Rapid mechanoenzymatic saccharification of lignocellulosic biomass without bulk water or chemical pre-treatment

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Lignocellulosic material is an abundant renewable resource with the potential to replace petroleum as a feedstock for the production of fuels and chemicals. The large scale deployment of biomass saccharification is, however, hampered by the necessity to use aggressive reagents and conditions, formation of side-products, and the difficulty to reach elevated monosaccharide concentrations in the crude product. Herein we report the high efficacy of Reactive Aging (or Raging, a technique where enzymatic reaction mixtures, without any bulk aqueous or organic solvent, are treated to multiple cycles of milling and aging) for gram-scale saccharification of raw lignocellulosic biomass samples from different agricultural sources (corn stover, wheat straw, and sugarcane bagasse). The solvent-free enzymatic conversion of lignocellulosic biomass was found to proceed in excellent yields (ca. 90%) at protein loadings as low as 2% w/w, without the need for any prior chemical pre-treatment or high temperatures, to produce highly concentrated (molar) monosaccharides. This crude product of mechanoenzymatic depolymerization is non-toxic to bacteria and can be used as a carbon source for bacterial growth.

Introduction

With the recognition that use of fossil resources is unsustainable, lignocellulosic biomass has been identified as a main candidate to fulfil the future needs for fuels and basic chemicals.[1–6] Saccharification of the biopolymer constituents of biomass affords monosaccharides - mainly glucose and xylose - which have been recognized as convenient platforms for the production of valued molecules such as ethanol,[7] polyhydroxyalkanoates,[8–10] succinate,[11] and itaconate.[12] Despite being produced at an estimated rate of 10^{12} ton per year,[13] lignocellulosic materials such as agricultural and forestry wastes remain underexploited because of their poor solubility and remarkably recalcitrant nature.[14] Lignocellulosic materials are composed of cellulose (a linear glucose polymer), hemicellulose (a branched xylose heteropolymer), and lignin (a heterogeneous polyphenolic branched polymer), which are closely intertwined and mostly unaccessible,[11] posing a persistent challenge for industrial applications.

As a result, chemical hydrolysis of cellulose and hemicellulose to form mono- or oligosaccharides typically requires aggressive chemicals (acids, bases, transition metals)[15–20] and harsh conditions (temperature, pressure),[21,22] leading to high energy demands, waste production,[23] as well as contamination with side reaction products[22,24–26] such as furfural, hydroxymethylfurfural (HMF), and acetic acid. Arguably the most important industrial application of glucose, its fermentation by yeasts to produce ethanol, is very sensitive to such impurities.

Whereas biocatalytic processes relying on the action of cellulase and/or hemicellulase enzymes offer a milder, promising alternative for depolymerisation of cellulosic biomass, they are notoriously slow,[27] and usually require biomass pre-treatment,[28] again under harsh conditions, to make the biopolymers more accessible to the enzymes.

Another important bottleneck specific to ethanol production from lignocellulosic material is the requirement for a high monosaccharide concentration in the biomass hydrolysate to optimize yeast growth.[7,29–31] Such concentrations can only be achieved in the presence of high initial amounts of biomass per sample volume (high solid loading), conditions under which the enzymatic saccharification yields are reported to drop significantly. This phenomenon is known as the “solids effect”,[32–34] and is in part linked to poor homogenization of the mixture and increased inhibition of cellulases by the reaction products.

Our group, and others, have recently demonstrated that enzymes can function surprisingly well in the absence of aqueous or organic solvent, and that their activity can be facilitated by mechanical mixing.[35–45] This emerging area of research, mechanoenzymology, may provide a solution to the solids effect challenge. We recently developed a mechanoenzymatic technique (Fig. 1) termed Reactive Aging (RAging), which proceeds in the absence of bulk water, and consists of cycles of alternating periods of brief (minutes) ball milling and longer periods (minutes or hours) of aging,[46,47] *i.e.* static incubation under controlled conditions. The RAging reactions of cellulases are conducted in the presence of only ca. 10-20 stoichiometric equivalents of water, which acts both as a substrate and likely as a reaction lubricant,[48] leading to a moist

solid reaction mixture. This corresponds to solid loadings of 50-100% w/v, which is, to our knowledge, higher than for any previously reported cellulase reactions (typically around 40% w/v).[29,32,34] At the laboratory scale, the methodology was found to be superior to traditional dilute aqueous reaction mixtures, not only for cellulases,[42,43] but also for chitinases,[44] and xylanases.[49] Although RAging allows direct and efficient depolymerization of microcrystalline cellulose (MCC), as well as cellulose in hay and tree saw dust, without any chemical pre-treatment, further optimization is warranted as earlier reports have been limited to monosaccharide yields not higher than 50%.[40,]

