Mechanoenzymology as a Novel Method for the Generation of Alginate Oligosaccharides from Alginate

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Abstract

Alginate, a biopolymer primarily derived from brown seaweed, holds significant potential for various industrial applications, such as food production, pharmaceutical treatments, and agriculture. In its natural polymeric form, however, it is somewhat restricted in its practical use due to its low water solubility. Its depolymerization products, alginate oligosaccharides, on the other hand are much more versatile in these industrial applications because of their greater water solubility and variability in molecular weight. While there are many established methods of producing alginate oligosaccharides, each has drawbacks in aspects like waste production, water consumption, and affordability. This thesis explores a novel, sustainable approach to produce these oligomers through alginate depolymerization by alginate lyase using a mechanoenzymatic method (*i.e.*; ball-milling). By employing the enzyme under moist-solid conditions the study aims to reduce water waste and energy consumption while maintaining enzymatic efficiency.

Key reaction parameters such as the liquid-to-solid ratio, milling time and frequency, incubation temperature, buffer pH, and enzyme loading were systematically optimized. The best mechanoenzymatic conditions yielded a reaction efficiency comparable to traditional aqueous methods, while reducing water consumption by ~680 times. The mechanoenzymatic method produced alginate oligosaccharides with narrow polydispersity and low molecular weights, comparable to those obtained in aqueous conditions.

Overall, this research demonstrates the viability of mechanoenzymatic depolymerization as a greener alternative for alginate depolymerization, offering a new method of efficient alginate oligosaccharide production for further applications in medicine, agriculture, and food science.

Résumé

L'alginate, un biopolymère principalement dérivé des algues brunes, présente un potentiel significatif dans diverses applications industrielles, telles que la production alimentaire, les traitements pharmaceutiques et l'agriculture. Cependant, sous sa forme polymère naturelle, son utilisation pratique est limitée en raison de sa faible solubilité dans l'eau. En revanche, ses produits de dépolymérisation, les oligosaccharides d'alginate, sont beaucoup plus polyvalents dans ces applications industrielles grâce à leur meilleure solubilité dans l'eau et à leur poids moléculaire variable.

Bien qu'il existe de nombreuses méthodes établies pour produire des oligosaccharides d'alginate, chacune présente des inconvénients en termes de production de déchets, de consommation d'eau et de coût. Cette thèse explore une approche nouvelle et durable pour produire ces oligomères par la dépolymérisation de l'alginate à l'aide de l'alginate lyase via une méthode mécanoenzymatique (c'est-à-dire avec un broyeur à boulets). En utilisant l'enzyme dans des solides-humides, l'étude vise à réduire le gaspillage d'eau et la consommation d'énergie tout en maintenant l'efficacité enzymatique.

Les paramètres clés de la réaction tels que le rapport liquide-solide, la durée de réaction, la fréquence de broyage, la température d'incubation, le pH, et la quantité d’enzyme ont été systématiquement optimisés. Les meilleures conditions mécanoenzymatiques ont permis d'obtenir un rendement de produit comparable aux méthodes aqueuses traditionnelles, tout en réduisant la consommation d'eau par un facteur d’environ 680. La méthode mécanoenzymatique

produit des oligosaccharides d'alginate avec une polydispersité restreinte et des poids moléculaires faibles, comparables à ceux obtenus avec les conditions aqueuses.

Dans l'ensemble, cette recherche démontre la viabilité de la dépolymérisation mécanoenzymatique comme une alternative plus écologique pour la dépolymérisation de l'alginate, offrant une nouvelle méthode de production efficace d'oligosaccharides d'alginate pour des applications ultérieures en médecine, agriculture et science alimentaire.

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Contribution of Authors

All sections of this thesis were written and assembled by Hannah Girard, with edits and feedback from Dr. Karine Auclair. All experiments and data analysis described in this thesis were performed by Hannah Girard, with exception to the GPC data collection which was performed by Dr. Violeta Toader.

Abbreviations

M - *β*-D-mannuronic acid
G - *α*-L-guluronic acid
AOS - alginate oligosaccharides
MW - molecular weight
DP - degree of polymerization
MOS - mannuronate oligosaccharides
AD - Alzheimer's disease
GOS - guluronate oligosaccharides
MBEC - minimum biofilm eradication concentrations
UV - ultraviolet
PL - polysaccharide lyase
LAG - liquid-assisted grinding
MAging - milling and aging
RAging - reactive aging
PET - polyethylene terephthalate
PEN - polyethylene naphthtalate
PLA - polylactic acid
MCC - microcrystalline cellulose
HIC - *Humicola insolens* cutinase
TPA - terephthalic acid
MHET - mono(2-hydroxyl) terephthalate
DNS – 3,5-dinitrosalycylic acid
PEG - polyethylene glycol
PS - polystyrene
GPC - gel permeation chromatography
Mw - weight average molecular weight
Mn - number average molecular weight
PD - polydispersity
HPLC - high-performance liquid chromatography
RO - reverse osmose

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Chapter 1 - Introduction and Literature Review

Biopolymers have long been exploited across a broad range of applications due to their abundant nature, low environmental impact, and unique properties. For example, cellulose, the world's most abundant biopolymer, has been relied upon for centuries for everyday use in textiles and stationery. Chitin, derived from the exoskeleton of arthropods, has uses as an additive in the food industry. In comparison, the applications of marine plant biopolymers have been somewhat underemphasized. Alginate, a naturally occurring polysaccharide, primarily obtained from brown seaweed, has been gaining popularity for its potential applications in the food, pharmaceutical, agricultural, and cosmetic industries, among many more.

1.1 Alginate

The cultivation of algae worldwide has increased exponentially over the past 70 years, and even more than doubled between 2000-2022, from 11 million tonnes to 37 million tonnes.[1] In 2019, the primary types of seaweeds employed were 51%, 48%, and 0.1% red, brown, and green seaweeds, respectively.[2] Of these seaweed varieties, the biopolymer alginate is primarily extracted from brown seaweed species (Phaeophyceae class) such as *Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Macrocystis pyrifera*, and *Ascophyllum* sp., and makes up about 40% of the plant's dry matter.[3,4] It is present in the hydrophilic anionic salt form and commonly paired with sodium, referred to as sodium alginate. Because of its anionic nature, alginate is easily cross-linked upon addition of divalent cations such as Ca^{2+} or Sr^{2+}, forming gels of customizable strengths which can be used in wide applications such as in wound dressings, drug delivery, and tissue engineering.[5,6] The polymer is composed of non-regular repeating units of uronic acid isomers, β-D-mannuronic acid (M) and α-L-guluronic acid (G), joined by 1,4 glycosidic linkages (Fig. 1.1). The distribution or arrangement of the M and G monomers

throughout the polymer is dependent on factors such as the source species, environmental conditions, extraction methods etc. They contain sub-blocks of homo-M (polyM), homo-G (polyG) linkages, and hetero-MG linkages (polyMG) (Fig. 1.1).[7] Alginates are often described by their M/G ratio, indicating which monomer the polymer is primarily enriched in. The majority of brown algae species are considered M-rich (*e.g. Macrocystis pyrifera, Fucus guiryi,* and *Bifurcaria bifurcate*); however, some species, such as *Lessonia trabeculata* and *Saccharina longicruris* have a higher ratio of G residues.[8] In its native form, alginate is somewhat limited in its scope of applications beyond gel formation because of its varying level of water solubility and stability under physiological conditions.[9]

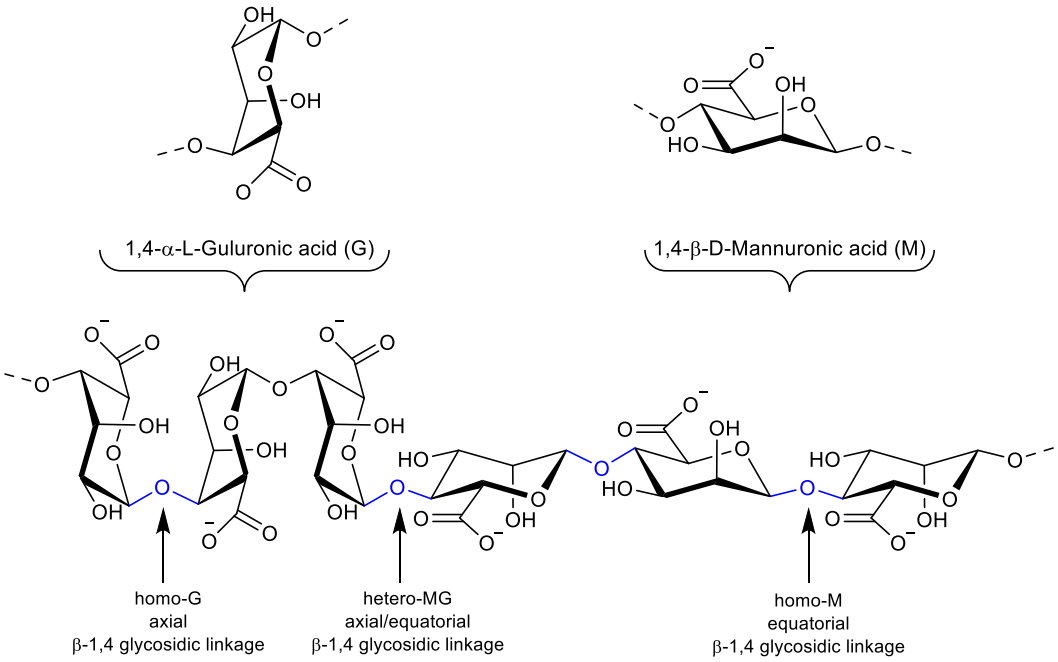

Figure 1.1: Monomers of the alginate polysaccharide and their respective linkage types within the chains

1.2 Alginate Oligosaccharides

Alginate oligosaccharides (AOS) produced from the degradation of alginate are the particular focus of interest when it comes to practical usages of the polymer because of their lower molecular weight (MW) and higher water solubility.[10] These oligosaccharides have a vast array of uses in areas ranging from agriculture to medicine, food, and even livestock and poultry. The applications of specific AOS depend on structural factors such as their monomer composition, MW, and degree of polymerization (DP). Although the β-D-mannuronic acids and α-L-guluronic acids that comprise the AOS are C-5 epimers, the resulting chain conformations experience different spatial structures and physicochemical properties, which, in turn, result in different composition-dependent functionality.[11] More specifically, β-D-mannuronic acids are linked equatorially, whereas α-L-guluronic acids are linked axially (Fig. 1.1). The equatorial conformation forces a more pliable stretched chain structure, while the axial conformation allows for a more rigid helical structure arising from hydrogen bond interactions between the carboxylate of one residue and the C_2 hydroxyl hydrogen of the adjacent residue.[11,12] A combination of these physical characteristics dictates the specific applications for which an oligomer is best suited. For example, AOS rich in guluronic acids are better suited for applications such as tissue scaffolding because of their more solid structure, whereas the more flexible AOS rich in mannuronic acids may be more applicable for drug delivery.[13,14]

1.2.1 Alginate Oligosaccharides – Applications

1.2.1.1 Sequence Specific Applications

The relationship between monomer sequence and function can be applied to maximize the useability of AOS. In a general sense, mannuronate oligosaccharides (MOS) have been found to influence gut microbiota and neuroinflammation. As such they have been used to treat symptoms of diabetes, Alzheimer's disease (AD), and Parkinson's disease.[15] For example, a study investigating AD contributors found a link between dysbiosis in the gut microbiota and neuroinflammation, and subsequently developed a clinical treatment using an MOS-based drug (GV-971).[16] The symptoms of AD may be exacerbated by irregular gut microbiota-related metabolites and their metabolic pathways. Specifically, heightened levels of phenylalanine and isoleucine may contribute to the progression of AD. Administration of GV-971 to patients effectively reduced gut phenylalanine and isoleucine levels by reconditioning the gut microbiota, thereby reducing the neuroinflammation that contributes to AD symptoms. In comparison, treatment with guluronate oligosaccharides (GOS) did not show any significant therapeutic benefits.[16] Instead, GOS are better applied for immunoregulation and pathogenic defense.[15] In one study, GOS were used to disrupt biofilm formation by *Pseudomonas aeruginosa in vitro*.[17] Bacterial biofilms contribute to bacterial resistance against antibiotics, disinfectants, and mammalian defense mechanisms. For example, lung infections caused by biofilm-associated bacteria, such as *P. aeruginosa*, often resist treatment and can lead to chronic disease, eventually causing permanent respiratory damage. GOS have been shown to disrupt biofilm production by inhibiting swarming and motility, and decreasing surface charge, all of which are factors contributing to biofilm growth.[18,19] *In vitro* treatment of bacteria with two different antibiotics

combined with the alginate oligomer OligoG CF-5/20, showed up to a 128-fold reduction in minimum biofilm eradication concentrations (MBEC) compared to treatment with the antibiotics alone. The results support the suggestion that incorporation of OligoG CF-5/20 would make the bacteria more susceptible to antibiotics.[17] This was further demonstrated in a mouse model of bacterial lung infection.[17] In similar studies, M-rich oligomers did not show the same disruptive effect on biofilms.[20]

1.2.1.2 Size-Specific Applications

In addition to their specific monomer sequence, the bioactivity of AOS can be influenced by their MW and DP. AOS are commonly used in agriculture to promote plant growth as they have been found to increase root and shoot development and to promote germination.[21] Many studies have observed a link between the DP and the resulting growth activities. For example, a study compared the effect of AOS of DP ranging from 2 to 7 on lettuce root growth elongation.[22] Root lengths of lettuce seedlings which had been treated with feed mixtures containing AOS of varying DP were compared to those of control seedlings with no AOS treatment. Hexasaccharide mixtures showed the greatest promotion of root growth, with tri-, tetra-, and pentasaccharides also demonstrating a positive effect on root growth. Interestingly, the di- and heptasaccharides had little to no effect on lettuce root growth.[22] Likewise, studies on barley root growth found that AOS with a DP of 3-4 had significant root growth activity.[23,24] The DP of AOS can also influence germination of plants. In a study investigating the effect of AOS on faba bean plant growth, AOS of MWs 1.25-1900 kDa were compared to native alginate. The findings were that, as the MW decreased, both the percent germination and speed of emergence increased, indicating a direct correlation between MW and germination, and between MW and growth rate of these plants.[25]

Other studies on maize, mustard, and lettuce plants using AOS of either 11-16 kDa or 1.4 kDa, further support the correlation between low MW AOS and improved germination.[26,27] These studies demonstrate the composition- and DP/MW-dependent applicability of AOS, and highlight the importance of controlled production methods.

1.2.2 Alginate Oligosaccharides - Preparation

1.2.2.1 Chemical Preparation

Current processes for preparing AOS include chemical methods (acid hydrolysis, alkali hydrolysis, or oxidative degradation), physical methods (heating, ultraviolet light or microwave radiation), and enzymatic methods (using alginate lyase). Acid hydrolysis, which is the most common chemical method, uses harsh reagents such as oxalic acid, HCl, H_2SO_4, or formic acid in boiling water, followed by neutralization with large amounts NaOH.[10,28,29] This results in hydrolysis of the 1-4 glycosidic bonds to generate saturated lower MW oligomers (Fig. 1.2).[30] Hydrochloric acid is the most commonly used acid (typically at concentrations of 0.1-1.0 M) for partial hydrolysis to AOS, but formic acid, although not as frequently used, has shown a better ability to generate the monomer.[29,31–33] Although quick, hydrolysis can be costly, requires harsh conditions that can corrode equipment, and involves a high energy consumption, in addition to generating environmentally damaging waste products.[11,34]

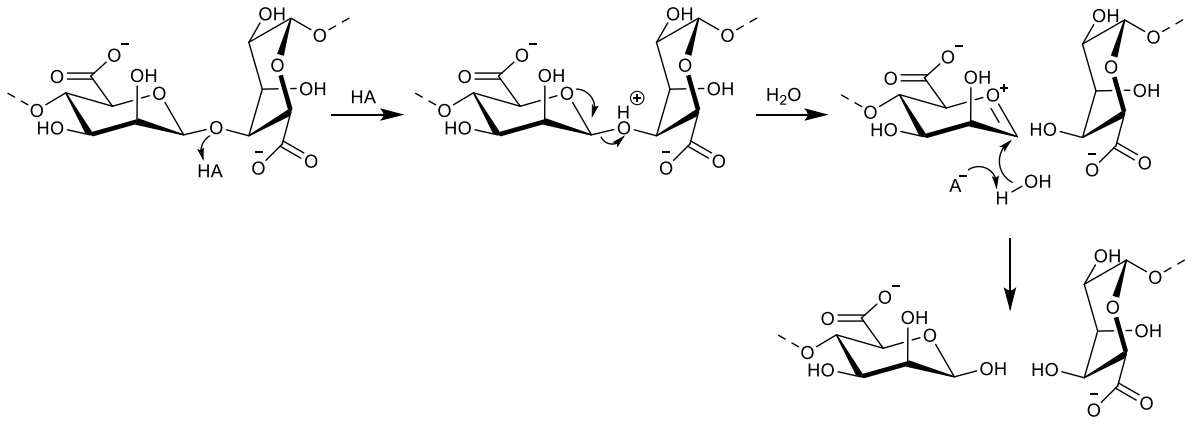

Figure 1.2: General mechanism for acid hydrolysis of alginate

Oxidative degradation using H_2O_2 is a more affordable method and has the advantage of only producing water as a by-product.[35] The reaction mechanism is unclear but is thought to proceed via hydroxyl radicals. The specific products of this reaction are dependent on the conditions, such as temperature and concentration of hydrogen peroxide.[35,36] Temperatures between 20°C to 30°C yield oligomers of similar MW (~75 kDa), whereas a negative exponential relationship between temperature and MW is seen when the temperature is increased from 40°C to 80°C.[35,37] Similarly, increasing the concentration of H_2O_2 from 0 to 0.6% w/v, then to 1.2% causes a sharp decrease in MW by about 80%. The MW then remains constant up to an H_2O_2 concentration of 8% where it decreases to about 14% of the original alginate MW, at which it remains up to an H_2O_2 concentration of 15%.[35] This method produces an open-ring end group with a carboxyl group at the C-1 reducing end (Fig. 1.3).[36,37] Although this chemical method is more environmentally friendly than acid hydrolysis, degradation using this process is often random and uncontrolled, and produces saturated AOS which have been shown to be less beneficial in biological applications.[38,39]

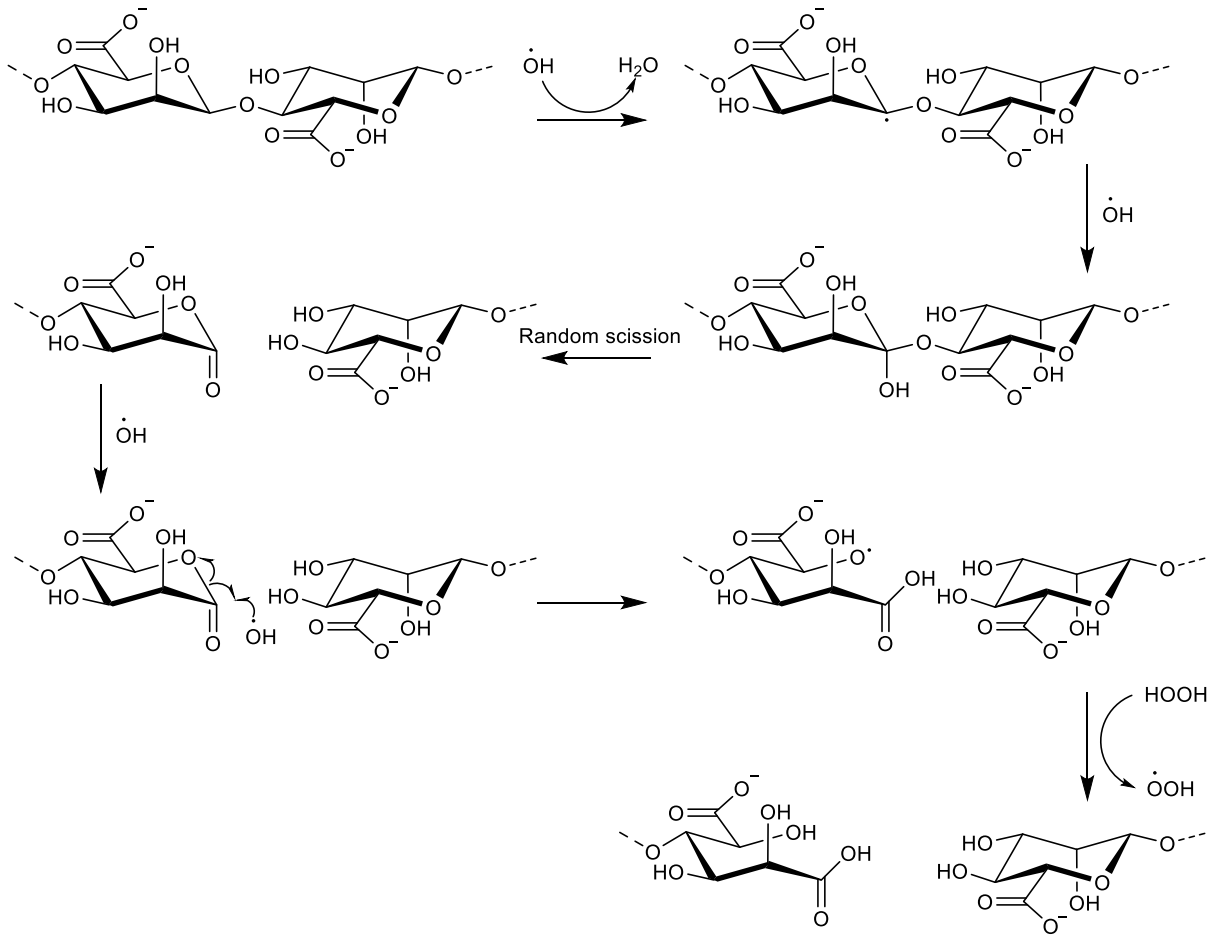

Figure 1.3: Potential mechanism for oxidative degradation of alginate by hydroxy radicals

1.2.2.2 Physical Preparation

An alternative to chemical preparation of AOS is physical degradation. This approach encompasses a variety of physical methods such as ultraviolet (UV), ultrasonic, or gamma (γ) irradiation, as well as microwave, plasma, and hydrothermal treatment.[40] Of these, the most widely used physical method is γ-irradiation because of its high energy-efficiency, ease of application, dose-dependent yield, and lack of by-products.[25] These methods proceed through free radical-induced scission to selectively break the glycosidic linkages and give the same products as hydrolysis.[41] Many studies have demonstrated a relationship between the applied

irradiation dose and the resulting reduction in MW. γ-Irradiation doses from a Co-60 source at a strength up to 200 kGy applied to liquid-state alginate samples, or up to 500 kGy applied to solid-state samples, both show reduction in overall MW as the dose increases.[42] While liquid-state degradation showed a greater response than solid-state samples, it has been suggested that the effectiveness of solid-state degradation has greater practical applications.[43] Typically, the MW decreases exponentially as the radiation dose increases within a low range (~0-100 kGy), but as the dose is increased beyond this range (~100-500 kGy), the MW of the oligosaccharides does not decline further.[25,41,43,44] Furthermore, upon structural analysis, it has been observed that the irradiation dose also influences the monomer composition of AOS, where relative amounts of homopolymeric blocks of both MM and GG increase while M/G blocks decrease with a rising dose. This suggests that M-G linkages are more easily cleaved by irradiation than their M-M or G-G counterparts.[41]

Microwave irradiation is another convenient physical degradation method for AOS production. It is fast, efficient, environmentally friendly, and avoids the need for a desalting step in the purification process. Polyguluronic acid, separated from alginate has been used to produce GOS with DP from 1-10 in only 15 minutes under microwave irradiation of 1600 W at 130°C. This method produced saturated GOS in high yields and low MW with no by-products or additional purification beyond standard chromatography methods.[45] This method, however, has not been explored to the same extent as the previously described physical methods. These physical methods are more economical than chemical methods but, like chemical methods, they produce only saturated AOS.[46]

1.2.2.3 Enzymatic Preparation

The enzymatic method is the most selective and generally more environmentally friendly approach. It uses mild conditions to produce highly specific AOS in high yields. Notably, the AOS produced by enzymatic degradation contain an unsaturated terminal saccharide at the non-reducing end, which has been found to be beneficial for biological activity. Alginate can be broken down into AOS by a class of enzymes called alginate lyases sourced from marine and terrestrial bacteria, marine algae, and marine mollusks. The degradation of alginate by this family of enzymes is characterized by β-elimination of the glycosidic bonds which results in a double bond on one side of the cleaved glycosidic linkage and a reducing sugar on the other side (Fig. 1.4).

