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Chitosan can be obtained from the deacetylation of chitin. This process is however difficult and usually accompanied by depolymerization, affording low molecular weight chitosan. We report a novel path, relying on a combination of mechanochemistry and aging, to afford high molecular weight chitosan with minimal use of energy and solvent. This method is versatile and applicable to a number of chitin sources, including crude crustaceans and insect shells, yielding deacetylation up to 98% and remarkably high molecular weights. Chitin deacetylation was measured by magic angle spinning nuclear magnetic resonance and molecular weight by viscometry. This process affords chitosan in a safer fashion and with less materials and energy usage than the classic hydrothermal one.

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Synthesis of high molecular weight chitosan from chitin by mechanochemistry and aging

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Chitosan can be obtained from the deacetylation of chitin. This process is however difficult, because of the low solubility of chitin in most solvents, and usually accompanied by depolymerization, which limits access to high molecular weight chitosan. We report herein a novel path, relying on a combination of mechanochemistry and aging, to afford high molecular weight chitosan with minimal use of energy and solvent. We demonstrate that this method is versatile and applicable to a number of chitin sources, including crude crustaceans and insect shells, yielding deacetylation up to 98% and remarkably high molecular weights. Chitin deacetylation was measured by magic angle spinning nuclear magnetic resonance and molecular weight by viscometry. This process affords chitosan in a less dangerous fashion and with less materials and energy usage than the classic hydrothermal one.

Introduction

Biopolymers are triggering intense research interest for they are envisaged as renewable sources for materials and molecules^{1,2}. Chitin is the second most abundant naturally produced polymer with yearly production levels in the billions of tonnes.^{3,4} It is a natural polysaccharide composed of β -(1–4)-linked 2-deoxy-2-acetamido-D-glucose units.⁵ Its amide functionality constitutes an interesting manifold for functionalization and applications.⁶ Chitin may be deacetylated to afford chitosan, an added-value polymer with greater manipulability and solubility in water than chitin due to its free amine group. Chitosan has been developed into many functional materials from biocompatible compounds to every day commodities. Chitosan membranes feature tunable permeability to sodium chloride, glucose, tyrosine and bovine serum protein.⁷ Chitosan has also been investigated for protein delivery, with both bovine⁸ and human serum albumin.¹⁵ Successful chondrocyte growth onto chitosan scaffolds¹⁰ as well as fibroblast cell attachment and proliferation¹¹ show promise for tissue growth. Water treatment with low concentrations of chitosan of 0.8 wt% or less, completely eliminated bacterial contaminations.^{12,13} The use of chitosan hydrogels in drug delivery,¹⁴ chitosan encapsulated quantum dots for anti-cancer drug delivery¹⁵ and controlled drug release¹⁶ has also been investigated. Carboxymethyl chitosan has been used in cosmetics as a thickening agent due to its high viscosity and large hydrodynamic volume.¹⁷ There is a need to replace polluting, non-biodegradable plastics¹⁸ and packaging made from chitosan shows great promise due to its biodegradability and improved tensile strength when prepared by evaporation method.¹⁹ Chitosan's ability to chelate many metals, due to its amine

functional group, allows it to be used as a heterogeneous catalyst support for organic transformations.²⁰ Across its many applications, the degree of deacetylation (DDA, or the percentage of amide functions converted to amine ones) and the molecular weight of chitosan played a major role in tune tuning its properties.^{7,12,21} Thus the ability to precisely control these two parameters is essential.

Commercial chitin is most commonly extracted from crustaceans by undergoing a heterogeneous deproteinization process, where the crustacean exoskeleton is treated with NaOH from mild to harsh conditions to remove proteins in the cuticle.²² The second step to chitin extraction is a demineralization process where the exoskeleton is exposed to dilute hydrochloric acid (HCl) at room temperature.²³ Finally chitin is deacetylated to afford chitosan. All these steps are complicated by the absence of solubility of chitin in most common solvents. Chitin is only slightly soluble in highly polar solvent systems such as N,N-dimethylacetamide/lithium chloride (DMAc/LiCl).²⁴ The most common and commercially-used chitin deacetylation method consists of a treatment in a highly concentrated NaOH solution ($\geq 50\%$) heated for several hours at temperatures above 100 °C and often repeated in multiple cycles to further deacetylation.^{25–28} This affords chitosan with good DDAs and medium sized molecular weights between 80 to 800kDa. Other methods have been explored in an effort to produce a greener and safer process: maceration,²⁹ steam explosion,³⁰ freeze-thaw cycles,³¹ high temperature and pressure,^{32,33} sonication,²⁹ microwaving,³⁴ and planetary milling.³⁵ These methods typically afford DDA in the 70 to high 90% values. Experimental details and results of these methods are detailed in Table S1. For these methods, molecular weights are not systematically reported. Yet when they are,

molecular weight typically under 500 kDa, with the exception of the high temperature and pressure method where the chitin is mixed with a 50% NaOH solution and heated to 120 °C from 3-24 hours in an autoclave at 15 psi. The DDA reaches up 90.4% with 50% NaOH solutions while maintaining MW of 1560 kDa.^{32,33}

Mechanochemistry, the use of mechanical energy to activate chemical transformations, is currently the topic of intense research effort,^{36,37} in particular for biomass conversion.^{38,39} Chitin is no exception and the group of Yan has recently revealed that mechanochemistry was particularly effective to this end *via* the use of planetary milling with a large number of balls resulting in chitosan oligomers with DDA of 76.4% and MW of 6.3 kDa.³⁵ They expanded this chemistry with the group of Kerton to achieve conversion of chitin into N-acetyl-D-glucosamine monomer and dimers with 5.1 and 3.9 wt% respectively *via* milling showing that harsh milling conditions can depolymerize chitin.⁴⁰ Accelerated aging has also emerged as a method to access inorganic and organic materials with very low energy input.⁴¹⁻⁴³ For chitin deacetylation, low temperature solution aging²⁸ has been reported and yields highly deacetylated chitosan with ~90% DDA, at the expenses of aging times close to a month. Also, although the MW was not formally measured, depolymerization levels above 70% were phenomenologically established by the excellent water solubility of the resulting samples.

In these methods, the depolymerization of chitin/chitosan by nucleophilic attack of the chitin glycosidic bonds by hydroxide ions occurs simultaneously to deacetylation. While depolymerization is a desired reaction outcome to afford low-molecular weight chitosan, and glucosamine monomers,⁴⁰ less work was done towards high MW chitosans (>1,000 kDa). Researchers have explored decreasing temperature and NaOH concentration, but it comes at the cost of a significant increase in time, and overall lower DDA.²⁹⁻³⁴ High temperature and pressure methods can yield highly deacetylated chitosan with high molecular weights although the process is energy intensive and potentially dangerous for scale-up.³³ As biopolymers are sought after as ideal precursors for high-end functional material, there is merit in exploring avenues to preserve the polymer chain as much as possible.⁴⁴ Indeed, high molecular weight (MW) polymers feature improved mechanical properties^{9,10,12} and glass transition temperature.⁴⁵ Despite its high added value applications, chitin remains a largely underutilized biomass waste: there is thus a dire need to develop simpler methods of extractions, and methods able to afford a broader range of products are needed to implement the *Shell Biorefinery* theorized by Yan.^{6,46} Such a method should also seek to reduce energy, chemical and solvent input for improved sustainability, while being safe.

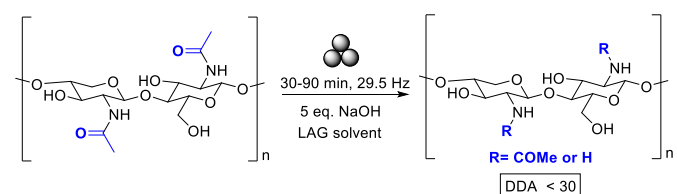
Below we present a study of chitin deacetylation in the solid state with NaOH with the aim to preserve the polymer chain. We have explored the use of low energy mechanochemistry, using mixer milling with only one ball, as well as aging. The role of time, quantity of NaOH, humidity, and the effect of an amorphization pre-treatment of chitin was carefully studied. We also used a number of chitin sources, including commercial chitin,

powdered shrimp shell, crab shell, lobster, black fly larvae and *Bombyx eri* larvae shells. Combining mechanochemistry, in absence of NaOH, with aging in the presence of the base, resulted in high MW chitosan with excellent DDA levels (73-95%). At these very high MW values, chitosans, even with DDA of 95% upwards, were not soluble under acidic aqueous conditions so we used a method based on viscometry with 1-ethyl-3-methylimidazolium acetate ([C₂mim][OAc]) as a proxy for estimating MW values. DDA was calculated by ¹³C magic angle spinning nuclear magnetic resonance (MAS NMR).

