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Mechanoenzymatic transformations in the absence of bulk water – a more natural way of using enzymes

Sandra Kaabel,[a] Tomislav Friščić,*[a] and Karine Auclair*[a]

Department of Chemistry, McGill University, 801 Sherbrooke St. W. H3A 0B8 Montreal, Canada
E-mail: tomislav.friscic@mcgill.ca, karine.auclair@mcgill.ca

Abstract: Mechanochemical enzymatic reactions without bulk water have emerged as low-waste, efficient method to access useful chemicals and depolymerize biomass components in a single step. This emergent mechanoenzymatic reaction strategy is able to take advantage of enzymes' stereospecificity, regio- and stereoselectivity, and renewability, while avoiding bulk solvents, offering the opportunity to control the direction of the reaction, bypassing reactant solubility issues, and enabling reactions with water-sensitive substrates or products. Enzymes are traditionally used in dilute aqueous solution, which is far different from their crowded, water-depleted natural environment. This review outlines recent work which demonstrates that enzymes can be equally or even more efficient under mechanochemical conditions, without bulk aqueous or organic solvent.

1. Introduction

Various enzymes have evolved to catalyze reactions in the absence of an aqueous solvent. For example, bacteria and fungi living on moist plant matter secrete enzymes to break down the polysaccharides of vegetal tissues into oligomers and monomers, which, unlike biopolymers, can be internalized and used as nutrients.[1–3] Similarly, the total molecule concentration in the cell cytoplasm is very high, with 30-40% of the volume taken up by macromolecules alone.[4–6] Taken together, this implies that the natural setting of enzymatic reactions is considerably more crowded,[6–8] with significantly less water molecules available, than the dilute aqueous *in vitro* conditions traditionally used for enzyme catalysis in research and industry. Therefore, reactions carried out without bulk water may better represent the natural environment of enzymes, and consequently improve their performance in various applications.

Solvent-free (neat) chemical transformations have received a lot of attention in recent years in part for their potential to greatly reduce the amount of waste generated.[9–14] One example is mechanochemistry, now an established, powerful tool to conduct solvent-free reactions, often reaching higher yields and/or faster rates compared to equivalent transformations in solution.[15–17] In addition, mechanochemical treatment, in the form of grinding, milling, extrusion, or acoustic mixing, has been shown to alter the selectivity of reactions and generate products unachievable by solution-based approaches.[18,19] Its impact on the chemical industry merited mechanochemistry a place on the IUPAC list of 10 chemical innovations that will change the world.[20]

In mechanochemistry, reactions are accelerated by impact with the milling media or shear forces, often using a ball mill (Figure 1c-e) or a twin screw extruder (Figure 1f,g).[21–24] Whereas mills are well suited for batch laboratory scale reactions (hundreds of milligrams to a few grams), and even production scale (kilograms to tons) with horizontal rotary ball mills, extrusion encompasses easily scalable mechanochemical flow methods utilizing co-rotating twin screws to create shear and compression forces.[24] The impact force of ball milling depends on the frequency used, the material and the size of the milling media, with milling jars and balls being commercially available from tungsten carbide (WC), different types of stainless steel, zirconium oxide, quartz, agate, polymethyl methacrylate (PMMA) and polytetrafluoroethylene (PTFE, or Teflon). In contrast, shear and compression forces in a twin screw extruder can be tuned by adjusting not only the rotating frequency and temperature, but also the configuration of kneading and conveying elements (Figure 1g).[25]

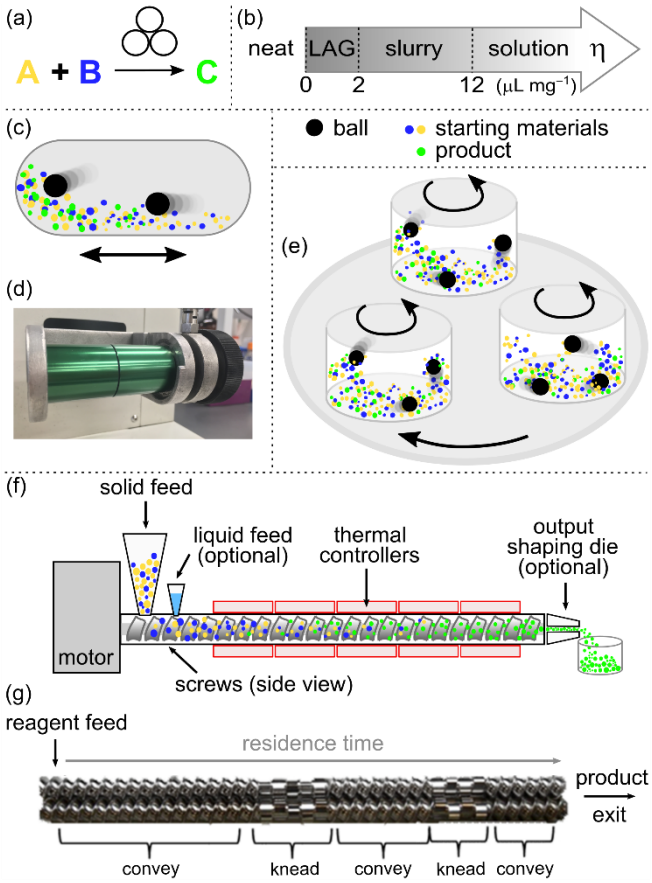


Figure 1. Selected definitions and methods in mechanochemistry. (a) The colour code of reactants (yellow, blue) and products (green) used in panels (c), (e) and (f), and the symbol for mechanochemical reactivity proposed by Rightmire and Hanusa.[26] (b) Neat, liquid-assisted, slurry and solution reactions with their approximate η range (μL mg^{-1}), introduced by Friščić *et al*.[27] (c-e) Mechanochemical reactions in a shaker (c,d) or a planetary (e) ball mill. (d) Picture of the jar mounted on a shaker ball mill. (f) Mechanochemical reaction carried out by twin-screw extrusion, showing the main components of an

extruder. (g) An example of the screw configuration, adapted with permission from [28].

A mechanochemical reaction generally involves mechanical agitation of two or more reactants (Figure 1b). In addition to reactions conducted by continuous application of such milling or grinding, in many cases a reaction of solids can be promoted through an initial short milling or grinding period, before static incubation. Such mechanically activated reactions are particularly interesting, as they offer the possibility of greatly reducing the energy consumption associated with a mechanochemical process.[29] Whereas many chemical and materials transformations can be achieved by simply milling or grinding starting materials, some of the most notable successes of mechanochemistry have recently been accomplished with mechanical agitation conducted in presence of catalytic additives. The most notable and widely used among such modified mechanochemical techniques is liquid-assisted grinding (LAG),[30] wherein minuscule amounts of a liquid additive provide a means to accelerate, direct or generally optimize mechanochemical reactivity.[31] Reactions employing LAG are distinguished from neat reactions or processes taking place in a slurry by the parameter η, which is defined as the ratio of the liquid volume to the weight of the solid reactants (Figure 1b).[27] The generally accepted η-range corresponding to LAG reactivity (ca. 0-2 μL mg^{-1}) was defined in a systematic study of co-crystal formation in the presence of different amounts of various liquid additives. Specifically, this study established LAG reaction conditions as those under which the reaction outcome remain independent of the solubility of the reactants.[27] Although the mechanism of action of the liquid in LAG is not fully elucidated, it is now known that a range of liquid properties, including polarity,[32] basicity[33] and even amount,[34] can play a significant but still unpredictable role in controlling the outcome of a mechanochemical process. The effectiveness of LAG has been demonstrated for organic and inorganic synthesis,[17,35] screening of polymorphism and cocrystals,[36,37] construction of metal-organic materials[26] and supramolecular complexes.[38] Fundamental principles and applications of LAG and related methodologies (e.g. ion- and liquid-assisted grinding or ILAG,[39] ionic liquid-assisted grinding or IL-AG,[40] polymer-assisted grinding or POLAG,[41] *etc.*) have been summarized in recent reviews.[17,42]

As summarized in this review, solvent-free mechanochemistry techniques have been applied to enzymatic transformations – giving rise to a growing field that may be termed mechanoenzymology. Beyond the already mentioned advantages of mechanochemistry, using neat grinding or LAG techniques with enzymes offers additional benefits. Perhaps the most appealing among these is that the absence of bulk water may better mimic enzymes' natural environment compared to dilute solution environments that have become ubiquitous since the beginning of enzymology. Furthermore, considering that many enzymes are known to display high substrate promiscuity, a solvent-free environment can bypass solubility issues, opening up a wealth of new reactivity.

This review will focus on solvent-free enzymatic reactions that can be initiated and/or sustained by kneading, milling, or extrusion,[16,43,44] as well as reactions that proceed by simple static incubation under controlled conditions, in a process known as aging.[45,46] The reports discussed below illustrate the unexpected robustness and efficiency of enzyme-catalyzed reactions under mechanochemical, water-depleted conditions, and the ability to conveniently favor a specific transformation by controlling the reaction environment. While the reports on the mechanochemical bioconjugation of enzymes can also elegantly address the stability of enzymes under ball-milling,[47] these chemical transformations will not be covered in this review. By bringing together the reported examples of solvent-less mechanoenzymatic transformations, the review aims to provide a useful guide through the techniques available and inspiration for further studies.