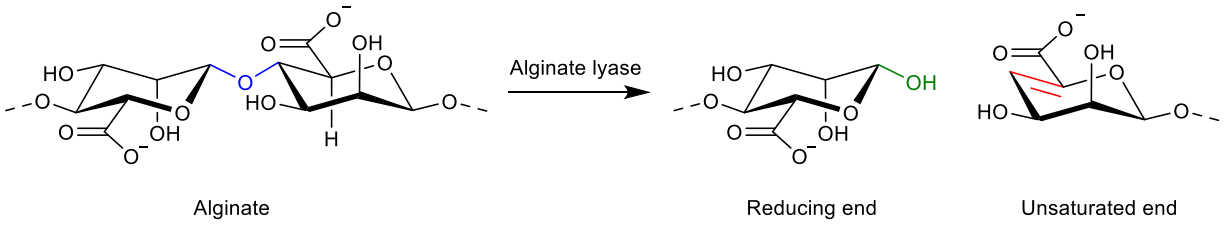

Figure 1.4: Reducing end and unsaturated end of the products of alginate depolymerization by alginate lyase

Alginate lyases can be categorized in a variety of ways, namely, substrate specificity, mode of action, and primary structure. The substrate specificity of alginate lyases is described by the enzyme's preference for either M-blocks or G-blocks. Depending on the enzyme's preference, the resulting AOS can be either M-rich or G-rich (Fig. 1.5). The specificity depends on the source organism and can even vary across different enzymes from the same source organism. Some enzymes can act on both M- and G-blocks, however this only applies to a minority of those that have been characterized. The mode of action can also differ from enzyme to enzyme. Some lyases are endolytic, meaning that they do not cleave the very first or very last ring of the polymer chain.

This produces unsaturated oligosaccharides of varying DP. Some lyases, on the other hand, although far less common, are exolytic, meaning that they cleave the glycosidic bonds in a stepwise fashion from the end of the polymer, producing monomers (Fig. 1.5). The lower DP and unsaturated nature of AOS produced by enzymatic depolymerization typically show better bioactivity compared to those produced by chemical or physical methods.[28,47,48]

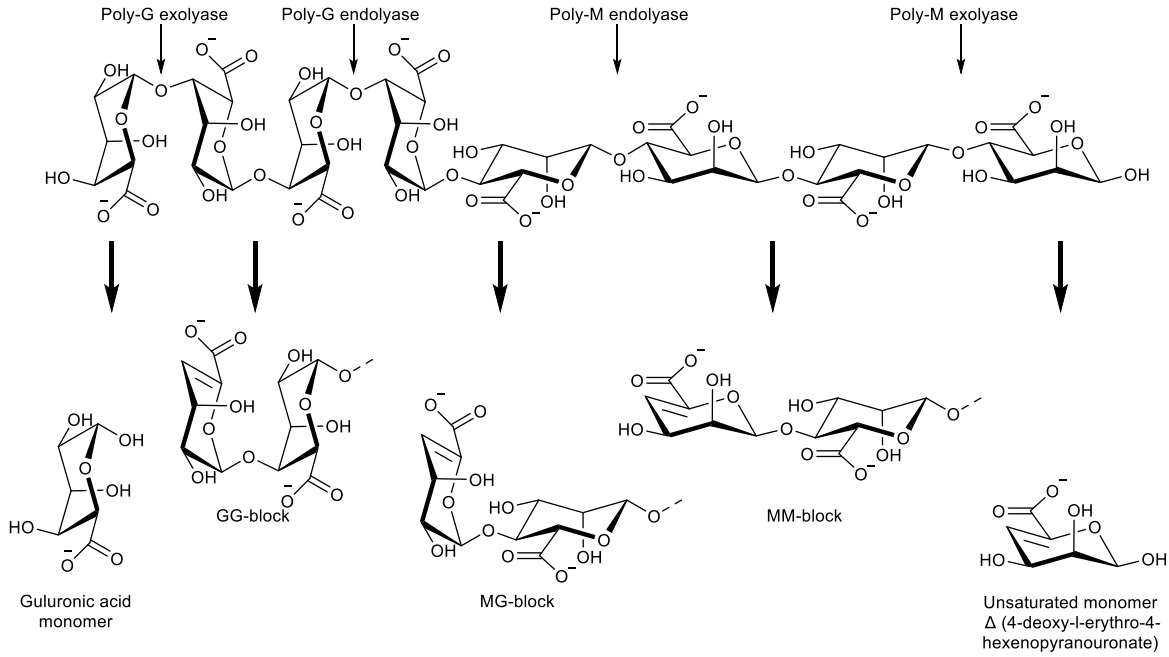

Figure 1.5: Examples of various alginate oligosaccharide products resulting from various modes of degradation from alginate lyases of different specificities

1.3 Alginate Lyases

Alginate lyases fall under the umbrella of polysaccharide lyases (PL) and are classified by PL families. This categorization is dependent on structural homology specifically in the enzyme's hydrophobic cluster.[49,50] The alginate lyases that have been characterized so far fall into one of the following families: PL-5, -6, -7, -14, -15, -17, -18, -31, -32, -34, -36, and -39, as presented in the CAZy database.[50–52] Further distinctions can be made based on the full three dimensional

structures of the enzymes. The alginate lyases for which a crystal structure has been determined harbor one of these tertiary structures: (α/α)n toroid fold (PL-5), β-jelly roll fold (PL-7, PL-14, PL-18, and PL-36), β-helix fold (PL-6 and PL-31), and a (α/α)n toroid + β-jelly roll fold hybrid (PL-15, PL-17, and PL-39) (Fig. 1.6).[53–55] The β-jelly roll is the most common fold across the alginate lyases that have been characterized. It is comprised of an outer concave β-sheet and an inner concave β-sheet running antiparallel with one another and bending almost 90° in the middle to form a globular shape. The catalytic site is housed in a cleft formed by the inner sheets wherein the alginate substrate can enter to interact with the active site.[56,57] The $(\alpha/\alpha)_n$ toroid fold is a barrel-like structure that is composed of many pairs of antiparallel α-helices (α-helical hairpins). Depending on their family, enzymes of this sub-class can harbor one or more domains. In the latter case, the other non-catalytic domains are present near either the N- or C-e terminus. The active site toroid domain contains both an inner and outer layer of helical arrangements, forming a tunnel wherein the substrate can bind.[53,56] The alginate lyases that adopt a β-helix fold typically consist of at least one trio of β-sheets, each separated by a turn with the first two sheets quasi-antiparallel and the third sheet perpendicular. The resulting domain can be described as a tower-like consecutive helical structure which provides structural stability to the enzyme's active site. While the active site is located at the N-terminus in a surface cleft, a C-terminus domain loop extends into the site and is essential in directing the substrate into the site.[53] The AlyGC enzyme in this class is a unique exception in that it is a homodimer with a β-helix fold on both the N- and C-termini, which are described as twin-towers (Fig. 1.6).[53,58] Finally, the (α/α)n toroid + β-jelly roll hybrid fold varies in the relative location of the different domains. For example, a PL-17 protein consists of an N-terminal $(\alpha/\alpha)_6$ toroid fold and a C-terminal β-jelly roll fold with additional helices

along the sides of the α/α barrel.[59] This differs from PL-15 proteins which contain an N-terminal β-sheet, a central $(\alpha/\alpha)_6$ toroid barrel, and another β-sheet on the C-terminus. The central barrel and C-terminal β-sheet form the active site via a deep substrate pocket.[60]

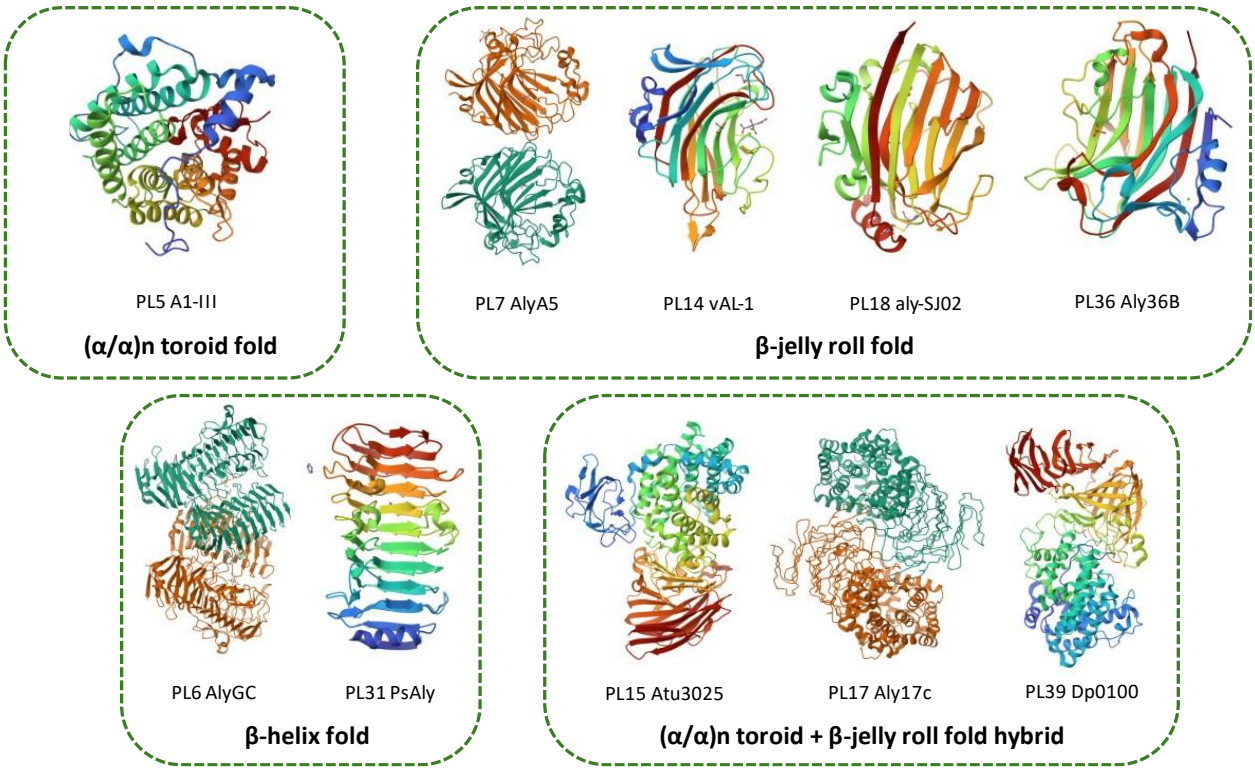

Figure 1.6: Structures of alginate lyases belonging to different families, categorized by fold type. Including the structures of PL5 A1-III [1HV6], PL7 AlyA5 [4BE3], PL14 vAL-1 [3GNE], PL18 aly-SJ02 [4Q8K], PL36 Aly36B [6KCW], PL6 AlyGC [5GKD], PL31 PsAly [6KFN], PL15 Atu3025 [3A0O], PL17 Aly17c [4NEI], PL39 Dp0100 [6JP4] from: https://www.rcsb.org/.

Despite the differences in the active site domains amongst alginate lyase enzymes, their general catalytic mechanism remains largely the same. The β-elimination which breaks the glycosidic bonds occurs in three major steps: 1) The C_5 proton is made more acidic by salt formation between the carboxyl anion and a positively charged residue or cation; 2) the C_5 proton is then abstracted by a general base; 3) next, elimination of the reducing sugar enables formation of the

C_4-C_5 double bond, thus cleaving the O-glycosidic bond; and 4) finally, a proton is exchanged to regenerate the enzyme resting state (Fig. 1.7).[53]

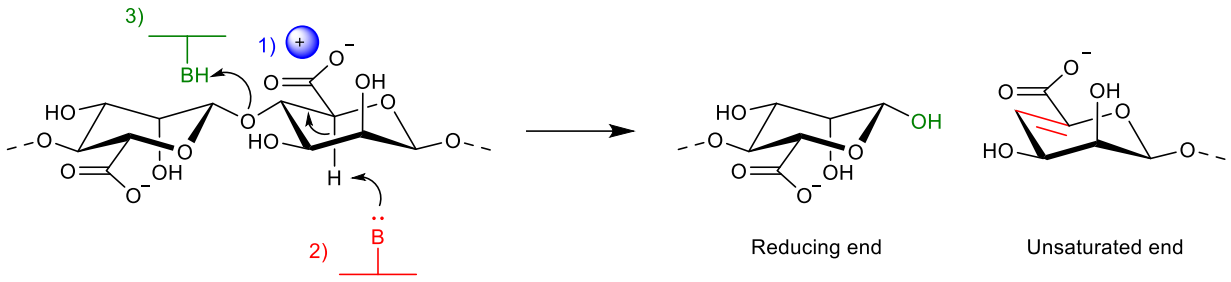

Figure 1.7: General mechanism of depolymerization of alginate polysaccharides through β-elimination by alginate lyase

The first step can be further described by the nature of the cation that forms a salt with the carboxyl anion. This can either be through metal-assisted β-elimination (with Ca^{2+}) or through His- or Tyr/Tyr-catalyzed β-elimination. The metal-assisted elimination mechanism is specific to members of the PL-6 family. Ca^{2+} is coordinated by conserved asparagine and glutamic acid residues and is used to interact with the -carboxylate groups of the alginate substrate, effectively neutralizing it to facilitate C_5 proton abstraction by a lysine residue. The result is a resonance-stabilized enolate intermediate with a weakened C_β-O bond. Once the C_5 proton is abstracted, the C_β-O bond is broken. This is also facilitated by protonation of this oxygen by a nearby protonated arginine, generating the reducing end product (often abbreviated as the A-1 product). Upon cleavage of the C_β-O bond, a C_4-C_5 double bond is formed on the other product (often referred to as the A+1 acid).[58] Instead of a metal ion, the His/Tyr and Tyr/Tyr β-elimination mechanism use a positively charged residue such as His, Arg, Asn, or Gln to form a salt with the carboxyl in the first step. In the His/Tyr mechanism, a His residue acts as a general base to remove the C_5 proton, whereas in the Tyr/Tyr mechanism, the base is a Tyr residue. In both cases, a Tyr acts as a general

acid to transfer a proton to the oxygen of the leaving -OH group, allowing for the β-elimination. Here again, a product with a reducing end and a product with a new unsaturated C_4-C_5 bond are formed, for both endotype and exotype activities (Fig. 1.7).[53,60]

1.4 Using Enzymes in Moist-solid Reaction Mixtures

1.4.1 Mechanoenzymology

Although enzymatic transformations typically do not use harsh reactants or conditions, they are typically performed in large volumes of aqueous solution, which generates a large amount of water waste, especially on scale-up. Recently, a new approach to enzymatic reactions has directly tackled this issue by using enzymes in moist-solid mixtures, which are mostly solid mixtures containing only a minimal amount of liquid component. These moist-solids are mixed mechanically, hence the term mechanoenzymology. This naming arises from mechanochemistry, which is a discipline that employs mechanical activation to induce chemical transformations in a nearly solvent-free environment. The mechanical force can be applied by a variety of equipment such as shaker, roller, or planetary mills, as well as twin screw extruders, and even mortar and pestles in the most rudimentary applications.[61] Milling is the most common approach and typically involves adding the reactants to jars made of stainless steel, Teflon, zirconia, tungsten, or some other durable material along with one or more small balls also made of one of these materials (Fig. 1.8).

A) B)

Figure 1.8: Equipment often used in mechanochemistry and mechanoenzymology. A) Standard benchtop shaker and B) typical milling jars and balls in a variety of sizes (images from: https://formtechscientific.com/)

Optionally, in mechanochemistry a very small amount of liquid can be added to the mixture in a process called liquid-assisted grinding (LAG) wherein the liquid can act as a lubricant to the otherwise dry reactants.[62,63] The amount of liquid added is reported as a ratio of the total liquid used (µL) to the total weight of solids (mg) in the mixture, and is denoted by η. The amount of liquid added defines the reaction mixture. Neat-grinding implies a solvent-free mixture with η = 0, while η ≤ 2 µL/mg is considered LAG, and depending on the reactants' solubility, typically η = 2-12 µL/mg could be a slurry, and mixtures with η > 12 µL/mg could be solutions.[64,65] Other additives such as salts and polymers can also be supplemented to the reaction mixture to further induce chemical transformations. Mechanochemical methods have been applied in areas such as synthesis of battery cathode materials, co-crystallization studies, generation of metal-organic frameworks, and vast array of organic reactions, just to name a few.[64–70]

Unlike in mechanochemistry, which uses mechanical forces to trigger reactions, in mechanoenzymology the mechanical mixing likely serves only to contribute to the homogeneity of the mixtures. One important motivation for using moist-solid mixtures with enzymes lies in the aspect of wastewater reduction. Additionally, it is expected that such conditions might improve enzyme efficiency by better mimicking the natural environment of many enzymes. In nature, enzymes can be produced either intra- or extra-cellularly, depending on their source and function. Many enzymes have evolved to function naturally on surfaces exposed to air moisture.[71] For example, bacteria and fungi that rely on plant nutrients must secrete enzymes to break down the surface tissues of the plants to gain access to cellulose and hemicellulose, which are depolymerized on the plant surface into smaller molecules to facilitate their absorption by the microorganism.[72–74] It was therefore envisaged, that moist-solid reaction mixtures would mimic these surfaces exposed to air moisture and be accelerated by mechanical mixing.

1.4.2 Applications of Mechanoenzymology

The first reported application of an enzyme in a mechanoenzymatic context was lipase B from *Candida antarctica* employed for the enantioselective resolution of secondary alcohols.[75] Using typical solvent-free mechanochemical conditions, the study demonstrated activity of the enzyme even under mechanical stress, suggesting potential for additional applications of enzymes in this context.[75] However, non-stop mechanical mixing requires that large amounts of enzyme be used to compensate for enzyme denaturation. To overcome this issue, the Auclair Lab demonstrated that either a single short period of initial mechanical mixing followed by static incubation (known as MAging) or multiple short periods of intermittent mixing between longer periods of static incubation (together known as RAging) proceed at lower enzyme loadings (Fig.

1.9).[76] To date, mechanoenzymology has shown success in the depolymerization of biological and synthetic polymers, including cellulose[76], hemicellulose[77], chitin[78], polyethylene terephthalate (PET)[79], polyethylene naphthalate (PEN)[80], and polylactic acid (PLA)[81].

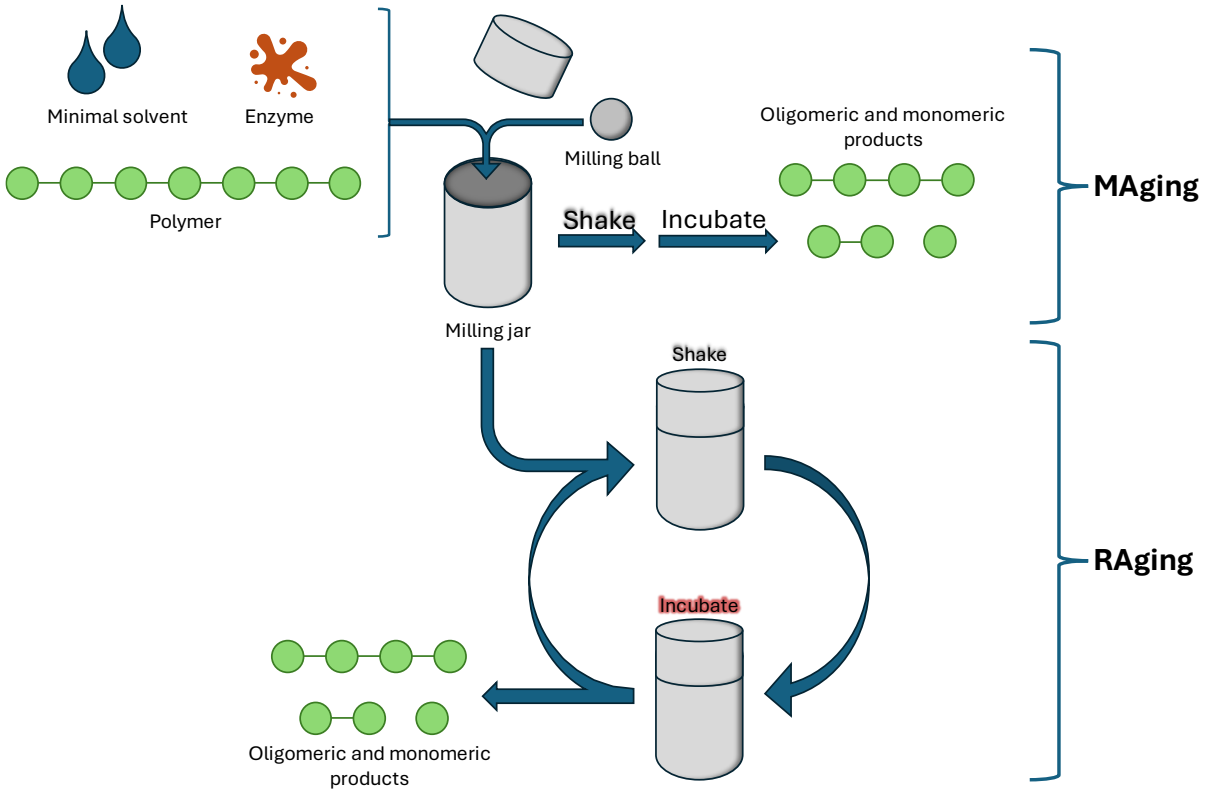

Figure 1.9: Workflow of general mechanoenzymatic MAging and RAging methods for depolymerization of natural and synthetic polymers

This was demonstrated first with the biopolymer cellulose. Using commercially available cellulases from *Trichoderma reesei*, *Trichoderma longibrachiatum*, or *Aspergillus niger*, combined with microcrystalline cellulose (MCC) and very minimal volumes of water (0.5-1.0 µL/mg) the reaction was subjected to repeated cycles of RAging (Fig. 1.9).[76] Interestingly, the authors reported that the conversion increased as η increased from 0.5 µL/mg to 1.0 µL/mg, but then decreased at greater η values. This formed the basis of evidence that the enzymes are indeed more active without the presence of bulk water. Additionally, while non-stop milling was found

to be highly detrimental to enzyme activity, RAging, using a regime of milling 5 minutes every hour, yielded 50% conversion of the MCC to glucose within 12 hours without pre-treatment of the substrate.[76] This is more than twice the yield obtained under conventional aqueous conditions. In addition to cellulose, the mechanoenzymatic approach showed success in the depolymerization of another plant polymer, hemicellulose, by xylanases with up to 70% yields.[77] The cellulases process was later adapted to biomass, producing even higher yields.[82,83] This area of research was further adapted to the depolymerization of chitin to *N*-acetylglucosamine by chitinase.[78] In this case, the optimal RAging conditions consisted of milling the mixture every 12 hours at η = 1.7 μL/mg. Under such conditions, commercial *Aspergillus niger* chitinases generated the monomeric product in yields 2-30 times greater than comparable reactions in conventional dilute aqueous conditions.[78] The improved yields consistently observed for enzymatic reactions in moist-solids across different substrate/enzyme systems attests to the robustness of the method.

The success obtained with biopolymer degradation led to further expansion of this technique to synthetic polymer hydrolysis. Because of its abundance in society and its ester bonds, PET was a highly attractive polymeric substrate for this new mechanoenzymatic methodology. Low-crystallinity PET has been depolymerized in solution by a variety of enzymes, but the high-crystalline regions require pre-treatment to amorphize the plastic. The use of moist-solid conditions, however, allowed the direct depolymerisation of PET, without the need for energy-intensive amorphization.[79] Using a commercial enzyme not optimized for PET depolymerization, *Thermomyces insolens* (formerly known as *Humicola insolens*) cutinase (HiC), on a PET powder of 36% crystallinity with a liquid-to-solid ratio η = 1.5 μL/mg and daily milling for 5 minutes, the

authors reported a 20% yield of terephthalic acid (TPA) in 3 days and less than 4% of the undesired product mono(2-hydroxyethyl) terephthalate (MHET). By adding the enzyme in batches every three day, the yield was further increased to 50%, far greater than when the enzyme was used under standard aqueous conditions.[79] Notably, the major product of the reaction in bulk water was the unwanted MHET. This method was later applied to polyester textile and polyester-cotton fabric.[84] These studies demonstrate that the mechanoenzymatic method is not only applicable to synthetic polymer depolymerization, but also superior to comparable aqueous methods, offering a low-waste alternative route for plastic recycling.[79]

Based on the success of these results, the mechanoenzymatic approach has also been applied to the synthetic polymers PLA and PEN.[80,81] PLA is claimed to be biodegradable; however, in nature its breakdown is extremely slow, warranting its recycling.[85,86] Upon subjecting untreated PLA to milling for 15 minutes followed by static incubation (MAging) in the presence of the HiC enzyme, the quantitative depolymerization to lactic acid was observed at double the rate of the equivalent aqueous conditions with a total yield that was 20% higher.[81] PEN is a highly recalcitrant synthetic polymer and therefore presents greater barriers to recycling. Remarkably, when reacted with HiC under mechanoenzymatic conditions, the reaction yield was 30-fold greater than under traditional aqueous conditions. Optimized RAging conditions afforded yields of up to 56%.[80] To our knowledge, this is the only reported example of enzymatic PEN depolymerization. Together, the results of these studies using enzymes in moist-solids are a testament to the applicability of this methodology in the depolymerization of a variety polymers, be they natural or synthetic. They show the potential of expanding the field of polymer recycling and functionalization in a low-energy, low-waste, and high-yielding way.

1.5 Research Objectives

The work presented in this thesis intends to demonstrate a new and alternative approach to the depolymerization of untreated alginate into AOS in a more efficient and more sustainable manner. This research employs commercial alginate lyase to degrade alginate under moist-solid conditions. The studies involve optimization of key variables such as η value, milling time, milling frequency, incubation temperature, buffer pH, and enzyme loading. Relative yields are compared to those obtained under traditional aqueous conditions and a preliminary characterization of the oligomeric products is reported. The goal herein is to present a straightforward, accessible method of producing valuable oligomeric products from an abundant biopolymer for use in fields such as food production, agriculture, and pharmaceuticals.