Results and discussions

Mixer-mill based chitin deacetylation experiments

High energy milling methods, such as the use of a planetary mill with several dozen balls, were previously reported to yield very efficient deacetylation of chitin, at the expense, however, of significantly reduced molecular weights.³⁵ We first decided to explore the use of lower energy milling mechanochemical methods, in an effort to obtain chitosan with distinct properties, namely high DDA and high MW. Milling using a Retsch MM 400 mixer mill with a 20 mL polytetrafluoroethylene (PTFE) jar and one 10 mm ZrO₂ ball was used as a soft solid-state reaction method (Scheme 1). In all methodology development experiments, commercial chitin (DDA=4%) was used as a starting material (Table S2). Initial attempts at milling chitin with 5 equivalents of NaOH resulted in minor deacetylation (7%) when the samples were milled for 30 minutes and worked up immediately with methanol to remove excess NaOH and sodium acetate by-product (Scheme 1). Increasing milling time, up to 90 min, yielded no greater degree of deacetylation (Table S3). In an effort to improve deacetylation, the addition of stoichiometric amounts of liquids of <1μL/mg,⁴⁷ also referred to as liquid assisted grinding⁴⁸ (LAG), was attempted. This method has proved useful for instance for co-crystal formation⁴⁹ or metal organic framework synthesis.⁵⁰ Dichloromethane, acetonitrile, ethyl acetate ethanol, methanol, and deionized water were tested for their effect in chitin deacetylation (Table S3).



Scheme 1: Mixer-mill based chitin deacetylation experiments. DDA = $[H]/([COMe]+[H]) \times 100$, LAG solvent 10 wt%: none, dichloromethane, acetonitrile, ethyl acetate, ethanol, methanol, deionized water. Typical experimental conditions: chitin (105 mg), NaOH, 5 eq. (95 mg) based on glucosamine unit, loaded to a PTFE jar with a ZrO₂ ball.

The best result obtained with this strategy was 23% DDA while using the 20% deionized water LAG 1:5 chitin:NaOH mixture (Table S3). In practice, chitin is considered to have been converted to chitosan once its DDA reaches 50%, yet, for application, chitosan featuring DDA greater than 70% is preferred for their improved solubility in dilute acid.^{8,10-17,19,20,51-53} This first attempt at soft mechanochemistry and LAG

was not conclusive, and we reasoned that the lack of amorphization could be problematic in favoring the reaction.

Mechanochemical amorphization of chitin as a pre-treatment to deacetylation

It is well-known that crystallinity in biopolymer restricts their reactivity. For instance, the acid hydrolysis of cellulose occurs more readily onto amorphous regions.⁵⁴ Commercial chitin itself is highly crystalline,⁵⁵ which has been shown to slow or even prevent significant deacetylation, including in liquid-phase enzymatic protocols.⁵⁶ Chitin can benefit from amorphization to improve access to acetyl sites^{31,55} through increased internal permeability.⁵⁷ We thus looked into the effect of dry milling pure chitin, in absence of any reagent, by probing the crystallinity index (CrI) of the product by X-ray diffraction (XRD). We first mixer-milled commercial chitin in PTFE using one ZrO₂ ball for 30 mins. This afforded very limited amorphization, yielding a crystallinity index (CrI) of 52.4% as compared to 65.8% for untreated practical grade (PG) chitin (Figure 1). Further milling in PTFE with a zirconia ball for 90 min yielded limited improvement with a CrI of 48.8%. In order to improve impact,⁵⁸ harder materials, i.e. steel and ZrO₂ jars equipped with one ball of the same material, were tested. In 30 min, significant amorphization had taken place with a crystallinity index of 14.3% for PG chitin milled in stainless steel, 19.7% for PG chitin mill in ZrO₂. Vickers hardness and density (ρ) can be commonly found in material technical data sheets. For PTFE (5-60 MPa, ρ =2.2 g/cm³), ZrO₂ (10-14 GPa, ρ =5.68 g/cm³) and stainless steel (1-2 GPa, ρ =7.7 g/cm³). Both the inverse of the Vickers hardness and the density were correlated to the amorphization measures as CrI (Figure 2). Indeed denser materials (ZrO₂ and steel) are better able to disrupt chitin, a material with high Vickers hardness (245-784 MPa) itself and fairly low density ρ =1.3 g/cm³.⁵⁹ Amorphization can also be correlated with FTIR, comparing commercial chitin and the zirconia amorphized chitin. The amorphized chitin IR shows loss of peak at ~3450 cm⁻¹(OH), merging of the amide peaks at 1663 cm⁻¹ and 1612 cm⁻¹ as well as loss of the out of plane N-H at 745 cm⁻¹ and 690 cm⁻¹(Figure S1)^{60,61}.

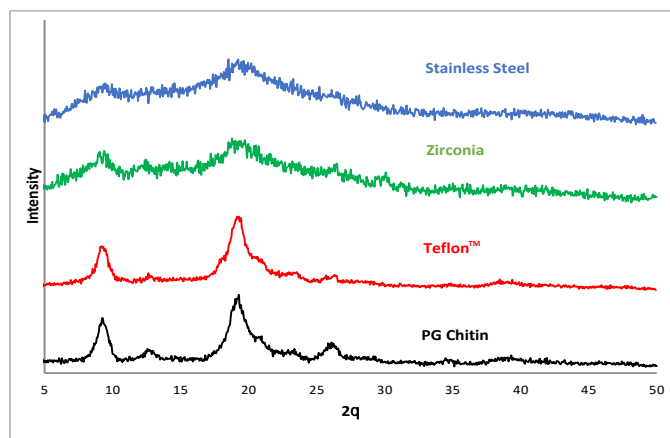


Figure 1 : XRD spectra of chitin and chitin milled in PTFE (90 min), Stainless steel (30 min) or ZrO₂ (30 min) jars.

While good amorphization was achieved in hard material jars, the impact on chitin MW is another important parameter to control. The intrinsic viscosity $[\eta]$ of a polymer in solution is classically used to determine its MW.⁶²⁻⁶⁵ DMAc/LiCl is a classic solvent system used in the literature for MW determination of chitin, yet it did not allow complete dissolution of our chitin samples, suggesting that any measure would underestimate MW.⁵¹ Additionally DMAc/LiCl is known to be corrosive, resulting in possible degradation of the polymer during the dissolution process.⁵¹ Chitin in the range of 5,000 kDa⁶⁵ have been reported previously using these suboptimal systems. [C₂mim][OAc] however has been shown to be a much better solvent for both chitin and chitosan.⁶⁶⁻⁶⁸ We successfully dissolved PG chitin completely, and found a $[\eta]$ of 7.23. Classically, polymer samples of known MW, typically determined by gel permeation chromatography (GPC), are used as reference to build a calibration curve correlating the intrinsic viscosity to MW. The Mark-Houwink parameters, α and K, are hence obtained from Equation 1.

$$[\eta] = K \cdot MW^\alpha$$

Equation 1 - Mark-Houwink equation to calculate Mw from intrinsic viscosity, where η is the intrinsic viscosity of the polymer solution and α and K are the Mark-Houwink parameters

At low MW regime, chitin samples of known MW could be easily obtained by acetylating reference chitosans. At high MW regime (> 1,500 kDa), we no longer had access to either chitin or chitosan samples for which the MW had been determined unequivocally. By using low MW regime calibration, we could obtain α and K values, which we used to estimate PG chitin MW to be 27,100 kDa. This value is much larger than what is usually reported, and the overestimation is likely caused by a change in viscosity behaviour between low and high MW regimes.

