Mechanoenzymatic Reactions Involving Polymeric Substrates or Products

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Abstract

Mechanoenzymology is an emerging field in which mechanical mixing is used to sustain enzymatic reactions in low-solvent or solvent-free mixtures. Many enzymes are reported that thrive under such conditions. Considering the central role of biopolymers and synthetic polymers in our society, this minireview highlights the use of mechanoenzymology for the synthesis or depolymerization of oligomeric or polymeric materials. In contrast to traditional in-solution reactions, solvent-free mechanoenzymology has the advantages of avoiding solubility issues, proceeding in a minimal volume, and reducing solvent waste, while potentially improving the reaction efficiency and accessing new reactivity. It is expected that this strategy will continue to gain popularity and find more applications.

Introduction

Mechanochemistry, the induction of a reaction between reagents via mechanical forces, is a very attractive branch of chemistry in part because of its low energy consumption and minimal solvent need.[1] This technique is particularly advantageous with poorly soluble substrates as it is compatible with solvent-free reaction conditions and liquid-assisted grinding (LAG)[2] in which small, often equimolar amounts of added liquid accelerate or enable reactions between solids, independently of solubility effects. LAG conditions are defined by $\eta \leq 2$ µL mg^{-1}, where η is the total volume of liquid added in µL per milligram of solid included in the mixture. Mechanochemistry uses brief or continuous periods of mechanical mixing, achievable for example by means of a shaker or planetary ball mill, a mortar and pestle, or extrusion. This can be paired with "aging"[3,4] *i.e.* periods of static incubation of the reaction mixture under mild temperatures to sustain reactivity. The application of mechanochemistry to synthesis has been extensively reviewed elsewhere.[1,5–10]

It has more recently been shown that mechanochemical conditions are perfectly suitable for many enzymes, giving rise to the growing field of mechanoenzymology. Recent studies suggest that mechanoenzymology in the absence of bulk water might actually favour enzyme activity by better mimicking the enzyme's natural environment than traditional dilute aqueous conditions typically used by scientists.[11] Whereas cellular enzymes operate in a highly concentrated milieu (50-200 mg mL^{-1}[12] in eukaryotic cells and 300-400 mg mL^{-1}[13] in bacteria), with little bulk water available, other enzymes are naturally secreted into the environment by microorganisms. The latter enzymes have evolved to operate on a surface exposed to air moisture, conditions that can be emulated using moist-solid reaction mixtures. Enzymes that have been successfully used in mechanochemistry to date are listed in Table 1. Benefits of mechanoenzymatic reactions include avoiding substrate solubility issues, accessing new

reactivity, altered product selectivity, limiting the use of bulk solvent and toxic reagents, and curtailing waste.

Table 1. Summary of the enzymes that have been employed in mechanoenzymology.

Enzyme	Source	Commerical Name	Ref.
Immobilized CALB	*Candida antarctica*	Novozyme® 435	Ref[14–20]
CALB	*Candida antarctica*		Ref[21,22]
Lipase A	*Candida antarctica*		Ref[16]
Lipase	Porcine pancreas		Ref[19]
Lipase	*Rhizomucor miehei*	Lipozyme ®RM IM	Ref[19]
Lipase	*Thermomyces lanuginosus*	Lipozyme®TL IM	Ref[19]
Lipase	*Burkholderia cepacia*	Amano lipase PS-IM	Ref[15,16]
Lipase	*Candida antarctica*	NovoCor	Ref[22]
Papain	*Carica papaya*		Ref[23,24]
Papain	Papaya latex		Ref[23]
Protease		Alcalase® CLEA	Ref[23]
Cellulases	*Trichoderma reesei*		Ref[25]
Cellulases	*Trichoderma longibrachiatum*		Ref[25–27]
Cellulases	*Aspergillus niger*		Ref[25,28]
Cellulases		Cellic® CTec2	Ref[26]
Xylanase	*Thermomyces lanuginosus*		Ref[29]
Chitanases	*Aspergillus niger*		Ref[11]
Cutinase	*Humicola insolens*	Novozym® 51032	Ref[30]

While several existing review articles discuss mechanoenzymatic reactions,[31–35] this minireview will focus specifically on those that involve polymeric substrates or products. Polymers are ubiquitous in society and central in a number of trending research areas such as peptide therapeutics,[36] the production of biofuels from lignocellulosic biomass,[37] and tackling the plastic pollution problem.[38] The emerging involvement of mechanoenzymology in these areas, and the widespread importance and impact of polymers make them an interesting area of focus. In summarizing the studies below, this review aims to highlight that not only do mechanoenzymatic techniques offer more sustainable routes to synthesizing or depolymerizing oligomeric/polymeric materials, but this approach also affords improvements in yield and/or efficiency over traditional techniques.