2. Lipase-Catalyzed Mechanoenzymatic Reactions by Ball Milling or Extrusion

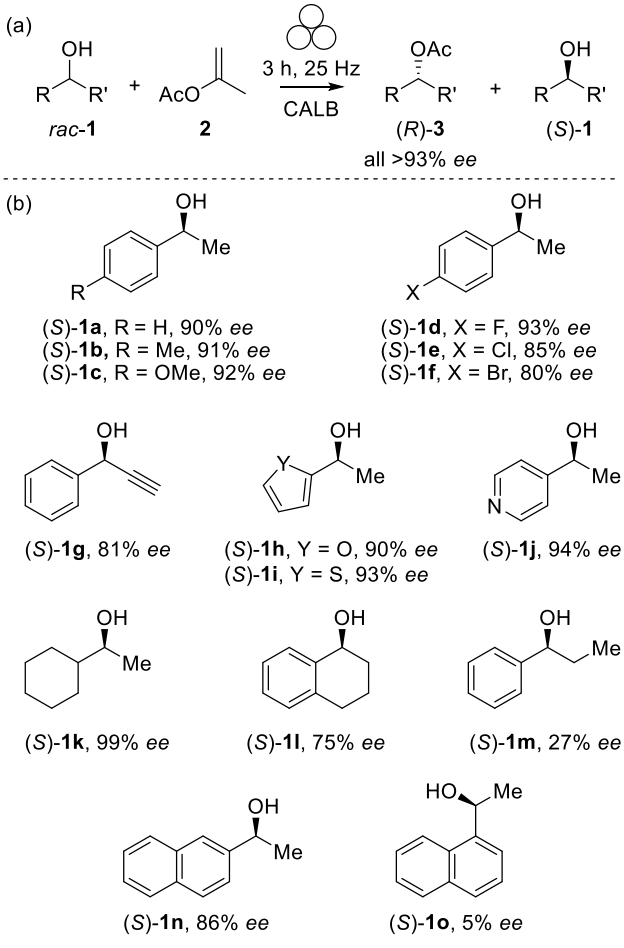
Lipases are among the most utilized biocatalysts, in academia and in industry,[48,49] owing to their high stereo- and regioselectivity, broad substrate specificity, and remarkable stability in aqueous, non-aqueous,[50] and even nonpolar media.[51–53] Indeed, from the perspective of biocatalysis, lipases have found applications also in highly unconventional environments, which include ionic liquids,[54] and supercritical fluids.[55] As a result of their ability to hydrolyse and process fats, lipases are widely used in detergents, food industry and have found utility in the production of various chemicals.[56] Immobilization of lipases on inert, insoluble supports was reported to improve their activity and stability, while at the same time allowing for convenient recycling of the biocatalyst.[57,58] For instance, the lipase B from *Candida antarctica* immobilized on acrylic resin (Novozyme 435, herein referred to as immobilized CALB) is one of the most applied biocatalysts, used in esterification, transesterification and hydrolysis reactions. Some of these applications have been detailed in a recent review by Fernandez-Lafuente and co-workers.[59]

2.1. CALB-Catalyzed Resolution of Secondary Alcohols by Acetylation

Hernández *et al*. reported the first mechanochemical reactions catalyzed by immobilized CALB without any aqueous or organic solvents.[60] The work demonstrated that racemic secondary alcohols such as **1** can be enantiospecifically acetylated with isopropenyl acetate (**2**) through ball milling in the presence of CALB, yielding (*R*)-**3** and the unmodified alcohol (*S*)-**1** in good-to-excellent stereoselectivity (Scheme 1a). The enzymatic reactions were performed by milling 50-82 mg of **1a**-**1o** with one equivalent of **2** and 30 mg of immobilized CALB beads (activity ≥ 5000 U/g) using a zirconia-based milling assembly (a 10 mL ZrO_2 milling jar loaded with a single 10 mm ZrO_2 milling ball) for 3 hours at a frequency of 25 Hz. Since many of the substrate alcohols (**1a**-**1d**, **1g**, **1k**, **1m**), the acetylating agent, and the products ((*S*)-**1e** and (*S*)-**1f**) are liquids, we refrain from using the conventional parameter η to describe these examples. They, however, illustrate well the resilience of an immobilized enzyme during extended ball milling and can provide fast kinetic resolution of *sec*-alcohols, comparable to those carried out in ionic liquids[61] and in bulk organic solvents[62]. In contrast, a CALB-catalyzed (immobilized, 25 mg) acylation of neat 1-phenylethanol (**1a**) with one equivalent of ethyl octanoate by conventional magnetic stirring was reported by Öhrner *et al.*,[63] yielding 50% conversion

in 90 hours. For most reactions, however, comparisons between matching solvent-free mechanochemical and stirred processes are lacking.

A wide scope of secondary alcohols (Scheme 1b), including aromatic (**1a**-**g**, **1n**), heterocyclic (**1h**-**j**) and aliphatic (**1k**, **1l**) alcohols were successfully resolved using the above mentioned mechanoenzymatic process. Sterically crowded secondary alcohols, such as 1-naphtyl ethanol (**1o**) and 1-phenylpropanol (**1m**) gave the corresponding esters (*R*)-**3o** and (*R*)-**3m** in equally high enantioselectivity, however in lower yield, thus resulting in a lesser enantiomeric excess of (*S*)-**1o** and (*S*)-**1m**, respectively. Notably, this enzymatic acetylation was also successful for the resolution of *rac*-**1a** generated *in situ* via mechanochemical reduction of acetophenone. An analogous lipase, *Burkholderia cepacia* lipase immobilized on diatomaceous earth (PS-IM), yielded (*R*)-**3a** with similar stereoselectivity (99% *ee*) as immobilized CALB, albeit in slightly lower yield (39%), and consequently generating (*S*)-**1a** in only 63% *ee*. Furthermore, the authors demonstrated the scalability of their method by preparing (*R*)-**3a** (44% yield, *ee* > 99%) and (*S*)-**1a** (44% yield, *ee* 98%) in gram quantities using a planetary ball mill.



Scheme 1. Mechanoenzymatic resolution of secondary alcohols by enantiospecific acetylation of racemic **1a**-**1o**, reported by Hernández *et al*.[60] (a) The overall reaction scheme. Note that the reactions also produce 2-propenol from **2**, which tautomerizes to acetone. (b) Resolved secondary alcohols (*S*)-**1**. The esters (*R*)-**3** were obtained with high enantiomeric excess (93%-99% ee) in all reactions, yet in variable yields, thus affording the shown enantiomeric excess for the unreacted alcohols.

The immobilized CALB was successfully recycled, as demonstrated with the resolution of *rac*-**1a**, notwithstanding a decrease in conversion to (*R*)-**3a** with each cycle, getting down to 27% (*i.e.* 37% *ee* of the unreacted alcohol (*S*)-**1a**) after the fourth round.[60] Based on previous reports that described the erosion of immobilized CALB by mechanical shear forces,[64,65] the gradual loss of enzymatic activity was ascribed to partial desorption of the enzyme from the acrylic resin upon milling. Alternative enzyme stabilization strategies[66] that may be more resistant to mechanical forces, such as covalent cross-linking or silicone coating of the beads,[65] may improve enzyme recyclability. Regardless of the lower conversion to (*R*)-**3a** with recycled CALB, the enantiospecificity of the esterification was not affected by the partial loss of enzyme.

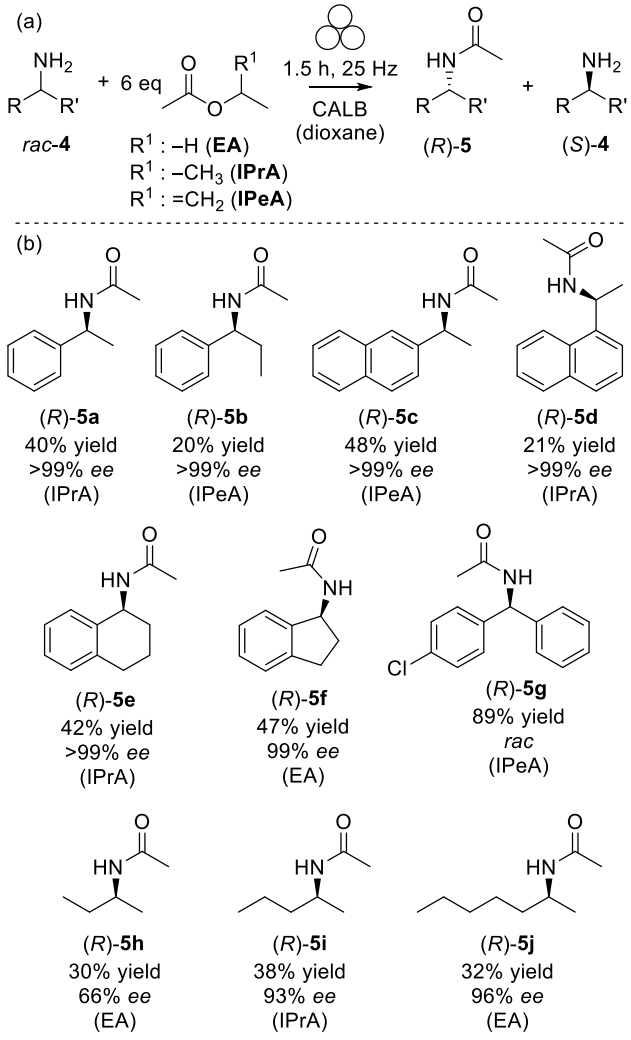
Kinetic resolution of secondary alcohols with immobilized enzymes in solution has been extensively studied.[67] The reactivity difference between the two enantiomers in solution is often not large enough to obtain good enantioselectivity at yields approaching 50%.[68] Coupling dynamic kinetic resolution (involving continuous racemization of the remaining alcohol) with enzymatic transformations, used to overcome yield limitations of enzymatic resolutions in solution,[69–71] has yet to be explored with mechanoenzymology.

2.2. CALB-Catalyzed Resolution of Primary Amines by Acylation

Pérez-Venegas and Juaristi have also tested immobilized CALB under ball milling without bulk water, as a catalyst for the enantioselective acetylation of racemic amines (Scheme 2a).[72] For example, milling of the racemic amine **4a** (100 mg) and six equivalents of either ethyl acetate (EA) or isopropyl acetate (IPrA) in the presence of immobilized CALB (100 mg) led to 25-40% conversion to (*R*)-**5a**. Similar to the acetylation of *sec*-alcohols, many of the amines used here and all acetylating agents are liquids. Milling of neat reactants in the presence of the immobilized enzyme proved optimal when using ethyl acetate as the acetylating agent, while isopropyl acetate afforded higher conversion to (*R*)-**5a** only when an additional 200 µL of 1,4-dioxane, toluene, diisopropyl ether or 2-methyl-2-butanol (2M2B) was introduced in the reaction mixture as a LAG additive. The highest conversion (40%) of (*R*)-**5a** was achieved with the addition of 1,4-dioxane (Scheme 2b).