Chapter 2 - Results and Discussion

2.1 Introduction to analysis method

In order to monitor AOS product formation throughout the course of the reaction, we needed a rapid but reliable characterization method conducive to the format of both the aqueous and the moist-solid mixtures. The generation of a double bond in the AOS products for each bond broken between monomers allows for detection by UV absorption at a wavelength of 235 nm. This method was used to monitor reaction progress, both in a continuous manner for reactions under standard aqueous conditions, and non-continuously, to collect data at specific time points for mechanoenzymatic reactions. Because the specific products of the reaction are non-commercial and challenging to access synthetically, the structurally similar monomeric molecule 1-cyclohexene-1-carboxylic acid was used to construct a calibration curve. The usability of this method was validated through secondary testing with the well-established 3,5-dinitrosalicylic acid (DNS) assay for reducing end sugars, one of which is produced at the opposite end of the unsaturated bond in the oligomers after each reaction.[87] Upon validation, most yields reported herein were calculated from the UV absorbances at 235 nm. It is important to note that the yields presented herein should be considered as underestimated. First, because the substrate is a mixture of polymers of different molecular weight, *i.e.* polydisperse, an assumption had to be made that the polymer is an infinitely long chain. Second, alginate consists of both G and M subunits, the exact sequence of which is unknown, whereas the alginate lyase used here cleaves only MM bonds (polyM specific), and not MG or GG bonds. Although the commercial alginate is listed as having an M:G ratio of 1.56 (meaning that it is only composed of approximately 60% M residues), which we took into consideration in our calculations, we could not account for the fact

that only M monomers that are adjacent to another M can be cleaved. The detailed method of calculation is described in section 4.5.3.

2.2 Enzyme assay

2.2.1 Testing the enzyme activity under standard aqueous conditions

Because solution-based methods are the current standard in the application of alginate lyase for the generation of alginate oligosaccharides, it was important to establish a benchmark of the enzyme's behaviour under varying parameters in aqueous conditions. We first performed a variety of aqueous enzymatic reactions with a polyM-selective alginate lyase (*Flavobacterium* sp) and M-rich sodium alginate to understand the enzyme's resilience under a range of different temperatures and pH levels. The activity of a given enzyme is greatly affected by the specific conditions and is typically optimal for a narrow range of temperature and pH values among other variables. Based on the manufacturer's recommendation for the standard assay of this alginate lyase, its activity is expected to be optimal at 37°C and at a pH of 6.3. Preliminary experiments were therefore performed under these conditions, at an enzyme loading of 0.04% w/w relative to the substrate, to confirm that the enzyme was active. Next, additional experiments under aqueous conditions were performed at 25°C, 50°C, and 57°C to further understand the working range of the enzyme (Fig. 2.1).

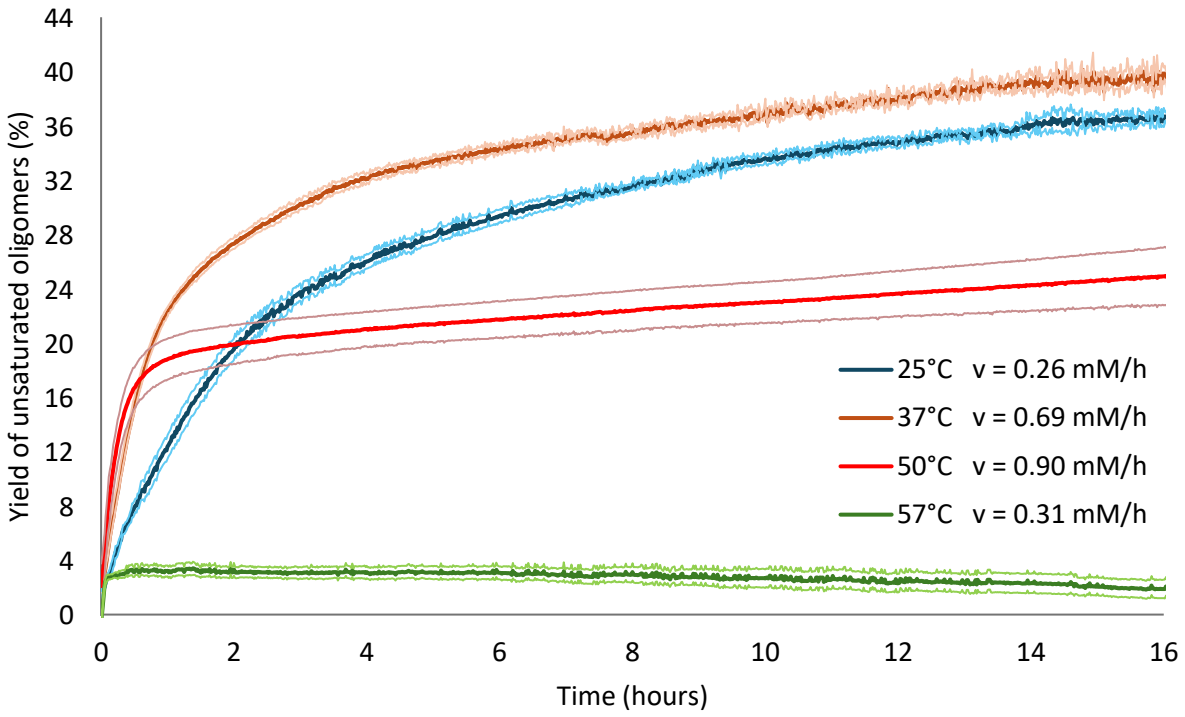

Figure 2.1: Kinetic profiles and initial rates (v) of the depolymerization of alginate by alginate lyase across a range of temperatures. An alginate lyase (10 µL, 0.1 mg/mL) solution was mixed by pipette to an alginate solution (450 µL, 1.0 mg/mL), both in phosphate buffer (0.1 M, pH 6.3) (η = 1022 µL/mg). The absorbance was measured continuously at 235 nm and converted to product concentration. Corresponding lighter-coloured curves represent standard deviations across triplicate.

At 50°C, enzyme activity was significantly reduced, and at 57°C, the enzyme had little to no activity. Although the enzyme still performed well at 25°C, the maximum yield was observed at 37°C, in addition to the initial rate being higher. Therefore, 37°C was confirmed as the optimal temperature under aqueous conditions.

Next, the enzyme activity was monitored at this temperature but for diverse pH values of 5.0, 6.3, 7.0, 8.0, and 10.0 (Fig. 2.2). The maximum yield was achieved at a pH of 6.3, as expected from the manufacturer's recommendations. Much lower activity was seen at pH values of 5.0, 7.0, and 8.0, and at a pH of 10.0, there was no activity at all. For these reasons, a pH of 6.3 was confirmed as optimal for the aqueous reaction.

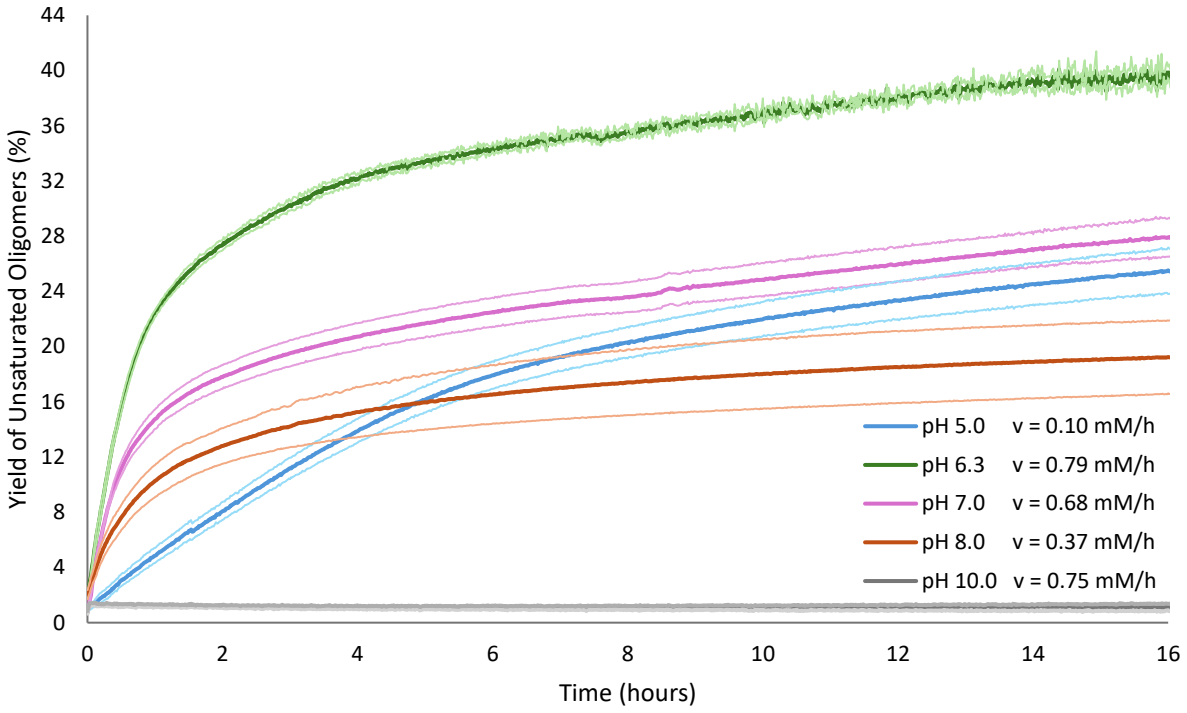

Figure 2.2: Kinetic profiles and initial rates (v) of the depolymerization of alginate by alginate lyase under aqueous conditions across a range of pH values. An alginate lyase solution (10 µL, 0.1 mg/mL) and an alginate solution (450 µL, 1.0 mg/mL), both in sodium phosphate (pH 6.3, 7.0), sodium acetate (pH 5.0), or sodium carbonate (pH 10.0) buffer at 0.1 M, were mixed by pipette (η = 1022 µL/mg). The progress of the reaction was monitored at 235 nm at 37°C. Corresponding light coloured curves represent standard deviations across triplicates.

2.3 Activity of the enzyme under mechanoenzymatic conditions

2.3.1 Activity optimization for MAging conditions

The goal of this study was to explore the ability of alginate lyase to produce oligosaccharides from its natural substrate, alginate, under mechanoenzymatic conditions, as previously shown for natural polymers such as chitin and cellulose.[76–78,88] Unless otherwise noted, all mechanoenzymatic experiments were performed with 150 mg of alginate substrate and 0.33 mg of alginate lyase enzyme (0.04% w/w protein loading relative to the substrate). Experiments were all performed in 10 mL zirconia jars with a single 10 mm zirconia ball and the mixtures were milled at 25 Hz for 5 min, unless otherwise stated, prior to static incubation. Factors such as enzyme

loading, milling frequency, milling duration, static incubation temperature, amount and pH of the liquid, each underwent optimization.

The initial mechanoenzymatic conditions were adapted from the best conditions found for the aqueous reactions above. Thus, sodium phosphate buffer (0.1 M at pH 6.3) was used to adjust the η value of the system, the static incubation was set to 37°C, and the enzyme loading was the same as in solution (0.04% w/w relative to substrate). Based on previous reports in this field and on instrumental capabilities,[76–78,88] we selected the initial mechanical mixing conditions to include 5 minutes of milling at a frequency of 25 Hz, in a 10 mL zirconia jar containing one 10 mm zirconia ball. First, we investigated the effect of varying the η value of the system, as this dictates the qualification of the system as "moist-solid", an important feature of mechanoenzymology.[89] Initial tests were conducted over 7 days to investigate the time course of the reaction under these preliminary conditions (Fig. 2.3).

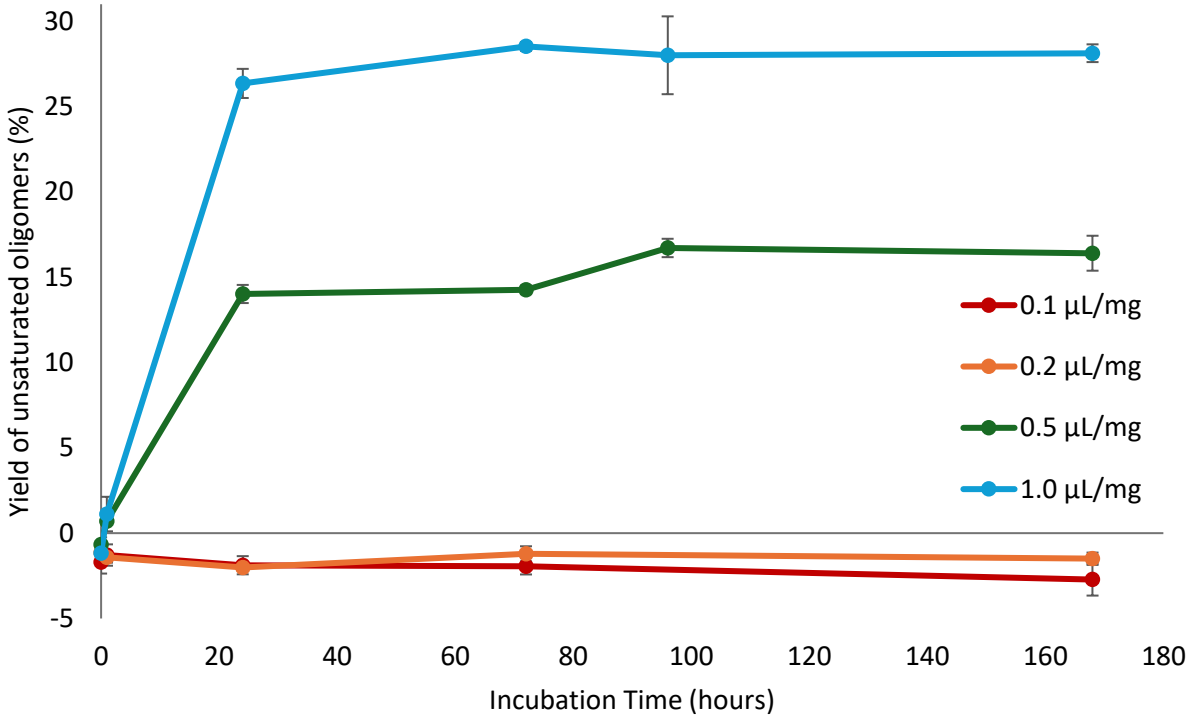

Figure 2.3: Yield for the mechanoenzymatic depolymerization of alginate by alginate lyase at different η values. Reaction mixtures contained alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate buffer (0.1 M, pH 6.3) to adjust η. The mixture was milled at 25 Hz for 5 min and incubated at 37°C for the indicated times. Data means are shown with error bars representing the standard deviation across triplicates.

Based on these initial experiments, the enzyme activity was below the detection limit for η = 0.1 and 0.2 µL/mg, however, activity was apparent at η = 0.5 µL/mg and further increased almost twofold as the η was increased to 1.0 µl/mg. In both cases where activity was observed, the reaction appeared to have completed within the first 24 hours. For this reason, all subsequent mechanoenzymatic reactions were performed over a 24-hour timeframe. Because the yield increased significantly between η = 0.5 and 1.0 µL/mg, higher η values were then tested to maximize the reaction yield, including moist-solid (η ≤ 2.0 µL/mg), slurry (η = 5.0 µL/mg), and aqueous (η = 1022 µL/mg) conditions (Fig. 2.4).

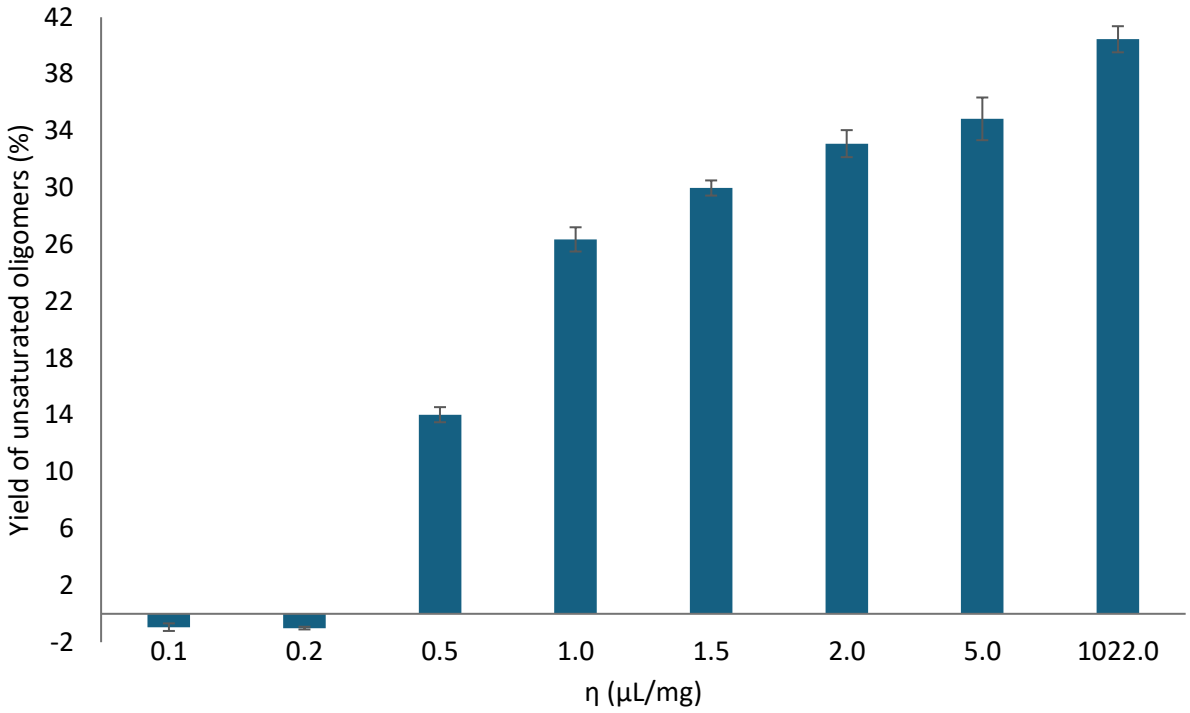

Figure 2.4: Reaction yields for alginate depolymerization by alginate lyase under mechanoenzymatic conditions at different η values after 24 hours of static incubation. Reaction mixtures contain alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate buffer (0.1 M, pH 6.3), milled at 25 Hz for 5 min and incubated at 37°C for 24 hours. Data means are shown with error bars representing the standard deviation across triplicates.

While the yield continued to increase as the η was increased, the relative difference between η values became less significant at higher η and the yield plateaued as the value of η approached traditional aqueous conditions (η > 5.0 µL/mg).[76,78] These result indicate that, unlike cellulases and cutinases, alginate lyase does not work better in moist-solid mixtures (η ≤ 2.0 µL/mg) than under conventional aqueous conditions, yet the gap is small enough that it might be bridged by further optimization. To remain within moist-solid conditions and thus minimizing the production of water waste, η = 1.5 µL/mg was selected for further experiments.

The milling duration is another fundamental parameter in mechanoenzymology as some enzymes are more sensitive than others to prolonged mechanical forces.[79,84] Milling durations ranging from

1 min to 60 min were tested to determine the resilience of the enzyme under mechanical stress, and to see if the activity could be further enhanced (Fig. 2.5).

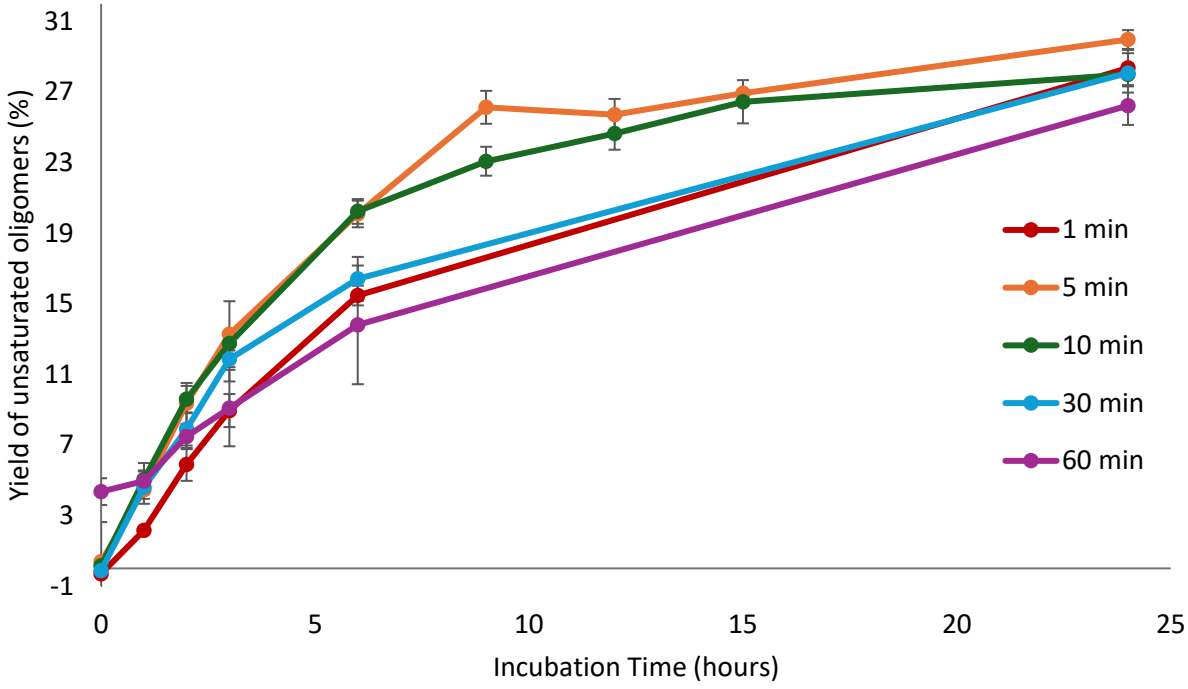

Figure 2.5: Reaction yield for the depolymerization of alginate by alginate lyase with varying milling durations before static incubation. The mixtures contained alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate buffer (0.1 M, pH 6.3, 225 µL) to adjust η = 1.5 µL/mg. The mixtures were milled at 25 Hz for the indicated time before static incubation at 37°C for up to 24 hours. Data means are shown with error bars representing the standard deviation across triplicates.

Interestingly, as the milling time increased beyond ten minutes, there appeared to only be a slight decrease in enzyme activity. A similar result was observed in the mechanoenzymatic depolymerization of cellulose where there was no loss of activity even in milling times of up to 90 minutes.[76] A milling time of 1 minute produced lower yields throughout the reaction and upon visual observation, it appeared insufficient to completely homogenize the mixture, unlike what was observed with longer milling durations. Five minutes was selected as optimal over 10 minutes because it required less energy to achieve a similar yield.

Similarly, the milling frequency can affect the activity of the enzyme and/or the progress of the reaction. One of the roles of mechanical mixing is to produce a homogenous solid mixture.[90] In testing frequencies of 5, 15, and 25 Hz (the maximum frequency of the instrument), we found that 5 minutes of milling at 5 or 15 Hz was insufficient to homogenize the mixture, and the maximum yield reached was still lower than that observed at 25 Hz (Fig. 2.6). Thus, 25 Hz was selected for all subsequent experiments.

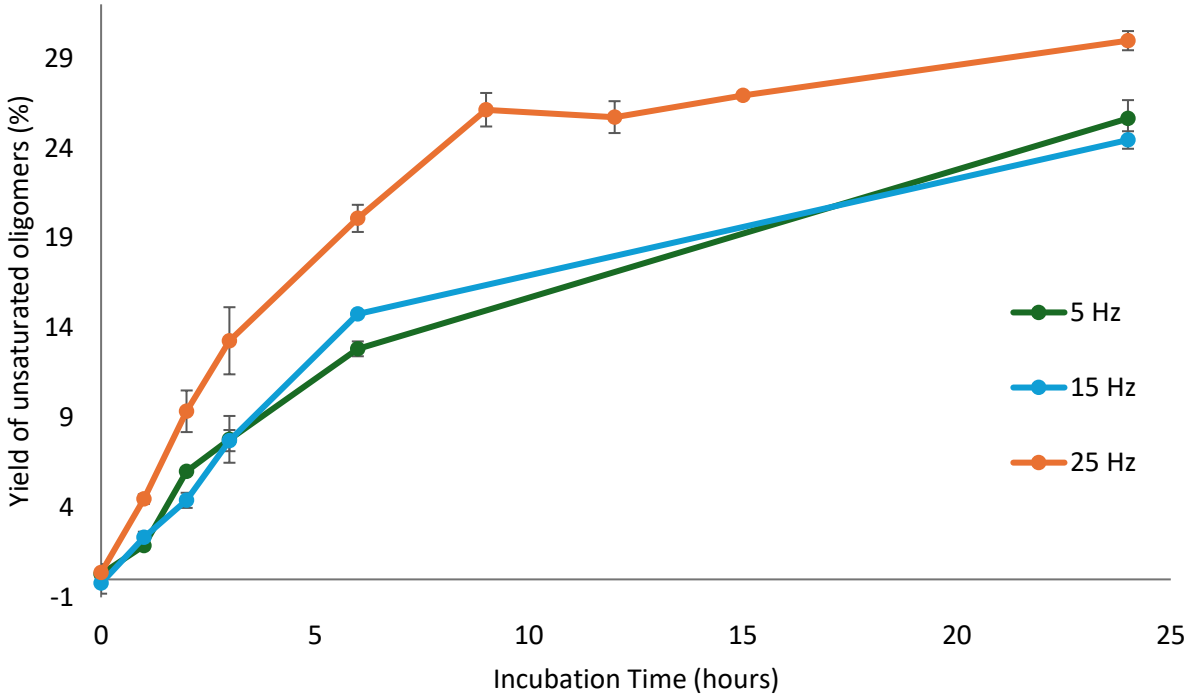

Figure 2.6: Yield for the mechanoenzymatic depolymerization of alginate by alginate lyase at varying milling frequencies. The reaction mixtures contained alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate buffer (0.1 M, pH 6.3, 225 µL) to adjust η = 1.5 µL/mg. The mixture was milled for 5 min at the indicated frequency and incubated statically at 37°C for up to 24 hours. Data means are shown with error bars representing the standard deviation across triplicates.