The studies reviewed here use milling, most commonly achieved via a shaker ball mill or a planetary ball mill (Figure 1 a,b). Whereas the former swings the milling jar back and forth at a desired frequency, the latter rotates the jar with "planetary motion" and is often available in larger sizes.[39,40] Both can be used with or without the addition of milling balls. Another common technique used in mechanochemistry is reactive extrusion: a continuous processing technique in which the reaction mixture is forced through a constrained space by one or two rotating screws at a desired temperature (Figure 1c).[41] The use of enzymes in extrusion processes has been designated as enzymatic reactive extrusion or "eREX",[14] and has found applications in the polymerization of ω-pentadecalactone,[14] the depolymerization of

polybutylene succinate,[21] and the depolymerization of many biopolymers including starch, proteins, and biomass.[42,43] This minireview will focus on studies that use milling as the primary means of mechanical mixing. A summary of the important reaction parameters in mechanoenzymology can be found in Table 2.

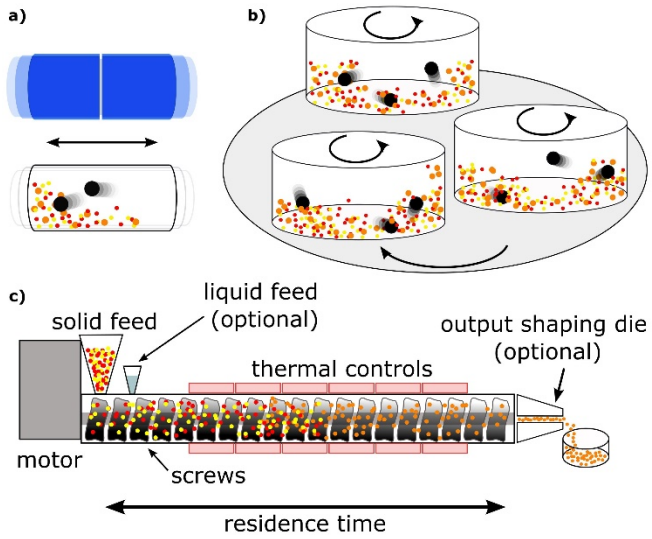

Figure 1. Some of the common techniques utilized in mechanochemistry: a) shaker ball milling; b) planetary ball milling; and c) reactive extrusion. Black circles represent the milling balls and colored circles depict reactants and products. Redrawn with permission from ref[34]. Copyright: 2020, John Wiley and Sons.

Table 2. Summary of the important parameters in mechanoenzymology

Parameter	Definition
enzyme loading (% w/w)	Percentage of the weight of enzyme used in the process compared to the weight of the main substrate
η (μL mg^{-1})	Liquid-to-solid ratio, with $\eta < 2$ corresponding to LAG conditions and $\eta > 2$ matching slurry or solution conditions, depending on solubility.
pre-milling	Milling of the substrate alone to decrease particle size
milling	Milling of all reaction components
aging	Static incubation of all reaction components

1. Mechanoenzymatic Polymerization

From the polymers found in nature such as nucleic acids, polypeptides, lignin, and polysaccharides, to the wide variety of synthetic plastics that have become essential to today's society, polymerization reactions are essential to obtaining these valuable materials. A growing recognition of the importance of

sustainability has encouraged the development of more environment-friendly processes. For example, mechanochemical synthesis has proven to be an alternative that satisfies many of the principles of Green Chemistry.[10] A number of mechanochemical polymerization reactions have been developed, including the synthesis of short DNA fragments[44] and peptides in the ball mill,[33] as well as reactive extrusion-based plastic polymerization.[45] The use of enzymes in mechanochemical conditions is an emerging technique in polymer synthesis with very few examples in the literature to date. This section discusses the efforts towards the mechanoenzymatic synthesis of polypeptides.

Peptides have applications in a diverse range of fields including drug discovery[36] and materials science.[46] Whereas molecules containing 50-2000 amino acids are often referred to as polypeptides, oligopeptides, or simply peptides, consists of 2-49 amino acids, all of which are referred to as peptides here.[47] Peptide synthesis typically uses solid-phase synthesis in a significant amount of solvent. There is interest in finding more environmentally friendly strategies to synthesize peptides given the large amounts of toxic reagents and/or solvents used.[48,49] Lamaty and coworkers were the first to demonstrate the compatibility between peptide synthesis and mechanochemistry,[50] pioneering the emergence of several new mechanochemical peptide synthesis methods.[33] These methodologies allow for the synthesis of peptides in the absence of bulk solvent, however, most of the approaches still suffer from the use of harmful reagents and additives. In more recent years, mechanoenzymatic peptide synthesis has been explored as summarized below.

1.1 Papain-Catalyzed Mechanoenzymatic Synthesis of Peptides

Following the successful mechanoenzymatic synthesis of α,α- and α,β-dipeptides by papain, a cysteine protease, in a ball mill,[23] Hernandez and coworkers next looked at the mechanoenzymatic homo-oligomerization of amino acids.[24] Using equimolar amounts of the solids HCl·L-Phe-OCH_3 and $Na_2CO_3 \cdot 10H_2O$, together with papain (8.6 % w/w) in the absence of organic or aqueous solvent, the mixture was ball milled for 2 hours at 25 Hz, affording an oligomerization yield of 23% and a degree of polymerization (DP) of 6.1 (Figure 2). Doubling the amount of enzyme increased the yield to 99%, while decreasing the DP to 5.0. Satisfyingly, this reaction could be scaled up 10-fold in a planetary ball mill, affording oligo(L-Phe) in quantitative yield and a DP of 8.0, suggesting that the less energetic milling technique (planetary versus shaker mill) might be superior to favour amide formation over hydrolysis.