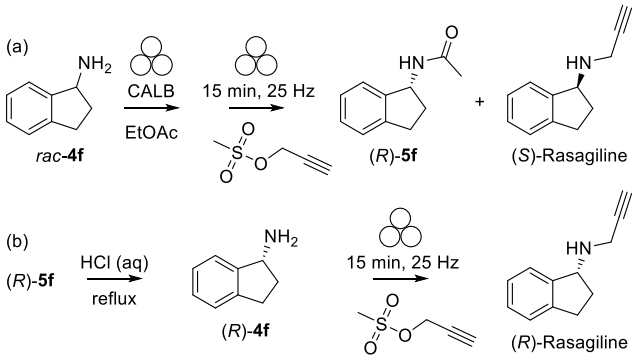
These optimized conditions were further studied for the acetylation of various racemic primary amines, such as 1-arylamines, amines on aliphatic rings and aliphatic amines, with three acetylating agents separately: EA, IPrA and isopropenyl acetate (IPeA). The amides (*R*)-**5** were obtained in excellent enantioselectivity (*ee* > 99%) in nearly all conditions tested, however the conversion to (*R*)-**5**, and consequently the enantiomeric excess of the amine (*S*)-**4**, varied with the acyl donor and specific milling conditions (Scheme 2b). Yields for the most advantageous conditions are shown on Scheme 2b for each product. The authors also carried out solution reactions in 2 mL of dioxane at 55°C or 80°C, which were shown to lead to lower conversion and enantiomeric excess. Furthermore, a non-mechanochemical solvent-free approach developed by Kanerva and co-workers, which involves reacting 2 mmol of *rac*-**4a** with one equivalent of isopropyl acetate (IPrA) in the presence of 25 mg of immobilized CALB, led only to 7-10% conversion of (*R*)-**5a**, when incubated in a shaker at 170 rpm for 6 hours.[73] Acetylation

proceeded to 50% conversion however when either isopropyl or ethyl methoxyacetate was used as the acyl donor.[73] Systematic studies comparing solvent-free reactions induced by milling, shaking and stirring at equal enzyme loading with a variety of amines would be valuable in determining differences between these approaches.



Scheme 2. Enzymatic enantiospecific acetylation of racemic amines by ball milling in the absence of bulk water.[72] (a) The overall reaction scheme. Note that the reactions also produce either ethanol (from EA), isopropanol (from IPrA) or 2-propenol (from IPeA, the product tautomerizes to acetone). (b) Resulting amides **5a**-**5j**, with the reaction yield and respective enantiomeric excess for optimal acetylating conditions.

Based on the high yield and excellent enantiomeric excess obtained for (*R*)-**5f**, Pérez-Venegas and Juaristi developed a simple and highly efficient synthesis of the enantiomerically pure drug (*R*)-rasagiline (Scheme 3). (*R*)-Rasagiline (Azilect®) is a monoamine oxidase B inhibitor, used to treat symptoms of Parkinson's disease.[74] The enantioenriched amine (*S*)-**4f** from the mechanoenzymatic acetylation reaction was converted *in situ* to (*S*)-rasagiline after addition of propargyl mesylate and 15 minutes of milling at 25 Hz (Scheme 3a). The biologically active (*R*)-enantiomer was obtained by chromatographic separation and hydrolysis of the (*R*)-**5f** amide (isolated in 44% yield from step shown on Scheme 3a), followed by a mechanochemical reaction with propargyl mesylate to afford (*R*)-rasagiline in > 99% *ee* and high yield (Scheme 3b, the mechanochemical step proceeds in 98% yield).



Scheme 3. Enantioselective synthesis of both (*R*)- and (*S*)-rasagiline by combination of a mechanoenzymatic acetylation with CALB and mechanochemical propargylation.[72]

2.3. CALB-Catalyzed Esterification of Lignin Model Compounds

In a first example of CALB-based mechanoenzymatic catalysis with solid substrates, Weißbach *et al.* have studied the regioselectivity of immobilized CALB in the acylation of lignin model compounds **6** (Scheme 4a).[75] Lignin is one of the most abundant biopolymers and a potential renewable feedstock for aromatic commodity chemicals.[76,77] Modifying the lipophilicity of lignin by acetylation[78] has been used to generate high-value materials, such as composite thermoplastics,[79,80] lignin carbon fibers,[81] and coating films.[82] Bridging solid-state mechanochemical approaches and biocatalysis to target poorly soluble or insoluble materials like lignin, while benefiting from the high selectivity of enzyme-catalyzed reactions, is an appealing concept for biomass valorization.

Owing to the large diversity of functional groups and linkages present in the three-dimensional polymeric structure of lignin,[83,84] dilignols such as **6** (Scheme 4) are commonly used as model compounds,[85–92] circumventing the challenges of analyzing the complex product mixtures obtained from the natural polymer. Weißbach *et al.* demonstrated that the primary hydroxyl group of dilignols **6** (50 mg) is selectively targeted by immobilized CALB (30 mg) under ball milling, yielding **8a**-**8e** when acetate donors **7a** is used (vinyl or phenyl acetate, **7b** and **7c** respectively, were also found suitable) or **8g**-**8i** when long-chain vinyl esters **7g**-**7i** are used as acyl group donors (Scheme 4b). The secondary hydroxyl group was preserved even after milling with a 4-fold excess of the acyl donor. The ratio of the liquid acetylating agents to the solid dilignols and catalyst was within η = 0.8-2.35 µL mg^{-1}, which thus sets these reactions within the LAG region on the η-scale (Figure 1). An equivalent mechanochemical base-catalyzed esterification without enzyme was shown to lead to a diacetylated product, which highlights the value of the mechanoenzymatic approach for controlling the degree of esterification, and consequently the properties of the product.

Furthermore, the enzymatic reaction proceeded faster with *erythro*-diastereomers than with the corresponding *threo*-

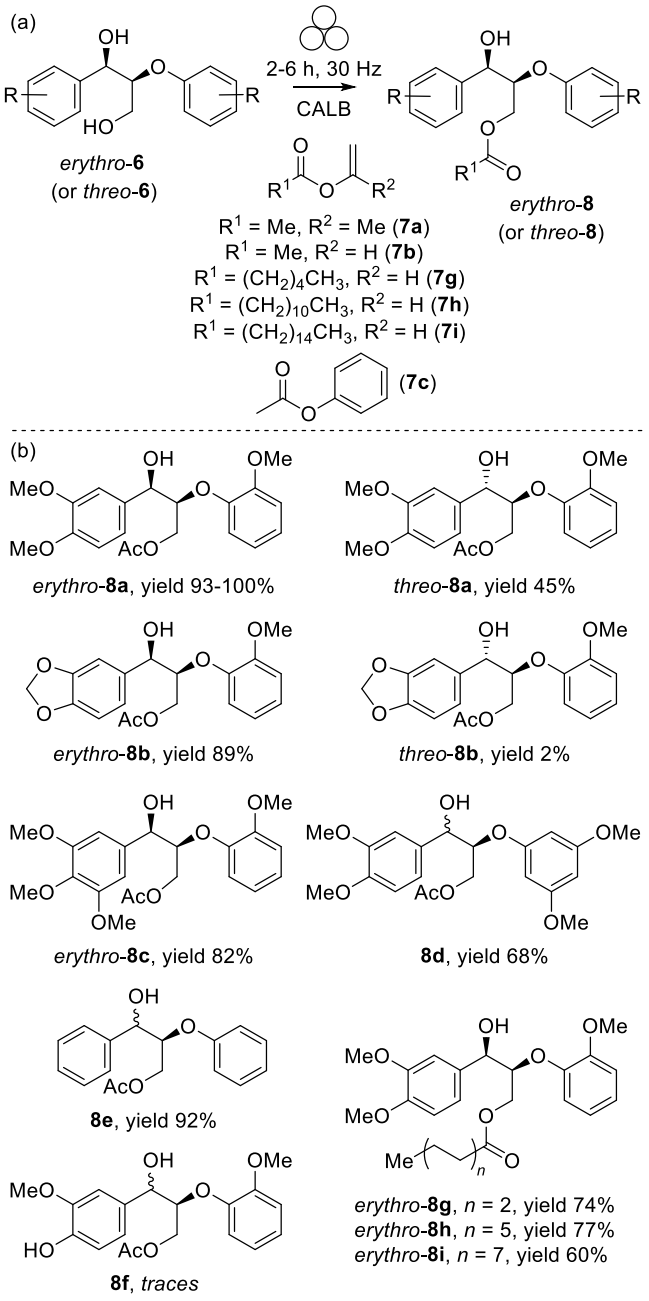
diastereomers (Scheme 4b), implying some diastereospecificity for this biotransformation. Ester **8f**, which mimics the terminal units of the lignin polymer by bearing a phenolic group, could however not be produced in significant yield with this method. The phenolic group was not acetylated either. This suggests that enzymatic esterification of lignin itself will likely show a complex regioselectivity pattern. The catalytic activity of immobilized CALB was unparalleled by alternative commercial lipases, namely lipase A from *Candida antarctica* immobilized on acrylic beads, *B. cepacia* lipase immobilized on diatomaceous earth (PS-IM), and the non-immobilized lipase A from *Aspergillus niger*.