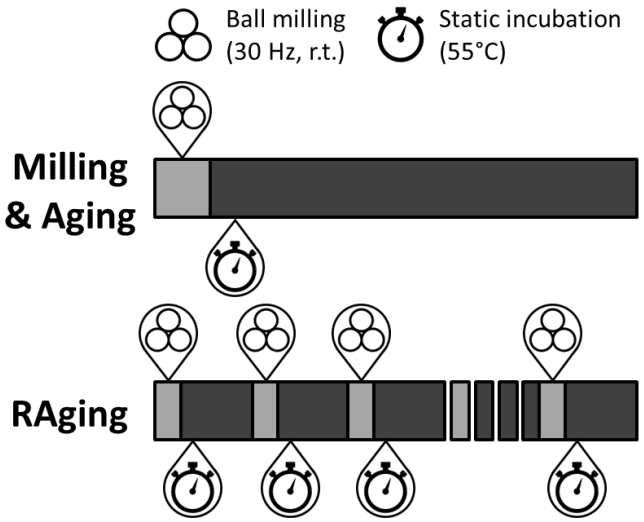

Figure 1. Representation of the mechanoenzymatic processes used in this work: milling followed by aging (top), and RAging (bottom). The commonly accepted symbol for ball milling is used,[50] while the clock symbol is used to represent aging (static incubation).

We now report the high efficacy, with up to 90% depolymerisation yield of monosaccharides, of cellulases under mechanoenzymatic, water-depleted conditions. This is illustrated in the saccharification of three distinct raw agricultural residues, notably wheat straw (WS), sugarcane bagasse (SB), and corn stover (CS), in a process involving biomass pre-milling and the use of the CTec2 cellulases cocktail (Novozymes). The herein presented methodology generates crude reaction mixtures of monosaccharides in molar concentrations, which we also show can be used directly as a source of carbon in the growth medium of bacterial cultures.

Results and discussion

Biomass composition.

The experimentally established average compositions (on a dry basis) of the three herein explored agricultural substrates, WS, SB and CS, are presented in Table 1.

Table 1. Dry basis composition of the biomass samples used and the maximum extractible monosaccharides.

	Biomass composition (%)			Total extractibles (mmol/g)
	Cellulose	Hemicellulose	Others	
Corn Stover (CS)	33.5	23.2	43.3	3.83
Wheat Straw (WS)	34.4	19.9	45.7	3.63
Sugarcane Bagasse (SB)	40.1	22.3	37.6	4.16

Water and protein loading in the mechanoenzymatic reactions.

The herein explored RAging mechanoenzymatic reactions proceed with the addition of a small amount of water, which also acts as a substrate. Following the terminology used in liquid-assisted mechanochemistry, the amount of water used corresponds to the η-parameter, *i.e.* volume of added liquid per sample weight (in µL/mg),[‡] between 0.5 and 1.5 µL/mg. These conditions meet the previously established regime of mechanochemical liquid-assisted grinding (LAG)[51] where, as long as η is maintained approximately below 2 µL/mg, the reactions can be accelerated or even catalysed[52] by the presence of a liquid additive, but proceed independent of the relative solubilities of reactants. Due to the absence of solubility limitations typical of reactivity in bulk solvent media,[51] transformations under LAG conditions are generally considered solvent-free. In our RAging reactions, the added water was completely adsorbed onto the biomass substrate, resulting in reaction mixtures with the appearance of a moist solid. As the reaction progressed, the mixtures were generally found to turn into soft solids with consistencies ranging from those of toothpaste to baking dough.