Oligomers of other amino acids were also successfully generated under these conditions, including L-leucine, L-alanine, and L-glycine, with interesting trends. The DP was found to decrease in the following trend L-Gly > L-Ala > L-Leu >LL-Phe. A similar trend is seen in solution chemistry, often with comparable DP values too.[51–54] This was unexpected based on prior attribution of low DP to the premature precipitation of the oligopeptides,[54] an issue that is circumvented under solvent-less conditions, implying that a decreasing affinity of the enzyme for the growing oligomer might instead be at play. An improved oligomerization of L-Phe and L-Leu was observed when $Na_2CO_3.10H_2O$ was used instead of anhydrous Na_2CO_3, whereas the opposite effect was seen for L-Ala and L-Gly, with water molecules suggested to promote the formation of hydrophobic pockets, favouring association of the lipophilic amino acid monomers with the enzyme active site. This effect, in addition to the higher concentration of the reactants in the absence of bulk solvent, is thought here to give mechanoenzymatic reactions an efficiency advantage over comparable in-solution reactions, given that similar/longer reaction times are often needed to produce the same oligopeptides in solution, in even lower yields.[51–58] Scaling-up of the

homo-oligomerization reactions was achieved using twin-screw extrusion, following similar reactivity and DP trends as comparable ball milling reactions.

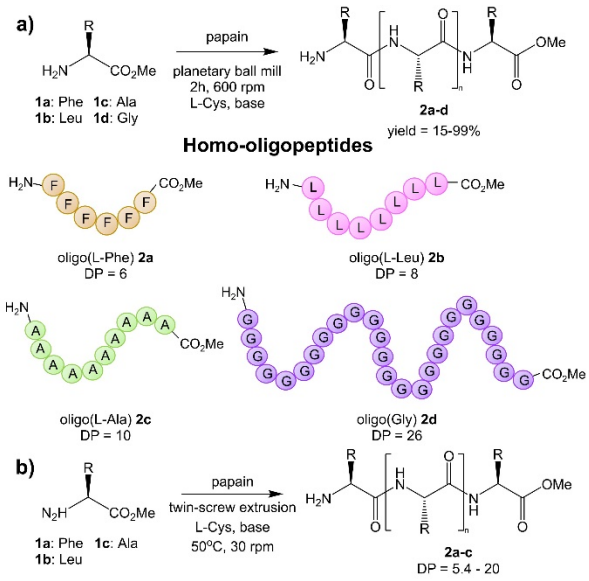

Figure 2. Mechanoenzymatic homo-oligomerization of amino acids by ball milling and extrusion. Redrawn with permission from ref[33]. Copyright: 2018, John Wiley and Sons.

1.2 Concluding Remarks

The use of enzymes in a ball mill is a recent concept in the field of polymer synthesis. The above study provides evidence for the benefits of mechanochemistry in peptide synthesis, most notably the possibility of avoiding solvent use. To further emphasize the advantages of their method, Hernandez *et al.* looked at the E-factor values (used to evaluate the environmental impact of a process and defined as the amount of product formed relative to the amount of waste generated[59,60]). When comparing the synthesis of oligo(L-Leu) via ball milling or extrusion to in-solution synthesis, they found that the mechanoenzymatic approaches had E-factor values two orders of magnitude lower than their in-solution counterpart.[24]

2. Mechanoenzymatic breakdown of natural polymers

While polymer synthesis is essential for obtaining many valuable materials, many polymers are present as recalcitrant waste products. The next two sections of this review will focus on the mechanoenzymatic depolymerization of polymers. Section 2 will touch on the depolymerization of natural polymers that accumulate as waste products from industries such as agriculture, forestry, and fishery, while Section 3 will discuss the depolymerization of synthetic polymers.

Fossil fuels are an unsustainable resource. There has been a lot of interest in finding renewable, non-fossil-based feedstocks to fulfil future needs for energy and basic chemicals.[61,62] Natural polymers such as cellulose, hemicellulose, and chitin; important components of lignocellulosic or chitinous biomass; could provide these feedstocks if they can be broken down into a clean, useful product.[63,64] Currently, biomass depolymerization remains challenging due to the constituent polymers' recalcitrance and poor solubility, as well as the complexity of the matrices.[65,66] For these reasons, the *chemical* degradation of

cellulose and chitin typically requires noxious reagents and conditions.[67,68] Alternatively, their enzymatic depolymerization has been demonstrated, however until recently, harsh chemical or thermal pre-treatments were still necessary to make the polymers accessible to the enzymes for degradation.[69,70] Interestingly, Auclair and coworkers demonstrated that mechanoenzymology allows for the production of sugar monomers from lignocellulosic and chitinous materials under mild conditions, without the need for a harsh pre-treatment, offering an exciting new and sustainable route to accessing these chemical feedstocks.[11,25,26,29]