2.4. CALB-Catalyzed Hydrolysis of Amino Esters

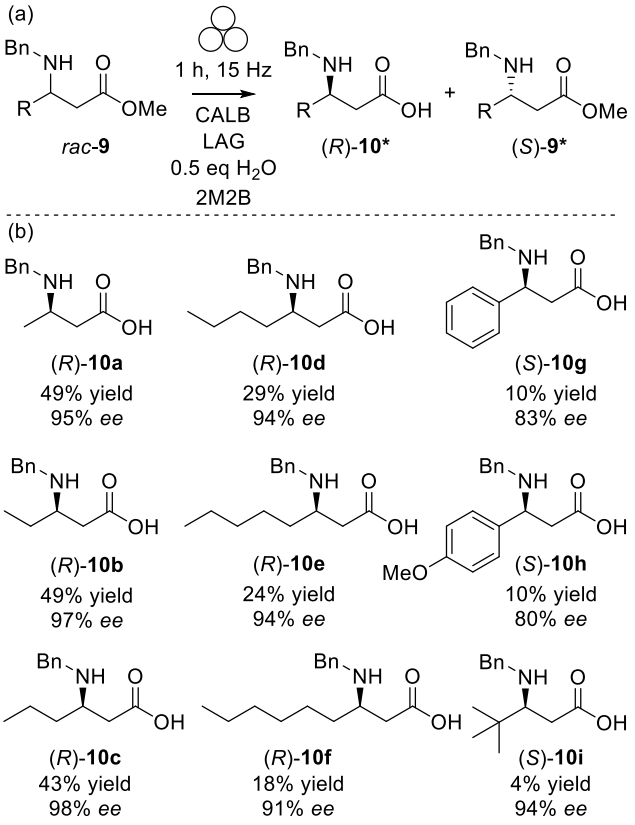
Many enzymes are known to be capable of catalyzing either direction of a reaction depending on the conditions. Juaristi and coworkers developed a lipase-catalyzed mechanochemical method for the resolution of racemic β-amino esters via enantioselective hydrolysis (Scheme 5).[93] All esters hydrolyzed in this study were again liquids. The authors report that ball milling *rac*-**9a** (82 mg) with half an equivalent of water in the presence of immobilized CALB (40 mg) afforded (*R*)-**10a** in 41% yield (not shown) and good enantioselectivity (*ee* 92%). Notably, the hydrolysis of *rac*-**9a** was scaled up 10-fold, with little loss in conversion or enantioselectivity. Furthermore, 40% conversion to (*R*)-**10a** was achieved from *rac*-**9a** even with 10-times less immobilized CALB (5 wt% relative to the substrate), without compromising the excellent 94% *ee* of the product.

Introducing the liquid additive 2-methyl-2-butanol (2M2B) to the reaction mixture increased the yield to 49% for the conversion of *rac*-**9a** to (*R*)-**10a**, thus affording the remaining (*S*)-**9a** in excellent 99% enantiomeric excess. These conditions were however less efficient for obtaining β-amino acids that contain aromatic (**10g**, **10h**) or bulky (**10i**) groups, or longer aliphatic chains (**10d**, **10e**, **10f**) from the corresponding esters (Scheme 5b). A similar trend was noticed previously in the enantiospecific enzymatic hydrolysis of **9a**, **9g** and **9i** with immobilized CALB in bulk *n*-hexane, affording 45% conversion to (*R*)-**10a** by stirring the solution for 2 h at 45°C, 23% conversion to (*S*)-**10g**, and undetectable conversion to (*S*)-**10i**, when stirred for 24 h at 60°C.[94] Surprisingly though, (*S*)-**10i** was obtained enantiomerically pure with full 50% conversion in bulk 2M2B.[94]

Methanol extraction of both the recovered (*S*)-**9** and the acid (*R*)-**10** from the crude milled reaction mixture allowed recovery of 90% of the immobilized enzyme, however with a significant loss in activity. The absence of other solids during milling, a situation which best describes the hydrolysis reaction of the liquid esters *rac*-**9** at low conversions to the solid (*R*)-**10** product, can lead to higher energy impact to the solid biocatalyst, and may accelerate the deactivation or desorption of the enzyme, compared to reactions where the kinetic energy of the ball can be dissipated between more solids in the jar. It would be interesting to investigate the effect of solid additives on the recyclability of immobilized CALB, and compare to reactions of solid reactants.



Scheme 4. Mechanochemical enzymatic esterification of lignin model compounds **6**.[75] (a) The overall reaction scheme. Note that the reactions also produce either 2-propenol (from **7a**, the product tautomerizes to acetone), vinyl alcohol (from **7b**, **7g**-**7i**) or phenol (from **7c**). (b) The resulting esters with corresponding yields.



Scheme 5. Enzymatic enantiospecific hydrolysis of racemic β-amino esters by ball milling.[93] (a) The overall reaction scheme. The reactions also produce methanol from rac-**9**. (b) The resulting β-amino acids **10a**-**10i**, with the respective reaction yield and enantiomeric excess of the product. *The nomenclature for the enantiomers of **10g**-**10i** changes to (*S*) due to opposite priority of the substituents on the stereocenter.

2.5. CALB-Catalyzed Ring-Opening Polymerization of ω-Pentadecalactone

It is likely that the first example of a neat enzymatic extrusion reaction was in a report by Gross and coworkers, on the ring-opening polymerization of ω-pentadecalactone (**11**, Figure 2a) using 10% (w/w) of the immobilized CALB.[95] The enzymatic activity was maintained even at high temperatures (90°C) and high shear forces (60 rpm), reaching 99% conversion of solid **11** to high molecular weight polymers (M_n = 90 000 g mol^{-1}) after 20 minutes of extrusion. In comparison, the polymerization reaction of **11** in a toluene solution at 80°C, at the same enzyme to substrate ratio, afforded similar length (M_n = 85 000 g mol^{-1}) and dispersity polymers, but only after 72 hours.[96] The progress of the mechanochemical polymerization reaction was monitored by measuring the vertical force exerted on the screws, which increases as the degree of polymerization leads to higher viscosity of the reaction mixture (Figure 2c). The authors found that the rate-limiting step was chain growth initiation, as lower enzyme loading prompted an initial lag phase. At 95% conversion of the monomer, the polymer length increased rapidly, suggesting that the lactone ring-opening process was outcompeted by polycondensation between the chain ends.

After 20 minutes of extrusion, the recovered immobilized CALB beads retained 75% of their activity, showing only some mechanical surface defects, without overall decrease in the bead size (Figure 2b). Based on elemental analysis (namely the percent nitrogen value determined by X-ray photon spectroscopy), the protein content of immobilized CALB beads decreased from 8.4% for the unused beads, to 4.5% after extrusion, suggesting that the lower activity of the recycled enzyme might be due to protein leaching and not protein denaturation.

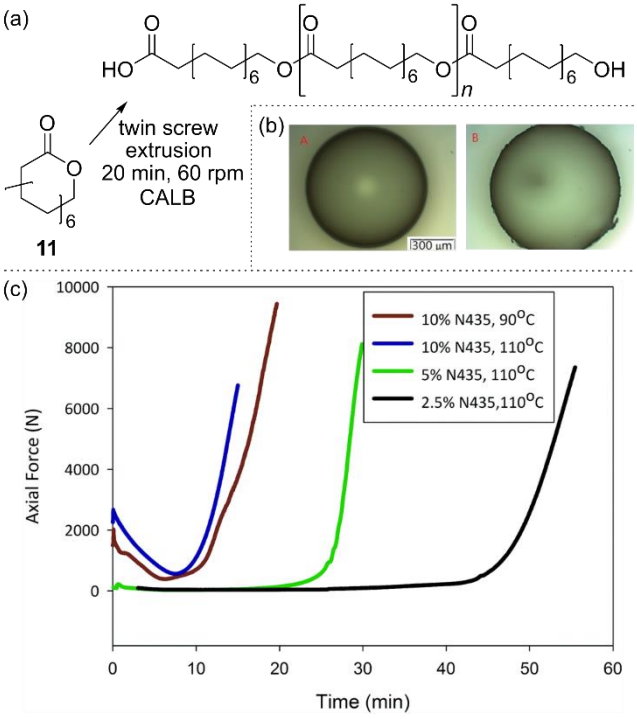


Figure 2. Ring-opening polymerization of ω-pentadecalactone (**11**) by twin screw extrusion catalyzed by immobilized CALB.[95] (a) Hiighlight of the overall reaction scheme and conditions used. (b) Image of a bead of immobilized CALB on acrylic support before (left) and after (right) extrusion, showing minute mechanical erosion at the surface of the bead. (b) Reaction monitoring based on the extruder force over time at different enzyme loading (2.5% black, 5% green and 10% brown) and at different temperatures (90°C brown and 110°C blue). Figures 2b and 2c are reproduced from ref. [95] with permission from The Royal Society of Chemistry.

2.6. Remarks on CALB-Catalyzed Mechanoenzymatic Reactions

Except for the latter example which constitutes a single solid substrate transformation, the lipase-catalyzed mechanochemical reactions reported to date occur either between solid and liquid reactants, or between two liquid reactants. While chemical reactions between liquid substrates are less common in the context of mechanochemistry, together the reported mechanoenzymatic CALB reactions clearly illustrate the benefit of milling and extrusion in reducing solvent waste compared to reactions in bulk solvent. Similar to lipase-catalyzed kinetic resolutions carried out in organic or aqueous solutions,[97] solvent-free mechanoenzymatic reactions proceed in high enantio- and regioselectivity and can be directed to esterification/amidation in water-depleted media, or to hydrolysis in the presence of water. Interestingly, the substrate preference of CALB seems mostly unaffected by mechanical stress or the solvent-free environment.