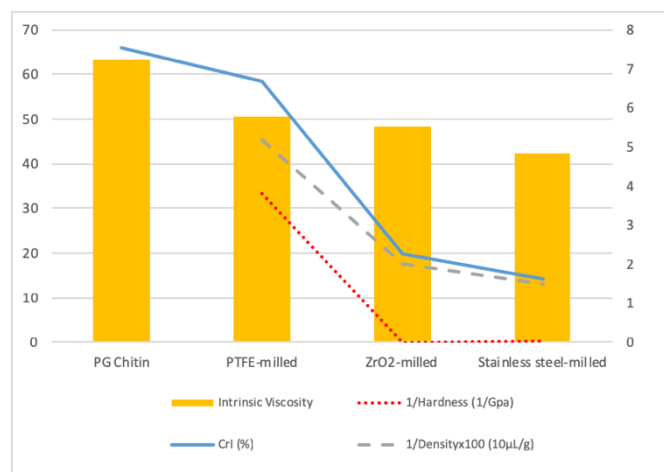


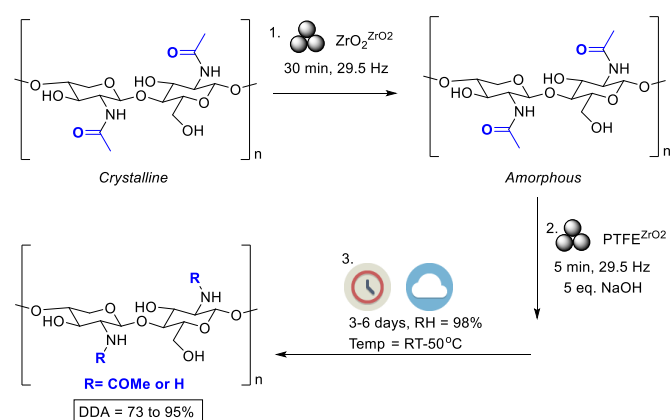
Figure 2 – Comparison chart of the $[\eta]$ (yellow bars – right ordinate) and CrI (blue line – left ordinate) for as-is and amorphized chitin samples with the [1/Vickers hardness] (dotted red line - left ordinate) and the [1/densityx100] (dashed gray line - left ordinate) of the apparatus material. Amorphization conditions: RT for 30 min in PTFE, ZrO₂ and stainless steel apparatus respectively.

With soft milling conditions (one ball, vibrational mill), we explored if we could maintain high viscosity - hence high MW - while amorphizing the material for further treatment. When milled for 30 min in PTFE, ZrO₂, and stainless steel, the $[\eta]$ were

5.80, 5.52 and 4.86 respectively (Figure 2). This method of milling with one ball thus afforded limited decrease in $[\eta]$. For reference, the Yan group used 100 ZrO_2 balls of 0.5 mm for 80 min to afford chitin of MW of 79.7 kDa³⁵, which would correspond to $[\eta]$ of 1.65. This exemplifies how careful selection of mechanochemical conditions may lead to vastly different results. In the following work, we used a ZrO_2 jar and one ZrO_2 ball for the amorphization step, as they provide an excellent compromise of high $[\eta]$ with good loss of crystallinity (19.7% of CrI).

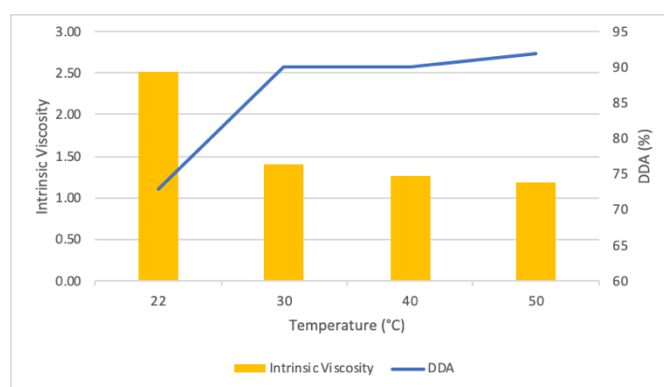
Chitin deacetylation by amorphization followed by aging studies

The amorphization treatment described above is still fairly intensive and we sought to use as mild as possible deacetylation conditions. We thus explored the use of aging, in dry and humid environments since it has been hypothesized that water plays a large role in depolymerization by activating the glycosidic bond for nucleophilic attack in a basic environment.⁶⁹ Humidity levels in the samples during aging were monitored thanks to controlled humidity chambers. Salt saturated aqueous solution prepared with K_2CO_3 , NaCl, and K_2SO_4 were able to afford stable 43, 75, and 98% relative humidity (RH) environments inside a sealed enclosure, respectively.⁴¹ Pure chitin samples were first amorphized by mixer milling in ZrO_2 apparatus for 30 min, before being mixed with NaOH and milled five minutes further in a PTFE jar with ZrO_2 ball to ensure homogeneous mixing. The sample was then aged in the various RH environment for 6 days at room temperature. Complete results of this studies are shown in Table S4. Interestingly RH of 98% afforded a DDA of 73% (Scheme 2). For this highly deacetylation sample, IR and ¹³C MAS NMR spectra are provided as Figure S2 and S3. To measure MW, GPC is commonly used for chitosan using an acetic acid buffer. One of the difficulties encountered in this project is that despite having high DDAs the solubility of the aged chitosan was low.



Scheme 2: Amorphization/aging based chitin deacetylation experiments. DDA = $[\text{H}]/([\text{COMe}] + [\text{H}]) \times 100$. Typical experimental conditions: chitin (200 mg) amorphized in a ZrO_2 jar with a ZrO_2 ball. Then chitin (105 mg), NaOH, 5 eq. (95 mg) based on glucosamine unit, loaded to a PTFE jar with a ZrO_2 ball, followed by aging at 98% RH for 6 days.

The material recovered from the reaction described in scheme 2 was left to dissolve in an acetic acid buffer (0.3M acetic acid, 0.25M sodium acetate, 0.8mM sodium azide), following literature dissolution methods,⁷⁰ and then filtered using a 0.45 μm PTFE filter. Less than 5% in weight was dissolved by this method, indicating that most the sample featured MW of high values. The soluble fraction was inspected with GPC, yielding MW of 4-20 kDa. This could be indicative of residual depolymerization. We thus turned again to viscometry to determine the MW of chitosan samples. Attempts at dissolution in buffers of 1% acetic acid, 2% acetic acid, 0.8% lactic acid and 5% DMAc/LiCl solutions also proved fruitless as can be seen in photos Figure S4. This is evidence that we made chitosan with higher MW than commercial ones. For reference, the highest molecular weight reported in the literature for chitosan samples was 1,560 kDa and these samples are soluble in acidic conditions.³³ This feature is particularly interesting since non-water soluble chitosans can be used towards application where water solubility is problematic such as metal polluted water remediation, or food packaging. Luckily, our samples were soluble in $[\text{C}_2\text{mim}][\text{OAc}]$ (Figure S4), so viscometry measurements were made with this solvent. Like with chitin,



Mark-Houwink parameters could be determined for the low MW regime, but not for the high one. Samples treated according to the method described in scheme 2 at RT afforded an $[\eta]$ of 2.52, corresponding to a MW of 4,040 kDa as calculated by the Mark-Houwink of the low MW regime. In order to further boost DDA while maintaining MW, optimization of reaction conditions was explored by modifying aging temperature, aging time, and NaOH equivalents (Table S5 and S6).

Figure 3: Effect of the temperature during the aging step in the amorphization/aging based chitin deacetylation experiments on Intrinsic Viscosity $[\eta]$ and DDA. Conditions of scheme 2.

Chitin deacetylation: reaction optimization

We first investigated the effect of the temperature during aging under RH of 98% for 6 days (Figure 3, Scheme 2). To modulate this parameter, the humidity chambers were placed in an oven. Raising aging temperatures was expected to accelerate all hydroxy-catalyzed reactions, deacetylation, but also depolymerization. From RT (22°C) to 30°C, DDA raised to 90%, while the $[\eta]$ was strongly affected and dropped from 2.52 to 1.41. Raising the temperature further up to 50 °C, marginally improved the DDA up to 92% and further eroded the $[\eta]$ down

to 1.18. Then we studied more the kinetics of the reaction by looking into aging times of 3, 4, 5 and 6 days, at RT, keeping all other conditions described in scheme 2. Under these conditions, fairly stable $[\eta]$ ranging from 2.52 to 2.76 were observed (Figure 4, orange solid columns). A sharp increase of DDA from 53 % at 3 days to 76% at 4 days was observed, followed by a plateau thereafter (Figure 4, brown solid line). At 50°C, DDAs were ranging between 90% and 95% from 3 to 6 days, revealing a much faster kinetic than at RT (Figure 4, green dashed line)). The $[\eta]$ was affected too, with values slowly decreasing from 1.27 to 1.18 from 3 to 6 days of aging.

The best $[\eta]$ value obtained for chitosan with good DDA, under these conditions, is 2.52. This is 1 $[\eta]$ unit higher than the largest commercial chitosan (MW=800 kDa). This method is also versatile since varying the reaction time and temperature afforded chitosans of a varied range of DDA and $[\eta]$, including ones comparable to commercial chitosans.