Building upon known techniques in mechanochemistry such as LAG and aging, Auclair and coworkers developed a new method, termed "RAging",[25] in which reactions are accelerated by repeating cycles of ball milling and aging, with the length of the cycles optimized based on reaction kinetics. Although RAging has proven superior to a single round of milling and aging for most enzymes tested, the latter is sometimes advantageous.[11] Most of the glycosyl hydrolases discussed below are naturally secreted by bacteria or fungi directly onto their solid substrates, *i.e.* in the absence of bulk water, where they catalyze the depolymerization of the substrate into smaller molecules that can then be absorbed into the cell.[71] It has been suggested that the ability of mechanoenzymatic reaction conditions to emulate such an environment may explain the higher efficiency of these enzymes in the absence of bulk aqueous solvent compared to the dilute aqueous reaction conditions typically used by scientists.[11,25]

2.1 Mechanoenzymatic Hydrolysis of Cellulose into Glucose by Cellulases

Auclair and coworkers sought to explore mechanoenzymatic methods to break down lignocellulosic biomass (Figure 3) starting with microcrystalline cellulose (MCC), a highly recalcitrant and crystalline, but well-defined model substrate of cellulose.[25] Commercial cellulase enzyme blends (*i.e.* including several cellobiohydrolases, endoglucanases, and β-glucosidases) available in the form of a lyophilized powder were employed. A few equivalents of water (a substrate) or buffer was added to every reaction mixture to obtain η values between 0.8 and 1.0 µL mg^{-1} (*i.e.* within the LAG regime), resulting in paste-like or "moist-solid" reaction mixtures.

Experimenting with *Aspergillus niger* cellulases (0.1 % w/w) yielded 20% of glucose after 20 days of RAging cycles composed of 5 minutes milling (30 Hz) and 24 hours aging (55°C). Moving to *Trichoderma longibrachiatum* cellulases (3 % w/w), the authors report that aging (55°C) for only 55 minutes between 5-minute milling cycles leads to 50% hydrolysis in a mere 12 hours (Figure 4). This yield is unprecedented for untreated MCC at such low enzyme loading. Interestingly, glucose was produced as a highly concentrated aqueous solution of ca. 3.2 M, *i.e.* more than 3 times higher than any other cellulose hydrolysis process, and interestingly, well above the inhibitory concentration of glucose for β-glucosidases.[72] At a space-time yield of 20 grams of glucose per liter per hour, this process was at least 20-fold superior to previously reported enzymatic digestions of chemically-untreated MCC. Additionally, analysis of the residual substrate revealed a transformation to cellulose nanocrystals, a valuable and renewable product with a wide range of applications.[73] Increasing the scale from 200 mg to 2 g, without optimization of the conditions, afforded a 43% yield of glucose after 36 hours of RAging.

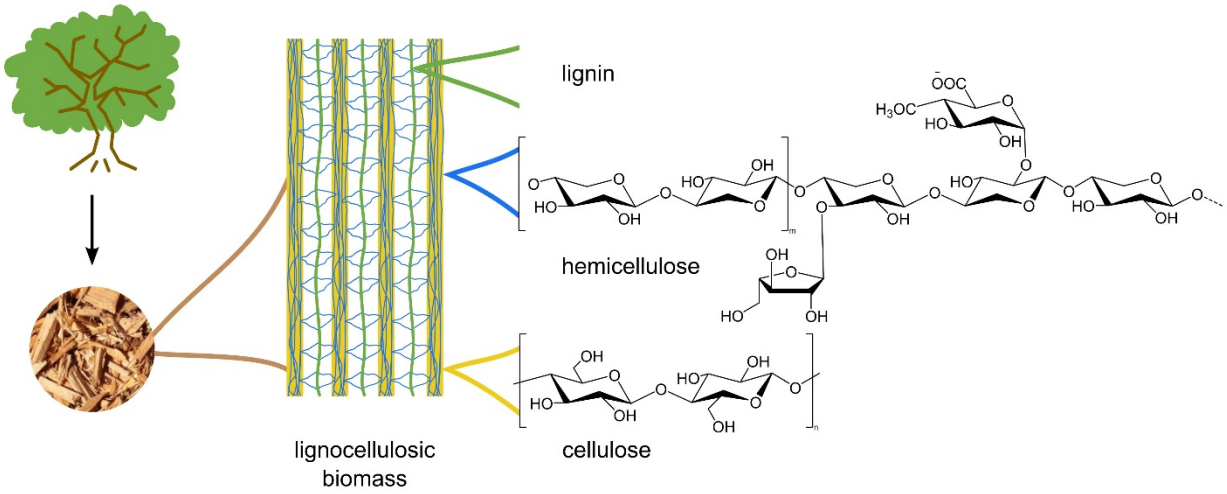

Figure 3. Composition of lignocellulosic biomass. Lignocellulosic biomass refers to dry plant matter and is composed primarily (35-50%) of cellulose[74] (yellow), *i.e.* a chain of β-(1-4)-linked glucose molecules.[75] Hemicellulose (blue) is the second most abundant component of lignocellulose (20-35%),[74] and consists primarily of xylans, *i.e.* D-xylopyranosyl units linked by β-(1-4)-glycosidic bonds, with branches of various monosaccharides.[76] Lignin is the other major component of lignocellulosic biomass (5-30%)[74] and consists of a complex polyphenolic polymer.[77]