Based on the protein content of the immobilized CALB beads measured by Gross and coworkers, the acrylic support accounts for most (around 90%) of the weight of the enzymatic preparation added, and therefore the enzyme loading is lower than apparent from the reaction descriptions,[95,98] but still significant in the examples discussed above. Strategies to achieve similar yields at lower enzyme loading would broaden applicability. Use of CALB immobilized on acrylic beads has so far been prevalent, but proved susceptible to mechanical erosion, limiting the recyclability of the catalyst compared to solution reactions. Further research is required to improve the resistance of CALB to mechanical shear forces. As an example, Jbilou *et al*. elegantly demonstrated that non-immobilized CALB could hydrolyse polybutylene succinate (PBS) during extrusion, even at temperatures above the melting point of PBS (110°C), when the enzyme was first entrapped into the PBS matrix and then reacted by extrusion at very low water content.[99] A recent example by Vaidya *et al*. reported a higher activity and recycling capacity of a thermostable lipase from *Candida antartica* (NovoCor) compared to CALB, for the grafting of caprolactone to chitosan by reactive extrusion.[100]

3. Protease-Catalyzed Mechanoenzymatic Reactions by Ball Milling or Extrusion

Proteases are enzymes that catalyze the catabolism of proteins through hydrolysis of peptide bonds. Similar to lipases, proteases can be substrate promiscuous and can catalyze either hydrolysis or formation of amide bonds, depending on the reaction conditions.[101,102] In aqueous media, the equilibrium of protease reactions favors peptide hydrolysis, but may be shifted towards peptide synthesis in concentrated substrate suspensions or with the addition of organic (co-)solvents.[101,103] Dipeptides and oligopeptides are frequent targets in the pharmaceutical industry,[104,105] with peptide-based drugs available for the treatment of diabetes, gastrointestinal disorders, osteoporosis, and immune system disorders, among others.[104] Chemoenzymatic peptide synthesis, known since the mid-20th century,[106] offers some advantages over standard chemical solid-phase peptide synthesis (SPPS), including the possibility to couple amino acids with unprotected side chains efficiently, without the use of harsh reaction conditions or toxic reagents.[107] High volumes of organic solvents and/or continuous removal of the water produced during synthesis are however often necessary to avoid the competing hydrolytic activity of proteases, thus introducing enzyme stability and substrate solubility issues.[108] Mechanochemical reactions, which proceed either in the absence or with very small quantities of a liquid additive, present a novel approach to control the reactivity of proteases. Mechanochemica non-enzymatic solid-state approaches for dipeptide and oligopeptide synthesis[109–113] have been shown to surpass solution synthesis and even SPPS in terms of shorter reaction times, improved product purity and reduced environmental impact.[114,115]

3.1. Protease-Catalyzed Dipeptide Synthesis

In 2017, Hernández and coworkers extended the scope of enzymes used in mechanoenzymatic reactions with the protease-catalyzed formation of amide bonds to yield α- and α/β-dipeptides (Scheme 6).[116] The enzymes studied were non-immobilized papain from *Carica papaya* (≥3000 U/g), papain from crude papaya latex (1500-10000 U/g), and the serine protease subtilisin from *Bacillus licheniformis* (Alcalase®, 653 U/g). Papain is a cysteine protease exhibiting endopeptidase, amidase and esterase activities.[117] Subtilisin is a serine endopeptidase widely used in detergents and household cleaning products.[118] All tested enzymes remained active during the 2-hour ball milling treatment and afforded good yields of the dipeptide **14a** in the benchmark reaction between *N*-protected L-alanine methyl ester (**12**) and the HCl salt of L-phenylalanine amide (**13**) in the presence of Na_2CO_3. The high efficiency of these reactions reveals that mechanoenzymatic catalysis is not limited to immobilized enzymes. A small amount of the protease activator L-cysteine was shown to increase the activity of papain in peptide bond formation, as did the introduction of hydrates to the reaction mixture. When the base was added as a monohydrate (η = 0.034 μL mg^{-1}), the yield of dipeptide **14a** augmented from 63% to 82%, and further increased to 90% with the corresponding decahydrate (η = 0.34 μL mg^{-1}). The water molecules were proposed to stabilize the active configuration of the enzyme, and improve mixing of the reactants by leading to a more homogeneous reaction mixture upon milling, which apparently surpasses the influence of water to drive the reaction towards hydrolysis.

Ultimately, quantitative conversion to the dipeptide was achieved by doubling the enzyme loading, from 10 to 20 wt% relative to the substrates. The optimized conditions allowed variations on both the amino acid ester (**12**) and the amino acid amide (**13**), enabling synthesis of a wide scope of dipeptides (**14a**-**14h, 14l**-**14n, 14p**-**14s**) (Scheme 6b). The D-alanine methyl ester was not well tolerated by papain, leading to significantly lower yields (**14n**), and D-phenylalanine phenyl ester did not react appreciably (*e.g.* **14j**, **14o** were not formed). Interestingly, alkylammonium chlorides were accepted as acyl acceptors (*e.g.* to generate **14t-14v**).

3.2. Protease-Catalyzed Oligopeptide Synthesis

Hernández and co-workers next applied the papain-catalyzed mechanoenzymatic process to the preparation of oligopeptides.[119] Oligomerization of the esters of either L-alanine, L-phenylalanine, L-leucine or glycine, in the presence of papain, afforded homopolypeptides with the degree of polymerization conveniently controlled by adjusting the duration of milling and the amount of water (Scheme 7). The yields with hydrophobic L-Phe and L-Leu esters were enhanced with additional water, whereas anhydrous conditions were necessary to obtain good yields with the more hydrophilic L-Ala and Gly esters (Scheme 7b). For all amino acid esters tested, the presence of water resulted in longer oligomers (DP > 8), compared to anhydrous conditions. Increasing polymer length was additionally correlated with decreasing hydrophobicity and steric bulkiness of the building block. Interestingly, with oligo(L-Phe) the longest oligomers (DP = 10) were observed after a short 30-minute milling (at low 33% conversion), while longer milling periods led to higher yields (99% at 2 h) but shorter oligomers (DP = 8), thus implying reversibility of the enzymatic oligomerization.

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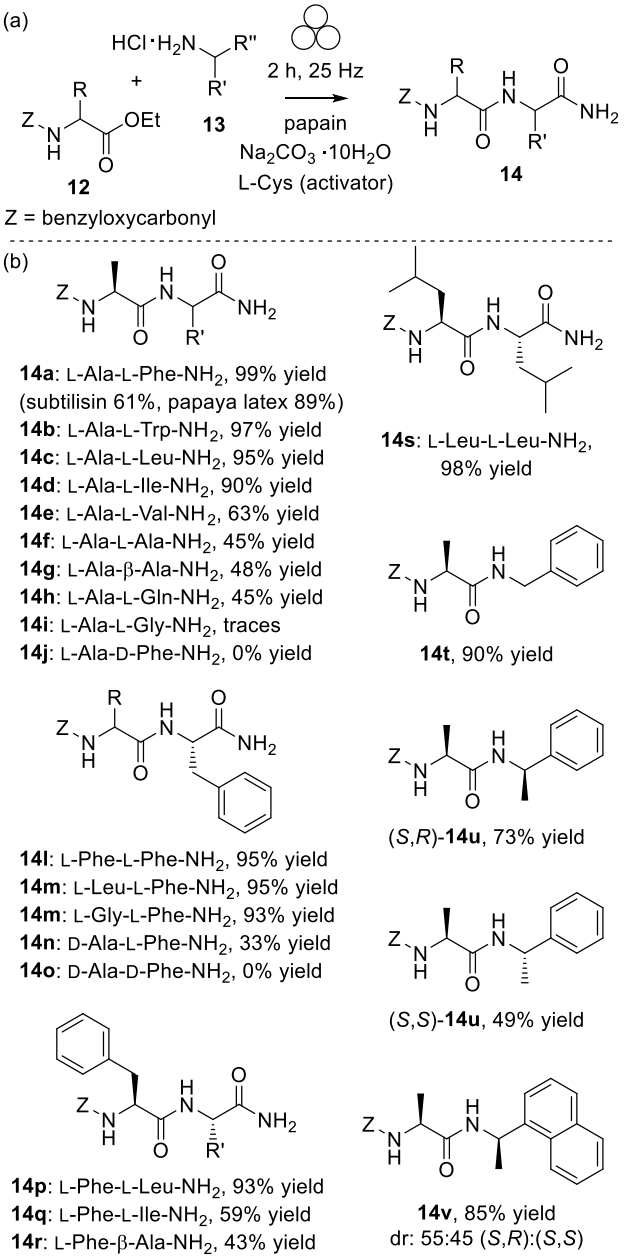
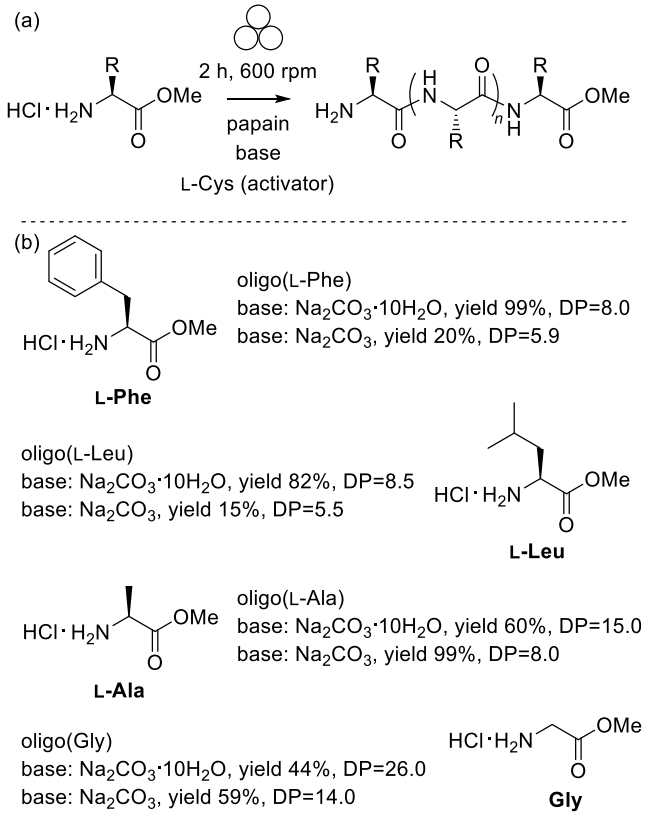


Scheme 6. Protease-catalyzed mechanoenzymatic synthesis of dipeptides **14a**-**14s** and amides **14t**-**14v**.[116] (a) Overall reaction scheme. Note that the reactions also produced NaCl, EtOH, water and CO_2. (b) Resulting products with the corresponding isolated yield.



Scheme 7. Mechanoenzymatic synthesis of oligopeptides from L-phenylalanine, L-leucine, L-alanine, and glycine methyl esters.[119] (a) Overall reaction scheme. Note that the reactions also produce NaCl, MeOH, water and CO_2. (b) Resulting oligopeptides showing the yield and average length of the resulting oligomers (DP; degree of polymerization) using either hydrated or anhydrous base in the reaction mixture.

