oligomers produced by enzymatic hydrolysis of chitin using commercial  
2 enzymes possessed obvious resonance peak at 1.9 ppm assigned to the acetyl  
3 group, 3.4 ppm detected for H-2 and 3.5 to 3.8 ppm corresponding to H-3, H-4, H-5  
4 and H-6 protons, meanwhile the H-1 ( $\beta$ ) and H-1 ( $\alpha$ ) protons generated peaks at  
5 around 4.6 to 4.7 ppm and 5.2 ppm, respectively.<sup>31</sup> Ngo et al.<sup>30</sup> reported the  
6 resonance of chitin oligomers hydrolyzed by acid (HCl) at 1.97 ppm is assigned to  
7 acetyl group, 3.34 ppm is corresponded to H-2 , 3.37 to 3.77 ppm are detected for H-  
8 3, 4, 5, 6, 4.5 to 4.6 ppm is assigned to H-1 ( $\beta$ ) and 5.0 ppm is corresponded to H-1  
9 ( $\alpha$ ). All spectra of chitin are essentially identical to the oligomers, regardless of the  
10 type of hydrolysis. It is caused by the occurrence of glycosidic linkages breakdown  
11 during the hydrolysis without the interference of deacetylation.<sup>41</sup>

## 12 **Degree of *N*-acetylation**

13 The degree of *N*-acetylation (DA) represents the molar fraction of *N*-acetylated units  
14 in the chitin polymer chain.<sup>76</sup> The DA is an important parameter influencing physico-  
15 chemical, electrostatic and biological properties of chitin.<sup>77</sup> Generally, the DA can be  
16 determined by the calculation of the absorbance and intensities generated by FT-IR  
17 and NMR, respectively. The absorbance values (A) obtained by FT-IR can be used  
18 to measure DA by using the following equation:

$$19 \quad \% \text{ N-acetylation} = (A_{1655}/A_{3450})(100/1.33) \quad (2)$$

20 where,  $A_{1655}$  and  $A_{3450}$  are the absorbances at  $1655 \text{ cm}^{-1}$  of the amide-I band and  
21  $3450 \text{ cm}^{-1}$  of the hydroxyl band, respectively; and the factor of 1.33 is the ratio of  
22  $A_{1655}/A_{3450}$  for fully *N*-acetylated chitin.<sup>55</sup> However, Chang et al.<sup>40</sup> determined the

1 percentage of DA by using the following equation, originally proposed by Baxter et  
2 *al.*<sup>78</sup>:

$$3 \qquad \qquad \qquad \% \text{ N-acetylation} = (A_{1655}/A_{3450})(115) \qquad \qquad \qquad (3)$$

4 The values of 100/1.33 (i.e. 75.2) and 115 are reciprocal values of the slope of the  
5 linear section of the plot of absorption ratio ( $A_{1655}/A_{3450}$ ) against DA, which depend  
6 on the baselines used.<sup>67,70</sup> Eq (3) is more reliable in comparison with Eq (2), since  
7 Eq (2) tended to overestimate values for DA > 20.<sup>67</sup>

8 The DA can also be quantitatively analysed by carbon nuclear resonance magnetic  
9 (C-NMR), where the relative intensities determined for the resonance of the ring  
10 carbon ( $I_{C1}$ ,  $I_{C2}$ ,  $I_{C3}$ ,  $I_{C4}$ ,  $I_{C5}$ ,  $I_{C6}$ ) and methyl carbon ( $I_{CH3}$ ) is used in the following  
11 equation proposed by Ottey et *al.*<sup>79</sup>:

$$12 \qquad \qquad \qquad \% \text{ N-acetylation} = \frac{I_{CH3}}{(I_{C1}+I_{C2}+I_{C3}+I_{C4}+I_{C5}+I_{C6})/6} \times 100 \qquad \qquad \qquad (4)$$