Another important parameter in enzymatic transformations is enzyme loading, described as the mass of protein per mass of cellulose in the biomass (in mg/g). Protein titers in the commercial *Trichoderma longibrachiatum* cellulases solid stock (13±1% w/w) and in the CTec2 cellulases solution (16±1% w/v) were determined using the Bradford assay. Both of these enzyme preparations contain several cellulases, including exoglucanases, endoglucanases, and β-glucosidases, as well as hemicellulases.

Unless specified otherwise, small-scale milling experiments were performed in 15 mL volume stainless steel jars containing two 7 mm stainless steel balls (1.3 grams each) mounted on a shaker mill operating at 30 Hz. Medium scale milling was accomplished using a 30 mL jar with one 15 mm ball (11.6 grams), both made from stainless steel. The aging part of the reactions was performed by incubation of the milled reaction mixture at 55°C in a closed container.

Biomass saccharification using *T. longibrachiatum* cellulases.

We have previously shown that *T. longibrachiatum* cellulases are superior to the corresponding *Trichoderma reesei* enzymes for cellulose cleavage under mechanochemical conditions.[42] Thus the latter enzyme preparation was not used here.

After milling raw WS (400 mg; small scale) for 15 min to reduce its size, lyophilized *T. longibrachiatum* cellulases preparation was added (86 mg protein per g cellulose, or 8.6% w/w) together with varying amounts of water. Efficient saccharification of this biomass required a slightly higher liquid/solid ratio (η = 1.34 μL/mL) than MCC (η = 0.9 μL/mL),[42] leading to 8% digestion of the glycosidic bonds after only 30 min of ball milling, and up to 25% when milling was followed by 3 days of aging at 55°C (Fig. S1; yield estimated using the classical dinitrosalicylic acid, or DNS, method which detects sugar reducing ends[53]). In contrast, RAging of the same reaction mixture, by alternating periods of 5 minutes milling and 55 minutes aging, afforded a yield of 37% in only 12 hours (Fig. 2). Saccharification of SB under the same RAging conditions afforded a 48% yield after 12 hours and 62% yield after 24 hours. When the raw biomass was not pre-milled, the yields went down to 16% and 28% after 12 hours of RAging for WS and SB, respectively.

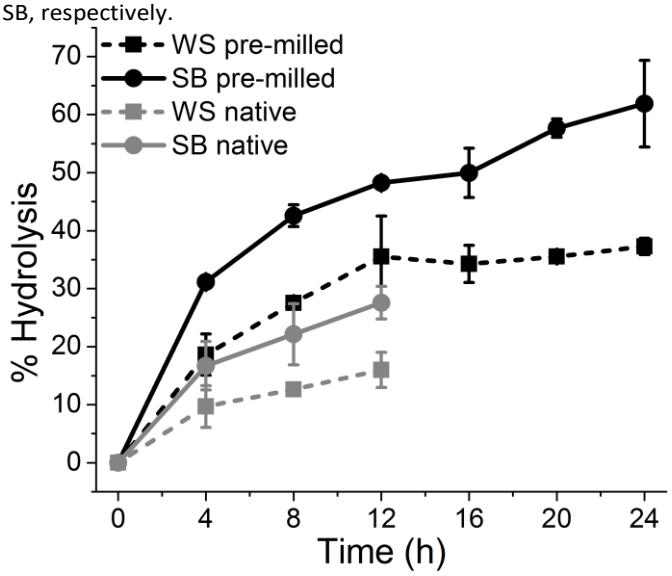

Figure 2. Digestion of native or pre-milled (400 mg, 15 min) WS and SB with the *T. longibrachiatum* enzyme preparation (86 mg/g cellulose) using RAging (cycles of 5 min milling and 55 min aging at 55°C). Yield is DNS-based; error bars are standard deviation from triplicates.

Biomass pre-milling enhances cellulase activity.

Compared to *T. longibrachiatum* cellulases, the commercial CTec2 cellulases blend was found to be more efficient (Fig. S2), and subsequent experiments were performed with this enzyme preparation.

