DEACETYLATION BY MECHANOCHEMISTRY AND AGING AS A PATHWAY TO HIGH MOLECULAR WEIGHT CHITOSAN FROM CHITIN

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Glossary of Abbreviations

$[C_2mim][OAc]$ – 1-ethyl-3-methylimidazolium acetate

aq – aqueous

BSA – bovine serum albumin

$CaCO_3$ – calcium carbonate

CP – cross polarized

CrI – crystallinity index

DDA – degree of deacetylation

DMAC – dimethylacetamide

eqs – equivalents

GPC – gel permeation chromatography

H_{ball} – hardness of ball

HCl – hydrochloric acid

hrs – hours

HSA – human serum albumin

Hz – hertz

I_{am} – intensity of amorphous phase

I_o – intensity of crystalline phase

IL – ionic liquid

Jar^{Ball} – indicates general jar and ball combination nomenclature, for example : $ZrO_2^{ZrO_2}$ – indicates ZrO_2 jar and ZrO_2 ball combination

K_2CO_3 – potassium carbonate

K_2SO_4 – potassium sulfate

KBr – potassium bromide

kDa – kilo Dalton

LiCl – lithitum chloride

MAS-NMR – magic angle spinning nuclear magnetic resonance

M_{ball} – mass of ball

mins – minutes

M_v – viscosity average molecular weight

MW – molecular weight

NaCl – sodium chloride

NaOH – sodium hydroxide

NMR – nuclear magnetic resonance

PMMA – polymethylmethacrylate

PTFE – polytetrafluoroethylene

pXRD – powder x-ray diffraction

RH – relative humidity

RT – room temperature

SS – stainless steel

temp – temperature

w/w – weight per weight

wt% - weight percent

Abstract/Resumé

English

Deacetylation of chitin to afford chitosan is a difficult reaction because of the low solubility of chitin in most solvents requiring excess quantities of sodium hydroxide and heat. Deacetylation of chitin often occurs alongside depolymerisation of the polymer chain, 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 in the solid state with limited needs in terms of energy and solvents. Commercial chitin is initially amorphized by milling in a zirconia milling jar with a zirconia ball to provide greater access to the N-acetyl groups for deacetylation. The deacetylation is activated further by milling the amorphized chitin with sodium hydroxide in a polytetrafluoroethylene jar with a zirconia ball to homogeneously mix the materials. Since excess water content has been hypothesized to activate the depolymerization of chitin/chitosan, an aging process using humidity-controlled chambers of 43, 75 and 98% relative humidity has been utilized. This allows for a controlled supply of water during the deacetylation step minimizing depolymerization. We demonstrate that this method is versatile and applicable to a number of chitin sources including shrimp shell, crab, lobster, fly larva and 1-ethyl-3methylimidazolium acetate treated fly larva. Chitin deacetylation was measured by magic angle spinning nuclear magnetic resonance and molecular weight by gel permeation chromatography. Amorphization of chitin was studied by milling chitin in stainless steel, zirconia, agate, polytetrafluoroethylene (PTFE), polymethylmethacrylate (PMMA), copper, brass, tungsten carbide, and aluminum jars with balls of the same material showing that material hardness and ball mass can be correlated to the crystallinity index of the milled chitin. Crystallinity was measured using powder x-ray diffraction (pXRD).

French

La désacétylation de la chitine pour obtenir le chitosane est une réaction difficile en raison de la faible solubilité de la chitine dans la plupart des solvants nécessitant l'utilisation d'un excès d'hydroxyde de sodium et de chauffage. La désacétylation de la chitine se produit souvent parallèlement à la dépolymérisation de la chaîne du polymère, ce qui limite l'accès à la chitosane de haute masse moléculaire. Nous rapportons ici une nouvelle méthode, donc une combinaison de mécanochimie et de vieillissement, pour donner une chitosane de haute masse moléculaire à l'état solide tout en minimisant l'emploi d'énergie et de solvants. La chitine commerciale est initialement amorphisée par moulage dans un pot en zirconium avec une bille de zirconium pour fournir un meilleur accès aux groupes N-acétyle pour la désacétylation. La désacétylation est davantage activée en moulant la chitine amorphisée avec de l'hydroxyde de sodium dans un pot en polytétrafluoroéthylène avec une bille de zirconium pour mélanger de manière homogène les matériaux. Puisque l'excès d'eau a été envisagé comme activant la dépolymérisation de la chitine/chitosane, un procédé de vieillissement utilisant des compartiments à humidité contrôlée de 43, 75 et 98% d'humidité relative a été utilisé. Ceci permet de fournir l'eau d'une manière contrôlée pendant l'étape de désacétylation minimisant la dépolymérisation. Nous démontrons que cette méthode est polyvalente et applicable à plusieurs sources de chitine, y compris la crevette, le crabe, le homard, la larve de mouche et la larve de mouche traitée à l'acétate de 1-éthyl-3-méthylimidazolium. La désacétylation de la chitine a été mesurée par résonance magnétique nucléaire en rotation à l'angle magique et la masse moléculaire est mesurée par chromatographie par perméation de gel. L'amorphisation de la chitine a été étudiée par moulage utilisant un pot d'acier inoxydable, zirconium, agate, polytétrafluoroéthylène, polyméthylméthacrylate, cuivre, laiton, carbure de tungstène et d'aluminium avec des billes de même matériau montrant que la dureté et la masse de la bille peuvent être corrélées à l'indice de cristallinité de la chitine moulée. La cristallinité a été mesurée en utilisant la diffraction des rayons X sur poudre.

Acknowledgements

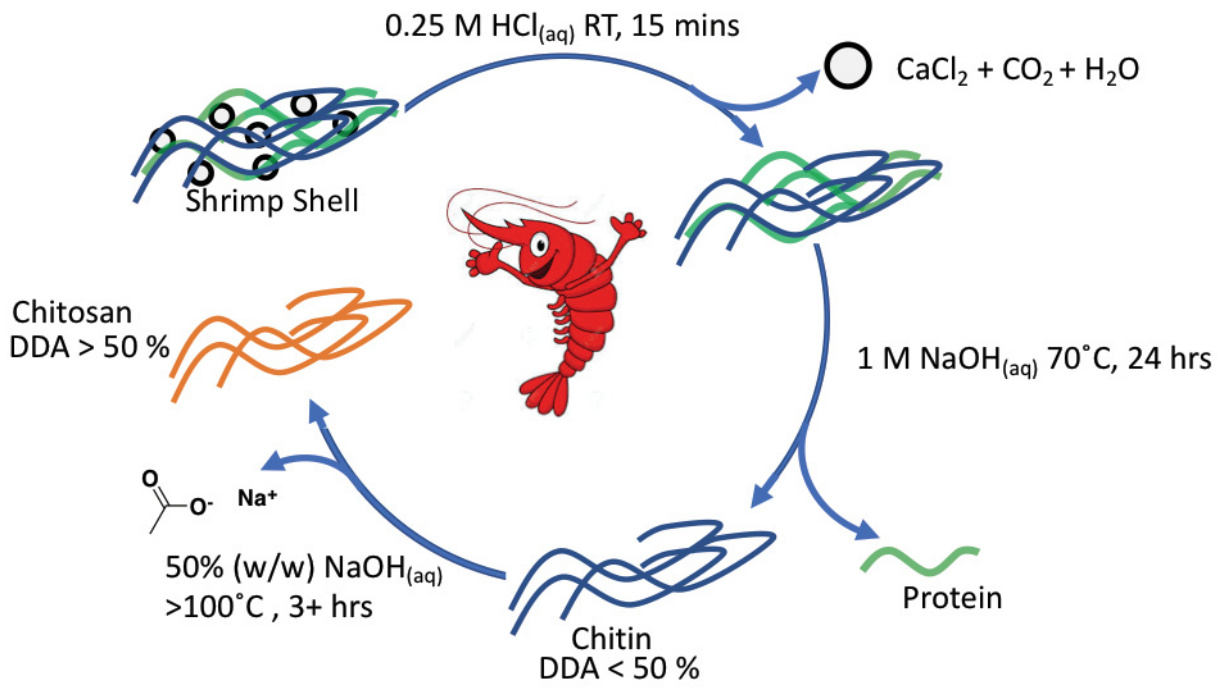
First and foremost, I would like to thank Dr. Audrey Moores for welcoming me into her group and guiding me for the past 2 years. Most importantly, I would like to thank my partner Jennifer for her patience and for all the extra work that she has taken on to enable me in my studies. To my daughters, Cecilia and Winnie, thank you for always making me smile and reminding me of the joy of curiosity, investigation and experimentation. We thank the Natural Science and Engineering Research Council of Canada (NSERC) Discovery Grant program, the Canada Foundation for Innovation (CFI), the Canada Research Chairs (CRC), the Fonds de Recherche du Québec – Nature et Technologies (FRQNT) Equipe program, the Centre for Green Chemistry and Catalysis (CGCC), McGill University for their financial support. We are grateful to Robin Stein for solid state and solution NMR help. We'd like to thank Catherine King, Jean-Louis Do, Hatem Titi, Alain Li and Petr Fiurasek for help and discussions, all well as Blaine Fiss for milling and biomass discussions.

Contribution of authors

This work will be submitted for publication in Green Chemistry by end of May. The authors on the paper, beyond Dr. Moores and myself, will include, Caroline Hadad and Albert Nguyen Van Nhien both from Laboratoire de Glycochimie des Antimicrobiens et des Agroresources in Amiens, France for supplying insect sourced chitin samples with and without pre-treatment with 1-ethyl-3-methylimidazolium acetate. Besides this, all the experimental work presented has been performed by myself. All chapters have been written by myself and edited by Dr. Moores

Introduction

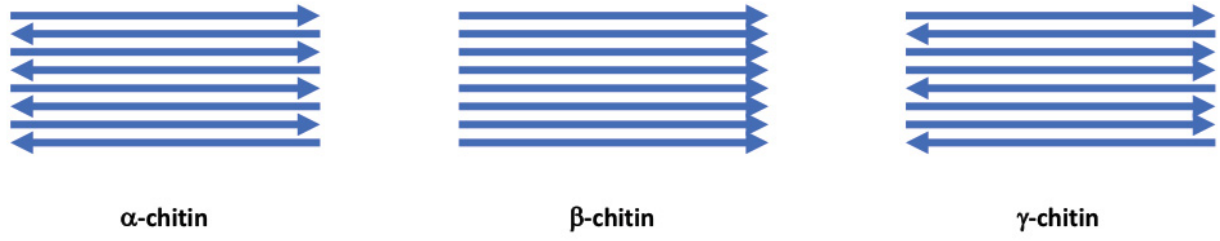
Biopolymers have recently triggered intense research interest and are envisaged as renewable sources for materials and molecules[1,2]. Cellulose and chitin are considered to be the most abundant naturally produced polymers with yearly production levels in the billions of tonnes.[3–5] This high availability, as well as sustainability considerations, explains the intense interest and extended research effort towards the transformation of these biopolymers into useful materials and small molecules. Chitin, which can be extracted from the exoskeleton of crustaceans or arthropods,[6] has a number of exciting properties, including the presence of an amide functionality, which is a useful functionalization manifold.[7] Chitin is a natural polysaccharide composed of β-(1–4)-linked 2- deoxy-2-acetamido-D-glucose units. Its extraction proceeds via two steps, Scheme 1. First the crustacean exoskeleton undergoes a demineralization process where the exoskeleton is exposed to dilute hydrochloric acid (HCl) at room temperature to remove calcium carbonate ($CaCO_3$). The second step to chitin extraction is a treated with NaOH under mild to harsh conditions to remove proteins in the cuticle.[8] Under harsher conditions, depolymerization will occur significantly, yielding lower molecular weight chitin as well as partial deacetylation of the N-acetyl group. At high temperature the acid treatment will also depolymerize the chitin yielding a lower molecular weight.[9]



Scheme 1 – Chitin/chitosan extraction from shrimp shell.