13 Eqn (4) has been applied in earlier work on chitin.<sup>68,70,80</sup>

14 Fully acetylated chitin provides DA of 100% while 0% corresponds to completely  
15 deacetylated chitin (chitosan).<sup>67</sup> In previous work, the DA of chitin decreased from  
16 98% to 93, 88 and 73% after exposure to ultrasonication, steam explosion and  
17 depressurization, respectively.<sup>36,38</sup> The lower DA values after adjunct treatment  
18 indicate deacetylation and modification of chitin structure. However, the lowest DA of  
19 depressurized chitin gave higher yield of the oligomers. It has been reported that the  
20 DA values of chitin between 45 and 55% provide excellent solubility in aqueous  
21 media.<sup>38</sup> Some authors have reported that the DA between 40 and 60% possessed

1 suitable characteristics for enzymatic hydrolysis, where the chitin solubility reached  
2 was greater than 60%.<sup>39,81</sup> It caused the polarity and electrostatic repulsion of the  
3 amino groups increased, thus increasing the accessibility of chitin to enzymatic  
4 attack.<sup>39</sup>

## 5 **Degree of polymerization**

6 Degree of polymerization (DP) is a significant parameter to identify the number of  
7 monomeric units in the oligomers. MALDI-TOF mass spectrometry is a powerful  
8 technique, which has been extensively applied to determine the DP of chitin and  
9 chitosan oligomers.<sup>6,36,42,82,83</sup> Figure 7 shows the MALDI-TOF spectra which consists  
10 of high intensity of a number of peaks assigned to a certain DP. The DP of the  
11 oligomers can be determined by the peak-to-peak mass difference of 203, which is  
12 the GlcNAc repeating unit ( $C_8H_{13}NO_5$ ). The end-groups of H and OH are deduced  
13 from monoisotopic mass. Each DP can be calculated from a peak with particular  
14 mass unit (m/z) as follows:

$$15 \quad DP = (\text{mass unit of a peak} - H - OH - Na) / C_8H_{13}NO_5 \quad (5)$$

16 where DP is the degree of polymerization. The molecular mass of  $C_8H_{13}NO_5$  is 203  
17 (i.e. mass unit of chitin oligomers); the atomic mass of H is 1 (hydrogen); and those  
18 of the OH group and Na are 17 and 22.99, respectively.<sup>42</sup>

19 The DP can also be determined by using high performance liquid chromatography  
20 (HPLC), where the concentration of each DP present can be calculated from the  
21 peak areas in the HPLC profile using the standard curve obtained from pure chitin  
22 monomer, dimer, trimer, tetramer, pentamer, and hexamer standard  
23 solution.<sup>6,11,12,40,57</sup> Moreover, the DP can be quantitatively analysed by integrating the

1 signals of the anomeric protons (H-1 ( $\alpha$ ) and H-1 ( $\beta$ )) obtained by  $^1\text{H}$  NMR using the  
2 following equation:

$$3 \quad \text{DP}_n = \frac{[\text{H-1 } (\alpha) + \text{H-1 } (\beta) + \text{H-1c}]}{[\text{H-1 } (\alpha) + \text{H-1 } (\beta)]} \quad (6)$$

4 where  $\int\text{H-1 } (\alpha)$  and  $\int\text{H-1 } (\beta)$  refer to the integral of the H1 protons of the chitin  
5 oligomers at terminal reducing end having  $\alpha$  and  $\beta$  configuration, respectively, and  
6  $\int\text{H-1c}$  is the sum of integrals of the H1 protons of all the central units.<sup>84</sup>