Prompted by the high oligomerization yield achieved in shaker and planetary ball mills, the enzymatic reaction was scaled up using twin-screw extrusion to demonstrate the potential of continuous flow mechanoenzymatic reactions (Figure 3). A solid mixture of 10 g of the desired amino acid ester, 0.086 g of papain, and 0.033 g of L-cysteine**,** together with 1 equivalent of $Na_2CO_3{\cdot}10H_2O$ was first manually mixed, and then fed to the twin-screw extruder inlet. Extrusion allowed for more accurate control over the temperature, which was fine-tuned together with varying the screw speed and configuration, affording near quantitative yields of oligo(L-Phe) and oligo(L-Leu) from the corresponding amino acid esters.[120] In contrast, oligo(L-Ala) was extruded in approximately 50% yield from the corresponding ester, thus agreeing well with the trends observed in ball milling, where the more hydrophilic amino acid esters led to lower conversions in the presence of water (introduced as hydrates). The degree of polymerization also followed the same trend as observed in the ball mill (*i.e.* L-Ala > L-Leu > L-Phe), however with a steeper incline. The oligopeptides obtained by twin screw extrusion were subsequently studied as catalysts in the asymmetric Julia-Colonna epoxidation of chalcones. Oligo(L-Leu) proved to be the most efficient organocatalyst.

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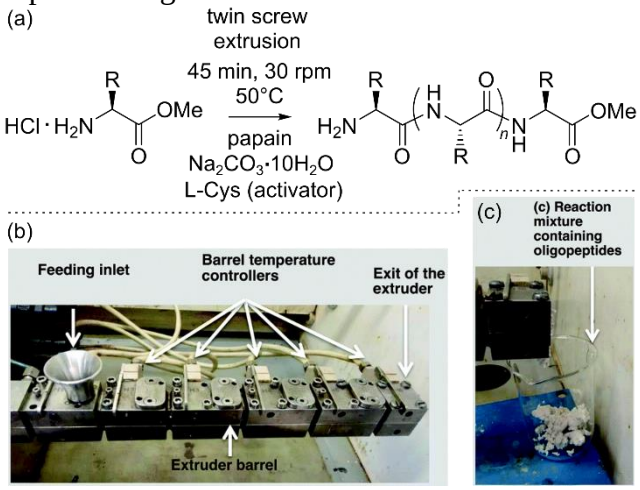

Figure 3. Papain-catalyzed mechanoenzymatic oligopeptide synthesis by twin-screw extrusion.[119] (a) The overall reaction scheme. (b) A photograph of the twin-screw extruder. (c) The extrudate mixture containing the oligopeptides. The photos (b) and (c) are reproduced from ref. [119] with permission from The Royal Society of Chemistry.

3.3. Remarks on Protease-Catalyzed Mechanoenzymatic Reactions

In summary, the above described two reports of protease-catalyzed reactions under mechanochemical conditions clearly testify that proteases can efficiently promote the formation of peptide bonds in the absence of bulk solvent. The mechanochemical approach offers new means to limit back-hydrolysis of the desired peptide, as showcased with the solvent-free mechanoenzymatic synthesis of oligopeptides, which has proven challenging to achieve in solution.[107] Examples of mechanoenzymatic peptide synthesis are thus far limited to substrates bearing hydrophobic side chains, and further research is necessary to extend the method to other amino acids present in relevant therapeutic oligopeptides. Mechanoenzymology can however alleviate limitations associated with solubility, thus potentially providing a useful technique for enzymatic condensation of larger peptide fragments[121] synthesized by SPPS or isolated by cleavage of natural proteins.

4. Mechanoenzymatic Reactions of Glycosyl Hydrolases

4.1 Lignocellulosic biomass as a chemical feedstock

As the most abundant natural polymer on earth, cellulose constitutes a substantial renewable resource.[122,123] Lignocellulosic biomass from forestry residues or agricultural waste provides an ideal feedstock for ethanol production as it does not compete with food production. The glycosidic bonds of cellulose are however highly recalcitrant, requiring harsh chemical reagents and conditions to produce fermentable sugars. Moreover, one of the leading problems associated with acid hydrolysis of lignocellulosic biomass is the poor fermentability of the obtained sugars by yeast, due to growth inhibition by side products, such as furfural and phenols formed during acidic treatment.[122,124] Whereas enzymatic hydrolysis proceeds under milder conditions and affords a cleaner and safer product, cellulase reactions are typically conducted in dilute aqueous solvent environments, which are not compatible with poorly soluble lignocellulosic biopolymers. Consequently, existing enzymatic approaches to hydrolytic cleavage of cellulose from biomass all depend on prior chemical pretreatment of the material, for example using strong acids or bases, in order to make the cellulose polymer more accessible to the enzyme. Notably, while twin screw extrusion has been investigated with cellulases by combining the steps of alkaline pre-treatment, neutralization and enzyme impregnation of lignocellulosic biomass,[125–128] dilute suspensions (*i.e*. 2.5% solids in water) remain the norm. A notable exception was reported by Duque *et al*. who evaluated the saccharification of the concentrated extrudate (30% solids in water, corresponding to a slurry with η = 3.3 μL mg^{-1}) upon incubation in the presence of a mixture of cellulases and xylanases, and reached a production of 32 g glucose and 18 g xylose per 100 g dry extrudate.[128]

4.2. Cellulase-Catalyzed Depolymerization of Cellulose by Reactive Aging (RAging)

Cellulases are often secreted in the environment by microorganisms such as bacteria, fungi and protozoa for the degradation of biomass cellulose into simple sugars that can penetrate cells and be used as nutrients.[129] Although not the sole enzymes responsible for cellulose degradation, cellulases include enzymes with three different types of activity: endoglucanase, exoglucanase and β-glucosidase (Figure 4). As glycosyl hydrolases, these enzymes use water, often only present as moisture on the surface of cellulose-containing materials. This has inspired a solvent-free mechanoenzymatic approach to cellulose saccharification, which avoids both the solubility restrictions of cellulose and the need for biomass pre-treatment.[130] Specifically, Hammerer *et al*, have shown that milling a mixture of lyophilized cellulases (5% loading), microcrystalline cellulose, and water (η = 0.95 μL mg^{-1}) in a shaker ball mill for 30 min yields 11% cellulose hydrolysis, implying that all three types of cellulase enzymes are resilient to ball milling. While milling led to the formation of both glucose and cellobiose (the glucose dimer), adding a period of aging (static incubation) at 45°C was found to stimulate β-glucosidase activity, leading to glucose as the main hydrolysis product in 23% yield after one week of aging with *Trichoderma reesei* cellulases. The enduring production of glucose after milling is significant, and demonstrates that cellulase activity is resilient to LAG. While this observation is in contrast with information found in literature,[131–133] which indicates that cellulases are deactivated upon milling with their substrates, it also opens up an opportunity to develop a highly energy- and materials-efficient approach to enzymatic cellulose saccharification.

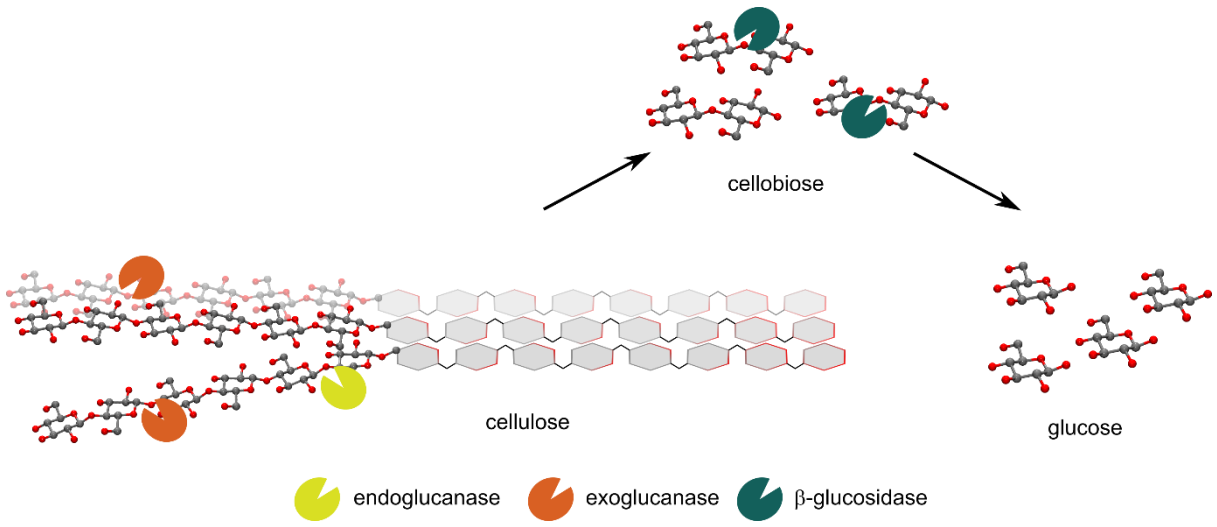

Figure 4. The three types of cellulase enzymes: endoglucanases (yellow), that cleave cellulose chains at random sites, generating oligosaccharides; exoglucanases (or cellobiohydrolases, orange) which progressively cleave cellobiose units from the ends of polysaccharide chains; and β-glucosidases (green) that hydrolyze soluble cellobiose to glucose.[134]

The observation that both milling and aging follow hyperbolic reaction kinetics led to the development of a novel process termed reactive aging (RAging), based on alternating cycles that combine mechanical activation by milling with subsequent aging (Figure 5). The application of RAging led to substantially higher conversion to glucose in a shorter period. Optimizing the RAging process with only 1.6 wt% of *T. reesei* cellulases led to 20% conversion to glucose in merely 12 hours. This was achieved by repeating one-hour cycles consisting of 5 minutes milling in a shaker mill operating at 30 Hz, and 55 minutes of aging at 55°C. This is a striking improvement compared to only one cycle of milling, followed by aging, which required 7 days to achieve a comparable conversion. Moreover, the reaction efficacy varied with the enzyme source and cellulases from *Trichoderma longibrachiatum* proved even better suited for RAging. The application of *T. longibrachiatum* cellulases afforded an unprecedented 50% conversion of microcrystalline cellulose to glucose in 12 hours at 3 wt% enzyme loading. This corresponds to a glucose concentration in the final reaction mixture of 3.2 M, which would be difficult to achieve under dilute aqueous conditions since β-glucosidases are typically inhibited by glucose at millimolar concentrations.[135]

In addition to microcrystalline cellulose, cellulose from raw lignocellulosic samples, such as hay and cedar wood sawdust, were similarly hydrolyzed to glucose by solvent-free RAging, directly without any chemical pre-treatment of the biomass. The RAging method generated three times more glucose than the comparable hydrolysis reactions carried out in the presence of bulk water (buffered or not) and at equivalent enzyme-to-substrate ratio. Interestingly, the remaining cellulose after the cellulases RAging reaction was mostly cellulose nanocrystals (CNCs), a high-value substrate for coatings,[136,137] membranes,[138,139] and tissue scaffolding,[140] used for example in medical devices and in biodegradable packaging materials.[141,142] With its versatility, unequalled space-time yield (20 g of glucose L^{-1} h^{-1}), and highly concentrated product, the RAging process is creating interesting opportunities in biocatalysis.