Chitin Crystallinity

Crystalline chitin exists in three known morphologies: α, β and γ forms as shown in Scheme 2.[8] The α form, which is the most common, is composed of antiparallel chains, the β form, which is the most reactive/soluble, is composed parallel chains and the γ form is composed of a sequence of three chains running antiparallel.



Scheme 2 – α, β and γ morphologies of chitin crystal structures.

Chitin can also undergo amorphization via mechanical routes[10–12] which transforms it from an ordered material to a disorganized one of low crystallinity (Figure 1).

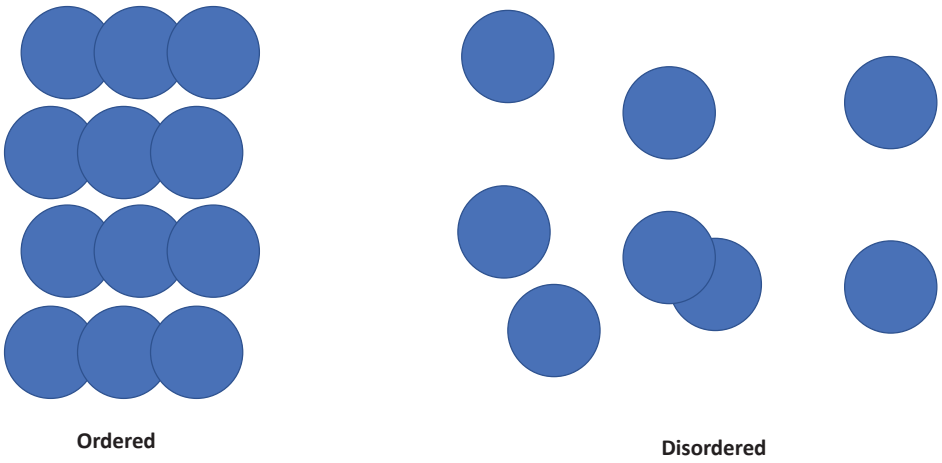
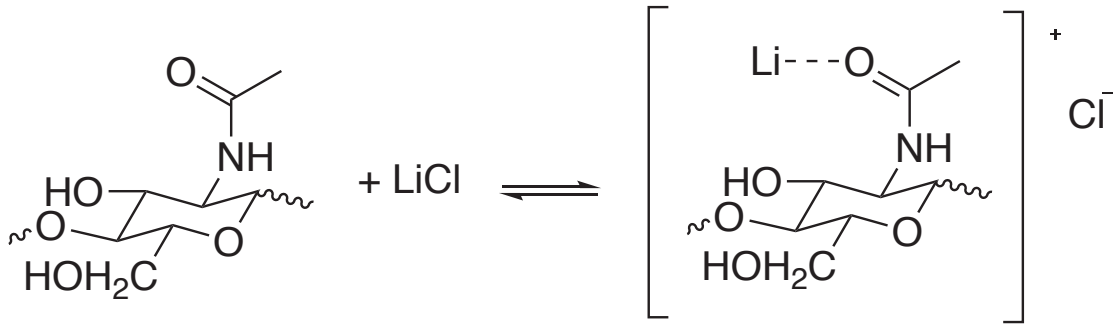


Figure 1 – Representation of crystalline materials as ordered and amorphous as disordered.

Chitin Solubility

Chitin is not readily soluble in most common solvents because of extensive intra and inter molecular hydrogen bonds in its crystalline structure and only slightly soluble in solvent systems like N,N-dimethylacetimide/lithium chloride (DMAc/LiCl) which can disrupt the hydrogen bonding where Lithium forms a complex with the acetyl group as shown in Scheme 3.[13]



Scheme 3 – Chitin dissolution model with LiCl in DMAc.

The use of ionic liquids (IL) for cellulose dissolution by Swatloski[14] ignited research in their use for chitin dissolution and extraction,[15,16] since both are polysaccharides. Chitin is typically extracted in a manner where the unwanted components are dissolved and removed to yield purified chitin, as described above. Alternatively, dissolving chitin with a number of ionic liquid simplifies the extraction process, while potentially preserving its MW.[16] Ionic liquids are defined as salts with a melting point below 100˚C,[16] which are valued for their low volatility, and their

capability to dissolve both organic and inorganic compounds.[17] Ionic liquids are composed of cation-anion pairs whose individual properties will affect the final properties of the ionic liquid, where for polysaccharide imidazolium based cations have been shown to be favourable[14] while the counter ions range from chloride, bromide, acetate, and thiocyanate anions.[14–17] Although during chitin extraction efforts are made to preserve the degree of acetylation (DDA),[6] much work has been conducted to fully deacetylate chitin into chitosan for its ease of manipulation by dissolution in dilute acids.[18–23]

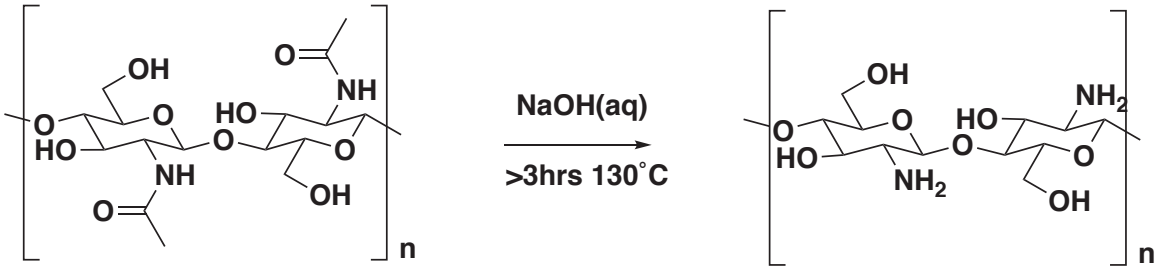
Chitosan applications

Deacetylation of chitin leads to 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 of varied molecular weight were studied for their permeability of sodium chloride, glucose, tyrosine and bovine serum protein,[24] where membranes prepared from higher molecular weight chitosan showed greater affinity to bovine serum proteins and lower molecular weight based membranes had higher throughput rate of sodium chloride, glucose, and tyrosine. Chitosan has also been investigated for protein delivery, where the loading and release efficiency of bovine serum albumin (BSA) into chitosan nanoparticles was studied and where BSA[25] and human serum albumin (HSA) affinity to chitosan were linked to the chitosan molecular weight.[15] Successful chondrocyte growth onto chitosan scaffolds[27] as well as fibroblast cell attachment and proliferation[28] show promise for tissue growth. Water treatment with low concentrations of chitosan of 0.8 wt% or less, completely eliminated bacterial contaminations.[29] Molecular weight was associated with antibacterial effect based on its solubility in water at varied pH, where lower molecular weight chitosan can dissolve more readily at higher pH and therefore have a greater ability to interfere with bacterial growth.[30] The use of chitosan hydrogels in drug delivery,[31] chitosan encapsulated quantum dots for anti-cancer drug delivery[32] and controlled drug release[33] has been investigated extensively. Cell growth occurs at a higher rate on chitosan with a higher degree of deacetylation.[34] Carboxymethyl chitosan has been used in cosmetics as a thickening agent due to its high viscosity and large hydrodynamic volume.[35] Packaging made from chitosan shows great

promise due to its biodegradability and improved tensile strength when prepared by evaporation method.[36] Chitosan ability to chelate many metals, due to its amine functional group, allows it to be used as a heterogeneous catalyst support for a multitude of reactions.[37]

From Chitin to Chitosan

The most common chitin deacetylation method consists of a treatment in a highly concentrated NaOH solution (≥12.5M) heated for several hours at temperatures above 100 ˚C and often repeated in multiple cycles to further deacetylation, where the acetyl group is removed to yield a primary amine in chitosan, Scheme 4.



Scheme 4 – Solution chitin deacetylation to yield chitosan.

Once the degree of deacetylation (DDA) reaches 50%, chitin is considered to be chitosan. In practice, chitosan featuring DDA greater than 70% are preferred for their improved solubility in dilute acid solutions. Beyond the typical solvo-thermal methods,[38–41] several methods have been developed to deacetylate chitin with the aim to create greener, safer processes and decrease depolymerization. Those of interest include maceration[18] steam explosion,[19] freeze-thaw cycles,[20] high temperature and pressure,[21,22] sonication,[18] microwaving,[42] and planetary milling.[11] In maceration, chitin is soaked in 60-80% NaOH solution at room temperature for 7 days, resulting in a nearly linear DDA of 60-80%.[18] For steam explosion humidity controlled chitin was sealed in a drum which was subsequently heated to 179˚C to a pressure of 9 kg/cm^2. The pressure is suddenly relieved to induce the steam explosion, yielding a DDA of 43.7% without the use of NaOH.[19] In the freeze-thaw cycle, chitin is suspended in 13-24% NaOH solution, frozen then thawed to room temperature, hence reducing its crystallinity. The solution is then heated to 75 ˚C reaching DDA of 80-95%.[20] High temperature and pressure methods

have been used to deacetylate chitin, where the chitin is mixed with a solution of NaOH 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.[21,22] Sonication has been shown to deacetylate chitin up to 77% using an 80% NaOH solution at 40˚C yet resulting in a greater loss of recoverable chitosan.[18] In microwaving, chitin is added to 45% NaOH solution and irradiated for up to 5.5 minutes at 900 watts resulting in a DDA of 85.3% while significantly decreasing the MW from ~400 kDa to 85 kDa.[42] Using a planetary mill, chitin was milled with 5 equivalents of NaOH in the solid state for 8 cycles of 10 minutes milling and 5 minutes rest to yield a low molecular weight chitosan, of 6.3 kDa with a DDA of 76.4% .[11] Table 1 shows a comparison of deacetylation methods, their molar equivalents of NaOH, DDA and MW, if known.

Table 1 – Comparison of deacetylation methods including NaOH equivalents, MW and DDA.

[11,18–23,38–40]

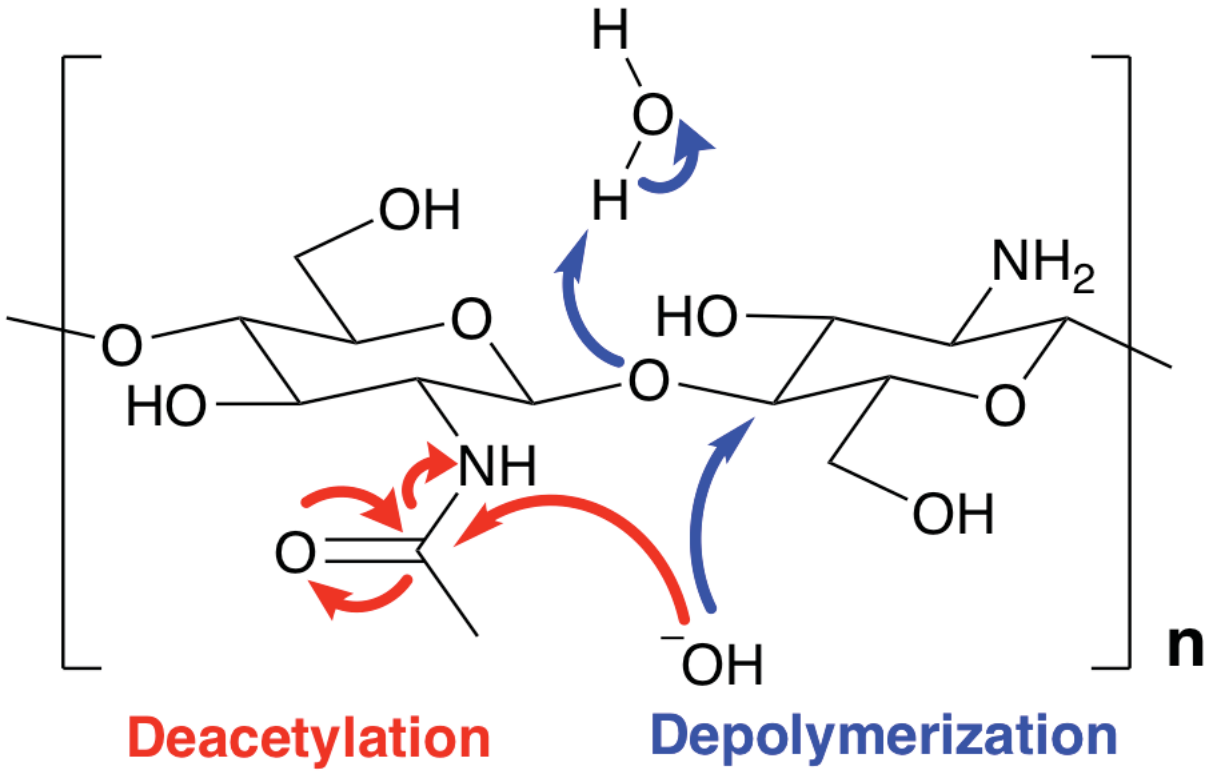
Method	**Conditions**	**NaOH equivalents**	**MW**	**DDA**
Solvo-Thermal	50% $NaOH_{(aq)}$, RT-140˚C, 3-540 Hrs	**42**	Varied	>70%
Planetary Milling	1:5 chitin:NaOH, 100 balls ZrO_2[ZrO2], 80 mins	**5**	1-13 kDa	80 %
Microwave	45% $NaOH_{(aq)}$, 5.5 Mins, 900 W	**31**	85 kDa	80 %
Sonication	60 % $NaOH_{(aq)}$, 30 mins, 500 W	**>16**	Degradation	73 %
High Pressure Run	50% $NaOH_{(aq)}$, 120˚C, 15 PSI	**41**	1466 kDa	90 %
Maceration	7 days, 80 % $NaOH_{(aq)}$	**22**	N/A	80 %