In a follow-up mechanistic study of β-glucosidases,[28] Auclair and coworkers were able to increase the yield of glucose from cellobiose (the glucose dimer) by adding an inert solid to the reaction mixture. RAging cycles similar to the above study (5 minutes milling and 55 minutes aging) yielded glucose in >70% after only 2 hours in the presence of an inert solid, whereas the activity of β-glucosidases on cellobiose alone was very slow. The effect was observed with inert additives as varied as chitin, lignin, synthetic polymers, and even inorganic compounds such as CeO_2. The authors propose that the additives may help maintain the solid consistency of the reaction mixture, which may protect the enzymes against the mechanical forces.

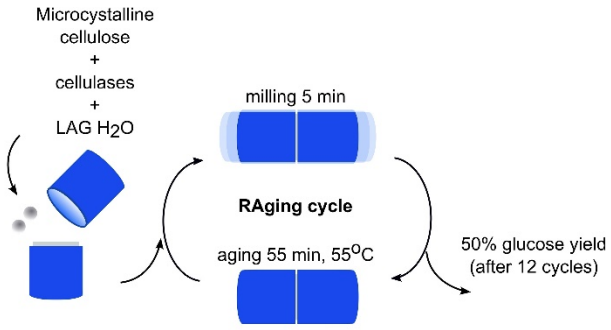

Figure 4. Schematic representation of the RAging process used for the hydrolysis of MCC by *Trichoderma longibrachiatum* cellulases.[25] Figure redrawn with permission from ref[34] Copyright: 2020, John Wiley and Sons.

Expanding their studies beyond purified cellulose, Auclair and coworkers explored the mechanoenzymatic saccharification of raw biomass, starting with cedar wood saw dust.[25] The commercial *T. longibrachiatum* cellulases blend used contained both cellulases and xylanases, resulting in the production of both glucose and xylose. At equal enzyme loadings, 12 hours of RAging (cycles of 5 minutes milling and 55 minutes aging) afforded a threefold increase in hydrolysis activity compared to analogous 12-hour reactions done in solution, demonstrating the superiority of the former process.[25]

They next turned to raw samples of wheat straw, sugarcane bagasse, and corn stover of known cellulose content.[26] Using an enzyme loading of 8.6 % w/w and water at η = 1.34 µL mg^{-1}, 25% hydrolysis of wheat straw was achieved in 30 minutes of milling (30 Hz) followed by 3 days of aging (55°C). When RAging (cycles of 5 minutes milling and 55 minutes aging) was applied to the same reaction mixture, hydrolysis of the wheat straw sample reached a yield of nearly 40%, while the sugarcane bagasse sample was >60% hydrolyzed in 24 hours. Brief pre-milling (15 minutes) of the biomass substrates was essential to accessing these yields.

Switching to the more efficient CTec2 cellulases (4.5 % w/w enzyme loading) with η = 1.5 µL of water per mg of solid in the reaction mixture, reducing sugars were produced in >70% yield for all three substrates (60 minutes pre-milled) after 12 hours of RAging (cycles of 5 minutes milling and 55 minutes aging).[26] An additional 12 hours of aging further increased the yield to >80%. Remarkably, not only does this methodology afford higher reaction rates than conventional slurry or solution processes,[78–88] but it does not require any chemical pre-treatment of the substrates, cleanly produces glucose and xylose in molar-level concentrations, and proceeds in higher space-time yield than previously reported methods.[81]

A recent study at larger scale (25 g) by Zhang *et al*. sought to produce cellulose nanocrystals from MCC using cellulases in a roller mill,[27] *i.e.* a rotated horizontally-oriented cylindrical jar containing balls. Here again, the *T. longibrachiatum* cellulose mixture was found to be most efficient among several cellulase blends. After 48 h of milling at 100 rpm (1.7 Hz) and η = 5 µL mg^{-1} (making this a slurry reaction rather than LAG), a maximum of 26% MCC hydrolysis was obtained at an enzyme loading of 35 U g^{-1} (the amount of protein per unit activity was not disclosed). At a lesser enzyme loading of 25 U g^{-1}, MCC hydrolysis decreased, but the authors observed a maximum yield of cellulose nanocrystals (49%). The production of cellulose nanocrystals via enzymatic MCC depolymerization is consistent with the results from Auclair and coworkers,[25] and demonstrates an additional application of this technology.