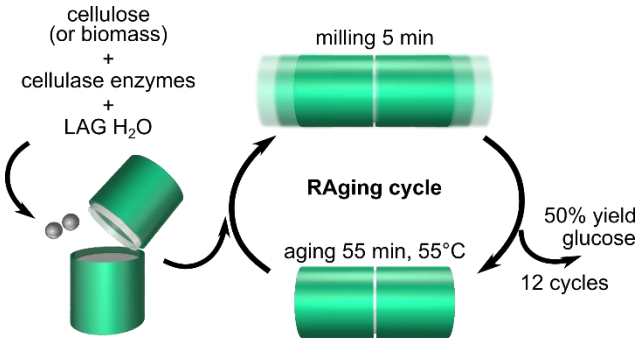

Figure 5. Schematic representation of the RAging process for the hydrolysis of microcrystalline cellulose (MCC) or biomass by cellulases. The scheme illustrates results from the conversion of MCC to glucose after 12 cycles of RAging (where the mechanical activation is based on LAG with water at η = 0.8 μL mg^{-1}), catalyzed by 3 wt% *T. longibrachiatum* cellulases.

Very recently Hammerer *et al*. demonstrated that enzyme reactivity can be improved by the presence of inert solid additives during milling.[143] Namely, cellulose, hemicellulose (xylans) and lignin, as well as synthetic polymers like polystyrene, polyethylene glycol 4000 and polymethylmethacrylate were shown to stabilize β-glucosidase (Figure 4, shown in green) during mechanoenzymatic hydrolysis of cellobiose into glucose. Furthermore, slight modifications to the amount of water in the reaction mixture (*i.e.* modifications to the η parameter) were found to allow favoring either cellobiose hydrolysis or glucose condensation. Such ability to direct the position of the enzymatic reaction equilibrium is not possible in dilute solutions, where there is always a significant excess of water, and is yet another example of additional synthetic opportunities being created through the mechanochemical reaction environment.

4.3. Chitinase-Catalyzed Depolymerization of Chitin by RAging

Chitin is the most abundant nitrogen-containing biopolymer on Earth, and therefore an attractive renewable feedstock to produce food supplements, agrochemicals and drugs.[144] The linear polymer of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc; Figure 6a) is a major component of crustacean shells, insect exoskeletons, and fungal cell walls, among others.[144,145] Producing high purity GlcNAc from chitinous biomass through chemical hydrolysis is, however, plagued by side reactions related to the harsh reagents needed to cleave the insoluble, unreactive polymer.[146] Use of chitinolytic enzymes, or chitinases,[147] in typical aqueous suspensions provides a more environment-friendly depolymerization route, but is slow and still requires chemical pre-treatment of the biomass.[148]

A recent study by Therien *et al*.[149] demonstrated that chitinases are not only several fold more efficient in the absence of bulk water, but are also more selective and generate a highly concentrated GlcNAc product. Under RAging conditions (η = 1.7 μL mg^{-1}), only 0.7 wt% of chitinases from a commonplace terrestrial fungus *Aspergillus niger* was necessary to reach >30% hydrolysis of chitin, of which 92% was GlcNAc. A comparable reaction in buffer (10 mg/ml chitin, 0.1 mg/ml of chitinase) proceeded to a 5-fold lower yield of GlcNAc. In contrast to cellulases,[130] the so far optimal RAging conditions for chitinases were found to consist of combining 5 minutes of milling, followed by 12 hours of aging, repeated over multiple cycles. Importantly, chitinases reactions under RAging conditions did not require chemical pre-treatment of the commercial purified shrimp shell chitin.

Waste shrimp and crab shells were also hydrolyzed in >40% yield by RAging with chitinases after a gentle wash with aqueous acetic acid (Figure 6b). Here again, GlcNAc accounted for ~90% of the soluble products, whereas the GlcNAc dimer and deacetylated glucosamine were obtained in minute quantities.

4.4. Amylase-Catalyzed Depolymerization of Starch by Extrusion and RAging

The existing literature provides strong evidence that α-amylases also have mechanoenzymatic reactivity. As early as 1978, Linko *et al*.,[150] noted that the activity of α-amylase can be fully retained in rye, wheat and malted barley flours at mild extrusion conditions, which inspired subsequent studies with extruding reactors in enzymatic starch depolymerization.[127,151–155] These, together with the examples of bioextrusion of lignocellulose have recently been described in detail in a review by Gatt *et al*.,[127] and are therefore only briefly discussed here. Linko *et al*. showed, that the saccharification of barley starch proceeded faster if the starch was liquefied to oligomers first by extrusion with thermostable Thermamyl α-amylases (η = 1.0 μL mg^{-1}) at 135°C and 150 rpm.[154] Saccharification of the resulting extrudate was performed in a slurry (η = 2.3 μL mg^{-1}) at 60°C, with the addition of 0.36 % w/w amylase, yielding 174 g/L of glucose after 5 hours. The ensuing syrup could be directly fermented with yeast to produce ethanol. Hakulin *et al*. showed that the enzymatic saccharification process could also be initiated in the extruder, by introducing glucoamylases to the screws at a lower temperature (70°C),[153] however, due to the slow reaction rate of the saccharification, incubation of the extrudate was still necessary to obtain high glucose yield.

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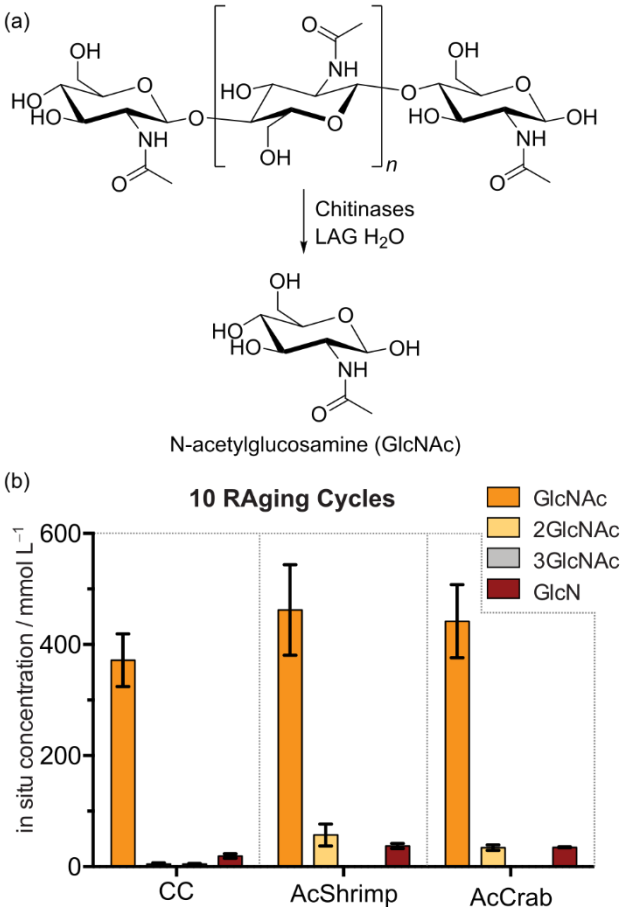

Figure 6. Chitinase-catalyzed chitin saccharification under LAG conditions. (a) Chitin hydrolysis catalyzed by chitinases. (b) Comparison of the yield and composition of the hydrolysis products from 10 cycles of RAging, for commercial practical-grade chitin (CC), biomass from shrimp (AcShrimp) and crab shells (AcCrab). The biomass was washed with dilute aqueous acetic acid prior to hydrolysis.[149]

Amylase-catalyzed saccharification has also been investigated on wheat,[156] corn,[157] sago,[152,155] and rice[158,159] starch by bioextrusion. Whereas the above described work by Therien *et al*. focused on chitinase activity, it also noted that the commercial enzyme preparation used for chitin hydrolysis contained, in addition to chitinases, α-amylases. During the RAging reactions, these α-amylases were found to hydrolyze the cryoprotectant present in the commercial chitinase preparation to glucose.[149] This fortuitous observation strongly suggests that amylases could also be compatible with RAging reaction conditions.

4.5. Remarks on Mechanoenzymatic Reactions of Glycosyl Hydrolases

The activity of glycosyl hydrolases such as cellulases, chitinases and amylases can be enhanced in the absence of bulk water, and even more so under RAging conditions. This may seem counterintuitive considering that these enzymes accelerate hydrolytic reactions. While the exact cause of this rate increase remains to be established, possible explanations may include a better match with the conditions under which the enzymes have evolved to thrive in, disruption and renewal of the biopolymer surface upon milling, and easier diffusion of enzymes towards unreacted substrates.