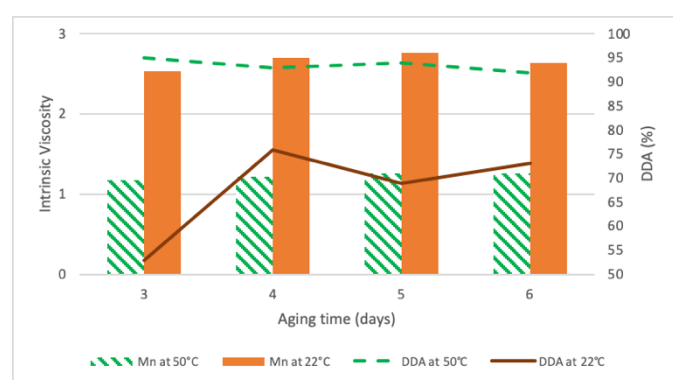


Figure 4: Reaction kinetics for the amorphization/aging based chitin deacetylation experiments. $[\eta]$ and DDA for reactions performed at 22 and 50°C. Conditions of scheme 2 additionally varying aging time.

We then explored the role of amorphization pretreatment in these aging experiments, at aging temperatures of 22 and 50°C, in 6 days (Figure 5). At room temperature, skipping the amorphization pre-treatment provided poor DDA of 59%, compared to 73% with amorphization. Conversely, no amorphization allowed to preserve a really high $[\eta]$ of 3.19, compared to 2.52 with the extra step. At 50°C interesting results are observed. No amorphization secured excellent $[\eta]$ of 3.01 and excellent DDA of 87%. This demonstrated that while amorphization is a good method for accelerating deacetylation, it is by no means a *sine qua none* condition to excellent DDAs. As absence of amorphization secured exceptionally high $[\eta]$ of 3.12 (Table S5, 3 days at 50°C), a value well above measured commercial chitosan sources, $[\eta]$ of 1.62. Finally, we were interested in limiting the chemical input in this reaction, while maintaining amorphization and explored the decrease of the amount of added NaOH. At 98% RH and 50°C aging, 1:5 chitin:NaOH afforded 95% DDA, while 1:4, 1:3, 1:2, 1:1, yielded 86, 68, 52, and 30% DDA respectively (Figure S5, Table S6). Reaching a DDA of 86% with 1:4 chitin:NaOH is an improvement in NaOH equivalents over recent milling deacetylation methodologies,^{35,71} where 1:5 chitin:NaOH mixture was used and a significant decrease compared to

conventional methods²⁶ with 1:40 chitin:NaOH (Table S1). At 1:4 ratio, the $[\eta]$ is also improved to 1.84 from 1.81 from 1:5 ratio.

Scale-up of chitin deacetylation by aging

To test scale-up feasibility of the process, 10 g of chitin was used in the simplified approach consisting of mixing commercial crystalline chitin with NaOH (1:5 molar ratio) and milled in a planetary mill for 5 mins, followed by aging for 3 and 6 days in 98% RH at 50°C was used. Either Stainless steel or ZrO₂ jars were used as indicated, each with 5 balls (10 mm) of the same material. The successful scale-up yielded chitosan up to 89% DDA and $[\eta]$ of 2.47, where longer aging times yielded higher DDA and lower $[\eta]$. Milling media also had an effect, where the higher density stainless steel improved DDA % but also decreased $[\eta]$ (table 2).

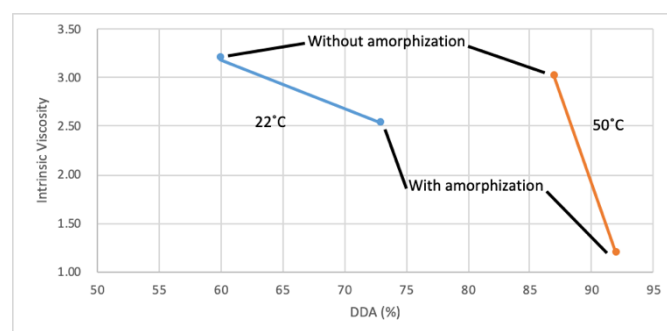


Figure 5: $[\eta]$ vs DDA graph for the aging based chitin deacetylation experiments, with or without amorphization pre-treatment, at 22 or 50°C. The reactions were performed of 6 days, at chitin:NaOH ratios of 1:5, with amorphization in ZrO₂ apparatus for 30 min.

Table 2: DDA and $[\eta]$ results on 10 g scaled-up deacetylation of commercial chitin, consisting of 5 min milling in a planetary mill, followed by aging at 50°C at 98%RH

Aging Time (days)	Milling apparatus	% DDA	Intrinsic Viscosity
3	ZrO ₂	75	2.47
	stainless steel	85	2.31
6	ZrO ₂	83	2.35
	stainless steel	89	2.28

Scope: other chitin sources

Besides commercial chitin, we were interested to study the synthetic method developed herein directly on untreated crustacean and arthropod shells. We thus tested shrimp, lobster, crab, fly larva larvae and Bombyx eri larvae (BEL) shell, which are all composed of chitin, as well as proteins and CaCO₃.⁷² All biomass samples were dried, ground and sieved to particles <125 μ m, then amorphized for 30 mins in a ZrO₂ jar with a ZrO₂ ball. We also explored BEL samples treated with ionic liquids in order to swell the chitin for better access and remove some from proteins and CaCO₃, according to a method we (Hadad, Nguyen Van Nhien) previously reported.⁷³ All these samples were mixed with NaOH, milled for 5 min in a PTFE jar with ZrO₂ ball, and aged for 6 days at 98% RH and 50 °C. For these tests, we used a

biomass:NaOH ratio of 1:5, which means that the chitin:NaOH ratio is higher, because of the presence of proteins and CaCO₃. Shrimp shell reached a DDA of 95%, while lobster, crab and fly larva reached 96%, 98% and 90% respectively (Table 3). The presence of protein and CaCO₃ within untreated biomass samples meant the true NaOH to chitin ratio was higher than 5:1 which can explain the higher deacetylation results as compared to our model reaction. Chitin samples extracted from fly larva reached their highest DDA of 78% (Table 3), when the biomass was pretreated with [C₂mim][OAc]. This lower DDA was rationalized by a higher crystallinity of the extracted BEL chitin. All these direct-from-biomass chitosan samples proved to feature excellent DDA and MW.

Table 3: DDA and [η] results on commercial chitin, crustacean and arthropod shells and ionic liquid treated shells.

Chitin source	% DDA	Intrinsic Viscosity [η]
shrimp ^a	95	1.61
lobster ^a	96	2.58
crab ^a	98	3.40
fly larva ^a	90	4.33
BEL ^b	74	2.84
Pretreated BEL ^b	78	3.00
Deproteinized BEL ^b	72	3.25
Pretreated deproteinized BEL ^b	60	1.96

^a Typical experimental conditions: Samples were dried, ground, sieved, before being amorphized by milling in ZrO₂ apparatus. Then 5 eq NaOH were added, and the mixture loaded to a PTFE jar with a ZrO₂ ball, followed by aging at 98% RH for 6 days at 50°C.

^b Typical experimental conditions: After ionic liquid treatment, samples were mixed with 5 eq NaOH, and loaded to a PTFE jar with a ZrO₂ ball, followed by aging at 98% RH for 6 days at 50°C.

Energy Comparison

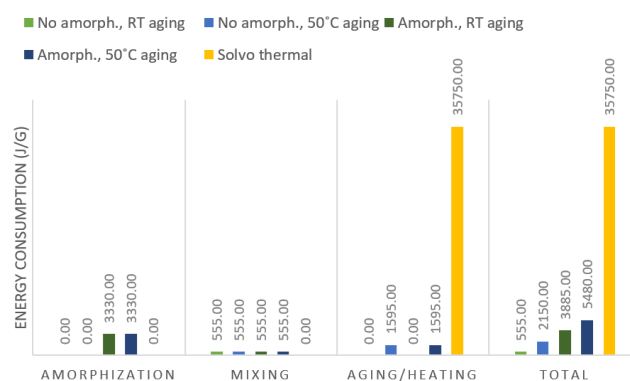
Finally, we compared the energy usage of the herein disclosed milling/aging processes with a conventional solvothermal. We measured experimentally the power consumption of the mixer mill, oven, and hot plate and normalized to Joules per gram (Figure 6). Due to the size of the oven, the batch size power consumption was measured for a 240 g batch, whereas the solvothermal process was measured for 1 g. When aging was conducted at RT only the milling power consumption was considered, resulting in a consumption of 3,885 J/g for the process. When aging without pre-milling (amorphization) is conducted at 50°C the power consumption was 3,739 J/g. On the other hand, the solvothermal process (3 hrs, 133°C) required 35,748 J/g in energy input. These values show significant favour and, potentially, bias to the aging/milling process in terms of power consumption.

To better understand the power consumption for scale-up, we theoretically modelled a batch conversion of chitin to chitosan using solvo-thermal and aging methods. We determined that solvo-thermal requires 11,467 KJ/Kg, whereas aging requires

between 269 and 3,885 KJ/Kg depending on conditions (Table S7).