Overall, these results demonstrate several advantages of using mechanoenzymatic reactions in moist-solid mixtures for the transformation of biomass, including not only the improved enzyme efficiency but also the production of highly concentrated products, while avoiding solubility issues and the "solids effect" that normally impairs cellulase activity at such high substrate content in solution.[89,90]

2.2 Mechanoenzymatic Hydrolysis of Hemicellulose by Xylanases

Following their studies on cellulose, Auclair and coworkers sought to explore mechanoenzymatic methods for the hydrolysis of xylans or hemicellulose, another major component of lignocellulosic biomass.[29] Purified xylans (birch wood xylans and oat spelt xylans) and raw biomass substrates (sugarcane bagasse and wheat straw) were hydrolyzed using the commercially available xylanase from *Thermomyces lanuginosus,* with the optimal amount of water corresponding to η = 0.6 µL mg^{-1} for purified xylans and η = 1 µL mg^{-1} for raw biomass. Interestingly, the RAging conditions that were

previously shown to improve the hydrolysis rate of cellulases, did not show the same improvement for xylanases. The xylanase was found to tolerate short milling periods (*e.g.,* 5 minutes) and then remain highly active for days under static incubation. Using a very low enzyme loading (0.08 % w/w), it was determined that 30 minutes of milling (30 Hz) followed by 72 hours of aging (55°C) resulted in the highest hydrolysis yields (>70%) for both the purified xylans and raw biomass. A single round of milling followed by static incubation was not only superior in terms of hydrolysis yield, but also generated a cleaner reaction product, producing predominantly the xylose monosaccharide and/or disaccharide, while milling alone or RAging produced mostly xylan oligomers. This demonstrates that enzyme selectivity can be altered by simply varying reaction conditions.

Compared to the analogous reactions in solution, this xylanase was much more efficient in the absence of bulk water, with mechanoenzymatic hydrolysis yields outperforming in-solution reactions by roughly 3 times for purified xylans, and almost 20 times for biomass (Table 3). This further confirms that moist-solid reaction conditions can provide a superior environment for many enzymes.

Table 3. Comparison of the percent hydrolysis yields (based on quantifying reducing sugars) for xylanase reactions under mechanoenzymatic or in-solution reaction conditions.

Hydrolysis yields[a]	**Mechanoenzymatic conditions** 125 µL water (η = 1 µL mg^{-1}), 30 min milling + 72 h aging (55°C)	**In-solution conditions** 10 mL buffer Shaking (55°C) 3 days
Purified xylans	67%	25%
Sugarcane bagasse	73%	4%
Wheat straw	84%	5%

[a]100 mg of substrate was used, and the enzyme loading was 0.08 % w/w

2.3 Mechanoenzymatic Breakdown of Chitin

Chitinous biomass is an attractive chemical feedstock for the production of drugs and agrochemicals due to its chitin component (Figure 5),[64] which is the most abundant nitrogen-containing biopolymer on earth.[91] In 2019, Therien *et al.*[11] reported the successful mechanoenzymatic breakdown of untreated, practical-grade chitin to *N*-acetylglucosamine (GlcNAc) by a commercial chitinases mixture from *A. niger* (lyophilized powder) at an enzyme loading as low as 0.7 % w/w and with water at η = 1.7 µL mg^{-1}. Interestingly, after a brief period of milling, the enzyme was found to be active under static incubation for more than 20 hours. RAging cycles were optimized to 5 minutes of milling (30 Hz) followed by 12 hours of aging (45°C). After 20 cycles, RAging yielded roughly 430 mM of GlcNAc, corresponding to >30% chitin hydrolysis, and GlcNAc accounted for >92% of the water-soluble products. Comparable in-solution reactions yielded approximately five times less GlcNAc.

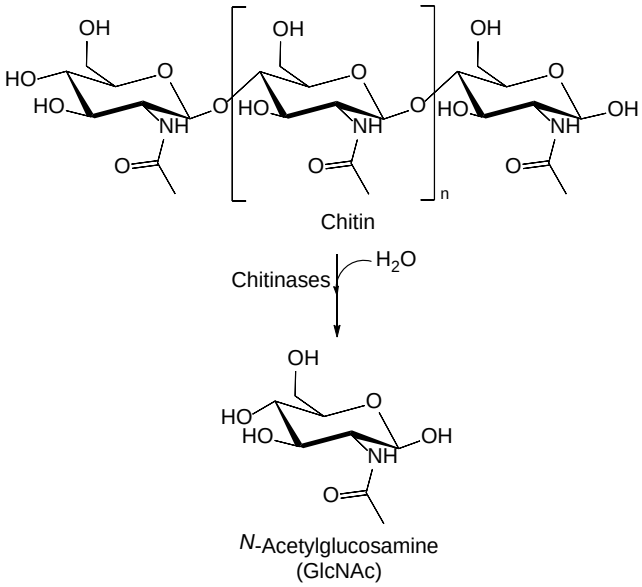

Figure 5. The hydrolysis of chitin by chitinases to generate GlcNAc.

Next, the group sought to hydrolyze raw chitinous biomass, including shrimp and crab shells.[11] This time the reaction proceeded best after a quick wash of the chitinous sample with dilute acetic acid.[92] The washed biomass was an even better substrate than the purified chitin, likely due to the additional protection of the enzyme offered by the other inert solids present in the substrate, as reported for cellulases.[28] With RAging (5 minutes milling and 12 hours aging cycles), GlcNAc was again the major reaction product (>80%), with yields 3-5 times higher than those from analogous reactions performed in solution (Table 4).

Table 4. Comparison of GlcNAc release for mechanoenzymatic versus in-solution reactions.