Next, we were interested in seeing if adjusting the enzyme loading could improve the reaction yield or reduce the amount of enzyme required. Ideally one would aim to use the least amount of enzyme necessary without compromising product formation. Tests were performed using both

half and double the amount of enzyme as in previous experiments under otherwise identical conditions (Fig. 2.7).

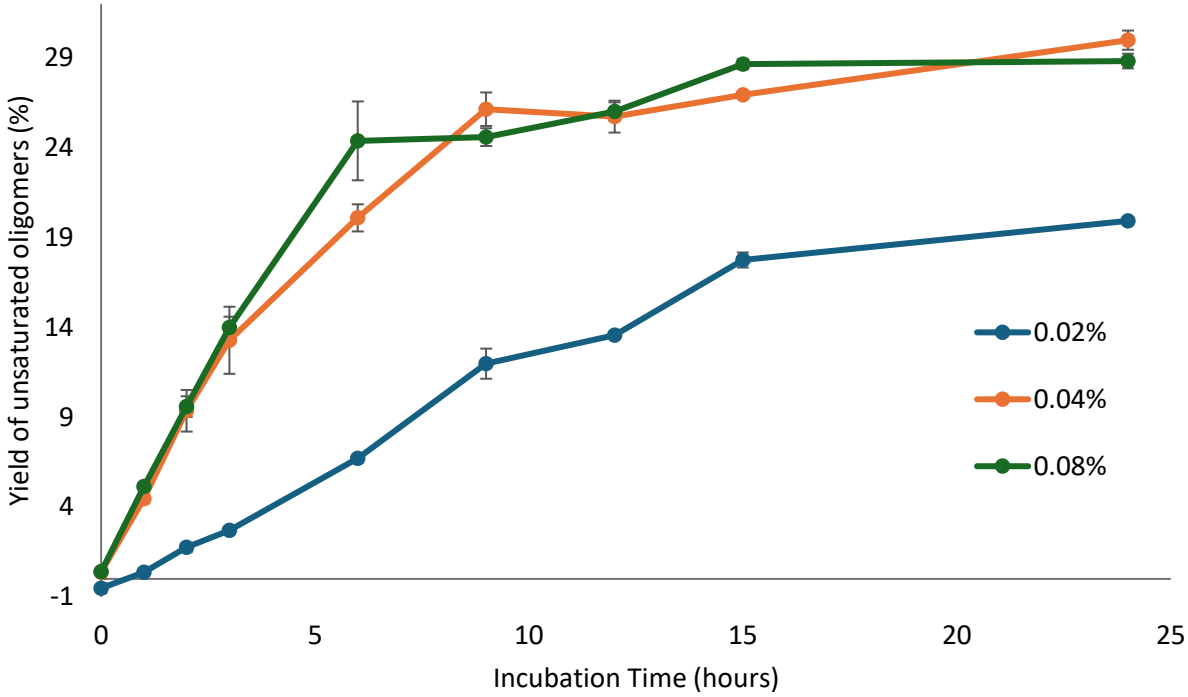

Figure 2.7: Yield for the mechanoenzymatic depolymerization of alginate by alginate lyase with varying enzyme loading reported as weight of enzyme per weight of substrate. The reaction mixtures contained alginate (150 mg), alginate lyase, and sodium phosphate buffer (0.1 M, pH 6.3, 225 µL) to adjust η = 1.5 µL/mg. The mixtures were milled at 25 Hz for 5 min and incubated at 37°C for up to 24 hours. Data means are shown with error bars representing the standard deviation across triplicates.

When the enzyme loading was reduced from 0.04% w/w to 0.02% w/w, the product formation was decreased by approximately half. Interestingly, doubling the enzyme loading from 0.04% w/w to 0.08% w/w had no significant impact on the kinetics of the reaction. This was also observed in previous studies with both cellulases and chitinases in mechanoenzymatic transformations, and has been explained by crowding of the enzyme on the polymeric substrate surface, reducing the enzyme's access to it.[76,78] In order to avoid any unnecessary excess enzyme consumption, an enzyme loading of 0.04% w/w was used in following reactions.

It is well known that enzyme activity is highly sensitive to temperature changes. Generally, low temperatures reduce the kinetic energy available to promote enzyme-substrate contacts and lower the overall mobility of the enzyme.[91] On the other hand, high temperatures lead to denaturing of the enzyme (*i.e.* loss of its 3-dimensional structure), either reversibly or irreversibly, and loss of activity.[92] We investigated a range of temperatures (Fig. 2.8) to determine how the optimal temperature of this enzyme in mechanoenzymology compares to that in aqueous solutions, which was determined to be 37°C (see Fig. 2.1).

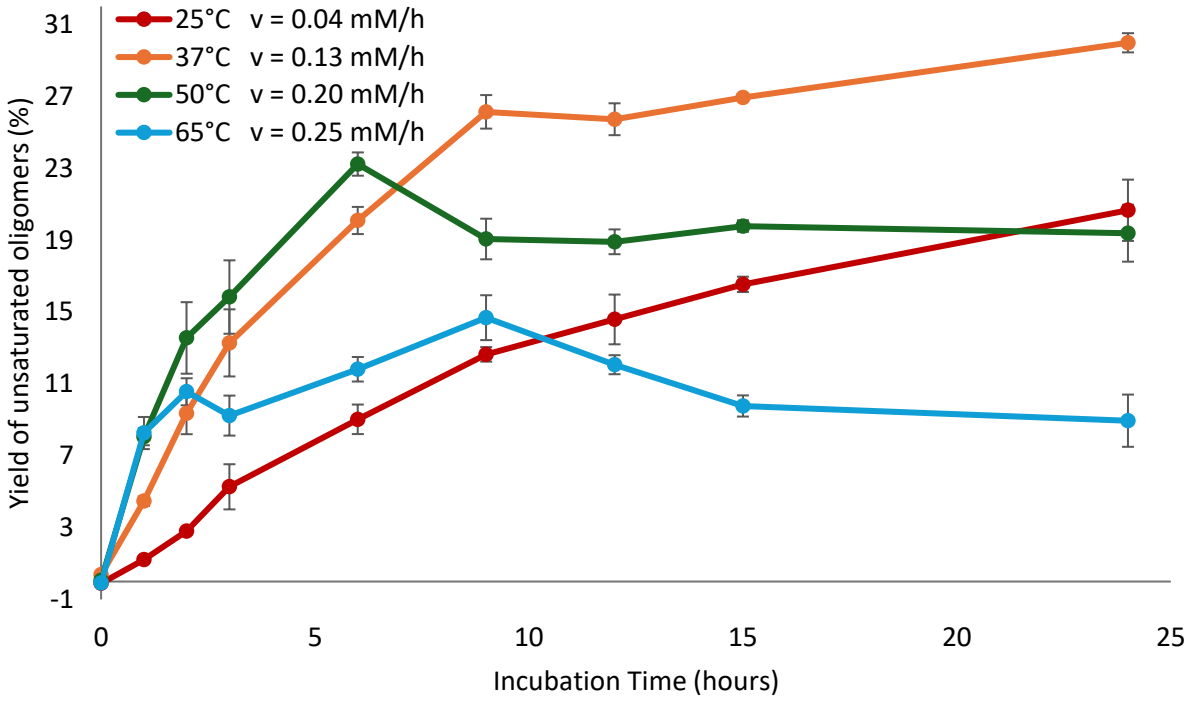

Figure 2.8: Yield and initial rates (v) for the mechanoenzymatic depolymerization of alginate by alginate lyase with varying incubation temperatures. The reaction mixtures contained alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate buffer (0.1 M, pH 6.3, 225 µL) to adjust η = 1.5 µL/mg. The mixture was milled at 25 Hz for 5 min before incubation at the indicated temperature for up to 24 hours. Data means are shown with error bars representing the standard deviation across triplicates.

From this data, it appeared that 37°C is also the optimal temperature under mechanoenzymatic conditions. Although higher temperatures typically lead to superior initial rates, they also cause

faster enzyme denaturation (*i.e.* the plateau in the reaction yield is reached sooner), as observed here (Fig. 2.8). As anticipated, at a temperature of 65°C, the activity is severely impeded, with product formation plateauing after only 2 hours at a final yield of $9 \pm 1\%$. In comparison, the 50°C trials experienced a rapid initial rate, slightly higher than that at 37°C, but the yield plateaued between 3-6 hours ($19 \pm 2\%$). Interestingly, the reaction was slower at 25°C but did not plateau, even after 24 hours. At 37°C, the initial rate is reasonable, and the reaction plateaus at a higher yield than any of the other temperatures tested. As such, 37°C was considered the optimal reaction temperature under these conditions with a final yield of $30 \pm 0.5\%$.

As with temperature, enzymes are typically highly susceptible to pH-dependent physical changes. For a given enzyme, there is a narrow working pH range within which the amino acids are in the necessary protonation state for substrate binding and to promote reactivity.[93] The optimal pH under aqueous conditions was found to be 6.3 (see Fig. 2.2), which was consistent with the recommendation from the manufacturer. For this reason, up until this point, the liquid component in all mechanoenzymatic experiments was sodium phosphate buffer at pH 6.3 (0.1 M). In order to understand how the pH can impact this mechanoenzymatic transformation, we tested a wide range of pH values (Fig. 2.9).

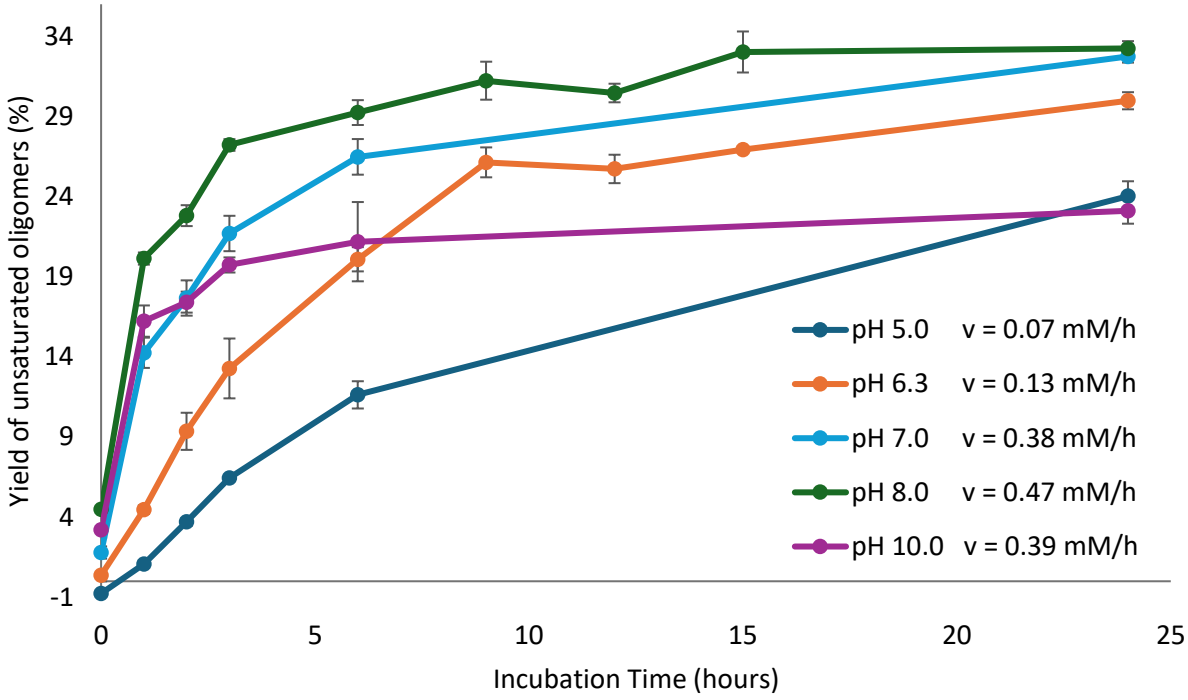

Figure 2.9: Yields and initial rates (v) for the mechanoenzymatic depolymerization of alginate by alginate lyase with varying buffer pHs. The reaction mixtures contained alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate (pH 6.3, 7.0, 8.0), acetate (pH 5.0), or carbonate (pH 10.0) buffer (0.1 M, 225 µL) to adjust η = 1.5 µL/mg. The mixture was milled at 25 Hz for 5 min and incubated at 37°C for up to 24 hours. Data means are shown with error bars representing the standard deviation across triplicates.

The reaction yield for both extremes of the range, pH 5.0 and pH 10.0, were nearly identical after the 24 hours of incubation (23 and 24 ± 1%, respectively). Whereas at pH 10.0 this yield was reached within the first three hours, at pH 5.0, product formation appeared to increase over the entire 24 hours and attained 23 ± 1% only after roughly 24 hours. Although the initial rate observed at pH 7.0, 8.0, and 10.0 were very similar, this rate was highest and maintained for longer at pH 8.0, affording a higher product yield (33 ± 0.5%). Interestingly, except for pH 10.0, all pHs above 6.3 gave higher yields. This was unexpected, considering that pH 6.3 is optimal under standard aqueous conditions. Because of the superior rate and yield, all subsequent mechanoenzymatic reactions were carried out at pH 8.0.

We then compared the reaction yields for the best mechanoenzymatic experimental conditions observed so far to those of the optimized dilute aqueous reaction (Fig. 2.10).

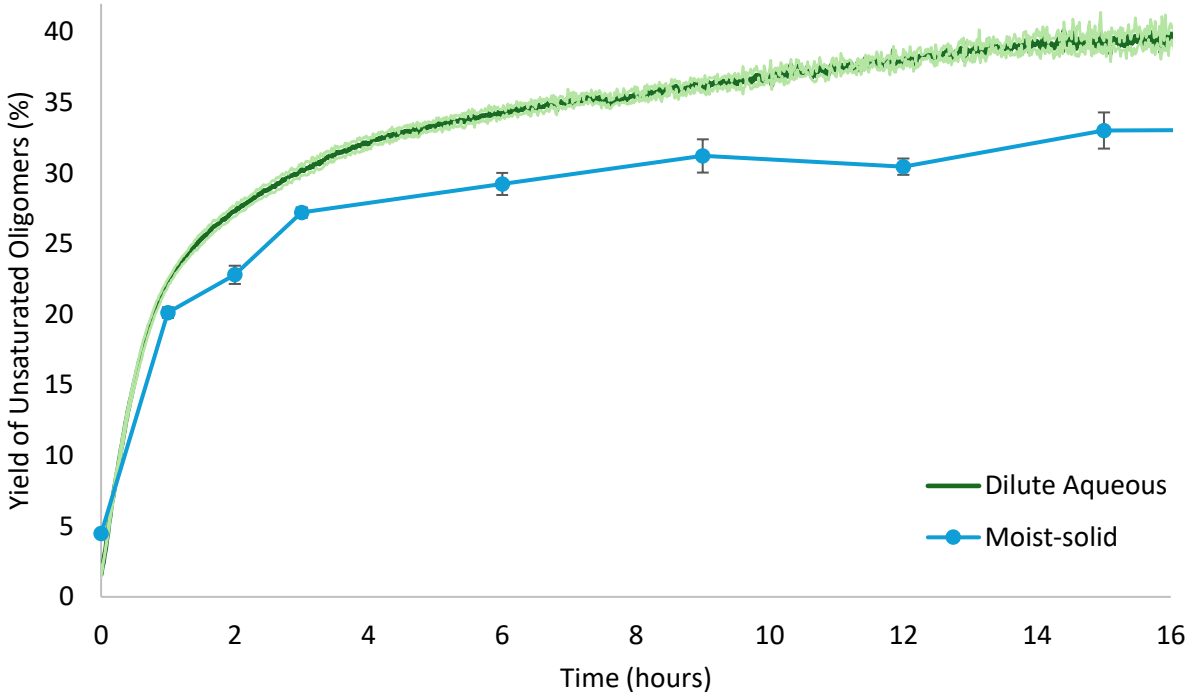

Figure 2.10: Comparison of the kinetics of both aqueous and mechanoenzymatic depolymerization of alginate by alginate lyase under respective optimal conditions. Both reactions were performed at 37°C at an enzyme loading of 0.04% w/w enzyme relative to alginate. Sodium phosphate buffer (0.1 M) was added to adjust the pH to 6.3 and η = 1022 µL/mg for the aqueous reaction, and to set the pH to 8.0 and η = 1.5 µL/mg for the mechanoenzymatic reaction. The later mixture was milled for 5 minutes at 25 Hz before static incubation, while the former was gently mixed by pipette. Light coloured curves and error bars shows standard deviations for aqueous and mechanoenzymatic triplicate experiments, respectively.

The results show that the kinetics of the mechanoenzymatic reaction follows the same hyperbolic trend as with the aqueous experiment, with comparable yields (33 ± 1% and 40 ± 1%, respectively). In each case, there is a rapid initial rate over the first hour, which then slows over the following 2-3 hours, eventually plateauing after approximately 6 hours. This comparison demonstrates the transferability of the reaction from conventional aqueous conditions to a mechanoenzymatic process, while providing a more sustainable alternative. Indeed, for the same

amount of alginate, the mechanoenzymatic reaction uses 681 times less water and therefore produces much less excess waste and proceeds in a 681 times smaller volume.

2.3.2 Further efforts beyond typical MAging

2.3.2.1 Applying the RAging strategy to further optimize the reaction

Still, even with these optimizations, the reaction yield of the mechanoenzymatic transformation is not superior to that of conventional aqueous mixtures, unlike what is seen with many other enzymes. Therefore, there is still a potential for further optimization of the mechanoenzymatic method. For example, the use of RAging is known to increase enzyme activity for many enzymes.[76,78,79] From the results of the above studies, it was interesting to observe that the milling frequency and duration did not have a large impact on the reaction yield. Thus, the reaction appears highly resistant to mechanical force, making it a good candidate for repeated cycles of milling and aging (or RAging) to further promote product conversion.[76] If an enzyme is too sensitive to milling, the repeated milling cycles can cause rapid enzyme denaturation. Preliminary exploration of RAging as a means of improving the yield was undertaken. The RAging regime selected consisted of milling for 5 minutes at the start of the reaction and milling again for 5 minutes after 6 hours of incubation (Fig. 2.11). The 6-hour time point was selected based on the kinetic plots, which show that the MAging reaction rate decreases dramatically at that point (see Fig. 2.10).

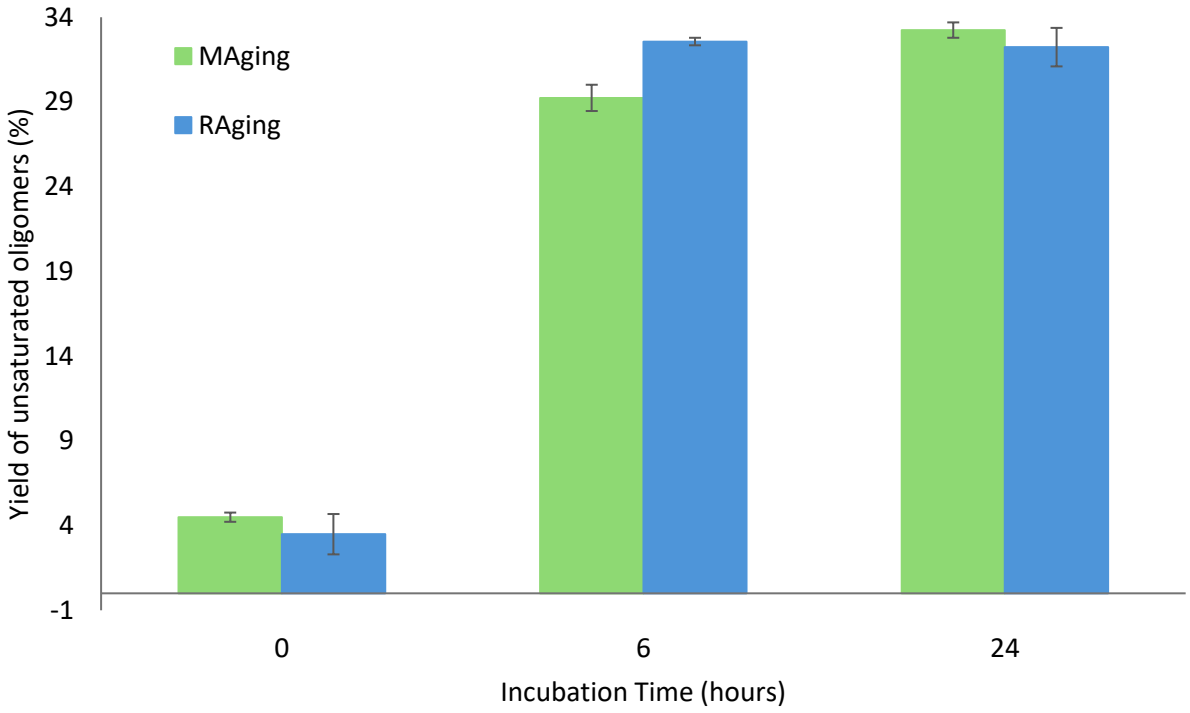

Figure 2.11: Yield for the mechanoenzymatic depolymerization of alginate by alginate lyase in a regular MAging experiment compared to a two-cycle RAging experiment with milling after 6 hours. Reaction mixtures contain alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate buffer (0.1 M, pH 8.0) to adjust η = 1.5 µL/mg. The mixture was milled at 25 Hz for 5 min at the start, with an additional 5 min of milling after 6 hours of incubation for RAging. The samples were incubated at 37°C for up to 24 hours. The means are shown with error bars representing the standard deviation across triplicates.

In other studies with biological and synthetic polymers, introducing RAging was reported to improve yields within even just one additional cycle.[76,78,82,88] With alginate lyase, however, the additional milling event had no noticeable impact on the final yield. MAging and RAging reactions displayed reaction yields that were within error after 24 hours (33 and 32 ± 1%, respectively) (Fig. 2.11). This result, however, cannot be used to conclude that the RAging strategy cannot be beneficial to this system as further testing of various RAging regimes is necessary to understand the real impact of RAging on this reaction.

2.3.2.2 Inclusion of a solid additive

Another option to attempt to improve product yield involves adding an inert solid into the reaction mixture. This is a concept previously demonstrated to improve activity especially when the substrate is liquid or very water soluble. In such a case, the solid additive gives a more solid texture to the reaction mixture.[94] For example, the mechanoenzymatic depolymerization of (water soluble) cellobiose by β-glucosidase was dramatically improved by the addition of solid additives.[94] It was found that, although not a participant in the reaction itself, the presence of solids such as microcrystalline cellulose, xylan, chitin, polyethylene glycol (PEG) and polystyrene (PS), significantly improved conversion to glucose, even beyond the yields observed in dilute aqueous conditions. Although not restrained to a specific physical characteristic of the solid, it was proposed that the solid may help protect the enzyme from mechanically-induced denaturation.[94] This concept was also considered for the improvement of alginate lyase activity, and a trial was performed with PEG as the solid additive. This synthetic polymer was selected because it was found to be one of the most effective additives in the cellulose study, in addition to being water soluble (facilitating work-up), and not absorbing at the analysis wavelength (235 nm). The experiment tested a ratio of 2:1 alginate to PEG (Fig. 2.12).