The development of these methods has progressively allowed the decrease of reaction time, temperature, or energy and the reduction of the number of necessary NaOH equivalents compared to conventional methods.

Importantly, the treatment of chitin with NaOH leads to two competing reactions: deacetylation, but also depolymerisation, by nucleophilic attack of the chitin glycosidic bonds by hydroxide ions. Depolymerisation may be a desired reaction outcome, to afford low-molecular weight chitosan, or even glucosamine monomers.[43] 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.[11] 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.[43]

Chitin Depolymerization during deacetylation

Chitin deacetylation occurs in an alkaline environment where there is a competition between deacetylation and depolymerization via glycosidic bond cleavage as depicted in Scheme 5. There is a trade-off, to maintain MW only lower DDA can be achieved but complete deacetylation comes at the cost of more significant depolymerization, where increased temperature and NaOH concentration have been shown to decrease MW. Chitin depolymerization in an alkaline environment has been ascribed to not only excess hydroxyl ions but more importantly to excess water which protonates and activates the glycosidic bond for cleavage.[44] Although water is essential in the deacetylation process controlling the amount of water available during the deacetylation process would protect the polymer from depolymerization.



Scheme 5 – Deacetylation/depolymerization trade-off in base catalyzed reactions.

Accelerated aging and mechanochemical methods

Accelerated aging has recently emerged as a method to access inorganic and organic materials with very low energy input, compounds can be mechanochemically activated and aged in a humidity chamber.[45–47] For chitin deacetylation, low temperature solution aging[41] 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, depolymerisation levels above 70% were phenomenologically established by the excellent water solubility of the resulting samples. Mechanochemical processes have been used to accelerate depolymerization of chitin, where first amorphizing the chitin allows greater access to the glycosidic bonds and increasing the yield of the N-acetyl-glucosamine monomer during a following catalytic process.[48] It has also been utilized to increase the reactivity of chitin to further the yield of 3-acetamido-5-acetylfuran.[49,50]

Chitin/chitosan molecular weight

Preservation of molecular weight is attempted in literature by decreasing temperature and NaOH concentration, but it comes at the cost of a significant increase in time, and overall lower DDA.[18–23] 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.[22] As biopolymers are sought after as ideal precursors for high-end functional materials, there is merit in exploring avenues to preserve the polymer chain as much as possible.[51] Indeed, high molecular weight (MW) polymers typically feature desirable mechanical properties[26,27,30] and improved glass transition temperatures.[52] There is thus a dire need to develop a method to afford in one-step and with reasonable scale-up prospect high MW chitosan from chitin. Such a method should also seek to reduce energy, chemical and solvent input for improved sustainability, while being safe.

Purpose of the MSc thesis

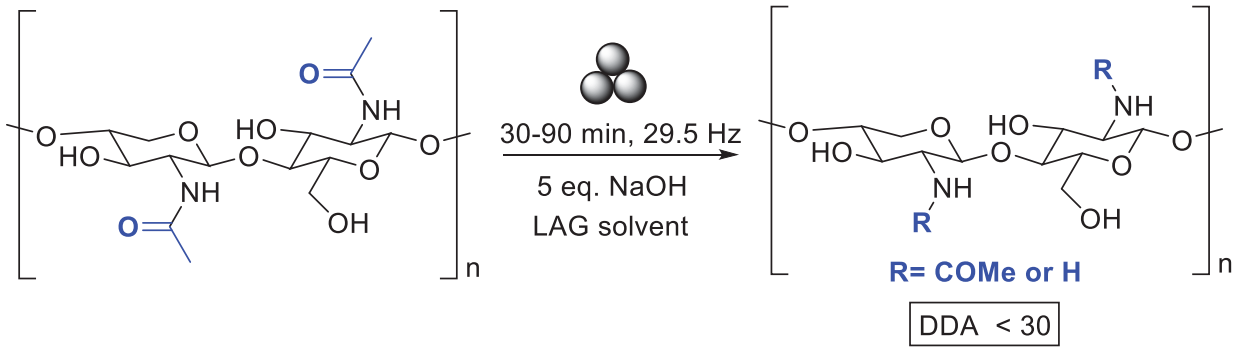
This thesis presents the work done towards preserving molecular weight of chitin during the deacetylation process while providing a safe, low energy alternative to methods found in literature. We present a study of chitin deacetylation in the solid state with NaOH with the aim to preserve the polymer chain. We have explored the use of low energy mechanochemistry, using mixer mill, as well as aging, the role of time, quantity of NaOH, humidity, as well as the effect of an amorphisation pre-treatment of chitin. A process controlling and restricting water availability, such as in a humidity chamber, could provide sufficient water necessary for deacetylation while minimizing its availability to activate the depolymerization process. At lower temperatures, access to internal N-acetyl sites are hindered, restricting the achievable DDA. To improve access to these sites, amorphization of the chitin has been utilized, which can be accomplished by milling or grinding in zirconia and stainless steel. It has also shown that chitin molecular weight decreases when milled extensively and furthermore when milled with NaOH. Amorphizing chitin to a nominal amount allows for greater retention of MW while providing sufficient access to N-acetyl sites to achieve higher DDA. We also studied a number of chitin sources, including commercial chitin, powdered shrimp, crab, and lobster shell as well black fly larva. And black fly larva treated with [C_2mim][OAc]DDA was calculated by ^{13}C magic

angle spinning nuclear magnetic resonance (MAS NMR), and ^{1}H solution NMR, MW was calculated by gel permeation chromatography (GPC) and viscometry. Optimized methods allowed for excellent DDA levels (73-95%).

Results and Discussion

Mixer-mill based chitin deacetylation experiments

High energy milling methods, such as the use of a planetary mill with 100 balls, were previously reported to yield very efficient deacetylation of chitin, at the expense however of significantly reduced molecular weights.[11] 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 zirconia (ZrO_2) ball was used as a soft solid-state reaction method(Scheme 6). In all methodology development experiments, commercial chitin (DDA=4%) was used as a starting material (Appendix 4). 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 6). Increasing milling time, up to 90 min, yielded no greater degree of deacetylation (Appendix 5). In an effort to improve deacetylation, the addition of stoichiometric amounts of liquids of <1µL/mg,[53] also referred to as liquid assisted grinding[54] (LAG), was attempted. This method has proved useful for instance for co-crystal formation[55] or metal organic framework synthesis.[56] Dichloromethane, acetonitrile, ethyl acetate ethanol, methanol, and deionized water were tested for their effect in chitin deacetylation (Appendix 6).



Scheme 6 – Mixer-mill based chitin deacetylation experiments.

DDA = [H]/([COMe]+[H])x100, LAG solvent 10 wt%: none, dichloromethane, acetonitrile, ethyl acetate Typical experimental conditions: chitin (105 mg), NaOH, 5 eq. (95 mg) based on glucosamine unit, loaded to a PTFE jar with a ZrO_2 ball

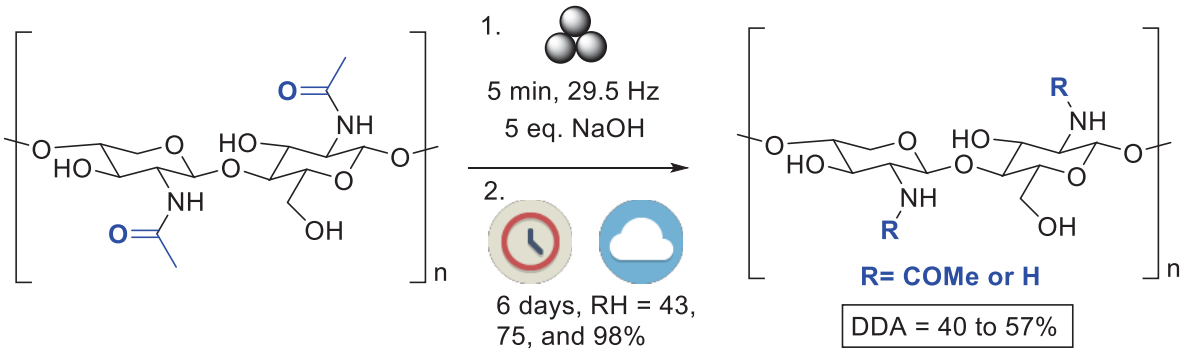
The best result obtained with this strategy was 23% DDA while using the 20% deionized water LAG 1:5 chitin:NaOH mixture (Appendix 7). As chitosan applications require a degree of deacetylation of at least 70%,[15,25,36,37,57,58,27–33,35] the soft solid state mechanochemical strategy did not prove useful to achieve our goal, and we turned to aging.

Chitin deacetylation experiments by aging

We reasoned that a longer contact period between the polymer and the NaOH were necessary to improve deacetylation. We thus explored the use of aging, in dry and humid environments. After a short milling period of 5 min in a mixer mill, in order to intimately mix the reagents, the samples were aged in a dry form in closed PTFE jars prior to workup. When aged at room temperatures (22 ˚C) and humidity (73-94% relative humidity), which were dependent on weather, we obtained promising results with DDA values varying between 10-30% and 15-50% DDA for 1:1 and 1:5 chitin:NaOH ratios respectively.

In an effort to rationalize the humidity levels in the samples during aging, we turned to controlled humidity chambers. Salt saturated aqueous solution prepared with K_2CO_3, NaCl, and K_2SO_4 are able to afford stable 43, 75, and 98% relative humidity (RH) environments inside a sealed enclosure,[45] respectively. Similarly, to experiments in ambient humidity, chitin was first milled for 5 mins with NaOH in a PTFE jar with a ZrO_2 ball, before being placed in a vial inside the humidity chamber for several days (Scheme 7). It should be noted that even with 5 minutes of milling chitin with PTFE^{ZrO_2} there were residues of PTFE found in the chitin as determined by X-ray photoelectron spectroscopy (XPS) (Appendix 3). Interestingly, at room temperature, the resulting degree of deacetylation improved greatly. When aged for 6 days with relative humidity (RH) of 43, 75, and 98% the DDA increased to 40, 43, and 57% respectively (Appendix 8). This showed that the availability of water vapor is an important factor in the deacetylation rate of chitin. To establish a comparison, we aged chitin samples in a 50% NaOH aqueous solution for 6 days with a 1:5 chitin:NaOH and achieved a lower DDA of 42% (Appendix 9). Maceration had previously been used to age chitin in concentrated NaOH solutions for 7 days at RT, where a DDA in the 60-81% range was achieved with reduction in recovered mass indicating significant depolymerization.[18] It can be hypothesized that solid state/high humidity environment experiments optimized the quantity of water available, while the high local

concentration of reagents was optimized, compared to the solution-based reaction.[59] In order to further boost DDA even further, we explored the possibility to mechanochemically amorphize chitin prior to reaction.