We next assessed the impact of biomass pre-milling on the ensuing activity of CTec2 cellulases. Whereas enzymatic hydrolysis typically relies on a chemical pre-treatment of the biomass,[28] chemical cellulose saccharification often involves mechanical pre-treatment.[28,54,55] Thus, CS alone (3 g; medium scale) was treated to various milling durations. The resulting powder was then submitted to a mechanoenzymatic reaction (milling for 30 minutes followed by aging for 3 days) in the presence of CTec2 cellulases (45 mg/g cellulose, 4.5% w/w) at η = 1.5 μL/mg. The duration of pre-milling was found to have a large impact on the subsequent mechanoenzymatic transformation (Fig. 3), with a maximum hydrolysis yield of 80±5% obtained when CS was pre-milled for 90 min. Similar results were obtained with the other two biomass substrates, with yields of 73±2% and 75±5% for WS and SB, respectively (Fig. S3). A pre-milling step of 60 minutes duration at a lower CS biomass loading (1.5 g instead of 3 g for the same jar) afforded an even higher yield of 88±2% after enzymatic transformation (Fig. 3).

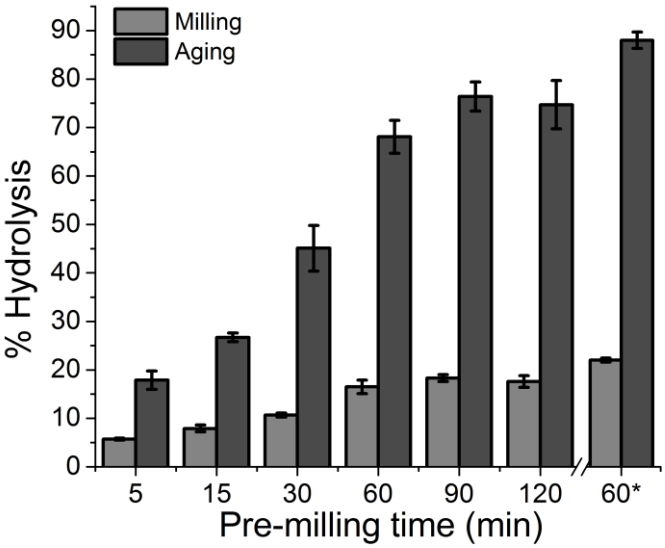

Figure 3. Influence of pre-milling time (3 g in 30 mL jars) on the outcome of milling (30 min) and aging (3 days) reactions of CS with CTec2 enzymes (45 mg/g, η = 1.5 μL/mg). Yield is DNS-based; error bars are standard deviation from triplicates. *Jar loading during pre-milling was reduced to 1.5 g.

Besides cellulases, commercial enzymatic preparations may also contain xylanases and other enzymes, indicating that biomass saccharification may lead to a mixture of oligo- and monosaccharides of different sugars, undistinguishable by the DNS assay. The glucose and xylose concentrations of the herein obtained crude reaction mixtures from CS were next measured using a sugar analyser. The glucose concentration was found to be 0.79 M (143 g/L), corresponding to a 57% yield, while the concentration of xylose produced was 0.39 M (60 g/L), corresponding to a yield of 24%. The generally observed variance between the DNS and sugar analysis methods can be explained by the partial digestion of biopolymers into soluble oligosaccharides,[49] detected by DNS but not with the sugar analyzer.

The crystallinity of CS during the pre-milling step (5-120 min, 3 grams at once) was investigated using powder X-ray diffraction (PXRD) at different time periods, and was found to decrease with milling time (Fig. S5A), with crystalline cellulose (characterized by the Bragg reflection at 2θ of ca. 22°) disappearing within 60 minutes of ball milling. Alone, this reduction in crystallinity cannot account for the increased enzymatic reaction yield, as the maximum conversion is only obtained with 90 minutes of pre-milling. Furthermore, no crystalline cellulose is detected after 60 min of pre-milling, regardless of biomass loading (3 or 1.5 grams; Fig. S5B), while the hydrolysis yield is higher at a 1.5 grams loading (Fig. S4), suggesting that an increase in surface area may also facilitate the enzymatic reaction. In support of this conclusion is the fact that when CS of 30% humidity (significantly less brittle and less efficiently comminuted) is pre-milled for 30 min, the subsequent enzymatic saccharification proceeds only to 25%

conversion (Fig. S6), compared to >60% from more brittle, dried CS upon similar treatment. Taken together, these results suggest that pre-milling the biomass enhances cellulases activity as a result of reducing both cellulose crystallinity and increasing substrate surface area.