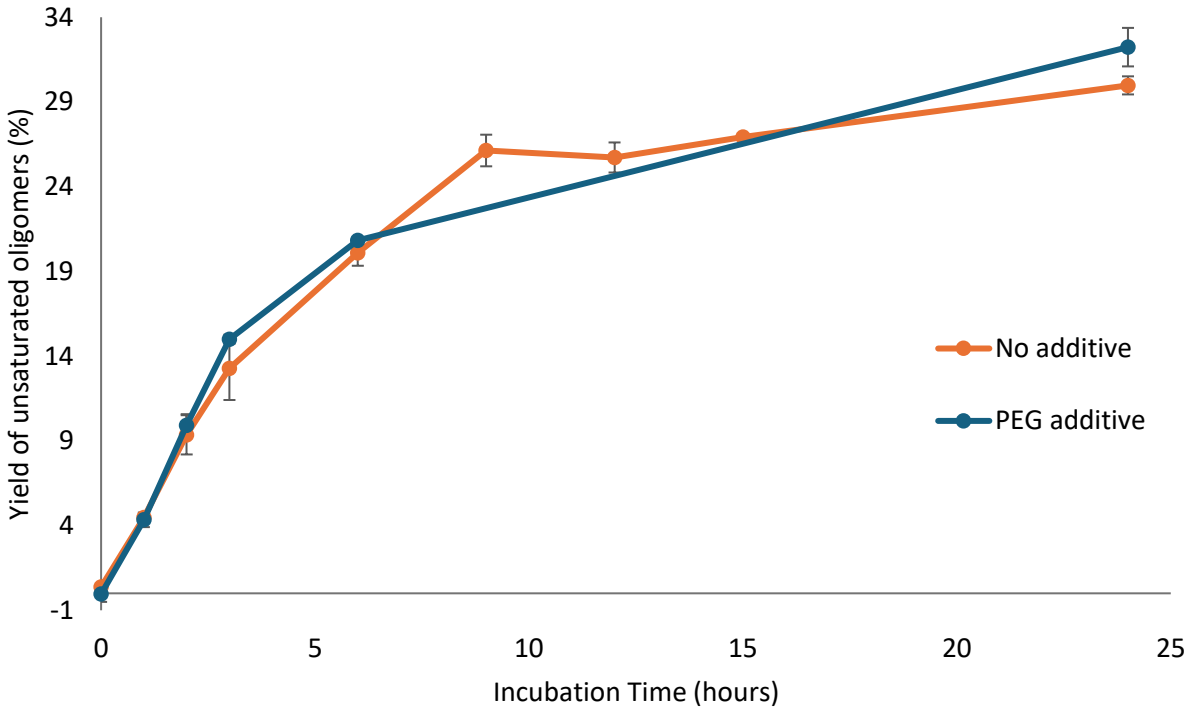

Figure 2.12: Yield for the mechanoenzymatic depolymerisation of alginate by alginate lyase with and without addition of the inert additive PEG. Reaction mixtures contain alginate (150 mg in the absence of PEG or 100 mg in the presence of PEG), alginate lyase (0.04% w/w enzyme loading), PEG (0 or 50 mg), and sodium phosphate buffer (0.1 M, pH 6.3) to adjust η = 1.5 µL/mg. The mixture was milled at 25 Hz for 5 min and incubated at 37°C for up to 24 hours. Data means are shown with error bars representing the standard deviation across triplicates.

The data revealed that the presence of the inert additive had no significant effect on the progress or yield of the reaction. The lack of improvement could indicate that the enzyme is not being limited by mechanical denaturation or that the effects seen previously were more specific to the nature of the particular enzyme or of the additive. To verify the latter hypothesis, tests with PS were also attempted, however, the additive was partially broken down by the mechanical process and caused interference with the absorbance measurements. Furthermore, removing all of the PS before analysis turned out to be challenging. It would be interesting, however, to further test if other types of inert solid additives, such as cellulose or inorganic oxides, could be beneficial to this reaction.

2.3.3 Investigating the reaction limiting factor

As mentioned above, the reaction of alginate lyase typically plateaus at a yield of approximately 33 ± 1% after 9 hours, whereas that under conventional aqueous conditions reaches 36 ± 0.2% in the same time, and even further increases to 40 ± 1% by 24 hours. Understanding why the yield plateaus could help further optimize the reaction. Some preliminary testing was performed with the hopes of determining if the reaction is being limited by protein denaturation, by substrate accessibility, or some other factor. It is important to note that the alginate lyase used throughout this study is polyM specific, meaning it has a significant preference for MM bonds over GG or MG bonds, but that the sodium alginate used is a mixture of M and G monomers at an M:G ratio of about 1.56, meaning it contains a mixture of both monomers.

In one experiment to verify if the reaction is limited by the substrate, the moist-solid samples were diluted in sodium phosphate buffer (0.1 M, pH 8.0) and aliquoted to a cuvette for analysis after 5 minutes of milling and 6 hours of static incubation (*i.e.* at the approximate time when the plateau is reached). An additional batch of alginate (about 2.5x the amount in the aliquot) was added to the reaction mixture, resulting in a new enzyme loading of approximately 0.01%. The absorbance was then monitored continuously for an additional 18 hours. The same experiment was repeated, but this time with addition of more enzyme (about 10x the amount in the aliquot), to give a new enzyme loading of approximately 0.5%. After correcting for the changes in concentration, both the samples with additional alginate and additional enzyme showed a small increase in yield following the additions (from 28 ± 1% to 37 ± 0.3% and 36 ± 2%, respectively), in contrast to the smaller increase in a comparable reaction mixture to which only buffer was added (from 28 ± 1% to 32 ± 1%) (Fig. 2.13).

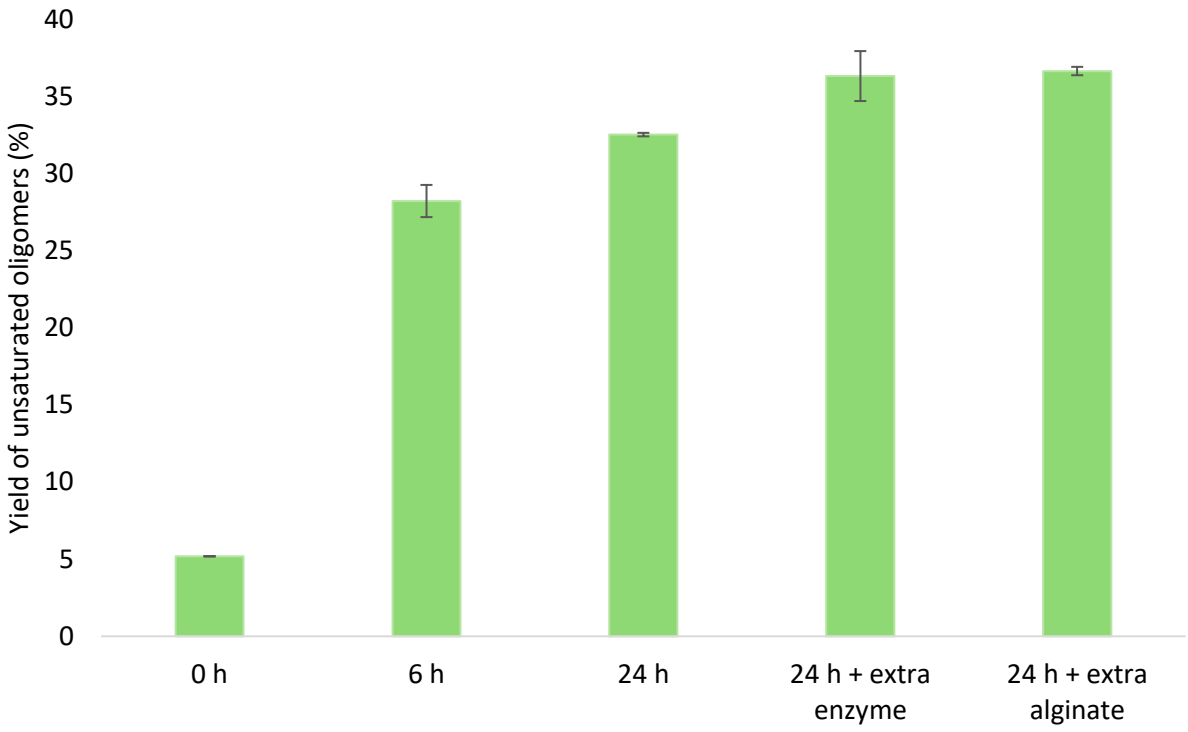

Figure 2.13: Yield of mechanoenzymatic depolymerisation of alginate by alginate lyase to investigate the limiting factors of the reaction. To begin, reaction mixtures contain alginate (150 mg), alginate lyase (0.33 mg), and sodium phosphate buffer (0.1 M, pH 8.0) to adjust η = 1.5 µL/mg. The mixture was milled at 25 Hz for 5 min at the start, followed by 6 hours of incubation at 37°C. After 6 hours, the moist-solid mixtures were diluted with the same buffer to 1 mg/mL and either only buffer (100 µL), alginate lyase (100 µL, 0.1 mg/mL in sodium phosphate buffer 0.1 M pH 8.0), or alginate (100 µL, 10 mg/mL in phosphate buffer, 0.1M pH 8.0) was added.

One possible explanation for this result is that during the first 6 hours, the enzyme may cleave most, but not all, of the MM bonds of the original mixture, leaving the GG and MG bonds untouched. It is also possible that part of the cleavable bonds may not be physically accessible to the enzyme (*e.g.* being covered by polymer chains containing only uncleavable GG and MG bonds). When fresh alginate is added to the mixture, new MM bonds are available for cleavage by the enzyme, further increasing product formation by whatever portion of enzyme is still active. The fact that the reaction rate undergoes a small burst after addition of fresh enzyme suggests that the originally added enzyme may have also been mostly inactivated after 6 hours before being able to act upon all of the accessible bonds. This is also supported by the fact that doubling

the enzyme loading from the beginning did not appear beneficial to the final yield, so in order to improve overall conversion, it may be necessary to introduce additional enzyme partway through the reaction. Based on this, it would appear that the reaction is nearing completion. This latter explanation is also supported by the GPC results which showed that there still remained some untouched polymer left at the end of the 24 hour-period (see Section 2.4). It would be interesting to test a pure (much more expensive) polyM substrate in the future.

2.4 Characterization of AOS products by gel permeation chromatography

We next performed some basic characterization of the reaction mixtures to compare the products yielded under moist-solid and aqueous conditions. The samples were analyzed using gel permeation chromatography (GPC) which provides information about the weight average molecular weight (Mw), the number average molecular weight (Mn), and the polydispersity (PD) of oligomeric and/or polymeric samples. The Mw is calculated from the mass of each molecule multiplied by the fraction of total mass of molecules with that mass and adding up all the masses together, whereas Mn is the total mass divided by the total amount of molecules, therefore is the average of the molecular weights of the molecules in a sample. The ratio of Mw/Mn is PD. As a general reference, an example of a chromatograms is shown in Fig. 2.14A for unreacted alginate with peaks assigned based on their retention times (RT). The broad peak seen in Fig. 2.14A at RT = 24 minutes represents a group of polymer chains of similar molecular weight, in this case having Mw ranging from ~50,000-200,000 g/mol. Going forward, when present, this peak will be referred to as RT24 in analysis. The peak around 31 minutes in each chromatogram is a system peak generated at the end of the run, regardless of the sample. Fig. 2.14B shows the mixture of products following the reaction under optimal dilute aqueous conditions.

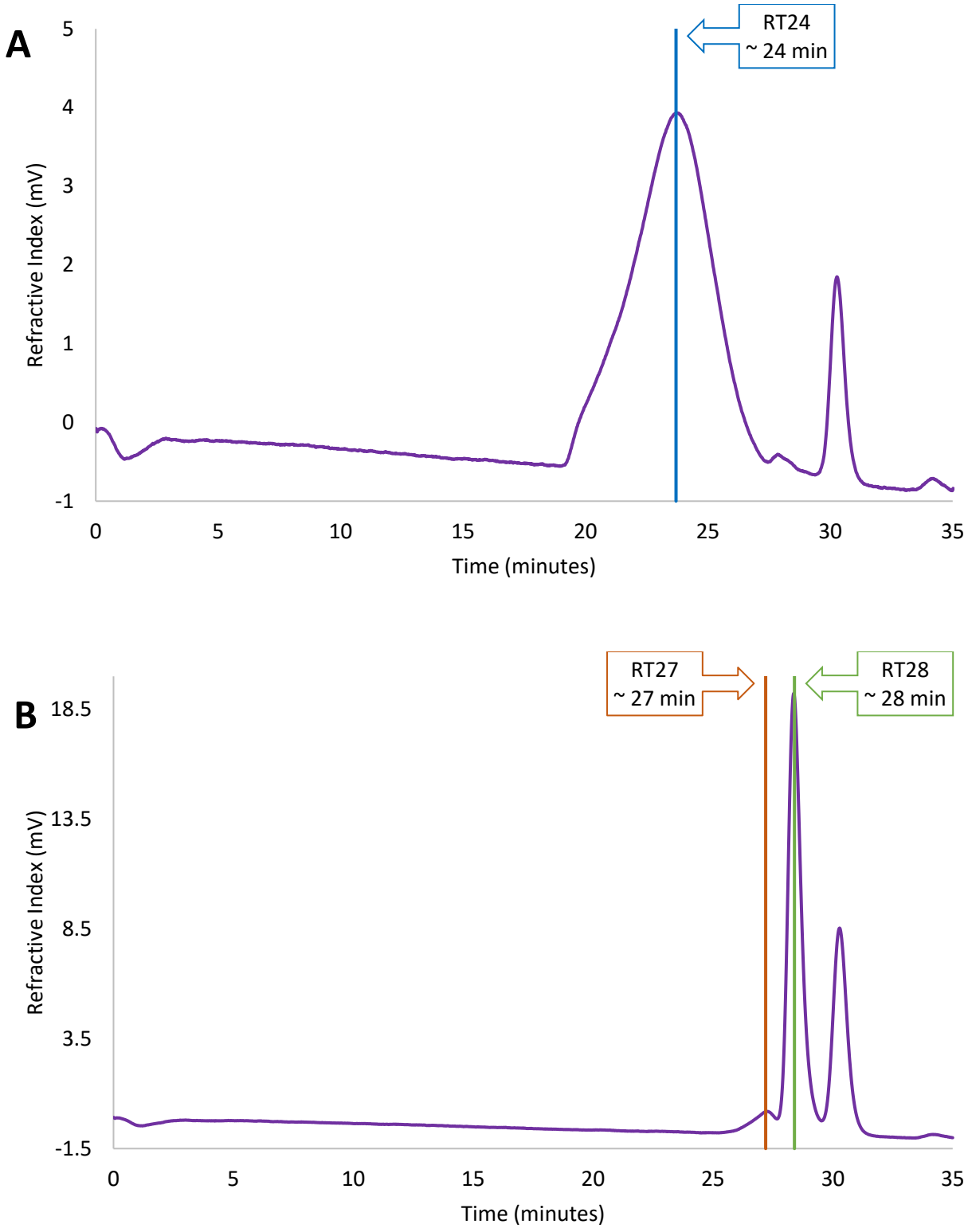

Figure 2.14: Sample GPC chromatograms with characteristic peaks representing unreacted commercial alginate (A) and depolymerization products of alginate (B). The dilute aqueous reaction consisted of alginate (450 µL of 1.0 mg/mL solution in buffer) and alginate lyase (10 µL of 0.1 mg/mL solution in buffer. The buffer was0.1 M sodium

phosphate pH 6.3. The reaction mixture was mixed by pipette and incubated at 37°C for 24 hours. The peak around 31 min in each chromatogram is a system peak generated at the end of the run, regardless of the sample. Experiments were conducted in single replicate..

The small peak around 27 minutes represents mid-sized oligomers of Mw 2000-5000 g/mol, while the larger peak around 28 minutes represents the smallest detectable oligomers for this machine, which range from between 500-700 g/mol. Analogous peaks, when present, will be referred to as RT27 and RT28, respectively.

Initially, comparisons were made between a reference sample of unreacted alginate, moist-solid reactions of alginate with and without enzyme (150 mg of alginate, 0.04% enzyme loading, η = 1.5 µL/mg with 0.1 M sodium phosphate buffer pH 8.0) after 5 minutes of milling and either 1 hour or 24 hours of incubation, and an aqueous enzymatic reaction mixture after 24 hours under the optimal conditions (Fig. 2.15). The unreacted alginate sample showed a Mw of 167,000 g/mol and a Mn of 47,000 g/mol, indicating a relatively high PD of 3.52 (Fig. 2.15A). Analysis of the products of a standard aqueous reaction showed a very tall narrow peak (RT28) of Mw 610 g/mol and Mn 522 g/mol (PD: 1.2) as well as a much smaller peak (RT27) of Mw 3700 g/mol and Mn 3100 g/mol (PD: 1.2). This suggests that in dilute aqueous conditions, the alginate present in the mixture underwent substantial depolymerization, to the point where no peak remained in the range of the original unreacted alginate (RT24). In addition, the small PD values of both reaction product peaks (both 1.2) indicate that the enzyme is capable of generating oligosaccharides of very narrow and specific distribution, with a significant preference for those in the 500-600 g/mol range (95%), as shown by their relative abundances (Fig. 2.15B).

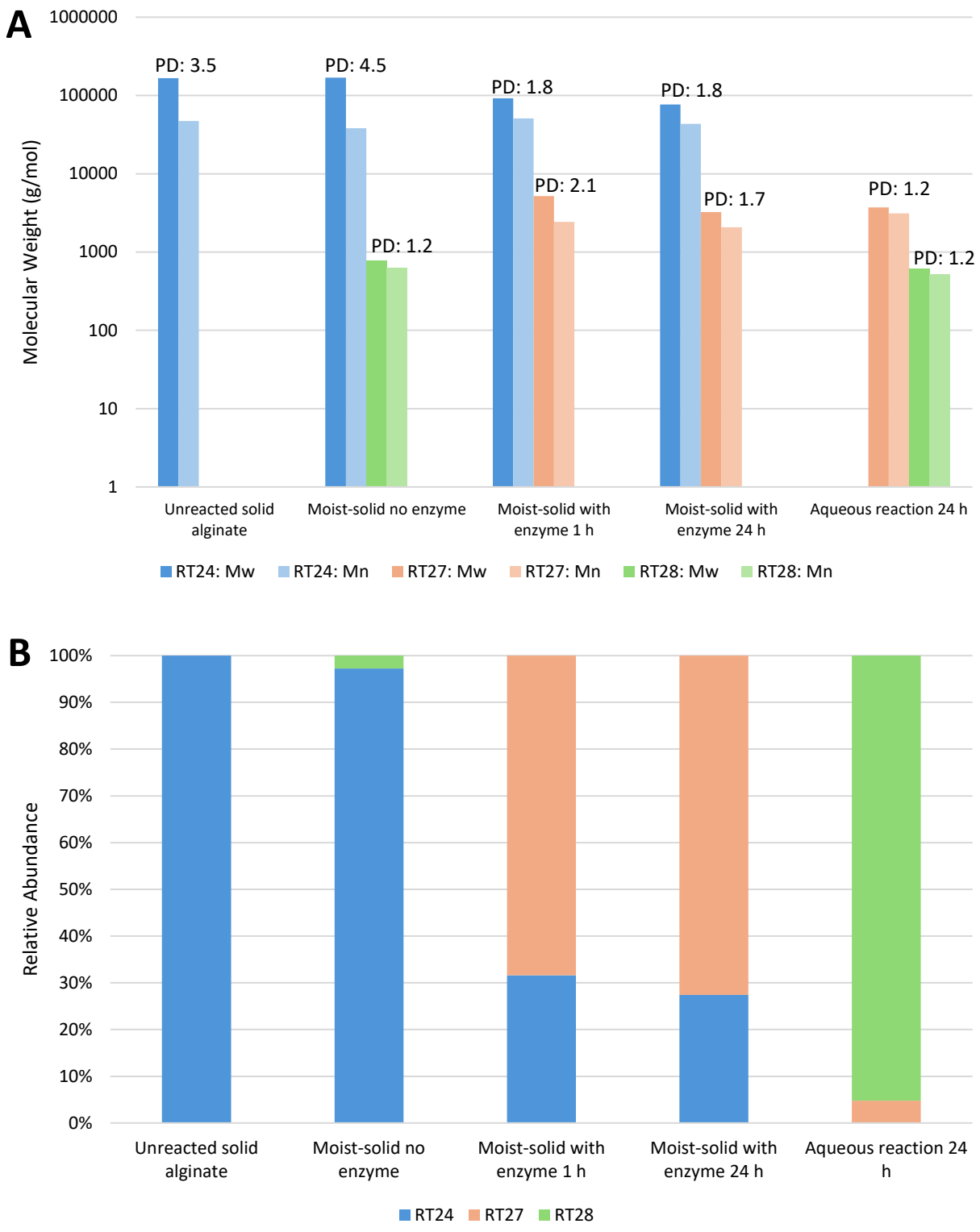

Figure 2.15: Results of GPC analysis for reaction mixtures and control samples. A) Comparison of the weight average molecular weights (Mw), number average molecular weights (Mn), and the corresponding polydispersities (PD) of the highest Mw oligosaccharide peak (RT24), mid-range Mw oligosaccharide peak (RT27) and of the lowest Mw

oligosaccharide peak (RT28) obtained from GPC analysis of untreated alginate, alginate under optimal mechanoenzymatic conditions without and with enzyme, and an optimal aqueous reaction mixture, each after 1 hour or 24 hours of incubation; B) Relative abundances of the highest Mw oligosaccharide peak (RT24), mid-range Mw oligosaccharide peak (RT27) and of the lowest Mw oligosaccharide peak (RT28) calculated from percentage of total area of the two peaks from the same experiments. Experiments were conducted in single replicate.

Following milling of an alginate sample for 5 minutes without enzyme, a large proportion (97%) of polymer remained that has Mw 170,000 g/mol and Mn 38,000 g/mol, indicating a mostly unchanged structure compared to the original substrate, but with slightly increased polydispersity (PD: 4.5). There was, however, a very small new peak (RT28) Mw 780 g/mol and Mn 631 g/mol (PD: 1.2), observed after milling the alginate (~3% of total peak area), suggesting that a very small quantity of oligomers of nearly uniform sizes, similar to those produced under aqueous conditions, were generated from the milling process alone. It is also possible that the same amount of these small oligomers is present in the commercial alginate but hidden under the main peak. The optimized mechanoenzymatic reaction with enzyme also gave two peaks (Fig. 2.15). Within the first hour of incubation with enzyme (after milling), the relative proportion of the larger oligomeric products (RT24) drastically drops to 32% from the 100% in the starting material with a Mw of the larger oligomers diminishing to an average of about 92,000 g/mol and the PD to 1.8. Interestingly, the smaller oligomeric product grouping (RT27) is composed of species with a Mw of 5200 g/mol, Mn of 2400, and PD of 2.1. The stark decrease in the proportion of large Mw oligomers and the rapid emergence of the mid-sized oligomers as well as their small PDs demonstrates both the high activity of the enzyme under these conditions, resulting in a highly specific oligomeric distribution within just this first hour of incubation. After 24 hours, these peaks consisted of a large, sharp peak, RT27, (~73%) of Mw 3434 g/mol and Mn 2065 g/mol (PD: 1.7) and a smaller peak, RT24, (27%) of Mw 76823 g/mol and Mn 43271 g/mol (PD: 1.8). The

emergence of such a large abundance of the RT27 oligosaccharides in only the samples with enzyme (both after 1 h and 24 h) indicates that the generation of these oligomers is the result of the enzymatic activity and not from the milling process. The Mw of these oligomers aligns with that seen for the minor product of the aqueous reaction, also indicating that the enzyme maintains some similar activity under mechanoenzmyatic conditions, however there is still some starting material remaining after 24 hours, which was not observed in the aqueous reaction mixture (Fig. 2.15). The Mw and PD of the RT24 peak in the moist-solid reaction are smaller than in both the unreacted alginate and in the mixture without enzyme, however, which confirms that there is significant depolymerization taking place. It is important to note that while the lowest Mw peak (RT28) seen in the optimal aqueous reaction is not seen in the moist-solid reaction, this does not necessarily imply the absence of oligomers in this range. The RT for both the mid-sized and smallest oligomers is very similar, and when there is a larger PD for either of these products, overlap is probable and the peaks cannot be resolved. It is therefore important to consider that there is some degree of intrinsic error involved in this analytical method, so it only serves as a preliminary understanding of the reaction mixture. The low PDs of the product peaks in both the aqueous and moist-solid reactions suggest that both methods generate oligosaccharides of highly specific sizes, and therefore a high degree of selectivity is possible for the mechanoenzymatic method, as previously reported for the aqueous method.

Although the peak for oligomeric products with molecular weights in the 10^2 scale (RT28) found in the aqueous reaction is not seen at either 1 h or 24 h, the 2000-5000 g/mol oligomeric products are consistent with those observed in both the moist-solid and dilute aqueous mixtures in the initial testing (Fig. 2.14A). As mentioned previously, this could be a result of broadening of the

RT27 peak in these mixtures causing overlap of a RT28 peak, or potentially further depolymerization of RT28 oligomers resulting in products below the Mw detection limit of the instrument. Figure 16 shows the chromatogram of the product mixture after 1 hour. The peaks identified and characterized as RT24 and RT27 are visible with a moderate degree of resolution, however, a shoulder can also be seen around 28 minutes that is consistent with that seen in the aqueous reaction mixture as well as with the moist-solid sample without enzyme, therefore it cannot be concluded that there are not also smaller oligomers present in these mixtures.