Scheme 7 – Aging based chitin deacetylation experiments.

DDA = [H]/([COMe]+[H])x100. Typical experimental conditions: 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 in humidity chamber.

Mechanochemical amorphization of chitin as a pre-treatment to deacetylation by aging.

It is well-known that crystallinity in materials restricts their reactivity, including biopolymers. For instance, the acid hydrolysis of cellulose occurs more effectively in amorphous regions.[60] Commercial chitin itself is highly crystalline,[10] which has been shown to slow or even prevent significant deacetylation, including in liquid-phase enzymatic protocoles.[61] Chitin can benefit from amorphization to improve access to acetyl sites[10,20] through increased internal permeability.[62] Mild amorphization can be achieved through a freeze thaw cycle[20] which helps to achieve a DDA of 80-95% while maintaining a good MW of 180-300 kDa in 24% NaOH solution (21 molar eqs., 75-85 ˚C, 3-5 Hrs). Amorphization has also been proposed as an important process taking place during mechanochemical deacetylation of chitin.[11] Under harsher mechanochemical conditions, amorphization occurs in conjunction to depolymerisation of the biopolymer yielding DDA of 76.4% and MW of 6.3 kDa, where 5 molar eqs. of NaOH were used with milling for a total of 80 minutes, in 10 minute cycles.[63] For this study, we explored the role of amorphization, as a pre-treatment prior to aging, to improve deacetylation, while limiting depolymerisation, and fine tune the properties of the end product.

We first looked into the effect of dry milling pure chitin, in absence of any reagent, by probing the crystallinity index (CrI) of the product by pXRD. We first mixer-milled commercial chitin in PTFE using one ZrO_2 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 2). In order to improve impact[64] and favor amorphization harder materials were investigated. Stainless steel and ZrO_2 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 ZrO_2. Further milling in PTFE with a zirconia ball for 90 mins yielded no significant further amorphization with a CrI of 48.8%. Comparing the Vickers hardness and density (ρ), which can be commonly found in material technical data sheets, of PTFE (5-60 MPa, ρ=2.2 g/cm3), ZrO_2 (10-14 GPa, ρ=5.68 g/cm3) and stainless steel (1-2 GPa, ρ=7.7 g/cm3) it can be correlated to the effect of amorphization of the milled chitin(Figure 3), which itself has been shown to exhibit high Vickers hardness (245-784 MPa, ρ=1.3 g/cm^3 in insect cuticles composed of chitin)[65] compared to some synthetic polymers. Crystallinity can be correlated to mass of the milling ball given that the hardness of the milling jar and ball is harder than the chitin. Figure 4 shows that regardless the ball used, when milling in PTFE, very little change in crystallinity occurs, and when milling in harder jars of ZrO_2 and SS, very little change in crystallinity occurs with the PTFE ball but significant decrease in crystallinity occurs with ZrO_2 and SS balls. This indicates that there is a threshold of hardness for energy transfer from milling media to the sample.

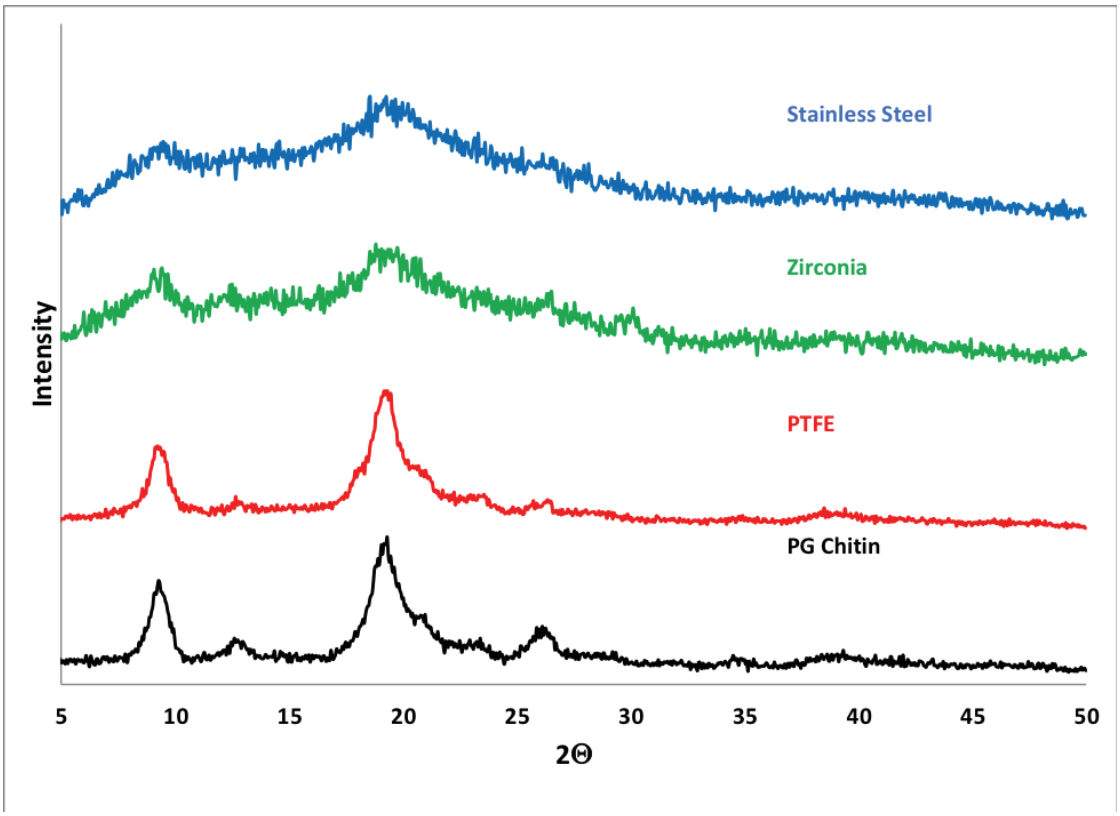


Figure 2 – pXRD of chitin and chitin milled for 30 mins in PTFE, Stainless steel, and ZrO_2 jars.

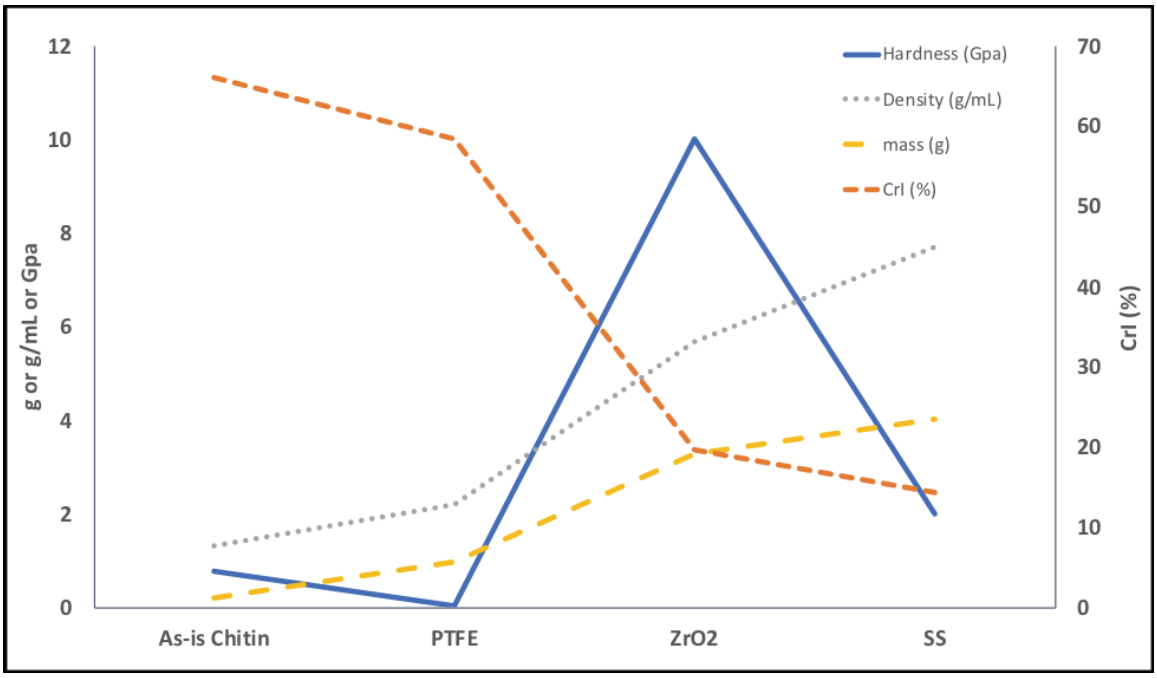


Figure 3 – Combined ball Density (g/mL), ball mass (g) and jar hardness (Gpa) effect on CrI (%).

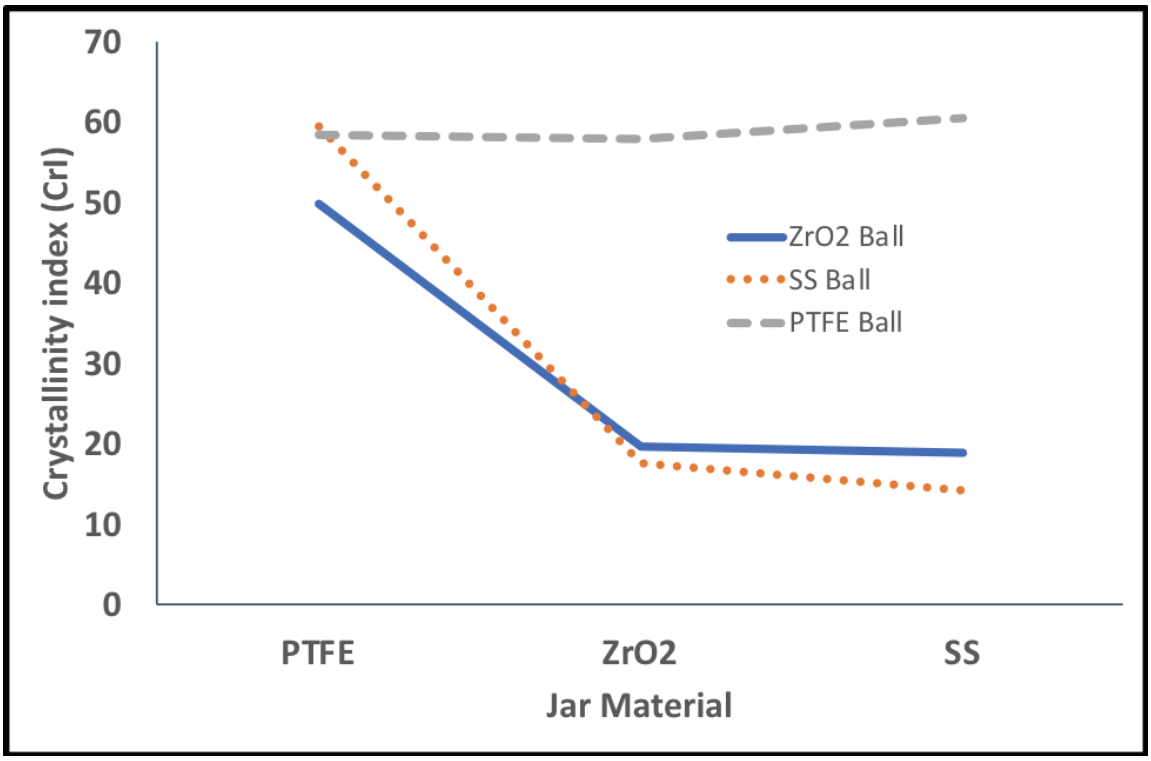


Figure 4 – CrI (%) of milled chitin comparing all three PTFE, ZrO_2 and SS balls in each of PTFE, ZrO_2 and SS jars.