7 As reported by Chang et al.<sup>40</sup>, the chitin hydrolysed in acid (HCl) produced oligomers  
8 with DP in the range of 2 to 6. Moreover, the oligomers with DP 4-6 were simply  
9 isolated from acetone-precipitation method.<sup>45</sup> In another study, the chitin oligomers  
10 produced by acetylation of chitosan oligomers with various DA up to 90% using  
11 acetic anhydride solution and isolated in HCl resulted in a value of DP ranging from 3  
12 to 7.<sup>42</sup> As an alternative to acid hydrolysis, enzymatic hydrolysis has been  
13 extensively used to obtain oligomers with higher DP. As observed by Wang et al.<sup>6</sup>  
14 and Purushotham et al.<sup>47</sup>, the oligomers obtained by hydrolysis with chitinases  
15 possessed DP in the range between 2 and 9. In theory, the hydrolysis must  
16 ultimately lead to the formation of chitobiose (DP 2). However, products with DP 4  
17 and higher show better functional properties as compared to DP 2. Chitinase and  
18 lysozyme from hevamine were reported to degrade chitin polymer to form pentamers  
19 (DP 5).<sup>70</sup> Commercial enzymes such as hemicellulase and pectinase have also  
20 successfully depolymerized chitin to DP 6.<sup>31</sup> Based on the above studies, it can be  
21 concluded that both acid and enzymatic hydrolysis result in higher DP of chitin  
22 oligomers. However, the enzymatic hydrolysis condition is milder than acid  
23 hydrolysis.

## 1 **Crystallinity**

2 Chitin has a highly ordered crystalline structure, and degrading into oligomers may  
3 reduce its crystallinity. Generally, the crystallinity of chitin and its oligomer can be  
4 evaluated using X-ray diffraction measurements. The peak intensity of chitin is  
5 recorded over the scattering range of  $4.5^\circ$  to  $50^\circ$  with scan steps of  $0.02^\circ$  at a speed  
6 of  $4.0^\circ \text{ min}^{-1}$ .<sup>36,45</sup> The crystallinity index ( $I_{CR}$ ) is generally measured by a method,  
7 which uses a maximum intensity of 110 ( $I_{110}$ ) and the intensity of amorphous halo  
8 contribution ( $I_{am}$ ),<sup>36,45,55,85</sup> as follows:

$$9 \quad I_{CR} = ((I_{110} - I_{am}) / I_{110}) \times 100 \quad (7)$$

10 The crystallinity index provides an idea on the crystalline fraction in chitin and its  
11 derivatives. The chitin exposed to adjunct treatment may be susceptible to  
12 depolymerization with crystalline fractions reduced. The crystallinity of steam  
13 explosion and depressurization treated chitin were reduced from 88 to 73%. These  
14 treated chitin samples were hydrolyzed with chitinases, and produced higher amount  
15 of oligomers compared to untreated chitin.<sup>38</sup> The chitin that has been treated with a  
16 combination of supercritical water and mechano-chemical grinding exhibited higher  
17 reduction of  $I_{CR}$  from 91 to 26%.<sup>35</sup> Ilankovan et al.<sup>12</sup> reported that chitin chemically  
18 treated with phosphoric acid, sodium hydroxide and methanol showed more  
19 amorphous nature with lower intensities of the 110 reflection. The adjunct treatment  
20 seems to result in chitin with lower crystallinity, which is more amenable to enzyme  
21 action in order to produce oligomers.

## 22 **Molecular weight**

1 Molecular weight (M) of chitin oligomers can be expressed in various ways, such as  
2 weight-average (Mw), number-average (Mn), and viscosity-average (Mv) molecular  
3 weights. Mn and Mw represent the total weight of the oligomers molecules, i.e  
4 GlcNAc and GlcN, divided by the total number of its constituting molecules and a  
5 sum of the weight fraction of each type of molecules multiplied by its molecular  
6 weight, respectively.<sup>35</sup> Mv can be determined from Mark-Houwink equation:

$$7 \quad [\eta] = k(Mv)^\alpha \quad (8)$$

8 where  $[\eta]$  ( $\text{cm}^3 \text{g}^{-1}$ ) is the intrinsic viscosity of chitin measured by viscometer, k is  
9  $0.24 \text{ cm}^3 \text{g}^{-1}$  and  $\alpha$  is 0.69, where, both k and  $\alpha$  are constant parameters of  
10 chitin.<sup>27,86</sup>