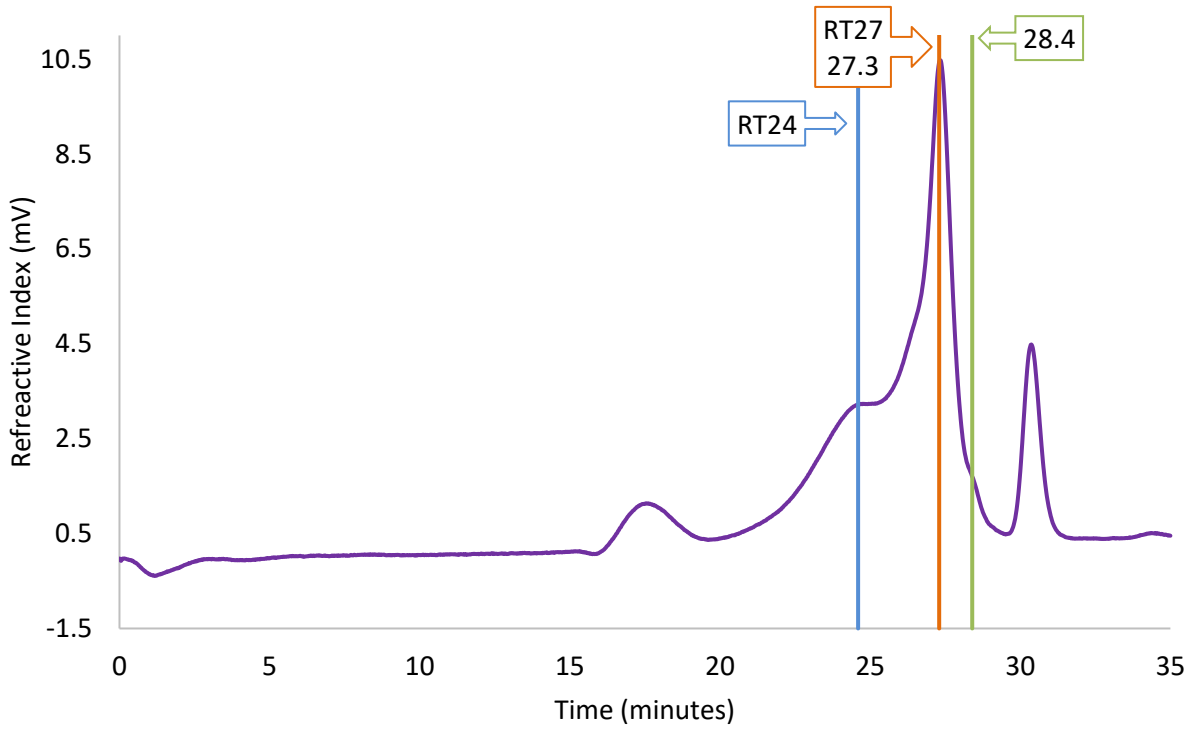

Figure 2.16: GPC chromatogram of mechanoenzymatic reaction mixture after 5 minutes of milling and 1 hour of incubation at 37°C. Mixture included 150 mg of alginate, 0.04% enzyme loading, η = 1.5 µL/mg with sodium phosphate buffer 0.1 M, pH 8.0. The peak around 31 min in each chromatogram is a system peak generated at the end of the run, regardless of the sample. Experiment was conducted in single replicate.

The aqueous reaction shows apparent complete depolymerization to oligomers of Mw ~500-600 g/mol (major product) and ~3100-3700 g/mol (minor product) with a respective abundance of ~95% and 5% , whereas the moist-solid reaction shows incomplete depolymerization to oligomers

of ~1800-3200 g/mol (major product) and ~44,000-83,000 g/mol (minor product) (~73% and 27%, respectively). Although the product distributions between the dilute aqueous conditions and moist-solid mechanoenzymatic conditions are different, there is consistency between the mid-sized oligomers produced in both conditions, especially considering the fact that these oligomers were not produced without enzyme. This is evidence that the enzyme is maintaining similar function under mechanoenzymatic conditions, although it is uncertain why the lowest Mw oligomers are so evident in the aqueous reaction and not in the moist-solid reaction. Of particular interest, the low PD (1.8) of the mid-sized oligomers produced in the moist-solid reaction also supports the evidence that the enzyme is highly selective towards these oligomeric products specifically under mechanoenzymatic conditions. This could be useful in applications where these specific Mw oligomers are desired. The presence of the high Mw oligomers remaining in the moist-solid mixture after the reaction is also consistent with the lower yields seen through the UV-vis analysis compared to the dilute aqueous conditions (Fig. 2.10).

Ideally, the lower the proportion of high Mw AOS in the product, the more useful the AOS in applications. Thus, an ideal alginate depolymerization method would produce mostly low Mw AOS. This is because of the somewhat limited applications of high Mw AOS resulting from their reduced water solubility and lack of physiological stability. For this reason, only the proportion of the mechanoenzymatic products composed of the ~1800-3200 g/mol AOS may be useful at present, and further optimization is needed to transform the higher Mw fraction to lower Mw. The traditional aqueous method produces two types of lower Mw oligomers (~500-600 g/mol and ~3100-3700 g/mol) which are both be useful in biological, environmental, or nutritional applications. Because the potential applications of AOS vary based on their Mws, it would be most

desirable if the mechanoenzymatic method produced a similar product Mw distribution as the in aqueous process (*i.e.* major product Mw in the range of 10^2 g/mol and minor product Mw 10^3 g/mol). Overall, although the results of the aqueous and mechanoenzymatic reactions show some differences, further optimization of the reaction in moist-solids may bridge the gap, or otherwise this method may give access to a new oligomeric product.

Chapter 3 – General Discussion and Conclusion

3.1 General Discussion

As has been demonstrated through the optimization of key mechanoenzymatic parameters, it is possible to achieve highly comparable alginate depolymerization yields to those obtained in conventional aqueous mixtures. Factors such as the milling duration or frequencies are expected to influence the enzyme stability during the reaction, because they can affect the temperature of the mixture, and extreme temperatures can lead to enzyme denaturation. In this study, none of the more aggressive milling conditions tested (higher duration and frequency) were found to have a significant impact on product formation, demonstrating the robustness of this enzyme.

Interestingly, the optimal temperature (37°C) was the same under both aqueous and mechanoenzymatic conditions. The results presented show that the enzyme activity at different temperatures follows the same trend in aqueous and moist-solid conditions. Under mechanoenzymatic conditions, some activity was still seen even at 50°C, however no activity was observed at 57-60°C. This was expected as enzymes typically display a narrow optimal temperature range. As expected, at 25°C (below the aqueous optimal temperature of 37°), only moderate activity was seen.

The other comparable parameter between aqueous and enzymatic conditions, the optimal pH of the buffer, did differ between mechanoenzymatic and aqueous conditions, being pH 8.0 and pH 6.3, respectively. For most other enzymes studied under mechanoenzymatic conditions, the pH has not typically been shown to have a significant impact on enzyme activity so the pH effects under mechanoenzymatic conditions have not been extensively studied.[76,78,79] In the case where an acidic, water-soluble product is generated, as in the depolymerization of polylactic acid by a

cutinase (*Humicola insolens*), a similar pH effect was seen where the optimal pH under mechanoenzymatic conditions (10.0) was higher than in solution (7.3).[79,81] This is explained by the fact that the lactic acid product (pK_a = 3.86) causes acidification of the moist-solid mixture by dissolution into the small amount of liquid component, causing the pH to fall below the enzyme's working range.[81] Interestingly, reaction products that are not water soluble (*e.g.* terepthalic acid from the depolymerization of PET plastics), do not seem to affect the enzyme activity.[79] In contrast to polylactic acid, both the starting polymer, alginate, and the alginate oligomer products all contain carboxylic acid groups (pK_a = 3.38 and 3.65 for β-D-mannuronic acid and α-L-guluronic acid, respectively), but the products are more water soluble than the starting material, thus potentially affecting the pH to a greater extent.[95] This would explain the influence of pH on the activity of alginate lyase under mechanoenzymatic conditions. It would be interesting to confirm these explanations by measuring the pH of moist-solid mixtures as the reaction progresses, but no pH meter for solid samples were available at the time.

As for further parameters to be investigated, the milling conditions could be further explored by, for example, using another type of mill, such as a roller mill, varying the ratio of ball-to-powder (w/w), testing reaction jars and balls of different material, or by testing other RAging regimes.

RAging has been shown to significantly improve product formation in previous studies, both with biopolymers and synthetic polymers. It has proven to show a wide scope in increasing yields under mechanoenzymatic conditions. For example, simply adding a RAging regime into the mechanoenzymatic depolymerization of MCC into glucose increased conversion by 20%.[76] Similarly, mechanoenzymatic conversion of chitin to *N*-acetylglucosamine by chitinase was

improved by 50% following introduction of RAging.[78] In the depolymerization of PET on the other hand, RAging improved the rate of the conversion by decreasing the time taken to reach the maximum yield from 7 days to 3 days.[79] It is for these reasons that we feel further testing of RAging regimes for the mechanoenzymatic depolymerization of alginate could be beneficial in reaching the yields achieved in aqueous conditions and even surpassing its rate.

The different types of mills available for mechanochemistry offer access to different kinds of motion or impact which could potentially influence the efficiency of the reaction. A roller mill, as the name suggests, rolls the jar in a continuous motion in the same direction, unlike the ball (shaker) mill which shakes the jar laterally back and forth. Another option is a planetary mill which has a central axis around which the jar rotates, providing additional centrifugal force to the motion. These are all commonly employed in mechanochemistry depending on accessibility of the instruments and applications of the reactions so it would be worthwhile seeing if the type of mechanical motion has an influence on the depolymerization of alginate.

The ratio of the ball's mass to the mass of the reaction mixture can also influence the type and scale of impacts between the ball and reactants, and between the reactants themselves, hence contributing another level of variability to the overall reaction conditions. In a similar vein, the material of the ball and jar *i.e.* the hardness of the material can also be manipulated to change the level of force or impact the reactants experience during mixing. Materials like agate or Teflon are less dense but have very low risk of sample contamination, whereas stainless steel is a harder material but can experience leeching after extended use. Zirconia is very durable and does not hold a risk of sample contamination, hence its selection for this study.

Nonetheless, it is important to consider all variables which can be tested to further improve the yield and efficiency of this method for alginate depolymerization in future studies.

Notably, the respective product composition of the final reaction mixtures for the mechanoenzymatic and the traditional aqueous reactions remain to be studied in more depth to determine if they are comparable or complementary. UV-Vis analysis provided information about the general emergence of products, while GPC analysis provided a global picture of product distribution and size, yet the characterization study reported herein was limited due to a few factors. Firstly, the standards required for calibration testing are not commercially available. The unsaturated monomer, Δ (4-deoxy-l-*erythro*-4-hexenopyranouronate), is difficult to synthesize and isolate for use as a standard so instead, a structurally similar molecule was used for the UV analysis. 1-Hexene-1-carboxylic acid was selected because of its similarity to the monomeric product, including its cyclic structure, single unsaturation, its hydroxy group, and its lack of other functional groups. These properties allow it to absorb light at the selected wavelength to the same level as for the unsaturated monomers and oligomers produced during alginate depolymerization. The GPC analysis was limited because it was only performed in single replicate and by its inability to completely resolve oligomers of similar retention times, therefore preventing more precise Mw and Mn measurements. Typically, for more detailed characterization, the primary method of analysis for alginate depolymerization is high-performance liquid chromatography (HPLC), which allows the separation of the oligomers and determination of their DP and their respective proportion in the mixture. This method is dependent on having an appropriate column for separation, which was not available at the time of this study. Because of their large molecular weight and anionic nature, alginate

oligosaccharides are limited to separation by a small selection of columns.[96,97] In addition, complete characterization would also require oligomer standards of known lengths which are also not easily obtainable commercially or synthetically. To complete further analysis on the mechanoenzymatic product mixtures, we could attempt to develop an HPLC method adapted from the previous characterization of glucose or *N*-acetylglucosamine reported by our group.[76,78] Alginate oligosaccharides can also be analyzed by NMR to elucidate their monomer sequences, which is another characteristic of interest for the specific applications of alginate oligosaccharides.[98] Characteristic shifts in proton NMR are seen based on whether the protons are part of a mannuronic acid or guluronic acid, in addition to the identities of the neighboring acids. Using 2D NMR could help to create an overall depiction of the oligomer sequences.[99,100] This could be employed after separation by HPLC to fully characterize the products obtained through mechanoenzymatic depolymerization and to discern their ideal applications.

Because this is the first use of mechanoenzymology for the production of alginate oligosaccharides, there still lies a vast range of options to explore. The specificity of the enzymes in aqueous methods typically limits the scope of alginate oligosaccharide products that can be generated from a given alginate sample, and often a combination of alginate lyases are used to achieve specific distributions, or necessary to reach full conversion.[100,101] Alginate lyase enzymes have preferences for cleaving certain bonds so the product mixtures often show prevalence of certain monomer sequences and DPs over others. This mechanoenzymatic method introduces the aspects of mechanical force as well as the solvent-free conditions, altering the environment in which the enzyme is acting. It is possible that further manipulation of the mechanoenzymatic conditions could enable access to novel oligosaccharide compositions without the need for

multiple enzymes or enzyme engineering. Already, interesting results were seen with different product distributions observed between the traditional aqueous methods and the mechanoenzymatic method. This would make novel alginate oligosaccharides more accessible for important applications such as therapeutics. The application of alginate oligosaccharides in such a wide variety of industries makes the introduction of this new production method a very valuable contribution to this area of research.

3.2 Conclusion

We have presented herein the first enzymatic depolymerization of alginate by alginate lyase through mechanoenzymology. This method employs mechanical mixing by a ball mill to homogenize a solvent-free mixture of substrate and enzyme with only a minimal amount of water acting as lubricant, followed by static incubation to allow the generation of alginate oligosaccharides. Previously, mechanoenzymology has been successfully used for the hydrolysis of biopolymers such as cellulose[76], hemicellulose[77] and chitin[78], as well as synthetic polymers such as PET[79], PLA[81], and PEN[80], but its application to the non-hydrolytic breakdown of natural or synthetic polymers had yet to be demonstrated. No mechanoenzymatic lyase-catalyzed reactions had been reported thus far. Through methodical optimization of the key parameters, such as the liquid-to-solid ratio, milling duration, incubation temperature, and pH, among others, we have shown that subjecting alginate lyase to the mechanoenzymatic approach can successfully generate AOS in comparable yields as in typical aqueous enzymatic conditions. Although further product characterization is necessary, GPC was used to demonstrate that the distribution of products generated through the mechanoenzymatic method showed some similarities and

differences to that of conventional aqueous enzymatic reactions, and importantly, that the mechanoenzymatic reaction is capable of producing AOS of highly specific Mw and low PD.

As the strain on Earth's resources escalates daily, it is vital to adapt our methods to reduce their negative impact on the environment. The approach presented in this thesis adheres directly to this goal by working with a biodegradable, renewable and non-toxic catalyst, the enzyme alginate lyase, and by employing it in the presence of minimal water, therefore generating minimal wastewater. By eliminating the need for bulk water, the reaction mixture has a greatly reduced volume and can use smaller facilities. Furthermore, the mechanoenzymatic method requires no additional pre-treatment of any of the reaction components and proceeds with mild conditions through a very simple workflow. Mechanoenzymology is an emergent field, and the complete range of possibilities is yet untapped, however, this research presents a very promising way to streamline the generation of alginate oligosaccharides and expand their applications, in addition to providing an accessible route to a greener future.

Chapter 4 - Materials and Methods

4.1 Materials

The water used was either from an in-lab reverse osmose (RO) water tap, or MilliQ water from a Millipore MilliQ system (specific resistance of ≤ 18.2 MΩ·cm at 25°C).

The sodium alginate powder (low viscosity, from brown algae, M:G ratio = 1.56) was purchased from Millipore Sigma (Oakville, ON, Canada), stored at 4°C and used as purchased. The alginate lyase enzyme was obtained as a lyophilized powder from Millipore Sigma (Oakville, ON, Canada) and was used as purchased. The enzyme originates from *Flavobacterium sp.* and is polyM-selective. The protein content of the commercial mixture was determined to be 18 ± 2% w/w by Bradford assay, and its molecular weight was confirmed by SDS-PAGE (expected ~40,000 kDa as noted by the manufacturer). The enzyme activity was verified using the method described in the section *Enzyme Characterization* from the manufacturer protocol.

The 0.1 M Na_2PO_4 buffers (pH 6.3, 7.0, 8.0) were prepared with the appropriate amounts of solid sodium phosphate monobasic and sodium phosphate dibasic, both purchased from Millipore Sigma (Oakville, ON, Canada). The 0.1 M Na_2CO_3 buffer (pH 10.0) was prepared using sodium carbonate and sodium bicarbonate from Thermo Fisher Scientific (Waltham, MA, US). The 0.1 M CH_3COONa buffer (pH 5.0) was prepared with sodium acetate and acetic acid from Millipore Sigma (Oakville, ON, Canada) and Thermo Fisher Scientific (Waltham, MA, US), respectively. MilliQ water was used for all of the above preparations.

The 1-cyclohexene-1-carboxylic acid used for the monomer calibration curve was purchased from Thermo Fisher Scientific (Waltham, MA, US). Reagents used for the 3,5-dinitrosalicylic acid (DNS)

calibration and assay (potassium sodium tartrate tetrahydrate, DNS, and sodium hydroxide), were all purchased from Millipore Sigma (Oakville, ON, Canada).

The Coomassie Blue solution was prepared by dissolving 0.25 g Coomassie Brilliant Blue G-250 (Waltham, MA, US) in 90 mL of methanol:H_2O (1:1, v/v).

4.2 Equipment

Samples were weighed either on a Mettler Toledo AB135-S/FACT DualRange analytical balance (linearity 0.2 mg, readability 0.01 mg/0.1 mg) or a Mettler Toledo XP105 DeltaRange analytical balance (linearity 0.15 mg, readability 0.01 mg/0.1 mg). Ball milling was performed using a Retsch MM 200 shaker mill from ATS Scientific, set to shake at frequencies of 5-25 Hz and equipped with two 10 mL zirconia SmartSnap^{TM} jars each containing one 10 mm zirconia ball, all purchased from FormTech Scientific. Static incubation of reaction mixtures was performed in a Thermo Scientific Heratherm incubator set to the desired reaction temperature (25-65°C). Product formation for both dilute aqueous and moist-solid mechanoenzymatic experiments was monitored using an Agilent Cary 60 UV-Vis spectrometer with a PCB 1500 water Peltier system accessory. Absorbance measurements were taken at a wavelength of 235 nm. Absorbance measurements for the Bradford assay and DNS calibration were conducted on a Molecular Devices SpectraMax i3x microplate reader equipped with clear-bottom 96-well microtiter plates. Lyophilization of samples for GPC analysis was achieved using a Labconco FreeZone 1 Liter Benchtop Freeze Dry System. GPC measurements were taken on an Agilent 1260 Infinity II GPC system equipped with a Shodex OHpak SB-G 6B (6 x 50 mm,10 µm) guard column, a Shodex OHpak LB 806M HQ (8 x

300 mm, 13 µm) analytical column and an Agilent 1260 Infinity dual, light scattering (LS) and refractive index (RI), detector.

4.3 Moist-solid Mechanoenzymatic Reactions

4.3.1 Method for MAging (milling + aging) reactions

The method for the optimized MAging conditions is described here. Sodium alginate powder (150 mg) was weighed into a 15 mL zirconia jar. To achieve an η of 1.5 µL/mg, 0.33 mg of the commercial enzyme powder (0.04% w/w enzyme:substrate loading) was dissolved in 225 µL of sodium phosphate buffer (0.1 M, pH 8.0) and added to the substrate. Control experiments were prepared the same way but without enzyme. One zirconia ball of 10 mm was added to the jar and the jar was closed and fixed to the ball mill before milling for 5 minutes at 25 Hz. The moist-solid reaction mixture was then aliquoted (~25-35 mg) by spatula into 2 mL Eppendorf tubes and then incubated statically at 37°C for 1, 2, 3, 6, 9, 12, 15, or 24 hours. A time zero sample was quenched by boiling for 5 minutes then subjected to the quantification procedure (see section 4.5.1), immediately after being aliquoted. The remaining reaction mixtures were rapidly quenched to deactivate the enzyme at the appropriate time points before quantification of the depolymerization products (see section 4.5.1). All experiments were conducted in triplicate.

4.3.2 Method for MAging (milling + aging) reactions in the presence of the solid additive PEG

Sodium alginate powder (100 mg) was weighed into a 15 mL zirconia jar with 50 mg of PEG powder (Millipore Sigma, Oakville, ON, Canada). To achieve an η of 1.5 µL/mg, 0.22 mg of the commercial enzyme powder (0.04% w/w enzyme:substrate loading) was dissolved in 225 µL of sodium phosphate buffer (0.1 M, pH 6.3) and added to the substrate. Control experiments were

prepared the same way but without enzyme. The remainder of the experiment proceeded as per the standard MAging method above (section 4.3.1).

4.3.3 Method for RAging (reactive aging) reactions

Sodium alginate powder (150 mg) was weighed into a 15 mL zirconia jar. To achieve an η of 1.5 µL/mg, 0.33 mg of the commercial enzyme powder (0.04% w/w enzyme:substrate loading) was dissolved in 225 µL of sodium phosphate buffer (0.1 M, pH 8.0) and added to the substrate. For control replicates, 225 µL of just buffer was added without enzyme. A 10 mm zirconia ball was added to the jar and the jar was fixed to the ball mill and set to shake at 25 Hz for 5 minutes. Three aliquots of the mixture (~25-35 mg) were added to 2 mL Eppendorf tubes using a spatula (time zero samples) before rapidly quenching the reaction and quantifying the product (see section 4.5.1). The jar was then sealed with electrical tape and then incubated at 37°C for 6 hours. The reaction mixture was then milled for another 5 minutes at 25 Hz before collecting aliquots (~25-35 mg), rapidly quenching the reaction and quantifying the products (section 4.5.1). The jar was then re-sealed with electrical tape and incubated at 37°C for an additional 18 hours (totalling 24 hours from the initial milling). After the final aging period, more aliquots were collected (~25-35 mg), before quenching the reaction and analyzing the products (section 4.5.1). The experiments were conducted in triplicate.

4.3.4 Method for MAging (milling + aging) reactions to determine reaction limiting components

Sodium alginate powder (150 mg) was weighed into a 15 mL zirconia jar. To achieve an η of 1.5 µL/mg, 0.33 mg of the commercial enzyme powder was dissolved in 225 µL of sodium phosphate buffer (0.1 M, pH 8.0) and added to the substrate. Control experiments were prepared the same

way but without enzyme. One zirconia ball of 10 mm was added to the jar and the jar was fixed to the ball mill and set to shake at 25 Hz for 5 minutes. The moist-solid reaction mixture was then aliquoted (~25-35 mg) by spatula into 4 x 2 mL Eppendorf tubes. The control reaction mixture was also aliquoted (3 x 2 mL Eppendorf tubes, ~25-35 mg each). One of each the enzyme-containing and control tubes were immediately subjected to the oligosaccharide quantification procedure (section 4.5.1) to obtain data at time 0. The remaining tubes were incubated at 37°C for 6 hours. After 6 hours, one of the enzyme-containing tubes was boiled for 5 minutes to deactivate the enzyme before product quantification. The contents of the 3 reaction tubes and 2 control tubes were used to prepare 1 mg/mL solutions (based on total alginate content) in sodium phosphate buffer (0.1 M, pH 8.0). To one control reaction and one reaction mixture, 100 µL of 10.0 mg/mL alginate solution (in 0.1 M sodium phosphate buffer at pH 8.0) was added. To one reaction mixture, 100 µL of 1.0 mg/mL of enzyme solution. To the remaining control and reaction mixtures, 100 µL of sodium phosphate buffer (0.1 M, pH 8.0) was added. The enzymatic reaction was monitored at 235 nm and 37°C (for all mixtures) over an additional 18 hours. The absorbance of the control reaction with additional alginate was subtracted from the absorbance of the enzymatic reaction mixture with additional alginate before product quantification. For product quantification of the reaction mixtures with additional enzyme and that with additional buffer, the absorbance of the reaction mixture with additional buffer was subtracted.

4.4 Method for enzyme reactions under traditional aqueous conditions

A sodium alginate solution (1 mg/mL) was prepared in sodium phosphate buffer (0.1 M) at the desired pH. A solution of the commercial alginate lyase enzyme (0.1 mg/mL) was also prepared in the same buffer. Some of the alginate solution (450 µL) was added into a masked quartz

cuvette, followed by some of the enzyme solution (10 µL) before gently mixing using a pipettor. Immediately after, the absorbance was measured continuously across the course of the reaction at a constant temperature of 37°C (or other designated temperature). The absorbance reading was used to quantify the products based on the 1-cyclohexene-1-carboxylic acid calibration curve (see section 4.6.1). All experiments were conducted in triplicate.

4.5 Characterization of Oligosaccharide Products

4.5.1 Quantification of alginate oligosaccharide products of mechanoenzymatic reactions by UV-Vis spectroscopy

To quench mechanoenzymatic reactions, 2 mL of RO water was added to each Eppendorf tube containing a pre-weighed moist-solid reaction mixture and the tubes were immediately immersed in boiling water for 5 minutes. To determine the mass of alginate and/or alginate oligosaccharides in the tube, the portion of the sample's mass composed of the buffer additive (60% w/w) was subtracted from the total mass of mixture. The content of each Eppendorf was then further diluted in vials with RO water to a concentration of 1 mg/mL (relative to the solids), accounting for the 2 mL of water already in the tube. The absorbance of the resulting solution was measured by UV-Vis spectroscopy at a wavelength of 235 nm. The absorbance of the control reactions without enzyme were subtracted from the absorbance of each reaction mixture, and the double bond concentration (one double bond is generated for each lyase reaction) was determined based on the 1-cyclohexene-1-carboxylic acid calibration curve (described in section 4.6.1).

Reaction samples prepared with the optimized conditions were also subjected to the DNS assay (see section 4.5.2) to validate the above product quantification method.

The % reaction yields were then calculated using the product concentration from these measurements and the theoretical yield calculated as described in section 4.5.3.