5. Conclusions and Outlook

Growing evidence from several research groups suggests that enzymatic reactions are compatible with a range of mechanochemical conditions, such as milling in the form of liquid-assisted grinding, and twin screw extrusion. In our view,

these observations are nothing short of remarkable, considering the well-established observation in enzymology that manual shaking of an aqueous enzyme solution will often denature the protein within seconds. As summarized in Table 1, successful mechanoenzymatic transformations have so far employed lipases, proteases, and glycosyl hydrolases. Although these enzymes are all hydrolases (E.C. 3), one can easily envisage that the list may eventually include proteins from all classes, and even ribozymes. Because they have evolved to act on moist surfaces, enzymes naturally secreted by microorganisms are especially likely to thrive in the absence of bulk water, whereas thermostable enzymes may better tolerate shear and milling forces. For the most sensitive enzymes, immobilization, encapsulation, or cross-linking may help preserve activity under mechanical stress. Interestingly, immobilization has been demonstrated to increase the stability of haloalkane dehalogenase specifically under gas-phase conditions.[160] Further studies looking at improving protein stability in the context of mechanoenzymology are warranted to improve yields and facilitate enzyme recycling.

The mechanical forces enabling mechanoenzymatic transformations have so far included batch processes in shaker or planetary mills, and continuous or batch extrusion . In principle, however, any physical means of mixing may be adaptable to mechanoenzymology. Furthermore, tuning mechanochemical reactions by cycling between milling and aging periods (the RAging technique) is a promising strategy to accelerate enzymatic reactions while reducing energy use and enzyme denaturation. Importantly, the scalability of mechanoenzymatic processes has been demonstrated with reactive extrusion techniques and planetary ball mills.

Mechanoenzymology has already produced several elegant examples of stereospecific, stereoselective and/or regioselective processes, meanwhile demonstrating that the absence of bulk water may enhance enzyme activity and conveniently allow control over the direction of a reaction. Yet this burgeoning field is mainly unexplored, with the possibility to further optimize the processes, expand them to other biocatalysts, transformations, or substrates, combine several transformations into enzymatic reaction cascades, and more.

Whereas mechanoenzymatic methods and applications are rapidly appearing in the scientific literature, enzyme mechanisms under such conditions, and how they relate to our knowledge of enzyme function in solution, are unknown. Given that classical enzyme kinetic methods and models are maladapted to solvent-free mixtures, new techniques and theories are needed to answer these questions. Only a better understanding of mechanoenzymology will allow the full deployment of biocatalytic mechanochemistry.

It is our view that solvent-less environments found in liquid-assisted grinding and/or aging in moist air, are more effective than conventional dilute solutions in replicating the natural environment of many enzymes. Much more akin to low η conditions of liquid-assisted mechanochemistry, enzymes secreted by microorganisms thrive on moist surfaces, and intracellular enzymes function in highly concentrated, organized environments. This observation is consistent with the ongoing change in our understanding of chemical transformations,[161] with the growing realization that many reactions readily proceed in solvent-less mechanochemical environments.[18,115,162–168] Indeed, mechanochemistry, which was recently proposed to be involved in the emergence of small organic molecules on the early Earth,[169] has successfully provided routes to chemical selectivity,[19,170,171] as well as reactions[172,173] or molecules[174–176] that have otherwise been considered difficult or even impractical to access. Although mechanoenzymatic reactivity is in a very early stage of development, it has already sparked a significant amount of interest,[177] and provided simple solutions to streamline industrial processing of, for example, lignocellulosic material. We hope that this overview will inspire the further development and practical deployment of mechanoenzymology, as the expected benefits can vastly overcome the current challenges.

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Table 1. Summary of enzyme-catalyzed mechanochemical transformations performed in the absence of bulk water.

Reaction	Enzyme	Product	η (μL mg^{-1})	Equipment	Duration and frequency	Ref.
Enantiospecific acetylation of secondary alcohols	Immobilized CALB [a]	(*R*)-acetate conversion 43-49% *ee* 93-99%	N/A [j]	shaker or a planetary ball mill	Milling: 3 h, 25 Hz or 3 h, 600 rpm	[60]
Enantiospecific acetylation of secondary alcohols	Lipase PS-IM [b]	(*R*)-acetate conversion 27-39% *ee* 99%	N/A [j]	shaker ball mill	Milling: 3 h, 20 Hz	[60]
Enantiospecific acetylation of primary amines	Immobilized CALB [a]	(*R*)-amide yield 20-48% *ee* 66-99%	N/A [j][k]	shaker ball mill	Milling: 1.5 h, 25 Hz	[72]
Regiospecific acetylation of lignin model compounds (diols)	Immobilized CALB [a]	erythro-monoacetates yield 82-100% threo-monoacetates yield 2-45%	0.8 - 2.35 [l]	shaker ball mill	Milling: 2-6 h, 30 Hz	[75]
Enantiospecific hydrolysis of β-amino esters	Immobilized CALB [a]	(*R*)- or (*S*)-β-amino acid conversion 4-49% *ee* 80-99%	N/A [k][m]	shaker ball mill	Milling: 1 h, 15 Hz	[93,178]
Polymerization of ω-pentadecalactone	Immobilized CALB [a]	99% conversion into polymers (M_n = 90 000 g mol^{-1})	0	twin screw extrusion	Extrusion: 15-20 min, 60 rpm, 90°C or 110°C	[95]
Hydrolysis of polubutylene succinate	CALB	Succinic acid yield 44%	0.03 [n]	twin screw extrusion	Extrusion: 5-30 min, 50 rpm, 120°C	[99]
Grafting of (poly)caprolactone to chitosan	NovoCor [c]	grafting yield 96.3%	0	Twin screw extrusion	Extrusion: 35 s, 300 rpm, 60°C	[100]
Peptide bond formation between *N*-protected L-amino acid esters and amino amides	Papain (cysteine protease) [d]	α-α and α-β-dipeptides yields up to 99%	0 - 0.34 [o]	shaker ball mill	Milling: 2 h, 25 Hz	[116]
Peptide bond formation between *N*-benzyloxy-carbonyl (Z) L-Ala ester and L-Phe-NH_2	Subtilisin (serine protease) [e]	Z- L-Ala-L-Phe-NH_2 yield 61%	0.34 [o]	shaker ball mill	Milling: 2 h, 25 Hz	[116]
Homopolymerization of amino acids	Papain (cysteine protease) [d]	oligopeptides, conversion 50-99%	0 - 0.34 [o]	shaker or a planetary ball mill, or twin screw extrusion	Milling: 2 h, 25 Hz or 2 h, 600 rpm Extrusion:45 min, 30 rpm, 45°C	[119]

Hydrolysis of cellulose	Cellulases [f]	glucose yield 50%	0.4 - 0.8 [p]	shaker and planetary ball mill	RAging: 5 min, 30Hz + 55 min 55°C 5 min, 600 rpm + 55 min 55°C	[130]
Hydrolysis of cellobiose	β-Glucosidase [g]	glucose yield 70%	0.95 [p]	shaker ball mill	RAging: 5 min, 30Hz + 55 min 55°C	[143]
Hydrolysis of chitin	Chitinases [h]	*N*-acetylglucosamine yield >40%	1.7 [p]	shaker ball mill	RAging: 5 min, 30Hz + 12 h 45°C	[149]
Hydrolysis of dextrose	α-Amylase [h]	glucose (side-product from cryoprotectant)	1.7 [p]	shaker ball mill	RAging: 5 min, 30Hz + 12 h 45°C	[149]
Hydrolysis of starch	α-Amylase [i]	glucose, 174 g/L	1.0 [p] (2.3 for slurry incubation)	twin screw extruder and incubator	Extrusion: 150 rpm, 135°C Incubation: 5 h 60°C	[154]

[a] Lipase B from *Candida antarctica* immobilized on acrylic beads, commercially available as Novozyme 435. [b] Lipase from *Burkholderia cepacia*, immobilized on diatomaceous earth. [c] NovoCor thermostable lipase from *Candida antarctica*. [d] Papain from *Carica papaya* powder or latex. [e] Alcalase-CLEA®, the commercial form of subtilisin from *Bacillus licheniformis* available as cross-linked enzyme aggregates. [f] *Trichoderma reesei* or *Trichoderma longibrachiatum* cellulases, containing endo- and exoglucanases and β-glucosidases. [g] β-glucosidase from *Aspergillus niger* [h] Chitinases from *Aspergillus niger* (enzyme preparation exhibits also α-amylase activity). [i] Thermostable Thermamyl 120 L or 60 L α-amylases. [j] Acetylating agent and substrates are liquids, with some exceptions. [k] In some examples 2-methyl-2-butanol or dioxane is used as a liquid additive. [l] The liquid added is the acetylating agent [m] The esters were described to be amber-colored liquids, to which 0.5 eq of water (reagent) is added. [n] Hydrolysis proceeded in presence of atmospheric humidity (water is a reactant), estimated at 3 wt% in the polybutylene succinate material. [o] In some reactions the liquid was added with the base as the mono- or decahydrate. [p] The liquid added is distilled water (reagent).

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Keywords: mechanochemistry • mechanoenzymology • solvent-free • milling • extrusion

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[120] The authors report that extrusion yielded 0.290 g of oligo(L-Phe), 0.214 g of oligo(L-Leu), and 0.074 g of oligo(L-Ala) per 1 g of the respective extruded mixture. Assuming only the loss of CO_2, the total weight of the extrudates is 26.77 g for oligo(L-Phe), 29.92 g for oligo(L-Leu) and 35.95 g for oligo(L-Ala) respectively, which includes the HCl salt of the corresponding amino acid methyl ester (10 g), papain (200 mg per 2.31 mmol of substrate), L-cysteine (77.8 mg per 200 mg of enzyme) and the base $Na_2CO_3{\cdot}10H_2O$ (1 equivalent). Based on the average length of each oligomer obtained and the weight of single chain capping HCl and methyl ester group, then quantitative yield from 10 g of the corresponding amino acid methyl ester HCl salt would give 7.46 g of oligo(L-Phe), 6.69 g of oligo(L-Leu) and 5.34 g of oligo(L-Ala). Therefore, quantitative conversion would afford 0.279 g of oligo(L-Phe), 0.223 g of oligo(L-Leu) and 0.148 g of oligo(L-Ala) per 1 g of the respective extruded mixtures. From here, the calculated conversions are 104% for oligo(L-Phe), 96% for oligo(L-Leu) and 50% for oligo(L-Ala).

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