Optimization of CTEc2 enzyme activity under milling & aging conditions.

Depolymerization of CS by CTec2 cellulases (45 mg/g cellulose) under conditions of milling only, exhibited the usual hyperbolic kinetic profile reported for other mechanoenzymatic reactions[42,44,49] (Fig. S7), confirming the emerging paradigm that hydrolytic enzymes can easily operate under mechanical agitation. This resilience was further highlighted during the subsequent aging step as shown on Fig. 4. Again, the reaction showed a hyperbolic kinetic profile, with a plateau in conversion appearing only after ca. 20 hours. The initial rate of hydrolysis during aging was measured to be 560±40 mM/h, which is 4 times faster than the initial rate of 130±40 mM/h observed during milling.

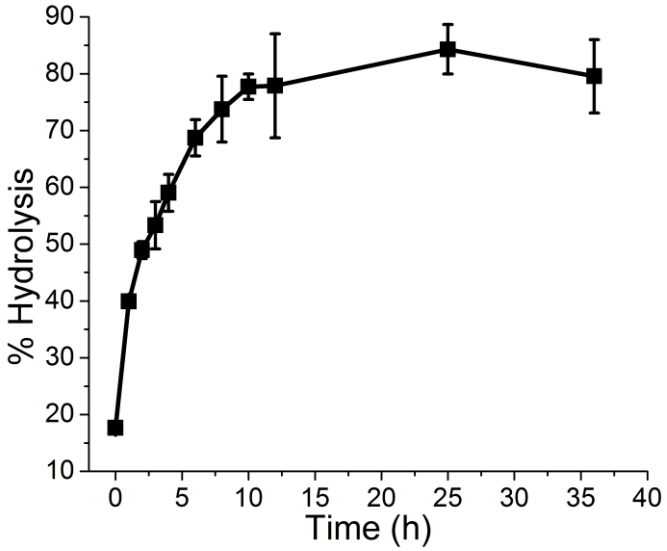

Figure 4: Kinetics of CTec2 enzymes (45 mg/g cellulose, η = 1.35 µL/mg) during aging (after 5 min milling) of a reaction mixture with pre-milled CS (1.5 g, 60 min). The yields were approximated using the DNS assay; error bars are standard deviation for triplicates.

The addition of sodium azide (NaN_3) – a common antibacterial agent used during enzymatic saccharification reactions – to the enzymatic reaction mixtures (0.04% w/v in the added water) had no significant effect on the reaction yield, even after 3 days of reaction (Fig. S8). In conventional solution-based processes, prolonged enzymatic reactions in dilute buffers are prone to contamination by bacterial or fungal growth. In contrast, the observation that NaN_3 addition does not affect the outcome of our experiments indicates that solvent-free conditions may not favour microbial growth. Sodium azide was nevertheless used in all subsequent experiments in order to eliminate any possibility of contamination during aging and sample handling.

The structure of biomass exhibits cellulose fibers fully surrounded by hemicellulose, which reduces their accessibility to enzymes. Speculating that this could be a potential limitation of our mechanoenzymatic process, we next explored the use of additional hemicellulase enzymes. The addition of the hemicellulase *Thermomyces lanuginosus* xylanase (1.5 mg/g cellulose) did not improve the yield of cellulose-catalyzed CS hydrolysis (Fig. S9), suggesting that milling and/or the low amount of hemicellulases already present in the CTec2 mixture might be sufficient to make cellulose available for reaction.

CTec2 cellulases exhibited high efficacy during aging, independent of the duration of the milling step (Fig. S10). By milling for only 5 min with the enzyme before aging, we were able to cut the total milling time significantly, without impacting the overall yield.

We also investigated the effect of varying enzyme loading on the efficacy of the mechanoenzymatic depolymerization of CS. Reactions containing 10-50 mg of protein per gram of cellulose were milled for 5 min and aged for 1 or 3 days (Table 2). As is often observed with cellulases in bulk water, higher loadings did not always improve reaction yields. This is usually attributed to limited substrate available at the surface of the biomass.[56,57] In this case, maximum efficacy was reached at 20 mg/g enzyme loading (or 2% w/w).