4.5.2 Quantification of alginate oligosaccharide products of mechanoenzymatic reactions by the DNS assay

Some of the DNS reagent solution (96 µL; see section 4.6.2.1) was transferred into a 2 mL Eppendorf, followed by a sample of the solution (128 µL) prepared from the moist-solid reaction as described in section 4.5.1. The Eppendorf tube was next immersed in boiling water for 10 minutes. Once they had cooled down to room temperature, RO water (1.38 mL) was added to the tube before inverting the tube a few times to ensure homogeneity. Some of this solution (400 µL) was transferred into a well of a 96-well plate and the absorbance was measured at 540 nm. The concentration was calculated from the DNS calibration curve presented in section 4.6.2: *Calibration curve preparation.* All reactions were prepared and measured in triplicate.

4.5.3 Approximate calculation of the theoretical yield

For the calculation of theoretical yield, the sodium alginate was first considered as an infinitely long chain of mannuronic (M) and guluronic (G) acid monomers. Using the molecular weight of the monomer unit (199.11 g/mol) and the mass of a molecule of monomer ($3.306 \cdot 10^{-22}$ g/molecule), the maximum yield of monomers possible from the complete depolymerization of the initial 0.150 g sample can be calculated as $4.537 \cdot 10^{20}$ molecules, or $7.534 \cdot 10^{-4}$ moles of monomer. This is, however, an overestimate of theoretical yield based on the following factors: 1) this method assumes complete depolymerization of the polymer, which is highly unlikely using this singular commercial enzyme that can only break bonds between two M monomers; 2) the

polymer used contains both M and G residues, and therefore MM, GG, and MG bonds, but the enzyme only acts on MM bonds; 3) the substrate is not composed of a singular infinitely long chain, but instead many polymers of varying lengths. To account for the third point, the approximate molar amount of polymer chains in the 0.150 g sample was calculated using Mw from the GPC measurement (167,000 g/mol), giving $8.982 \cdot 10^{-7}$ moles. This was subtracted from the $7.534 \cdot 10^{-4}$ moles to adjust for the infinite chain assumption, giving $7.525 \cdot 10^{-4}$ moles. The difference between the calculations with alginate as a single molecule or as a polymer of 167,000 g/mol is therefore considered negligible. To account for the fact that the enzyme only acts on MM bonds in the polymer, considering that the commercial alginate is labelled as containing a M:G ratio of 1.56, which translated to approximately 60% M, the $7.525 \cdot 10^{-4}$ moles were then multiplied by the 0.6. This gives $4.515 \cdot 10^{-4}$ moles. Considering that a reaction mixture of 0.150 g was diluted by 0.150 L to achieve the 1 mg/mL concentration for analysis (in sections 4.3.4 and 4.5.1), this equates to a concentration of 3.01 mM as the theoretical yield. However, not all M monomers are adjacent to another M unit, and since MG bonds are not cleaved by this enzyme, this calculation still underestimates the possible theoretical yield for this enzyme. Therefore, all percent yields reported herein should be considered largely underestimated.

4.5.4 GPC characterization of alginate polymers and oligosaccharides

For GPC analyses, experimental reaction mixtures or unreacted alginate were lyophilized overnight. The solid samples were then used to prepare aqueous solutions with a concentration of 1 mg/mL in 200 mM $NaNO_3$ and 10 mM sodium phosphate. They were allowed to dissolve overnight at room temperature, before filtration with 0.22 µm filters and transfer into autosampler vials. Standard solutions of pullulan ranging from 180 g/mol to 404,000 g/mol were

prepared by the same method. They were injected (100 µL) and analyzed at a flow rate of 0.4 mL/mL and at 40°C. A calibration curve was generated from the pullulan standards and used to calculate the weight average molecular weight (Mw), the number average molecular weight (Mn), and the polydispersity (PD) of the polymer and oligosaccharide samples.

4.6 Calibration Curve Preparations

4.6.1 Preparation of the calibration curve for 1-cyclohexene-1-carboxylic acid

The calibration curve for calculation of double bond concentration (each enzymatic reaction produces one double bond) was prepared using the commercially available 1-cyclohexene-1-carboxylic acid, which is expected to absorb UV/Vis light like the alginate monomer or oligomers. Standard solutions ranging in concentration from 0-1.0 mM were prepared by serial dilution of a 2.0 mM stock solution of 1-cyclohexene-1-carboxylic acid dissolved in phosphate buffer (0.1 M, pH 7.0). The absorbance of each standard solution was measured at 235 nm and plotted against the concentrations as shown in Figure 4.1 below. Each standard was prepared and measured in triplicate.

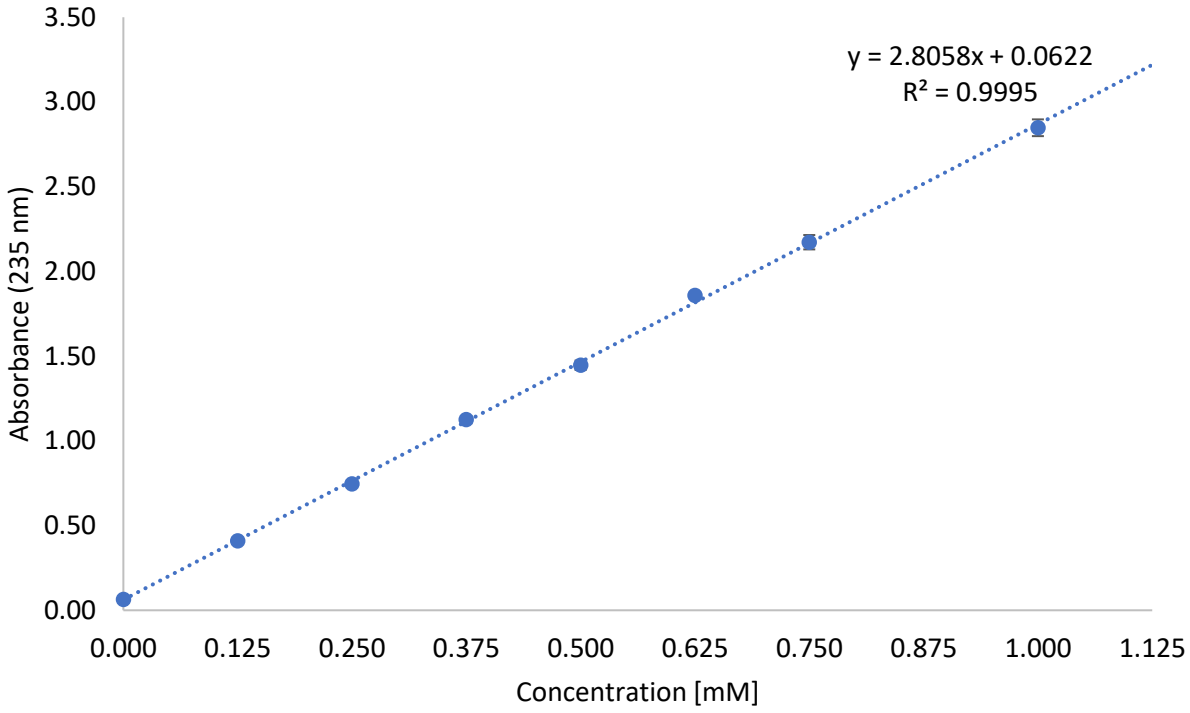

Figure 4.1: Calibration curve for the calculation of alginate oligosaccharide double bond concentration by UV/Vis spectroscopy. The curve was prepared from standards of 1-cyclohexene-1-carboxylic acid. The data shows the means with error bars representing the standard deviation for measurements performed in triplicate.

4.6.2 Preparation of the calibration curve for the DNS assay

The calibration curve for the DNS assay was constructed using standards of glucose dissolved in sodium phosphate buffer (0.1 M, pH 7.0) ranging in concentrations from 0-1.0 mM. Some of the DNS reagent solution (96 µL; see section 4.6.2.1) was first transferred into a 2 mL Eppendorf, followed by a known amount (128 µL) of a glucose standard solution. The Eppendorf tube was next immersed in boiling water for 10 minutes. Once the mixtures had cooled to room temperature, RO water (1.38 mL) was added to the tubes before inverting them a few times to ensure homogenous dilution. Some of each standard (400 µL) was next transferred into a 96-well plate. The absorbance was measured at 540 nm and plotted against concentration as shown in Figure 4.2 below. All standards were prepared and measured in triplicate.

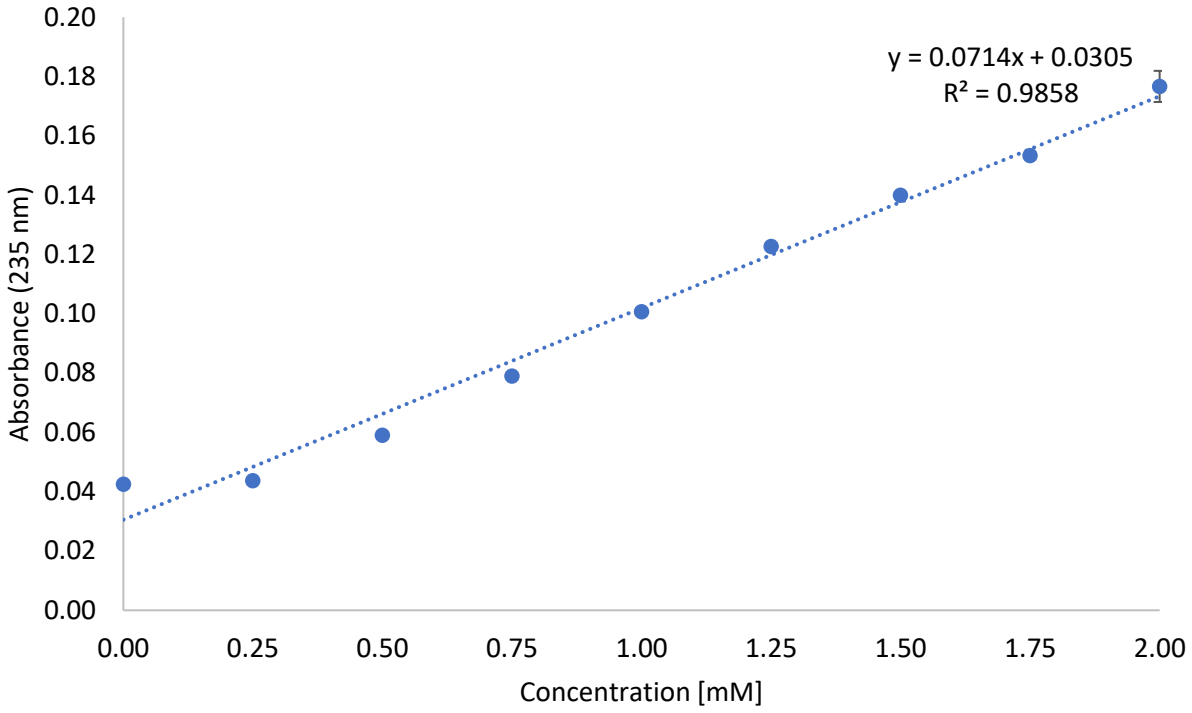

Figure 4.2: Calibration curve for quantifying the reducing ends of alginate oligosaccharides. Standards of glucose dissolved in sodium phosphate buffer (0.1 M, pH 7.0) were used at different concentrations. The data shows the means with error bars representing the standard deviation for measurements performed in triplicate.

4.6.2.1 DNS Reagent Preparation

DNS (1 g) was dissolved into 50 mL of MilliQ water. Sodium potassium tartrate tetrahydrate (30 g) was introduced to the DNS solution in small portions while stirring continuously. A sodium hydroxide solution (20 mL, 2 M) was added. Next, the mixture was pipetted into a 100 mL volumetric flask and RO water was added to the 100 mL mark. The mixture was stirred for 10 minutes then filtered through cotton into an amber bottle and stored at 4°C.

4.7 Enzyme Characterization

4.7.1 Protein content of the commercial alginate lyase

Total protein concentration of the commercial enzyme powder was determined by Bradford assay. The commercial Pierce™ Bovine Serum Albumin Standard Ampule (2 mg/mL) was used to prepare the protein standards at concentrations of 0-2000 µg/mL in sodium phosphate buffer (0.1 M, pH 7.3). An aliquot of each (10 µL) was mixed with Coomassie blue reagent (300 µL) in a well of a 96-well plate and left standing for 10 minutes before measurement. The plate was shaken for 30 seconds in the plate reader before measuring the absorbance at 595 nm. The absorbance was plotted as a function of the concentration to give the calibration curve of Figure 4.3. Triplicate samples of enzyme solution (1 mg/mL) were subjected to the same treatment and the protein content was calculated from the standard curve.

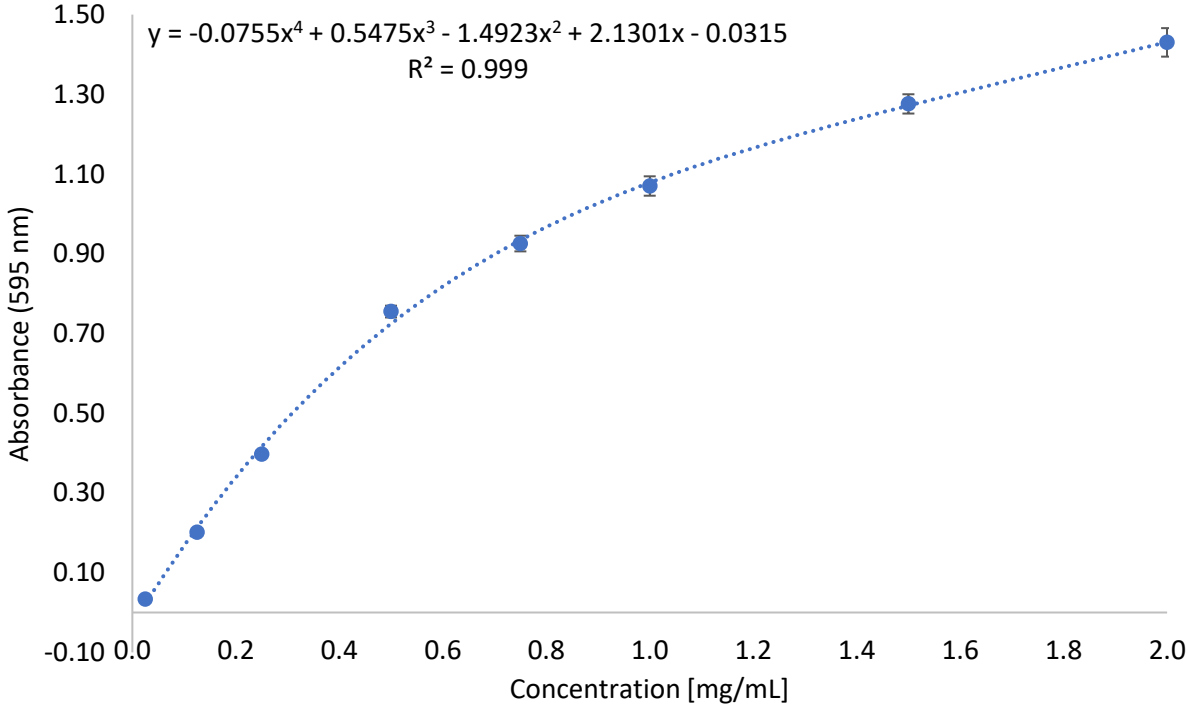

Figure 4.3: Calibration curve of bovine albumin serum standards for the calculation of protein content in commercial alginate lyase. The data shows the means with error bars representing the standard deviation for measurements performed in triplicate.

4.7.2 Confirmation that the commercial alginate lyase is active

The activity assay was performed by preparing a solution of the sodium alginate powder in phosphate buffer (0.1 M, pH 6.3) to a concentration of 1 mg/mL (0.1% w/v) and a solution of the alginate lyase powder to 0.03 mg/mL (~ 1 U/mL). In a quartz cuvette, 450 µL of the sodium alginate solution was pipetted followed by 15 µL of the enzyme solution. The absorbance was measured continuously at 235 nm and at 37°C for 30 minutes.

4.7.3 SDS-PAGE analysis of the commercial alginate lyase

SDS-PAGE was performed to confirm the identity and analyse the purity of the commercial alginate lyase. Enzyme solutions (10 µL of ~4 µM, ~2 µM, and ~1 µM) in sodium phosphate buffer (0.1 M, pH 7.0) were each mixed with 4x Laemmli buffer (15 µL, 0.25 M Tris base, 0.28 M SDS, 40% v/v glycerol, and 20% v/v β-mercaptoethanol). The samples were boiled for 10 minutes, and then a sample of each (10 µL) was loaded onto a 4-20% mini-PROTEAN® TGXTM 12-well pre-cast protein gel (Biorad). Some (4 µL) Thermo Scientific™ PageRuler™ Prestained Protein Ladder (10 to 180 kDa) was loaded into the first well. Electrophoresis was conducted at 120 V for 1.5 h before staining with the Coomassie blue solution (see section 4.1) for 20 minutes with gentle shaking. Two rounds of 30 minutes destaining were performed with destaining solution (50% v/v methanol, 10% v/v acetic acid, and 40% v/v MilliQ). Finally, the gel was left in MilliQ water overnight to remove excess dye before imaging with a Bio-Rad ChemiDoc MP imaging system (Hercules, California, USA).

References

(1) FAO. *The State of World Fisheries and Aquaculture 2024: Blue Transformation in Action*; Food & Agriculture Org.: Rome, Italy, 2024.

(2) Zhang, L.; Liao, W.; Huang, Y.; Wen, Y.; Chu, Y.; Zhao, C. Global Seaweed Farming and Processing in the Past 20 Years. *Food Prod. Process. Nutr.* **2022**, *4* (1), 23. https://doi.org/10.1186/s43014-022-00103-2.

(3) Horn, S. J.; Moen, E.; Østgaard, K. Direct Determination of Alginate Content in Brown Algae by near Infra-Red (NIR) Spectroscopy. *J. Appl. Phycol.* **1999**, *11* (1), 9–13. https://doi.org/10.1023/A:1008024009954.

(4) Chowdhury, S.; Chakraborty, S.; Maity, M.; Hasnain, M. S.; Nayak, A. K. Chapter 7 - Biocomposites of Alginates in Drug Delivery. In *Alginates in Drug Delivery*; Nayak, A. K., Hasnain, M. S., Eds.; Academic Press, 2020; pp 153–185. https://doi.org/10.1016/B978-0-12-817640-5.00007-8.

(5) Rhim, J.-W. Physical and Mechanical Properties of Water Resistant Sodium Alginate Films. *LWT - Food Sci. Technol.* **2004**, *37* (3), 323–330. https://doi.org/10.1016/j.lwt.2003.09.008.

(6) Ren, Y.; Wang, Q.; Xu, W.; Yang, M.; Guo, W.; He, S.; Liu, W. Alginate-Based Hydrogels Mediated Biomedical Applications: A Review. *Int. J. Biol. Macromol.* **2024**, *279*, 135019. https://doi.org/10.1016/j.ijbiomac.2024.135019.

(7) Abka-khajouei, R.; Tounsi, L.; Shahabi, N.; Patel, A. K.; Abdelkafi, S.; Michaud, P. Structures, Properties and Applications of Alginates. *Mar. Drugs* **2022**, *20* (6), 364. https://doi.org/10.3390/md20060364.

(8) Saji, S.; Hebden, A.; Goswami, P.; Du, C. A Brief Review on the Development of Alginate Extraction Process and Its Sustainability. *Sustainability* **2022**, *14* (9), 5181. https://doi.org/10.3390/su14095181.

(9) Lee, K. Y.; Mooney, D. J. Alginate: Properties and Biomedical Applications. *Prog. Polym. Sci.* **2012**, *37* (1), 106–126. https://doi.org/10.1016/j.progpolymsci.2011.06.003.

(10) Liu, J.; Yang, S.; Li, X.; Yan, Q.; Reaney, M. J. T.; Jiang, Z. Alginate Oligosaccharides: Production, Biological Activities, and Potential Applications. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18* (6), 1859–1881. https://doi.org/10.1111/1541-4337.12494.

(11) Wang, M.; Chen, L.; Zhang, Z. Potential Applications of Alginate Oligosaccharides for Biomedicine – A Mini Review. *Carbohydr. Polym.* **2021**, *271*, 118408. https://doi.org/10.1016/j.carbpol.2021.118408.

(12) Mackie, W.; Perez, S.; Rizzo, R.; Taravel, F.; Vignon, M. Aspects of the Conformation of Polyguluronate in the Solid State and in Solution. *Int. J. Biol. Macromol.* **1983**, *5* (6), 329–341. https://doi.org/10.1016/0141-8130(83)90056-9.

(13) Tomlins, P. 1 - Material Types for Tissue Scaffolds. In *Characterisation and Design of Tissue Scaffolds*; Tomlins, P., Ed.; Woodhead Publishing Series in Biomaterials; Woodhead Publishing, 2016; pp 1–21. https://doi.org/10.1016/B978-1-78242-087-3.00001-8.

(14) Datta, N.; Jinan, T.; Wong, S. Y.; Chakravarty, S.; Li, X.; Anwar, I.; Arafat, M. T. Self-Assembled Sodium Alginate Polymannuronate Nanoparticles for Synergistic Treatment of Ophthalmic Infection and Inflammation: Preparation Optimization and *in Vitro*/*Vivo* Evaluation. *Int. J. Biol. Macromol.* **2024**, *262*, 130038. https://doi.org/10.1016/j.ijbiomac.2024.130038.

(15) Lu, S.; Na, K.; Wei, J.; Zhang, L.; Guo, X. Alginate Oligosaccharides: The Structure-Function Relationships and the Directional Preparation for Application. *Carbohydr. Polym.* **2022**, *284*, 119225. https://doi.org/10.1016/j.carbpol.2022.119225.

(16) Wang, X.; Sun, G.; Feng, T.; Zhang, J.; Huang, X.; Wang, T.; Xie, Z.; Chu, X.; Yang, J.; Wang, H.; Chang, S.; Gong, Y.; Ruan, L.; Zhang, G.; Yan, S.; Lian, W.; Du, C.; Yang, D.; Zhang, Q.; Lin, F.; Liu, J.; Zhang,

H.; Ge, C.; Xiao, S.; Ding, J.; Geng, M. Sodium Oligomannate Therapeutically Remodels Gut Microbiota and Suppresses Gut Bacterial Amino Acids-Shaped Neuroinflammation to Inhibit Alzheimer's Disease Progression. *Cell Res.* **2019**, *29* (10), 787–803. https://doi.org/10.1038/s41422-019-0216-x.

(17) Hengzhuang, W.; Song, Z.; Ciofu, O.; Onsøyen, E.; Rye, P. D.; Høiby, N. OligoG CF-5/20 Disruption of Mucoid Pseudomonas Aeruginosa Biofilm in a Murine Lung Infection Model. *Antimicrob. Agents Chemother.* **2016**, *60* (5), 2620–2626. https://doi.org/10.1128/aac.01721-15.

(18) Powell, L. C.; Pritchard, M. F.; Emanuel, C.; Onsøyen, E.; Rye, P. D.; Wright, C. J.; Hill, K. E.; Thomas, D. W. A Nanoscale Characterization of the Interaction of a Novel Alginate Oligomer with the Cell Surface and Motility of Pseudomonas Aeruginosa. *Am. J. Respir. Cell Mol. Biol.* **2014**, *50* (3), 483–492. https://doi.org/10.1165/rcmb.2013-0287OC.

(19) Powell, L. C.; Sowedan, A.; Khan, S.; Wright, C. J.; Hawkins, K.; Onsøyen, E.; Myrvold, R.; Hill, K. E.; Thomas, D. W. The Effect of Alginate Oligosaccharides on the Mechanical Properties of Gram-Negative Biofilms. *Biofouling* **2013**, *29* (4), 413–421. https://doi.org/10.1080/08927014.2013.777954.

(20) Khan, S.; Tøndervik, A.; Sletta, H.; Klinkenberg, G.; Emanuel, C.; Onsøyen, E.; Myrvold, R.; Howe, R. A.; Walsh, T. R.; Hill, K. E.; Thomas, D. W. Overcoming Drug Resistance with Alginate Oligosaccharides Able To Potentiate the Action of Selected Antibiotics. *Antimicrob. Agents Chemother.* **2012**, *56* (10), 5134. https://doi.org/10.1128/AAC.00525-12.

(21) Zhang, C.; Wang, W.; Zhao, X.; Wang, H.; Yin, H. Preparation of Alginate Oligosaccharides and Their Biological Activities in Plants: A Review. *Carbohydr. Res.* **2020**, *494*, 108056. https://doi.org/10.1016/j.carres.2020.108056.

(22) Iwasaki, K.; Matsubara, Y. Purification of Alginate Oligosaccharides with Root Growth-Promoting Activity toward Lettuce. *Biosci. Biotechnol. Biochem.* **2000**, *64* (5), 1067–1070. https://doi.org/10.1271/bbb.64.1067.

(23) Natsume, M.; Kamo, Y.; Hirayama, M.; Adachi, T. Isolation and Characterization of Alginate-Derived Oligosaccharides with Root Growth-Promoting Activities. *Carbohydr. Res.* **1994**, *258*, 187–197. https://doi.org/10.1016/0008-6215(94)84085-7.

(24) Tomoda, Y.; Umemura, K.; Adachi, T. Promotion of Barley Root Elongation under Hypoxic Conditions by Alginate Lyase-Lysate (A.L.L.). *Biosci. Biotechnol. Biochem.* **1994**, *58* (1), 202–203. https://doi.org/10.1271/bbb.58.202.