While good amorphization was achieved in hard material jars, the impact on chitin MW is another important parameter to control. Considering planetary ball milling with 100 - 0.5 mm ZrO_2 balls for 80 mins yielded a MW of 79.7 kDa[11] and that ball mass affects amorphization, it is highly possible that depolymerization can be greatly limited with lower milling time and mass of milling media. These amorphized samples were further studied for subsequent deacetylation by aging, with the expectation that DDA yields should be improved since access to N-Acetyl sites is greater.[10]

Jar and Ball Material Milling Study

Further investigation with different jar and ball materials where in each trial, 200 mg of PG chitin was milled for 30 minutes in each jar: stainless steel, copper, brass, PMMA, PTFE, ZrO_2, aluminum, tungsten carbide, and agate, with balls of the same material in approximately 10 mm diameter (Figure 5) and separately with a ball of the same material weighing approximately 2 g (Figure 6). This study showed correlation between milling media hardness, mass and effect on CrI.

CrI difference is calculated by the following equation:

Equation 1 - Crystallinity difference.

$CrI_{diff} = CrI_{PGchitin} - CrI_{milled\ sample}$

Where,

$CrI_{PGchitin}$ is the measured CrI of commercial PG chitin and,

$CrI_{milled\ sample}$ is the measured CrI of the milled chitin.

Mass and hardness corrected CrI difference was calculated by the following equations

Equation 2 – Mass and Hardness correction of CrI.

$CrI_{MHdiff} = CrI_{diff}/(M_{ball} \bullet H_{ball})$

Where,

M_{ball} is the mass of the ball in g, and

H_{ball} is the Vickers hardness of the ball in GPa

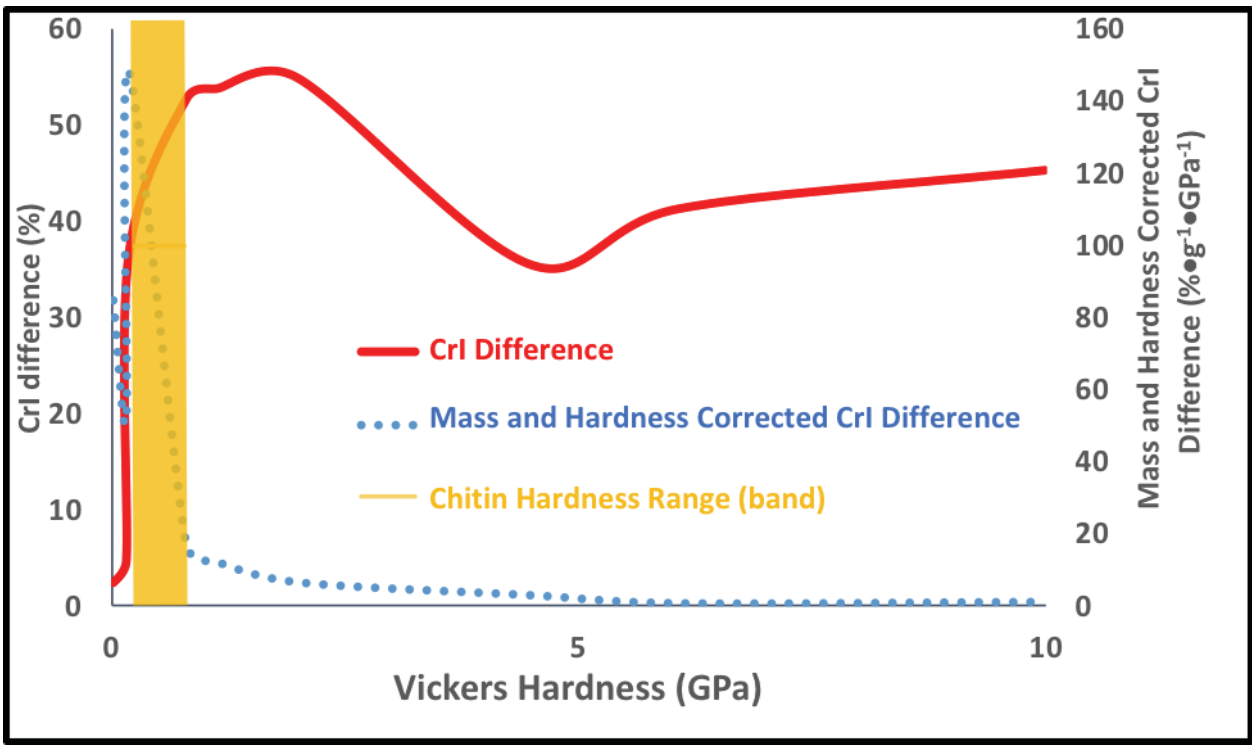


Figure 5 – CrI difference and Corrected CrI difference of chitin milled in stainless steel, copper, brass, PMMA, PTFE, ZrO_2, aluminum, tungsten carbide, and agate, with balls of the same material with approximately 10 mm diameter balls of the same material.

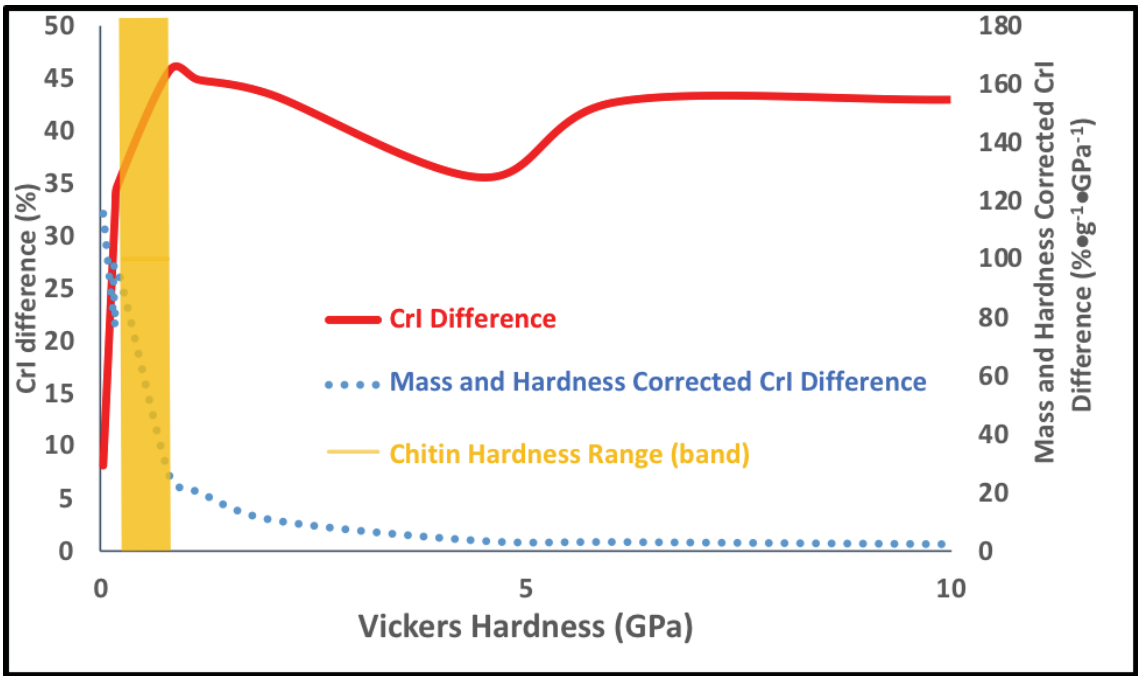


Figure 6 – CrI difference and Corrected CrI difference of chitin milled in stainless steel, copper, brass, PMMA, PTFE, ZrO_2, aluminum, tungsten carbide, and agate, with balls of the same material with approximately 2 g balls of the same material.

In another trial 200 mg of chitin was milled for 30 minutes with balls of different material, (PTFE, PMMA, aluminum, brass, copper, stainless steel, agate, tungsten carbide, ZrO_2, silicon nitride, and alumina each weighing approximately 2 g in both aluminum (Figure 7) and copper () jars for comparison. Aluminum and copper jars were chosen for their Vickers hardness, 196 MPa and 1.15 GPa respectively, being similar yet outside of the Vickers hardness range of chitin (245 – 784 MPa). This lead to interesting correlations where mass of ball as well as materials Vickers hardness shows a crossover point of ball hardness, where CrI changes significantly (Figure 7 and Figure 8), which overlaps with published chitin Vickers hardness range.[65]

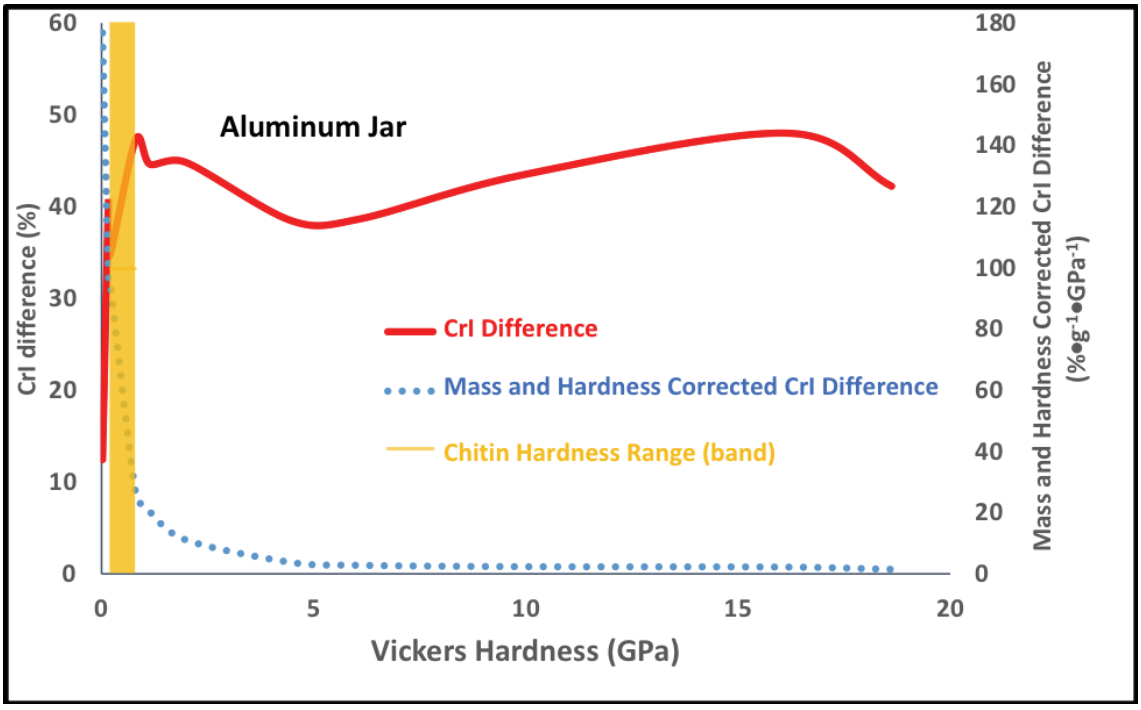


Figure 7 – Chitin CrI difference vs Vickers hardness of milling balls in an aluminum jar.