Table 2: Influence of enzyme loading on milling, and milling & aging reactions. Reaction mixtures contained 400 mg of CS (pre-milled for 60 min at 1.5 g scale) combined with a 600 µL aqueous solution of NaN_3 (0.04% w/v) and CTec2 cellulases. Mixtures were milled for 5 min at 30 Hz and aged for 1 or 3 days at 55°C.

	Reducing sugar yield (%)[a]		
Enzyme loading[b]	Milling (5 min)	Aging (1 day)	Aging (3 days)
10	6.5 ± 0.3	61 ± 1	71 ± 2
20	12.3 ± 1.2	69 ± 5	79 ± 2
30	15.1 ± 1.0	68 ± 2	80 ± 5
40	15.8 ± 0.9	71 ± 4	79 ± 6
50	14.8 ± 1.1	64 ± 2	79 ± 3

a) DNS-based. Error is standard deviation from triplicates. b) Reported in mg protein/g cellulose, corresponding to 1-5% w/w.

Remarkably, the reaction can be conveniently scaled up from 400 mg to 1.5 g, affording 81±5%, 73±3% and 83.1±0.8% after 3 days of aging for CS, WS and SB respectively, similar to smaller scale reactions (Fig. S11).

Enhanced reactivity under RAging conditions.

We further looked to accelerate biomass hydrolysis by CTec2 using RAging. Pre-milled CS was hydrolysed enzymatically via multiple 1-hour cycles, each consisting of 5 min of milling and 55 min of aging. Saccharification reached 83% after only twelve cycles (Fig. 5, Table 3). Even though the yield (as measured by the DNS assay) was roughly the same as with milling only once followed by 3 days of aging, it was reached much faster (6 times). Furthermore, detailed analysis revealed a glucose content ~20% higher with RAging than through a combination of a single milling step followed by aging, and a slightly improved xylose content. Reproducibility was also significantly improved (comparing Tables 2 and 3).

Enzymatic hydrolysis of WS and SB under the same RAging conditions proceeded in 70% and 76% yields, respectively.

These values, however, rose to 84% or 86% if the 12-hour RAging period was followed by an additional 12 hours of aging (Table 3).

Table 3: RAging pre-milled biomass. Reaction mixtures contained 400 mg pre-milled substrate (1.5 g, 60 min) combined with 600 µL of an aqueous solution of NaN_3 (0.04% w/v) and CTec2 cellulases (45 mg protein/g cellulose). Reactions were submitted to 12 cycles of 5 min milling at 30 Hz and 55 min aging at 55°C followed by another 12 h of aging at 55°C.

Substrate	Technique	Reducing sugars[a]		Glucose[b]		Xylose[b]	
		Yield	Final concentration (M)	Yield	Final concentration (M)	Yield	Final concentration (M)
CS	milling & aging (72h)	88 ± 2%	2.22 ± 0.04	57 ± 1%	0.79 ± 0.02	24 ± 2%	0.39
CS	RAging (12h)	83 ± 3%	2.09 ± 0.06	77 ± 1%	1.05 ± 0.01	39 ± 2%	0.4 ± 0.1
WS		70 ± 1%	1.61 ± 0.01	65 ± 4%	0.85 ± 0.05	39 ± 3%	0.4 ± 0.1
SB		76 ± 4%	1.96 ± 0.09	66 ± 2%	1.0 ± 0.2	37 ± 5%	0.3 ± 0.1
CS	RAging (12h) + Aging (12h)	86 ± 2%	2.16 ± 0.03	--	--	--	--
WS		84 ± 4%	1.90 ± 0.09	--	--	--	--
SB		84 ± 4%	2.14 ± 0.01	--	--	--	--

a) Based on the DNS assay. b) Measured by sugar analysis. Error is the standard deviation for triplicates.