Conclusion

In this paper we present a solvent-free method to deacetylate chitin by first amorphizing then aging, or aging alone, with NaOH in a humidity chamber. Increasing NaOH molar equivalents or temperature or time increased degree of deacetylation. We concluded that amorphization of chitin prior to deacetylation increased the extent of deacetylation under the same conditions, showing that greater access to N-acetyl sites on chitin increases the rate of deacetylation, but that is also resulted in lower MW. While amorphisation accelerated deacetylation, it was not essential to achieve high DDA. Greater than 70% DDA can be achieved in 6 days at room temperature or in 24 hrs of aging at 50 °C while 95% DDA can be achieved after 3 days at 50°C. [η] of the order of 2.52 could be reached with amorphization (at RT) while absence of amorphization allowed to reach [η] of 3.04 and 3.19 at 50 °C and RT respectively. Similarly, chitin can be directly deacetylated from shrimp, crab and lobster shell as well as fly larvae and BEL that has been pretreated with [C₂mim][OAc] in the same aging process,



allowing to reach values for [η] up to 4.33 with fly larva.

Figure 6: Breakdown of energy consumption (J/g) for mechanochemical/aging and solvothermal reactions. Reaction conditions: No amorph. corresponds to absence of amorphization step, Amorph. is with this step; aging is performed at RT or 50°C; All aging reactions are done for 3 days; solvothermal is done for 3 h at 133 °C.

This method is completely novel and opens access to really large MW chitosan. Compared to known methods, it limits the reliance on materials (with excellent results at NaOH:chitin ratios of 4 or 5), no solvent use during reaction, and very low energy input, especially for the pure aging experiments. This method is also particularly simple to implement, requiring limited technical equipment, which is very well adapted to the *Shell Biorefinery* challenge of delivering solutions for the local production of high added value products close to fisheries and insect farms. In this article we also provide a proof of concept for a 10g scale up of the process with excellent results. Overall this affords a new type of chitosan materials able to resist water dissolution and open access to new applications, in pollution remediation, or food packaging.

Experimental

Chemicals and methods

Practical grade chitin, low, medium, and high molecular weight chitosan, acetic acid-d₄ (≥99.5% D) were purchased from Sigma-Aldrich Co. LLC (St-Louis, MO). Medical grade chitosans were purchased from Bonding Chemical (Katy, TX). Atlantic sourced shrimp, lobster, and crab shells were purchased from a local fishery cleaned with DI water and air dried before further processing. Bombyx eri larvae (BEL) were produced from private insects rearing. After separation by extrusion, the raw chitin was cleaned with DI water, ethanol and acetone and air dried. Deproteinized BEL were obtained with NaOH 1M overnight under reflux and washed with DI water until neutral pH. NaOH micropearls were purchased from Acros Organics (Belgium). Deuterium oxide (99.9% D) was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). Methanol, sodium chloride, sodium acetate, and acetic acid (all reagent grade) were purchased from ACP (Montreal, Qc., Canada). Potassium bromide Spectrosol was purchased from VWR (Mount-Royal, Quebec). Potassium sulfate and potassium carbonate were purchased from Alfa Aesar (Ward Hill, MA). [C₂mim][OAc] was purchased from Iolitech (Tuscaloosa, AL). Pullulan calibration ReadyCal Kit (Mp 180 – 1 220 000 Da) was purchased from PSS polymers (Germany).

Milling

In the following procedures, a Retsch MM 400 was used as mixer mill, with jars made of polytetrafluoroethylene (PTFE), 20 mL, stainless steel (10 mL) or zirconia (10 mL), equipped with balls of zirconia or steel.

Controlled humidity chamber

In aging experiments described below, controlled humidity chambers were used. Three relative humidity (RH) levels were tested (43%, 75%, 98%). The chamber consisted of a 100 mL glass screw top jar. The chamber is filled with 20 mL of saturated aqueous solutions of K₂CO₃, NaCl and K₂SO₄, to access 43%, 75%, and 98% RH respectively. An open 4 dram vial, containing the solid state sample, was placed inside the chamber. The overall chamber could be heated by placing in a Fisher Scientific Isotemp oven.

Conventional solvothermal chitin deacetylation experiments

For comparison purposes, chitosan samples were prepared by a traditional method²⁷ for comparison by heating for 3 hours in 50% NaOH solution at 133°C.

Mixer-mill based chitin deacetylation experiments

In a typical experiment, 105 mg of chitin and 95 mg of NaOH (1:5 chitin:NaOH ratio) were combined in a PTFE jar and milled for 30, 60 or 90 minutes with one zirconia ball (10 mm). Milling mixture mass was always maintained to be 200 mg. For example, with a 1:1 chitin:NaOH ratio, 170 mg of chitin and 30 mg of NaOH were used. Post milling, the samples were worked up by washing and filtering with methanol until neutral pH using Whatman filter paper (# 1, 55mm), then air dried.

Mechanochemical amorphization of chitin

In a typical experiment, commercial chitin (200 mg) was placed in a zirconia jar equipped with one zirconia ball (10 mm) and milled in a mixer mill for 30 mins in a at 29.5 Hz. The resulting powder was used as is for analysis or further mechanochemical or aging treatments.

Chitin deacetylation experiments by aging

In the following, chitin samples are used as is, or after a first step of mechanochemical amorphization (*vide supra*). In a typical experiment, 105 mg of chitin and 95 mg of NaOH (1:5 chitin:NaOH ratio) were combined in a PTFE jar and milled for 5 minutes with one zirconia ball (10 mm). The procedure was similar with other chitin:NaOH ratios, and the total reagent mass of solid was kept at 200mg. The mixture, a very pale-yellow powder was transferred from the jar to an open 4 dram glass vial and aged in a controlled humidity chamber for 1 to 6 days at constant temperatures from room temperature to 50°C. Post aging, the samples were worked up by washing and filtering with 100 mL of methanol until neutral pH using Whatman filter paper (# 1, 55mm), then air dried. Controlled experiments were run exactly as stated above, in absence of NaOH, or in absence of controlled humidity (achieved by sealing the sample in a glass vial during the aging period).

Scale-Up - Chitin deacetylation experiments by aging

For 100x scale-up, commercial chitin (10 g) was milled with NaOH (9 g) in a planetary mill for 5 minutes in ZrO₂ (50 mL) and stainless steel (80 mL) with 5 balls (10 mm) of the same material as the jar. The mixture was then placed in a petri dish (115 mm x 65 mm) which was placed in a sealed 1 L container containing 100 mL of saturated K₂SO₄ salt solution with excess salt. The containers were placed in the oven to age at 50°C for 3 and 6 days. Post aging, the samples were worked up by washing and filtering with methanol until neutral pH using Whatman filter paper (# 1, 55mm), then air dried.

Pre-treatment of biomass samples

Shrimp, lobster, crab and fly larva shell samples were ground using a Bel-Art Products micro-mill for 2 mins then sieved using U.S.A standard test sieves, where particle <125 μm were collected and used for the deacetylation process. The resulting powder was used as is for further treatment, namely mechanochemical amorphization and aging-based deacetylation.

Ionic liquid pretreatment of biomass sample

Biomass of BEL were treated with [C₂mim][OAc] for 40 mins at 110°C. The chitin suspension was then cooled in an ice bath and washed with deionized water, centrifuged at 10 733 g for 20 mins. The supernatant was removed, the resulting mass was then filtered, re-washed and refiltered.⁷³

Control experiments

Manual grinding

A 1:5 chitin:NaOH mixture containing 105 mg of chitin and 95 mg of NaOH was manually ground in an agate mortar and pestle

for 1 minute to homogenize. The mixture was then aged in an open vial in 43, 75, and 98% RH at 50°C and worked up as described previously.

No NaOH

A 200 mg chitin samples was milled for 5 minutes in an PTFE jar with ZrO₂ ball. The mixture was then aged in an open vial in 98% RH at 50°C and worked up as described previously.

No humidity

A 1:5 chitin:NaOH mixture containing 105 mg of chitin and 95 mg of NaOH was milled for 5 minutes in an PTFE jar with ZrO₂ ball. The mixture was then aged in a sealed vial at 50°C and worked up as described previously.

Analysis - Characterization – equipment details and methods

¹³C Magic Angle Spinning nuclear magnetic resonance (MAS-NMR)

NMR spectra were recorded on a Varian VNMRS operating at 400 MHz for the solid-state ¹³C acquisition using a 4mm double-resonance Varian Chemagnetics T3 probe. A contact time of 1000 μs and a recycle delay of 3 s were used to acquire quantitative spectra.⁷⁴ 2872 scans were acquired of each sample for a total time of 2.5 hrs. DDA was calculated using a known method.⁷⁵

¹H NMR

NMR spectra were recorded on a Varian VNMRS spectrometer operating at 500 MHz for ¹H acquisitions. DDA was calculated using a known method⁶³ when samples were soluble in dilute acetic acid.