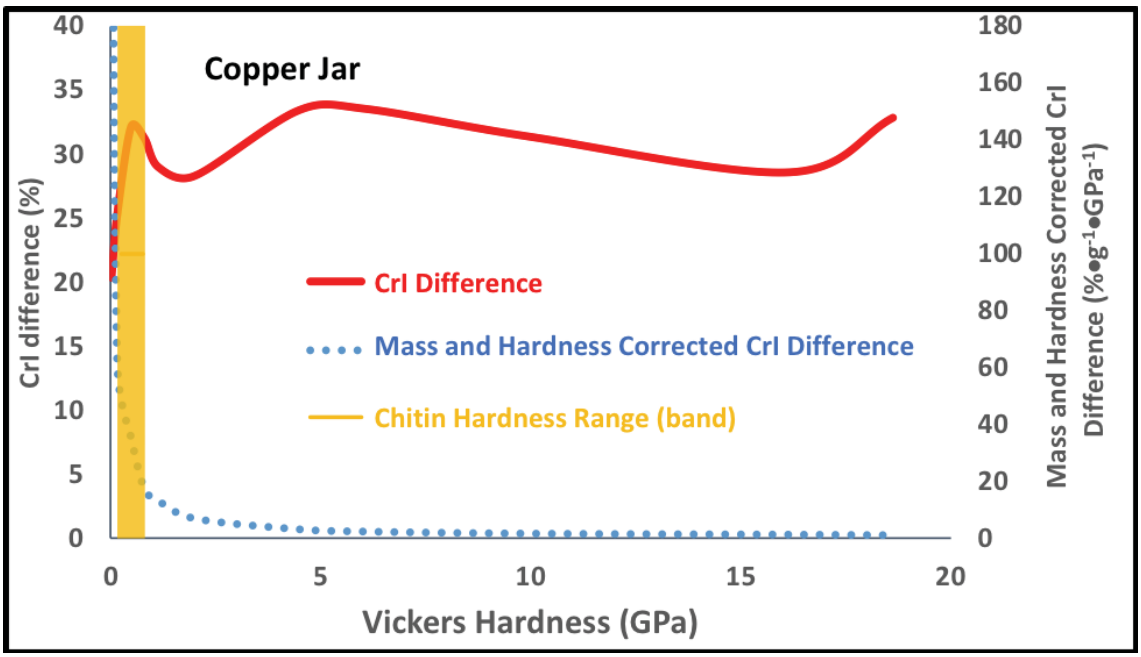
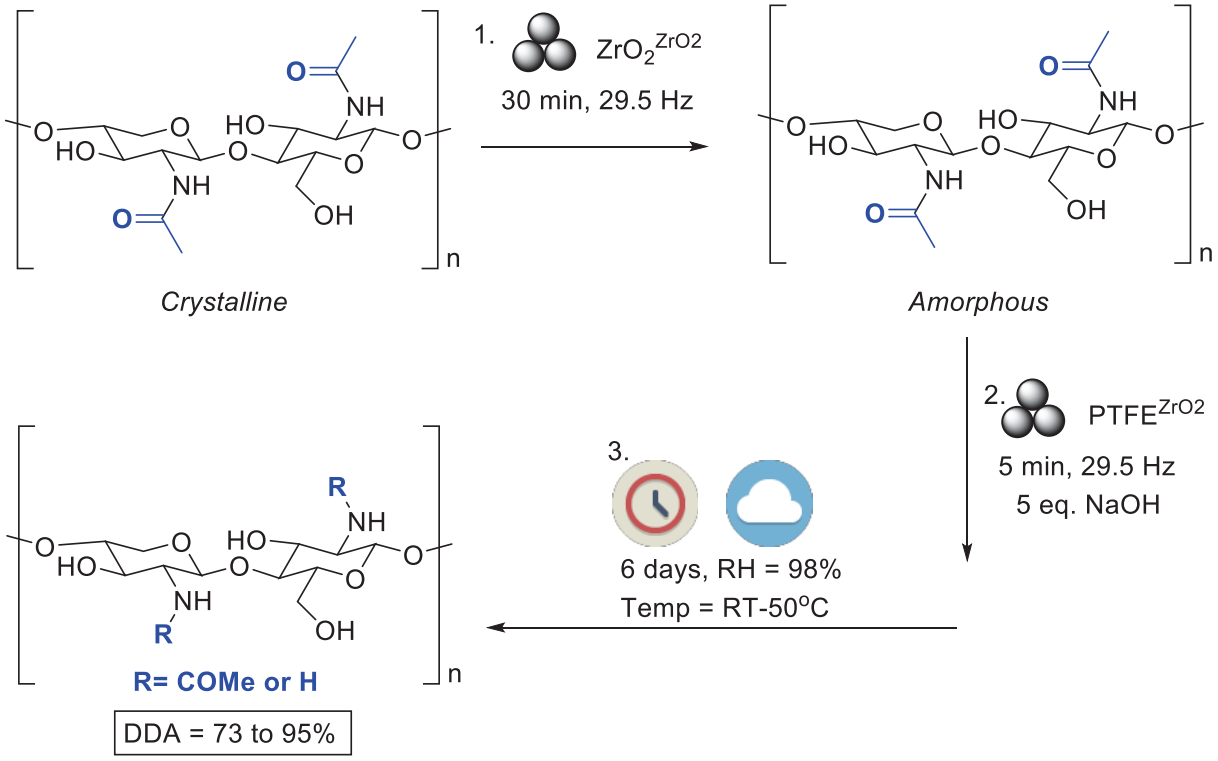


Figure 8 – Chitin CrI difference vs Vickers hardness of milling balls in a copper jar.



Scheme 8 – Amorphization/aging based chitin deacetylation experiments.

DDA = [H]/([COMe]+[H])x100. 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.

For aging reactions, only chitin milled in ZrO_2 was used. Chitin samples, which were first amorphized by mixer milling in ZrO_2 apparatus for 30 mins, were then 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 98% RH for 6 days. This afforded

DDA of 73% (Appendix 8). DDA was confirmed using IR (Appendix 1) and ^{13}C MAS NMR (Appendix 2). In order to further boost DDA while maintaining MW, optimization of reaction conditions was explored by modifying aging temperature, aging time, and NaOH equivalents.

Reaction optimization

Raising aging temperatures was expected to accelerate all hydroxyl-catalyzed reactions, not just deacetylation, but also depolymerisation. We first launched a study of the aging temperature where the humidity chambers were placed in an oven. Raising the temperature from RT (22 ˚C)

to 30 ˚C allowed to improve the DDA from 73% to 84%, while 40 and 50 ˚C afforded DDAs of 90% and 95% DDA, respectively (Figure 9).

Decreasing the amount of added NaOH is also important in order to lower the footprint of chitin to chitosan transformation. 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 10). Reaching a DDA of 86% with 1:4 chitin:NaOH is an improvement in NaOH equivalents over recent milling deacetylation methodologies,[11,63] where 1:5 chitin:NaOH mixture was used and a significant decrease compared to conventional methods with 1:40 chitin:NaOH.[39]

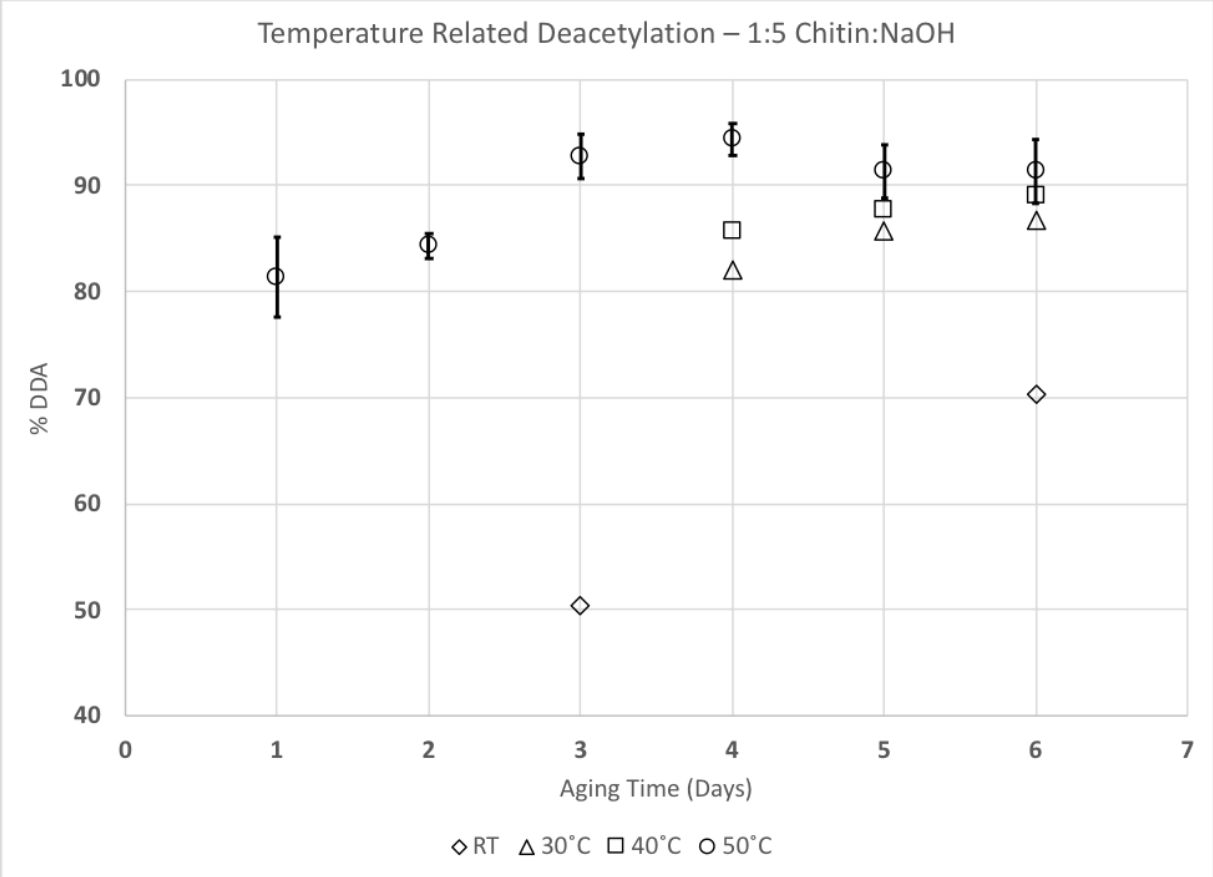


Figure 9 – Effect of temperature and duration of aging on DDA of chitin.

Since, both 1:4 and 1:5 chitin:NaOH conditions result in highly deacetylated chitin suitable for most applications, their aging time conditions were investigated since decreasing the overall reaction time while achieving high DDA could result in higher MW retention. For 1:4

chitin:NaOH, after 1 day 70% DDA is achieved, increasing to 86% after 5 days. For 1:5 chitin:NaOH, after 1 day 77% DDA is achieved, increasing to 83% after 2 days and 95% after 3 days. Although the reaction times are long, for higher DDA, they offer a very low energy methodology while still providing high DDA after 1 day of aging.

Scope: other sources of chitin

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 shells and fly larva, which are all composed of chitin, as well as proteins and $CaCO_3$.[66] All biomass samples were dried, ground and sieved to particles <125 µm, then amorphized for 30 mins in a ZrO_2 jar with a ZrO_2 ball. We also explored fly larva samples treated with ionic liquids in order to purify the chitin from proteins and $CaCO_3$, according to a method we (Hadad, Nguyen Van Nhien) previously reported.[67] All these samples were mixed with NaOH, milled for 5 min in a PTFE jar with ZrO_2 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 $CaCO_3$. Shrimp shell reached a DDA of 95%, while lobster, crab and fly larva reached 96%, 98% and 90% respectively (Table 2). Besides having higher ratios of NaOH to chitin, the protein, $CaCO_3$ matrix allows for better access to chitin for deacetylation. Chitin samples extracted from fly larva reached their highest DDA of 78% (Table 2), when the biomass was pretreated with 1-ethyl-3-methylimidazolium acetate. This lower DDA is expected since the crystallinity of the extracted fly larva chitin is higher.