(25) El-Mohdy, H. L. A. Radiation-Induced Degradation of Sodium Alginate and Its Plant Growth Promotion Effect. *Arab. J. Chem.* **2017**, *S1* (10), S431–S438. https://doi.org/10.1016/j.arabjc.2012.10.003.

(26) Hu, X.; Jiang, X.; Hwang, H.; Liu, S.; Guan, H. Promotive Effects of Alginate-Derived Oligosaccharide on Maize Seed Germination. *J. Appl. Phycol.* **2004**, *16* (1), 73–76. https://doi.org/10.1023/B:JAPH.0000019139.35046.0c.

(27) Luan, L. Q.; Ha, V. T. T.; Uyen, N. H. P.; Trang, L. T. T.; Hien, N. Q.; Luan, L. Q.; Ha, V. T. T.; Uyen, N. H. P.; Trang, L. T. T.; Hien, N. Q. Preparation of Oligoalginate Plant Growth Promoter by γ Irradiation of Alginate Solution Containing Hydrogen Peroxide. *J. Agric. Food Chem.* **2012**, *60* (7), 1737–1741. https://doi.org/10.1021/jf204469p.

(28) Xu, X.; Bi, D.; Wan, M. Characterization and Immunological Evaluation of Low-Molecular- Weight Alginate Derivatives. *Curr. Top. Med. Chem.* **2016**, *16* (8), 874–887. https://doi.org/10.2174/1568026615666150827101239.

(29) Chandía, N. P.; Matsuhiro, B.; Vásquez, A. E. Alginic Acids in *Lessonia Trabeculata*: Characterization by Formic Acid Hydrolysis and FT-IR Spectroscopy. *Carbohydr. Polym.* **2001**, *46* (1), 81–87. https://doi.org/10.1016/S0144-8617(00)00286-1.

(30) Haug, A.; Larsen, B. A Study on the Constitution of Alginic Acid by Partial Acid Hydrolysis. In *Proceedings of the Fifth International Seaweed Symposium, Halifax, August 25–28, 1965*; Young, E. G., McLachlan, J. L., Eds.; Pergamon, 1966; pp 271–277. https://doi.org/10.1016/B978-0-08-011841-3.50043-4.
(31) Ariyo, B. T.; Bucke, C.; Keshaverz, T. Alginate Oligosaccharides as Enhancers of Penicillin Production in Cultures of Penicillium Chrysogenum. *Biotechnol. Bioeng.* **1997**, *53* (1), 17–20. https://doi.org/10.1002/(SICI)1097-0290(19970105)53:1<17::AID-BIT3>3.0.CO;2-1.
(32) Chandía, N. P.; Matsuhiro, B.; Mejías, E.; Moenne, A. Alginic Acids in Lessonia Vadosa: Partial Hydrolysis and Elicitor Properties of the Polymannuronic Acid Fraction. *J. Appl. Phycol.* **2004**, *16* (2), 127–133. https://doi.org/10.1023/B:JAPH.0000044778.44193.a8.
(33) Wang, W.; Fu, Y.; Xu, J.; Gao, X.; Fu, X.; Wang, L. Optimization of Hydrolysis Conditions of Alginate Based on High Performance Liquid Chromatography. *Heliyon* **2024**, *10* (9). https://doi.org/10.1016/j.heliyon.2024.e29738.
(34) Noach, M. N.; Mampana, R. L.; Rensburg, E. V.; Goosen, N. J.; Pott, R. W. M. Chemical and Enzymatic Hydrolysis of Alginate: A Review. *Bot. Mar.* **2024**, *67* (5), 487–511. https://doi.org/10.1515/bot-2023-0056.
(35) Mao, S.; Zhang, T.; Sun, W.; Ren, X. The Depolymerization of Sodium Alginate by Oxidative Degradation. *Pharm. Dev. Technol.* **2012**, *17* (6), 763–769. https://doi.org/10.3109/10837450.2011.583927.
(36) Yang, Z.; Li, J.-P.; Guan, H. Preparation and Characterization of Oligomannuronates from Alginate Degraded by Hydrogen Peroxide. *Carbohydr. Polym.* **2004**, *58* (2), 115–121. https://doi.org/10.1016/j.carbpol.2004.04.022.
(37) Li, X.; Xu, A.; Xie, H.; Yu, W.; Xie, W.; Ma, X. Preparation of Low Molecular Weight Alginate by Hydrogen Peroxide Depolymerization for Tissue Engineering. *Carbohydr. Polym.* **2010**, *79* (3), 660–664. https://doi.org/10.1016/j.carbpol.2009.09.020.
(38) Iwamoto, M.; Kurachi, M.; Nakashima, T.; Kim, D.; Yamaguchi, K.; Oda, T.; Iwamoto, Y.; Muramatsu, T. Structure-Activity Relationship of Alginate Oligosaccharides in the Induction of Cytokine Production from RAW264.7 Cells. *FEBS Lett.* **2005**, *579* (20), 4423–4429. https://doi.org/10.1016/j.febslet.2005.07.007.
(39) Li, S.; He, N.; Wang, L. Efficiently Anti-Obesity Effects of Unsaturated Alginate Oligosaccharides (UAOS) in High-Fat Diet (HFD)-Fed Mice. *Mar. Drugs* **2019**, *17* (9), 540. https://doi.org/10.3390/md17090540.
(40) Li, L.; Jiang, J.; Yao, Z.; Zhu, B. Recent Advances in the Production, Properties and Applications of Alginate Oligosaccharides - a Mini Review. *World J. Microbiol. Biotechnol.* **2023**, *39* (8), 207. https://doi.org/10.1007/s11274-023-03658-5.
(41) Lee, D. W.; Choi, W. S.; Byun, M. W.; Park, H. J.; Yu, Y.-M.; Lee, C. M. Effect of γ-Irradiation on Degradation of Alginate. *J. Agric. Food Chem.* **2003**, *51* (16), 4819–4823. https://doi.org/10.1021/jf021053y.
(42) Nagasawa, N.; Mitomo, H.; Yoshii, F.; Kume, T. Radiation-Induced Degradation of Sodium Alginate. *Polym. Degrad. Stab.* **2000**, *69* (3), 279–285. https://doi.org/10.1016/S0141-3910(00)00070-7.
(43) Hien, N. Q.; Nagasawa, N.; Tham, L. X.; Yoshii, F.; Dang, V. H.; Mitomo, H.; Makuuchi, K.; Kume, T. Growth-Promotion of Plants with Depolymerized Alginates by Irradiation. *Radiat. Phys. Chem.* **2000**, *59* (1), 97–101. https://doi.org/10.1016/S0969-806X(99)00522-8.
(44) Luan, L. Q.; Nagasawa, N.; Ha, V. T. T.; Hien, N. Q.; Nakanishi, T. M. Enhancement of Plant Growth Stimulation Activity of Irradiated Alginate by Fractionation. *Radiat. Phys. Chem.* **2009**, *78* (9), 796–799. https://doi.org/10.1016/j.radphyschem.2009.05.001.

(45) Hu, T.; Li, C.; Zhao, X.; Li, G.; Yu, G.; Guan, H. Preparation and Characterization of Guluronic Acid Oligosaccharides Degraded by a Rapid Microwave Irradiation Method. *Carbohydr. Res.* **2013**, *373*, 53–58. https://doi.org/10.1016/j.carres.2013.03.014.
(46) Bi, D.; Yang, X.; Lu, J.; Xu, X. Preparation and Potential Applications of Alginate Oligosaccharides. *Crit. Rev. Food Sci. Nutr.* **2023**, *63* (29), 10130–10147. https://doi.org/10.1080/10408398.2022.2067832.
(47) Xu, X.; Wu, X.; Wang, Q.; Cai, N.; Zhang, H.; Jiang, Z.; Wan, M.; Oda, T. Immunomodulatory Effects of Alginate Oligosaccharides on Murine Macrophage RAW264.7 Cells and Their Structure–Activity Relationships. *J. Agric. Food Chem.* **2014**, *62* (14), 3168–3176. https://doi.org/10.1021/jf405633n.
(48) Zhang, C.; Li, M.; Rauf, A.; Khalil, A. A.; Shan, Z.; Chen, C.; Rengasamy, K. R. R.; Wan, C. Process and Applications of Alginate Oligosaccharides with Emphasis on Health Beneficial Perspectives. *Crit. Rev. Food Sci. Nutr.* **2023**, *63* (3), 303–329. https://doi.org/10.1080/10408398.2021.1946008.
(49) Zhu, B.; Yin, H. Alginate Lyase: Review of Major Sources and Classification, Properties, Structure-Function Analysis and Applications. *Bioengineered* **2015**, *6* (3), 125–131. https://doi.org/10.1080/21655979.2015.1030543.
(50) Lombard, V.; Bernard, T.; Rancurel, C.; Brumer, H.; Coutinho, P. M.; Henrissat, B. A Hierarchical Classification of Polysaccharide Lyases for Glycogenomics. *Biochem. J.* **2010**, *432* (3), 437–444. https://doi.org/10.1042/BJ20101185.
(51) Li, Q.; Hu, F.; Wang, M.; Zhu, B.; Ni, F.; Yao, Z. Elucidation of Degradation Pattern and Immobilization of a Novel Alginate Lyase for Preparation of Alginate Oligosaccharides. *Int. J. Biol. Macromol.* **2020**, *146*, 579–587. https://doi.org/10.1016/j.ijbiomac.2019.12.238.
(52) *CAZy - PL*. https://www.cazy.org/Polysaccharide-Lyases.html (accessed 2025-01-16).
(53) Xu, F.; Wang, P.; Zhang, Y.-Z.; Chen, X.-L. Diversity of Three-Dimensional Structures and Catalytic Mechanisms of Alginate Lyases. *Appl. Environ. Microbiol.* **2018**, *84* (3), e02040-17. https://doi.org/10.1128/AEM.02040-17.
(54) Dong, F.; Xu, F.; Chen, X.-L.; Li, P.-Y.; Li, C.-Y.; Li, F.; Chen, Y.; Wang, P.; Zhang, Y.-Z. Alginate Lyase Aly36B Is a New Bacterial Member of the Polysaccharide Lyase Family 36 and Catalyzes by a Novel Mechanism With Lysine as Both the Catalytic Base and Catalytic Acid. *J. Mol. Biol.* **2019**, *431* (24), 4897–4909. https://doi.org/10.1016/j.jmb.2019.10.023.
(55) Itoh, T.; Nakagawa, E.; Yoda, M.; Nakaichi, A.; Hibi, T.; Kimoto, H. Structural and Biochemical Characterisation of a Novel Alginate Lyase from Paenibacillus Sp. Str. FPU-7. *Sci. Rep.* **2019**, *9* (1), 14870. https://doi.org/10.1038/s41598-019-51006-1.
(56) Garron, M.-L.; Cygler, M. Structural and Mechanistic Classification of Uronic Acid-Containing Polysaccharide Lyases. *Glycobiology* **2010**, *20* (12), 1547–1573. https://doi.org/10.1093/glycob/cwq122.
(57) Yamasaki, M.; Moriwaki, S.; Miyake, O.; Hashimoto, W.; Murata, K.; Mikami, B. Structure and Function of a Hypothetical *Pseudomonas Aeruginosa* Protein PA1167 Classified into Family PL-7. *J. Biol. Chem.* **2004**, *279* (30), 31863–31872. https://doi.org/10.1074/jbc.M402466200.
(58) Xu, F.; Dong, F.; Wang, P.; Cao, H.-Y.; Li, C.-Y.; Li, P.-Y.; Pang, X.-H.; Zhang, Y.-Z.; Chen, X.-L. Novel Molecular Insights into the Catalytic Mechanism of Marine Bacterial Alginate Lyase AlyGC from Polysaccharide Lyase Family 6*. *J. Biol. Chem.* **2017**, *292* (11), 4457–4468. https://doi.org/10.1074/jbc.M116.766030.
(59) Park, D.; Jagtap, S.; Nair, S. K. Structure of a PL17 Family Alginate Lyase Demonstrates Functional Similarities among Exotype Depolymerases. *J. Biol. Chem.* **2014**, *289* (12), 8645–8655. https://doi.org/10.1074/jbc.M113.531111.
(60) Ochiai, A.; Yamasaki, M.; Mikami, B.; Hashimoto, W.; Murata, K. Crystal Structure of Exotype Alginate Lyase Atu3025 from *Agrobacterium Tumefaciens*. *J. Biol. Chem.* **2010**, *285* (32), 24519–24528. https://doi.org/10.1074/jbc.M110.125450.

(61) Takacs, L. The Historical Development of Mechanochemistry. *Chem. Soc. Rev.* **2013**, *42* (18), 7649–7659. https://doi.org/10.1039/C2CS35442J.
(62) Do, J.-L.; Friščić, T. Mechanochemistry: A Force of Synthesis. *ACS Cent. Sci.* **2017**, *3* (1), 13–19. https://doi.org/10.1021/acscentsci.6b00277.
(63) Liquid-Assisted Grinding Mechanochemistry in the Synthesis of Pharmaceuticals. *Adv. Synth. Catal.* **2021**, *363* (5), 1246–1271. https://doi.org/10.1002/adsc.202001245.
(64) Hasa, D.; Schneider Rauber, G.; Voinovich, D.; Jones, W. Cocrystal Formation through Mechanochemistry: From Neat and Liquid-Assisted Grinding to Polymer-Assisted Grinding. *Angew. Chem. Int. Ed.* **2015**, *54* (25), 7371–7375. https://doi.org/10.1002/anie.201501638.
(65) Wang, G.-W. Mechanochemical Organic Synthesis. *Chem. Soc. Rev.* **2013**, *42* (18), 7668–7700. https://doi.org/10.1039/C3CS35526H.
(66) Klimakow, M.; Klobes, P.; Thünemann, A. F.; Rademann, K.; Emmerling, F. Mechanochemical Synthesis of Metal−Organic Frameworks: A Fast and Facile Approach toward Quantitative Yields and High Specific Surface Areas. *Chem. Mater.* **2010**, *22* (18), 5216–5221. https://doi.org/10.1021/cm1012119.
(67) Dong, L.; Li, L.; Chen, H.; Cao, Y.; Lei, H. Mechanochemistry: Fundamental Principles and Applications. *Adv. Sci. n/a* (n/a), 2403949. https://doi.org/10.1002/advs.202403949.
(68) Liu, X.; Li, Y.; Zeng, L.; Li, X.; Chen, N.; Bai, S.; He, H.; Wang, Q.; Zhang, C. A Review on Mechanochemistry: Approaching Advanced Energy Materials with Greener Force. *Adv. Mater.* **2022**, *34* (46), 2108327. https://doi.org/10.1002/adma.202108327.
(69) Reynes, J. F.; Leon, F.; García, F. Mechanochemistry for Organic and Inorganic Synthesis. *ACS Org. Inorg. Au* **2024**, *4* (5), 432–470. https://doi.org/10.1021/acsorginorgau.4c00001.
(70) Muñoz-Batista, M. J.; Rodriguez-Padron, D.; Puente-Santiago, A. R.; Luque, R. Mechanochemistry: Toward Sustainable Design of Advanced Nanomaterials for Electrochemical Energy Storage and Catalytic Applications. *ACS Sustain. Chem. Eng.* **2018**, *6* (8), 9530–9544. https://doi.org/10.1021/acssuschemeng.8b01716.
(71) Kaabel, S.; Friščić, T.; Auclair, K. Mechanoenzymatic Transformations in the Absence of Bulk Water: A More Natural Way of Using Enzymes. *Chembiochem Eur. J. Chem. Biol.* **2020**, *21* (6), 742–758. https://doi.org/10.1002/cbic.201900567.
(72) de Gonzalo, G.; Colpa, D. I.; Habib, M. H. M.; Fraaije, M. W. Bacterial Enzymes Involved in Lignin Degradation. *J. Biotechnol.* **2016**, *236*, 110–119. https://doi.org/10.1016/j.jbiotec.2016.08.011.
(73) Pettipher, G. L.; Latham, M. J. Characteristics of Enzymes Produced by Ruminococcus Flavefaciens Which Degrade Plant Cell Walls. *Microbiology* **1979**, *110* (1), 21–27. https://doi.org/10.1099/00221287-110-1-21.
(74) Wilson, D. B. Three Microbial Strategies for Plant Cell Wall Degradation. *Ann. N. Y. Acad. Sci.* **2008**, *1125* (1), 289–297. https://doi.org/10.1196/annals.1419.026.
(75) Hernández, J. G.; Frings, M.; Bolm, C. Mechanochemical Enzymatic Kinetic Resolution of Secondary Alcohols under Ball-Milling Conditions. *ChemCatChem* **2016**, *8* (10), 1769–1772. https://doi.org/10.1002/cctc.201600455.
(76) Hammerer, F.; Loots, L.; Do, J.-L.; Therien, J. P. D.; Nickels, C. W.; Friščić, T.; Auclair, K. Solvent-Free Enzyme Activity: Quick, High-Yielding Mechanoenzymatic Hydrolysis of Cellulose into Glucose. *Angew. Chem.* **2018**, *130* (10), 2651–2654. https://doi.org/10.1002/ange.201711643.
(77) Ostadjoo, S.; Hammerer, F.; Dietrich, K.; Dumont, M.-J.; Friscic, T.; Auclair, K. Efficient Enzymatic Hydrolysis of Biomass Hemicellulose in the Absence of Bulk Water. *Molecules* **2019**, *24* (23), 4206. https://doi.org/10.3390/molecules24234206.
(78) Therien, J. P. D.; Hammerer, F.; Friščić, T.; Auclair, K. Mechanoenzymatic Breakdown of Chitinous Material to N-Acetylglucosamine: The Benefits of a Solventless Environment. *ChemSusChem* **2019**, *12* (15), 3481–3490. https://doi.org/10.1002/cssc.201901310.

(79) Kaabel, S.; Therien, J. P. D.; Deschênes, C. E.; Duncan, D.; Friščić, T.; Auclair, K. Enzymatic Depolymerization of Highly Crystalline Polyethylene Terephthalate Enabled in Moist-Solid Reaction Mixtures. *Proc. Natl. Acad. Sci. U. S. A.* **2021**, *118* (29), e2026452118. https://doi.org/10.1073/pnas.2026452118.
(80) Xia, Y.; Auclair, K. Mechanoenzymatic Depolymerization of Highly Crystalline Polyethylene Naphthalate under Moist-Solid Conditions. *ACS Sustain. Chem. Eng.* **2024**, *12* (40), 14832–14840. https://doi.org/10.1021/acssuschemeng.4c05734.
(81) Pérez-Venegas, M.; Friščić, T.; Auclair, K. Efficient Mechano-Enzymatic Hydrolysis of Polylactic Acid under Moist-Solid Conditions. *ACS Sustain. Chem. Eng.* **2023**, *11* (27), 9924–9931. https://doi.org/10.1021/acssuschemeng.2c06847.
(82) Al Amin Leamon, A. K. M.; Venegas, M. P.; Orsat, V.; Auclair, K.; Dumont, M.-J. Semisynthetic Transformation of Banana Peel to Enhance the Conversion of Sugars to 5-Hydroxymethylfurfural. *Bioresour. Technol.* **2022**, *362*, 127782. https://doi.org/10.1016/j.biortech.2022.127782.
(83) Nzediegwu, E.; Pérez-Venegas, M.; Auclair, K.; Dumont, M.-J. Semisynthetic Production of Hydroxymethylfurfural and Furfural: The Benefits of an Integrated Approach. *J. Environ. Chem. Eng.* **2022**, *10* (5), 108515. https://doi.org/10.1016/j.jece.2022.108515.
(84) Kaabel, S.; Arciszewski, J.; Borchers, T. H.; Therien, J. P. D.; Friščić, T.; Auclair, K. Solid-State Enzymatic Hydrolysis of Mixed PET/Cotton Textiles. *ChemSusChem* **2023**, *16* (1), e202201613. https://doi.org/10.1002/cssc.202201613.
(85) Ali, W.; Ali, H.; Gillani, S.; Zinck, P.; Souissi, S. Polylactic Acid Synthesis, Biodegradability, Conversion to Microplastics and Toxicity: A Review. *Environ. Chem. Lett.* **2023**, *21* (3), 1761–1786. https://doi.org/10.1007/s10311-023-01564-8.
(86) Folino, A.; Karageorgiou, A.; Calabrò, P. S.; Komilis, D. Biodegradation of Wasted Bioplastics in Natural and Industrial Environments: A Review. *Sustainability* **2020**, *12* (15), 6030. https://doi.org/10.3390/su12156030.
(87) Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31* (3), 426–428. https://doi.org/10.1021/ac60147a030.
(88) Hammerer, F.; Ostadjoo, S.; Dietrich, K.; Dumont, M.-J.; Rio, L. F. D.; Friščić, T.; Auclair, K. Rapid Mechanoenzymatic Saccharification of Lignocellulosic Biomass without Bulk Water or Chemical Pre-Treatment. *Green Chem.* **2020**, *22* (12), 3877–3884. https://doi.org/10.1039/D0GC00903B.
(89) Friščić, T.; Childs, S. L.; Rizvi, S. A. A.; Jones, W. The Role of Solvent in Mechanochemical and Sonochemical Cocrystal Formation: A Solubility-Based Approach for Predicting Cocrystallisation Outcome. *CrystEngComm* **2009**, *11* (3), 418–426. https://doi.org/10.1039/B815174A.
(90) Pladevall, B. S.; de Aguirre, A.; Maseras, F. Understanding Ball Milling Mechanochemical Processes with DFT Calculations and Microkinetic Modeling. *ChemSusChem* **2021**, *14* (13), 2763–2768. https://doi.org/10.1002/cssc.202100497.
(91) More, N.; Daniel, R. M.; Petach, H. H. The Effect of Low Temperatures on Enzyme Activity. *Biochem. J.* **1995**, *305 (Pt 1)* (Pt 1), 17–20. https://doi.org/10.1042/bj3050017.
(92) Peterson, M. E.; Daniel, R. M.; Danson, M. J.; Eisenthal, R. The Dependence of Enzyme Activity on Temperature: Determination and Validation of Parameters. *Biochem. J.* **2007**, *402* (2), 331–337. https://doi.org/10.1042/BJ20061143.
(93) Dixon, M. The Effect of pH on the Affinities of Enzymes for Substrates and Inhibitors. *Biochem. J.* **1953**, *55* (1), 161–170. https://doi.org/10.1042/bj0550161.
(94) Hammerer, F.; Ostadjoo, S.; Friščić, T.; Auclair, K. Towards Controlling the Reactivity of Enzymes in Mechanochemistry: Inert Surfaces Protect β-Glucosidase Activity During Ball Milling. *ChemSusChem* **2020**, *13* (1), 106–110. https://doi.org/10.1002/cssc.201902752.

(95) Draget, K. I.; Skjåk Bræk, G.; Smidsrød, O. Alginic Acid Gels: The Effect of Alginate Chemical Composition and Molecular Weight. *Carbohydr. Polym.* **1994**, *25* (1), 31–38. https://doi.org/10.1016/0144-8617(94)90159-7.
(96) Li, J.; Sun, M.; Song, X.; Chen, G.; Zhou, J.; Chang, Y.; Xue, C. Analysis of Unsaturated Alginate Oligosaccharides Using High-Performance Anion Exchange Chromatography Coupled with Mass Spectrometry. *Anal. Bioanal. Chem.* **2024**, *416* (15), 3501–3508. https://doi.org/10.1007/s00216-024-05299-5.
(97) Heyraud, A.; Colin-Morel, P.; Girond, S.; Richard, C.; Kloareg, B. HPLC Analysis of Saturated or Unsaturated Oligoguluronates and Oligomannuronates. Application to the Determination of the Action Pattern of *Haliotis Tuberculata* Alginate Lyase. *Carbohydr. Res.* **1996**, *291*, 115–126. https://doi.org/10.1016/S0008-6215(96)00138-3.
(98) Heyraud, A.; Gey, C.; Leonard, C.; Rochas, C.; Girond, S.; Kloareg, B. NMR Spectroscopy Analysis of Oligoguluronates and Oligomannuronates Prepared by Acid or Enzymatic Hydrolysis of Homopolymeric Blocks of Alginic Acid. Application to the Determination of the Substrate Specificity of *Haliotis Tuberculata* Alginate Lyase. *Carbohydr. Res.* **1996**, *289*, 11–23. https://doi.org/10.1016/0008-6215(96)00060-2.
(99) Zhang, Z.; Yu, G.; Zhao, X.; Liu, H.; Guan, H.; Lawson, A. M.; Chai, W. Sequence Analysis of Alginate-Derived Oligosaccharides by Negative-Ion Electrospray Tandem Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2006**, *17* (4), 621–630. https://doi.org/10.1016/j.jasms.2006.01.002.
(100) Aarstad, O. A.; Tøndervik, A.; Sletta, H.; Skjåk-Bræk, G. Alginate Sequencing: An Analysis of Block Distribution in Alginates Using Specific Alginate Degrading Enzymes. *Biomacromolecules* **2012**, *13* (1), 106–116. https://doi.org/10.1021/bm2013026.
(101) Inoue, A.; Nishiyama, R.; Ojima, T. The Alginate Lyases FlAlyA, FlAlyB, FlAlyC, and FlAlex from Flavobacterium Sp. UMI-01 Have Distinct Roles in the Complete Degradation of Alginate. *Algal Res.* **2016**, *19*, 355–362. https://doi.org/10.1016/j.algal.2016.03.008.