IR

IR spectra were recorded using a Perkin-Elmer Spectrum 400 for 64 scans from 4000 cm⁻¹ to 400 cm⁻¹ in transmission mode from pressed KBr discs where, 2 mg of sample was mixed with 200 mg of KBr. DDA was calculated by comparing the by comparing the absorption of the amide band at 1655 cm⁻¹ and the hydroxyl absorption band at 3450 cm⁻¹ as the reference peak using the equation, % N-acetylation = (A₁₆₅₅/A₃₄₅₀) × 115.⁷⁶

pXRD

Sample diffractogram was recorded from 5° to 40° on a zero-background plate using a Bruker D8 ADVANCE X-Ray Diffractometer equipped using Cu-Kα (λ = 1.54 Å) source. Chitin crystallinity was determined by comparing the entire area of the diffractogram (global area) and the area of the peaks (reduced area). Where %Crystallinity = 100-%Amorphous and %Amorphous = [(Global Area – Reduced Area)/Global Area] × 100.⁷⁷

GPC

We used an Agilent Technologies 1260 Infinity II GPC triple detection equipped with Shodex OHpak SB-804 HQ 300 × 8 mm, 10 μm column which was calibrated with a PSS Polymers ReadyCal Kit pullulan standard in an acetic acid buffer system

(0.3M Acetic Acid, 0.2M Sodium Acetate and 0.8mM Sodium Azide).

Samples were left to solubilize in an acetic acid buffer by shaking at room temperature for two days then filtered through a 0.45 μm PTFE filter. The filter was pre-weighed prior to filtration, then dried and weighed post filtration to determine the amount of insoluble material.

Viscosity

Viscosity was measured as an average of 6 repetitions at 40° incline using Anton Paar AMVn automated micro viscometer with 1.6 mm capillary and 1.5 mm stainless steel ball at 35°C. Sample viscosity was measured and used to determine intrinsic viscosity [η]. A typical sample was prepared by dissolving chitosan or chitin in [C₂mim][OAc] in concentrations of 3 mg/mL and lower at 80°C for two days in a sand bath, yielding a golden amber solution. Since filtration was not possible due to viscosity, the samples were centrifuged at 3000 RPM for 10 minutes to remove any undissolved material. Each sample was measured at 5 decreasing concentrations for determination of intrinsic viscosity.

Density

Density was measured using Anton Paar DMA 38 density meter using the method described by the company in the user manual.

Energy Measurements

Power consumption was measured using a P3 P3IP4400, Kill A Watt electricity usage monitor. Several hours or days of run time power consumption were measured for each, the mill, hot plate and the oven, while in experimental use. The overall power consumption was averaged to W/min/g based on experimental conditions of each process.

Energy Calculations

Theoretical power consumption was calculated based on heating the required amounts of water, NaOH and chitin, using heat capacity, to the final desired temperatures for each process, including the enthalpy of dissolution for NaOH and without heat losses of the system. For mechanochemical energy, we did not have any scale-up data so we used values obtained experimentally from the mixer-mill which may yield higher energy consumption than if appropriate scale equipment is used. The energy difference for chitin deacetylation was ignored as its effect is considered the same for all situations.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

§
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etc.

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Deacetylation by mechanochemistry and aging as a pathway to high molecular weight chitosan from chitin

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Table S1: Comparison of known deacetylation techniques.

Method	Conditions	NaOH equivalents	MW	DDA	Ref
Solvo-Thermal	50% NaOH _(aq) , RT-140°C, 3 ^a -540 Hrs ^b	42	Varied	70-99%	C. Rong Huei, Carbohydr. Polym., 1996, 29 , 353–358. A. Domard and M. Rinaudo, Int. J. Biol. Macromol., 1983, 5 , 49–52. A. Alimuniar, and R. Zainuddin, In Advances in Chitin and Chitosan, C.J. Beine, P.A. Sanford and J.P. Zikakis (eds.), Elsevier Applied Science, London and New York, pp. 627-632, 1992.

Planetary Milling	1:5 chitin:NaOH, 100 balls ZrO ₂ ^{ZrO₂} , 80 mins	5	1-13 kDa	80 %	X. Chen, H. Yang, Z. Zhong and N.Yan, <i>Green Chem.</i> , 2017, 19 , 2783–2792.
Microwave	45% NaOH _(aq) , 5.5 Mins, 900 W	31	85 kDa	80 %	A. Sahu, P. Goswami and U. Bora, <i>J. Mater. Sci. Mater. Med.</i> , , DOI:10.1007/s10856-008-3549-4.
Sonication	60 % NaOH _(aq) , 30 mins, 500 W	>16	Degradation	73 %	M. Anwar, A. S. Anggraeni and M. H. Al Amin, <i>AIP Conf. Proc.</i> DOI:10.1063/1.4978144.
High Pressure Run	50% NaOH _(aq) , 120 °C, 15 PSI	41	1466 kDa	90 %	H. K. No, Y. I. Cho, H. R. Kim and S. P. Meyers, <i>J. Agric. Food Chem.</i> , 2000, 48 , 2625–2627.
Maceration	7 days, 80 % NaOH _(aq)	22	N/A	80 %	M. Anwar, A. S. Anggraeni and M. H. Al Amin, <i>AIP Conf. Proc.</i> DOI:10.1063/1.4978144.
Freeze thaw	24% NaOH	>13	180-300 kDa	80-95%	S. V. Nemtsev, A. I. Gamzazade, S. V. Rogozhin, V. M. Bykova and V. P. Bykov, <i>Appl. Biochem. Microbiol.</i> , 2002, 38 , 521–526.
<i>This work</i>	Milling for 30 min followed by 6 days of aging	5	N/A	80-95%	

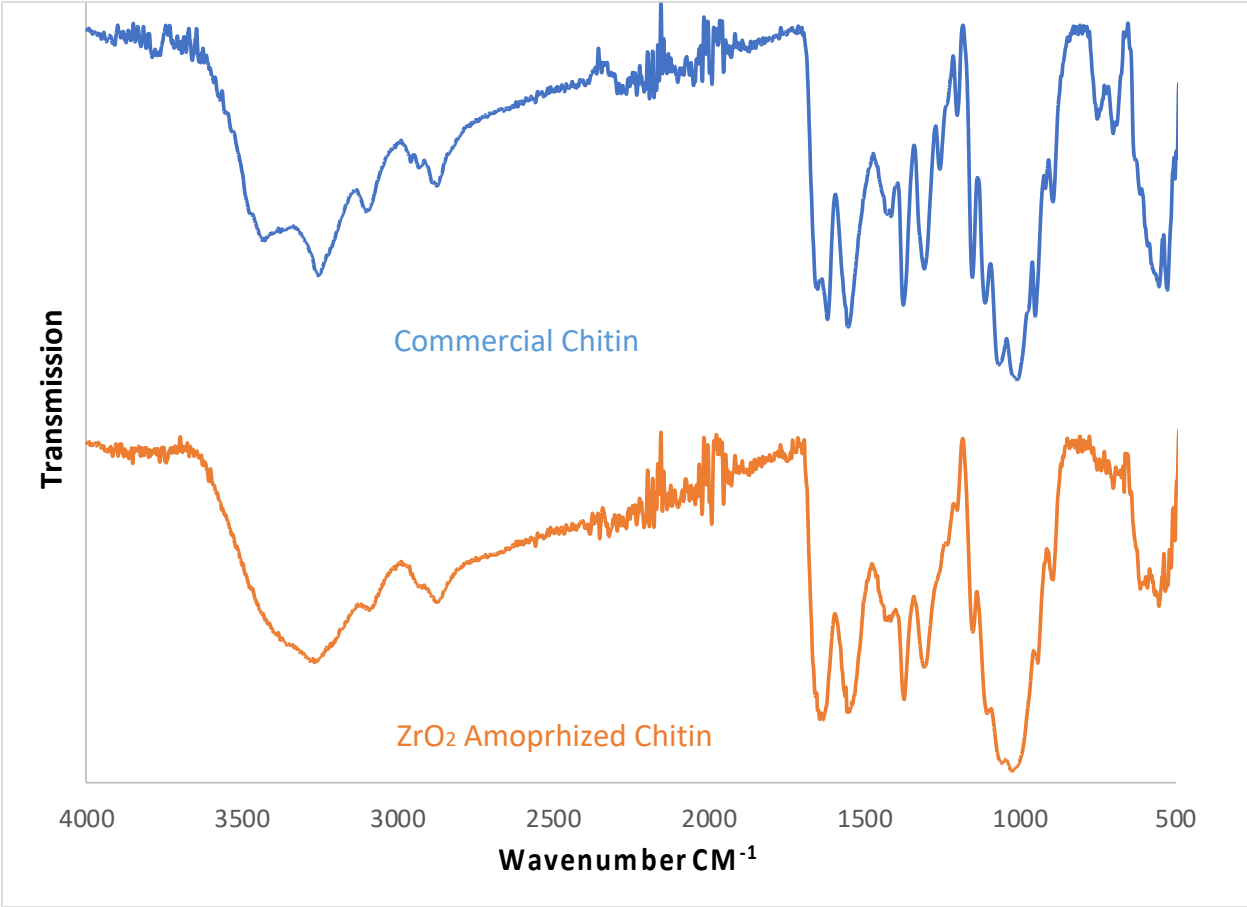


Figure S1: FTIR spectra of commercial chitin pre and post amorphization in ZrO_2 jar with ZrO_2 ball for 30 minutes milling.