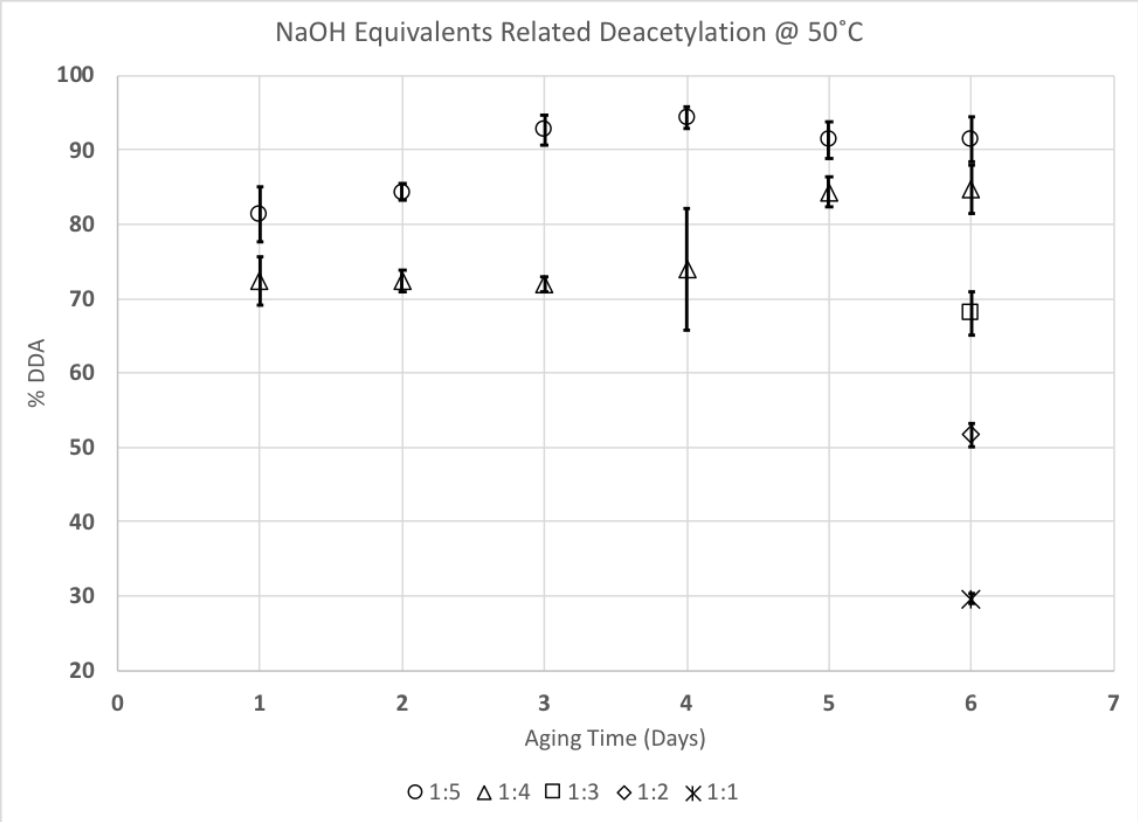


Figure 10 – Effect of duration of aging and number of equivalent of NaOH on DDA of chitin.

Table 2 – DDA results on crustacean shells, arthropod larva and ionic liquid treated larva. a Typical experimental conditions: Samples were dried, ground, sieved, before being amorphized by milling in ZrO_2 apparatus. Then 5 eq NaOH were added, and the mixture loaded to a PTFE jar with a ZrO_2 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 ZrO_2 ball, followed by aging at 98% RH for 6 days at 50˚C.

Shell source	% DDA (^{13}C CP MAS NMR)
shrimp[a]	95
lobster[a]	96
crab[a]	98
fly larva[a]	90
Chitin 0 Steps[b]	74
Pretreated Chitin 0 Steps[b]	78
Chitin 1 Steps[b]	72
Pretreated Chitin 1 Steps[b]	60

Molecular weight analysis

Part of the intention for this project was to probe for the effect on MW of aged chitosan. Commonly, gel permeation chromatography is used to measure the MW of chitosan using. One of the difficulties encountered in this project is that despite having very high DDA, the solubility of the aged chitosan was low. It is understood that higher molecular weight polymer as less stable or soluble in solution. In our case, less than 5% of the aged chitosan was soluble in acetic acid buffer, as determined by following literature dissolution methods then filtering using 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. The portion of aged chitosan which was soluble was analyzed using GPC yielding lower MW of 10-40 kDa regardless of treatment method conditions. This could be indicative of low depolymerization. Further investigation is currently being conducted using 1-ethyl-3-methylimidazolium acetate to dissolve the aged chitosan and determine its molecular weight using viscometric studies. With the use of GPC, we have measured the MW of the commercial chitosan samples we have. These samples can be used to build a calibration curve for the viscometric measurements. These will be used to determine the Mark-Houwink paramaters, α and K.[68] With these parameters the unknown molecular weight of the aged chitosan can be calculated using the Mark-Houwink equation,

Equation 3 - Mark-Houwink equation to calculate MW from intrinsic viscosity.

$$[\eta] = K{M_v}^{\alpha}$$

Where,

η, is the intrinsic viscosity of the polymer solution

And M_v is the viscosity molecular weight

For chitin the same viscometric method will be used, where the known molecular weight chitosan will be reacetylated then used to build a calibration curve in the same manner. This method will also be compared to a known viscometric method using DMAc/LiCl to correlate the data. The use of ionic liquid would be favourable in these cases since they favour chitin and chitosan dissolution.

Experimental

Chemicals and methods

Practical grade chitin, low, medium, and high molecular weight chitosan, lithium chloride (anhydrous, ≥99.0%), acetic acid-d4 (≥99.5% D), N,N-dimethylacetamide (HPLC grade ≥99.9%) were purchased from Sigma-Aldrich Co. LLC (St-Louis, MO). 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). Pullulan calibration Readycal Kit (Mp 180 – 1 220 000 Da) was purchased from PSS polymers (Germany). Homemade chitosan was prepared by a traditional method[40] for comparison by heating for 3 hours in 50% NaOH solution at 120˚C.

Milling

In the following procedures, a Retsch MM 400 was used as mixer mill, with jars made of PTFE, 20 mL, stainless steel (10 mL), ZrO_2 (10 mL) equipped with balls of ZrO_2 or stainless 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 K_2CO_3, NaCl and K_2SO_4, 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 an oven, Fisher Scientific Isotemp.

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 100 mL of methanol until neutral pH using Whatman filter paper (# 1, 55 mm), 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 in 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).

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 pre-treatment of biomass sample

Biomass of fly larva were treated with 1-ethyl-3-methylimidazoliumm acetate 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.[67]

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 ZrO_2 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 ZrO_2 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

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

NMR spectra were recorded on a Varian VNMRS operating at 400 MHz for the solid-state ^{13}C acquisition using a 4mm double-resonance Varian Chemagnetics T3 probe (Appendix 2). A contact time of 1000 µs and a recycle delay of 3 s were used to acquire quantitative spectra, where care must be taken to assure max magnetization is the same for the carbons used for quantitative calculations since carbonyl, methyl and polysaccharidic backbone are in different chemical environments.[69,70] 2872 scans were acquired of each sample for a total time of 2.5

hrs. DDA was calculated using a known method of comparing the integration of the methyl peak to the integration of the C1 carbon of the polysaccharidic backbone.[71]

^{1}H NMR

NMR spectra were recorded on a Varian VNMRS spectrometer operating at 500 MHz for ^{1}H acquisitions. DDA was calculated using a known method[72] when samples were soluble in dilute acetic acid.

IR

IR spectra were recorded using a Perkin-Elmer Spectrum 400 for 64 scans from 4000cm^{-1} to 450cm^{-1} in transmission mode from pressed KBr disc 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.[73]

pXRD

Sample diffractogram was recorded from 5° to 50° 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] x 100.[74]

XPS

XPS was performed on a VG ESCALAB 3 MKII spectrometer (VG, Thermo Electron Corporation, UK) equipped with an Mg Kα source using a spot size of 100 µm, running 5 survey scans at 200 mV for 50 ms residence times, and 10 scans for specific elements, also at residence times of 50 ms.

GPC

Molecular weight was measured using Agilent Technologies 1260 Infinity II GPC triple detection equipped with Shodex OHpak SB-804 HQ 300 x 8 mm, 10 µm column which was calibrated with a PSS Polymers Readycal Kit pullulan standard.

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 ad 1.5 mm stainless steel ball.

Density

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

Conclusion

In this thesis we present a solvent-free method to deacetylate chitin by first amorphizing then aging with NaOH in a humidity chamber. Where Increasing NaOH molar equivalents or temperature or time increases degree of deacetylation. 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. Similarly, chitin can be directly deacetylated from shrimp, crab and lobster shell as well as fly larva and fly larva that has been pretreated with [C_2mim][OAc] in the same aging process. Initial dry and lag milling methods showed to be ineffective at deacetylating chitin. Aging of crystalline chitin in humidity significantly improved deacetylation to 57% in 98% RH. 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.

A degree of deacetylation of 86% is also achieved with a 1:4 molar ratio of chitin to NaOH. This is 20 % improvement over other methods found in literature. Using the aging method, DDA is easily tunable from 29 to 95% by changing the NaOH eqs during the aging process at 50˚C. Aging for deacetylation is a soft method which helps preserve MW of chitosan by selectively deacetylating rather than depolymerizing. At this time all indications show that the chitosan produced from aging maintains a high MW although MW has not been formally measured. Next, we will be using viscometric methods to determine the MW of chitosan as well as the MW of the chitin starting material by first determining the α and K Mark-Houwink parameters for chitosan and chitin in [C_2mim][OAc]. In conjunction to this, a GPC technique will be attempted using [C_2mim][OAc] as a mobile phase because of its ability to dissolve both chitin and chitosan effectively. It is possible that the use of a co-solvent would be required such as dimethyl sulfoxide to reduce the viscosity of the biopolymer-ionic liquid solution without precipitating the biopolymer. Beyond this work solid state functionalization of the chitosan can be attempted by milling to continue research of solid-state reactions of biopolymers, bypassing they're insoluble nature and improving the potential uses. One aspect that could be interesting to follow up on is that when milling chitin solo in metal and plastic jars, particles of the jar and or ball were found in the chitin. This could be interesting in two tracks, one would be measuring

the size of the particles being removed to see on what order they are and see if it could be controlled to produce nanoparticles. Another aspect would be to use their abrasive nature to scavenge metals.