11 Measurement of the molecular weight of chitin and its oligomers commonly can be  
12 done by gel permeation chromatography (GPC) or size exclusion chromatography  
13 (SEC), which is generally equipped with refractive index detector, a GPC (gel  
14 permeation chromatography) column, and a guard column.<sup>35,87-89</sup> The relative mean  
15 molecular weight of the chitin is estimated by the Pullulan standard curve.<sup>35</sup> Prior to  
16 measurement, samples for GPC require microfiltration (with  $0.45 \mu\text{m}$  filters) .<sup>13,90</sup>

17 The molecular weight is a significant parameter for effective hydrolysis of chitin.  
18 Kurita et al.<sup>81</sup> reported that the chitin molecular weight of 300 kDa was effective for  
19 enzymatic hydrolysis. It has already been mentioned that the adjunct treatments  
20 applied may reduce the molecular weight of chitin prior to hydrolysis. Dziril et al.<sup>37</sup>  
21 reported that the molecular weight of gamma-radiated chitin decreased to 60% when  
22 irradiated with 50 kGy, and it further decreased to 90% when the applied dose was  
23 210 kGy. The application of supercritical water treatment combined with mechano-

1 chemical grinding reduced the molecular weight of chitin from 800 kDa to 10 kDa.<sup>35</sup>  
2 Ramírez-Coutiño et al.<sup>39</sup> obtained the molecular weight of 343.5 kDa for α-chitin with  
3 90% of solubility after treating it with alkali. This molecular weight decrease is mainly  
4 due to the glycoside bond breaking caused by the treatment of chitin.<sup>37</sup>

5 On the other hand, the molecular weight of chitin oligomers produced by hydrolysis  
6 is lower than native chitin. Kazami et al.<sup>45</sup> reported that the number-average  
7 molecular weight of both α- and β-chitin rapidly decreased to 13 kDa and 10 kDa  
8 from 241 kDa and 90 kDa, respectively, after 15 min of hydrolysis in HCl. At the  
9 same time, the weight-average molecular weight of α- and β-chitin also reduced to  
10 approximately 16 kDa from 330 kDa and 250 kDa, respectively. Previous study  
11 reported that the chitin oligomers with molecular weight of 1 to 3 kDa was more  
12 effective as antioxidant agents those with molecular weight of 1 kDa and lower.<sup>32</sup>  
13 Thus, the biological effect of chitin oligomers is significantly dependent on the  
14 molecular weight.<sup>91</sup>

## 15 **Oligomers yield**

16 Yield of the oligomers after hydrolysis is a significant parameter because it  
17 determines the economic viability of the process. The yield can be expressed simply  
18 as the percentage of the chitin hydrolyzed as follows:

$$19 \quad \% \text{ Yield} = \frac{W_1 - W_2}{W_1} \times 100 \quad (9)$$

20 where,  $W_1$  and  $W_2$  are the initial weight and weight of chitin after hydrolysis,  
21 respectively.<sup>31,40</sup> The yields reported earlier for acidic hydrolysis of chitin lie between  
22 10 and 21%.<sup>40,45</sup> These values are comparable with enzymatic hydrolysis. Ilankovan

1 et al.<sup>12</sup> and Hongkulsup<sup>31</sup> reported the yield of chitin oligomers hydrolyzed by  
2 commercial enzymes were in a range of 10% to 13%. Although this is a low yield, it  
3 can be increased by modifying chitin structure prior to hydrolysis by employing some  
4 of the adjunct methods discussed in this paper.