GlcNAc yield [mg][a]	**Mechanoenzymatic conditions** 500 µL water (η = 1 µL mg^{-1}), RAging[c][5 min mill + 12 h aging (45°C)]	**In-solution conditions** 20 mL buffer Shaking[d] (45°C)
Chitin	47	9
Shrimp[b]	51	18
Crab[b]	49	11

[a]200 mg of substrate and 2 mg of chitinases were used.
[b]Powdered shrimp and crab samples were first washed with diluted aqueous acetic acid.
[c]Total of 20 (chitin) or 10 (shrimp and crab) cycles
[d]Shaking for 10 days (chitin) or 5 days (shrimp and crab)

Concluding Remarks

Together these studies demonstrate how moist-solid reaction mixtures treated with gentle and/or intermittent mechanical mixing offer a way to improve the enzymatic activity of many glycosyl hydrolases, affording yields that far outperform those of analogous reactions in dilute aqueous solution or suspension. It may also modify enzyme selectivity, as suggested by the activity observed on untreated

biomass samples and product selectivity. Not only does the absence of bulk solvent represent an environmental benefit, but it also translates into smaller reaction volumes, which facilitate handling. The possibility of avoiding chemical pre-treatment of the samples also limits the use of toxic reagents and/or harsh temperatures and pressures.

3. Mechanoenzymatic Hydrolysis of Synthetic Polymers

Although there has recently been increasing interest in natural polymers (mainly as a renewable feedstock), synthetic polymers such as plastics have been playing a central role in our society for nearly a century. Currently, this demands a global annual plastic production of almost 320 M tons (2018).[93] Unfortunately, only about 9% of this plastic is recycled after use, and almost 80% of it accumulates in landfills or leaks into the environment.[94] Mechanical recycling is currently the only widely applied recycling method and consists of mechanically processing the plastics to a desired particle size and then melting and moulding the plastic into pellets for re-use.[95,96] This generates lower quality products that ultimately end up in landfill.[97] Recycling through plastic depolymerization offers an opportunity to recover valuable chemicals or generate virgin-quality recycled plastics.[98] Biocatalysts capable of depolymerizing plastic materials offer a sustainable route to plastic recycling since they work under mild conditions and without the use of harsh chemicals. The study summarized below demonstrates how mechanoenzymology can be applied to the depolymerization of synthetic polymers, offering a novel approach to plastic recycling that works under mild conditions, without harsh chemicals, and without any need for amorphization of the material.

3.1 Mechanoenzymatic Depolymerization of Highly Crystalline PET

Several enzymes capable of depolymerizing low crystallinity PET have been reported,[99] but until recently, none of these used mechanoenzymology, and efficient depolymerization of highly crystalline forms of PET typically found in consumer products had remained elusive. Using the thermostable, commercially available *Humicola insolens* cutinase (HiC, Novozym® 51032), Kaabel *et al.* reported that mechanoenzymatic conditions can enable the direct depolymerization of high crystallinity PET plastics.[30] At an enzyme loading of 0.6 % w/w and with buffer at η = 1.5 µL mg^{-1} , commercial PET powder (36% crystallinity, similar to the crystallinity of a post-consumer PET water bottle) was hydrolyzed to 20% terephthalic acid (TPA) after 5 minutes of milling (30Hz) followed by 7 days of aging (55°C). This process showed a 20-fold selectivity for TPA over mono(2-hydroxyethyl) terephthalate (MHET) and generated no detectable amounts of bis(2-hydroxyethyl) terephthalate (BHET) or oligomers. This was remarkable given that prior in-solution enzymatic PET depolymerization methods reported are only efficient on low-crystallinity PET and produce a mixture of products. Gratifyingly, although optimized for PET, this process worked with other plastics such as polybutylene terephthalate (PBT), polycarbonate (PC), and polylactic acid (PLA), with yields consistently outperforming comparable in-solution reactions, demonstrating the potential for this technology to be applied more widely to other plastics.

The efficiency of PET hydrolysis was further improved via the implementation of RAging[25] (cycles of 5 minutes milling and 24 hours aging), affording TPA yields of roughly 25% in only 3 days. Mechanistic studies revealed that activity-induced denaturation of HiC caused the yield plateau at 25%. To circumvent this issue, the authors added fresh enzyme in batches every 3 days, affording an additional 10% yield (from the remaining PET) for each subsequent round (Figure 6). An overall yield of 50% was reached after 7 days, using only 3 % w/w enzyme in total; by far the highest yield, space-time yield, and

yield per gram of enzyme reported to date for the direct depolymerization of highly crystalline PET. It is expected that this yield could be further improved by using a different enzyme isoform or after enzyme engineering to increase protein stability. Notably, the authors clearly demonstrated that both amorphous and crystalline regions of PET were depolymerized to the same extent. Overall, this study offers an alternative strategy for the true recycling of PET under more sustainable conditions and without thermal pre-treatment.