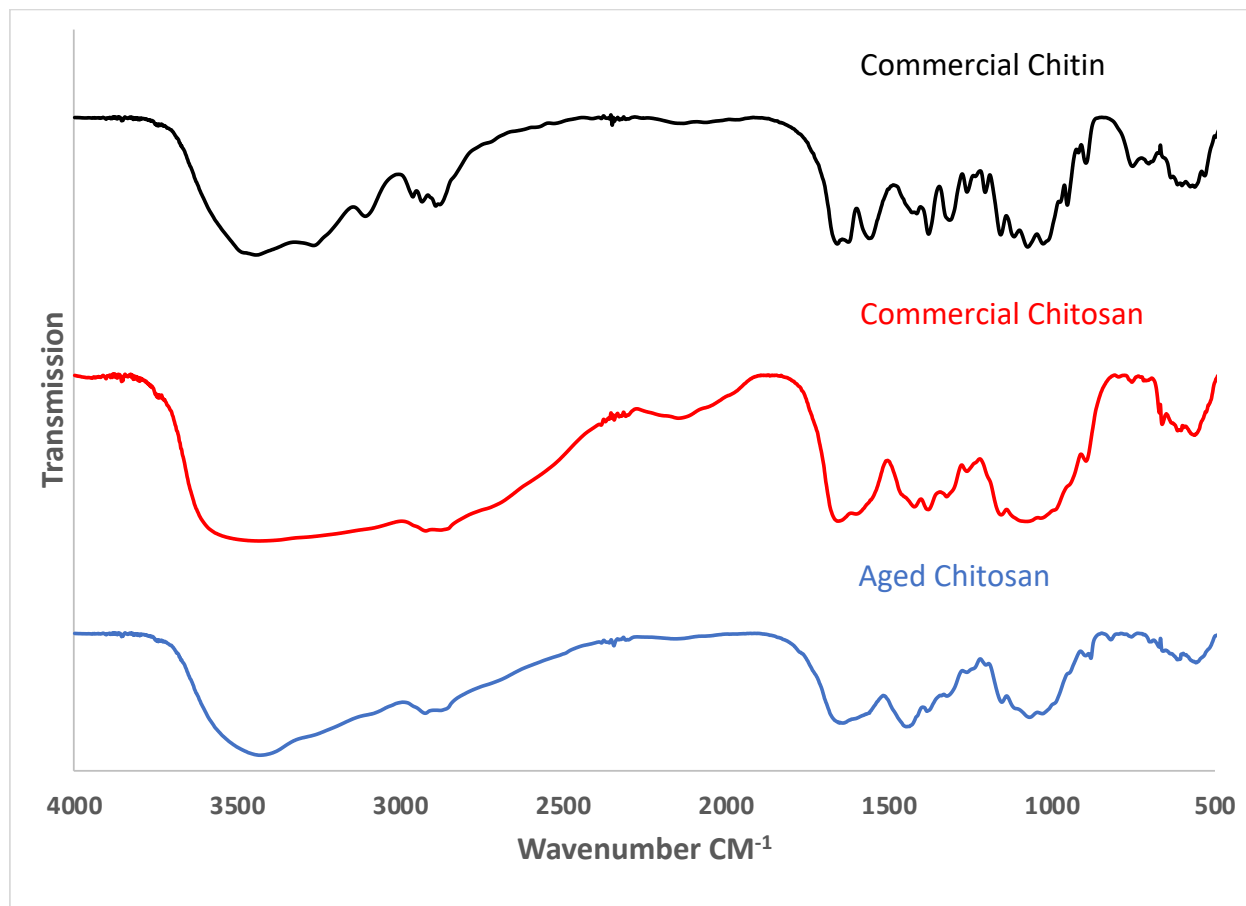


Figure S2: IR spectra of commercial chitin, commercial chitosan, and chitosan produced with the aging process at room temperature, 98% RH 1:5 chitin:NaOH. The amide peak at 1655 cm^{-1} is measured in reference to the hydroxyl peak at 3450 cm^{-1} .

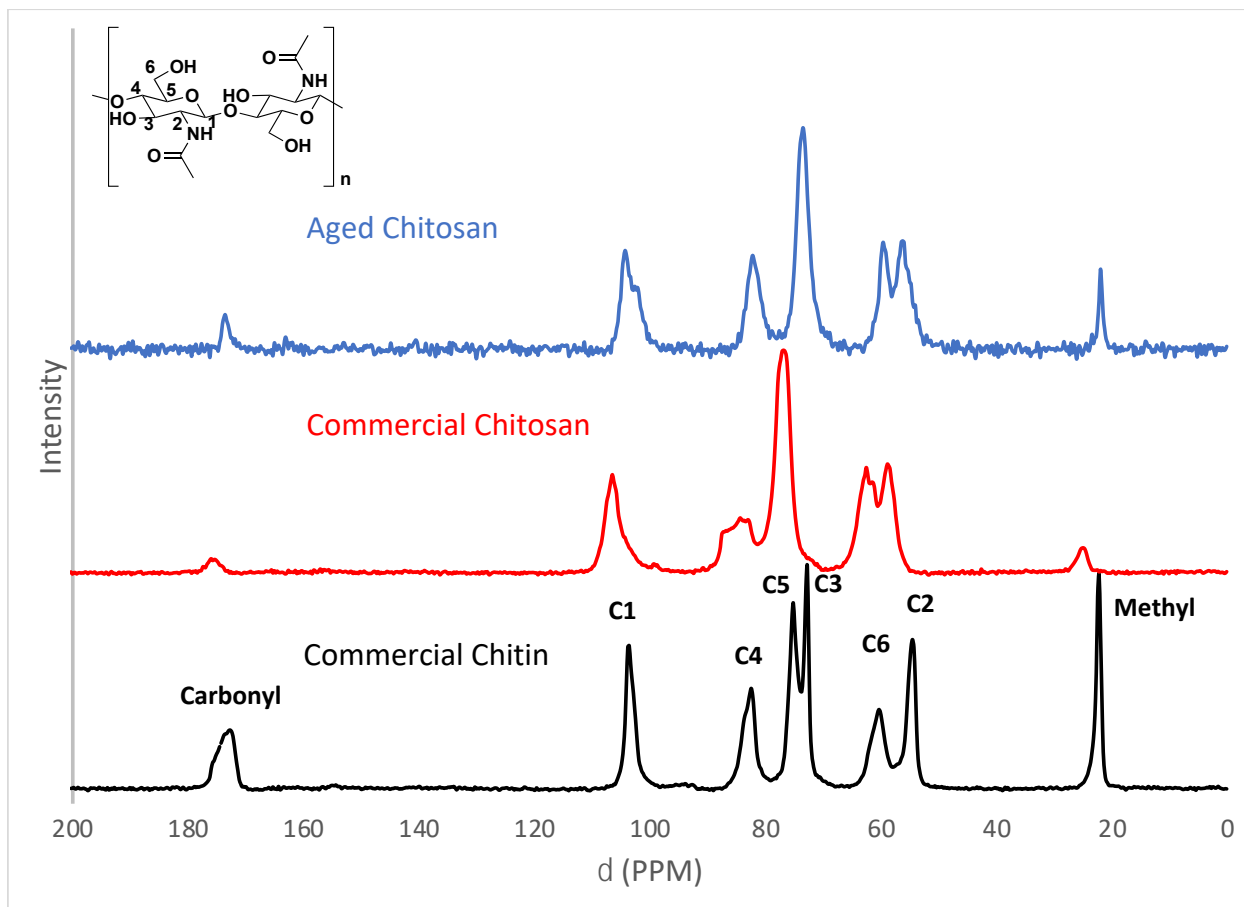


Figure S3: Solid-State ^{13}C NMR spectra of commercial chitin, commercial chitosan, and chitosan produced with the aging process at room temperature. DDA is calculated by comparing the methyl peak at 22 ppm to a reference C1 carbon peak at 104 ppm.