5 High performance liquid chromatography (HPLC) is an efficient technique for  
6 quantification of the oligomers according to each DP.<sup>6,11,31</sup> The amount of each DP  
7 (GlcNAc<sub>n</sub>) can be estimated with the calibration curve, as shown in the following  
8 equation:

$$9 \qquad C_1 = C_2 \times (A_1/A_2) \qquad (10)$$

10 where, C<sub>1</sub> is the sample concentration (mg/mL), C<sub>2</sub> is the standard concentration  
11 (mg/mL), A<sub>1</sub> is the peak area of sample and A<sub>2</sub> is the peak area of standard.<sup>6</sup> Wang  
12 et al.<sup>6</sup> found the yield of the GlcNAc<sub>2</sub>, GlcNAc<sub>3</sub>, GlcNAc<sub>4</sub> and GlcNAc<sub>5</sub> were 0.44  
13 mg/mL, 0.08 mg/mL, 0.09 mg/mL and 0.43 mg/mL, respectively, after chitin  
14 depolymerizing under fermentation condition for two days. The results obtained show  
15 that the concentrations of GlcNAc<sub>2</sub> and GlcNAc<sub>5</sub> are significantly higher than  
16 GlcNAc<sub>3</sub> and GlcNAc<sub>4</sub>. Unlike fermentation, the hydrolysis of chitin by commercial  
17 enzymes like hemicellulase and pectinase produce higher amounts of GlcNAc<sub>3</sub>,  
18 GlcNAc<sub>4</sub> and GlcNAc<sub>5</sub> as reported by Hongkulsup<sup>31</sup>. In acid hydrolysis, higher acid  
19 concentration and temperature can influence the amount of chitin oligomers formed.  
20 The amount of GlcNAc<sub>2</sub>, GlcNAc<sub>3</sub> and GlcNAc<sub>5</sub> apparently increased when the acid  
21 concentration increased from 4 N to 7 N during hydrolysis at 70 °C.<sup>40</sup> The amount of  
22 GlcNAc<sub>2</sub> produced can be 30 times greater when the hydrolysis temperature is  
23 raised to 90 °C.<sup>40</sup>

## 1 **CONCLUSION**

2 Chitin subjected to adjunct treatments allows modification of the native structure, and  
3 improve the hydrolysis rate and product functionality. Microwave irradiation on the  
4 chitin enhances access to the susceptible bonds for enzymatic hydrolysis and  
5 reduces the reaction time for hydrolysis. Crystallinity and DA of the chitin reduced  
6 when steam explosion was applied prior enzymatic hydrolysis. Furthermore, gamma  
7 irradiation, and the combination of supercritical water and grinding, can reduce chitin  
8 molecular weight and partially depolymerize it in order to facilitate enzyme attack on  
9 the substrate. It is somewhat unfortunate that the literature reviewed in this study  
10 does not specifically state the extent of yield improvement with statistical confidence,  
11 which would have enabled us to establish commercial and economic viability of  
12 using adjunct treatments. Nevertheless, one can conclude that chitin polymer  
13 reacting with acid or enzymes, particularly HCl or chitinases, can successfully  
14 produce oligomers with DP between 2 and 6. The chitin oligomers are also reported  
15 to have a potential to be commercialized for further applications, specifically as  
16 antimicrobial agents. This is due to the simple preparation method and the quality of  
17 oligomers produced without the need for deacetylation into chitosan. Unlike acid, the  
18 enzymatic hydrolysis may be considered to be more favourable due to the use of  
19 milder reaction conditions and environmental compatibility. Commercial enzymes  
20 may also be used to simplify the process and improve economics by reducing the  
21 cost of specific enzymes extraction and purification.

22 In conclusion, all adjunct treatments reported so far, enhance the hydrolysis of chitin,  
23 regardless of the hydrolysis method employed. For future research, it would be worth  
24 trying pre-treatments like the application of high-pressure to chitin in order to lower

1 its crystallinity. At the same time, it is also important to statistically quantify the  
2 improvements produced by such treatments so that their viability can be conclusively  
3 established.

4

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