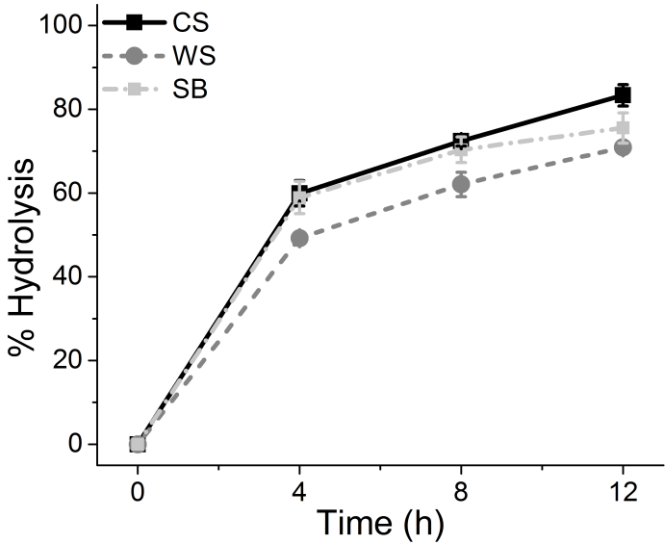

Figure 5: RAging pre-milled CS (full black line), WS (dashed, dark grey), and SB (dashed, light grey). Reaction mixtures contained biomass (400 mg, pre-milled for 60 min) combined with 600 µL of an aqueous solution containing NaN_3 (0.04% w/v) and CTec2 (45 mg protein/g cellulose). Mixtures were submitted to 12 cycles of 5 min milling and 55 min aging. Yields are approximated with the DNS assay. Error bars are standard deviations from triplicates.

Compared to conventional slurry or solution processes which require a harsh pre-treatment, RAging does not require any chemical pre-treatment, while leading to higher reaction rates (Tables S1, S2).[31,34,58–66] In addition to providing the crude product with the highest reported monosaccharide concentration (second highest for glucose alone), the space-time yield (mass of sugar produced per litre of reaction per hour, see Tables S1 and S2) of our enzymatic RAging process is at least twice higher than that of any other reported method (Tables S1 and S2).[59]

We have previously established that during RAging reactions, the cellulolytic enzymes remain in the solid fraction and can be recycled.[42] Centrifugation allows convenient separation of the sugars (highly concentrated aqueous solution) from the solid lignin, which is expected to be mostly unreacted based on prior reports demonstrating that ball milling of lignin in the absence of a base, an acid, or a catalyst leads to negligible depolymerisation.[67–69] This is a significant asset for the inclusion of our method within comprehensive lignocellulose degradation processes. Furthermore, the high-lignin content residue produced (theoretically 80-90% dry weight) is valuable for further physicochemical,[70–72] enzymatic,[73] or microbiologic[74] transformation, thus mitigating waste production.

Several reports have reported an inhibitory effect of lignin on holocellulose hydrolysis, with yields reduction as high as 65%.[75–79] Whereas many strategies have been explored to alleviate this inhibition (*e.g.* lignin removal or alteration[79–81]), they generate downstream complications like additional processing steps, increased energy consumption and waste, use of additional reagents, and byproduct generation.[82,83] In contrast, our RAging method on moist solid reaction mixtures proceeds as well in the presence (biomass) than in the absence (MCC, Fig. S2) of lignin.

Crude RAging products as a carbon source for bacterial growth.

Next, we evaluated the potential of our biomass hydrolysates for use as the carbon source in bacterial growth media. Thus, from crude RAging reaction mixtures, the resulting sugar-rich supernatant was diluted to ca. 0.3% w/v based on monosaccharides[§], supplemented with standard mineral salts (carbon-free), and directly used as a bacterial culture medium using standard protocols. Both *Escherichia coli* and *Salmonella enterica* ser. Typhimurium were found to proliferate equally well on agar gels derived from either standard lysogeny broth (LB) or media prepared from crude saccharification reaction mixtures from either CS, WS, or SB (Fig S12).

We next looked at planktonic growth of a strain of *Paraburkholderia sacchari*[10] known to metabolize both glucose and xylose, and to produce polyhydroxybutyrate, a highly valued polymer and important candidate for large scale deployment of biodegradable plastics.[9] The hydrolysate resulting from milling & aging SB was used as the sole carbon source of the growth medium, adjusted to final concentrations of 11.6 g/L glucose and 5.7 g/L xylose. Bacteria were inoculated in this medium, and after 24 hours of incubation, they

proliferated to 6.9±0.3 g/L cell dry mass (ΔCDM), which is higher than for a control experiment containing 20 g/L of pure glucose instead of biomass hydrolysate (Fig. 6A). While the glucose consumption was similar in both experiments (around 11 g/L) the hydrolysate allowed the consumption of an extra 3.5 g/L of xylose (Fig. 6B). These experiments demonstrate that the crude products of our mechanoenzymatic reactions can be used directly as the only carbon source in fermentation processes without toxicity.