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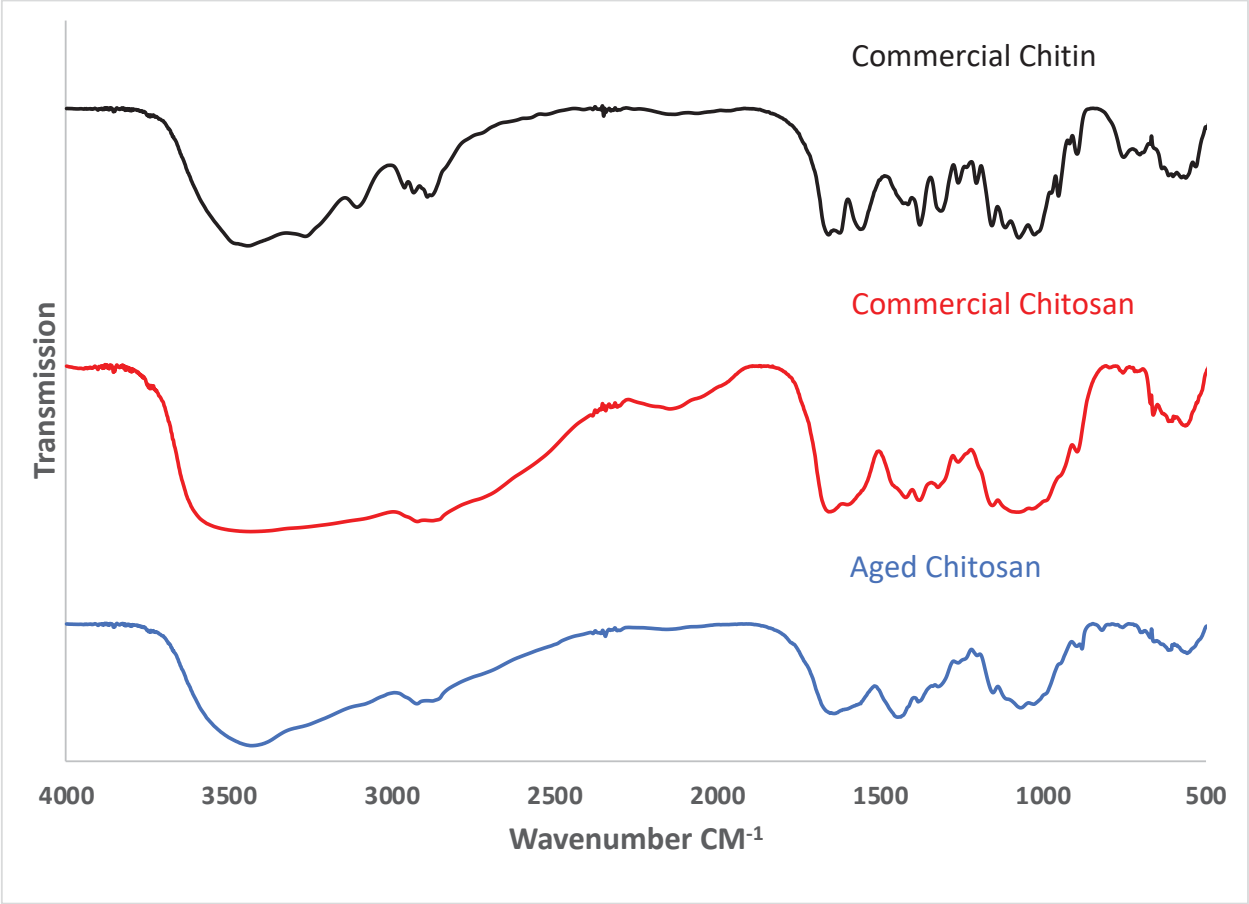
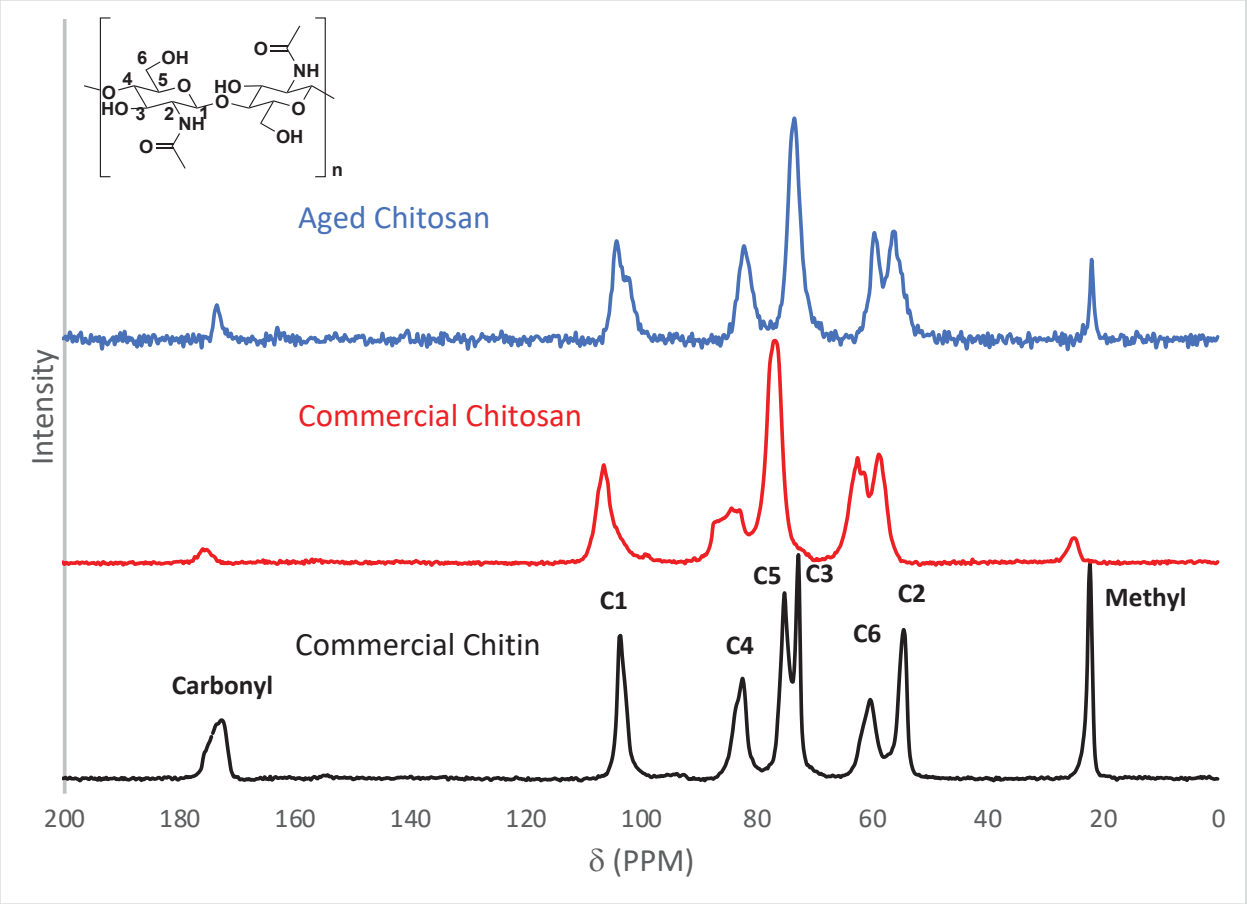
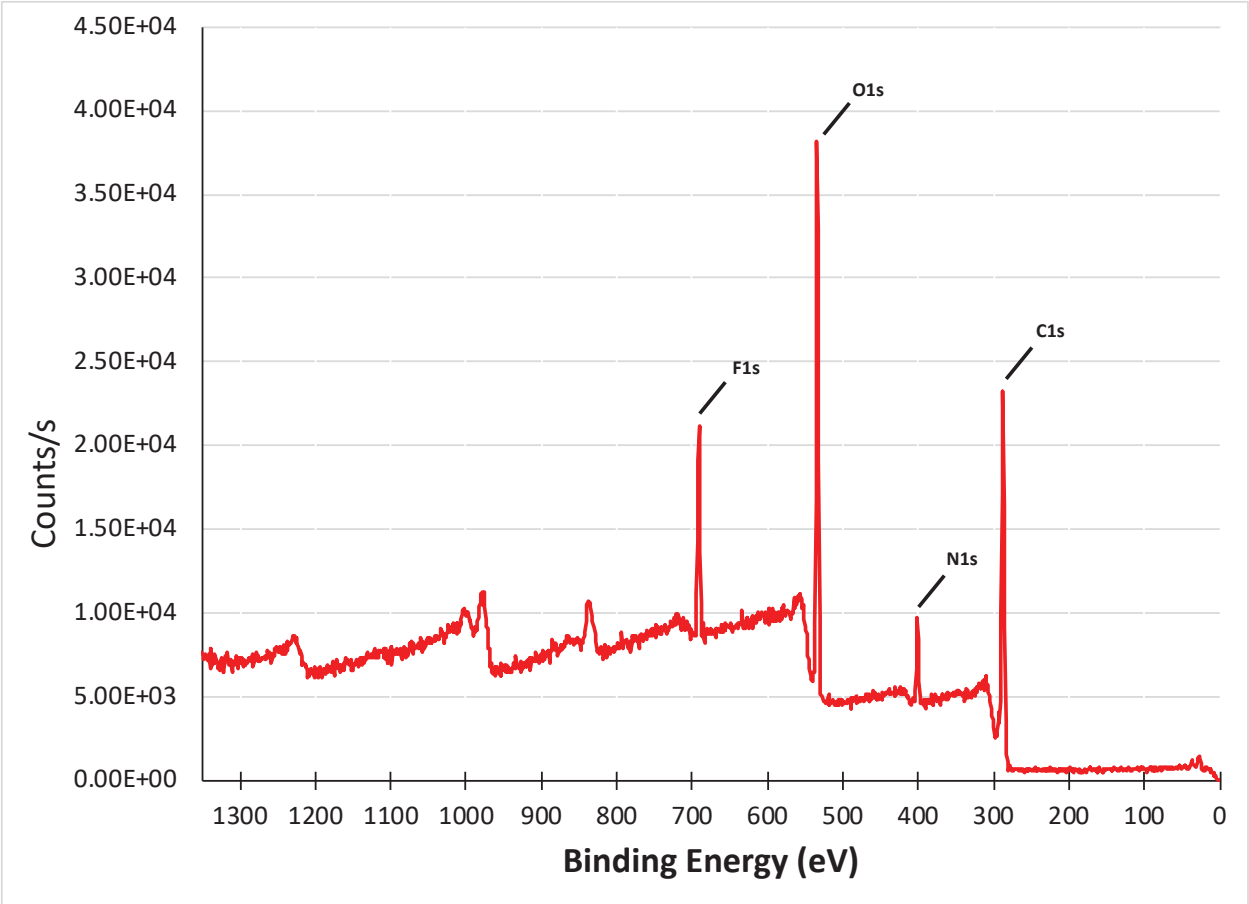
Appendices



Appendix 1 – 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}.



Appendix 2 – 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.



Appendix 3 – XPS of chitin milled 5 mins in PTFE jar showing the presence of fluorine in the sample indicating PTFE was present.

Appendix 4 – Commercial chitin, commercial chitosan and solution deacetylated chitosan.

	% DDA (^{13}C CP MAS NMR)
PG-Chitin	4
Commercial Chitosan LMW	89
Commercial Chitosan MMW	96
Commercial Chitosan HMW	85
Commercial Chitosan LMW 2	82
Solution Deacetylated Chitosan	76

Appendix 5 – Time and NaOH equivalents varied milling, PTFE^{ZrO_2}.

Milling Time	**NaOH Eqs.**	**% DDA (^{13}C CP MAS NMR)**
30 mins	1:1	5
	1:2	6
	1:3	6

	1:4	7
	1:5	7
60 mins	1:5	6
90 mins	1:5	7

Appendix 6 – Lag milling for chitin deacetylation, 10% liquid, 1:1 chitin:NaOH, 30 min mill PTFE^{ZrO_2}.

	% DDA (^{13}C CP MAS NMR)
Water	7
Ethanol	7
Methanol	6
Dichloromethane	6
Ethyl Acetate	6
Acetonitrile	4

Appendix 7 – Lag milling for chitin deacetylation, water, 1:1 and 1:5 chitin:NaOH, 30 min mill PTFE^{ZrO_2}.

	% DDA (^{13}C CP MAS NMR)	
% water	1 eq NaOH	5 eq NaOH
10	9.9	12.4
20	14	23.8
30	11.76	20.56
50	11.65	19

Appendix 8 – Initial aging of crystalline and amorphous chitin at room temperature.

Method	**Relative Humidity**	**Time**	**% DDA (^{13}C CP MAS NMR)**
Milled Dry Aging	N/A	6 days	6
Crystalline Chitin	43%	1 day	18
	43%	6 days	40
	75%	6 days	43
	98%	6 days	57
Amorphized Chitin	98%	3 days	56
	98%	6 days	73
	98%	10 days	78

Appendix 9 – Control experiments at room temperature and 50˚C with 1:5 amorphous Chitin:NaOH and 98% RH (unless otherwise noted), without NaOH, without humidity, without amorphization, manual grinding, and solution aging.

Controls	RT	50˚C
No NaOH amorphized 6 days	4	4
No humidity amorphized 6 days	6	82
Aging Crystalline 98%	57	82
Manual Grinding 43%	54	–
Manual Grinding 73%	58	–
Manual Grinding 98%	59	–
Crystalline chitin solution aging 50% NaOH 6 days	45	–
Amorphous chitin solution aging 50% NaOH solution 1:5 6 days	62	–