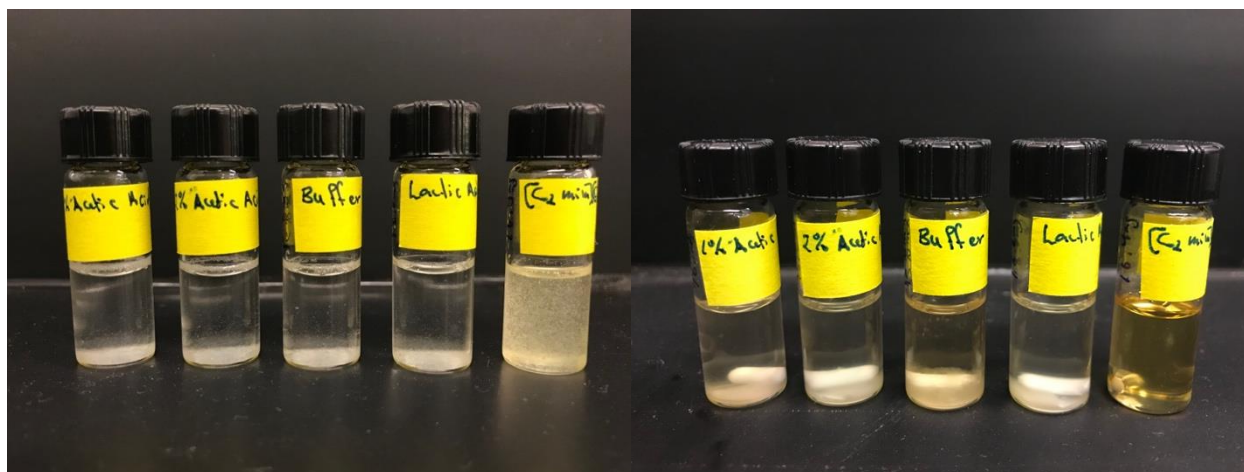


Figure S4: Pictures of aged chitosan (from left to right) in 1% acetic acid, 2% acetic acid, buffer solution (0.3M acetic acid, 0.25M sodium acetate, 0.8mM sodium azide), 0.8% lactic acid and $[C_2mim][OAc]$ pre (left) and post (right) heating (80°C. Images show that the aged chitosan only dissolves in $[C_2mim][OAc]$.

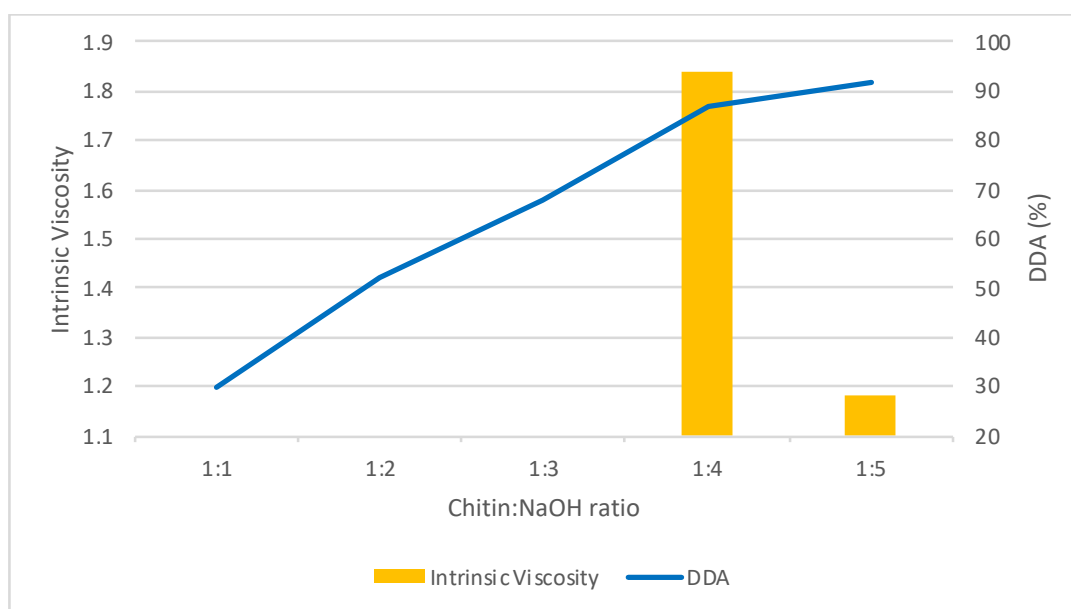


Figure S5: Effect of the chitin:NaOH ratio on $[\eta]$ and DDA in the amorphization/aging based chitin deacetylation experiments. Conditions of scheme 2 with aging at 50°C for 6 days. $[\eta]$ not measured for 1:1, 1:2 and 1:3 chitin:NaOH ratios.

Table S2: Initial DDA of commercial chitin, commercial chitosan and solution deacetylated chitosan acquired by ^{13}C CP MAS NMR

Sample	DDA (%)
PG-Chitin	4
Commercial Chitosan LMW	89
Commercial Chitosan MMW	96

Commercial Chitosan HMW	85
Solution Deacetylated Chitosan	76

Table S3: Mixer-mill based commercial chitin deacetylation experiments in PTFE jar with a ZrO₂ ball. DDA = $[H]/([COMe]+[H]) \times 100$, determined by ¹³C CP MAS NMR. LAG solvent 10 wt%: none, dichloromethane, acetonitrile, ethyl acetate, methanol, ethanol and deionized water.

Milling time (min)	NaOH:chitin ratio	DDA (%)	LAG solvent
30	1	5	none
30	2	6	none
30	3	6	none
30	4	7	none
30	5	7	none
60	5	6	none
90	5	7	none
30	1	7	Water
30	1	7	Ethanol
30	1	6	Methanol
30	1	6	Dichloromethane
30	1	6	Ethyl Acetate
30	1	4	Acetonitrile
30	1	9.9	Water (10%)
30	1	14	Water (20%)
30	1	11.76	Water (30%)
30	1	11.65	Water (50%)
30	5	12.4	Water (10%)
30	5	23.8	Water (20%)
30	5	20.56	Water (30%)
30	5	19	Water (50%)

Table S4: Table of the relative humidity (RH) study of aging of crystalline chitin for 6 days at 22°C with a chitin to NaOH ratio of 1:5.

RH(%)	DDA(%)
43	40
75	43
98	60

Table S5: DDA and $[\eta]$ of commercial chitin treated with or without amorphization pre-treatment (30 min milling in ZrO₂ apparatus) followed by 3 to 6 days aging at 22 to 50°C, at 98% humidity and with a chitin to NaOH ratio of 1:5.

Amorphization pre treatment	Temp. (°C)	Aging time (days)	DDA(%)	Intrinsic Viscosity $[\eta]$
Yes	22	6	73	2.52
	30	6	90	1.41
	40	6	90	1.27

	50	6	92	1.18
No	50	3	83	3.12
		4	84	3.04
		5	84	3.04
		6	87	3.01
	22	3	50	3.23
		4	51	3.22
		5	65	3.12
		6	60	3.19
yes	50	3	95	1.27
		4	93	1.26
		5	94	1.22
		6	92	1.18
	22	3	53	2.64
		4	76	2.76
		5	69	2.69
		6	73	2.52

Table S6: DDA and $[\eta]$ of commercial chitin treated with amorphization pre-treatment (30 min milling in ZrO_2 apparatus) followed by 6 days aging at $50^\circ C$, at 98% humidity and with a chitin to NaOH ratio ranging from 1:1 to 1:5.

Chitin:NaOH ratio	DDA(%)	Intrinsic Viscosity $[\eta]$
1:1	30	Not measured
1:2	52	Not measured
1:3	68	Not measured
1:4	87	1.84
1:5	92	1.18

Table S7: Energy consumption comparison of solvo-thermal and aging methods for deacetylation of 1 Kg of chitin into chitosan. Solvo-thermal was calculated for heating 1 Kg of chitin, 18.5 Kg of water, and 18.5 Kg of NaOH to $133^\circ C$. Aging was calculated for heating 1 Kg of chitin, 2 Kg of water, and 0.9 Kg of NaOH. Amorphization includes 30 mins of milling in ZrO_2 jar with ZrO_2 ball. Mixing requires 5 mins milling in PTFE jar with one ZrO_2 ball.

Method	Solvo-thermal	Amorphization	Mixing	Aging
Energy (KJ/Kg)	11,467	3,330	555	269

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