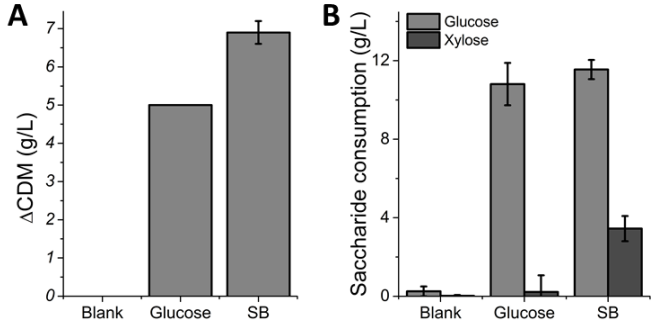

Figure 6: A) *P. sacchari* growth in a culture medium either without a carbon source (blank), prepared from commercial glucose (20 g/L), or from a RAging-derived SB hydrolysate (final titer: 11.6 g/L glucose and 5.7 g/L xylose). B) Saccharide consumption after 24 hours of *P. sacchari* growth using commercial glucose or SB hydrolysate as the main carbon source.

Conclusions

We report here that both RAging, as well as aging after a brief period of mechanochemical activation, enable the enzymatic breakdown of cellulose and xylan in the absence of bulk water, directly from different types of biomass, without any need for chemical pre-treatment. Simply pre-milling of the raw lignocellulosic material for 60-90 min in order to obtain a fine powder, before enzyme addition, was sufficient to ensure subsequent mechanoenzymatic saccharification yields of ca. 90% within 12 to 24 hours on a gram scale. Moreover, the enzymatic depolymerization of all three biomass substrates proceeded to yield molar-level concentrations of glucose and xylose monosaccharides. After separation from the solids, the crude sugars were efficiently used as a carbon source for bacterial growth on agar gels or in a bioreactor, demonstrating their low toxicity and biocompatibility.

We previously validated the possibility of using RAging on a planetary mill, a more scalable technique.[42] Several other scalable mechanochemical techniques are well-established, including both batch (e.g. horizontal rotary ball mills) and flow (e.g. twin screw extruders) methods.[84–87]

Biocatalysis is appreciated for its selectivity, mild conditions, low toxicity, and catalyst renewability. The growing field of mechanoenzymology provides new exciting opportunities for biocatalytic transformations. Not only were enzymes reported to tolerate mechanical stress,[33-42] but they were also found to remain active in moist solid mixtures.[37] Static incubation of enzymatic reaction mixtures without bulk water may provide a better mimic of the natural environment of enzymes, and especially so for enzymes that are secreted by soil microorganisms that thrive on moist surfaces rather than dilute aqueous solutions.[88,89] By minimizing the total volume of the reaction mixture, the herein presented mechanochemically-activated enzymatic processes greatly facilitate handling and mixing, and curtail waste associated with processing and depolymerization of polysaccharide biomass. Furthermore, as highlighted in this study, mechanoenzymology avoids solubility issues and the solids effect which normally impairs enzymatic saccharification of lignocellulosic materials at high solid loading. Consequently, we believe that this non-traditional way of using enzymes should find broad application as a cleaner, simpler and more efficient route for converting biomass into well-defined small molecules, without requiring bulk solvents, strong mechanical impact or high temperatures.

Conflicts of interest

Some of the herein presented work is a part of the patent application US 62/465,443 filed 1 March 2017.

Acknowledgements

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Notes and references

‡ Enzyme mass was taken into account only when it was loaded as a solid (*T. longibrachiatum*). The CTec2 cellulases mix was purchased as a solution, and the liquid volume measured was used in the calculation of the *η* parameter.

§ calculated based on the average molecular weight of glucose and xylose theoretically released after quantitative hydrolysis of cellulose and xylan in CS, *i.e.* 167.6 g.mol^{-1}.

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