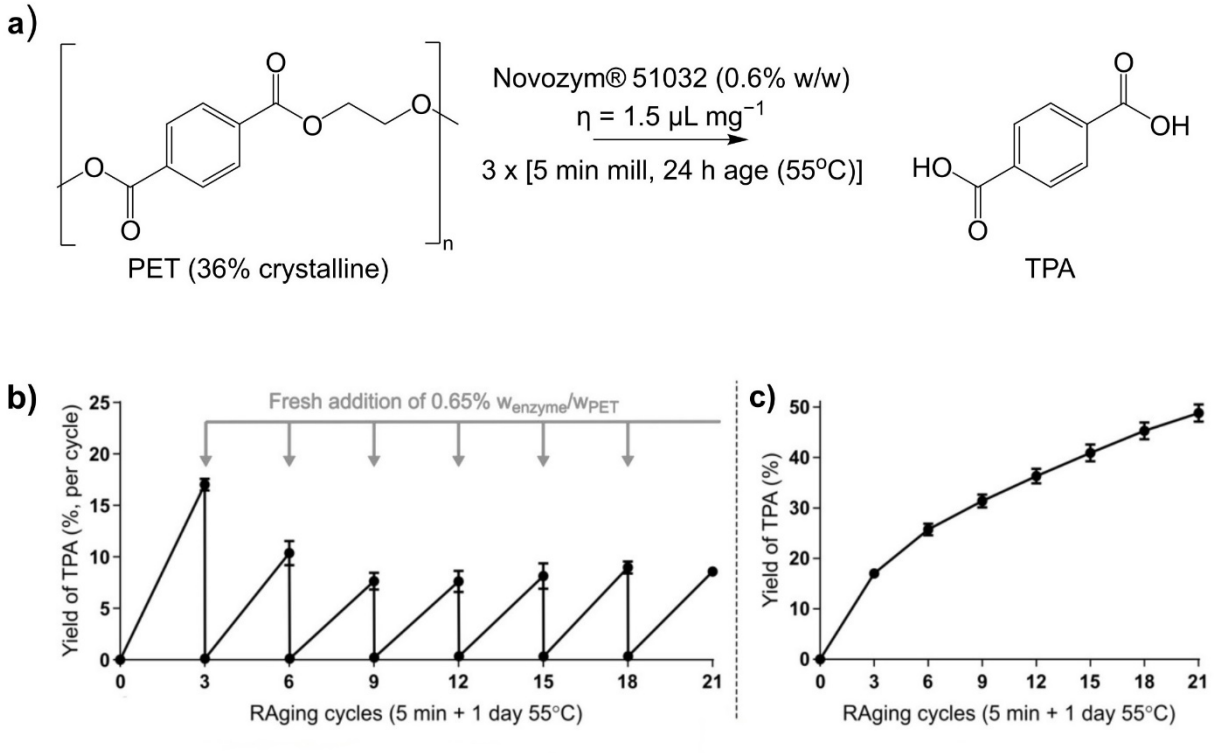

Figure 6. Optimized mechanoenzymatic procedure for PET hydrolysis to TPA. (A) Each round consisted of 3 x (5 minutes milling and 24 hours aging), followed by TPA extraction and addition of new enzyme. (B) TPA yield obtained per round. (C) Cumulative TPA yield over time. Figure adapted with permission from Kaabel *et al*. 2021.[30]

4.0 Conclusion and Outlook

Although enzymes have been reported to show activity in non-conventional media such as organic solvents,[100] emulsions,[101] and ionic liquids[102] to name a few, it is far more common to use them in dilute aqueous conditions, a practice that has remained unquestioned. In nature, however, enzymes rarely operate in such an environment. Whereas cellular enzymes exist in a highly concentrated aqueous medium with little bulk water available (which scientists have attempted to mimic using molecular crowding agents with mixed success[103]), some secreted enzymes have evolved to function on surfaces exposed only to air moisture. It is proposed that the success of mechanoenzymology may originate from its ability to better mimic enzyme natural environments.[11]

Given the prevalence of polymers in society, it is encouraging to see the development of more sustainable technologies, such as mechanoenzymology, for both their synthesis and depolymerization.

Mechanoenzymology is a versatile approach, with ample room for optimization. While enzymes can evidently tolerate the mechanical forces applied by the ball mill to some extent, continuous milling may not be necessary to achieve the desired reactivity. Instead, static incubation at temperatures easily accessible, for example, in a greenhouse, allows for sustained reactivity. LAG amounts of liquid are typically sufficient, while some reactions even proceed in the absence of liquid. Finally, very low enzyme loadings are often enough to access very high reactions yields. Parameters like the RAging cycles, solid additives, and assisting liquids (as in LAG) can also be adjusted to tune enzyme efficiency and selectivity.

To date, only a small collection of enzymes has been employed under mechanochemical conditions, including proteases, lipases, glycosyl hydrolases, and cutinases, warranting further investigations with other classes of enzymes. Additionally, very few techniques have been explored to provide the mechanical mixing, and the field might benefit from additional, complementary options. With a growing acknowledgement of the need for greener synthetic methods, the exploration of mechanoenzymology offers exciting opportunities. The current success of this emerging technique should urge scientists to seriously consider this alternative.

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Keywords: green chemistry • enzymes • mechanoenzymology • ball milling • solvent-free

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